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Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants



Edited by Yvonne Will & James A. Dykens

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Volume I

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Foreword

The field of mitochondrial medicine is enjoying a renaissance driven largely by advances in molecular biology and genetics. The first draft sequence of the human mitochondrial genome in 1981 provided the critical blueprint that enabled the identification of the first point and single large-scale deletion mutations of mitochondrial DNA (mtDNA) in 1988. To date, more than 270 distinct mtDNA point mutations and hundreds of mtDNA deletions have been identified. Subsequent sequencing of the human nuclear genome in the early 2000s helped to catalyze the discovery of approximately 1000 nuclear genes that, together with the mtDNA, encode the mitochondrial proteome. With a complete parts list, it has been possible to delve deep into the molecular basis of Mendelian mitochondrial disorders, with more than 200 nuclear disease genes now identified. There is now widespread consensus that mitochondrial dysfunction contributes to a spectrum of human conditions, ranging from rare syndromes to common degenerative diseases to the aging process itself.

Today, there is great excitement that in the coming decade, new medicines will become available that alleviate disease by targeting mitochondria. At the same time, there is widespread appreciation that many drugs fail clinical trials because of their mitochondrial liabilities. *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* represents one of the most important textbooks for those hoping to target mitochondria, as well as for those wanting to avoid mitochondrial side effects. It is a deep and thoughtful resource that will appeal to basic scientists, clinicians, and professional drug developers.

Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants provides ample reminders of the intimate connections between mitochondria, pharmacology, and toxicology. Some of the most widely used tool compounds for investigating mitochondrial physiology, such as antimycin and oligomycin, are indeed natural products that serve as a chemical warfare in the microbial world. The fact that antimicrobial agents are often toxic to mitochondria is not surprising given the hypothesized proto-bacterial origin of mitochondria. These overlapping effects of such drugs are perhaps best

illustrated by aminoglycosides and linezolid antibiotics, which not only inhibit bacterial protein synthesis but are also well known to cause neurotoxicity such as hearing loss, peripheral neuropathy, and optic neuropathy through impairment of mitochondrial translation. Pharmacogenetics contributes to these toxicities with the well-established link between the m.1555A>G variant that predisposes to aminoglycoside-induced deafness.

Toxic side effects of clinically important and investigational new drugs for viruses have historically provided fundamentally new insights into the replication of mtDNA. One of the earliest anti-HIV agents, zidovudine (azidothymidine (AZT)), is a nucleoside analogue that effectively inhibits viral reverse transcriptase but, in some patients, inhibits the mitochondrial polymerase gamma, leading to depletion of mtDNA particularly in muscle and causing myopathic weakness. These mitochondrial toxicities exposed the reliance and vulnerability of the mitochondrial genome to disruptions of the deoxynucleotide pool substrates for mtDNA replication. These toxicities also serve as a reminder that the mtDNA replication machinery of mitochondria actually resembles that of viruses.

Mitochondrial toxicity is such a common side effect in humans; a thorough understanding and surveillance of these off-target effects are required for the successful development of new medicines. A vivid case in point is fialuridine or 1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil (FIAU), a nucleoside analogue that was tested for therapeutic efficacy for hepatitis B infection but tragically caused fatal liver failure and death in 5 of 15 patients and forced liver transplantations in two other patients.

Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants takes a rather systematic approach to mitochondrial pharmacology and toxicology and for this reason will be of use to even those outside of strict drug discovery. It begins with a scholarly introduction to the nuances of mitochondrial drug transport and detoxification systems, illustrated with specific case studies (Chapters 1–5). It then reviews cardinal features of mitochondrial toxicity at the organ level, highlighting some of the dose-limiting toxicities of very commonly used

and lifesaving drugs (Chapter 6–12). One of the greatest challenges in our field lies in measuring mitochondrial function. *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* dedicates many chapters (Chapters 13–29) to reviewing modern technologies for measuring mitochondrial function *in vitro*, *ex vivo*, and *in vivo*. Although these technologies represent the current state of the art, they have their limitations, and much research is required to pioneer new, facile biomarkers and technologies that are sensitive, specific, and minimally invasive. The text then progresses to reports from the clinic (Chapters 30–40) as well as from environmental biology (Chapters 41–45) that offer additional vignettes and examples of drug–mitochondria interactions.

The book *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* is very timely. While genetics and genomics have driven much progress in mitochondrial medicine for the past few decades, we anticipate that chemical biology may represent one of the most exciting new frontiers. We applaud Yvonne Will, James Dykens, and all of their contributors for assembling this new two volume book entitled: *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants*. This textbook will be an important canon in the future of mitochondrial medicine and more broadly in modern drug discovery.

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Part 1

Basic Concepts

1

Contributions of Plasma Protein Binding and Membrane Transporters to Drug-Induced Mitochondrial Toxicity

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CHAPTER MENU

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1.1 Drug Accumulation

Successful pharmaceutical treatment of disease requires molecules with chemical properties that allow for entry into the human circulation and delivery to the intended site for effective binding to the target molecule. When the small molecule arrives at its target, modulation of a disease process is achieved that results in alleviation of the disease symptoms. Many years of research are dedicated to optimization of the chemical properties to ensure a small molecule is effective. However, small molecules rarely affect a single target, and unwanted side effects can arise due to inappropriate tissue accumulation or the presence of a target in other tissues. More recently, it has become increasingly apparent that this is particularly the case for side effects due to accumulation and deleterious consequences on mitochondrial processes.

Small molecules that are used as treatments for many types of diseases have been observed as having effects on mitochondrial processes—often inhibiting crucial functions such as electron transport or increasing production of mitochondrial reactive oxygen species (ROS). These off-target effects are especially important when considering tissues with a high demand for mitochondrial function, such as the heart and brain, or those having roles in

general metabolism, such as the liver. The optimal properties of a drug often promote the undesired effects associated with mitochondrial toxicity. Small molecules are optimally lipophilic (displaying a high partition coefficient— $\log P$) in nature, which allows for transit through the circulation by binding to plasma proteins and passage through cellular membranes to gain access to intracellular targets. The molecular composition and architecture of mitochondria is also responsible for attracting certain small molecules that results in accumulation and impact on mitochondrial function. The mitochondrial membrane environment is unique because of the presence of specific phospholipids, such as the atypical cardiolipin with four acyl chains, as well as highly folded membrane structures particularly in the inner mitochondrial membrane (IMM) where specific geometry exists with certain structures such as the tips of the inner membrane cristae protrusions. These specific features will allow for certain molecules to accumulate due to affinity for cardiolipin and/or fitting into a specific geometry associated with inherent mitochondrial membrane arrangements. Mitochondrial function is also dependent on an electrochemical gradient across the IMM. This gradient is responsible for driving metabolite and ion transport across the IMM to power

processes such as ATP synthesis. Anabolic substrates, such as heme synthesis precursors, and other metabolites are transported across the IMM through specific transporters that are directly or indirectly driven by the mitochondrial membrane potential. However, certain lipophilic molecules are able to traverse the IMM by passive diffusion due to their chemical properties, and this is driven by the charge and pH differences across the IMM. The mitochondrial matrix is an overall negatively charged environment, with a -180 mV potential across the IMM, as well as being slightly alkaline compared with the intermembrane space and cytosol. This attracts positively charged molecules to the matrix and has been exploited by attaching positively charged moieties onto small molecules to target them to mitochondria, such as triphenylphosphine (TPP), a lipophilic and cationic moiety (Smith et al., 2011).

Impacts on mitochondrial function happen either directly or indirectly. Direct impacts involve small molecules binding to specific mitochondrial targets and impacting directly on a particular mitochondrial process, such as inhibition of mitochondrial DNA replication, modification of mitochondrial resident enzymes, and uncoupling of the electron transport chain. Indirect mechanisms involve small molecules modulating processes within the cell (not in the mitochondria) that eventually impact mitochondrial function. For example, potential indirect effects can be inhibition of fatty acid metabolism by carnitine sequestration in the cytoplasm, general oxidizing agents such as ROS and hydrogen peroxide, or inhibition of HMG-CoA reductase, an important enzyme in the biosynthesis of the mitochondrial electron carrier coenzyme Q. Therefore, a certain subset of drugs with mitochondrial toxicity act by accumulating in mitochondria, while other drugs only need to gain entry to the cytoplasm to exert their effects. Delivery into these different cellular compartments affects the extent of toxicity by controlling the concentration of drug.

1.2 Small Molecule Delivery to Tissues

Small molecules that enter into the bloodstream are transported through the circulatory system in one of two ways depending on their chemical properties. Plasma proteins in the circulation will have a certain affinity for small molecules depending on complementary interaction sites. Plasma proteins that interact readily with small molecules are albumins, lipoproteins, and alpha-1 acid glycoprotein (AGP-1)/orosomucoid (Pike et al., 1983). Drugs with more hydrophilic properties will be soluble in the aqueous environment of the blood and be transported around the circulation until delivered to

target tissues and/or metabolized before clearance from the body. Drugs that interact with a plasma protein will bind to these proteins and be transported to tissues, whereas plasma soluble drugs will be freely delivered to target tissues. Interactions between drugs and plasma proteins are mostly determined by the chemical nature of a drug including hydrophobicity and charge at plasma pH, measured by $\log P$ and pK_a , respectively. Binding to plasma protein provides a reservoir of the drug capable of providing a longer-lasting reservoir of the drug compared with those more freely soluble in the bloodstream and may eventually accumulate in target tissues to a greater extent than the latter more hydrophilic drugs (Figure 1.1).

Human serum albumin is the most abundant protein in human blood ($35\text{--}50\text{ g/L}$ $\sim 640\text{ }\mu\text{M}$) and is responsible for carrying lipid-soluble molecules such as hormones, steroids, and fatty acids in the circulation. Albumin tends to interact with acidic and neutral drugs at three specific sites that overlap with natural ligand binding sites (Sudlow et al., 1975; Ghuman et al., 2005). Due to the high concentration of albumin in the blood, this is the primary plasma transporter of acidic and neutral drugs systemically. Molecules transported by albumin are released in regions of low drug concentration. This mechanism enables drugs to be delivered to target tissues and enter into cells either by transport through specific plasma membrane transporters or via diffusion through the plasma membrane (see Section 1.3). Albumin bound drugs capable of inducing mitochondrial toxicity are listed in Table 1.1.

Lipoprotein particles are also responsible for carrying hydrophobic drugs in the circulation, especially when albumin has been saturated. Acidic and neutral drugs can be bound by lipoprotein particles while being transported throughout the circulation. Lipoprotein particles are typically responsible for the transport of hydrophobic molecules, such as triglycerides and cholesterol, in the circulation to target tissues. The lipoprotein particle structure is made up of a polar surface composed of phospholipid, cholesterol, and apolipoprotein on the exterior allowing for solubility in the aqueous bloodstream and a hydrophobic core composed of triglycerides and cholesteryl esters sequestered away from the aqueous environment for delivery to target tissues. The hydrophobic core of lipoprotein particles is therefore the appropriate environment for hydrophobic drugs to be sequestered and transported to various tissues. Several varieties of lipoprotein particles exist that vary in the composition of protein and lipid. High-density lipoprotein (HDL) particles have the highest protein-to-lipid ratio, while very low-density lipoprotein (VLDL) particles contain the lowest ratio. Both intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL) particles have

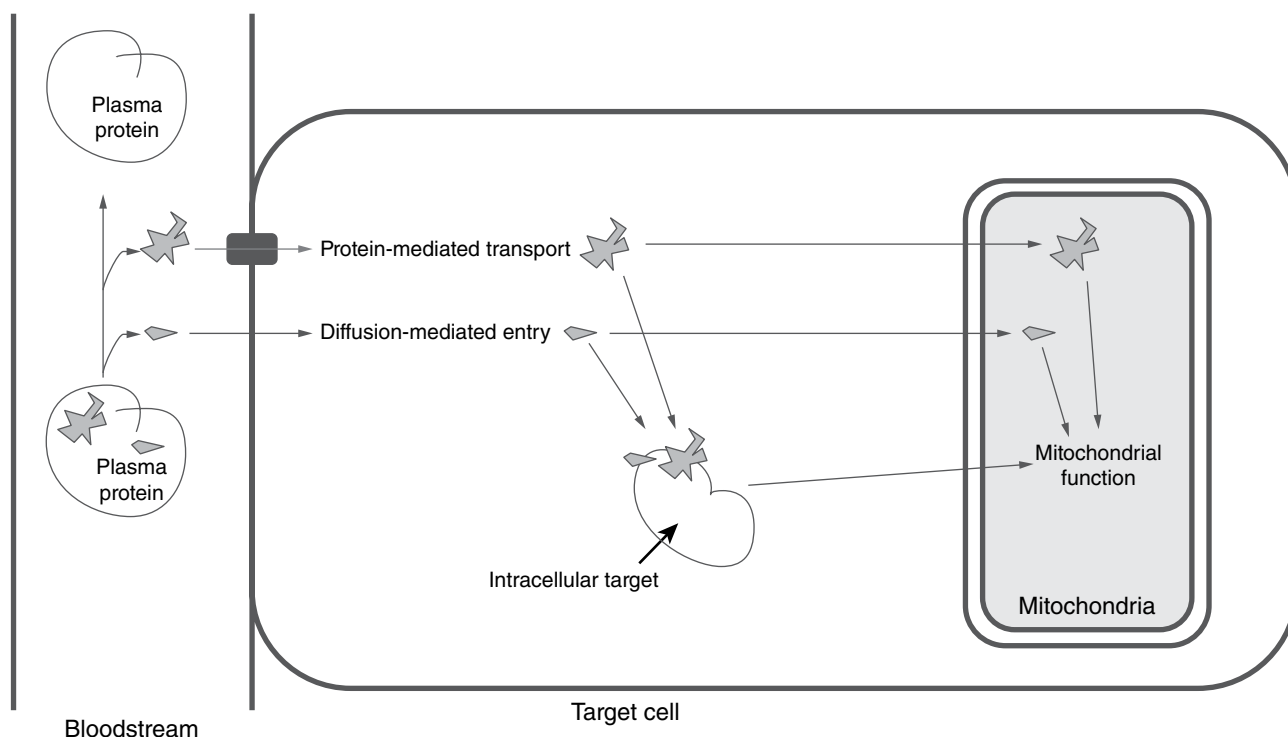


Figure 1.1 General mechanisms of drug delivery and impacts on mitochondrial functions in target cells. Drugs are carried by plasma proteins in the bloodstream. Drugs then enter into target cells using specific plasma membrane transporters or via passive diffusion. Drugs with targets in the cytoplasm bind to these target molecules, which then impact mitochondrial function. Drugs with target within mitochondria then enter into mitochondria to bind to the target and impact mitochondrial function.

Table 1.1 Drugs that bind to serum albumin.

Drug	Plasma protein binding	Therapy	pK _a	log P	References
Acetylsalicylic acid	50–80%	Analgesic/anti-inflammatory	3.49	1.19	Sułkowska et al. (2006)
Amitriptyline	96%	Antidepressant	9.4	4.92	Brinkschulte and Breyer-Pfaff (1980)
Chlorpromazine	>90%	Antipsychotic	9.3	5.41	Kitamura et al. (2006), Rukhadze et al. (2001), and Silva et al. (2004) all bovine serum albumin
Diazepam	>90%	Antianxiety	3.4	2.82	Brodersen and Honoré (1989)
Diclofenac		Analgesic/anti-inflammatory	4.15	4.51	Yamasaki et al. (2000)
Fluoxetine	94.5%	Antidepressant	9.8 (predicted)	4.05	
Flurbiprofen	99%	Analgesic/anti-inflammatory	4.42 (predicted)	4.16	Aarons et al. (1985) and Takla et al. (1985)
Ibuprofen		Analgesic/anti-inflammatory	4.91	3.97	Yamasaki et al. (2000) and Galantini et al. (2010)
Indomethacin		Analgesic/anti-inflammatory	4.5	4.27	
Naproxen		Analgesic/anti-inflammatory	4.15	3.18	Banerjee et al. (2006) bovine serum albumin
Piroxicam	99%	Analgesic/anti-inflammatory	6.3	3.06	
Tetracycline		Antibiotic	3.3	–1.30	Bratlid and Bergan (1976)
Zidovudine	30–38%	Antiretroviral		0.05	Quevedo et al. (2001)

ratios between these two extremes. Lipoprotein particles are taken up by LDL receptors present on every nucleated cell type, but especially by liver cells that take up the vast majority of LDL particles. LDL-bound receptors undergo endocytosis, and eventually the LDL particle is trafficked to the lysosome by endosome fusion. In the lysosome the LDL particle is broken down into constituents, releasing free cholesterol, amino acids, and fatty acids for use in the cell. During the process of LDL particle internalization and transit to the lysosome, the drug may be able to exit into the cytoplasm via passive diffusion or facilitated transport through the endosomal or lysosomal membranes to gain access to the cellular target. There are reports of physical contacts between lysosomes and mitochondria-derived vesicles (MDVs) where mitochondrial components are directed to lysosomes presumably for degradation. A reverse pathway has not been described, but could potentially exist, which could supply drugs directly from lysosomes to mitochondria. One drug that binds to lipoprotein particles is amitriptyline (log *P*, 4.92; *pK_a*, 9.4), indicating a preference for hydrophobic and basic molecules (Brinkschulte and Breyer-Pfaff, 1980).

AGP-1 is a plasma protein present at about 1–3% of total plasma protein and is responsible for transporting basic and neutral lipophilic molecules in the bloodstream. This allows AGP-1 to associate with basic and neutral lipophilic drugs that would not normally associate with albumin or lipoproteins. However, the serum concentration of AGP-1 is much lower than albumin so that it becomes saturated at lower drug concentrations. AGP-1 has a beta-barrel cavity that is both hydrophobic and acidic, allowing for a variety of substrates to bind, and this cavity is sealed with sugar side chains covalently attached to the polypeptide backbone. Mitochondrial toxins that bind to AGP-1 are listed in Table 1.2.

In some cases, drugs are able to associate with more than one plasma protein. The antidepressants amitriptyline and fluoxetine associate with serum albumin and AGP-1. This would allow for an increased plasma

accumulation of these drugs, thereby increasing exposure (Brinkschulte and Breyer-Pfaff, 1980).

There are some other plasma proteins that are less abundant that are also able to interact with drugs. For example, transthyretin—the thyroxine and retinol-transporting protein—interacts with diclofenac to stabilize the active form of the protein (Almeida et al., 2004; Miller et al., 2004). Other plasma proteins are likely to associate with drugs, but the vast majority of plasma protein binding is through albumin, LDL particles, and AGP-1. Alterations in protein levels of either of these three can lead to alterations in drug transport in the circulation. Diseases associated with altered levels of albumin will alter saturation of drug binding sites. Hypoalbuminemia is caused by liver and kidney malfunctions, while hyperalbuminemia can be caused by dehydration. Decreased levels of albumin will result in a higher concentration of free drug that can lead to increased exposure to the drug and potentially increased mitochondrial toxicity. Elevated albumin levels would allow for more drugs to be sequestered from the circulation; however this could lead to increased persistence of the drug in the circulation and result in a delay in toxic effects. AGP-1 plasma concentrations can be dramatically altered after infection, physiological changes such as pregnancy, and physical traumas such as burns. Also, lipoprotein particle concentrations vary in the population due to disease and diet, and so drug association with these particles will vary depending on the subject. Therefore, it can be seen that determination of the amounts of plasma proteins is an important consideration when administering any drug, especially those that are associated with toxicity through impacts on mitochondria.

A second consideration is when more than one drug is present in the circulation. As many drugs bind to similar regions on the various plasma proteins, the drug with the higher affinity for the plasma protein will displace that with lower affinity. This displacement will cause an effective increase in the plasma concentration of the lower affinity drug and increase the likelihood of toxicity.

Table 1.2 Drugs that bind to alpha-1 glycoprotein.

Drug	Plasma protein binding	Therapy	<i>pK_a</i>	log <i>P</i>	References
Amitriptyline	96%	Antidepressant	9.4	4.92	Brinkschulte and Breyer-Pfaff (1980) and Ferry et al. (1986)
Amoxapine	90%	Antipsychotic	8.83 (predicted)	3.4	Ferry et al. (1986)
Fluoxetine	94.5%	Antidepressant	9.8 (predicted)	4.05	
Propranolol	90%	Anti-arrhythmia	9.42	3.48	Ferry et al. (1986)

1.3 Entry into Cells

Passage of small molecules from the circulation to the interior of a cell relies on two pathways. The chemical properties of the small molecules determine whether they will enter through plasma membrane transporters or pass freely without the aid of transporters via diffusion. Molecules with lipophilic properties are able to pass freely without membrane transporters and gain access to their sites of action. However, some molecules enter cells through plasma membrane transporters due to their physicochemical properties or similarity to membrane transporter substrates. The cellular repertoire of plasma membrane transporters will therefore govern which small molecules will accumulate within a specific cell type or tissue and so yield organ-specific toxicity.

Several plasma membrane transporter families are involved in the entry of drugs into the cells. These transporters rely on plasma membrane solute gradients or binding and hydrolysis of ATP to power the entry of the molecule. The solute carrier family (SLC) of proteins is the main family of proteins responsible for transporting drugs into the cells. Substrates of these proteins are transported through facilitated transport or secondary active transport through coupled transport of a substrate going down the concentration gradient. Proteins not part of the SLC family of plasma membrane transporters are also capable of drug transport. These include members of the monocarboxylate transporter (MCT) family and Ral-binding proteins. The MCT family of proteins is responsible for the transport of organic ions across the plasma membrane. Ral-binding proteins associate with and regulate the activated GTP-bound form of the G protein Ral that is involved in signal transduction pathways regulating gene expression, cell migration, cell proliferation, and membrane trafficking (Mott and Owen, 2014).

The SLC family of transporters transports a wide array of substrates ranging from anions to cations and from small inorganic ions to amino acid-derived hormones and metabolic carriers, such as carnitine. Transport of these substrates can also be dependent on counterions such as sodium and chloride. These transporters also display varying tissue distributions providing a mechanism for tissue-specific accumulation of drugs, leading to tissue-specific toxicity that is seen with drug-induced mitochondrial toxicity. The specific types of transporters that allow for drug entry into cells include uptake receptors of neurotransmitters such as serotonin, noradrenaline, and dopamine in the synapse, carnitine transporters in fatty acid-metabolizing tissues, and copper and steroid hormone and steroid conjugate transporters in tissues with these requirements.

The majority of transporters of drugs into cells are members of the SLC22 family. This includes transporters of organic anions and cations. SLC22A6 (organic anion transporter 1 (OAT-1)) and SLC22A7 (organic anion transporter 2 (OAT-2)) are the main transporters of endogenous organic anions such as dicarboxylic acids, prostaglandins, and cyclic nucleotides. These transporters are highly expressed in the basolateral membrane of epithelial kidney proximal tubule cells (Lopez-Nieto et al., 1997). These transporters function as antiporters with endogenous dicarboxylic acids cotransported. Increased activity of these transporters can deplete cellular dicarboxylic acids, such as α -ketoglutarate, which are important components of the citric acid cycle. Organic anion transporters (OATs) are responsible for the transport of several nonsteroidal anti-inflammatory drugs and antiretroviral drugs such as zidovudine (Takeda et al., 2002). As the kidney is one of the main routes of drug excretion, drugs can accumulate in the kidneys through these transporters, resulting in kidney-specific mitochondrial dysfunction. Interestingly, as dicarboxylic acids are removed from kidney epithelial cells upon drug entry through OATs, this may act as a double hit to these cell types as critical substrates for the citric acid cycle are depleted along with the toxic effects of drugs on mitochondria such as ROS generation, mitochondrial membrane depolarization, and mitochondrial DNA replication inhibition. A related organic anion transporter, SLC02B1 (OATP-2), is also capable of transporting ibuprofen into the cells (Satoh et al., 2005). SLC02B1 is highly expressed in the liver and functions as a transporter for steroids and steroid conjugates.

The organic cation transporters SLC22A5 (OCTN-2) and SLC22A16 are sodium-dependent carnitine transporters that are widely expressed to deliver carnitine to fatty acid-metabolizing tissue. Carnitine is a quaternary ammonium molecule with an overall positive charge, and molecules with similar chemical properties can be substrates of these transporters. Doxorubicin is reported to be a substrate of SLC22A16 (Okabe et al., 2005). SLC22A2 (organic cation transporter 2 (OCT-2)) is expressed in the basolateral membrane of kidney proximal tubules and transports cations from the blood into the kidney epithelium for excretion. The chemotherapeutic agent cisplatin is transported via OCT-2 into kidneys where accumulation can lead to nephrotoxicity (Burger et al., 2010).

The neurotransmitter reuptake transporters SLC6A2, SLC6A3, and SLC6A4 are specific for noradrenaline, dopamine, and serotonin, respectively. These transporters are all symporters by transporting sodium simultaneously with the neurotransmitter. These neurotransmitters are part of the monoamine family of molecules and all contain modified aromatic rings, so that drugs with

related structures will be substrates for these transporters. Inhibitors of the reuptake transporters are commonly used as antidepressants. These drugs function by preventing the neurotransmitters from binding to the receptor, thereby extending their synaptic dwell times. For example, citalopram is a potent inhibitor of the serotonin reuptake transporter, resulting in increased neuronal accumulation (Bareggi et al., 2007).

The high affinity copper transporters SLC31A1 and SLC31A2 (copper transporter 1 and 2, respectively (CTR-1 and CTR-2)) are responsible for the uptake of drugs like *cis*-platinum, carboplatin, and oxaliplatin (Song et al., 2004). As copper is an essential cofactor for many enzymes, copper transporters are ubiquitously expressed and will render all cell types susceptible to platinum-based drug accumulation and toxicity.

The MCT family of plasma membrane proton- and ion-linked transporters is part of the SLC family and has an important role in the transport of small charged molecules across the plasma membrane. This family of transporters is involved in the distribution of drugs to various tissues and organs such as the brain (Vijay and Morris, 2014). The anticonvulsant valproate (Depakote) is likely a substrate for MCT-1 (Fischer et al., 2008). This transporter is ubiquitously expressed and has substrate specificity for short-chain aliphatic monocarboxylic acids, such as pyruvate and acetoacetate, and short-chain fatty acids (up to 6 carbon atoms), which are structurally related to valproate (Halestrap and Meredith, 2004). Valproate is a putative carnitine and intramitochondrial sequestering agent, and therefore accumulation of the drug will have impact on cells and tissues that have high metabolic dependency on carnitine for oxidation of fatty acids in mitochondria. Due to these properties, valproate results in severe hepatotoxicity as the liver expresses MCT-1 and is also a site of both carnitine biosynthesis and coenzyme A-dependent metabolism (Coulter, 1991; Fromenty and Pessayre, 1995). The carnitine-binding activity of valproate would be relevant in the cytoplasm and so would not need to gain access to mitochondria to yield toxic side effects. However, the coenzyme A-dependent effects would require access to the mitochondrial matrix. How valproate enters the mitochondrial matrix is not well described; however, as it has structural similarity to short-chain fatty acids, it may be able enter simply by diffusion.

Lovastatin, a small molecule that inhibits cholesterol synthesis, is a substrate for MCT-4 in cultured mesangial cells from rats (Nagasawa et al., 2002). It is associated with kidney failure as well as myopathy. It also displays both direct and indirect effects on mitochondrial physiology by inhibiting and uncoupling OXPHOS and via decreasing coenzyme Q levels. Skeletal muscle expresses MCT-4 (Pilegaard et al., 1999), and this would be in

accord with lovastatin accumulation in the muscle by entry via MCT-4, so affecting coenzyme Q levels (Folkers et al., 1990). It should be noted in this context that the abundance of this transporter is higher in slow twitch muscle fibers, likely resulting in their greater susceptibility to statin-induced rhabdomyolysis. Statins are also responsible for lowering plasma LDL, which are used to transport coenzyme Q in the circulation. Therefore, the effects of statins on coenzyme Q levels could also be due to systemic changes in general lipid transport in the circulation (Littarru and Langsjoen, 2007).

The analgesics ibuprofen, aspirin, and diclofenac are also substrates for MCTs, as they are low molecular weight monocarboxylic acids (Tamai et al., 1995; Choi et al., 2005). They are transported by MCT-1, a ubiquitously expressed member of this family. These molecules are associated with a variety of toxicities due to impacts on mitochondrial function including hepatotoxicity.

An unusual transporter of certain drugs into cells is RALBP1 (RalA-binding protein 1). This protein functions mostly during receptor-mediated endocytosis by acting as an effector molecule for the small GTPase RalA. RALBP1 has been identified as a transporter of lipid peroxidation-derived glutathione conjugates that mediates transport of the chemotherapeutic agent doxorubicin (Awasthi et al., 2000). There are also suggestions that the ATP-binding cassette (ABC) transporter, ABCG2, that is conventionally associated with drug efflux can transport doxorubicin into the cells (Kawabata et al., 2003; Singhal et al., 2007).

Several drugs are transported by transporters from different families, such as valproate, ibuprofen, and acetylsalicylic acid (Table 1.3). This allows a greater range of tissue exposure of these drugs and therefore increases the likelihood of any toxicity.

1.4 Transport Out of Cells

Cells have a variety of mechanisms that remove drugs and endogenous molecules that may accumulate to yield toxicity. These efflux pumps are usually coupled to ATP hydrolysis and are therefore active transporters. The main transporter types that remove drugs from cells are the ABCB- and ABCC-type ABC transporters, plus the ATPase coupled transporters (ATP7 family), as well as some of the SLC family.

The largest family of membrane transporters—the ATP ABC transporters—transports the majority of drugs out of cells. These transporters have a cytosolic ATPase domain that is required for efflux out of the cell. This transporter family recognizes a wide variety of substrates to rid cells of toxic levels of molecules (Chen et al., 2016). ABCB1 is the most common

Table 1.3 Transporters of mitochondrial toxic drugs.

Drug class	Drug name	Transporter family					References
		ABC	ATP	MCT	SLC	Other transporter	
Anticonvulsants	Valproate			MCT-1	SLC22A6 SLC22A7		Ohashi et al. (1999) and Kobayashi et al. (2002)
Antidepressants	Amitriptyline	ABCB1					Faassen et al. (2003) and Mahar Doan et al. (2002)
	Fluoxetine	ABCB1					Weiss et al. (2003)
	Citalopram	ABCB1			SLC6A2 SLC6A3 SLC6A4		Bareggi et al. (2007)
Antipsychotics	Chlorpromazine	ABCB1					Polli et al. (2001) and Boulton et al. (2002)
	Haloperidol	ABCB1					Mahar Doan et al. (2002), Boulton et al. (2002), and Nagy et al. (2004)
	Risperidone	ABCB1					Faassen et al. (2003) and Boulton et al. (2002)
Barbiturates	Phenobarbital	ABCB1					Schuetz et al. (1996) and Luna-Tortós et al. (2008)
Antianxiety	Diazepam	ABCB1					Yamazaki et al. (2001) and Adachi et al. (2001)
Cholesterol	Statins—lovastatin	ABCC2		MCT-4			Nagasawa et al. (2002)
Analgesic/ anti-inflammatory	Ibuprofen	ABCB1		MCT-1	SLCO2B1 SLC22A6 SLC22A8		Khamdang et al. (2002), Choi et al. (2005) and Satoh et al. (2005)
	Acetylsalicylic acid	ABCB1		MCT-1	SLC22A7		Choi et al. (2005)
	Acetaminophen	ABCB1			SLC22A6		Faassen et al. (2003) and Wang et al. (2001)
	Diclofenac			MCT-1			Choi et al. (2005)
	Indomethacin	ABCB1			SLC22A6 SLC22A8		Khamdang et al. (2002)
Antibiotics	Minocycline					Active transport Sodium dependent	Brayton et al. (2002)
Antiretroviral	Zidovudine	ABCB1 ABCC4 ABCC5 ABCG2			SLC22A6		Anderson et al. (2006), Abia et al. (2008), Jorajuria et al. (2004), Pan et al. (2007), and Takeda et al. (2002)
Anticancer	Doxorubicin	ABCB1 ABCB8 (mitochondrial) ABCB11 ABCC1 ABCC2 ABCC6 ABCG2			SLC22A16	RALBP1	Singhal et al. (2007), Kawabata et al. (2003), and Okabe et al. (2005)
	Cis-platinum	ABCB1 ABCC6 ABCG2	ATP7A ATP7B		SLC22A2 SLC31A1 SLC31A2		Song et al. (2004), Burger et al. (2010), Howell et al. (2010), and Holzer et al. (2004)
	Tamoxifen	ABCB1 ABCC2 ABCG2					Janvilisri et al. (2003)

multidrug resistance protein (MDR-1) and has a wide array of substrates with almost every class of drug being exported by this protein. There are also transporters related to ABCB1 capable of drug efflux including ABCB11, ABCC1, ABCC4, ABCC5, and ABCC6 that transport molecules such as bile salts, organic anions, and cyclic nucleotides, among others. Some of these transporters have wide tissue expression, while some have cell-type-specific expression such as ABCB11 that is expressed only in the liver and little elsewhere (Vasiliou et al., 2009).

1.5 Entry into Mitochondria

Several mitochondrial toxins are weak acids that, when protonated, are lipid soluble. These molecules act as protonophores and result in dissipation of the mitochondrial membrane potential, thereby uncoupling electron transport from ATP synthesis. These uncoupling agents cause an increased rate of respiration, effectively causing both heat dissipation and substantial increase in oxygen radicals that can overwhelm the oxidative stress resistance capacity. If this is the case, ROS are able to damage macromolecules in the mitochondria such as mitochondrial DNA, phospholipids, and proteins. A general stress of this nature results in overall mitochondrial dysfunction as ATP synthesis and the mitochondrial membrane potential are not maintained with an overall outcome of cellular dysfunction and tissue failure. Examples of mitochondrial toxins in this class are several small molecules

used as analgesics and anti-inflammatory molecules such as acetylsalicylic acid (aspirin), mefenamic acid, nabumetone, naproxen, diclofenac, and dipyrene in the rat intestine (Somasundaram et al., 1997) and kidney (Mingatto et al., 1996). Evidence for the direct action of these small molecules as uncoupling agents is widely available when using isolated mitochondria as well as in isolated cells and when administered to rodents (Mingatto et al., 1996; Somasundaram et al., 1997). In the cytoplasm these molecules exist in their ionized anion state; in the intermembrane space the concentration gradient of protons across the IMM creates an environment with a lower pH than that in the matrix. The ionized form becomes protonated due to the high concentration of protons, rendering the small molecule unionized and hence much more lipophilic. In this state the small molecule can pass through the IMM via diffusion. In the matrix the small molecule deprotonates as protons are used by the electron transport system to attempt to restore the membrane potential. The rapid translocation of the protons by the small molecule dissipates the mitochondrial membrane potential and prevents proton translocation through ATP synthase, thereby diminishing ATP generation (Figure 1.2).

Positively charged amphipathic molecules are able to enter the mitochondrial matrix once they arrive at the mitochondrial intermembrane space. The positively charged molecule then accumulates in the mitochondrial matrix due to the difference in charge across the membrane (Figure 1.2). This phenomenon is aided by the lipophilic nature of the molecule, allowing it to pass

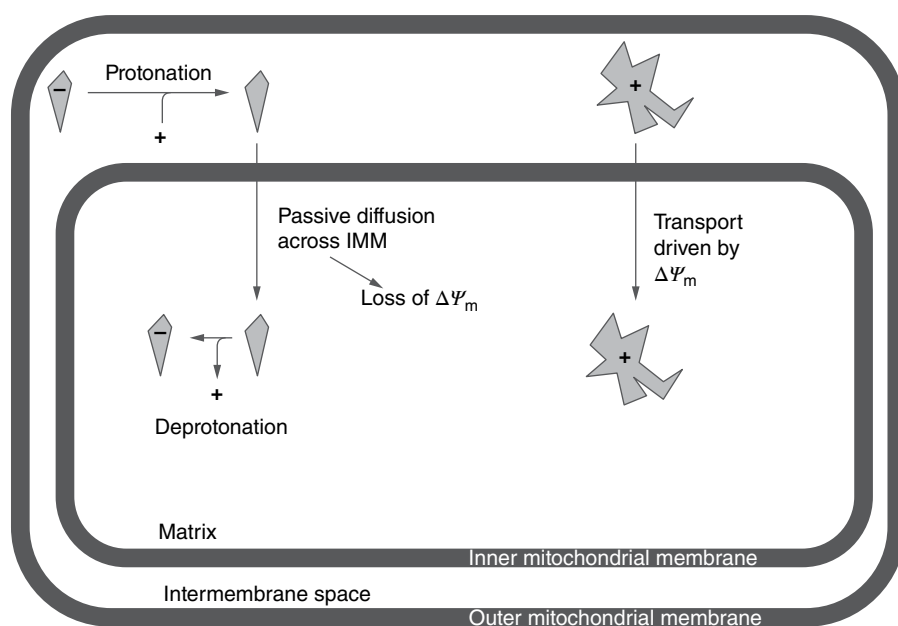


Figure 1.2 Entry of drugs into the mitochondria occurs by two mechanisms. Negatively charged amphipathic molecules are protonated in the intermembrane space. The drug now has no charge and is maximally able to pass through the IMM via diffusion. In the matrix the drug becomes deprotonated. This process results in the dissipation of the mitochondrial membrane potential and can result in mitochondrial dysfunction.

through the IMM without the aid of a protein channel of transporter. Small molecules such as the antiarrhythmic drug amiodarone, the anticancer drug tamoxifen, the antianginal agent perhexiline, and the opioid buprenorphine accumulate in liver mitochondria through this mechanism (Fromenty et al., 1990; Larosche et al., 2007; Begriche et al., 2011).

Short- to medium-chain fatty acids up to 12 carbons in length are able to cross the IMM freely to enter into the matrix for β -oxidation without the aid of protein transporters. Small molecules that resemble these types of fatty acids are able to pass through the IMM because of the shared properties. Examples of drugs using this mechanism are valproate (see above).

1.6 Export from Mitochondria

The ABC transporter ABCB8 is localized to the IMM (Hogue et al., 1999) and is a putative exporter of iron–sulfur clusters from the mitochondrial matrix. ABCB8 acts as an exporter of doxorubicin from mitochondria and provides protection for the mitochondrial genome from intercalation and inhibition of DNA replication (Elliott and Al-Hajj, 2009). ABCB8 is ubiquitously expressed, and although its exact function is not known, it has been shown to protect cardiomyocytes against oxidative stress (Ardehali et al., 2005).

1.7 Concluding Remarks

As mitochondria are the primary source of ATP in most tissues and organs, any impact on mitochondrial function caused by a drug can be severely detrimental. Drugs can be transported systemically through the circulation while liganded to a variety of plasma proteins and so delivered to almost every tissue. Plasma protein binding prolongs the persistence of a drug in the circulation, potentially increasing toxicity. Drugs toxic to mitochondria can enter the cells through diffusion through the plasma membrane or via transport facilitated by transporters and channels. Many members of membrane transport protein families, such as SLC, MCT, and ATP, are able to transport drugs toxic to mitochondria both into and out of cells. Once in the cytoplasm, drugs toxic to mitochondria can elicit effects by interacting with cytoplasmic or non-mitochondrial targets. Some drugs toxic to mitochondria enter into mitochondria to elicit their effects, which can occur via passive or facilitated transport across the two mitochondrial membranes. Cells have mechanisms to remove toxic molecules, and these are able to reduce the intracellular concentration of a drug to minimize toxic effects. Therefore, considerations of the pharmacokinetic properties of drugs at more complex and tissue-specific levels are increasingly illuminating efficacy as well as toxicity, so offering potential avenues to increase the former while decreasing the latter.

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2

The Role of Transporters in Drug Accumulation and Mitochondrial Toxicity

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CHAPTER MENU

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2.1 Introduction to Chapter

In this chapter, an introduction to transporters that play a role in the accumulation of solutes in tissues, cells, and subcellular compartments is presented. The focus of the chapter is on transporters in the solute carrier (SLC) superfamily. Following a brief introduction on the SLC superfamily and an overview of how transporters can mediate toxicity as well as detoxification, the chapter focuses on transporters in the intestine, liver, and kidney. These tissues are sites of toxicity because of their role in absorption and elimination of xenobiotics including chemical carcinogens and many environmental toxins. Transporters in these tissues work with enzymes as the body's primary detoxification mechanism. For example, toxins absorbed in the intestine through various mechanisms may enter the liver through hepatic transporters and be subsequently metabolized via various enzymes. Hydrophilic metabolites of many toxic substances may be eliminated in the kidney by secretory transporters that mediate transepithelial flux into the tubule lumen and urine. Following an overview of transporters in these major absorption and elimination organs, a discussion of mitochondrial transporters is presented. These highly

conserved transporters in the SLC25 family move their substrates in and out of mitochondria and play vital roles in mitochondrial toxicity of many substances. Finally, a short conclusion is presented, which highlights areas that need further study.

2.2 The Solute Carrier (SLC) Superfamily

Transporters on cell membranes play critical roles in the disposition of endogenous molecules, macro- and micro-nutrients from dietary sources, and xenobiotics including environmental toxins, chemical carcinogens, and prescription drugs (Giacomini et al., 2010). Of the two major superfamilies of transporters that have received wide attention in the disposition of xenobiotics, the ATP-binding cassette superfamily (ABC superfamily) and SLC superfamily (Lin et al., 2015; Nigam, 2014), this chapter will focus on the latter, which includes mitochondrial transporters as well as transporters on the plasma membrane and on membranes of subcellular organelles. Human ABC transporters, which are efflux pumps, driven by ATP hydrolysis are reviewed elsewhere

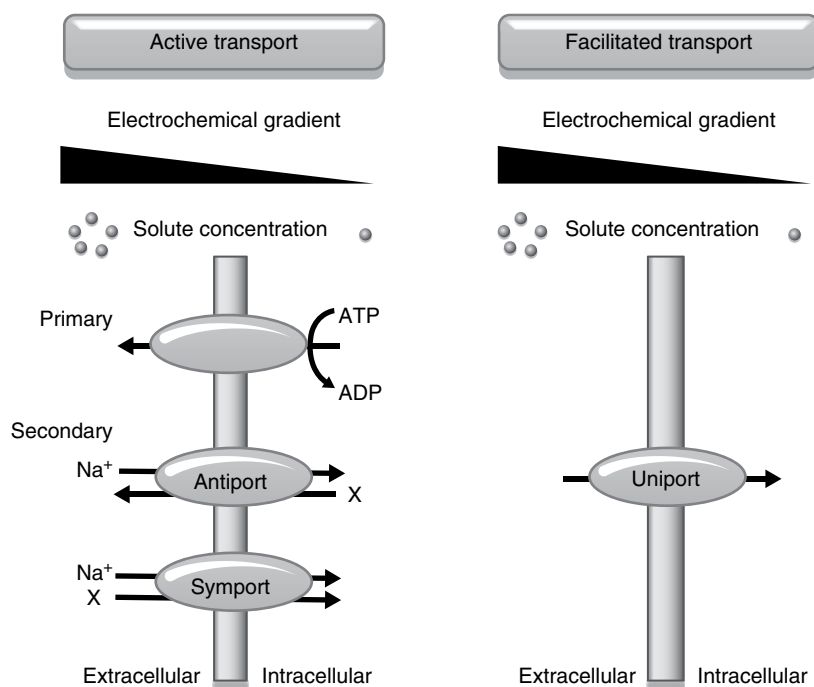
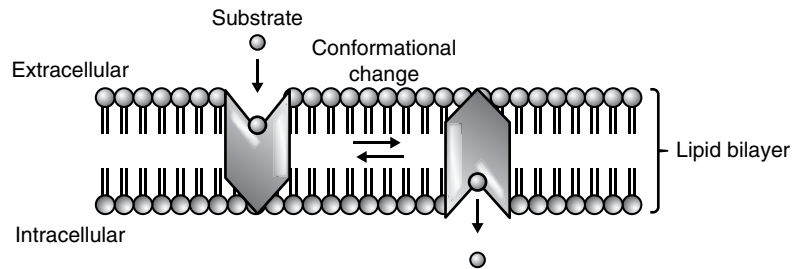


Figure 2.1 Active transport and facilitated transport. Active transport is either primary or secondary. In primary active transport, ATP is hydrolyzed to provide the free energy needed for transport against electrochemical gradient. Secondary active transport uses the energy stored in the concentration gradient of a driven ion (i.e., Na^+) and then couples the movement of another molecule or ion with that gradient. When the driving ion and driven ions move in opposite directions, the process is termed an antiport mechanism. When the driving and driven ions move in the same direction, the process is termed a symport mechanism. Facilitated transport is a process in which molecules or ions are transported across the plasma membrane with the help of membrane proteins. Transporters in the SLC superfamily are facilitated or secondary active transporters. ADP, adenosine diphosphate; ATP, adenosine triphosphate; X, transporter substrates.

(Rees et al., 2009; Szakács et al., 2006). The SLC superfamily currently consists of 52 families organized by sequence homology and approximately 395 transporters. Transporters in the SLC superfamily are facilitated or secondary active transporters (Figure 2.1). Facilitated transporters move their substrates across cellular membranes in accordance with the electrochemical gradient. In contrast, secondary active transporters may move their substrates across the plasma membrane against the concentration gradient. They rely on ion gradients across cellular membranes, which have been created by primary active proteins that directly use ATP. For example, Na^+/K^+ ATPase, which uses ATP to actively pump Na^+ and K^+ , creates an Na^+ gradient across the plasma membrane, which is harnessed by many Na^+ -dependent secondary active transporters to actively move their substrates from extracellular to intracellular spaces (Morth et al., 2011). For example, the mitochondrial toxin *N*-methyl-4-phenylpyridinium (MPP^+) is transported across the plasma membrane of dopaminergic neurons by the Na^+ -dependent dopamine transporter (DAT) SLC6A3 and accumulates against its concentration gradient in dopaminergic neurons, ultimately causing cell death (Storch et al., 2004).

Membrane transporters in the SLC superfamily are generally multi-pass membrane proteins. Secondary structures of the transporters, based on hydropathy analyses, have been predicted and in many cases validated (César-Razquin et al., 2015; Schlessinger et al., 2013). However, to understand the mechanisms by which the transporters actually translocate their substrates across plasma membranes, crystal structures are needed, in particular structures of the transporters in multiple conformations. Unfortunately, as with all membrane proteins, crystal structures of SLC transporters have been difficult to obtain. Thus, the molecular mechanisms by which SLC transporters mediate transmembrane flux of their substrates are poorly understood. Currently, a limited number of prokaryotic and eukaryotic transporters have been crystallized to high resolutions to enable some understanding of their molecular mechanisms. Two major protein folds or structural classes have become apparent. One is the major facilitator family (SLC2, SLC22) (Koepsell, 2013; Mueckler and Thorens, 2013), and the other is the neurotransmitter: sodium symporter (LeuT-like fold) (Perez and Ziegler, 2013). A so-called rocker-switch model appears to characterize solute movement by proteins in the major facilitator family

Figure 2.2 Rocker-switch model. The rocker-switch mechanism involves protein rearrangements around a substrate binding site that results in the substrate being alternately exposed to either side of the membrane.



(Quistgaard et al., 2016) (Figure 2.2). In this model, the transporter alternates its open face between intracellular and extracellular spaces, allowing substrates to bind and be released. Transporters in the SLC2 family (or glucose transporter (GLUT) family) appear to work in this way, and data suggest that transporters in the SLC22 family are distantly related to SLC2 and share the major facilitator fold. With time, more structures will become available and lead to an increased understanding of the atomic structure of transporters and their molecular mechanisms. These structures will provide information for molecular docking, which can help predict the interaction of mitochondrial toxins and various xenobiotics with transporters.

2.3 Transporters as Determinants of Drug Levels in Tissues and Subcellular Compartments

The tissue distribution of each transporter in the SLC superfamily is a key determinant of its biological, pharmacological, and toxicological roles. For example, the tissue distribution of the organic cation transporters (OCTs) (OCT1, OCT2, and OCT3) in the SLC22 family,

the organic ion transporter family, determines the physiologic and pharmacologic roles of the three paralogs (Table 2.1). OCTs have strongly overlapping substrate specificities, mediating the transmembrane flux of low molecular weight hydrophilic organic cations such as the highly prescribed antidiabetic drug metformin; the neurotransmitters serotonin and norepinephrine; and the mitochondrial toxin MPP⁺. Though substrate selectivity overlaps among the three transporters, they have unique roles because of their tissue distribution. In particular, OCT1 (SLC22A1) is highly expressed in the human liver, with much lower levels in other tissues, and as such plays a major role in the delivery of its substrates to the human liver, which may result in biological action, toxicity, or metabolism and biliary elimination in the liver. However, OCT2 (SLC22A2) is highly expressed in the kidney, where it plays a role in the nephrotoxicity of platinum drugs and in the renal secretion of its substrates including thiamine (vitamin B1) and many cationic drugs such as metformin. Finally, OCT3 (SLC22A3) is ubiquitously expressed at lower levels in multiple tissues and as such plays a role in tissue distribution and pharmacologic/biological activity of its substrates in many tissues including the intestine, liver, kidney, salivary glands, placenta, and blood–brain barrier. Though much attention has been paid to plasma membrane

Table 2.1 Selected transporters in the SLC22 family that play roles in the disposition of xenobiotics.

Transporter	Gene	Major tissue distribution	Selected substrates	Selected inhibitors
OCT1	SLC22A1	Liver hepatocyte (sinusoidal)	Metformin, <i>N</i> -methyl-4-phenylpyridinium (MPP ⁺), tetraethylammonium (TEA)	Quinine, quinidine, disopyramide, verapamil
OCT2	SLC22A2	Kidney (proximal tubule)	Metformin, MPP ⁺ , TEA	Cimetidine, quinidine, quinine, rifampicin, testosterone
OCT3	SLC22A3	Ubiquitous (intestine, liver, kidney)	Metformin, MPP ⁺ , TEA, Ketamine	Cimetidine, quinidine, rifampicin, progesterone
OAT1	SLC22A6	Kidney (proximal tubule), placenta	Para-aminohippurate, adefovir, cidofovir, methotrexate	Probenecid, novobiocin
OAT2	SLC22A7	Liver (sinusoidal)	2-Deoxyguanosine, tetracycline, bumetanide	Indomethacin, cyclic GMP
OAT3	SLC22A8	Kidney (proximal tubule), placenta	Nonsteroidal anti-inflammatory drugs (NSAID), furosemide, estrone-3-sulfate	Probenecid, novobiocin

transporters, transporters on subcellular organelles have also been characterized. For example, the vesicular monoamine transporters (VMATs) in the SLC18 family have been well characterized (Lawal and Krantz, 2013). These transporters, which take up neurotransmitters such as serotonin, norepinephrine, and dopamine, cycle from intracellular vesicles to the plasma membrane where they release neurotransmitters into the synaptic cleft for action at their receptors. In addition to interacting with endogenous neurotransmitters, the transporters also take up other compounds such as MPP⁺. In fact, through transport and storage in intracellular vesicles (and away from mitochondria), VMATs protect cells from the potent mitochondrial toxicity of MPP⁺.

2.4 Drug Transporters in the Intestine

For an orally administered drug to reach a tissue, it must be taken into the bloodstream via intestinal absorption. Several factors such as compound solubility, chemical stability, and ability to permeate the intestinal surface (polarized enterocytes) will affect drug absorption and systemic bioavailability (the fraction of the dose that reaches the systemic circulation as intact drug). In addition, uptake transporters in the SLC superfamily localized on the apical (brush border) or basolateral membrane of enterocytes facilitate the entry of poorly permeable molecules into the systemic circulation. Such transporters including PEPT1 (SLC15A1), OCTN1 (SLC22A4), and CNTs and ENTs (SLC28 and SLC29 superfamilies) play important roles in the absorption of drugs. ABC efflux transporters such as P-gp (ABCB1), BCRP (ABCG2), and MRP2 (ABCC2) may limit the absorption of many prescribed drugs and other xenobiotics (Giacomini et al., 2010). Inhibition of these transporters in the intestine can improve oral drug bioavailability (e.g., verapamil or quinidine increases the plasma levels of digoxin by inhibiting P-gp (Fromm et al., 1999; Verschraagen et al., 1999)).

In 2012, the US Food Drug Administration (FDA) proposed that drug developers use a decision tree to determine whether an investigational drug is a substrate (or inhibitor) of P-gp or BCRP and to aid in determining whether an *in vivo* clinical drug–drug interaction (DDI) study is needed (FDA Guidance, 2012). As part of the proposed decision trees, the use of *in vitro* assays such as Caco-2 cells, a colon adenocarcinoma cell line, was recommended as a model to test both intestinal permeability and efflux activity. Polarized cell lines such as Madin–Darby canine kidney cells (MDCK II), porcine kidney cells (LLC-PK1), or MDR-1-overexpressing cells can also be used for *in vitro* bidirectional transport

assays. In their excellent review, Estudante et al. (2013) cataloged substrates, inhibitors, and inducers for intestinal efflux and uptake transporters important in drug absorption. In addition, Wu and Benet (2005) proposed a Biopharmaceutics Drug Disposition Classification System (BDDCS) that categorized drugs into four classes based on the drug's solubility and extent of metabolism. The review and classification system may be helpful in predicting oral drug disposition including the potential of DDI in the intestine.

2.5 Drug Transporters in the Liver

After entry into the systemic circulation, drugs or compounds may distribute into the muscles and other organs such as the liver. Since the liver is the major organ responsible for drug metabolism and indeed for the metabolism of most xenobiotics, hepatic drug transporters can significantly modulate drug metabolism. In addition, hepatic blood flow rate, molecular size and polarity of compounds, binding of compounds to serum proteins, and transporter activity in the hepatocyte will affect the distribution of drugs into the hepatocyte. Figure 2.3 summarizes the uptake and efflux transporters on the basolateral/sinusoidal and apical/canalicular membranes. Uptake transporters such as OCT1 and OCT3 (SLC22A1 and A3), NTCP (SLC10A1), and OATP1B1 and OATP1B3 (SLCO1B1 and SLCO1B3) are localized to the sinusoidal (or basolateral) membrane where they mediate drug entry into the hepatocyte. Two hepatic uptake transporters, OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3), are responsible for the uptake of many anionic drugs and play a role in DDI (FDA Guidance, 2012). Efflux transporters including BCRP (ABCG2), P-gp (ABCB1), MRP2 (ABCC2), BSEP (ABCB11), and MATE1 (SLC47A1) located on the apical membrane transport drugs, drug metabolites, and bile salts across the canalicular membrane into the bile. MRP3 and MRP4 are efflux transporters on the basolateral membrane, which bring their substrates back into the systemic circulation (Funk, 2008; Köck and Brouwer, 2012; Russel et al., 2008). Once drugs or compounds enter the hepatocytes, they may undergo phase I and/or phase II metabolism (Xu et al., 2005; Yoshida et al., 2013). Cytochrome P450 enzymes (CYPs) regulate phase I (oxidative metabolism), and UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), and glutathione S-transferase (GST) regulate phase II (conjugation reaction), respectively. Numerous studies have shown that metabolites of drugs or various xenobiotics may also be pharmacologically active and sometimes more than the original compounds (Fura, 2006; Obach, 2013). Thus, membrane transporters together with enzymes in

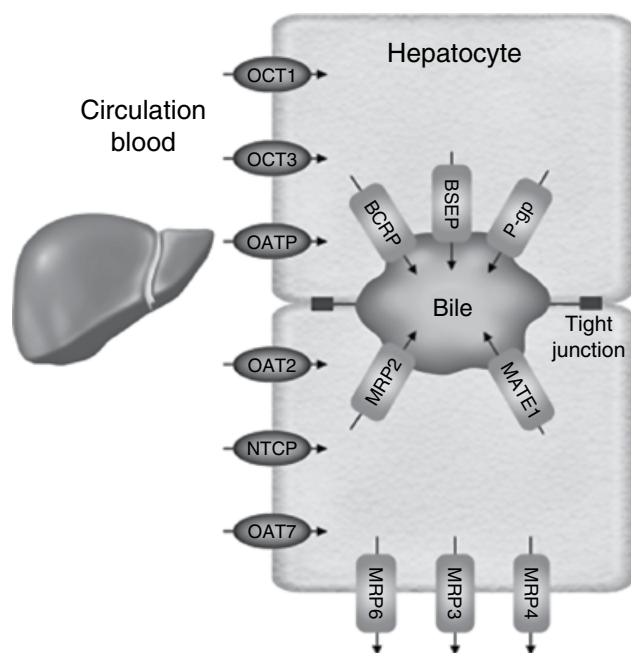


Figure 2.3 Selected hepatic transporters for drugs and endogenous substances. Human hepatocyte uptake transporters in the basolateral (sinusoidal) membrane include organic cation transporters, OCT1 (SLC22A1) and OCT3 (SLC22A3); organic anion transporters, OAT2 (SLC22A7) and OAT7 (SLC22A9); organic anion-transporting polypeptides, OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1); and sodium-taurocholate cotransporting polypeptide (NTCP, SLC10A1). Efflux pumps in the basolateral membrane include multidrug resistance-associated protein 3 (MRP3, ABCC3), MRP4 (ABCC4), and MRP6 (ABCC6). Apical (canalicular) efflux pumps including P-gp (ABCB1), bile salt export pump (BSEP, ABCB11), breast cancer resistance protein (BCRP, ABCG2), MRP2 (ABCC2), and multidrug and toxin extrusion protein 1 (MATE1, SLC47A1).

hepatocytes contribute to the disposition of drugs and other xenobiotics and may also be sites of DDI. For example, lovastatin or simvastatin, a HMG-CoA reductase inhibitor, that is metabolized by CYP3A can have more than 10-fold increase in the blood when coadministered with CYP3A inhibitors such as ketoconazole or erythromycin (Williams and Feely, 2002).

Pharmacogenetics of major hepatic transporters have been reported (Franke et al., 2010; Kerb, 2006). For example, non-synonymous single nucleotide polymorphisms (SNPs) found in SLC22A1 either completely abolish or partially reduce its transport function (Koepsell et al., 2007). Furthermore, polymorphisms of OATPs affect statins' pharmacokinetics and efficacy (Kallioikoski and Niemi, 2009). SNPs in several ABC transporters including BSEP (ABCB11) and BCRP (ABCG2) result in lower efflux activities and had been associated with an increase in the systemic exposure of various compounds such as uric acid and statins (Ishikawa et al., 2005; Lang et al., 2007). *In vitro* models

including *Xenopus laevis* oocytes, membrane vesicles, various human embryonic kidney (HEK293) cell lines overexpressing transporters, and primary hepatocytes are commonly used to address questions about potential drug–drug and drug-transporter interactions. Several issues may complicate *in vitro* transporter studies (i.e., lack of selective inhibitor/substrates and drug-transporter/drug metabolism interplay), and combining *in silico* modeling with *in vitro* studies will greatly accelerate our understanding of transporters in the liver (Barton et al., 2013; Li et al., 2014; Yoshida et al., 2013).

2.6 Drug Transporters in the Kidney

SLC transporters in the kidney play vital roles in three of the kidney's major functions: electrolyte balance, detoxification, and conservation of nutrients (Morrissey et al., 2013). In this section, an overview of SLC transporters involved in detoxification and conservation mechanisms will be presented.

2.6.1 Conservation Mechanisms

The kidney plays a critical role in the reabsorption of essential solutes including macronutrients such as glucose and amino acids, as well as micronutrients such as various vitamins and heavy metals. As many macro- and micronutrients are filtered in the glomerulus, reabsorptive transporters, many in the proximal tubule, serve to transport these molecules back into the systemic circulation, playing a vital role in the conservation of glucose, amino acids, oligopeptides, heavy metals, and vitamins. For transepithelial flux across the proximal tubule of the kidney from tubule lumen to systemic circulation (reabsorptive direction), distinct transporters are expressed on the apical and basolateral membranes. For example, transporters in the SLC5 family, notably SGLT2 (SLC5A2), reabsorb glucose across the apical membrane into the proximal tubule cell. SLC5 transporters work in concert with transporters in the SLC2 family, notably GLUT2, which are involved in moving glucose from the intracellular space across the basolateral membrane into the extracellular spaces and ultimately into the systemic circulation (Scheepers et al., 2004; Wright, 2013). Similar mechanisms are involved in reabsorptive flux of other compounds such as oligopeptides and amino acids, where transporters on the apical membrane involved in the transmembrane flux from lumen to cell are distinct from basolateral membrane transporters (Silbernagl, 1988). Often a secondary active transporter is involved in the uphill movement from lumen to cell (e.g., SGLT2

for glucose), and a facilitative transporter (e.g., GLUT2 for glucose) is present on the basolateral membrane serving in the downhill flux of substrates from cell to extracellular fluid.

2.6.2 Detoxification Mechanisms

The kidney is the key organ involved in the body's defense mechanisms against chemical toxins. In particular, the kidney serves to eliminate many toxic substances and their metabolites through filtration in the glomerulus and secretory transporters in the proximal tubule. These transporters, which are generally classified as OCT and organic anion transporters (OATs), include transporters in the SLC22 family (OAT) as well as transporters in the SLC47 family (multidrug toxin and extrusion proteins (MATEs)) (Figure 2.4). The two most well-studied OATs are OATs 1 and 3 (OAT1, SLC22A6 and OAT3, SLC22A8), which are both expressed on the basolateral membrane and mediate the first step in active tubular secretion of their substrates (Morrissey et al., 2013). Substrates of these transporters include structurally diverse organic anions including many prescription drugs, for example, antibiotic agents such as penicillins and cephalosporins and antiviral agents such as adefovir and tenofovir (for review see (Burckhardt, 2012)). In addition, OATs are known to transport highly nephrotoxic compounds such as the nephrotoxin; ochratoxin A, which is a substrate of OAT3; and the antiviral drug cidofovir, which must be administered with probenecid, an inhibitor of OAT1 and OAT3, to reduce its accumulation and thus toxicity in the kidney (Burckhardt, 2012;

Hagos and Wolff, 2010). For basic compounds and organic cations, the major transporter on the basolateral membrane that mediates renal secretion is OCT2 (SLC22A2), which plays a key role in renal elimination of drugs and structurally diverse molecules. OCT2 has been implicated in both the nephrotoxicity and ototoxicity of cisplatin (Ciarimboli, 2012, 2014). Working in concert with OCT2 are two transporters in the SLC47 family, MATE1 and MATE2 (Motohashi and Inui 2013), on the apical membrane. These transporters mediate the electroneutral exchange of protons with organic cations and serve to move their substrates from tubule cell to lumen. Several studies have shown that there is much less nephrotoxicity of compounds that are substrates of both OCT2 and MATEs, because these dual substrates are easily secreted into the tubule lumen (Ciarimboli, 2012, 2014; Motohashi and Inui, 2013). However, for some compounds, such as cisplatin, which is an excellent substrate of OCT2, but not a substrate of MATEs, accumulation of the compound in the proximal tubule occurs with resulting nephrotoxicity.

2.7 Mitochondrial Transporters

Mitochondria are double membrane-bound organelles that are essential to the aerobic eukaryotic cells. The number of mitochondria in a cell can vary from 0 (red blood cells) to more than 2000 (liver cells) (Alberts et al., 2002; Voet et al., 2016). The outer membrane is freely permeable to small molecules such as sucrose, salt, and nucleotides but not to larger molecules like polyglucans, albumin, and cytochrome c (Salazar-Roa and Malumbres, 2016). Because many metabolic processes occur in the cytosol followed by the mitochondrial matrix, mitochondrial transporters (or mitochondrial carriers) can transport metabolites formed in the cytosol across the inner mitochondrial membrane and, as such, serve to catalyze several important mitochondrial functions such as oxidative phosphorylation, citric acid cycle, fatty acid oxidation, and apoptosis (Chipuk et al., 2006; Wang and Youle, 2009). Mitochondrial transporters must ensure sufficient rate of solute flux to fulfill the needs of various metabolic pathways.

Transport of solutes across the inner mitochondrial membrane is facilitated by transporters encoded by genes in the SLC25 family, which are present on chromosomal DNA. To date, 53 members have been reported in this family from SLC25A1 to SLC25A53 (Palmieri, 2013, 2014). They have similar molecular characteristics, that is, a tripartite structure, six transmembrane alpha-helices, and threefold repeat signature motifs that are different from any other transporter families (Berardi et al., 2011; Palmieri, 2013, 2014; Pebay-Peyroula et al., 2003).

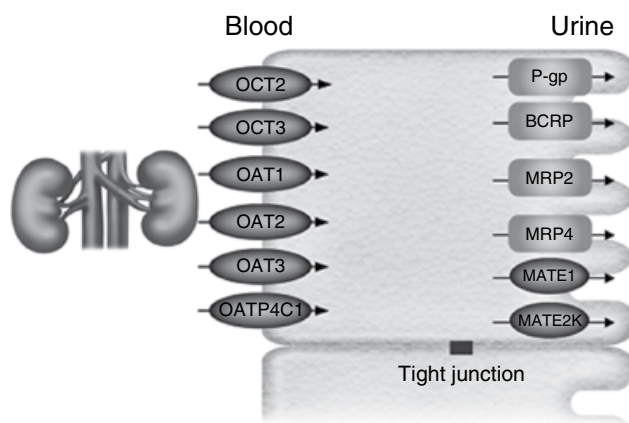


Figure 2.4 Selected renal transporters for drugs and endogenous substances. Kidney basolateral uptake transporters including OCT2 (SLC22A2) and OCT3 (SLC22A3) and OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SLC22A8), and OATP4C1 (SLC47C1). Apical (luminal) transporters including P-gp (ABCB1), BCRP (ABCG2), MRP2 (ABCC2), and MRP4 (ABCC4) and MATE1 (SLC47A1) and MATE2-K (SLC47A2).

These transporters have different tissue distributions and cellular expression; however, they may have overlapping selectivity for substrates (Palmieri, 2013, 2014). For example, SLC25A4, SLC25A5, and SLC25A6 (adenine nucleotide translocase 1-3 (ANT1-3)) are expressed at different levels in various organs, but ADP and ATP are the predominant substrates of all three transporters (Table 2.2, modified from reference (Palmieri, 2013)). Furthermore, several mitochondrial transporters are tissue specific (e.g., SLC25A27 in the brain), suggesting that they play important roles in special functions (Anitha et al., 2012). Transporters of SLC25 family are not restricted to the mitochondria. For example, several SLC25 family members are located in other organelles such as peroxisomes (e.g., SLC25A17 in humans) (Agrimi et al., 2012), chloroplasts (plant, ortholog of SLC25A42) (Zallot et al., 2013), and mitosomes (parasite, ADP/ATP transporter) (Chan et al., 2005). If classified by substrates, mitochondrial transporters can be divided into carriers for carboxylates, nucleotides, amino acids, keto acid, and other substrates. Uncoupling proteins (UCPs) (i.e., UCP 1–3, SLC25A7-9; Table 2.2) are transmembrane proteins that decrease proton ions generated from oxidative phosphorylation. This reaction facilitates the movement of protons into the intermembrane space to return to the mitochondrial matrix. UCP1 is activated in the brown adipose tissue and inhibited by purine nucleotide such as ADP. It plays a critical role in thermogenesis (Krauss et al., 2005; Rousset et al., 2004). In this reaction, norepinephrine released by the sympathetic nervous

system triggers a signaling cascade: conversion of ATP to ADP, followed by catalysis of triglycerides into free fatty acids, which, in turn, activate UCP1 and thermogenin to generate heat.

Many mitochondrial DNA mutations cause respiratory chain and oxidative phosphorylation defects. Only a few diseases that are caused by mutations in mitochondrial transporters have been found (Cl  men  on et al., 2013; Guti  rrez-Aguilar and Baines, 2013; Palmieri, 2014). These mitochondrial carrier-related diseases can be divided into two categories: (1) diseases directly linked to oxidative phosphorylation or (2) diseases associated with mitochondrial carrier gene mutation (Palmieri, 2004, 2008). The first group of disorders include AAC1 deficiency (Palmieri et al., 2005), adPEO (van Goethem et al., 2001; Kaukonen et al., 2000), and PiC deficiency (Mayr et al., 2007) and are caused by defects either in ADP/ATP translocase (SLC25A4) or PiC phosphate carrier (SLC25A3). These disorders are characterized by insufficient energy production. CAC deficiency (Huizing et al., 1997) (defect of SLC25A20), HHH syndrome (Camacho et al., 1999) (defect of SLC25A15), and Amish microcephaly (Kelley et al., 2002) (defect of SLC25A19) belong to the second group. These transporters are responsible for transporting carnitine, ornithine, and thiamine pyrophosphate, respectively, as needed for cellular metabolism. Though much is known about several SLC25 family members, several still have not been characterized, and their functions remain to be determined.

Table 2.2 Selected transporters in the SLC25 family that move substrates across the inner mitochondrial membrane.

Transporter	Gene	Major tissue distribution	Predominant substrates
CIC (citrate carrier)	SLC25A1	Liver, kidney, pancreas	Citrate, isocitrate, malate
ORC2 (ornithine carrier)	SLC25A2	Liver, testis, spleen	Ornithine, citrulline, lysine, arginine
PHC (phosphate carrier)	SLC25A3	Isoform A: heart, muscle Isoform B: liver, kidney, brain	Phosphate
ANT1 (adenine nucleotide translocase-1)	SLC25A4	Heart, skeletal muscle, much less in brain, kidney, lung	ADP, ATP
ANT2 (adenine nucleotide translocase-2)	SLC25A5	Brain, lung, kidney, pancreas	ADP, ATP
ANT3 (adenine nucleotide translocase-3)	SLC25A6	Brain, lung, kidney, liver, pancreas	ADP, ATP
UCP1 (uncoupling protein 1)	SLC25A7	Brown adipose tissue	H ⁺
UCP2 (uncoupling protein 2)	SLC25A8	Lung, kidney, spleen	H ⁺
UCP3 (uncoupling protein 3)	SLC25A9	Skeletal muscle, lung	H ⁺
DNC (deoxynucleotide carrier)	SLC25A19	Brain, testis, lung, kidney, liver, skeletal muscle, heart	Thiamine pyrophosphate (TPP), thiamine monophosphate (TMP), deoxynucleotides
CAC (carnitine/ acylcarnitine carrier)	SLC25A20	Heart, skeletal muscle, liver	Carnitine, acylcarnitine

2.8 Conclusions

Transporters in the SLC superfamily play critical roles in the accumulation of xenobiotics including mitochondrial toxins in cells, tissues, and organs. Working together, transporters in epithelia with distinct mechanisms mediate transepithelial flux of substrates, which generally promotes detoxification, for example, moving substrates from the blood to the tubule lumen and urine. Further, with enzymes, transporters play a major role in detoxification mechanisms in the body. That is, enzymes metabolize hydrophobic toxins to more hydrophilic molecules, which then interact with organic ion transporters in the kidney and undergo renal secretion. Mitochondrial transporters in the SLC25 family have a special role in the accumulation

of chemical substances within the mitochondria and have a direct role in mitochondrial toxicities. Currently there are 53 transporters in the SLC25 family, several of which are still orphan transporters with unknown substrates. These transporters are highly conserved across species, and polymorphisms are generally present at low allele frequencies. Mutations, which are generally rare, in mitochondrial transporters lead to many genetic disorders such as optic atrophy spectrum disorder. For mitochondrial toxins to exert their deleterious effects, they must first gain entry into the tissue of toxicity and then the mitochondria. Clearly, transporters in the SLC superfamily are involved in both the detoxification and toxicity of many mitochondrial toxins by mediating entry and exit from cells and subcellular compartments.

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3

Structure–Activity Modeling of Mitochondrial Dysfunction

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3.1 Introduction

Computational toxicology plays a key role in helping to reduce drug attrition in the pharmaceutical industry by aiming to identify problematic chemicals early in the drug development phase (Naven and Louise-May, 2015). In addition, it also plays an important role in a number of chemical industries, including the cosmetic sector, due to European Union (EU) law aimed at replacing the use of animals in chemical risk assessment (European Commission, 2003, 2007). Increasingly, the methods used in computational toxicology have become a key part of the adverse outcome pathway (AOP) approach to toxicology (Ankley et al., 2010; Landesmann et al., 2013; Vinken, 2013). An AOP is a framework that means to establish a mechanistic connection between an upstream molecular initiating event (MIE) and a downstream adverse effect (e.g., organ toxicity) through a series of testable key events. The MIE is the critical event in the progression of an AOP as it provides insight into the initial interaction(s) between the chemical behavior of the xenobiotic and the biological system that initiates the perturbation of the normal pathway. Elucidation of the mechanistic information relating to specific MIEs enables the identification of

structural (and physicochemical) features of chemicals that are responsible for the interaction with biological macromolecules, thus facilitating the development of structural alerts and/or (quantitative) structure–activity relationship ((Q)SAR) models. Importantly, these computational tools enable a mechanistic link between chemistry and biology to be established.

Research has shown that a range of chemicals can induce mitochondrial dysfunction, leading to organ toxicity in the kidney, liver, cardiac, and nervous tissues (Amacher, 2005; Begriche et al., 2011; Chan et al., 2005; Dykens and Will, 2008; Esposti et al., 2012; Krahenbuhl, 2001; Masubuchi et al., 2000; Montaigne et al., 2012; Nadanaciva and Will, 2011; Pessayre et al., 2012; Rolo et al., 2004). These tissues are most susceptible to mitochondrial dysfunction as they contain high concentrations of mitochondria and, in the case of the liver and kidney, are exposed to higher concentrations of chemicals (or their metabolites). In order to be able to relate these organ effects to chemistry and thus build predictive structure–activity relationship models, it is important to understand the structure and function of the mitochondria and the possible mechanisms by which toxicity can occur.

3.1.1 Mitochondrial Structure and Function

Mitochondria consist of two membranes, the outer and inner membrane, enclosing three compartments, the intermembrane space, the cristae, and the mitochondrial matrix (Mannella, 2008). The outer membrane is relatively smooth and permeable to molecules that are less than 5 kDa in size. In contrast, the inner membrane containing multiple invaginations (cristae) is impermeable to all molecules except O₂, CO₂, and H₂O and contains each of the protein complexes within the electron transport chain, ATP synthase (complex V), and various electron carriers. The mitochondria are responsible for a number of tasks vital to a cell's normal functioning and survival. These tasks include the production of approximately 95% of the total amount of adenosine triphosphate (ATP) generated by cells during oxidative phosphorylation. Oxidative phosphorylation is a process whereby energy from the transfer of electrons, generated by the oxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂), along various complexes of the electron transport chain is utilized to pump protons across the inner mitochondrial membrane, generating an electrochemical gradient. The electron transport chain comprises four complexes situated within the inner mitochondrial membrane. Complexes I and II are involved in the oxidation of NADH and FADH₂ respectively, providing the input of electrons into the respiratory chain. Complexes I, III, and IV use the energy released from the transfer of electrons along the electron transport chain to pump protons out of the mitochondrial matrix into the intermembrane space. Complex V, the terminal complex involved in oxidative phosphorylation, utilizes the electrochemical gradient produced to transfer protons from the intermembrane space back into the mitochondrial matrix. The energy released from this action is utilized to phosphorylate adenosine diphosphate into ATP.

3.1.2 Mechanisms of Mitochondrial Toxicity

Five general mechanisms of mitochondrial dysfunction have been identified: (i) inhibition of the electron transport chain and ATP synthase (complex V), (ii) uncoupling of oxidative phosphorylation, (iii) opening of the membrane permeability transition pore, (iv) inhibition of fatty acid β -oxidation, and (v) disruption of mitochondrial DNA (mtDNA) (Chan et al., 2005; Dykens and Will, 2008; Krahenbuhl, 2001; Nadanaciva and Will, 2011; Scatena et al., 2007; Wallace and Starkov, 2000). Briefly stated, chemicals that inhibit the electron transport chain can do so by either direct binding to the complexes of the electron transport chain or ATP synthase or by acting as an alternative electron acceptor. The inhibition

of electron flow along the electron transport chain by both of these mechanisms may induce the formation of reactive oxygen species, resulting in oxidative stress. Uncouplers of oxidative phosphorylation induce mitochondrial toxicity by shuttling protons into the mitochondrial matrix, via the inner mitochondrial membrane, bypassing ATP synthase. This assisted transport of protons back into the matrix dissipates the electrochemical potential, resulting in the loss of ATP production and, ultimately, cell death. Induction of the membrane permeability transition increases the permeability of the inner mitochondrial membrane to low molecular weight solutes (<1500 Da), which may lead to a disruption of the electron transport chain, loss of membrane potential, and/or swelling of both the inner and outer mitochondrial membranes. Inhibition of β -oxidation of mitochondrial fatty acids reduces the amount of NADH and FADH₂ available for oxidative phosphorylation that, in turn, reduces ATP production. Finally, mtDNA encodes 13 components of the electron transport chain, and damage that occurs to mtDNA can have a variety of downstream effects depending upon where such damage occurs. It should be noted, however, that the mitochondrial toxicity observed for a single (group of) chemical(s) could be initiated through multiple competing mechanisms, that is, one chemical may induce several MIEs.

The link that has been established between mitochondrial dysfunction and organ-level toxicity is an important breakthrough for the assessment of human health risks in drug discovery, the cosmetics industry, and other chemical sectors. The ability to identify potential problematic chemicals as early as possible in the development phase is a key requirement for these industries, and the aim of this chapter is to outline *in silico* methods that enable a mechanistic link between mitochondrial toxicity and chemistry to be established.

3.2 Mitochondrial Toxicity Data Sources

Experimental data is critical for the development of computational tools for the prediction of mitochondrial toxicity. This often requires significant additional investment or the use of publicly available data described hereafter.

3.2.1 Zhang Dataset

This dataset was compiled by Zhang and colleagues as part of the development of a support vector machine (SVM) QSAR model for the binary classification of drug-induced mitochondrial toxicity (Zhang et al., 2009).

The dataset consists of 171 drug-like molecules that are reported as causing mitochondrial toxicity, the data being collected from eight different reference sources. In order to develop the SVM model (an automated machine learning approach to QSAR modeling), the authors selected 117 chemicals from the Food and Drug Administration (FDA) list of approved drugs, with the assumption that drugs on this list do not cause mitochondrial toxicity. This assumption, along with the use of multiple data sources for the identification of mitochondrial toxicants, is the key limitation of this dataset. Additionally, no information about the mechanism (potential MIE) leading to mitochondrial toxicity is included for any of the chemicals in the dataset.

3.2.2 ToxCast Data

There are a number of *in vitro* assays for mitochondrial toxicity among the extensive ToxCast dataset (available from <http://epa.gov/ncct/toxcast>). These assays target different organs, organisms, biological processes, and durations of exposure. In contrast to the data within the Zhang dataset, the ToxCast assays focus on specific biological mechanisms. For example, there are six assays relating to mitochondrial depolarization in human livers covering 1, 24, and 72 h exposures. There are a further six assays for the same biological process gathered in rat liver (exposure times of 1, 24, and 48 h). At the time of writing (August 2016), the number of chemicals tested in these assays ranges from 300 to over 1000, covering drugs, pesticides, cosmetic ingredients, and industrial chemicals. This data source is the single largest repository of freely available *in vitro* data related to mitochondrial toxicity. However, its use in structure–activity studies may be limited as unpublished analysis by the authors of this chapter have shown it to lack significant clusters of chemicals from which structural alerts could be developed. Instead, the ToxCast data covers a broad range of chemical space, with no specific area of chemistry being represented in significant detail.

3.3 In Silico Modeling of Mitochondrial Toxicity

There has been limited structure–activity models (quantitative and/or qualitative) published in the scientific literature for mitochondrial toxicity, presumably due to the lack of publically available data. These studies relate to a statistical model aimed at classifying mitochondrial toxicants from non-mitochondrial toxicants, and three studies that have developed mechanistically based structural alerts related to some of the mechanisms outlined in Section 3.1.

3.3.1 Statistical Modeling

As mentioned at the time of writing, there is only a single published statistical quantitative structure–activity model in the literature—the work of Zhang et al. published in 2009 (Zhang et al., 2009). This work utilized the dataset of 288 drug molecules outlined previously to develop an SVM model capable of classifying chemicals as either being mitochondrial toxicants or not. A set of 253 chemicals and an initial set of 179 molecular descriptors (covering a range of descriptor types from 2D structural indices to traditional physicochemical properties) were utilized to develop the model. A genetic algorithm was utilized to select the best subset of these descriptors for the SVM approach, resulting in the final model containing 27 molecular descriptors. An additional 35 chemicals were utilized to validate the model. The authors reported that the best performing model had a prediction accuracy of 85% for the training set and 77% for the external test set. Clearly, these are excellent classification statistics (likely in line with the experimental error of the *in vitro* data used to train the model), making this approach very useful for high-throughput screening of chemical databases. However, this model offers no mechanistic rationale behind the classification, making it difficult to rationalize the predicted mitochondrial toxicity for a given chemical. This is additionally important if one wishes to use such models as part of a non-animal risk assessment strategy—something that is particularly relevant to the non-pharmaceutical sectors such as the cosmetics industry given recent EU legislation (European Commission, 2003, 2007).

3.3.2 Structural Alert Modeling

In contrast to the work of Zhang et al., there have been several studies that have developed structural alerts from a mechanistic point of view. The first of these outlined 11 structural alerts (termed “toxicophores” by the authors) related to the ability of weak acids to cause an uncoupling of oxidative phosphorylation in mitochondria from an analysis of 2085 drug-like molecules (Naven et al., 2013). The authors utilized the collection of 11 alerts in an in-house structure–activity model that enabled the identification of 68% of the chemicals in the dataset with potent uncoupling activity (alerts 1–11; Table 3.1). The study also detailed the predictive ability of each structural alert along with an analysis of *clog P* and *pK_a* ranges associated with potent uncouplers. This study provided a clear mechanistic insight into each alert, enabling the definition of chemistry associated with an MIE for weak acid respiratory uncouplers. This has the advantage of making the alerts useful in both screening and as part of a chemical safety assessment testing

Table 3.1 Published structural alerts for mitochondrial toxicity.

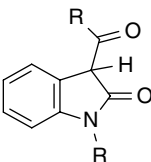
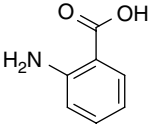
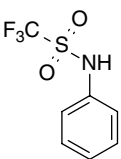
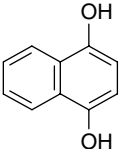
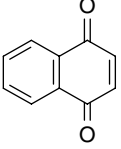
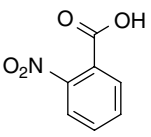
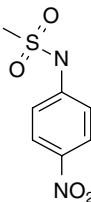
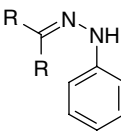
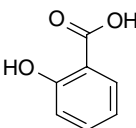
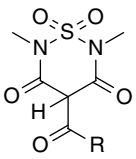
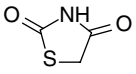
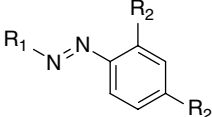
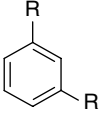
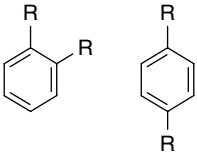
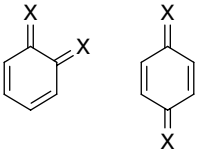
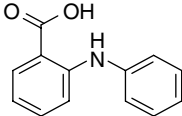
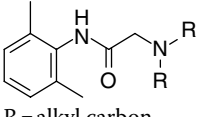
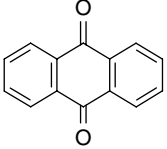
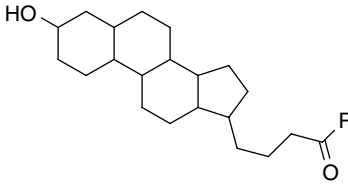
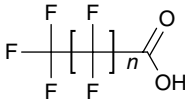
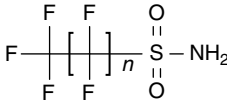
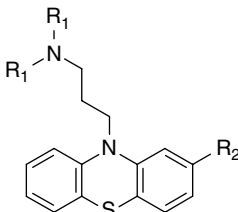
Alert	Structural alert	Reference	Mechanism	Structural alert
1	Acyindolones	Naven et al. (2013)	Protonophore	 R = alkyl carbon
2	Anthranilic acids	Naven et al. (2013)	Protonophore	
3	Fluoromethylsulfanilides	Naven et al. (2013)	Protonophore	
4	Hydroxynaphthoquinones	Naven et al. (2013)	Redox cycling	
5	Naphthoquinones	Naven et al. (2013)	Redox cycling	
6	Nitrophenols	Naven et al. (2013)	Protonophore	
7	Nitrosulfonanilides	Naven et al. (2013)	Protonophore	
8	Phenylhydrazones	Naven et al. (2013)	Protonophore	 R = any carbon
9	Salicylates	Naven et al. (2013)	Protonophore	

Table 3.1 (Continued)

Alert	Structural alert	Reference	Mechanism	Structural alert
10	Thiadiazinedione dioxides	Naven et al. (2013)	Protonophore	 R = any carbon
11	Thiazolidinediones	Nelms et al. (2015b) and Naven et al. (2013)	Protonophore	
12	Aromatic azos	Nelms et al. (2015a)	Redox cycling	 (R₁ = aromatic carbon) (R₂ = NH₂, NH, OH)
13	Meta-substituted benzenes	Nelms et al. (2015a)	Redox cycling	 (R = NH₂, OH)
14	Pro-quinones	Nelms et al. (2015a)	Redox cycling	 (R = OH, NH₂, NH, NO₂)
15	Quinones	Nelms et al. (2015a)	Redox cycling	 (X = NH, O)
16	2-Anilinobenzoic acids	Nelms et al. (2015b)	Protonophore	
17	2-(Dialkylamino)-N-(2,6-dimethylphenyl)acetamides	Nelms et al. (2015b)	Protonophore	 R = alkyl carbon
18	Anthracene-9,10-diones	Nelms et al. (2015b)	Redox cycling	

(Continued)

Table 3.1 (Continued)

Alert	Structural alert	Reference	Mechanism	Structural alert
19	Bile acids	Nelms et al. (2015b)	Unclear (possible inhibition of complex III)	 R = alkyl carbon
20	Perfluorinated carboxylic acids	Nelms et al. (2015b)	Protonophore	 $n = 5-11$
21	Perfluorinated sulfonamides	Nelms et al. (2015b)	Protonophore	 $n = 5-11$
22	Phenothiazines	Nelms et al. (2015b)	Inhibition of complexes I–IV	 $R_1 = \text{CH}_2, \text{CH}_3$ $R_2 = \text{Cl}, \text{CF}_3$

strategy aimed at reducing animal usage (or replacing it altogether, depending on the industrial sector). However, the defined alerts do not address the numerous other MIEs outlined in Section 3.1 that can lead to mitochondrial toxicity. In addition, the associated database was not published along with the alerts (as would be expected given the confidential nature of the drug-like chemicals), making further analysis (in the public domain) of the dataset by other researchers impossible.

Two additional studies have also developed structural alerts for mitochondrial toxicity (Nelms et al., 2015a, b). The first of these analyzed a set of chemical structures typically used in hair dye products from a hypothesis that mitochondrial toxicity is a key driving force in determining chronic toxicity (Nelms et al., 2015a). The premise of this paper was to group “similar” chemicals into categories to enable the prediction of toxicity for chemicals with no data via read-across. Read-across is simply a structure–activity model in a localized area of chemical space that has found utility in regulatory toxicology for the non-animal prediction of toxicity (Cronin et al., 2013; Dimitrov and Mekenyan, 2010; Przybylak and Schultz,

2013). It has been shown in the literature that the best way to define chemical similarity when making read-across predictions is in terms of a clear MIE (Enoch, 2010; Enoch and Cronin, 2012; Enoch et al., 2009, 2010, 2011; Hewitt et al., 2013; Przybylak and Schultz, 2013; Roberts et al., 2015; Sakuratani et al., 2008; Schultz et al., 2009, 2015; Yamada et al., 2013). The authors of this paper used structural similarity (atom environments and fingerprints coupled with the Tanimoto coefficient) to cluster a dataset of 94 chemicals used in hair dyes into four similarity-based categories. The authors then undertook a structure–activity analysis of the chemicals within each category, enabling them to establish mitochondrial toxicity as a potential driving force for chronic toxicity. This analysis resulted in the definition of four structural alerts related to proton and electron cycling (alerts 12–15; Table 3.1). Importantly, the authors demonstrated that within these well-defined mechanistic categories, it was possible to predict chronic toxicity for 56 of the 94 chemicals in the dataset via read-across (chronic toxicity defined by no observed adverse effect levels (NOAELs) extracted from 90-day rat studies).

Several of the same authors recently followed this study uptaking the same approach to develop structural alerts from the 288 drug-like chemicals published as part of the Zhang et al. GA–SVM model (Nelms et al., 2015b). This analysis defined a further eight structural alerts related to several key mechanisms of mitochondrial toxicity (alerts 11, 16–22; Table 3.1). This paper also brought together the mechanistic knowledge from the previously discussed work of Naven et al. (2013), enabling the definition of a set of 17 structural alerts based on chemicals tested in an *in vitro* mitochondrial toxicity assay. Inclusion of the structural alerts discussed previously derived from chronic toxicity data expands the total number of publically available (unique) structural alerts for mitochondrial toxicity to 22. All structural alerts discussed in this section are as shown in Table 3.1.

3.4 Mechanistic Chemistry Covered by the Existing Structural Alerts

The remainder of this chapter will focus on detailing the mechanistic chemistry associated with the structural alerts outlined in Table 3.1. This focus is due to the importance of mechanism-derived structural alerts toward the prediction of toxicology in drug discovery, the cosmetics sector, and the wider chemicals industry. As previously outlined in Section 3.1, there are at least five biological mechanisms that may lead to mitochondrial dysfunction:

- Inhibition the electron transport chain and ATP synthase (complex V)
- Uncoupling of oxidative phosphorylation
- Opening of the membrane permeability transition pore
- Inhibition of fatty acid β -oxidation
- Disruption of mtDNA synthesis and translation

The structural alerts shown in Table 3.1 only cover two of these mechanisms—uncoupling of oxidative

phosphorylation (13 alerts) and inhibition of the electron transport chain (eight alerts). The final alert, relating to bile acids, has an unclear mechanism of action according to the authors of the study in which it was published (Nelms et al., 2015b). One example of the type of mechanistic chemistry covered by these two classes of alerts is related to the ability of chemicals to act as alternate electron acceptors enabling them to transport electrons directly from complex I into complex IV (or oxygen). These chemicals typically have to be capable of undergoing facile reduction in order to accept an electron (or electrons) from complex I and then be capable of being easily oxidized in order to release electrons directly into complex IV or oxygen (summarized in Figure 3.1). This results in effective structural alert “pairs” in which either the oxidized or reduced chemical is capable of causing mitochondrial toxicity—presumably due to the fact that the reduced form is readily oxidized prior to acting as a mitochondrial toxicant. The requirement for the prior oxidation to the ultimate mitochondrial toxicant is summarized in Figure 3.2 for chemicals containing a 2-nitroaniline moiety (covered by alert 14 in Table 3.1). For such chemicals it has been suggested that 2-phenylenediamine is the alternate electron acceptor (this chemical is reduced to 1,2-diaminobenzene upon accepting two electrons from the electron transport chain). Similar analysis is able to rationalize the presence of structural alerts for hydroxynaphthoquinones/naphthoquinones (alerts 4 and 5; Table 3.1) and hydroquinones/quinones (alerts 14 and 15; Table 3.1).

The second class of alerts covers chemicals capable of uncoupling oxidative phosphorylation due to their ability to transport hydrogen ions across the inner mitochondrial membrane into the mitochondrial matrix. The mechanistic chemistry associated with this class of structural alerts is due to their ability to act as weak acids within the intermembrane space. The deprotonated forms of chemicals containing these structural alerts scavenge free protons from the intermembrane space. Upon protonation, the chemical migrates across the inner mitochondrial membrane into the mitochondrial

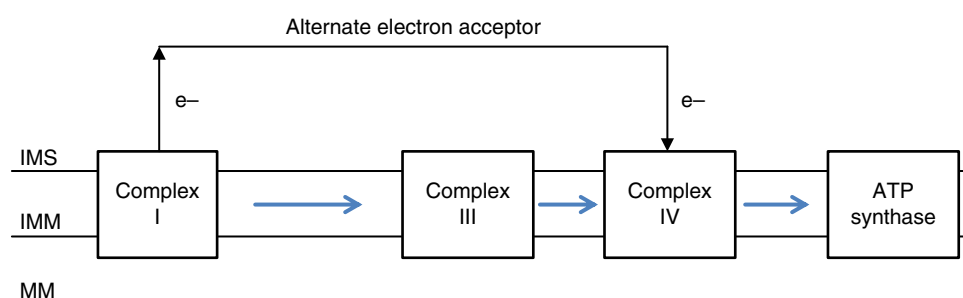


Figure 3.1 Schematic of how a chemical capable of facile reduction can act as an alternate electron acceptor leading to disruption of the electron transport chain.

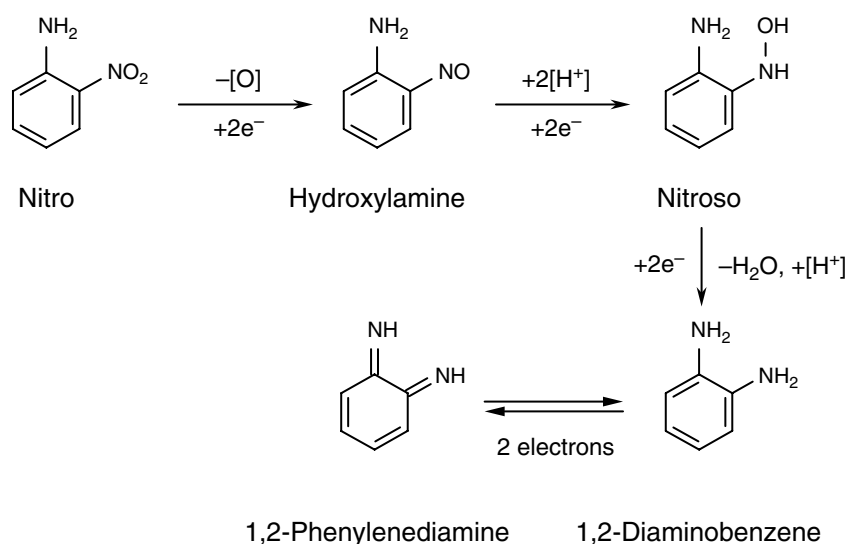


Figure 3.2 Reduction of 2-nitroaniline leading to the production of the alternate electron acceptor 1,2-phenylenediamine.

matrix, where the increased alkaline environment results in proton dissociation. The newly deprotonated chemical is then able to return to the intermembrane space to collect a second proton, enabling the cycle of proton transport to continue. This cycle increases oxygen consumption and heat production, alongside a reduction in the electrochemical gradient and ATP production. This process is summarized in Figure 3.3.

The presence of a pH gradient between the intermembrane space (pH $\approx$ 7) and the mitochondrial matrix (pH $\approx$ 8) enables weak acids to cycle protons across the inner mitochondrial membrane. Thus, acids that are partially ionized between the pH values of approximately

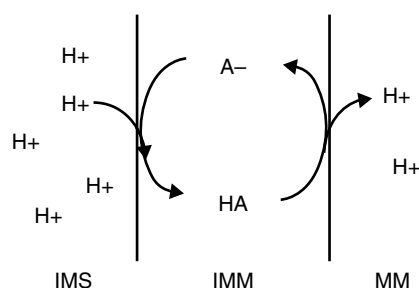


Figure 3.3 Schematic showing the ability of weak acids to transport protons across the inner mitochondrial membrane (IMM) from the intermembrane space (IMS) to the mitochondrial matrix (MM).

5 and 8 are likely to be able to cycle protons in this manner. This enables predictive structure–activity models to incorporate pK_a as a descriptor, that is, identifying those weak acids with pK_a values around six being particularly potent uncouplers of oxidative phosphorylation (Naven et al., 2013). In terms of structural alert development, this has been rationalized in the need for resonance stabilization in the conjugate base (e.g., thiazolidinedione as shown in Figure 3.4). Inspection of the structural alerts in this class (labeled protonophores illustrated in Table 3.1) shows them all to contain moieties capable of resonance stabilization of the acidic functional group.

Interestingly, there has only been two structural alerts published relating to the inhibition of complexes I–IV (alerts 19 and 22; Table 3.1), and no alerts published for toxicity to mtDNA. The difficulty in identifying potential ligand binding sites for these large protein complexes, coupled with the lack of publically available data being key to this gap in the structure–toxicity relationship for such chemicals. This is an area where future research is clearly required, with a need for targeted work utilizing a combination of *in vitro* technologies coupled with *in silico* analysis. In terms of toxicity to mtDNA, it is likely that a subset of the structural alerts that have been published for covalent and non-covalent genotoxicity are likely to be applicable (Benigni and Bossa, 2008, 2011; Enoch and Cronin, 2010). However, the exact nature of these alerts remains to be defined.

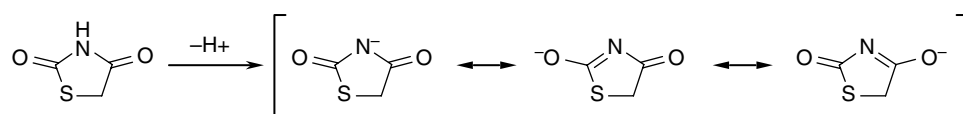


Figure 3.4 Resonance stabilized weak acid behavior for chemicals containing a thiazolidinedione structural alert.

3.5 Structural Alert Applicability Domains: Physicochemical Properties

The majority of the structural alerts defined in the previous section do not have well-defined applicability domains in terms of the associated physicochemical properties with individual alerts. However, Naven and coworkers (2013) performed an analysis of the physicochemical properties of the entire dataset that was tested in the RST assay from which structural alerts for uncoupling of oxidative phosphorylation were derived. This analysis showed that strong and moderately potent uncouplers were acidic rather than neutral, zwitterionic, or basic. In addition, this analysis also showed that uncoupling potency increased as hydrophobicity increased, with chemicals with a cLogP in excess of four being the most potent in the RST assay. This latter finding clearly provides additional information that could be combined with the structural alerts in Table 3.1 as part of a tiered approach to the *in silico* screening for mitochondrial toxicants—the presence of an alert combined with a cLogP value in excess of four potentially being a good indicator of toxicity.

3.6 Future Direction: Structure–Activity Studies for Other Mechanisms of Mitochondrial Toxicity

Due to the limited amount of publically available data suitable for structure–activity analysis, the published structural alerts are mainly focused around the ability of chemicals to act as either protonophores or redox cyclers. There is a clear need to develop structural alerts (and/or structure–activity models) to other mechanisms capable of causing mitochondrial toxicity. For example, currently there are no structural alerts generated for chemicals that act to inhibit mtDNA synthesis. As inhibition of mtDNA is demonstrated to induce mitochondrial dysfunction, this area is likely to be a good source of data for the development of structural alerts, especially where evidence is already collated for specific drug classes, such as nucleoside reverse transcriptase inhibitors (NRTIs), which are antiviral drugs that act via the inhibition of a viral enzyme, reverse transcriptase. Evidence has shown that this class of drugs is a potent inhibitor of mtDNA synthesis.

This class of drugs presents an ideal opportunity for structural alert development as much is understood at the pathogenesis of NRTI-induced mitochondrial

dysfunction. Briefly, there are three key steps. Firstly, competition takes place in the mitochondria between the NRTI triphosphate groups with the naturally occurring nucleoside triphosphate bases within the cell. Secondly, if competition is successful, this leads to incorporation of the NRTIs into the growing mtDNA strand, thus resulting in chain termination due to the lack of a second hydroxyl group within the NRTI chemical structure, which is required for elongation of the mtDNA strand. Finally, chain termination induces impaired mitochondrial polymerase γ , leading to mtDNA depletion and ultimately mitochondrial dysfunction (Birkus et al., 2002; Koizumi et al., 2006; Lewis et al., 2001).

In terms of other potential mechanisms of mitochondrial dysfunction, new *in vitro* data need to be generated that is suitable for structure–activity studies. It is likely that the generation of such data in combination with bioenergetic mathematical modeling will be needed to identify key proteins that when inhibited lead to mitochondrial dysfunction (Bazil et al., 2010; Dash et al., 2009; Gille et al., 2010). The discussion of such methods is outside the scope and expertise of the authors of this chapter; however, it is clear that the development of such mathematical models is an active area of research that will hopefully further the understanding of potential targets of chemical-induced mitochondrial dysfunction. Given a potential protein target (or targets), informatics approaches could be used to identify the key binding features associated with each protein, leading to the development of structural alerts and/or structure–activity models.

3.7 Concluding Remarks

This chapter has outlined the published structure–activity modeling studies for mitochondrial toxicity. Due to the limited amount of publically available data suitable for modeling, these studies have focused on a single machine learning classification model and the publication of three sets of structural alerts. Inspection of these structural alerts shows them to be mainly related to proton and electron cycling mechanisms, with there being a distinct lack of alerts relating to other mechanisms leading to mitochondrial toxicity. While the structure–activity relationship for proton and redox cyclers is relatively well defined, it is clear that much work is needed to elucidate equivalent structure–activity relationships for other mechanisms leading to mitochondrial toxicity. It is the opinion of the authors of this chapter that only a combination of targeted *in vitro* biology and *in silico* approaches is going to generate necessary data required for this challenge.

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4

Mitochondria-Targeted Cytochromes P450 Modulate Adverse Drug Metabolism and Xenobiotic-Induced Toxicity

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CHAPTER MENU

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4.1 Introduction

Mitochondrial genome, membrane complexes, and metabolic enzyme systems are highly sensitive to changes in intramitochondrial as well as extramitochondrial environments (Dorn and Kitsis, 2015; Hill et al., 2012; Valero, 2014). Additionally, alterations in mitochondrial dynamics (such as biogenesis, fission, fusion, mass, mitophagy, etc.) have implications in organ-specific physiology, pathology, pharmacology, and toxicity (Bravo-Sagua et al., 2013; Kotiadis et al., 2014; Sivitz and Yorek, 2010). Mitochondrial dynamics may change in response to physiological (exercise, starvation, hormonal changes), pathological (diabetes, infection, inflammation, cancer, neuro/cardiovascular disorders), and toxicological (drugs, carcinogens, chemicals) stress. Altered mitochondrial functions have been implicated in diseases and toxicities (Boland et al., 2013; Calkins et al., 2011; Seo et al., 2010; Yoon et al., 2011). Mitochondria are the major producer of ATP and reactive oxygen

species (ROS) and a key player in buffering of intracellular calcium, maintenance of the metabolite pool, and regulation of NAD⁺/NADH ratio. Mitochondria also play critical roles in cell signaling and integration of signals for autophagy and apoptosis (Dorn and Kitsis, 2015; Hill et al., 2012). Consequently, in addition to physiological significance, mitochondria play an important role in toxicity, oxidative stress, and complications associated with targeted drug therapies (Gupta et al., 2009; Tang et al., 2014). Mitochondrial dysfunction and mitochondrial toxicity (mitotoxicity) have been reported in a number of pathophysiological conditions and drug-induced inflammation and oxidative stress (Apostolova and Victor, 2015; de Castro et al., 2010; Javadov et al., 2014). Therefore, mitochondrial function and toxicity should be considered as an important factor in any pharmacological or toxicological studies.

Cytochrome P450s (CYPs) are a multigene family of heme–thiolate enzymes having thousands of constitutively expressed or xenobiotic (drug and chemical)-induced isoenzymes located mainly in the membrane of the endoplasmic reticulum (ER) as well as in mitochondria, cytosol, peroxisomes, cell membrane, and other organelles (Johnson and Stout, 2013; Nebert et al., 2013; Neve and Ingelman-Sundberg, 2010).

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These drug-metabolizing enzymes are mainly involved in the metabolic conversion of both endogenous physiological substrates and xenobiotic drugs and chemicals mostly for excretion. The detoxification capacities of CYPs vary widely depending on the CYP type and the metabolites it produces. For example, the reactive metabolite of acetaminophen, a benzoquinoneimine, produced by CYPs 2E1, 3A4/5, and 1A2 is largely attributed to Tylenol®-induced liver damage and mitochondrial dysfunction (Neve and Ingelman-Sundberg, 2010). In addition, CYPs are also involved in the generation of ROS and maintenance of redox homeostasis (Bajpai et al., 2013, 2014; Bansal et al., 2013, 2014b; Bhattacharyya et al., 2014; Sangar et al., 2010b). Therefore, targeting of CYPs by drugs and nutraceuticals (phytochemicals) and the manipulation of their catalytic activities are potentially significant in determining the biological responses toward toxicities and diseases. Drug-induced mitochondrial toxicity leading to a wide range of pathological conditions is seen in neuropathy, myopathy, pancreatitis, and several other disorders. Our objective in this chapter is to discuss the molecular mechanism(s) of targeting of some of the CYPs to mitochondria, then assess their roles in the metabolism of diverse drugs, and finally discuss the significance of mitochondrial drug metabolism in mitochondrial toxicity, oxidative stress, and inflammatory-associated tissue injury.

4.2 Multiplicity of Mitochondrial CYPs

Mitochondria from mammalian and other species contain drug-metabolizing CYPs that cross-react with antibodies to a number of microsomal CYPs belonging to the subfamilies 1A, 1B, 2B, 2C, 2D, 2E, 3A, and others (Avadhani et al., 2011; Sangar et al., 2010b). Mitochondrial CYPs, like microsomal CYPs, are also inducible and require NADPH/NADH and/or adrenodoxin (Adx) and adrenodoxin reductase (Adr) as electron donors. However, mitochondria-targeted CYPs prefer Adx and Adr as electron donors over the NAD(P)H-CYP reductase enzyme and exhibit altered substrate specificity and catalytic efficiency (Avadhani et al., 2011; Sangar et al., 2010b). This may be because mitochondria-targeted CYPs lack the transmembrane topology that is essential for interaction with NADPH-CYP reductase (CYPR). The membrane extrinsic topology likely facilitates efficient interaction with soluble Adx and Adr proteins. Crystal structure analysis of a number of CYPs reveals a highly conserved structural fold and membrane topography with remarkably high degree of genetic and amino acid sequence homology among the different families and yet with a highly divergent substrate specificity and

catalytic functions (Johnson and Stout, 2013; Omura, 2006; Werck-Reichhart and Feyereisen, 2000). The most conserved region among all the CYPs is the heme-binding domain, and the most variable regions are the substrate recognition site and the amino-terminal or chimeric signal domains for alternate translocation of these proteins (Omura, 2006; Werck-Reichhart and Feyereisen, 2000). Occurrence of multiple forms of constitutive as well as inducible CYPs in different tissues and in different organelles with a wide array of differential substrate specificity and catalytic efficiencies itself implies the significance of CYP pools in physiology and pathophysiology (Nebert and Dalton, 2006; Pikuleva and Waterman, 2013). Although most of drug bioavailability, metabolism, distribution (pharmacodynamic and pharmacogenetic), and drug toxicity studies are mainly focused on the microsomal CYPs, the emerging evidence suggests the relevance of alternative intracellular organelle-specific distribution of CYPs in maintaining the physiological functions of organisms. Mitochondria-targeted CYPs may also have critical roles in determining the physiological, pharmacological, and toxicological consequences of drugs. As mentioned earlier, mitochondrial DNA and respiratory chain function as well as mitochondrial membrane permeability are highly susceptible to intramitochondrial and extramitochondrial environments, and, in this regard, mitochondrial CYPs play important roles in metabolic and redox homeostasis that are critical determinants of bioenergetics (survival) and apoptosis (death). We, therefore, will focus on highlighting the multimodal targeting mechanisms of CYPs to mitochondria and their role in drug metabolism and toxicity under physiological and pathophysiological conditions. Several studies by our laboratories and others have demonstrated that a number of ER-associated CYPs from the subfamilies 1A, 1B, 2B, 2C, 2D, 2E, and 3A are also located inside the inner membrane compartment of mitochondria facing the matrix (Addya et al., 1997; Anandatheerthavarada et al., 1997, 1999a, b; Bhagwat et al., 2000, 1999b; Boopathi et al., 2000; Dasari et al., 2006; Neve and Ingelman-Sundberg, 1999, 2001; Raza and Avadhani, 1988; Robin et al., 2001, 2002, 2005; Shayiq et al., 1991). This alternate translocation of mitochondrial CYPs may establish a pool of about one-third of total cellular CYPs (Anandatheerthavarada et al., 1999b).

4.3 Targeting and Significance of Multiple Forms of Mitochondrial CYPs

The majority of mitochondria-directed proteins follow the mechanism of amino-terminal mitochondria-specific targeting signals that are removed inside the

mitochondria (Attardi and Schatz, 1988; Blobel, 2000; Neupert and Herrmann, 2007; Wickner and Schekman, 2005). However, some of the mitochondria-imported proteins contain noncanonical signals that are not processed after their translocation inside the mitochondria (Anandatheerthavarada et al., 1999a, b; Bhagwat et al., 1999b; Boopathi et al., 2000; Dasari et al., 2006; Robin et al., 2001). These signals have been characterized as a terminal amphipathic helix with diverse secondary structure and also as non-cleaved internal signals (Attardi and Schatz, 1988; Bolender et al., 2008; Neupert and Herrmann, 2007; Wickner and Schekman, 2005). The positively charged residues in the mitochondrial targeting signal, along with hydrophobic domains, interact with negatively charged residues on the translocase of the outer membrane of mitochondria (TOM) complexes (Abe et al., 2000; Anandatheerthavarada et al., 2009; Hildenbeutel et al., 2008; Neve and Ingelman-Sundberg, 2008; Suzuki et al., 2004). After moving through the outer membrane channel comprising TOM40 channel-forming protein and other regulatory proteins, the pre-proteins interact with the translocase of the inner membrane of the mitochondria (TIM) complexes in order to cross the inner membrane. As the protein advances into the matrix, the pre-sequence is generally cleaved by mitochondria-specific metalloproteases to form the mature proteins, which is then released (facilitated by local chaperons to fold into its final

conformation) (Abe et al., 2000; Anandatheerthavarada et al., 2009; Hildenbeutel et al., 2008; Neve and Ingelman-Sundberg, 2008; Suzuki et al., 2004). Similarly, constitutively expressed mitochondrial CYPs involved in steroid metabolism (e.g., CYP 27A1, CYP 24A1, CYP 11A1, and CYP 11B1,2) have canonical and cleavable mitochondrial targeting signals at their N-termini that are different from the signal sequence of microsomal CYPs (Engelman et al., 1986; Morohashi et al., 1987). On the other hand, some of the CYPs imported into the mitochondria contain a more dynamic chimeric signal that directs the CYP both to ER and into mitochondria, depending upon the demand of the cell under physiological and pathological conditions. The cryptic mitochondrial targeting signal requires activation by a cytosolic endoprotease (e.g., CYP1A1, CYP1B1) or protein kinase A (PKA)-mediated phosphorylation (e.g., CYP2B1, CYP 2E1, and CYP2D6) (Figures 4.1 and 4.2). Our laboratories have demonstrated that multiple xenobiotic-metabolizing CYPs, as well as cytosolic glutathione (GSH)-drug conjugating enzymes (glutathione *S*-transferase (GSTs)), are present in the mitochondria and exhibit differential induction and translocation properties in response to physiological and chemical stress when compared with ER-expressed counterparts (Addya et al., 1997; Anandatheerthavarada et al., 1997, 1999a, b; Bhagwat et al., 2000, 1999b; Boopathi et al., 2000; Dasari et al., 2006; Nebert and Dalton, 2006; Neve and

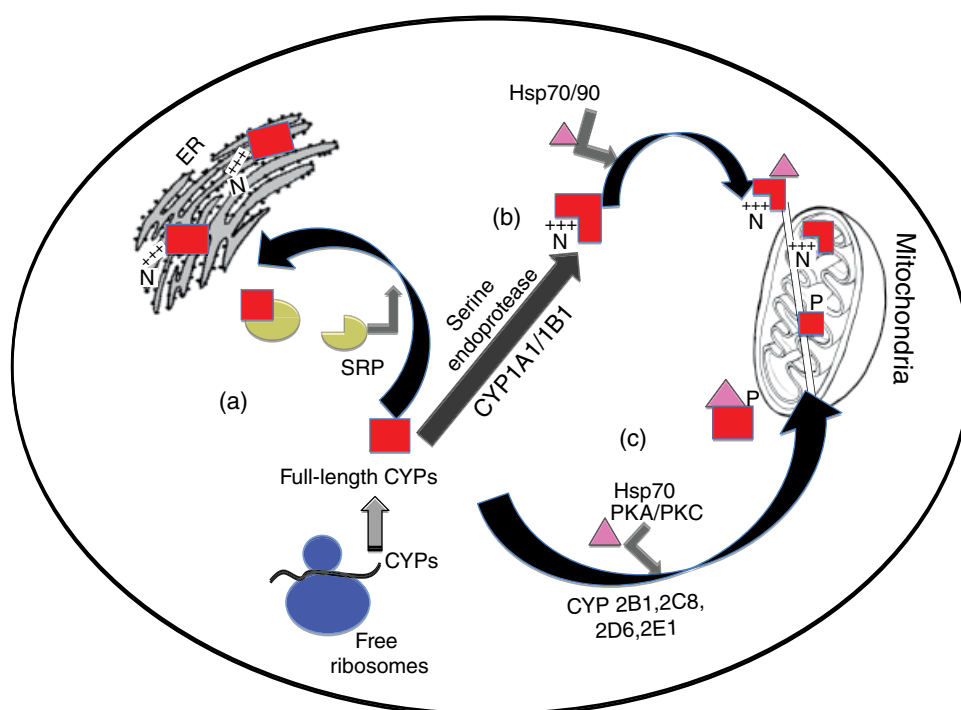


Figure 4.1 Schematic representation of bimodal targeting of CYPs. A: SRP-dependent microsomal translocation of CYPs. B: Mitochondrial translocation after N-terminal truncation (e.g., CYP1A1, CYP1B1). C: Mitochondrial translocation after PKA/PKC phosphorylation and activation of chimeric signals.

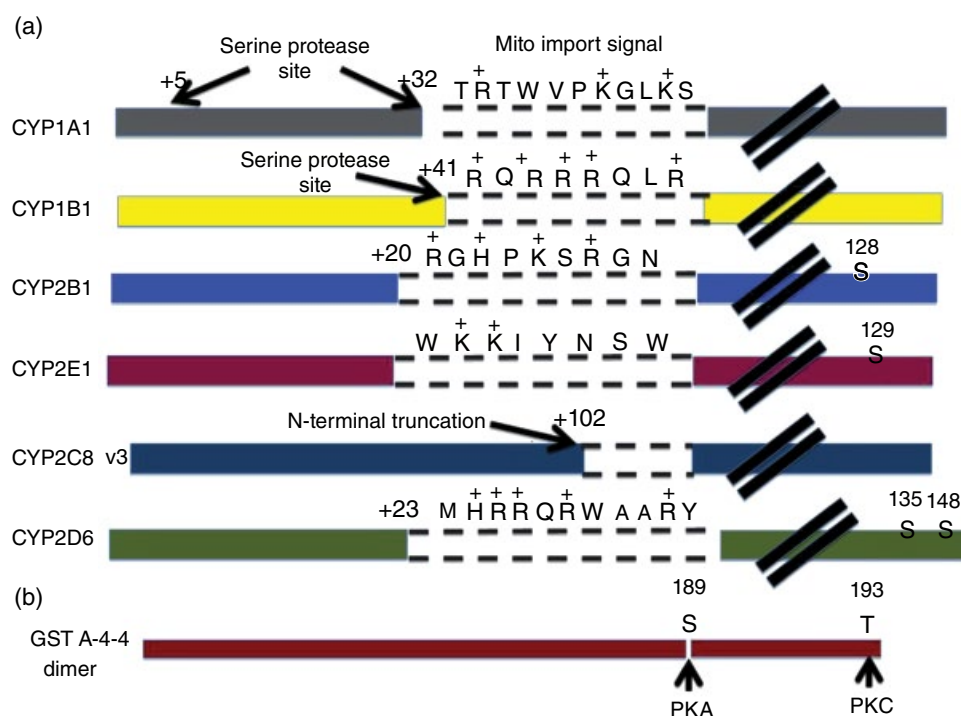


Figure 4.2 Specific targeting signal for mitochondrial import of CYPs and GSTA4-4. (a) CYP signals. (b) GST signal.

Ingelman-Sundberg, 1999, 2001; Pikuleva and Waterman, 2013; Raza and Avadhani, 1988; Robin et al., 2001, 2002, 2003, 2005; Shayiq et al., 1991).

4.3.1 Mitochondrial Import of CYP1A1

The bimodal targeting of CYP1A1 to the ER and mitochondria involves a noncanonical N-terminal chimeric signal, which can target the passenger protein of both subcellular compartments (Addya et al., 1997; Anandatheerthavarada et al., 1999b; Dasari et al., 2006). The N-terminal end of the protein contains signal for targeting to the ER, while internal sequences from amino acids 33–48 contain information for targeting the protein to mitochondria. Thus, the cryptic mitochondrial signal sequence at 33–48 needs to be activated by a sequence-specific serine protease-mediated processing at amino acid 33 of the rat CYP1A1. The mitochondrial targeting mechanism and the protease processing site are conserved in all mammalian genes including the human CYP1A1 (Boopathi et al., 2008). The mechanism has been summarized in Figure 4.1b, and the conserved serine protease processing site has been shown in Figure 4.2. In the rat, +33/1A1 (MT-CYP1A1) is the major mitochondrial CYP1A1, with a minor +5/1A1 component detected by N-terminal sequencing of purified mitochondrial CYP1A1 (Addya et al., 1997;

Anandatheerthavarada et al., 1999b; Bhagwat et al., 1999a, b; Boopathi et al., 2000, 2008; Dasari et al., 2006). We have shown that about 25% nascent CYP1A1 chains escape ER signal recognition particle (SRP) binding and processed for mitochondrial import in both mouse and rat liver systems. Cytosolic chaperons Hsp70 and Hsp90, peripheral TOM receptors, and channels are required for mitochondrial import (Anandatheerthavarada et al., 2009). We have generated CYP mutants that modulate SRP binding and accordingly direct CYP1A1 proteins for alternate translocations. Stable cell lines expressing these mutant CYPs show marked differences in the levels of mitochondrial- and ER-associated CYP1A1 that provide excellent models for toxicological studies (Dasari et al., 2006). Similar signal remodeling strategies were used for developing transgenic mouse lines (Dong et al., 2009), which express either MT-CYP1A1 or (mainly) ER-CYP1A1. Mice expressing microsomal CYP1A1 play a more important role in the elimination of benzo[a]pyrene (B[a]P) from the liver and the intestine. Mitochondrial CYP1A1, on the other hand, appears to play a major role in B[a]P- and 2,3,7,8-tetrachlorobenzo-*p*-dioxin (TCDD)-induced mitochondrial toxicity and oxidative stress (Bansal et al., 2014b; Dong et al., 2009, 2013; Raza and Avadhani, 1988). Mouse mitochondrial MT-CYP1A1 exhibits preferential *N*-demethylation of wide spectrum of compounds including an antibiotic

Table 4.1 Altered substrate metabolism by mitochondria-targeted CYPs.

MT CYPs	Altered substrate specificity	ROS production
CYP1A1	<i>N</i> -Demethylation of erythromycin diazepam, lidocaine, imipramine, amitriptyline, morphine, etc.	Unclear
CYP2E1	Alcohol-induced toxicity	Increased ROS
CYP2C8	High eicosanoid synthesis, paclitaxel oxidation	Increased ROS
CYP2D6	Metabolism of MPTP and other monomethylamine toxins	Increased reactive metabolites and ROS

(erythromycin), an opioid (morphine), a local anesthetic (lidocaine), and several anticardiac and antipsychotic drugs (diazepam, imipramine, and amitriptyline). These drugs are poor substrates of microsomal CYP1A1 supported by microsomal CYPR, suggesting subtle differences in substrate specificity (Table 4.1). These compounds are classical substrates of microsomal CYP2D6 or CYP3A2, which contain large substrate binding grooves. In this respect, mitochondrial CYP1A1 shows distinct physiological and pharmacological roles.

4.3.2 Mitochondrial Import of CYP1B1

Similar to CYP1A1, another polycyclic aromatic hydrocarbon (PAH)-inducible family 1 CYP, CYP1B1, is also targeted to mitochondria by sequence-specific cleavage at the N-terminus. Similar to CYP1A1, the rat, mouse, and human CYP1B1 proteins are processed by a cytosolic serine protease, resulting in the activation of a positively charged domain at the 41–48 amino acid sequence of human CYP1B1 (Bansal et al., 2014b; Dong et al., 2013). Mitochondrial CYP1B1 plays an important role in inducing PAH toxicity in the lung and also in a number of other cells as reflected in mitochondrial oxidative and metabolic functions. B[a]P, an environmental PAH carcinogen, abundantly present in cigarette smoke, induce mitochondrial toxicity and tissue damage in wild-type (WT) mice but at markedly low levels in CYP1B1-null mice (Bansal et al., 2014b). These results suggest the importance of mitochondrial CYP1B1 in mediating toxicity. Similarly, cells expressing mitochondrial predominant CYP1B1 show high level of B[a]P- and TCDD-induced toxicity and mitochondrial DNA depletion, while cells expressing mostly ER-targeted CYP1B1 show markedly reduced toxicity (Bansal et al., 2014b). These effects appear to be independent of CYP 1A1 expression in the lung (Bansal et al., 2014b; Dong et al., 2009, 2013).

4.3.3 Mitochondrial Import of CYP2C8

Analysis of human liver samples showed the presence of both full-length and an N-terminal truncated splice variant (Bajpai et al., 2014). Based on sequencing of RT-PCR amplified DNA, the splice variant lacked the N-terminal 102-amino acid coding region of the WT full-length mRNA. Based on three-dimensional molecular modeling, the splice variant found mostly in mitochondria retained nearly complete substrate and heme-binding domains. However, substrate specificity of truncated form was different from that of full-length microsomal/mitochondrial CYP2C8. HepG2 cells, stably expressing mitochondrial variant-3 CYP2C8, exhibited higher catalytic activity with arachidonic acid as a substrate that is accompanied by an increased production of ROS and mitochondrial dysfunction, suggesting its role in oxidative stress (Bajpai et al., 2014). Cells expressing mitochondrial predominant splice variant also showed high activity for paclitaxel oxidation but relatively lower levels of dibenzylfluorescence oxidation compared with microsomal CYP2C8 supported by POR.

4.3.4 Mitochondrial Import of CYP2D6

Studies in our laboratory show that human CYP2D6, which accounts for the metabolism of approximately 20% of clinical drugs, is also transported to mitochondria. In this case, intact unprocessed protein is involved in mitochondrial targeting, and very likely, PKA-mediated phosphorylation at Ser-148 may be involved in the activation of cryptic mitochondrial signal (Sangar et al., 2009, 2010a, b). Sequencing of the N-terminal 200-amino acid coding region of CYP2D6 from human liver samples showed mutations that possibly affect the extent of mitochondrial targeting and the interindividual variations and alternate distribution of CYP2D6 in these livers (Sangar et al., 2009, 2010a). We have determined that mitochondrial CYP2D6 requires an N-terminal chimeric signal located between residues 23 and 33 and that the positively charged residue at positions 24, 25, 26, 28, and 32 are essential for mitochondrial targeting (Sangar et al., 2009). These results highlight the importance of interindividual variations (pharmacogenetics) in the level of hepatic mitochondrial CYP2D6 in metabolizing xenobiotics and commonly used clinical drugs. The role of mitochondrial CYP2D6 in drug-induced toxicity is evident from results showing that mitochondria-targeted CYP2D6 can actively oxidize 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to a toxic pyridinium ion, which induces mitochondrial toxicity through inhibition of complex I activity (Bajpai et al., 2013). Mitochondrial CYP2D6 in the presence of Adx and Adr oxidized MPTP to MPP⁺ at a rate higher than

monoamine oxidase (MAO)-B, suggesting its importance in toxicity of dopaminergic neurons (Bajpai et al., 2013). Our studies have also shown that PKA mediated phosphorylation (Sangar et al., 2010a).

4.3.5 Mitochondrial Import of CYP2B1 and CYP2E1

Bimodal targeting of CYP2B1 to ER and mitochondria involves novel signal activation by cAMP-dependent phosphorylation. Nearly intact CYP2B1 is translocated to mitochondria following PKA-mediated phosphorylation at Ser-128, which likely reduces interaction of nascent chain with SRP, which primarily traffics the complex to the ER. The reduced SRP binding also makes the protein mitochondrial import competent (Anandatheerthavarada et al., 1999a). Similarly, CYP2E1 import to mitochondria is also facilitated by the activation of a cryptic signal domain from N-terminal amino acid sequence 21–31 of the protein after PKA phosphorylation at the Ser-129 residue. It has also been shown that N-terminal chimeric signal of CYP2B1 has a significantly higher affinity for SRP than that of CYP2E1. However, in the case of CYP2E1, phosphorylation increases the affinity of the nascent proteins for Hsp70/Hsp90 chaperons, facilitating the import of the protein to mitochondria (Robin et al., 2001, 2002). It has been shown that the phosphorylated CYP2B1 and CYP2E1 interact strongly with mitochondrial translocases and matrix Hsp70/Hsp90, facilitating the efficient binding of the cryptic mitochondrial targeting signal to the TOM40 complex (Robin et al., 2001, 2002). These results suggest that SRP binding to nascent CYP chains is a major regulatory step in the bimodal protein targeting process and nascent chains that escape SRP binding for mitochondrial import, either because of inherent lower affinity of the N-terminal chimeric signals or lowering of affinity by internal phosphorylation (Anandatheerthavarada et al., 1999a, 2009; Bansal et al., 2013, 2014b; Robin et al., 2001). In support of this hypothesis, mutations at the N-terminal protein targeting regions of CYP2E1 affect the subcellular distribution of the protein that can have physiological and pathophysiological consequences (Bansal et al., 2013). In support, the signal modification studies show that a more hydrophobic N-terminal 1–20 region of the chimeric signal exhibits higher affinity to SRP and thus higher ER targeting. Nascent CYP2E1, with a more hydrophilic N-terminal end, exhibiting lower affinity for SRP binding, is targeted more preferentially to mitochondria (Bansal et al., 2010). Screening of human liver samples showed marked variations in the mitochondrial levels of CYP2E1, with some samples showing predominantly the mitochondrial form and others showing predominantly the ER form (Bansal et al., 2013). Sequencing of N-terminal coding regions of human liver CYP2E1 showed single nucleotide variants,

namely, L32N, W30R, W23R, and W23/30R. The L32N form was predominantly targeted to the ER with very low levels of mitochondrial targeting. The remaining variants showed higher levels of mitochondrial targeting. In particular, the W23/30R form showed the highest level of mitochondrial targeting and highest level of alcohol-induced ROS production in HepG2 cells. Thus mitochondria-targeted CYP2E1 is associated with increased ROS production, mitochondrial dysfunction, and responses in alcohol-induced liver and brain toxicity (Bansal et al., 2010, 2012; Knockaert et al., 2011; Raza et al., 2004), suggesting its role in physiology and pathophysiology.

4.3.6 Import Mechanism of GSH-Conjugating GSTA4-4

The cytosolic GSH-conjugating enzymes GSTs (isoenzymes alpha, mu, and pi) are also targeted to mitochondria differentially under physiological and oxidative stress conditions (such as in diabetes) (Raza et al., 2002, 2004). Using a combination of *in vitro* mitochondrial import assay and *in vivo* targeting in COS cells transfected with GSTA4-4-cDNA, we demonstrated that, similar to some of the CYPs, mitochondrial import signal of GSTA4-4 is activated after cAMP-dependent phosphorylation (Robin et al., 2003). Consistent with the import mechanisms of CYP2E1/2B1, and possibly CYP 2D6, GSTA4-4 import into mitochondria is also facilitated by binding with the Hsp70 chaperon. Interestingly, mitochondrial targeting signal in GSTA4-4 resides within C-terminal 20-amino acid residues of the protein and that the signal is activated by PKA-dependent phosphorylation at Ser-189 or PKC-dependent phosphorylation at Thr-193. Our results suggest that hyperphosphorylated GSTA4-4 has a higher affinity for Hsp70, making this protein mitochondrial import competent, while hypophosphorylated GSTA4-4, with lower affinity for chaperon Hsp70, remains in the cytosol (Robin et al., 2003).

4.4 Variations in Mitochondrial CYPs and Drug Metabolism

Multiplicities and tissue-specific and organelle-specific localization of CYPs have consequences in determining the pharmacokinetics and pharmacodynamics as well as toxicity of xenobiotics and therapeutic drugs. Mutations in CYPs coding and noncoding genes and their transcription regulation may result in altered CYP activities, which render individuals with increased or decreased CYP activities (Tang et al., 2005). The metabolism of over 95% of clinically prescribed drugs has been attributed mainly to a limited number of CYPs: CYP 3A4/5, CYP2C8, CYP 2C9, CYP 2D6, CYP 2C19, and

CYP1A2 (Gonzalez et al., 1986; Guengerich, 2008; Zanger et al., 2008). Therefore genetic polymorphism in these CYPs, their alternate localization, substrate specificity, and their ability to be induced or inhibited by xenobiotics have great significance in determining drug-induced pharmacological and toxicological effects. Interestingly, most of the CYPs, with the exception of CYP2D6, are induced by xenobiotic chemicals (Lin and Lu, 2001). Polymorphism and differential expression in human liver mitochondrial CYP2D6 have been reported (Neve and Ingelman-Sundberg, 1999). Recent studies from our laboratory have indicated that mutations at the targeting region of human CYP2D6 may influence mitochondrial targeting and metabolic efficiency. Since there is a relatively steady mitochondrial CYP2D6 content (4–6%) in inbred rats and mouse livers (Sangar et al., 2009, 2010a), it is unlikely that variations observed in human livers is observed in wild rodents. There is evidence suggesting polymorphic expression and other interindividual variations in the expression and activities of other mitochondrial CYPs (e.g., CYP 2C8, CYP2E1, and CYP 3A1/2) (Bajpai et al., 2014; Bansal et al., 2013; Sangar et al., 2009) and their significance in determining xenobiotic-induced toxicity and oxidative stress.

4.5 Physiological and Toxicological Significance of Mitochondria-Targeted CYPs

Mitochondrial bioenergetics and other physiological functions are coupled with oxygen utilization, heme and protein turnover, and integrity of membranes, as well as mitochondrial biogenesis (Suliman and Piantadosi, 2016). Mitochondrial dysfunction is also prevalent in aging, apoptosis, “off-target” pharmacotherapy or collateral adverse effects, oxidative stress, and inflammation. Therefore, mitochondria are currently a major focus for toxicological and pharmacological studies as well as drug development. Mitochondrial CYPs are therefore important factors to consider since they share metabolites and biochemical required for the normal function of respiratory complexes. Alterations in CYP expression and/or catalytic activities are therefore important in maintaining mitochondrial quality control (Suliman and Piantadosi, 2016). Increased levels of mitochondrial CYPs under xenobiotic exposure or other physiological conditions alter cytochrome *c* oxidase structure and function, which is the major use of cellular O₂. Mitochondrial biogenesis is linked to the cell cycle, as well as in response to energy demand and mitochondrial dysfunction associated with drug-induced toxicities and oxidative stress (Dorn and Kitsis, 2015; Hill et al., 2012; Moren et al., 2016; Valero, 2014; Wallace, 2015).

Mitochondrial dysfunction may lead to shortage of ATP, a key factor for cell survival and apoptosis. In addition, abnormal oxygen utilization by mitochondrial respiratory complexes may induce leakage of harmful ROS and induction of inflammatory responses from accumulated fatty acids (e.g., reduction in mitochondrial β -oxidation). Increased oxidative stress associated with mitochondrial dysfunction has been reported in functional defects of hepatic, cardiovascular, renal, and neural systems (Auger et al., 2015; Baranyai et al., 2015; Liu et al., 2016; Luo et al., 2015).

Unlike microsomal CYPs, mitochondrial CYPs are mainly supported by Adx and Adr electron transport system for their catalytic functions. The mitochondria-targeted CYPs preferentially interact with Adx and Adr. Additionally, mitochondrial CYPs may be folded somewhat differently as shown by circular dichroism spectroscopy and UV/Vis spectral analysis of CYP1A1, CYP2B1, and CYP2E1. It was shown that mitochondrial CYPs contain less α -helical and more β -sheet content, indicative of more open folding (Anandatheerthavarada et al., 1999b, 2009; Robin et al., 2001). This may be the reason for altered substrate specificities observed in some cases (see Table 4.1). For example, with use of transgenic mice with either ER-CYP1A1 or MT-CYP1A1, the detoxification of orally induced BaP was more efficient in mouse expressing ER-CYP1A1, not the MT-CYP1A1 (Dong et al., 2009). Mitochondrial CYP1A1, on the other hand, was shown to metabolize relatively large substrates such as erythromycin and various antipsychotic and epileptic drugs listed in Section 4.3.1 (Anandatheerthavarada et al., 1999b; Dasari et al., 2006). Similarly, altered expression of mitochondrial CYP2E1 has been reported in oxidative stress-induced toxicity (Bansal et al., 2010, 2012, 2013; Knockaert et al., 2011; Raza et al., 2004; Robin et al., 2001, 2002, 2005).

Results also show that mitochondria-imported CYPs and heme oxygenase (HO-1) induce oxidative stress and mitochondrial dysfunction (Bajpai et al., 2014; Bansal et al., 2010, 2012, 2014a; Knockaert et al., 2011). Thus, unlike microsomal HO-1 that is protective, mitochondrial HO-1 induces mitochondrial dysfunction under physiological conditions. Some studies report that inhibition of cellular autophagy promotes CYP2E1-induced oxidative stress and mitochondrial dysfunction in HepG2 cells (Wu and Cederbaum, 2013). Reports also show that increased mitochondrial ROS formation related to acetaminophen metabolism is associated with gene expression changes, causing dysfunction of mitochondrial electron transport chain and drug toxicity (Jiang et al., 2015). Reactive metabolites generated by MT-CYP2E1 can also deplete the mitochondrial antioxidant pool and GSH, causing increased oxidative stress (Bansal et al., 2010, 2012; Knockaert et al., 2011; Raza et al., 2004). Therefore,

in pharmacotherapy, “off-target/collateral” mitochondrial toxicity causing mitochondrial dysfunction may be an important factor in determining the efficacy of drugs. This emerging role of mitochondrial drug metabolism should be an important factor to consider during drug development and drug screening.

4.6 Mitochondrial CYPs and Cell Signaling

There are 18 mammalian CYP families that encode 57 genes in the human genome and express a number of CYP enzymes catalyzing the metabolism of a wide array of bioactive endogenous (hormones, vitamins, lipid derivatives, etc.) and exogenous substrate (Neve and Ingelman-Sundberg, 2010). CYP-mediated metabolism is the most prevalent pathway for clearance of more than 200 most prescribed clinical drugs, and over 95% of the drugs are metabolized in order of frequency by microsomal CYPs 3A4, CYP2C9, CYP2D6, CYP2C19, CYP1A2, CYP2C8, and CYP2B6 (Guengerich, 2008; Zanger et al., 2008). Direct CYP-mediated diseases comprise those related to steroidogenesis, defects in fatty acid, cholesterol, vitamin D, and retinoid and eicosanoid metabolism (Miller and Auchus, 2011). Indirectly, as discussed earlier, CYPs play important metabolic roles in cancer, diabetes, cardiovascular, neurodegenerative, and other oxidative stress-associated diseases. Increasing evidence from our laboratory and others has suggested the specialized role of mitochondria-targeted CYPs in the metabolism of selected drugs with unique characteristics. More than 85% of tissue oxygen is utilized by mitochondrial respiratory chain enzymes and other heme proteins, including mitochondria-targeted CYPs. During physiological oxygen metabolism, some leakage of electrons may result in ROS generation, which is further increased under disease and toxicity conditions that contribute to mitochondrial genetic (mtDNA defects) and metabolic stress (mitochondrial dysfunction). As discussed earlier, in addition to metabolizing xenobiotics to reactive electrophiles, mitochondria-targeted CYPs play a significant role in the generation of ROS. At low levels, mitochondrial ROS (mainly superoxide and hydrogen peroxide) are key signaling molecules that connect mitochondrial respiratory chain to communicate with the remainder of the cell to control numerous physiological and pathological responses (Patterson et al., 2015). Therefore, the expanded role of mitochondria is not limited to energy metabolism and cell survival (ATP production), but in cellular protection/repair mechanisms (autophagy/mitophagy) and in cell death (apoptosis) by controlling the signal transduction mechanisms and communication with other organelles.

In particular, mitochondrial dysfunction due to metabolic or genetic stress, as seen in diseases and toxicities, are communicated to the nucleus as an adaptive response through retrograde signaling (Amuthan et al., 2001; Biswas et al., 1999, 2005, 2008; Guha and Avadhani, 2013; Guha et al., 2014; Srinivasan et al., 2016). Retrograde signaling from mitochondria to the nucleus or cytosol controls cell growth and differentiation and hence may play a critical role in toxicity. Alterations in mitochondrial structural and functional dynamics have significant implications in retrograde signaling. Mitochondrial GSH pool and targeted GSH-metabolizing enzymes, for example, GSTA4-4, may also play a role in sending retrograde signaling to cytosol to control thiol-reactive species/ROS-dependent redox signaling pathways (Dodson et al., 2013).

4.7 Conclusion

Mitochondria-based drug metabolisms are involved in controlling the physiological processes as well as in a number of toxicities and diseases. Both mitochondrial DNA and metabolic systems are highly sensitive to changes in the intramitochondrial and extramitochondrial environments. Therefore, alterations in mitochondrial function and communication with other organelles are key factor in diseases and toxicities. Mitochondrial dysfunction has been implicated in many human diseases, including, but not limited to, diabetes, cancer, obesity, cardiovascular, and neurodegenerative disorders. Mitochondrial dysfunction contributes to both the etiology and progression of the diseases and toxicities. Hence, mitochondria have emerged as a potential therapeutic target against numerous human diseases. The emerging therapeutic strategies directed to mitochondria and understanding the mechanisms of drug metabolism and their effects on mitochondrial function in diseases and toxicities are being implemented by researchers in academia and pharmaceuticals companies. Therapeutic application of mitochondria-targeted antioxidants and drugs are increasingly tried experimentally and clinically. There are potential competing metabolic activation and detoxification reactions in mitochondria and extramitochondrial compartments, which lead to the differential generation/clearance of reactive metabolites and, hence, have consequences in drug-induced toxicity. Mitochondrial CYP-dependent drug metabolism, mitochondrial ROS, and redox-regulated cell signaling and communications with other organelles (e.g., retrograde signaling) may exert an integrated response that helps in understanding the critical role of mitochondria in the etiology and progress of diseases and drug-induced toxicities.

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Part 2

Organ Drug Toxicity

Mitochondrial Etiology

5

Mitochondrial Dysfunction in Drug-Induced Liver Injury

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5.1 Introduction

More than 350 drugs of the modern pharmacopoeia have been reported to induce liver injury such as hepatic cytolysis, steatosis, and cholestasis (Björnsson and Hoofnagle, 2016; Weng et al., 2015). In the most severe cases, drug-induced liver injury (DILI) can require liver transplantation or lead to the death of the patient (Björnsson and Hoofnagle, 2016; Robles-Diaz et al., 2014). Moreover, DILI can lead to the withdrawal of drugs from the market, or earlier during clinical trials, thus causing important financial losses (Labbe et al., 2008). Thus, DILI is a major issue for public health and pharmaceutical companies.

Although the mechanisms of DILI are not always known, mitochondrial liability is often present when drug-induced impairment of mitochondrial function is investigated (Labbe et al., 2008; Pessayre et al., 2010; Porceddu et al., 2012; Schumacher and Guo, 2015). However, it is noteworthy that mitochondrial dysfunction is a generic term that includes alteration of several metabolic pathways and damage to different mitochondrial components. Thus, these mitochondrial disturbances can have a variety of deleterious consequences such as oxidative stress, energy shortage, accumulation of triglycerides (steatosis), and cell death. It is also important to underline that drug-induced mitochondrial

dysfunction can be due to the drug itself and/or to reactive metabolites generated through cytochrome P450 (CYP)-mediated biotransformation (Begrache et al., 2011; Fromenty and Pessayre 1995).

In the present chapter, we first briefly recall the major roles of mitochondria in fuel oxidation, energy homeostasis, reactive oxygen species (ROS) production, and cell death. We then describe the main biochemical, histological, and clinical manifestations arising from the different forms of drug-induced mitochondrial dysfunction. Finally, we provide information about different drugs for which enough clinical and experimental data indicate the potential role of mitochondrial dysfunction in the pathogenesis of liver injury.

5.2 Structure and Physiological Role of Mitochondria

5.2.1 Structure and Main Components of Mitochondria

Mitochondria are intracellular organelles with two membranes, namely, the outer and the inner membranes. Because the inner membrane is poorly permeable to various molecules, it contains transporters allowing the entry of many endogenous compounds (e.g., ADP, fatty

acids (FAs), glutathione (GSH), pyruvic acid) and possibly xenobiotics as well (Begrache et al., 2011; Palmieri, 2004; Solazzo et al., 2006). The inner membrane also harbors all the polypeptides mandatory for the mitochondrial respiratory chain (MRC) activity and ATP synthesis (Schon and Fromenty, 2015; Wallace et al., 2010). The outer and inner mitochondrial membranes surround the matrix, a space containing various enzymes involved in different key oxidative pathways such as mitochondrial fatty acid oxidation (mtFAO) and pyruvate oxidation via the tricarboxylic acid (TCA) cycle (Bénit et al., 2014; Houten et al., 2016).

The mitochondrial matrix also contains the mitochondrial DNA (mtDNA) and all the components (e.g., enzymes and transcription factors) mandatory for its replication, transcription, translation, and repair (Taanman, 1999; Wallace et al., 2010). This 16.6 kb circular genome is present within the mitochondrion in several copies and encodes 13 polypeptides of the MRC (Schon and Fromenty, 2015; Wallace et al., 2010). These polypeptides are then inserted within the MRC complexes I, III, IV (cytochrome *c* oxidase), and V (ATP synthase), along with dozens of nuclear DNA-encoded proteins. Permanent mtDNA replication by the DNA polymerase γ allows the maintenance of constant mtDNA levels in cells despite continuous removal of the most dysfunctional and/or damaged mitochondria. PrimPol, a second mtDNA polymerase, has been recently identified, but this enzyme is specifically involved in the bypass of different oxidative and UV-induced lesions (Garcia-Gomez et al., 2013; Mislak and Anderson, 2015). Notably, mtDNA is particularly prone to oxidative damage compared with nuclear DNA, possibly because of its proximity to the MRC (the main cellular source of ROS) and the absence of protective histones (Begrache et al., 2011; Yakes and Van Houten, 1997). Moreover, the repertoire of mtDNA repair enzymes seems to be less complete compared with nuclear DNA. For instance, mitochondria lack a nucleotide excision repair (NER) system (Akhmedov and Marin-Garcia, 2015). The presence of mutations in a significant proportion of mtDNA copies, or a strong reduction of the mtDNA copy number, can lead to mitochondrial dysfunction and severe diseases (Schon and Fromenty, 2015; Wallace et al., 2010).

5.2.2 Oxidation of Pyruvate and Fatty Acids

Mitochondria are able to oxidize many substrates including amino acids (e.g., glutamine, alanine, and valine), pyruvate, and different types of FAs (Rui, 2014; Wallace et al., 2010). Notably, a major aim of such substrate oxidation is to provide enough energy required for cell homeostasis and function.

Pyruvate, which is provided by the glycolysis of glucose, is oxidized by the TCA cycle after its transformation into acetyl-CoA by the mitochondrial enzyme pyruvate dehydrogenase. Succinate dehydrogenase, a FAD-dependent enzyme of the TCA cycle, is one of the five complexes of the MRC and is referred to as complex II. The TCA cycle also includes three different NAD⁺-dependent dehydrogenases.

Mitochondria are also able to oxidize short-chain, medium-chain, and long-chain FAs (Fromenty and Pessayre, 1995; Houten et al., 2016). Whereas short-chain FAs (SCFAs) and medium-chain FAs (MCFAs) freely enter mitochondria, the entry of long-chain FAs (LCFAs) into mitochondria depends on several enzymes, including carnitine palmitoyltransferase 1 (CPT1). During their β -oxidation in mitochondria, SCFAs, MCFAs, and LCFAs undergo four sequential reactions, leading to the release of one acetyl-CoA molecule and a shortened FA. Notably, two of these reactions are catalyzed by different FAD-dependent and NAD⁺-dependent dehydrogenases that have specific activities for SCFAs, MCFAs, or LCFAs (Fromenty and Pessayre, 1995; Houten et al., 2016). The mtFAO cycle is subsequently repeated to split FAs into several acetyl-CoA subunits, which generate acetoacetate and β -hydroxybutyrate. These ketone bodies (KB) are then oxidized in extrahepatic tissues by the TCA cycle in order to generate ATP. mtFAO is regulated by several transcription factors, such as forkhead box A2 (FoxA2), cAMP response element-binding protein (CREB), and peroxisome proliferator-activated receptor α (PPAR α) (Begrache et al., 2013; Massart et al., 2013).

5.2.3 Production of ATP

Mitochondrial pyruvate oxidation and FAO generate FADH₂ and NADH. These reduced cofactors then transfer their electrons to the MRC, thus regenerating the NAD⁺ and FAD necessary for other cycles of fuel oxidation (Begrache et al., 2013; Wallace et al., 2010). The electrons provided by NADH or FADH₂ migrate all the way along the respiratory chain up to cytochrome *c* oxidase (COX), where they safely react with oxygen and protons to form water. The transfer of electrons across MRC complexes I, III, and IV is coupled with the extrusion of protons from the mitochondrial matrix into the intermembrane space of mitochondria, thus creating a large electrochemical potential ($\Delta\psi$) across the inner membrane. When ADP is high, protons reenter the matrix through the F₀ portion of ATP synthase, causing the rotation of a molecular rotor within the F₁ portion of ATP synthase and the conversion of ADP into ATP. The biochemical process linking substrate oxidation to ATP generation is referred to as oxidative phosphorylation (OXPHOS).

5.2.4 Production of ROS as Signaling Molecules

During OXPHOS, a small fraction of the electrons going through the MRC complexes I and III can leak from these complexes and react with oxygen to form the superoxide anion radical ($O_2^{\cdot-}$) (Begrache et al., 2013; Wallace et al., 2010). This ROS is however mostly dismutated by manganese superoxide dismutase (MnSOD) into hydrogen peroxide (H_2O_2). Notably, some enzymes of β -oxidation pathway can also produce significant amount of hydrogen peroxide within mitochondria (Kakimoto et al., 2015; Seifert et al., 2010). Hydrogen peroxide can be subsequently detoxified into water by mitochondrial GSH peroxidases, which require reduced GSH as cofactor (Pessayre et al., 2010; Wallace et al., 2010). The physiological production of mitochondrial ROS serves as key signaling molecules, for instance, by activating transcription factors such as NF-E2-related factor 2 (NRF2) (Hamanaka and Chandel, 2010; Rigoulet et al., 2011). However, the excessive production of mitochondrial ROS eventually leads to oxidative stress, as discussed later on.

5.3 Main Consequences of Hepatic Mitochondrial Dysfunction

5.3.1 Consequences of Mitochondrial β -Oxidation Inhibition

Because mtFAO is providing a major part of the ATP required for cell homeostasis and function (especially in the fasted state), any significant impairment of this metabolic pathway can lead to energy shortage, hepatocyte necrosis, and cytolytic hepatitis (Figure 5.1) (Begrache et al., 2011; Fromenty and Pessayre, 1995). This liver lesion encompasses a large spectrum of liver injury of different severity because the destruction of hepatocytes (i.e., cytotoxicity) can involve a variable amount of the hepatic mass. While the mildest forms of cytolytic hepatitis are characterized by an isolated increase in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the most severe cases can be associated with fulminant hepatitis and severe hepatic dysfunction.

Inhibition of the mtFAO also leads to the accumulation of lipids, which is referred to as hepatic steatosis (Figure 5.1). Although most of the accumulated lipids are

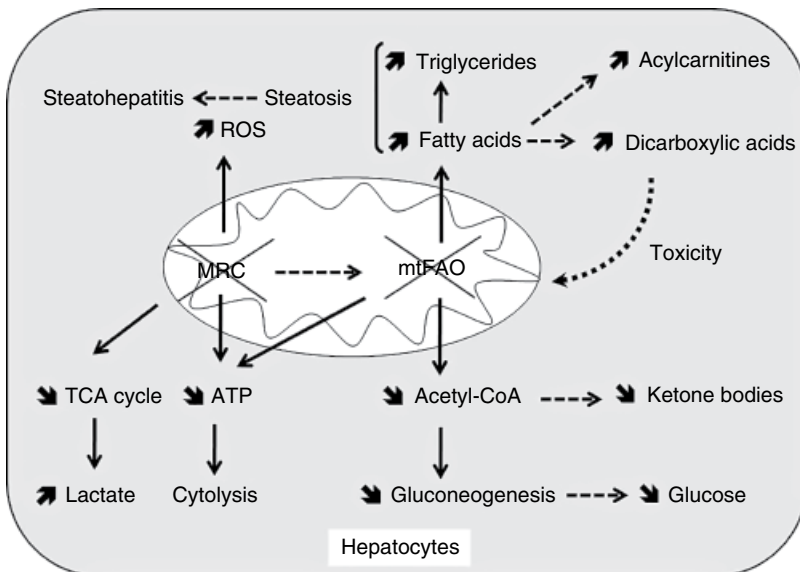


Figure 5.1 Consequences of severe inhibition of mitochondrial fatty acid oxidation and respiratory chain. A severe inhibition of mitochondrial fatty acid oxidation (mtFAO) can induce different deleterious effects for the hepatocytes. (i) Accumulation of free fatty acids and triglycerides, thus explaining the occurrence of steatosis. Free fatty acids are also metabolized into acylcarnitine derivatives and dicarboxylic acids, which can be detected in plasma and urine. Free fatty acids and dicarboxylic acids can be toxic for mitochondria, thus reinforcing mitochondrial dysfunction. (ii) Reduction of ATP synthesis, which can induce energy shortage and hepatic cytotoxicity. (iii) Decreased ketogenesis with lower generation of ketone bodies (mainly acetoacetate and β -hydroxybutyrate). (iv) Inhibition of gluconeogenesis with decreased hepatic glucose production. Low plasma levels of ketone bodies and glucose can be responsible for a profound energy deficiency in extrahepatic tissues. A severe impairment of the mitochondrial respiratory chain (MRC) activity can also have different harmful consequences: (i) reduction of ATP synthesis, thus inducing energy shortage and hepatic cytotoxicity; (ii) a secondary inhibition of the tricarboxylic acid cycle (TCA), thus leading to an accumulation of lactate and subsequent lactic acidosis; (iii) a secondary inhibition of mtFAO with the previously mentioned consequences; and (iv) increased generation of mitochondrial ROS, which could favor the progression of steatosis to steatohepatitis.

triglycerides, other lipid derivatives can be present in a significant amount such as free FAs, dicarboxylic acids, and acylcarnitine intermediates (Figure 5.1) (Fromenty and Pessayre, 1995; Labbe et al., 2008). Accumulation of free FAs and dicarboxylic acids could reinforce drug-induced mitochondrial dysfunction because these lipid derivatives are able to impair key biochemical processes such as OXPHOS and TCA cycle (Begrache et al., 2011; Fromenty and Pessayre, 1995). It has been postulated that severe inhibition of mitochondrial β -oxidation induces microvesicular steatosis, whereas milder inhibition of this metabolic pathway leads to macrovacuolar steatosis (Fromenty and Pessayre, 1995; Massart et al., 2013). The size of the fat droplets could also depend on the nature and/or the abundance of some proteins wrapping the lipids (Bickel et al., 2009; Massart et al., 2013). Finally, inhibition of mitochondrial β -oxidation can lead to hypoglycemia in particular because lower acetyl-CoA levels reduce the activity of pyruvate carboxylase, a rate-limiting enzyme in gluconeogenesis (Figure 5.1) (Fromenty and Pessayre, 1995).

5.3.2 Consequences of MRC Inhibition

MRC inhibition can induce a significant ATP shortage, thus leading to hepatocyte necrosis (Figure 5.1) (Begrache et al., 2011; Pessayre et al., 2010). Another important consequence of MRC inhibition is the secondary impairment of mitochondrial β -oxidation and TCA cycle. This is because the lack of NADH and FADH₂ oxidation strongly reduces FAD and NAD⁺ levels, thus impeding the activity of the different FAD-dependent and NAD⁺-dependent dehydrogenases of the β -oxidation and TCA cycle pathways (Fromenty and Pessayre, 1995; Massart et al., 2013). Secondary impairment of mtFAO due to MRC inhibition explains why some drugs such as nucleoside reverse transcriptase inhibitors (NRTIs) are able to induce steatosis, as discussed later on. Impairment of the TCA cycle can lead to hyperlactatemia and lactic acidosis because the conversion of unmetabolized pyruvate to lactate by lactate dehydrogenase (LDH) is favored by NADH accumulation (Igoudjil et al., 2006; Margolis et al., 2014).

Another important consequence of MRC inhibition can be ROS overproduction (Figure 5.1) (Li et al., 2003; Pessayre et al., 2010). Indeed, when the flow of electrons is blocked at some point downstream to complexes I and III, these MRC complexes become overly reduced, thus leading to higher leakage of electrons and superoxide anion overproduction (Begrache et al., 2011; Pessayre et al., 2010). Notably, drug-induced mitochondrial ROS overproduction can favor lipid peroxidation and different types of mtDNA oxidative damage (Berson et al., 1998; Igoudjil et al., 2006; Lewis et al., 2001; Schon and

Fromenty, 2015). Because MRC inhibition can secondarily induce steatosis and oxidative stress, it has been proposed that drugs targeting this mitochondrial component could be more likely to favor steatohepatitis, a liver disease characterized by steatosis, necroinflammation, and fibrosis (Berson et al., 1998; Pessayre et al., 2010; Massart et al., 2013).

Finally, it is possible that MRC inhibition and ATP shortage could participate to the occurrence of hepatic cholestasis. Indeed, several bile acid transporters are ATP dependent (Aleo et al., 2014; Erlinger, 1996; Hirohashi et al., 2000), so that any significant energy deficiency is expected to hamper bile acid secretion from the hepatocytes. Notably, several mitochondrial genetic diseases with hepatic dysfunction are characterized by the frequent occurrence of cholestasis in addition to steatosis and ballooning degeneration of hepatocytes (Bianchi et al., 2011; El-Hattab and Scaglia, 2013; Schon and Fromenty, 2015).

5.3.3 Consequences of Mitochondrial Membrane Permeabilization

In some pathophysiological circumstances, mitochondrial membranes can lose their structural and functional integrity after the opening of the mitochondrial permeability transition (MPT) pores. MPT pore opening is leading to the swelling of the mitochondria because of water accumulation in the matrix and to the rupture of the outer mitochondrial membrane (Marroquin et al., 2014; Pessayre et al., 2010). Cyclophilin D and the c subunit of mitochondrial ATP synthase have been shown to be key components of the MPT pores, although other proteins could be involved such as adenine nucleotide transporter (ANT) (Bernardi, 2013; Bonora et al., 2015; Pessayre et al., 2010). Notably, the immunosuppressive drug cyclosporine A is able to reduce the opening probability of the MPT pores by specifically binding to cyclophilin D.

Whatever the triggering factor, MPT pore opening can profoundly disturb ATP synthesis through the loss of inner mitochondrial membrane integrity and OXPHOS impairment. If numerous mitochondria present opened MPT pores, ATP stores will collapse rapidly and necrosis will occur (Begrache et al., 2011; Pessayre et al., 2010). In contrast, if MPT pore opening takes place only in some mitochondria, ATP levels will be maintained thanks to undamaged organelles. However, the rare swollen mitochondria involved in MPT pore opening will release different proapoptotic proteins including the apoptosis-inducing factor (AIF) and cytochrome *c* (Begrache et al., 2011; Pessayre et al., 2010). Hence, MPT pore opening could be one important mechanism whereby some drugs can induce

cytolytic hepatitis (Begrache et al., 2011; Jaeschke et al., 2002; Pessayre et al., 2010). However, it should be underscored that studies pertaining to drug-induced MPT pore opening are sometimes performed in mitochondria de-energized with oligomycin and in the presence of high concentrations of calcium (e.g., from 10 to 50 μ M). Since these conditions have a profound impact on MPT pore opening (Bernardi et al., 2006), it is difficult to extrapolate some data to the *in vivo* situation.

Finally, it is noteworthy that other modes of mitochondrial membrane permeabilization not involving MPT pores have been described (Begrache et al., 2011; Belosludtsev et al., 2009; Lei et al., 2006). For instance, the proapoptotic proteins Bax and Bak can oligomerize into proteolipid pores within the mitochondrial outer membrane, thus promoting its permeabilization and cytochrome *c* release (Luna-Vargas and Chipuk, 2016; Pessayre et al., 2010). Such mechanisms of mitochondrial membrane permeabilization are thus insensitive to cyclosporine A (Marroquin et al., 2014). Nonetheless, MPT-independent mitochondrial membrane permeabilization can eventually induce cell death (Belosludtsev et al., 2009; Luna-Vargas and Chipuk, 2016; Pessayre et al., 2010).

5.4 Main Hepatotoxic Drugs Inducing Mitochondrial Dysfunction

Numerous hepatotoxic drugs have been reported to impair mitochondrial function via different mechanisms (Fromenty and Pessayre, 1995; Massart et al., 2013; Nadanaciva and Will, 2011; Nadanaciva et al., 2012; O'Brien et al., 2006; Porceddu et al., 2012). Interestingly, our recent investigations with 124 drugs revealed a highly significant relationship between drug-induced mitochondrial toxicity and DILI occurrence in patients (Porceddu et al., 2012). However, when considering each drug individually, such ability to impair mitochondrial function does not necessarily mean that mitochondrial liability is a key mechanism leading to liver injury. Indeed, mitochondrial dysfunction could occur at suprapharmacological concentrations, and furthermore, other mechanisms of liver toxicity could also play a significant role such as endoplasmic reticulum (ER) stress, lysosomal permeabilization, and nuclear DNA damage (Foufelle and Fromenty, 2016; Hu et al., 2016; Pessayre et al., 2010). In addition, cellular events such as ER stress and lysosomal permeabilization can secondarily impair mitochondrial function (Foufelle and Fromenty, 2016; Kon et al., 2010). It is also noteworthy that mitochondrial dysfunction in DILI can occur secondary to the activation of the apoptotic cascade by Fas ligand, tumor

necrosis factor- α (TNF α), or other cytokines (Pessayre et al., 1999, 2010). Thus, it is sometimes difficult to ascertain whether mitochondrial dysfunction is due to a direct effect of the drug on mitochondria or a secondary consequence of extramitochondrial events. More information regarding the role of inflammation and immunity in DILI can be found in several excellent recent reviews (Jaeschke et al., 2012; Ju and Reilly, 2012; Luedde et al., 2014). Hence, the present chapter will deal only with drugs for which different experimental and/or clinical investigations strongly support a role of direct mitochondrial dysfunction in DILI pathogenesis.

5.4.1 Acetaminophen

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP), also called paracetamol, is one of the most widely prescribed drugs for the management of pain and hyperthermia (Table 5.1). Although APAP is usually considered as safe, the inadvertent or intentional ingestion of an excessive dose of this drug can cause massive hepatocellular necrosis and acute liver failure, which can be fatal (Michaut et al., 2014; Yoon et al., 2016). Hepatic steatosis can also be observed after acetaminophen intoxication (Biour et al., 2004; Ramachandran and Kakar, 2009). Besides the context of APAP overdose, it is noteworthy that therapeutic dose of APAP (i.e., 3–4 g/day) can induce mild to moderate hepatic cytolysis and even fulminant hepatitis in a few patients (Claridge et al., 2010; van Rongen et al., 2016). Such therapeutic misadventures seem to occur preferentially in patients with predisposing factors such as malnutrition, comedication with different drugs, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD) (Claridge et al., 2010; Michaut et al., 2014).

APAP is mainly metabolized in the liver into harmless glucuronide and sulfate conjugates. However, a small amount of APAP is oxidized to the highly toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) by several CYPs, in particular CYP2E1 and CYP3A4 (Michaut et al., 2016; Yoon et al., 2016). Interestingly, increased hepatic CYP2E1 expression in chronic alcoholic liver disease and NAFLD could be an important mechanism explaining why these diseases are associated with a higher risk of APAP-induced hepatotoxicity (Michaut et al., 2014; Schmidt et al., 2002).

NAPQI is usually safely detoxified by hepatic GSH when APAP is taken at the recommended dose. However, high levels of NAPQI after an acute overdose consistently induce a fall in the GSH stores, thus promoting the covalent binding of free NAPQI to different cellular proteins, in particular at the mitochondrial level (McGill and Jaeschke, 2013; Xie et al., 2015). The covalent binding of NAPQI to critical mitochondrial proteins is deemed

Table 5.1 Mechanisms of mitochondrial dysfunction induced by different hepatotoxic drugs.

Drug(s)	Therapeutic class	Main liver injury and lesions	Mechanism(s) of mitochondrial dysfunction	Additional information
Acetaminophen (APAP)	Antalgic, antipyretic	Hepatic cytolysis, steatosis	Inhibition of MRC activity by NAPQI, a CYP-generated reactive metabolite	The presence of CYP2E1 in mitochondria could favor the local generation of NAPQI. Refer to the text for further details
Amineptine	Antidepressant	Hepatic cytolysis, cholestasis, steatosis	Inhibition of mtFAO	Amineptine was withdrawn from the market, in particular because of its hepatotoxicity. Refer to Le Dinh et al. (1988) and Fromenty and Pessayre (1995) for further details
Alpidem	Anxiolytic	Hepatic cytolysis	Inhibition of MRC activity, MPT pore opening	Alpidem was withdrawn from the market because of its hepatotoxicity. Refer to Berson et al. (2001a) for further details
Amiodarone	Antiarrhythmic, antianginal	Hepatic cytolysis, steatosis, steatohepatitis, cirrhosis, phospholipidosis, cholestasis	Dual effect on OXPHOS (uncoupling and inhibition) depending on the concentrations. Direct inhibition of mtFAO	Some of the amiodarone-induced mitochondrial effects can be explained by its “cationic amphiphilic” structure (Figure 5.2). Refer to the text for further details
Aspirin (acetylsalicylic acid)	Antalgic, antipyretic, NSAID	Hepatic cytolysis, steatosis, cholestasis	OXPHOS uncoupling (mild). Inhibition of mtFAO by sequestration of CoA and possibly L-carnitine	The use of aspirin could favor the development of Reye syndrome, a severe condition characterized by microvesicular steatosis and encephalopathy. Refer to Deschamps et al. (1991) and Fromenty and Pessayre (1995) for further details
Buprenorphine	Antalgic agent used to treat opioid addiction	Hepatic cytolysis, steatosis	Inhibition of MRC activity and mtFAO	Refer to Berson et al. (2001b) and Pessayre et al. (2010) for further details
Ciprofloxacin	Antibiotic	Hepatic cytolysis, cholestasis, steatosis	Possible involvement of mtDNA depletion	Refer to Pessayre et al. (2010) and Schon and Fromenty (2015) for further details
Diclofenac	NSAID	Hepatic cytolysis, cholestasis, steatosis	Dual effect on OXPHOS (uncoupling and inhibition) depending on the concentrations. OXPHOS inhibition could be due to an impairment of complex V (ATP synthase) and adenine nucleotide translocase (ANT) activity	Refer to Moreno-Sanchez et al. (1999), Nadanaciva et al. (2007b), Nadanaciva et al. (2013) and Syed et al. (2016) for further details
Fialuridine	Antiviral (anti-HBV)	Steatosis, cholestasis	Inhibition of mtDNA replication leading to mtDNA depletion (Figure 5.3)	Refer to the text for further details
Ganciclovir	Antiviral	Hepatic cytolysis, cholestasis, diffuse fibrosis	Possible involvement of mtDNA depletion	Refer to Pessayre et al. (2010) and Schon and Fromenty (2015) for further details
Ibuprofen	NSAID	Hepatic cytolysis, cholestasis, steatosis	OXPHOS uncoupling (mild). Inhibition of mtFAO	Refer to Browne et al. (1999), Fréneaux et al. (1990) and Fromenty and Pessayre (1995) for further details
Linezolid	Antibiotic	Hepatic cytolysis, steatosis	Inhibition of mitochondrial protein synthesis	Refer to the text for further details

Nimesulide	NSAID	Hepatic cytolysis, cholestasis	Dual effect on OXPHOS (uncoupling and inhibition) depending on the concentrations. OXPHOS inhibition could be due to an impairment of adenine nucleotide translocase (ANT) activity	Refer to Berson et al. (2006), Moreno-Sanchez et al. (1999) and Nadanaciva et al. (2013) for further details
Nucleoside reverse transcriptase inhibitors (NRTIs)	Antiretroviral (anti-HIV)	Hepatic cytolysis, steatosis, steatohepatitis, cirrhosis, cholestasis	Inhibition of mtDNA replication leading to mtDNA depletion (Figure 5.3). mtDNA-independent mitochondrial dysfunction could occur with some NRTIs	Refer to the text for further details
Perhexiline	Antianginal	Hepatic cytolysis, steatosis, steatohepatitis, cirrhosis, phospholipidosis, cholestasis	Dual effect on OXPHOS (uncoupling and inhibition) depending on the concentrations. Direct inhibition of mtFAO	This cationic amphiphilic drug impairs mitochondrial function in a similar way than amiodarone. Refer to Deschamps et al. (1994), Fromenty and Pessayre (1995), Hamdan et al. (2001), Kennedy et al. (1996) and Schumacher and Guo (2015) for further details
Tacrine	Anti-Alzheimer	Hepatic cytolysis, steatosis	OXPHOS uncoupling and possible involvement of mtDNA depletion	Refer to Berson et al. (1996), Mansouri et al. (2003) and Schon and Fromenty (2015) for further details
Tamoxifen	Anticancer	Steatosis, steatohepatitis, fibrosis, cirrhosis	Dual effect on OXPHOS (uncoupling and inhibition) depending on the concentrations. Direct inhibition of mtFAO. Possible involvement of mtDNA depletion	Refer to the text for further details
Tianeptine	Antidepressant	Hepatic cytolysis, cholestasis, steatosis	Inhibition of mtFAO	Refer to Fromenty et al. (1989) and Fromenty and Pessayre (1995) for further details
Tetracycline	Antibiotic	Steatosis, cholestasis, hepatic cytolysis	Inhibition of mtFAO. Possible involvement of the inhibition of mitochondrial protein synthesis	Refer to the text for further details
Troglitazone	Antidiabetic	Hepatic cytolysis, cholestasis, steatosis	Inhibition of different MRC complexes	Refer to the text for further details
Valproic acid	Antiepileptic	Hepatic cytolysis, steatosis, cholestasis	Inhibition of mtFAO via several mechanisms (Figure 5.4)	Refer to the text for further details

to be a major mechanism leading to profound OXPHOS impairment, ATP shortage, and subsequent hepatocellular necrosis (McGill and Jaeschke, 2013; Xie et al., 2015). Among the different components of the MRC, complex II (succinate dehydrogenase) seems to be very sensitive to NAPQI (Burcham and Harman, 1991; Lee et al., 2015). ROS-induced activation of c-Jun N-terminal kinase (JNK) might also play a significant role in APAP-induced mitochondrial dysfunction and liver injury (Hinson et al., 2010; McGill and Jaeschke, 2013), although some investigations do not support such role (Cubero et al., 2016; Michaut et al., 2016).

Some data strongly suggest that APAP-induced OXPHOS inhibition can secondarily impair mtFAO. First, APAP can induce steatosis in intoxicated patients and rodents (Aubert et al., 2012; Biour et al., 2004; Ramachandran and Kakar, 2009). Second, APAP liver injury in humans and mice is associated with the presence of circulating acylcarnitine derivatives (Bhattacharyya et al., 2014; McGill et al., 2014). Although investigations reported that NAPQI covalently inhibited MRC activity, in particular at the level of complex II (Burcham and Harman, 1991; Lee et al., 2015), it would be interesting to determine whether this reactive metabolite could also be able to inactivate one or several enzymes involved in the mtFAO pathway.

Different investigations performed in rodent and human livers consistently showed that CYP2E1 is located not only in the ER compartment but also within mitochondria (Bansal et al., 2013; Knockaert et al., 2011a; Robin et al., 2005). The presence of CYP2E1 within mitochondria might play a significant role in APAP-induced mitochondrial dysfunction and liver injury because intramitochondrial CYP2E1 is expected to generate NAPQI close to the MRC complexes and other possible key NAPQI mitochondrial targets. In keeping with this assumption, investigations performed in COS-7 cells expressing CYP2E1 only in mitochondria showed that APAP treatment was associated with ROS overproduction, GSH depletion, mitochondrial dysfunction, and cytotoxicity (Knockaert et al., 2011b).

5.4.2 Amiodarone

Amiodarone is a broad-spectrum antiarrhythmic drug that also presents an antianginal effect (Table 5.1). It can induce different types of liver lesions including hepatic cytolysis, steatosis, steatohepatitis, cirrhosis, phospholipidosis, and cholestasis (Biour et al., 2004; Fromenty and Pessayre, 1995; Wang et al., 2013).

Different investigations provided evidence that mitochondrial dysfunction is a major mechanism of amiodarone-induced liver toxicity, in particular regarding steatosis and steatohepatitis (Card et al., 1998;

Felser et al., 2013; Fromenty and Pessayre, 1995; Fromenty et al., 1990a, b, 1993; Kennedy et al., 1996; Schumacher and Guo, 2015; Serviddio et al., 2011; Spaniol et al., 2001; Waldhauser et al., 2006). Actually, investigations performed in isolated mouse liver mitochondria disclosed that this cationic amphiphilic drug presents a dual effect on mitochondrial respiration and OXPHOS depending on its concentration. Indeed, at relatively low concentrations (20–100 μ M), amiodarone is able to uncouple OXPHOS and stimulate mitochondrial respiration via a protonophoric effect (Figure 5.2) (Fromenty et al., 1990b). However, higher concentrations lead to an intramitochondrial accumulation of the drug that rapidly induces an inhibition of MRC activity, especially at the level of MRC complexes I and II (Figure 5.2). Hence, stimulation of mitochondrial respiration (i.e., OXPHOS uncoupling) is only transient at high amiodarone concentrations (Fromenty et al., 1990b). Subsequent studies performed by other investigators confirmed that amiodarone presents such dual effect on OXPHOS depending on its concentration (Felser et al., 2013; Serviddio et al., 2011; Spaniol et al., 2001). Notably, both OXPHOS uncoupling and MRC inhibition can impair ATP synthesis so that amiodarone-induced energy shortage could occur for low intracellular concentrations of this drug. The ability of amiodarone to inhibit ATP synthesis might explain the occurrence of acute hepatic cytolysis in some treated patients (Biour et al., 2004; Verhovez et al., 2011; von Vital et al., 2011).

Amiodarone is also able to inhibit mtFAO (Fromenty and Pessayre, 1995; Fromenty et al., 1990a). Although this effect could be secondary to MRC inhibition, some investigations also suggested that mtFAO could be directly inhibited by amiodarone. In particular, long-chain acyl-CoA dehydrogenase (LCAD) and CPT1 are two major mtFAO enzymes that could be targeted by amiodarone (Hamdan et al., 2001; Kennedy et al., 1996; Serviddio et al., 2011; Spaniol et al., 2001). The inhibitory effect of amiodarone on both mtFAO and MRC might explain why this drug can cause not only steatosis but also steatohepatitis in some patients (Begrache et al., 2011; Berson et al., 1998; Fromenty and Pessayre, 1995).

The ability of amiodarone to impair mitochondrial function is linked, at least in part, to its accumulation within the mitochondrial matrix (Fromenty and Pessayre, 1995; Fromenty et al., 1990b; Spaniol et al., 2001). Interestingly, amiodarone can also accumulate inside the lysosomes where it impairs phospholipase activity and alters other key lysosomal components (Ikeda et al., 2008; Kodavanti and Mehendale, 1990). These lysosomal effects explain why amiodarone can frequently cause phospholipidosis, a liver lesion that seems to have limited biochemical or clinical consequence (Fromenty and Pessayre, 1995).

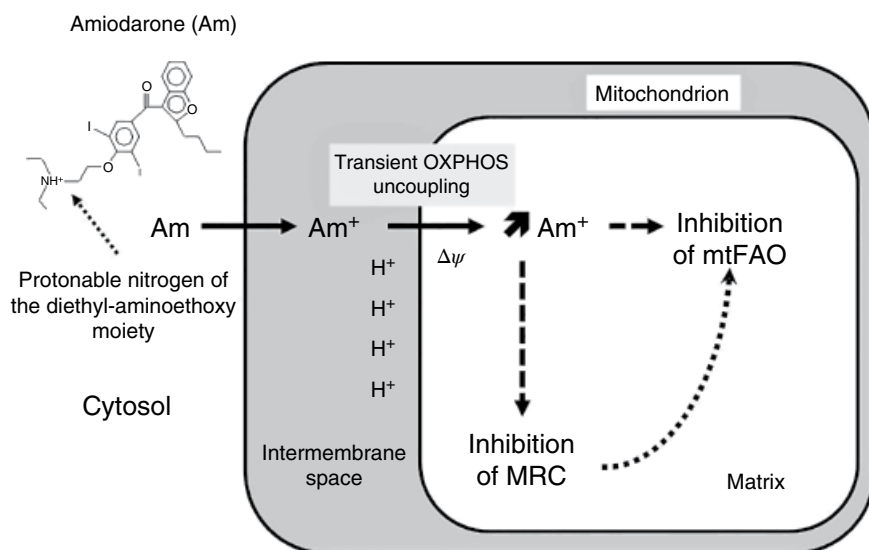


Figure 5.2 Mechanisms of amiodarone-induced impairment of oxidative phosphorylation and mitochondrial fatty acid oxidation. Amiodarone (Am) is an amphiphilic compound that harbors a protonable nitrogen within its diethyl-aminoethoxy moiety. In the intermembrane space of mitochondria, which is an acidic milieu, Am undergoes a protonation to generate Am⁺. This cationic derivative thus freely enters the mitochondrion thanks to the mitochondrial transmembrane potential $\Delta\psi$. The entry of the protonated molecule Am⁺ has two major consequences regarding oxidative phosphorylation (OXPHOS), mitochondrial fatty acid oxidation (mtFAO), and the mitochondrial respiratory chain (MRC): (i) a transient uncoupling of OXPHOS since protons are not entering the matrix by the use of the ATP synthase and (ii) a progressive accumulation of Am⁺ in the mitochondrial matrix, which induces the subsequent inhibition of different enzymes involved in MRC and mtFAO. Hence, amiodarone-induced inhibition of mtFAO could be due to direct impairment of FAO enzymes (e.g., CPT1 and LCAD) and to MRC inhibition, in particular at the level of complexes I and II.

5.4.3 Fialuridine

Fialuridine (FIAU) is a nucleoside analogue that has been developed in the early 1990s for the treatment of chronic hepatitis B virus (HBV) infection (Table 5.1). However, clinical trials were prematurely interrupted because FIAU induced several cases of lactic acidosis, microvesicular steatosis, and severe hepatic failure requiring liver transplantation or leading to death (Fromenty and Pessayre, 1995; McKenzie et al. 1995; Schon and Fromenty, 2015). Besides steatosis, the histopathologic changes could also include marked bile ductular proliferation and cholestasis (Kleiner et al., 1997; McKenzie et al. 1995). FIAU hepatotoxicity was sometimes associated with other adverse effects such as pancreatitis, neuropathy, and myopathy (McKenzie et al. 1995).

New experimental investigations after this therapeutic misadventure showed that FIAU strongly inhibits DNA polymerase γ and reduces mtDNA levels by an unusual mechanism (Figure 5.3) (Lewis et al., 1996, 1997). Indeed, unlike antiretroviral drugs such as stavudine (d4T) and zidovudine (AZT) whose incorporation into a growing chain of mtDNA blocks mtDNA replication (see following text), FIAU can be incorporated into the mitochondrial genome without immediately terminating its replication. This is because FIAU presents a 3' hydroxyl group on the sugar moiety, thus allowing the subsequent insertion of other nucleotides. Nevertheless, when

several adjacent molecules of FIAU are successively incorporated into the mitochondrial genome, DNA polymerase γ is strongly inhibited (Figure 5.3). This subsequently blocks further mtDNA replication and leads to severe mtDNA depletion, destroyed mitochondrial cristae, and OXPHOS deficiency (Lewis et al., 1996, 1997).

It is still unclear why FIAU toxicity was not detected during the preclinical safety studies performed in rats and dogs (Labbe et al., 2008). A possible explanation is that these animal species do not express the specific nucleoside transporter responsible for the mitochondrial entry of this drug (Lee et al., 2006). The human mtDNA polymerase γ and PrimPol might also have a higher capacity to incorporate FIAU into mtDNA. Whatever the mechanism of this interspecies difference, the FIAU story underscores the difficulty to detect drug-induced hepatotoxicity during preclinical safety studies (Labbe et al., 2008). For some drugs such as FIAU, the use of chimeric mice with humanized livers could be of great interest (Xu et al., 2014).

5.4.4 Linezolid

The oxazolidinone linezolid is an antibiotic used against drug-resistant Gram-positive pathogens (Table 5.1). Like many other antibiotics, linezolid impairs bacterial growth by inhibiting protein synthesis. Moderate to

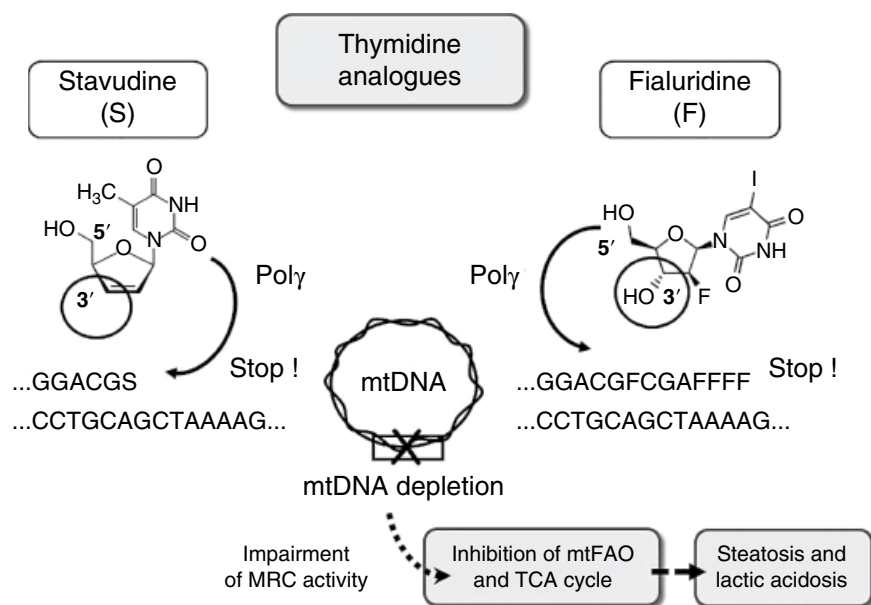


Figure 5.3 Mechanisms of inhibition of mitochondrial DNA replication by the thymidine analogues stavudine and fialuridine. The antiviral stavudine (d4T) and fialuridine (FIAU) are able to impair mitochondrial DNA (mtDNA) replication by two different mechanisms. The antiretroviral nucleoside reverse transcriptase inhibitor (NRTI) d4T is a thymidine analogue in which the hydroxyl group (OH) in the 3' position on the sugar ring is replaced by a hydrogen atom. D4T (S) can be incorporated into the growing chain of mtDNA by the DNA polymerase γ (Poly). However, if d4T is not removed by the proofreading activity of this DNA polymerase, mtDNA replication is blocked. Indeed, no other nucleotides can be incorporated after d4T because the DNA chain now lacks a 3'OH end. Note that other NRTIs such as zidovudine (AZT) and didanosine (ddI) are also able to inhibit mtDNA replication via a similar mechanism. The anti-HBV FIAU is also a thymidine analogue, but it was never marketed because of severe hepatotoxicity. Unlike d4T, FIAU (F) can be incorporated into mtDNA without immediately stopping mtDNA replication since this drug carries a 3'OH group on the sugar moiety. However, when several adjacent molecules of FIAU are successively incorporated into a growing chain of mtDNA, DNA Poly is strongly inhibited. D4T- and FIAU-induced blockage of mtDNA replication can lead to severe mtDNA depletion and impairment of mitochondrial respiratory chain (MRC) activity, which subsequently inhibits mitochondrial fatty acid oxidation (mtFAO) and tricarboxylic acid (TCA) cycle. Impairment of mtFAO and TCA cycle can secondarily induce severe hepatic steatosis and lactic acidosis, respectively.

severe liver injury can occur in some patients after several weeks of treatment, with the occurrence of increased plasma transaminases and several types of hepatic lesions such as macrovacuolar and microvesicular steatosis as well as bile duct damages (De Bus et al., 2010; De Vriese et al., 2006; Garazzino et al., 2011). In addition, severe lactic acidosis has been observed in some of these patients and in others (Carson et al., 2007; De Bus et al., 2010; Velez and Janech, 2010). Prolonged administration of linezolid can also induce peripheral and optic neuropathy, skeletal myopathy, thrombocytopenia, bone marrow suppression, and renal failure (Schon and Fromenty, 2015; Song et al., 2015; Takahashi et al., 2011). Long-term treatment, high doses, and elevated linezolid blood concentrations seem to favor the occurrence of adverse effects (Song et al., 2015; Takahashi et al., 2011). Interestingly, the spectrum of linezolid-induced adverse effects is similar to the one observed with the antiviral NRTIs, as mentioned in the following text.

Linezolid is a potent inhibitor of mitochondrial protein synthesis via an interaction with the mitochondrial ribosomes (Flanagan et al., 2015; Leach et al., 2007;

McKee et al., 2006). This mitochondrial effect secondarily reduces the activity of MRC complexes that contain mtDNA-encoded proteins (De Vriese et al., 2006; Garrabou et al., 2007; Nagiec et al., 2005). Interestingly, the mtDNA A2706G polymorphism is suspected to favor linezolid-induced lactic acidosis (Carson et al., 2007; Del Pozo et al., 2014; Velez and Janech, 2010). However, further investigations will be needed to decipher the mechanism whereby the mtDNA A2706G polymorphism might favor linezolid-induced mitochondrial dysfunction and to determine whether this mtDNA polymorphism also increases the risk of liver injury.

5.4.5 Nucleoside Reverse Transcriptase Inhibitors

The NRTIs are the first antiretroviral drugs marketed for the treatment of human immunodeficiency virus (HIV) infection (Table 5.1). NRTIs include AZT, d4T, lamivudine (3TC), didanosine (ddI), and abacavir (ABC). Notably, these drugs are 2',3'-dideoxynucleoside analogues in which the hydroxyl group in the 3' position on the sugar

ring is replaced by either an hydrogen atom or another group unable to form a phosphodiester linkage. The lack of a 3' hydroxyl group is required for the inhibition of HIV reverse transcriptase activity.

NRTI-induced liver injury includes hepatic cytolysis, microvesicular and/or macrovacuolar steatosis, steatohepatitis, cirrhosis, and cholestasis (Biour et al., 2004; Massart et al., 2013; Wang et al., 2013). Such hepatotoxicity is particularly observed with d4T, ddI, and AZT. In addition to hepatotoxicity, NRTIs can induce other adverse effects such as renal dysfunction, myopathy, pancreatitis, peripheral neuropathy, bone marrow suppression, lipodystrophy, and lactic acidosis (Caron-Debarle et al., 2010; Fromenty and Pessayre, 1995; Margolis et al., 2014).

It is now acknowledged that most of the side effects induced by NRTIs are the consequence of mitochondrial dysfunction (Fromenty and Pessayre, 1995; Gardner et al., 2014; Igoudjil et al., 2006). Indeed, these drugs are able to impair mtDNA replication, thus inducing mtDNA depletion and OXPHOS impairment (Arnaudo et al., 1991; Igoudjil et al., 2006). More precisely, NRTIs are acting as chain terminators because their incorporation into the growing chain of mtDNA does not allow the addition of other nucleotides by the DNA polymerase γ (Figure 5.3) (Fromenty and Pessayre, 1995; Gardner et al., 2014; Igoudjil et al., 2006). Hence, the lack of a 3' hydroxyl group is responsible not only for the antiretroviral activity of NRTIs but also explains their mitochondrial toxicity. Actually, the ability of NRTIs to deplete hepatic mtDNA in treated patients greatly varies among the analogues. In particular, the so-called D-drugs (ddC, ddI, d4T) reduce liver mtDNA levels more strongly than other NRTIs, possibly because of their potent inhibitory effect on DNA polymerase γ (Igoudjil et al., 2006; Walker et al., 2004). mtDNA depletion and OXPHOS impairment in other mtDNA-containing tissues most probably explain the occurrence of a large array of adverse effects (Fromenty and Pessayre, 1995; Gardner et al., 2014; Igoudjil et al., 2006). Interestingly, a mutation (R964C) in the gene encoding the DNA polymerase γ (POLG) might favor NRTI-induced mitochondrial toxicity, possibly by enhancing the probability of NRTI incorporation within the mtDNA molecules (Bailey et al., 2009; Yamanaka et al., 2007). POLG polymorphisms might also favor NRTI-induced mitochondrial toxicity (Baruffini et al., 2015).

Some NRTIs such as AZT and d4T could have mitochondrial and metabolic effects through mechanisms unrelated to the inhibition of DNA polymerase γ and mtDNA depletion (Apostolova et al., 2011; Igoudjil et al., 2006, 2007; 2008; Lund et al., 2007). For instance, both NRTIs were reported to reduce the activity of succinate dehydrogenase (Gerschenson et al., 2001; Igoudjil et al., 2006), an MRC complex that does not include

mtDNA-encoded polypeptides. Treatment of rat hepatocytes with high concentrations of d4T (i.e., 0.75 and 1.0 mM) induced a significant impairment of mtFAO after 48 and 72 h, which was alleviated by a cotreatment with L-carnitine and clofibrate (Igoudjil et al., 2008). Importantly, impairment of mtFAO in d4T-treated rat hepatocytes was associated with an accumulation of neutral lipids, but mtDNA levels were unchanged (Igoudjil et al., 2008). Administration of high doses of d4T in mice for 2 weeks also impaired mtFAO and induced steatosis in liver via an mtDNA-independent mechanism (Igoudjil et al., 2007). Interestingly, a cotreatment with L-carnitine prevented different deleterious effects in d4T-treated mice including impairment of mtFAO, hepatic steatosis, and increased levels of plasma ALT (Igoudjil et al., 2007). Hence, the aforementioned data and others (Apostolova et al., 2011; Igoudjil et al., 2006; Lund et al., 2007) suggest that mtDNA-independent mitochondrial dysfunction could also participate to the pathogenesis of NRTI-induced hepatotoxicity, in addition to mtDNA depletion.

5.4.6 Tamoxifen

Tamoxifen is a selective estrogen receptor modulator used for the treatment of estrogen receptor-positive breast cancer (Table 5.1). It can induce different types of chronic liver lesion including steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (Biour et al., 2004; Wang et al., 2013; Yang et al., 2016). In contrast to steatosis, tamoxifen-induced acute hepatitis and cholestasis seem to be less frequent (Biour et al., 2004).

Investigations performed in isolated mouse liver mitochondria have shown that tamoxifen is able to impair OXPHOS and MRC activity in a similar manner to amiodarone, with OXPHOS uncoupling for relatively low tamoxifen concentrations (50–100 μ M) and inhibition of MRC activity for higher concentrations (Larosche et al., 2007). This dual effect of tamoxifen on OXPHOS was observed in other investigations carried out in mitochondria isolated from rat liver (Cardoso et al., 2001, 2003; Tuquet et al., 2000). Interestingly, similarly to amiodarone (Figure 5.2), tamoxifen is a cationic amphiphilic molecule that can be protonated within the mitochondrial intermembrane space and be electrophoretically transported into the mitochondrial matrix by using the membrane potential $\Delta\psi$, thus achieving high intramitochondrial concentrations (Larosche et al., 2007). Besides its effect on OXPHOS, tamoxifen is also able to directly inhibit mtFAO, in particular at the level of CPT1 (Larosche et al., 2007). In addition to these effects, investigations in mice showed that chronic tamoxifen treatment (i.e., 28 days) induced progressive hepatic mtDNA depletion, possibly via an impairment of mitochondrial

topoisomerase activity (Larosche et al., 2007). Although all these mitochondrial effects most probably explain why tamoxifen is able to induce hepatic steatosis in a significant number of patients (Begriche et al., 2011; Larosche et al., 2007; Schumacher and Guo, 2015), some investigations suggested that increased lipogenesis could also be involved in the accumulation of liver triglycerides (Cole et al., 2010; Gudbrandsen et al., 2006).

5.4.7 Tetracycline

Tetracycline is a broad-spectrum antibiotic that prevent bacterial growth by inhibiting bacterial protein synthesis (Table 5.1). However, its clinical use declined because the high doses required to treat infections induced numerous adverse effects including gastrointestinal disturbances, skin reactions, blood disorders, renal dysfunction, and liver injury (Fromenty and Pessayre, 1995). In particular, tetracycline has been responsible for several cases of severe hepatotoxicity characterized by microvesicular steatosis and fulminant liver failure (Farrell, 1997; Fromenty and Pessayre, 1995). Several cases of cholestasis have also been reported (Biour et al., 2004; Hunt and Washington, 1994). Synthetic tetracyclines such as doxycycline and minocycline are now preferred to tetracycline because of easier dose schedules and faster gastrointestinal absorption when taken with food. Nevertheless, these tetracycline derivatives have been reported to induce hepatic steatosis in a few patients (Biour et al., 2004; Burette et al., 1984).

Tetracycline-induced inhibition of mtFAO seems to be an important mechanism leading to hepatic steatosis (Amacher and Martin, 1997; Fréneaux et al., 1988; Yu et al., 2009). A recent study suggested that one mechanism of tetracycline-induced impairment of mtFAO could be due to the oxidative alteration of LCAD (Deng et al., 2015). In addition to these effects, tetracycline is able to inhibit mitochondrial protein synthesis (McKee et al., 2006; Moullan et al., 2016; Zhang et al., 2005). Interestingly, other tetracycline derivatives such as doxycycline, minocycline, and rolitetracycline are able to induce mitochondrial dysfunction (Cuenca-Lopez et al., 2012; Labbe et al., 1991; Moullan et al., 2016). Finally, it is noteworthy that tetracycline derivatives present other metabolic effects that could also favor hepatic lipid accumulation including impairment of triglyceride secretion and stimulation of *de novo* lipogenesis (Anthérieu et al., 2011; Labbe et al., 1991; Lettéron et al., 2003).

5.4.8 Troglitazone

The thiazolidinedione (TZD) troglitazone is the first PPAR γ agonist used for the treatment of type 2 diabetes (Table 5.1). TZD derivatives enhance insulin sensitivity by different mechanisms including reduction of circulating

free FAs, increased adiponectin secretion, and stimulation of FAO and energy expenditure (Begriche et al., 2006; Soccio et al., 2014). However, troglitazone has been withdrawn from the market in 2000 because of the occurrence of several cases of severe (sometimes fatal) liver injury (Ikeda, 2011; Kohlroser et al., 2000). In most patients, the histopathologic changes included massive necrosis, but other lesions such as cholestasis, steatosis, fibrosis, and cirrhosis have also been reported (Biour et al., 2004; Julie et al., 2008; Kohlroser et al., 2000). Other TZD derivatives such as rosiglitazone and pioglitazone have subsequently been developed and marketed for the treatment of type 2 diabetes. These newer TZDs are less hepatotoxic, but several cases of liver injury have nevertheless been reported (Biour et al., 2004; Forman et al., 2000).

Mitochondrial dysfunction seems to be an important mechanism whereby troglitazone is able to induce severe liver injury (Bavli et al., 2016; Pessayre et al., 2010). Notably, troglitazone is able to significantly inhibit MRC activity and mitochondrial respiration for concentrations lower than 20 μ M (Hu et al., 2015; Porceddu et al., 2012). All the MRC complexes are inhibited by troglitazone although complex II (succinate dehydrogenase) seems to be less sensitive (Hu et al., 2015; Nadanaciva et al., 2007a). Troglitazone-induced mitochondrial impairment might be reinforced by a reduction of mitochondrial GSH levels, possibly due to lower GSH import into mitochondria (Lee et al., 2013). Interestingly, the less hepatotoxic TZD derivatives rosiglitazone and pioglitazone induce lower MRC inhibition compared with troglitazone (Hu et al., 2015; Nadanaciva et al., 2007a). Troglitazone was also shown to induce MPT pore opening in isolated rodent liver mitochondria in condition of calcium pulse (Masubuchi et al., 2006; Okuda et al., 2010). However, troglitazone-induced mitochondrial membrane permeabilization could not be observed in mouse liver mitochondria in the absence of this calcium pulse (Porceddu et al., 2012). Interestingly, investigations in the human hepatic cell line HC-04 treated by troglitazone suggested that MPT pore opening was induced by ROS generated as a consequence of MRC inhibition (Lim et al., 2008). Hence, although MPT pore opening appears to be an important mechanism of troglitazone-induced cell death (Lim et al., 2008; Tirmenstein et al., 2002), the previously mentioned data strongly suggest that troglitazone could not be able to directly induce MPT pore opening in hepatocytes.

CYP-mediated metabolic activation of troglitazone generates several highly reactive metabolites that have been postulated to be involved in troglitazone-induced hepatotoxicity (Kassahun et al. 2001; Smith, 2003). However, different investigations carried out in HepG2 cells did not support a pathogenic role of these metabolites (Bae and Song, 2003; Tirmenstein et al., 2002). Finally, it is noteworthy that some of these reactive

metabolites could undergo GSH conjugation, in particular by the glutathione *S*-transferase (GST) isoforms A1, A2, M1, and P1 (Kassahun et al. 2001; Okada et al., 2011). In contrast, GSTT1 was not involved in this metabolic pathway (Okada et al., 2011). Thus, when these data are taken into consideration, it remains unclear why individuals with defective GST activity (in particular in those presenting the combined GSTT1-GSTM1 null genotype) seem to present a higher risk of troglitazone-induced liver injury (Watanabe et al., 2003).

5.4.9 Valproic Acid

Valproic acid (VPA), or 2-propylpentanoic acid, is a broad-spectrum antiepileptic drug used in the treatment of different forms of epilepsy (Table 5.1). Two clinical forms of hepatotoxicity may occur during VPA therapy. In most cases, hepatotoxicity merely consists of an asymptomatic increase in serum transaminase activity that normalizes with dose reduction or drug discontinuation. A much less frequent hepatotoxic effect is severe Reye-like syndrome occurring mainly in young children. Microvesicular steatosis and necrosis are salient pathological features in this form of VPA-induced hepatotoxicity, whereas cholestasis and inflammatory infiltrates can also be observed in some patients. Biochemical abnormalities include elevated aminotransferase

levels, hypoglycemia, hyperammonemia, and prolonged prothrombin time (Fromenty and Pessayre, 1995; Powell-Jackson et al., 1984; Zimmerman and Ishak, 1982). Notably, three major predisposing factors can increase the risk of VPA-induced Reye-like syndrome, namely, young age of the patients, polytherapy with other antiepileptic drugs (e.g., phenytoin, phenobarbital, and carbamazepine), and some underlying genetic diseases (Fromenty and Pessayre, 1995; Star et al., 2014; Zimmerman and Ishak, 1982). In particular, genetic mutations affecting mtFAO and MRC activity could greatly favor VPA-induced severe liver injury (Fromenty and Pessayre, 1995; Krähenbühl et al., 2000; Li et al., 2015; Njolstad et al., 1997; Stewart et al., 2010).

It is noteworthy that VPA frequently induces obesity in treated patients, but the involved mechanisms are still unclear (Rauchenzauner et al., 2008; Verrotti et al., 2011). Similarly to lifestyle-induced obesity, VPA-induced weight gain is associated with insulin resistance, dyslipidemia, and fatty liver disease (Luef et al., 2004; Verrotti et al., 2010, 2011). Thus, VPA-induced obesity and fatty liver disease could explain, at least in part, the occurrence of asymptomatic increase in serum transaminase activity in some treated patients.

VPA is metabolized in the liver by two major pathways (Figure 5.4). First, this branched-chain FA is activated into VPA-coenzyme A (VPA-CoA), which undergoes

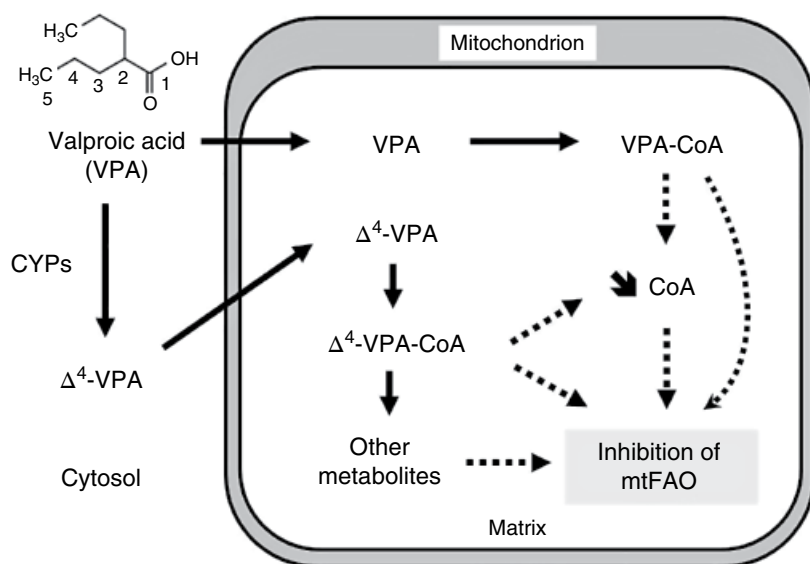


Figure 5.4 Mechanisms of valproic acid (VPA)-induced inhibition of mitochondrial fatty acid β -oxidation. VPA is an analogue of medium-chain fatty acid that freely enters the mitochondrion and generates a coenzyme A ester (VPA-CoA) within the mitochondrial matrix. VPA is also metabolized by cytochromes P450 (CYPs) into Δ^4 -VPA, a VPA metabolite that presents a double bond between carbons 4 and 5. Like VPA, Δ^4 -VPA is able to freely enter the mitochondrion and to generate a coenzyme A ester (Δ^4 -VPA-CoA) and other metabolites. Both VPA-CoA and Δ^4 -VPA-CoA can inhibit the mitochondrial fatty acid oxidation (mtFAO) by two different mechanisms: (i) a sequestration of CoA, which is a cofactor mandatory for fatty acid activation and subsequent mtFAO, and (ii) a direct inhibition of different mtFAO enzymes such as SCAD, MCAD, and CPT1. Note that Δ^4 -VPA-CoA and other CYP-generated VPA metabolites (e.g., Δ^2 -VPA-CoA) could be particularly deleterious for the mtFAO pathway. This may explain why CYP inducers such as phenobarbital and phenytoin significantly increase the risk of VPA-induced severe hepatotoxicity.

complete mitochondrial β -oxidation generating propionyl-CoA and acetyl-CoA (Luis et al., 2011; Silva et al., 2008). Notably, like natural MCFAs and SCFAs, VPA can freely enter the mitochondria (Fromenty and Pessayre, 1995). Second, VPA can be metabolized via CYP-mediated dehydrogenation, leading to the formation of 4-ene-valproate (also referred to as Δ^4 -VPA). Inside the mitochondria, 4-ene-valproate is then activated into 4-ene-valproyl-CoA that undergoes mtFAO to form 2,4-diene-valproyl-CoA (also referred to as $\Delta^{2,4}$ -VPA-CoA) (Fromenty and Pessayre, 1995; Kassahun et al. 1991). VPA structure and metabolism could explain why this drug is interacting with the mitochondrial β -oxidation pathway, as mentioned in the following text.

Numerous *in vitro* and *in vivo* investigations have been performed in order to determine the exact mechanism of VPA-induced Reye-like syndrome (reviewed in Baillie (1988), Coulter (1991), Fromenty and Pessayre (1995), Ponchaut and Veitch (1993), and Silva et al. (2008)). Notably, these investigations consistently showed that VPA is able to inhibit the mtFAO. Actually, VPA-induced inhibition of mtFAO could be due to at least three different mechanisms: (i) sequestration of CoA (a cofactor mandatory for FA activation and oxidation) by VPA and different VPA metabolites (Δ^4 -VPA and $\Delta^{2,4}$ -VPA); (ii) depletion of carnitine (another key cofactor necessary for FA activation and oxidation), in particular due to reduced biosynthesis and increased urinary excretion; and (iii) direct inhibition of several enzymes of the β -oxidation pathway. More specifically, VPA and/or different VPA metabolites (Δ^4 -VPA-CoA and $\Delta^{2,4}$ -VPA-CoA) are able to inhibit short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), CPT1, and the trifunctional enzyme (Aires et al., 2010; Fromenty and Pessayre, 1995; Silva et al., 2008). Notably, the CYP-generated VPA metabolites could be particularly deleterious for mitochondrial β -oxidation, and this could explain why CYP inducers such as phenobarbital and phenytoin increase the risk of VPA-induced severe hepatotoxicity (Figure 5.4) (Baillie, 1988; Fromenty and Pessayre, 1995). Finally, it is noteworthy that different dicarboxylic acids have been detected in the urine of treated patients, thus reflecting VPA-induced inhibition of mitochondrial β -oxidation (Fromenty and Pessayre, 1995; Mortensen et al., 1980; Silva et al., 2008).

In addition to mtFAO, VPA can impair other mitochondrial metabolic pathways. VPA is able to inhibit ureagenesis, and this could explain, at least in part, why VPA can induce asymptomatic or severe hyperammonemia in treated patients (Coude et al., 1983; Fromenty and Pessayre, 1995). Chronic VPA administration in rat was shown to reduce MRC activity in liver at the level of

complex IV (Fromenty and Pessayre, 1995; Ponchaut et al., 1992). VPA is also able to directly inhibit mitochondrial respiration stimulated by succinate (Porceddu et al., 2012), but this effect could be secondary to an impairment of succinate transport into mitochondria (Rumbach et al., 1989). Finally, investigations in isolated rat liver mitochondria showed that VPA was able to induce MPT pore opening in condition of calcium pulse (Trost and Lemasters, 1996). However, VPA-induced mitochondrial membrane permeabilization could not be observed in mouse liver mitochondria in the absence of this calcium pulse (Porceddu et al., 2012).

5.4.10 Other Hepatotoxic Drugs

Table 5.1 provides key information and references regarding mitochondrial dysfunction induced by different hepatotoxic drugs that have not been discussed in the previous sections, namely, amineptine, alpidem, aspirin, buprenorphine, ciprofloxacin, diclofenac, ganciclovir, ibuprofen, nimesulide, perhexiline, tacrine, and tianeptine. Although these drugs are likely to be hepatotoxic via mitochondrial function impairment, they are not treated in detail in the present chapter because the mechanisms of mitochondrial dysfunction are identical, or similar to those reported for drugs such as amiodarone, NRTIs, or VPA (Table 5.1). It is also noteworthy that many other hepatotoxic drugs have been shown to impair mitochondrial function (O'Brien et al., 2006; Porceddu et al., 2012). However, further investigations will be needed in order to ascertain that mitochondrial dysfunction plays a central role in their hepatotoxicity.

5.5 Conclusion

It is now acknowledged that mitochondrial dysfunction is a key mechanism leading to DILI, in addition to the metabolic activation of drugs into reactive metabolites. Notably, mitochondrial dysfunction could also be involved in the occurrence of other types of drug-induced adverse effects such as cardiomyopathy, myopathy, lactic acidosis, bone marrow suppression, and neuropathy (Igoudjil et al., 2006; Nadanaciva and Will, 2011; Varga et al., 2015). Notably, adverse effects secondary to mitochondrial dysfunction can be severe and threaten the life of patients because mitochondria are the main source of energy in most cells. Moreover, these serious adverse events can lead to the premature interruption of clinical trials or to the withdrawal of drugs from the market (Labbe et al., 2008; Nadanaciva and Will, 2011). Hence, a major challenge for the pharmaceutical industry is

to be able to detect drug-induced mitochondrial dysfunction during preclinical studies. This can be performed with different *in vitro* methods that allow to determine whether drug candidates are able to rapidly impair OXPHOS and MRC activity or lead to MPT opening (Marroquin et al., 2014; Nadanaciva et al., 2012; Porceddu et al., 2012). However, it should be kept in mind that these methods are not able to detect late-onset mitochondrial dysfunction that is, for instance, induced by an inhibition of mtDNA replication or mitochondrial protein synthesis. Hence, long-term treatment in relevant experimental models is required to detect such deleterious effects (Gerschenson et al., 2001; Lebrecht et al., 2007; Setzer et al., 2008). Finally, it should be recalled that the detection of mitochondrial dysfunction can be complicated by interspecies differences, as already underlined with FIAU.

Another major challenge is to determine the main factors that might predispose to drug-induced mitochondrial dysfunction and related side effects. Indeed, drug-induced mitochondrial dysfunction can be silent in most patients but can become severe and life threatening in a few individuals with different predisposing factors (Begrache et al., 2011; Labbe et al., 2008). Recent investigations suggested that some polymorphisms in the human POLG gene could favor mitochondrial

dysfunction induced by VPA and some NRTIs (Baruffini et al., 2015; Stewart et al., 2010). The Ala16Val genetic dimorphism of MnSOD (also referred to as SOD2) could increase the susceptibility to drug-induced mitochondrial dysfunction and hepatotoxicity (Huang et al., 2007; Lucena et al., 2010), possibly by favoring mitochondrial oxidative stress (Degoul et al., 2001; Sutton et al. 2003). It will be important in the future to determine the list of drugs that can pose a significant risk in patients harboring such DNA polymorphisms. If a specific DNA polymorphism significantly enhances the risk of mitochondrial dysfunction and DILI for some drugs, thus they must be avoided in any patients harboring such polymorphism. Obesity-related NAFLD might also favor drug-induced mitochondrial dysfunction and DILI (Fromenty, 2013) because NAFLD is associated with OXPHOS and MRC impairment, in particular when nonalcoholic steatohepatitis (NASH) has developed (Begrache et al., 2013; Koliaki et al., 2015). To determine which drugs can pose a significant risk in obese patients is also an important challenge for the future. Hence, animal models such as the *sod2*^{+/-} and obese ob/ob mice could be useful to identify which drugs are able to be particularly hepatotoxic in the presence of underlying mitochondrial dysfunction (Ballet, 2015; Buron et al., 2017; Labbe et al., 2008; Lee et al., 2013).

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6

Evaluating Mitotoxicity as Either a Single or Multi-Mechanistic Insult in the Context of Hepatotoxicity

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6.1 Introduction

Drug-induced liver injury (DILI) is a major clinical problem. Over 1000 drugs are known to induce DILI, a condition that mimics the whole spectra of liver disease. It causes the withdrawal of 3–4% of new chemical entities from the market, with hepatic adverse drug reactions (ADRs) having a global incidence of 13.9 per 100,000 inhabitants/year (Friis and Andreasen 1992). In many cases it is not predictable from the primary pharmacology of a drug and is not detected preclinically, and the onset can be delayed and idiosyncratic in nature. Several major mechanisms are considered to be of most importance in underlying the onset of DILI; this includes mitochondrial toxicity. It is however becoming increasingly clear that in many cases the clinical and experimental evidence support a multi-mechanistic origin for hepatotoxicity over a single pathway and/or direct target origin. This multi-mechanistic hypothesis is of particular interest in understanding the hepatotoxic potential of drugs that are known to contain a mitochondrial liability. Specifically, it has been acknowledged that when tested up to 50% of drugs have mitotoxic potential; however this does not translate to known clinical toxicity (Dykens and Will 2007).

The disconnect between *in vitro* screening and clinical outcome is underpinned by a lack of clear clinical evidence for the role of mitotoxicity in DILI. Together these two factors form one of the major unknowns in drug-induced mitotoxicity with implications in the preclinical assessment of potential toxicity. This has had a major impact on the development of suitable models to allow the translation of *in vitro* and *in vivo* results to the clinic. In terms of hepatotoxicity, recent progress has been made in filling these crucial gaps in knowledge by work undertaken jointly by pharma and academia. For example, the Innovative Medicines Initiative (IMI) funded Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury (MIP-DILI). This project was designed to provide a better understanding of the utility of current test systems and to facilitate the emergence of novel, more physiologically relevant models and testing strategies more able to replicate the multi-mechanistic nature of toxicity. In this chapter we will discuss examples of important chemical and molecular mechanisms that interplay with mitotoxicity in the onset of DILI with associated drug case studies and also the state of the current and emerging model systems and testing strategies to assess this phenomenon.

6.2 Important Considerations When Studying Drug-Induced Mitochondrial Toxicity in the Liver

Although mitochondrial toxicity is a well-accepted cause of DILI and the subject of many reviews, there are still several key questions to be answered regarding the importance of mitochondrial liability to the onset of clinical DILI. This is brought into sharp focus by the figures that show over 50% of drugs with a black box warning for hepatotoxicity are known to contain a mitochondrial liability (Dyken and Will 2007). Moreover, it is established that the traditional preclinical toxicity screens do not adequately report mitochondrial toxicity; this is due to changes in the bioenergetic phenotype of cancerous cells used combined with the unsuitability of animal models for detection of mitotoxicity (Marroquin et al. 2007). Thus, the ability to predict ADR based upon screening for a mitochondrial liability is poor. It is therefore of paramount importance to understand the reasons behind this lack of translatability between preclinical screens of mitotoxicity and clinical toxicity by defining the limitations of the current screening methods and using this to design better testing models and strategies. Some of this disconnect arises from the inability of many models to replicate the complex physiology of a hepatocyte. This chapter will address this important issue by describing the key non-mitochondrial processes and mechanisms that should be considered when assessing the roles of mitotoxicity pathways in DILI (Figure 6.1). This knowledge will then be used to discuss the current and emerging models of

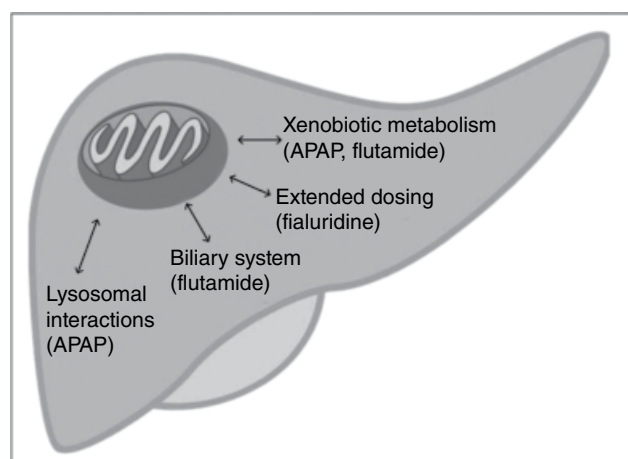


Figure 6.1 Graphical representation of the important mechanisms to consider when assessing the importance of mitochondrial dysfunction in the onset of DILI. Representative drugs associated with each mechanism, discussed in the detailed case studies, are included in brackets.

hepatotoxicity and their suitability for the prediction and examination of drug-induced mitotoxicity.

6.2.1 Xenobiotic Metabolism

Xenobiotic metabolism refers to the metabolic pathways responsible for the modification of chemicals, such as drugs or poisons, that are foreign to the human body. These mechanisms are in place in order to detoxify the xenobiotics and to safely excrete them from the body.

Although the liver is not the sole site in the human body where drug metabolism is performed (Pavek and Dvorak 2008; Xie et al. 2016), it is the main organ where the processing of xenobiotics takes place during first-pass metabolism. As such, the liver among all the organs contains the highest concentration of enzymes required to catalyze drug metabolism (Pavek and Dvorak 2008). In general, the process of drug metabolism in the liver is divided into three phases (I, II, and III), with the primary aim being the conversion of lipophilic compounds into hydrophilic compounds, through the addition of charged polar groups, to enable their excretion from the body in the urine or bile (Andrade et al. 2009).

The reactions catalyzed by the phase I enzymes include hydroxylation, deamination, dehalogenation, desulfuration, epoxidation, peroxygenation, and reduction (Hannemann et al. 2007). The majority of oxidation reactions are catalyzed by a family of monooxygenase heme-thiolate enzymes, known as cytochrome P450 (CYP450) enzymes that are located in cytoplasm, microsomes, and mitochondria of hepatocytes. CYP450s are found more abundantly in the centrilobular rather than periportal region of the liver; this centralized region experiences lower oxygen tension, and hence cells are more susceptible to necrosis as a result of cell toxicity (Andrade et al. 2009). To date more than 2700 CYP450 enzymes have been identified, of which 57 have been detected in humans. 41 of these human-associated CYP enzymes have been functionally characterized (Lewis 2004). However, only 10 of these are known to contribute to drug metabolism, with CYP1, CYP2, and CYP3 families found to be associated with phase I metabolism in the human liver (Daly 2004). Importantly, from more than 2000 compounds that have been recognized as substrates of CYP450s, 68% are clinically utilized drugs. CYP3A4 is the most widely involved in drug metabolism (28.6%), most commonly performing *N*-demethylation, whereas the next most involved CYPs, 1A2 (11.6%), 2D6 (11.1%), and 2E1 (6.9%), largely react with their substrates by hydroxylation (Lewis 2004).

Phase I metabolism can often lead to the formation of active metabolites, that is, those capable of having an effect on a pharmacological or toxicological target within the body. In most cases, these active metabolites are

either excreted or further metabolized in phase II reactions. During this secondary processing, xenobiotics become conjugated with charged groups such as glutathione (GSH), sulfate, glycine, or glucuronic acid, thus reducing their pharmacological or toxicological effects to negligible levels. One of the most important groups of enzymes in phase II conjugation is the GSH transferases, which play an important role in many drug toxicities, for example, acetaminophen (APAP) (Park et al. 2011). In general, the products of phase II metabolism are less active than the products of phase I, so while phase I reactions can produce chemicals that are more toxic than the parental, phase II metabolism usually detoxifies those chemicals. Finally phase III reactions enable further modification and excretion of the compound (Park et al. 2011).

Phase I and phase II reactions of drug metabolism are the most important in hepatotoxicity due to their function in producing and/or detoxifying active metabolites. These metabolites may have the same target as the parent compound, for example, the phase I metabolites of flutamide and amiodarone (Dragovic et al. 2016). In other cases they are termed chemically reactive metabolites (CRM), which means a reactive metabolite is capable of forming an irreversible covalent bond with a biological target, for example, a protein adduct. In many cases the CRM are deactivated by phase II enzymes. However, toxicity can occur if phase II enzymes are depleted, allowing excess reactive metabolites to covalently bind to intracellular proteins. This can trigger pathways within hepatocytes, leading to cell death and subsequent liver damage (Dragovic et al. 2016). The pathways most affected in this way are those involving the mitochondria and immune response pathways, both of which lead to the overproduction of reactive oxygen species (ROS), further damage to other organelles, and hepatocyte death (Feng and He 2013).

The importance of drug bioactivation via phase I/II metabolism in the induction of clinical DILI varies depending on the chemical structure of the compound and the CYP450 isoforms that are involved in the metabolism. APAP is an important example as the clinical evidence indicates that almost all the toxicity is due to its metabolite, *N*-acetyl-*p*-aminoquinone imine (NAPQI). However, in the case of amiodarone, both the parent drug and primary metabolite (mono-*N*-desethylamiodarone) play a role in hepatotoxicity (Dragovic et al. 2016).

Although drug bioactivation, particularly the formation of CRM, can act as one of the main mechanisms of DILI, it is not always required, nor is every drug that is chemically capable of producing CRM associated with clinical DILI. The mechanisms of toxicity associated with protein adduct formation by CRM and their role in DILI remain unclear, in part due to the fact that their chemical reactivity often leads to a high degree of

biological promiscuity and the formation of multiple protein adducts (Park et al. 2011). Furthermore, CRM can also act indirectly, such as in the activation of inflammatory pathways during the induction of DILI. It has been postulated that CRM may target the resident macrophages of the liver, Kupffer cells, as has been speculated to be the case in APAP toxicity, or alternatively it could be that CRM affects blood-driven natural killer cells or neutrophils, as in the case of alcohol-induced liver injury (Woolbright and Jaeschke 2015).

Considering the importance of drug metabolism in DILI, it is vital to choose the test system that most closely resembles the xenobiotic capacity of the liver. This ensures that the chemical pathways that occur clinically are recapitulated entirely so that the parental compound and active metabolites are present at their physiological levels and that toxicity is neither under- or overestimated. However, to date the current test systems are not capable of achieving this level of clinical relevance. Therefore, it is crucial that researchers have an awareness of the specific activation/detoxification pathways that are important for the compound(s) under investigation, particularly the CYP enzymes or phase II conjugates, so that the best test system can be selected based on phenotypic characteristics.

6.2.2 Biliary System

Cholestatic injury, resulting from a failure in the flow of bile from the liver to the duodenum, comprises one of the most severe manifestations of DILI, alongside hepatocellular injury (Padda et al. 2011). This severity stems in part from the fact that cholestatic injury takes longer to resolve than hepatocellular injury and that these patients are also at a higher risk of developing chronic liver injury (Chalasani et al. 2014). One Swedish study of DILI cases between 1970 and 2004 found that over half of the patients examined had cholestatic or mixed cholestatic and hepatocellular injury (Björnsson and Olsson 2005).

Bile acids, the main constituent of bile, are synthesized from cholesterol in hepatocytes and must be conjugated to taurine or glycine to form bile salts prior to secretion (Hofmann 2009). Secretion of bile salts from the canalicular or apical membrane of hepatocytes occurs against a steep concentration gradient, thus requiring active transport mediated by the bile salt export pump (BSEP) (ABCB11) (Dawson et al. 2009). This requirement for active transport creates a potentially detrimental scenario where bile salt export inhibition can easily occur (Stieger 2009). Adaptive mechanisms have been identified to facilitate bile salt secretion, namely, via an increased glucuronidation and sulfation of bile salts, enabling excretion into the bile canaliculi via MRP2 or

back into sinusoids by ABCC3/4, although given the prevalence of cholestatic injury these adaptive mechanisms fail to recapitulate the BSEP-mediated route (Ballatori et al. 2009).

6.2.2.1 Mechanisms of Bile Acid and Bile Salt-Mediated Mitochondrial Toxicity

Given the severe clinical manifestations of cholestasis, bile acid-induced mitochondrial toxicity and its contribution toward this injury have become an important area of research within the field. While all bile acids are amphipathic, there remains a great deal of variation in their hydrophobicity, with studies revealing clear distinctions between hydrophobic and hydrophilic bile acids (Palmeira and Rolo 2004). This appears to play an important role in their mitotoxic potential as the toxicity of each bile acid has been found to positively correlate with their hydrophobicity (Mehta et al. 2008).

Bile acids have been found to decrease mitochondrial membrane potential and state 3 respiration while increasing state 4 respiration, which is indicative of an impairment of oxidative phosphorylation (OXPHOS) and uncoupling, respectively. The precise mechanisms remain to be fully elucidated, yet given the ability of cyclosporine A to block mitochondrial permeability transition pore (MPTP) opening and prevent subsequent mitochondrial swelling and the induction of cytochrome *c* release by the hydrophobic bile acid deoxycholic acid, it is thought that the mitochondrial toxicity of hydrophobic bile acids is primarily via induction of MPTP prior to cytochrome *c* release. The induction of MPTP by bile acids results from the binding of bile acids to the outer mitochondrial membrane, with the adenine nucleotide translocator (ANT) implicated as a primary binding target (Lemasters et al. 1998; Schulz et al. 2013). Glycochenodeoxycholic and tauroursodeoxycholic acids have been found to preferentially permeabilize liposomes containing ANT, a process that resembles MPTP opening in intact mitochondria (Schulz et al. 2013). As would be expected from the impairment of mitochondrial membrane potential, concomitant increases in ROS generation and cytochrome *c* release have been identified following induction of MPTP (Rolo et al. 2000).

Although unconjugated bile acids are weak acids, they do not possess the usual structural requirements for uncoupling activity as shown by the classical uncouplers such as FCCP, including the presence of an acid-dissociable group, bulky lipophilic groups, and a strong electron-withdrawing moiety (Palmeira and Rolo 2004).

In contrast to hydrophobic bile acids, hydrophilic bile acids are known to inhibit apoptosis via stabilization of the mitochondrial membrane independent of the MPTP. The hydrophilic bile acid ursodeoxycholic acid (UDCA) has been shown to interfere with the transcriptional activation of apoptosis, resulting in a modulation of p53

stability. In addition, UDCA has also been found to reduce hydrophobic bile acid-induced disruption of mitochondrial membrane potential, to cause Bax localization to the mitochondria, and to forestall ROS production while also preventing the release of cytochrome *c* from the mitochondria and subsequent cytosolic caspase activation (Solá et al. 2003; Perez and Briz 2009).

6.2.2.2 Bile Acid Accumulation Following Mitochondrial Perturbation

It is important to remember that under physiological conditions, intracellular concentrations of bile acids should not reach concentrations able to induce the aforementioned perturbations to a degree that compromises cell viability. However, due to the active nature of bile acid secretion, compounds that diminish ATP production, including inhibitors of mitochondrial electron transporters and uncouplers, are associated with reduced active bile acid excretion via the BSEP. The resultant intracellular accumulation of mitotoxic bile acids causes further deficits in mitochondrial function, compromising bile acid export further and heightening bile acid-induced mitochondrial toxicity (Aleo et al. 2014). Due to the need for ATP to actively export bile salts from hepatocytes, any drug able to impair mitochondrial OXPHOS and ATP production has the potential to create this vicious circle of increasing bile acid levels and hence yielding mitochondrial dysfunction.

6.2.2.3 Bile Acid Toxicity Resulting from Dual Inhibition of Mitochondrial Function and Bile Salt Export

Two different mechanisms for the inhibition of bile acid export play a role in bile acid-associated toxicity, either as an indirect consequence of mitochondrial liabilities alone or in combination with the inhibition of the BSEP transporter. These two different mechanisms of toxicity in isolation are potentially lethal in their own right; however compounds capable of inducing both modes of toxicity have a significantly greater risk of drug-induced hepatotoxicity. This concept has been supported by the work of Aleo et al. (2014) who tested compounds with high (24), less (28), and no DILI concern (20). Compounds that exhibited dual liabilities toward both the mitochondria and BSEP comprise 50% in the high DILI concern category, 29% in the less DILI, and 0% in the no DILI category, whereas BSEP inhibitors alone were equally distributed across each category.

6.2.3 Lysosome/Mitochondria Interplay

The generation of reactive species within biological systems, as described by the Haber–Weiss reaction, can be harmful to cells via the disturbance of many cellular functions, including DNA repair, lipid homeostasis,

membrane integrity and mitochondrial function, and the subsequent induction of apoptosis. This is due to the production of the highly reactive hydroxyl radical ($\text{OH}\cdot$) from a reaction between superoxide ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Haber and Weiss 1934).

ROS can be derived from numerous sources *in vivo*. These include many enzymatic reactions, of which the CYP450-catalyzed metabolism of xenobiotics is probably the most relevant to drug hepatotoxicity. However, it is widely believed that the most important source of ROS in living organisms is the mitochondria (Loschen et al. 1971; Boveris and Cadenas 1975). The main sites of generation for mitochondrial ROS, predominantly H_2O_2 and $\text{O}_2^{\cdot-}$, are respiratory complexes I and III of the electron transport chain plus the autoxidation of ubiquinone (Kehrer 2000).

The generation of $\text{OH}\cdot$, the most highly reactive oxygen radical, is catalyzed by a variety of transition metals including ferrous iron (Fe^{2+}) (Liochev 1999). Although iron is an essential nutrient, when in excess within the liver cells due to accidental overdose or chronic disease, it can induce hepatocellular necrosis. Two pools of iron are present within cells: The “non-chelatable” or “structural iron”, such as that sequestered within ferritin or heme molecules, is less involved in oxidative stress. The “chelatable iron” pool is located predominantly in the lysosomes and is the major cellular source of free reactive Fe^{2+} (Kehrer 2000; Uchiyama et al. 2008).

Chelatable iron plays an important role in the pathophysiology of cell necrosis in conditions such as hepatic ischemia and reperfusion injury, which leads to massive oxidative injury. What ultimately kills the cells however is mitochondrial failure following ROS-mediated initiation of mitochondrial permeability transition (MPT). The resultant uncoupling of OXPHOS leads to mitochondrial depolarization, mitochondrial swelling, and ultimately cell necrosis due to depletion of cellular ATP. It has been demonstrated that there is a synergistic effect between chelatable iron and mitochondrial oxidative stress; increased levels of chelatable iron alone are not sufficient to enhance ROS production or result in cellular necrosis. Conversely, if mitochondrial oxidative stress is initiated, but no chelatable iron is available from the lysosome, events leading to MPT and cell death are attenuated (Uchiyama et al. 2008).

Based upon these findings, a hypothesis has been tested recently, proposing that a “two-hit” process occurs during iron-catalyzed ROS-mediated cell necrosis. According to this hypothesis, the first hit is provided by oxidative stress, such as drug-induced mitochondrial injury, for example, the NAPQI–protein adducts produced from APAP toxicity. In this scenario the increase in oxidative stress (H_2O_2 , $\text{O}_2^{\cdot-}$), arising from a dysfunctional electron transport chain, leads to the release of free Fe^{2+} iron from the lysosome, which provides fuel for the second hit. This

free chelatable iron is transported into the mitochondria via the mitochondrial Ca^{2+} , Fe^{2+} uniporter (MCFU). In the presence of H_2O_2 and $\text{O}_2^{\cdot-}$, this free iron will catalyze the formation of $\text{OH}\cdot$, which will initiate further and final mitochondrial damage, onset of MPT, and eventually cell necrosis (Uchiyama et al. 2008; Hu et al. 2016). This exemplifies the importance of considering the interplay between mitochondria and other cellular organelles when investigating drug-induced toxicity in order to select the appropriate model system.

6.2.4 Chronic Toxicity

Mitochondrial injury in the clinic is often associated with delayed-onset ADRs. This is a consequence of an extended timeline of cell death, arising from an accumulation of mitochondrial injury against a backdrop of compensatory energy production pathways and active mitochondrial quality control processes including fusion, fission, and mitophagy. Furthermore, the accumulation of damage is slowed by the cellular multiplicity and heteroplasmy of mitochondria, where multiple versions of the mitochondrial genome exist within the same cell, which delays the onset of toxicity until a critical threshold of damage is reached and cell death ensues (Ni et al. 2015; Stewart and Chinnery 2015). In the liver this chronic onset is compounded by the liver’s natural repair and protection mechanisms, which can lead to the active clearance of damaged hepatocytes and liver regeneration until these protective mechanisms are also overwhelmed (Fausto 2004).

A genetic basis of the mitochondrial threshold effect during drug-induced mitotoxicity has been proposed (Boelsterli and Lim 2007). In this model, drug-induced oxidative stress occurs as a consequence of electron transport chain dysfunction or direct effects of drugs upon mitochondrial DNA (mtDNA), which accumulates over time. Studies have shown that a low degree of mitochondrial heteroplasmy leads to no noticeable clinical effect. However, mutations can continue to accumulate until a threshold level is reached, where approximately 60–90% of the mtDNA is mutated, and this threshold point is dependent upon the specific mutations occurring and tissue location. At this point ATP production is no longer capable of supporting cell viability, and cell death rapidly accelerates. Increasingly, the usage of the term “threshold effect” has expanded to include cases of drug-induced mitochondrial toxicity where a genetic basis of mitochondrial damage has not been proven, instead referring to a bioenergetic threshold. In this context it is commonly stated that a cell can survive a loss of up to 70% of its bioenergetics capacity, defined as ATP production capacity. However, although an emerging topic of discussion, there is a lack of evidence to support this theory.

A clear example of a threshold effect due to direct drug-induced mtDNA damage in hepatotoxicity comes from the drug fialuridine. Fialuridine is a nucleoside reverse transcriptase inhibitor, developed as an antiviral, which demonstrated potential clinical efficacy in the treatment of hepatitis B. However, during clinical trials, extended dosing led to the death of five patients following hepatic failure and pancreatitis (McKenzie et al. 1995). Strikingly, this massive hepatic failure was unexpected based upon previous patient exposure and occurred despite continual liver monitoring. Importantly, the rapid onset and acceleration of damage mirrors the predicted late onset and acceleration due to the threshold effect. The clinical symptoms of DILI were consistent with a mitochondrial etiology, and detailed retrospective studies have supported this mechanism, supplying further detail regarding its effect upon mtDNA (see case study). Although extreme, the case of fialuridine exemplifies the need to consider chronic toxicity and organ-specific processes when assessing drug-induced mitotoxicity *in vitro*.

6.3 Current and Emerging Model Systems and Testing Strategies to Identify Hepatotoxic Mitotoxics

6.3.1 Mitochondrial Models

The study of mitochondrial function *in vitro* has usually been undertaken on either isolated mitochondria or whole cells. Each method has advantages and disadvantages; most notably isolated mitochondria allow direct application of substrates but limited physiological relevance, whereas intact cells have greater physiological relevance but are impermeable to many mitochondrial substrates. In order to select the most suitable model for a specific mitochondrial study, one must therefore carefully consider both the chemical characteristics of the drug being studied and the overall aim of the study. In this way, by appreciating and understanding the potential benefits and limitations of the experimental setup, the results are able to be correctly interpreted in a chemical and clinical context (Figure 6.2a).

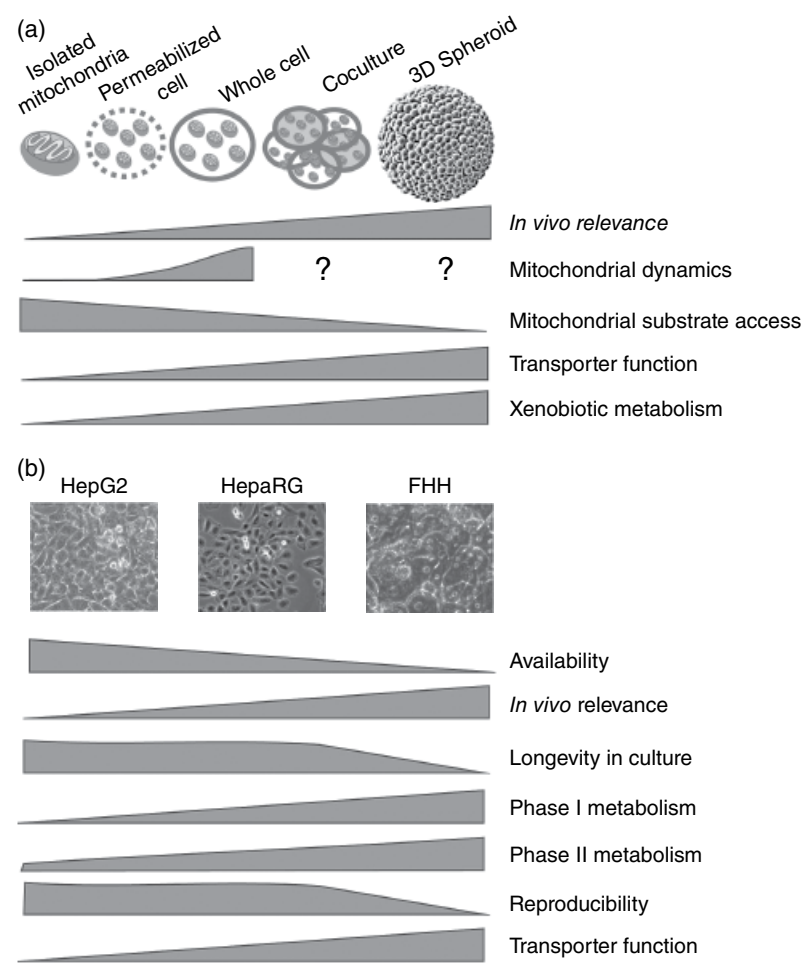


Figure 6.2 Comparison of *in vitro* models for the investigation of mitotoxicity in DILI. (a) Comparison of multiple cell models from which mitotoxicity can be assessed: from isolated mitochondria to 3D cell models such as spheroids. (b) Comparison of three commonly used liver cell types for mitotoxicity-DILI investigation: HepG2 and HepaRG cell lines as well as fresh human hepatocytes (FHH).

6.3.1.1 Whole Cell Models

Whole cell models for the assessment of mitochondrial dysfunction are commonly used for indirect studies, such as the measurement of cellular ATP levels. They also have the notable benefit of only requiring a small number of cells to perform a variety of plate-based assays. Importantly, whole cells provide increased physiological relevance as the mitochondria are in an *in situ* environment, enabling continued interaction with other mitochondria and cellular organelles, as well as a myriad of intracellular signaling including changes in cytosolic calcium, thus allowing the assessment of multi-mechanistic toxicity. In line with this, alterations in cellular function due to changes in mitochondrial localization, dynamics, and number are preserved. Mitochondria are dynamic organelles, with the energy demand of the cell dictating the level of fusion and fission; indeed many inhibitors can be overcome by the increased fusion of mitochondria to “share the load.” When mitochondria are isolated, these protective mechanisms are lost, providing potential for artifactual toxicity. Furthermore, using intact cells obviates isolation artifacts. The critical hindrance of using whole cells is the inability of many compounds to permeate the cell membrane, necessitating the use of isolated mitochondria in some scenarios.

6.3.1.2 Isolated Mitochondria

Isolated mitochondria from hepatocytes have long been a model of choice for the assessment of drug-induced mitochondrial dysfunction, necessitated by the inability of many mitochondrial substrates to freely permeate the cell membrane (Salabei et al. 2014). Practical limitations of this method include the high number of cells (200×10^6 cells) required to generate a sufficient yield of mitochondria and the limited time they remain functional for post-isolation (Kuznetsov et al. 2008). Furthermore, there is the strong possibility of structural disruption upon mitochondrial isolation and a lack of relevance as a result of removing mitochondria from the cellular environment (Kuznetsov et al. 2008). Using isolated mitochondria does indeed allow the determination of direct mitochondrial interactions; however these interactions are not as relevant as if the mitochondria were in the whole cell due to the lack of competing toxicity or protective mechanisms. Conversely, toxicity testing using isolated organelles also increases the likelihood of drug-induced dysfunction going unnoticed in situations where multiple mechanisms of toxicity or xenobiotic activation of drugs are required to produce a phenotypic effect.

6.3.1.3 Permeabilized Cells

The disadvantages of both isolated mitochondria and whole cell model systems can be alleviated in part by the

use of permeabilized cells. This method enables many mitochondria-associated proteins and organelles to remain intact, thereby increasing physiological relevance in comparison with isolated mitochondria but still allowing mitochondrial access to a variety of substrates. In this way cell permeabilization permits assays that investigate the location of compound-mediated mitochondrial respiratory chain inhibition (Brand and Nicholls 2011). Far fewer cells are required than would be needed for comparable studies using isolated mitochondria. Furthermore any bias occurring due to sub-selection of the mitochondrial population during isolation and limitations in quality due to the time taken for isolation can also be avoided (Kuznetsov et al. 2008; Brand and Nicholls 2011). Permeabilization is thought to retain many cellular interactions with proteins and other organelle membranes, particularly those of the mitochondrially associated endoplasmic reticulum, thereby increasing physiological relevance.

6.3.2 Cell Models

The development of *in vitro* models able to recapitulate the complex *in vivo* architecture of the liver is vital in increasing the identification of mitochondrial perturbations that occur alongside or as a result of multiple mechanisms of toxicity. Though freshly isolated human hepatocytes are the gold standard for *in vitro* liver toxicity testing, their limited availability and lack of longevity in culture have necessitated the use of liver cell lines such as HepG2 and HepaRG, which are less phenotypically similar to that of a functioning human liver. Due to the limitations of these primary and carcinoma cell types, many studies have aimed to improve these models by the development of coculture methods, whereby multiple liver cell types are combined, and also 3D culture. Both of these culture systems allow the complex network of intra- and intercellular interactions within the liver to be more accurately represented, thus providing a more accurate prediction of *in vivo* hepatotoxicity (Figure 6.2b).

6.3.2.1 Primary Human Hepatocytes

Primary human hepatocytes (PHHs) are not without their limitations; complicated isolation procedures, restricted availability, and interindividual variability reduce their utility as an *in vitro* screening model (Gerets et al. 2012). One of the main advantages is the retention of xenobiotic metabolism that is relatively similar to that of a whole human liver; nevertheless current protocols do not enable the maintenance of this function for more than a few hours (Levy et al. 2015). PHHs also express a multitude of transporters including the BSEP; however, due to current limitations in the longevity of PHHs, the ability of these cells to act as a model for chronic toxicity

induced by inhibition of BSEP or other transporters is notably restricted. One important features of the PHH is their utility in studying the effects of interindividual variation. However, while representative of the clinical situation, this effect can reduce the reproducibility of results, and so some researchers choose to utilize frozen PHH as a pooled mixture from several donors to decrease variability.

The non-tumorigenic phenotype of PHHs means that the Warburg effect, which confers tumorigenic cells with the ability to use glycolysis when required, does not occur to the same extent. Resultantly, although PHHs can be used to determine toxicity that may have been caused by mitochondrial dysfunction, they lack the advantage of tumorigenic cells in determining whether cytotoxicity is due to mitochondrial dysfunction using the technique of metabolic modification as first described by Marroquin et al. (Marroquin et al. 2007; Kamalian et al. 2015). Therefore, a vital piece of information when considering multi-mechanistic toxicity is absent.

In summary, PHHs provide an excellent *in vitro* representation of xenobiotic metabolism and transport in the liver, enabling their use in detecting both direct and multi-mechanistic mitochondrial toxicity. Incorporation of multiple cell types in 3D culture systems is likely needed to enable this model to recapitulate chronic mitochondrial toxicity.

6.3.2.2 HepG2 Cells

HepG2 hepatoma cells are the most widely used preclinical model for hepatotoxicity due to availability, reproducibility, and expression of many hepatocyte features. However, the primary caveat for the use of these cells in investigating potential DILI is their extremely low xenobiotic metabolic capacity, specifically the limited expression of phase I CYP450 enzymes, thus reducing their suitability for studying metabolite-mediated toxicity. In contrast, the vast majority of phase II detoxification enzymes are expressed at a normal level in HepG2 cells (Gerets et al. 2012). The lack of mitochondrial CYP450s, the activity of which generates ROS, reduces the propensity for ROS production upon drug treatment and so could lead to an underestimation of drug-induced toxicity induced by or exaggerated by oxidative stress.

There is also differential expression of nearly all key transporters in HepG2 cells compared with PHHs. The 16-fold reduction in the expression of the BSEP in HepG2 cells is just one example, and this significantly reduces the ability of this cell line to detect synergistic toxicity arising from compounds with liabilities in both the mitochondria and the BSEP. However, the comparative absence of this export pump has been advantageous in scenarios where direct inhibition of mitochondrial respiratory complexes is to be identified in a situation of

low active bile salt export, providing a baseline from which to compare with a situation of active bile transport (Sison-Young et al. 2015; Ball et al. 2016).

In addition to HepG2 cells' lack of reactive metabolite-mediated and synergistic toxicity arising from lack of bile salt accumulation, their ability to switch to glycolysis as their main source of ATP following a mitochondrial insult can also reduce their utility in the detection of direct mitochondrial toxicity. As a result, HepG2 cells require manipulation to increase their ability to detect direct mitochondrial toxicity. This is easily achieved via long-term or acute galactose conditioning of HepG2 cells; the use of galactose as a substrate drastically reduces the ability of glycolysis to compensate for perturbation(s) in OXPHOS, thus making mitochondrial toxicity easier to detect (Marroquin et al. 2007).

HepG2 cells offer a widely available alternative to PHH, and with the direct testing of both a parent and its primary metabolites in combination with metabolic manipulation, these cells can be used to detect both direct and metabolite-mediated mitochondrial toxicity.

6.3.2.3 HepaRG Cells

HepaRG cells were developed to circumvent the limitations presented by the use of PHH and HepG2 cells, providing a higher metabolic profile than HepG2 cells and a wider availability and longevity than PHH. Although HepaRG cells are based on a hepatocellular carcinoma cell line, they are in fact composed of a mixture of hepatocyte-like and biliary-like cells (Gerets et al. 2012).

HepaRG cells express a large panel of liver-specific genes, including several CYP450 enzymes, specifically CYP1A2 and CYP2E1, required for the generation of reactive metabolites from flutamide and APAP, respectively (Kanebratt and Andersson 2008). The CYP3A4 gene, one of the most important CYP450s for drug metabolism, has 19.1-fold increased expression in HepaRG cells compared with HepG2 cells (Gerets et al. 2012). The expression of multiple additional functions, such as phase II metabolizing enzymes, apical and canalicular ABC transporters, and basolateral solute carrier transporters, is also found within these cells. The combination of metabolizing enzymes and transporters provides HepaRG cells with a phenotype that is able to much more accurately mimic the function of PHHs than can be achieved using HepG2 cells.

Another important advantage of HepaRG cells compared with HepG2 cells is the expression of BSEP and the possession of functional bile canalicular networks, leading to hepatobiliary transporter activity comparable with PHH, despite a threefold reduction in BSEP expression in HepaRG cells (Guillouzo et al. 2007). When used with labeled bile acid derivatives, drug-induced perturbations of bile salt export can be identified alongside inhibition of

mitochondrial function. Prediction of toxicity induced by compounds with both mitochondrial and BSEP liabilities can also be increased by a short-term metabolic switch via the substitution of glucose for galactose media, as is routinely performed to increase HepG2 susceptibility to mitotoxins (Guillouzo et al. 2007; Hendriks et al. 2016).

Overall, HepaRG cells have been shown to provide a viable alternative to PHHs, with fewer concerns over longevity while retaining many hepatocyte-specific functions. Crucially, once in their differentiated form, they can be cultured for up to 4 weeks *in situ*, thus providing the possibility of extended toxicity studies. Metabolic manipulation to increase reliance upon OXPHOS in combination with active bile salt transport and *in vivo*-like xenobiotic metabolism allows the detection of synergistic toxicity by both parent drug and metabolite-mediated mitochondrial toxicity or via dual BSEP/mitochondrial inhibition.

6.3.2.4 Coculture of Multiple Cell Types

Given the multiple cell types found within the liver, it is reasonable to assume that the incorporation of multiple liver cell types into *in vitro* culture has the ability to better recapitulate the *in vivo* functionality by permitting complex heterotypic cell–cell interactions. Examples of such cocultures are hepatocytes grown with the resident macrophages of the liver, Kupffer cells, as well as sinusoidal endothelium and stellate cells; these cultures have been shown to be successful in maintaining hepatocyte function under *in vitro* conditions (Soldatow et al. 2013).

Coculture of PHHs with liver sinusoidal endothelial cells (LSECs) has been shown to alleviate the decline of hepatic functions, including xenobiotic metabolism, usually associated with PHH. Despite LSECs having negligible CYP activity themselves, their presence in coculture is associated with a 7–10 times higher CYP1A activity level within the PHHs compared with native hepatocyte cultures, thus increasing the spectrum of potentially reactive metabolites generated upon parental drug treatment (Bale et al. 2015).

Despite some coculture models enhancing reactive metabolite-mediated mitochondrial toxicity, APAP-induced oxidative stress and ATP depletion have been found to be reduced in cocultures of C3A cells (derivative of HepG2) and human vascular endothelial cells. This indicates the ability of coculture to enable bidirectional stabilization between different cell types, and this resistance importantly closely resembles the resistance seen in PHHs (Nelson et al. 2015). The coculture of multiple cell types has also been found to increase the stability of expression of multiple transporters including BSEP (Maschmeyer et al. 2015).

There is an array of hepatic coculture methods, with many proving to enhance hepatic function, though this

varies largely between cell types. Any coculture that is capable of increasing hepatocyte-specific function reduces the probability of missing mitochondrial toxicity induced or enhanced by multi-mechanistic perturbations.

6.3.2.5 3D Culture

Normal cellular function strongly depends upon interactions with both other cells and extracellular matrix in a 3D tissue environment. In an attempt to mimic the hepatic microenvironment, various more complex culture systems have been developed including sandwich cultures and 3D models such as scaffold-based systems and bioreactors.

The major drawbacks of these culture systems however include the binding of drugs to scaffold and a lack of scalability. To circumvent these problems, hepatocytes can be cultured as 3D microtissues termed spheroids. In spheroid culture, it has been shown that PHH can be maintained for up to 32 days with stable viability as well as the presence of cellular polarity and formation of functional bile ducts (Bell et al. 2016). The reduction in metabolic activity associated with the long-term culture of PHH has also been found to be eased in part by incorporation of PHH into 3D spheroids. The activities of the phase I CYP1A2, CYP2D6, and CYP3A4 enzymes are found not to significantly change over 5 weeks when PHHs are incorporated into this model (Bell et al. 2016).

Despite the improved ability of HepaRG cells in 2D culture to mimic the metabolic capacity of PHHs over HepG2 cells, their incorporation into HepaRG spheroids can further enhance expression of liver-specific genes and activity of drug-metabolizing enzymes. Specifically, three major CYP450 enzymes—CYP1A2, CYP2B6, and CYP3A4—as well as phase II enzymes GSH S-transferase a1 and UDP-glucuronosyltransferase family 2 member B7, were all found to be improved in tethered spheroids, with responses to typical CYP450 inducers such as rifampicin (Tostoes et al. 2012; Wang et al. 2015).

The analysis of bile transport in PHH spheroids has found that the expression of BSEP and the bile exporter MRP2 were not significantly different when compared with liver tissue, suggesting that the molecular machinery involved in bile acid circulation is preserved in spheroid culture. This was supported by the identification of a downregulation of BSEP mRNA induced by chlorpromazine, indicating that the mechanisms attributed to chlorpromazine-induced cholestasis are reflected in the spheroid system (Bell et al. 2016).

In summary, spheroid cultures appear to be a feasible, relatively high-throughput method of 3D culture, able to enhance the function of both PHHs and hepatocarcinoma cell lines. This enhancement, particularly in the polarization of cells, more accurately reflects the *in vitro*

flow of bile salts and production of metabolites, increasing the identification of toxicity that is exaggerated by compounds with multiple liabilities.

6.3.3 The Development and Validation of Testing Strategies

The *in vitro* detection of compounds that induce mitotoxicity was revolutionized by the work of Will and Dykens and the publication of the paper describing the use of metabolic modification to circumvent the Crabtree effect in HepG2 cells (Marroquin et al. 2007). Before this seminal finding, mitotoxicity was underestimated in many preclinical testing assays due to the use of tumorigenic cells, which are able to generate ATP from glycolysis in times when OXPHOS is compromised. The concept of the glucose–galactose assay quickly became incorporated into pharmaceutical testing strategies and is now a cornerstone of mitochondrial testing as it can be more amenable to high-throughput testing than the use of isolated mitochondria. Indeed, in 2013 a survey of eight pharmaceutical companies in the IMI-funded MIP-DILI project on their use of HepG2 cells preclinically revealed that 75% routinely used the glucose–galactose screen.

As the importance of mitotoxicity in DILI continues to be recognized, the search for the most predictive methods to screen for mitochondrial liabilities at an early stage of drug development has continued. This research effort has been mainly driven by industry, with the key objectives of improving sensitivity, specificity, and accuracy alongside the ultimate aims of utilizing such screening assays to provide mechanistic information and prediction of DILI severity (Will and Dykens 2014).

Table 6.1 provides a summary of the key advances in developing preclinical screening strategies to predict mitotoxicity. The earlier papers focus upon the development and assessment of the utility of novel methodologies, for example, extracellular flux analysis and the glucose–galactose screen (Marroquin et al. 2007; Nadanaciva et al. 2012). As the field has progressed, it has become clear that mitochondrial toxicity is most often part of a multi-mechanistic pathway of hepatotoxicity, and so screening strategies are evolving to try to recapitulate this. Several strategies have been reported to combine the use of several screens of mitotoxicity, and these report an improvement in specificity and sensitivity (Porceddu et al. 2012; Hynes et al. 2013; Swiss et al. 2013; Kamalian et al. 2015; Eakins et al. 2016). However these approaches are limited by their focus upon mitochondrial dysfunction, neglecting other pathways that may be important in the development of DILI clinically. For example, in the study by Hynes et al., the glucose–galactose screen only correctly predicted 2–5% of

mitotoxins (Hynes et al. 2006; Will and Dykens 2014). The importance of multiple mechanisms in the onset of hepatotoxicity was exemplified by the work by Aleo et al. (2014) to combine measurements of both mitotoxicity, in whole cells, and BSEP activity, in vesicles, in order to categorize compounds in terms of predicted DILI severity.

Despite the constant improvement in methods available to predict mitotoxicity to date, all strategies have been limited by the constraints of model systems to recapitulate the complex physiology of a human hepatocyte and beyond that the whole liver. However, rapid advancements in 3D models and coculture systems present the opportunity to examine mitotoxicity in a more relevant physiological setting in concert with other key mechanisms in hepatotoxicity, for example, biliary system and xenobiotic metabolism, with the first reports of their use in examining drug-induced mitochondrial dysfunction now becoming available (Bell et al. 2016; Hendriks et al. 2016). In addition to the limitations of current *in vitro* models, the translation of *in vitro* findings to predicting clinical DILI is hindered by the almost complete lack of *in vivo* models for the study of mitotoxicity. However, mathematical modeling may provide the key to bridging preclinical mitotoxicity assessment and the prediction of DILI in man; such has been begun by the MITOsym project within the DILIsym framework (Yang et al. 2015).

6.4 Case Studies

6.4.1 Acetaminophen: Multi-Mechanistic Mitochondrial Hepatotoxicity

APAP overdose is one of the most common causes of acute liver failure in the United States, responsible for more than 48% of acute liver failure from which 29% will undergo liver transplant with a 28% fatality rate (Blieden et al. 2014; Yoon et al. 2016).

APAP is a very good example of a dose-dependent hepatotoxin, with a toxicity profile that involves multiple mechanisms and pathways within different organelles prior to hitting its final target, the mitochondria, whose failure is the determinant factor for cell death (Figure 6.3). Although the initial and terminal phases of APAP toxicity have been examined widely, the intermediate events that lead to cellular necrosis have only recently begun to unfold.

APAP toxicity is a xenobiotic process, with about 90% of the drug detoxified by phase II metabolic pathways, conjugating to glucuronate or sulfate molecules before excretion in the urine. However, a small proportion of APAP (10%) is metabolized by phase I hepatic enzymes, mostly CYP2E1 and to a lesser extent by CYP1A2 and 3A4. As a result of APAP oxidation by the phase II enzymes, a highly reactive metabolite, *N*-acetyl-*p*-benzoquinone

Table 6.1 A summary of the development of preclinical testing strategies to identify compounds with mitochondrial liabilities and to predict compounds capable of inducing DILI.

	Model	Methodology	Outcomes
Hynes et al. (2006)	RLM	Fluorescence-based plate oxygen consumption analysis	Amenable to screening ~200 compounds per day
Marroquin et al. (2007)	HepG2	Cells cultured in galactose and mitotoxicity assessed by ATP and respirometry	Culture in galactose prior to screening increases cell susceptibility to mitotoxics
Nadanaciva et al. (2010)	THLE-2	High-content screening of mitochondria DNA-encoded proteins versus nuclear DNA-encoded proteins	Enables screening for compounds that affect mitochondrial DNA, typically over a longer period
Nadanaciva et al. (2012)	HepG2	Extracellular flux analyzer to monitor oxygen consumption and extracellular acidification (glycolytic function) 16 therapeutics were assessed; some were known mitotoxics	Technology shown to be a robust technique for screening The ability to rank compounds in terms of clinical mitotoxic potential was suggested
Porceddu et al. (2012)	MLM	Screening platform based on the combined measurement of mitochondrial membrane permeabilization, inner membrane permeabilization, outer membrane permeabilization, and alteration of mitochondrial respiration 124 chemicals were screened, 87 with documented DILI and 37 without DILI	94–92% sensitivity for clinical outcome of DILI and 82–89% predictivity A significant relationship between drug-induced mitochondrial toxicity and DILI in patients was demonstrated
Swiss et al. (2013)	K562, stem cell-derived cardiomyocytes and hepatocytes	Dual assessment of cellular ATP and cell death (2h) in glucose and galactose media Validated with classic mitotoxins, subsequently used for 74 test compounds	Proposal that this assay could be used as a first screen of mitochondrial and cellular toxicity. Further assays may be required as some mitotoxic compounds were not identified
Hynes et al. (2013)	HL60, HepG2, RLM, THLE-2	Three methods were compared; 384-well dual-parameter plate-based assay to measure simultaneous oxygen consumption and extracellular acidification; oxygen consumption in RLM and glucose–galactose assay in HepG2 cells 200 commercially available drugs were assessed	The dual-parameter assay and the isolated mitochondrial oxygen consumption assay identified the highest number of mitotoxic compounds. The combination of these assays provides the best identification rate of positive compounds
Aleo et al. (2014)	RLM	Oxygen consumption rates were measured in RLM, and BSEP activity was measured in SB-BSEP-Sf9-VT vesicles Three different categories of drug were investigated: 24 (most DILI concern), 28 (less DILI concern), and 20 (no DILI concern)	Dual consideration of BSEP activity and mitochondrial function provides better prediction of DILI severity Compounds that are positive for mitochondrial and BSEP inhibition comprise 50% of the most DILI concern category, yet only 29% and 0% of the less DILI and no DILI concern categories, respectively. In comparison, BSEP inhibitors alone had an equal distribution across the most, less, and no DILI concern categories
Yang et al. (2015)	<i>In silico</i>	MITOsym mathematical model of hepatocellular respiration within DILIsym To simulate changes in oxygen consumption, extracellular acidification, proton leak, and ATP concentration induced by classical mitotoxics	MITOsym can be used to model mitotoxicity, assisting in bridging the gap between <i>in vitro</i> and <i>in vivo</i> studies
Kamalian et al. (2015)	HepG2	Glucose–galactose assay (short-term) dual assessment of cellular ATP and cell death (2h) 13 test compounds of known mitotoxics and non-mitotoxics	Short assay with dual assessment correctly identifies most mitotoxics Limitations identified of HepG2 cell system
Eakins et al. (2016)	HepG2	Combined glucose–galactose assay (short-term) and extracellular flux analysis 39 known mitotoxics, 33 non-mitotoxics	Prediction of mitotoxicity with 91% sensitivity, 100% specificity, and 92% accuracy

MLM, isolated mouse liver mitochondria; RLM, isolated rat liver mitochondria.

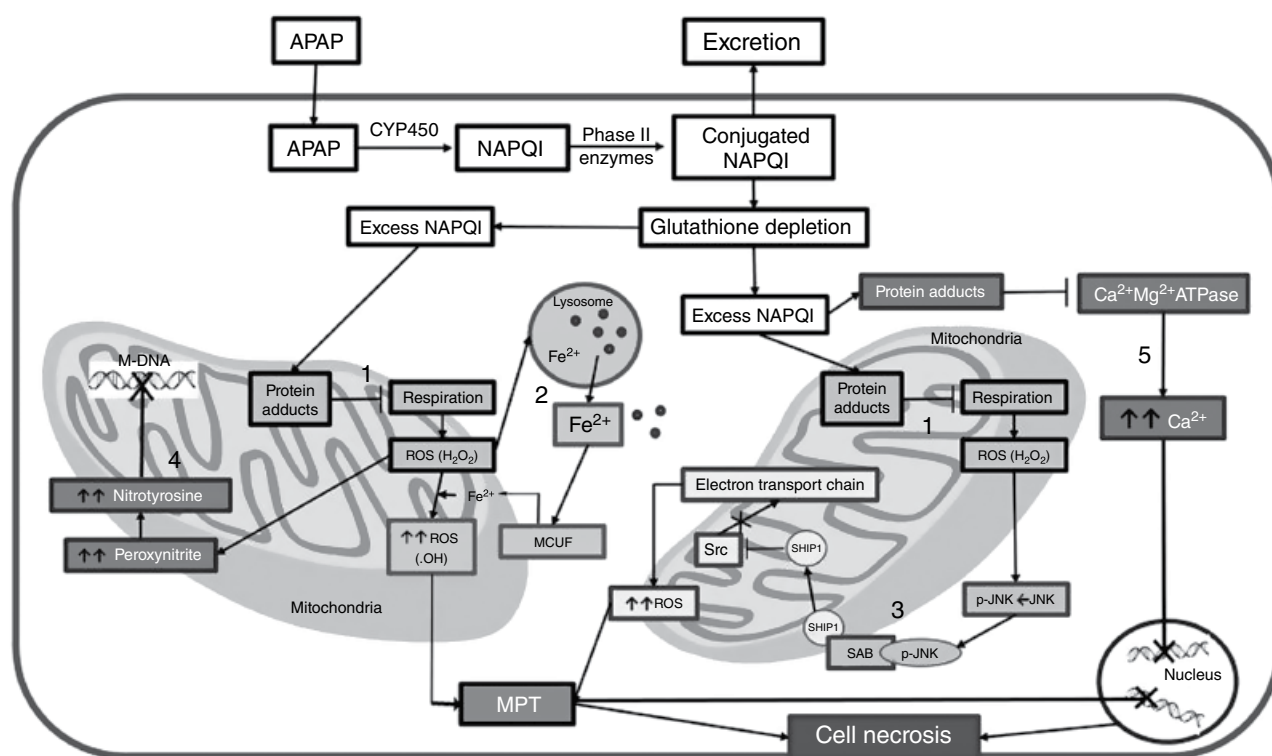


Figure 6.3 The multiple mechanisms involved in APAP mitochondrial and subsequent hepatocyte toxicity. Formation of protein adducts within mitochondria and initial oxidative stress (1), which results in lysosomal free iron–mitochondria interplay (2) and activation of JNK pathway (3). The excessive ROS formation due to mechanisms 2 and 3 will initiate onset of MPT and cell necrosis. The initial ROS formation within the mitochondria also triggers the formation of reactive nitrogen species, mainly nitrotyrosine (4), resulting in mitochondrial DNA damage and accumulation of Ca^{2+} within the cell (5), both of which contribute to further oxidative stress, nuclear DNA damage, and cell necrosis.

imine (NAPQI), is formed. This metabolite, when in excess, is considered to be the main culprit in APAP hepatotoxicity. This was the first major breakthrough regarding mechanism of APAP toxicity (Potter et al. 1973; Fernando et al. 1980; Jaeschke and McGill 2015).

At nontoxic doses of APAP, NAPQI formed is scavenged and detoxified by GSH, forming APAP–GSH adducts (Hodgman and Garrard 2012), which are safely excreted as cysteine and mercapturic acid conjugates. It has been experimentally and clinically demonstrated that GSH has a protective effect on paracetamol toxicity (Mitchell et al. 1973). In the case of APAP overdose, once the GSH supply is depleted by excessive amount of NAPQI, the reactive metabolite covalently binds to sulfhydryl groups within various hepatic proteins, including mitochondrial ones. However, this initial formation of protein adducts within mitochondria would not be sufficient on their own to cause cellular necrosis if it were not for the events that follow (Du et al. 2015; Hu et al. 2016).

One of these important events is the formation of nitrated tyrosine residues. It has been shown that nitrotyrosine, as well as APAP adducts, accumulates in the

necrotic cells of the centrilobular region of the liver of the APAP-treated mice (Hinson et al. 1998). Nitrotyrosine formation is mediated by peroxynitrite, a highly reactive molecule that is a product of a reaction between superoxide and nitric oxide (James et al. 2003). Peroxynitrite is normally detoxified by GSH; however this is depleted during APAP toxicity (James et al. 2003). Mitochondria have been shown to be the main site for peroxynitrite production, which in turn is partially responsible for mtDNA loss that is observed in APAP toxicity. Furthermore, peroxynitrite may cause nuclear DNA fragmentation during APAP toxicity through its indirect effects on the activation of DNase(s) such as lysosomal or mitochondrial endonucleases (Cover et al. 2005).

The homeostasis of important intracellular ions has also been shown to be dysregulated during the course of APAP-induced hepatotoxicity. Although accumulation of Ca^{2+} is known to occur in the cytoplasm and nuclei of pre-necrotic hepatocytes when exposed to an overdose of APAP, the mechanism by which this occurs has remained unclear. Although nonselective Ca^{2+} blockers chlorpromazine and verapamil have been shown to reduce APAP liver injury in mice (Tsutsui et al. 2003), it

has been suggested that it is mainly due to the inhibition of Ca^{2+} – Mg^{2+} ATPases arising due to the formation of critical protein adducts. A recent study has postulated that the specific channel responsible for this Ca^{2+} accumulation might be the transient receptor potential melastatin 2 (TRPM2) cation channel (Kheradpezhohu et al. 2014). The resultant APAP-induced necrosis is shown to be due to accumulation of Ca^{2+} within the nuclei of hepatocytes, which then initiates a calcium-dependent endonuclease-mediated DNA degradation (Shen et al. 1992; Salas and Corcoran 1997).

The role of Fe^{2+} dysregulation in amplification of mitochondrial oxidative stress has been discussed in Section 6.2.3. Briefly, the initial generation of protein adducts by NAPQI within mitochondria causes disruption in OXPHOS, inducing oxidative stress. It is believed that free iron molecules are released from damaged lysosomes and translocate into mitochondria to induce mitochondrial dysfunction, further oxidative stress, the generation of the highly reactive hydroxyl group ($\cdot\text{OH}$), MPT onset, mitochondrial depolarization, and ultimately cellular necrosis.

The role of c-Jun N-terminal kinase (JNK), a protein kinase from mitogen-activated protein kinase (MAPK) superfamily, in APAP hepatotoxicity has been the subject of discussion (Bourdi et al. 2008; Du et al. 2015; Cubero et al. 2016). Most of the evidence for the involvement of JNK activation in APAP toxicity have been gathered using either animals lacking this kinase (Gunawan et al. 2006) or via inhibitors of the pathway in both *in vitro* and *in vivo* models (Latchoumycandane et al. 2007; Xie et al. 2014). This pathway is believed to be activated in various liver pathologies, mostly in response to oxidative stress (Seki et al. 2012). In APAP toxicity the first stage of oxidative stress is due to NAPQI formation and GSH depletion in both the cytosol and mitochondria; this alone is insufficient to initiate cell death. However, this initial process activates JNK signaling pathway through the formation of p-JNK, which translocates to the mitochondria where it binds to its essential ligand SAB (SH3 domain-binding protein) located on the cytoplasmic side of the outer mitochondrial membrane (Win et al. 2011). This signaling pathway results in the further amplification of oxidative stress and ultimately the formation of MPTP, depletion of mitochondrial membrane potential, and cellular necrosis (Du et al. 2015). Although the exact intramitochondrial events are unclear, one study using isolated mitochondria has suggested that JNK activation and translocation are mediated via SHP1, which is normally sequestered by SAB (Win et al. 2016). The subsequent cascade of molecular events leads to the inactivation of the tyrosine kinase Src, which has important roles in the activation and function of many proteins within electron transport chain. The resultant vicious

cycle of ROS formation, sustained activation of JNK pathway, and mitochondrial dysfunction finalizes the fate of cell by initiating the opening of the MPTP and cell necrosis (Win et al. 2016).

Despite intense investigation of APAP hepatotoxicity over the last several decades, the detailed mechanisms that connect the key events in this process remain unknown. However, the details that have been established are sufficient to give clues as to which *in vitro* or *in vivo* models are fit for further study of APAP toxicity. For instance, the metabolic capability of the model should be similar to that of human liver cells, that is, it should have phase I and phase II metabolic pathway enzymes available, especially the relevant CYP450 enzymes. Also, iron and calcium homeostasis regulation and the bioenergetic phenotype of the cell system should mimic human physiology as closely as possible. This renders cell models such as HepG2 cells completely unsuitable due to an almost complete lack of CYP450 enzymes (Sison-Young et al. 2015). Furthermore detailed characterization of the cell or animal model is critical when delineating toxic mechanisms. For example, HepaRG cells express most of the toxicity pathways found within the PHH. However, when present, the level of CYP2E1 is severalfold lower in the HepaRG cells, while CYP3A4 is similar or higher (Sison-Young et al. 2015). In addition, the JNK pathway activation does not seem to be initiated by NAPQI within these cells (Xie et al. 2014), although it has been shown to do so by other compounds such as furosemide (McGill et al. 2015). These differences may not inhibit APAP toxicity completely in HepaRG cells, but may explain the delayed response observed in peak toxicity in this cell line (McGill et al. 2011).

6.4.2 Flutamide: Multi-Mechanistic Mitochondrial Hepatotoxicity

Flutamide is a nonsteroidal antiandrogen that blocks the binding of 5α -dihydrotestosterone to androgen receptors (Gao et al. 2006). It is most commonly used in combination with luteinizing hormone-releasing hormone agonists in the treatment of prostate cancer (Coe et al. 2007).

Since first being marketed to treat prostate cancer in 1989, there have been reports of idiosyncratic DILI associated with flutamide treatment. However, it was not until 1999 that the FDA applied a black box warning regarding the risk of hepatic necrosis and cholestasis in flutamide-treated patients. These cases collectively resulted in hospitalization or death in 0.03% of flutamide-treated patients and have driven the preferential use of alternative antiandrogen therapies such as bicalutamide. Despite bicalutamide being associated with mild, asymptomatic, and transient elevations in

serum aminotransferase levels in approximately 6% of patients, there are only scarce reports of idiosyncratic hepatotoxicity, and thus this drug has become one of the first-line treatments for advanced prostate cancer (Hussain et al. 2014).

Many metabolites of flutamide have been detected in the form of GSH adducts (Kostrubsky et al. 2007), but the primary and most extensively studied metabolite is the α -hydroxylation product 2-hydroxyflutamide (Kostrubsky et al. 2007). The CYP1A2-mediated conversion to generate this metabolite results in a C_{max} of $4.4\mu\text{M}$, greatly exceeding the 72.2nM C_{max} of flutamide. Following production of 2-hydroxyflutamide, there is glucuronidation step prior to its excretion (Shet et al. 1997; Coe et al. 2007; Kostrubsky et al. 2007). Unsurprisingly, given the chemical similarity of flutamide and its metabolite, 2-hydroxyflutamide has been found to have similar liabilities to its parent compound in terms of both direct mitochondrial inhibition and inhibition of biliary function.

When assessed in HepG2 cells, a model with little or no active bile salt transport, flutamide, has been found to induce a significant reduction in both cellular ATP content and the proportion of oxygen consumption used to generate a proton gradient for ATP production. Furthermore, flutamide inhibits respiratory complex I in both permeabilized HepG2 cells and isolated rat mitochondria (Fau et al. 1994; Ball et al. 2016). Given the rapid first-pass metabolism of flutamide, an increased physiological relevance can be provided by examining the effects of the first-pass metabolite 2-hydroxyflutamide, which was found to harbor additional mitochondrial liabilities in HepG2 cells, inhibiting respiratory complex II and ATP synthase, as well as respiratory complex I (Ball et al. 2016). This inhibition at multiple points of the electron transport chain may prove more damaging to mitochondrial function, particularly as both sites of electron entry to the mitochondrial respiratory chain are functionally reduced. The significant reduction in cellular ATP production induced by both flutamide and its metabolite confers liability to cholestasis due to the ATP-dependent mechanism of bile salt export previously discussed.

In PHH, flutamide and 2-hydroxyflutamide have been found to inhibit the efflux of the bile acid taurocholate with an IC_{50} of 75 and $110\mu\text{M}$, respectively (Kostrubsky et al. 2007). This is consistent with research by Gerets et al. (2012) where under the same conditions flutamide was able to induce significant cytotoxicity in PHHs, but the same effect was not observed in HepG2 or HepaRG cells. This may suggest that there is an underestimation of toxicity in cell lines lacking a functional biliary system and hence the positive feedback loop that lies between mitochondrial toxicity and BSEP inhibition. Importantly,

for flutamide, the toxic concentrations reported to inhibit complex I activity and bile acid efflux in PHHs overlap, increasing the risk of a synergistic effect.

The concentrations of flutamide and 2-hydroxyflutamide found to inhibit mitochondrial electron transport and BSEP function *in vitro* greatly exceed their *in vivo* C_{max} ; however the intra-hepatocyte concentrations of these compounds may be much higher. Furthermore, the intra-hepatocyte generation of 2-hydroxyflutamide may result in particularly high concentrations of this metabolite in close proximity to hepatic mitochondria. Importantly, despite differences between clinical and *in vitro* exposure, the finding of mitochondrial toxicity and BSEP inhibition within the same concentration range in multiple models suggests increased probability for the simultaneous, multiple toxicities of flutamide and its primary metabolite. This may subsequently translate to a synergistic effect, lowering the concentration of compound required to induce hepatotoxicity to below the concentration required for specific mechanisms of toxicity.

6.4.3 Fialuridine: A Case of Chronic, Direct Mitochondrial Toxicity

Fialuridine, developed as an antiviral agent, is one of the clearest examples of where clinical hepatotoxicity can be attributed solely to direct mitochondrial toxicity. It remains one of the most infamous cases of clinical trial failure due to the severity of the adverse effects: 7 of the 15 patients developed hepatotoxicity, which was fatal in five cases, with the remaining two patients surviving only following liver transplant (McKenzie et al. 1995). The clinical etiology is representative of the classical hallmarks of mitochondrial toxicity, particularly the threshold effect. Toxicity was only observed when patients were exposed to the drug for extended periods during week 13 of treatment. In two previous shorter trials, no adverse effects were noted. The first sign of toxicity was lactic acidosis and dysfunctional respiratory function. Thymidine and uridine supplementation of the patients failed to reduce toxicity; however, glucose treatment did have positive effects on lactic acidosis. Once toxicity was reported, liver damage was seen to accelerate despite the discontinuation of fialuridine treatment, and histopathological analysis revealed evidence of hepatic steatosis and abnormal mitochondria within the damaged livers.

Subsequent research has established that fialuridine targets mtDNA, an effect arising from its pharmacology as a nucleoside analogue. It has been postulated that once phosphorylated to the triphosphate form, fialuridine can inhibit mtDNA replication by its insertion into the growing chain, with the eventual presence of several

consecutive fialuridine units, leading to the inability of mtDNA polymerase γ to replicate the DNA (Lewis et al. 1996). Ultimately, this effect on mtDNA led to a reduction in ATP-generating activity via the electron transport chain and the subsequent increase in lactate production due to the compensatory upregulation in glycolysis.

One of the most important aspects of fialuridine toxicity to highlight is the complete lack of its prediction, despite full preclinical *in vitro* and *in vivo* testing and several prior clinical trials, and as such it has important implications in the selection of appropriate models for safety testing. It has been discovered that the *in vivo* failure to predict toxicity was due to the uptake of fialuridine into the mitochondria via the human equilibrative nucleoside transporter 1 (hENT-1) (SLC29A1) (Lai et al. 2004). In humans hENT-1 is found on both the outer mitochondrial membrane and the plasma membrane; however there is low homology across species for the mitochondrial localization signal (PEXN) (Lee et al. 2006). Therefore, in most animals apart from the woodchuck, fialuridine cannot enter the mitochondria, leading to a complete lack of adverse effects. An advanced *in vivo* model has been generated to overcome this human selectivity, namely, a humanized mouse model utilizing chimeric TK-NOG mice (Xu et al. 2014).

By examining the molecular mechanisms of fialuridine-induced hepatotoxicity, it is possible to define key cellular characteristics that must be present in order to successfully study fialuridine toxicity *in vitro* with clinical relevance. These include the presence of a functional hENT1 transporter, active mitochondrial thymidine kinases, and the ability to perform extended toxicological studies that mirror the delayed toxicity that developed

in humans. This is exemplified by the failure in HepG2 cells to replicate fialuridine-induced mitochondrial dysfunction over a 6-day period (Colacino et al. 1994), whereas a study reporting 14 days of exposure (20 μ M) observed mitochondrial ultrastructural defects (Lewis et al. 1996). The more physiologically relevant 3D culture systems may be, the more suitable they are for studying fialuridine toxicity, particularly as they are more amenable to extended exposure. Indeed, it has been demonstrated that PHH spheroids mimic the delayed toxicity of fialuridine in humans, with no toxicity evident after 48 h, but with an IC₅₀ approaching 100 nM calculated after 4 weeks of repeated dosing (Bell et al. 2016).

6.5 Concluding Remarks

Mitochondrial dysfunction as a fundamental mechanism of DILI has been increasingly recognized to play an important role in drug discovery, and as such testing for mitochondrial affects has become commonplace in pre-clinical screening. However, many of the most commonly used methods do not fully recapitulate the complex role of the mitochondria in hepatotoxicity, thus reducing their effectiveness. Already, the generation of *in vitro* models with a more faithful hepatocyte phenotype, such as 3D structures and coculturing, has led to exciting new discoveries and advancements in the field. It is only by acknowledging and addressing the limitations associated with mitochondria-associated DILI screening that mitotoxicity research can more fully play a role in facilitating advances in drug discovery and positive patient outcomes.

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7

Cardiotoxicity of Drugs: Role of Mitochondria

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CHAPTER MENU

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7.1 Introduction

Adverse cardiac effects (most commonly cardiac arrhythmia and impaired myocardial contractile function) are the leading cause of drug discontinuation and failure of clinical trials. Cardiotoxicity accounted for almost half of all drug withdrawal cases between 1994 and 2006 (Dyken and Will, 2007). It may develop in a predictable dose- and time-dependent manner (e.g., in case of doxorubicin); in other cases adverse consequences develop in an unpredictable fashion (often years after the drug treatment), more frequently in patients with cardiovascular comorbidities. While cardiac adverse effects of widely used drugs have already been recognized, the cellular mechanisms of their cardiotoxicities are still under extensive investigations.

Ten percent of drugs in the last four decades have been recalled from the clinical market worldwide due to cardiovascular safety concerns. It received high media attention, when already marketed drugs are withdrawn. This was the case with the selective COX2 inhibitor rofecoxib in 2004, used for the treatment of inflammatory conditions, due to significantly increased risk of acute myocardial infarction (Juni et al., 2004). Similarly in 2007 the serotonin 4 receptor agonist tegaserod was withdrawn, originally used for irritable bowel syndrome (Tack et al., 2012). In 2010 an antiobesity drug, sibutramine, failed to remain on market, followed in the same year by the peroxisome proliferator-activated

receptor gamma (PPAR γ) agonist antidiabetic drug rosiglitazone (James et al., 2010; Kerr et al., 2007; Nissen and Wolski, 2007).

In spite of significant efforts in preclinical development to utilize new toxicity screens to reveal cardiotoxicity, safety concerns still remain (Madonna et al., 2015). This is most likely due to the lack of sufficient understanding of the molecular pathways leading to cardiotoxicity (Kang, 2001).

Mitochondria-related cardiotoxicity results from the interference of drugs with processes of normal mitochondrial homeostasis (see Table 7.1). This may involve direct effects on the electron transport chain, promotion of mitochondrial redox imbalance (favoring mitochondrial oxidative stress), and effects on complex signaling pathways that influence mitochondrial biogenesis and the clearance of damaged mitochondria (known as mitophagy) (Gottlieb and Gustafsson, 2011). To facilitate better understand the cardiotoxic effect of individual compounds, first we will overview the major off-targets of mitochondria-related toxicity.

7.1.1 Mitochondrial Energy Homeostasis in Cardiomyocytes

Mitochondria have a crucial role in myocardial tissue homeostasis, providing ATP for the contractile apparatus and critical enzymes in intermediate metabolism and maintaining ion homeostasis (e.g., SERCA and Na⁺/K⁺-ATPase). Consequently, deterioration in mitochondrial

Table 7.1 Putative mechanism of mitochondrial toxicity.

Drug	Mitochondrial off-target/effect	References
Doxorubicin	mtDNA adduct formation	Serrano et al. (1999)
	Redox cycling-dependent reactive oxygen species production	Davies and Doroshov (1986)
	Fe ²⁺ /Fe ³⁺ cycling and reactive oxygen species production	Myers (1998)
	Cardiolipin binding–oxidation–cytochrome <i>c</i> release	Pereira et al. (2016)
	Oxidative/nitrative PARP activation	Pacher et al. (2003)
	Antioxidant enzyme inhibition	Gustafson et al. (1993)
	DNA topoisomerase II beta inhibition	Pointon et al. (2010)
Cisplatin	Possible reactive oxygen species inducer; direct mechanism is unknown	Ma et al. (2010)
Trastuzumab	Inhibition of mitochondrial biogenesis possibly through Her2-dependent ERR activation	Chang et al. (2011)
	Inhibition of prosurvival Akt activation	Negro et al. (2004)
Arsenic trioxide	Possible reactive oxygen species inducer; direct mechanism is unknown	Zhao et al. (2008)
	Parkin-dependent UPS activation	Watanabe et al. (2014)
Mitoxantrone	Inhibition of ATP synthase expression	Rossato et al. (2014)
	DNA topoisomerase II beta inhibition	Douarre et al. (2012)
Imatinib mesylate	Induction of mitochondrial proteostasis	Kerkela et al. (2006)
Sunitinib, sorafenib	Impairment of autophagy/mitophagy	
	via inhibition of ribosomal S6 kinase and AMP-activated protein kinase	Hasinoff et al. (2008) and Jacob et al. (2016)
Azidothymidine	Inhibition of mitochondrial ADP/ATP transport	Barile et al. (1997)
	Inhibition of mitochondrial DNA polymerase	Lewis et al. (1994)
	Increase in mitochondrial reactive oxygen species production	Gao et al. (2011)
Ethanol	Uncoupling of the respiratory chain from ATP synthesis	Lange and Sobel (1983)
	Inhibition of the respiratory chain	Mihailovic et al. (1999)
	Inhibition of mitochondrial biogenesis	Hu et al. (2013)
Cocaine	Intramitochondrial redox cycling of aminochromes	Behonick et al. (2001)
	Complex I inhibition	Yuan and Acosta (2000)
Ecstasy/MDMA	Possible reactive oxygen species inducer; direct mechanism is unknown	
	Lysosomal destabilization; mitochondrial lysis	Shintani-Ishida et al. (2014)
Cannabinoids	Possible reactive oxygen species inducer; direct mechanism is unknown	

function eventually leads to energetic and contractile imbalance and cardiomyocyte death. In normal healthy cells mitochondria form complex networks inside myocytes and differentiate into two distinct populations, one located between the myofibrils (i.e., interfibrillar mitochondria) and another one below the sarcolemma (i.e., subsarcolemmal mitochondria). This spatial distribution and large number of cardiac mitochondria ensure efficient and localized ATP delivery to

support contraction, metabolism, and ion homeostasis at the same time.

7.1.2 Mitochondrial Oxidative Stress in Cardiomyocytes

Modifications of mitochondrial proteins due to increased oxidative/nitrative stress play a crucial role in the development of myocardial dysfunction. Oxidative/nitrative

modifications may lead to harmful events including dissociation of catalytic subunits of enzymes, unfolding, aggregation or fragmentation of proteins, and formation of new antigen epitopes, all facilitating degradation of the modified proteins, leading to autophagy/mitophagy and endoplasmic reticulum stress (Bayeva and Ardehali, 2010; Castro et al., 2011; Varga et al., 2015). Oxidation/nitration might be directly caused by reactive oxygen and/or nitrogen species or triggered by secondary events via the products of subsequent oxidation reactions produced during peroxidation of membrane lipids (e.g., by malondialdehyde or 4-hydroxynonenal (Marin-Corral et al., 2010; Zhao et al., 2014)).

The free radicals inside the mitochondria may directly target and inactivate the electron transport complexes via interactions with the iron–sulfur moieties of the complexes (especially in complex I and II). There is substantial evidence for the inhibition of the energetically indispensable tricarboxylic acid cycle (TCA cycle) due to excessive production of mitochondrial reactive oxygen species (Bulteau et al., 2005; Kil and Park, 2005; McLain et al., 2011). Increased reactive oxygen species generation in cardiomyocytes may further trigger the initiation and activation of various cell death pathways involved in apoptotic and necrotic cell death (e.g., activation of caspases and poly(ADP-ribose) polymerases (PARP)) (Pacher and Szabo, 2007). Furthermore, superoxide may react with nitric oxide to generate a highly reactive oxidant, peroxynitrite (Pacher et al., 2003, 2007), which may impair cellular function and lead to apoptotic and necrotic cell death (Mukhopadhyay et al., 2009; Pacher et al., 2005) in cardiomyocytes and in endothelial cells via multiple interrelated mechanisms involving PARP (Pacher et al., 2002) and matrix metalloprotease (MMP) activation (Bai et al., 2004). Mitochondrial proteins are also susceptible to peroxynitrite-induced nitration, leading to irreversible functional loss (Pacher et al., 2007; Radi et al., 2002; Szabo et al., 2007).

7.1.2.1 Sources of Mitochondrial Reactive Oxygen Species

Oxidative stress is generally a result of a redox imbalance favoring production of reactive oxygen species over antioxidant defense mechanisms. The majority of reactive oxygen species are produced as a by-product of mitochondrial respiration. In healthy cells about 2% of the molecular oxygen consumed by mitochondria is converted into superoxide radicals, a relatively stable intermediate, serving as a precursor for further reactive species production (i.e., hydrogen peroxide, hydroxyl radical, peroxynitrite, etc.) (Chance et al., 1979). Major sites of superoxide generation by intact mitochondria are complex I and complex III (Chen et al., 2003). However, in addition to complex I and III, complex II (aka the succinate dehydrogenase complex) is also considered to

be an important site of excessive superoxide production, especially when mitochondrial respiration is suppressed and there is an excess of succinate levels (e.g., during ischemia (Chouchani et al., 2014)). In these situations, the accumulated succinate is rapidly reoxidized by complex II, driving extensive reactive oxygen species generation. In parallel, reverse electron transport occurs to complex I, leading to further uncontrolled reactive oxygen species production.

Although in recent years the electron transport chain components were the main focus of mitochondrial oxidative stress research, there are other potential sources of reactive oxygen species that are located within the mitochondria including p66Shc, monoamine oxidases (MAO-A and -B), and NADPH oxidase 4 (Di Lisa et al., 2009). Nevertheless, the role of non-electron transport chain-derived reactive oxygen species in drug-induced cardiotoxicity has not been studied in detail so far.

p66Shc is a variant of cytoplasmic adaptor protein family that is described to be involved in the activation process of Ras (Pelicci et al., 1992). It contains an additional N-terminal region compared with other Ras members and rather functions as a key regulator of reactive oxygen species metabolism and is not involved in Ras regulation (Migliaccio et al., 1997, 1999). In addition, it is also clear now that p66Shc directly interacts with cytochrome *c* and is able to transfer electrons to cytochrome *c* and thereby generate hydrogen peroxide (Giorgio et al., 2005).

Monoamine oxidases are flavo-enzymes localized within the outer mitochondrial membrane and responsible for the oxidative deamination of neurotransmitters and dietary amines, producing hydrogen peroxide as a by-product (Pizzinat et al., 1999). To date, two major isoforms have been identified, MAO-A and MAO-B, differing mainly in substrate specificity and inhibitor sensitivity. In conditions when the heart undergoes hemodynamic stress (e.g., during heart failure), the maladaptive neurohumoral changes (activation of the sympathoadrenal system) and additional local synthesis of monoamines drive MAO-derived hydrogen peroxide production that may further contribute to cardiac tissue injury (Kaludercic et al., 2014a, b).

NADPH oxidase enzymes (NOX) generate reactive oxygen species as their primary function, catalyzing electron transfer from NADPH to oxygen and thereby producing superoxide anion. Phagocytic cells express primarily NOX2, while other cell types including cardiomyocytes show high expression of NOX4 (Varga et al., 2013). Although NOX4 is described in many conditions as a major source of reactive oxygen species, its physiologic role is still controversial (Sirokmany et al., 2016). Based on recent reports, it seems that NOX4 isoform is localized to the outer mitochondrial membrane (Hirschhauser et al., 2015). It has also been suggested recently that NOX4 may function to control

by redox-dependent mechanisms cellular metabolism (e.g., mitochondrial fatty acid oxidation in macrophages (Moon et al., 2016) and cardiomyocytes (Nabeebaccus et al., 2015)).

7.1.2.2 Mitochondrial Antioxidant Defense

In the development of mitochondrial oxidative stress and subsequent mitochondrial functional deficit, the role of the malfunctioning mitochondrial antioxidant system is critical. There are several tightly organized enzymatic and nonenzymatic mechanisms that are responsible for the neutralization of reactive oxygen species produced inside the mitochondria.

Manganese-dependent superoxide dismutase (also known as mitochondrial superoxide dismutase) is localized within the mitochondrial matrix and converts the highly reactive superoxide to hydrogen peroxide that is capable of diffusing out from the mitochondria to the cytosol, where hydrogen peroxide can be further decomposed to water and oxygen (e.g., by the enzyme catalase).

There are further important nonenzymatic antioxidant component of mitochondria, for example, the tripeptide glutathione (γ -L-glutamyl-L-cysteinyl-glycine), abbreviated as GSH. In our current understanding GSH is synthesized in the cytoplasm and transported to the endoplasmic reticulum, nucleus, and the mitochondria. Its transport to the mitochondria requires a specialized machinery (Chen and Lash, 1998), since reduced GSH (having anionic nature) has to enter the inner mitochondrial matrix, owing a highly negative charge as well. Once inside the mitochondria, reduced GSH is able to undergo oxidation and subsequent dimerization by the formation of cysteine cross-bridges (GSSG). The reduced GSH can be regenerated from the oxidized GSSG with the action of multiple GSH-linked antioxidant enzymes (e.g., by glutathione reductase, glutathione peroxidases, or glutathione *S*-transferase or by the complex redox system of glutaredoxins, thioredoxins, and peroxiredoxins) being equally important in antioxidant defense. Glutathione peroxidase enzyme family (GPx) consists of eight different isoenzymes. GPx1 is the most abundant enzyme, expressed in many cell types of the body. Gpx4 (i.e., also known as phospholipid hydroperoxide glutathione peroxidase) is a membrane-associated isoenzyme that is also localized in the mitochondria (Pushpa-Rekha et al., 1995). The major function of Gpx4 is to detoxify membrane lipid hydroperoxides; thus it is considered the most important enzymatic defense mechanism against oxidative membrane damage. Cytochrome *c* is one of the most potent proapoptotic factors that preferentially binds to cardiolipin (the most abundant complex phospholipid in cardiac mitochondria), but not to cardiolipin hydroperoxide. Thus Gpx4 plays a key role in

mitochondrial cell death regulation by maintaining normal cardiolipin pool, thereby keeping cytochrome *c* membrane bound (Mari et al., 2009; Nomura et al., 2000).

Glutaredoxins, thioredoxins, and peroxiredoxins are further members of GSH-linked mitochondrial redox enzymes that are able to reduce GSSG, in addition to other mitochondrial thiols. Glutaredoxin 2 is present in both mitochondrial and nuclear fractions (Mailloux et al., 2014) and is able to prevent doxorubicin-induced cardiac injury (Diotte et al., 2009). The mitochondrial thioredoxin system, including thioredoxin 2 and thioredoxin reductase 2, is also an important member of disulfide reductases, maintaining mitochondrial proteins in reduced state (Arner and Holmgren, 2000). The thioredoxin system interacts with the peroxiredoxins, relying on thioredoxins as hydrogen donors (Chae et al., 1999).

7.1.3 Birth and Death of Cardiac Mitochondria

7.1.3.1 Mitochondrial Biogenesis

Mitochondrial biogenesis is defined as a process whereby preexisting mitochondria grow and divide. This process involves tight spatiotemporal coordination of mitochondrial DNA replication and transcription, intramitochondrial protein synthesis, and the nuclear-encoded protein import and assembly with mitochondrial-encoded proteins. These processes occur in tight regulation with the synthesis of complex membrane phospholipids (e.g., mitochondria-enriched cardiolipin). In addition, successful mitochondrial biogenesis is highly dependent on proper organization of the mitochondrial network by subsequent mitochondrial fission/fusion. Among the complex processes outlined, understanding the complex transcriptional control of mitochondrial biogenesis is critical, for example, to understanding the molecular basis of trastuzumab-induced cardiotoxic events.

Mitochondrial DNA replication and transcription is driven by nuclear-encoded transcription factors, like the mitochondrial transcription factor A and two transcription specific factors (TFB1M and TFB2M), a single RNA polymerase, and a termination factor (Scarpulla, 2008). Further coordination between mitochondrial and nuclear gene expression is regulated by a complex interplay between specific transcription factors (i.e., nuclear respiratory factors (NRFs), peroxisome proliferator-activated receptors (PPARs), estrogen-related receptors (ERR), and members of the transcriptional coactivator of PPAR γ (PGC-1 α and β) family). Among these, ERRs are of special interest, being indirectly targeted by trastuzumab. ERRs orchestrate a complex cardiac transcriptional program in tight relation with the PGC-1 family members, affecting uptake and processing of

energy-rich substrates (fatty acids, glucose), production and transport of ATP, and intracellular fuel sensing (AMP-activated protein kinase pathway) (Dufour et al., 2007). In recent years, however, upstream regulators of ERR activity have been identified, linking tightly both the receptor tyrosine protein kinase erbB-2 (Her2) and the insulin-like growth factor 1 receptor (IGF-1R) pathways to the regulation of ERR (Chang et al., 2011). Thus pharmacological manipulation of EGFR signaling by trastuzumab blocks Her2 dimerization or Her2/Her4 heterodimerization and thereby may interfere with ERR-related processes of mitochondrial biogenesis (Nemeth et al., 2016).

7.1.3.2 Mitophagy and Mitochondrial Apoptosis

In comparison with healthy, normal mitochondria, damaged ones have reduced capacity to maintain effective oxidative phosphorylation; therefore the production of ATP is impaired. In addition damaged mitochondria release proapoptotic molecules and generate high amounts of reactive oxygen species. Thus deterioration of mitochondrial function further promotes oxidative damage to mitochondrial lipids, proteins, and DNA, which further decreases the efficiency of electron transport chain, making mitochondria more prone to reactive oxygen species production, leading to a vicious cycle known as “reactive oxygen species-stimulated reactive oxygen species release.” Thus, the timely cleaning of damaged mitochondria is a pivotal mechanism in preventing and interrupting this vicious self-promoting cycle of reactive oxygen species generation.

Given the importance of mitochondria in cellular metabolism, several tightly controlled quality checking mechanisms have evolved to maintain a pool of healthy, functioning mitochondria. These processes include the mitochondrial ATP-dependent protease pathway, by which unfolded or misfolded proteins are degraded by two ATP-dependent proteases (Smakowska et al., 2014). Similarly the vesicular transport pathway delivers damaged proteins to lysosomes for degradation via sequestration into vesicles from mitochondria. In case of irreversible mitochondrial damage, mitophagy is responsible for bulk mitochondrial degradation. Electron microscopy studies clearly showed that entire mitochondria are often observed and likely degraded in lysosomes (Narendra et al., 2008).

Mitophagy is specifically activated to eliminate damaged mitochondria in response to various stress conditions. Besides being involved in mitochondrial clearance, mitophagy is also coupled to mitochondrial biogenesis, resulting in parallel removal of old and synthesis of new mitochondrial populations.

Mitochondria are also important regulators of cell death, responding to a wide variety of stress signals,

including loss of growth factors, toxic drugs, hypoxia, oxidative stress, and DNA damage. The switch to a cell death program can be mediated by opening of the mitochondrial permeability transition pore in the inner mitochondrial membrane, leading to a rapid collapse of the mitochondrial membrane potential subsequent swelling of the mitochondria that often culminates into necrotic cell death. In other cases the permeability transition of the mitochondrial outer membrane leads to the release of proapoptotic proteins such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor to activate an energy-dependent apoptotic cell death pathway (Lemasters et al., 2002).

7.2 Cardiotoxic Drugs That Cause Mitochondrial Dysfunction

7.2.1 Cardiotoxicity During Cancer Chemotherapy

7.2.1.1 Doxorubicin-Induced Cardiotoxicity

Doxorubicin is still one of the most effective chemotherapeutic drugs, but its use is limited by cumulative cardiotoxicity that restricts the applicable lifetime dose patients may receive.

It has been noted in the early 1970s that a group of patients who underwent doxorubicin treatment developed unexpected cardiomyopathy a few years later (Lefrak et al., 1973; Steinherz et al., 1991). In spite of its cardiotoxicity, doxorubicin is still widely used as it is one of the most potent and broad-spectrum chemotherapeutics available for different forms of leukemias and lymphomas, sarcomas, and various solid tumors (Hortobagyi, 1997).

The mechanisms by which doxorubicin induces cardiotoxicity are complex and involve numerous mitochondrial and non-mitochondrial processes. During the past decades several hypotheses have been proposed to explain the doxorubicin-induced cardiotoxicity.

The majority of studies identified mitochondrial involvement in the toxic effects and focused on the elucidation of molecular mechanisms of the overt mitochondrial abnormalities. It has been detected that doxorubicin is capable of adduct formation with mitochondrial DNA and directly binds to other biomolecules like the mitochondrial abundant phospholipid cardiolipin (Pereira et al., 2016). Mitochondrial DNA adduct formation and oxidation is a selective process for cardiomyocytes and cumulative with the dose applied (Serrano et al., 1999). Once doxorubicin accumulates in mitochondria, it can initiate intramitochondrial reactive oxygen species and reactive nitrogen species production (Mukhopadhyay et al., 2007b) by various mainly

nonenzymatic mechanisms (e.g., by redox cycling-dependent production of superoxide anion (Davies and Doroshow, 1986; Doroshow and Davies, 1986; Kumar et al., 2002; Ravi and Das, 2004; Singal and Iliskovic, 1998)), leading to activation of multiple cell death pathways (e.g., caspase and PARP-dependent cell death (Pacher et al., 2002, 2006)). The diffusion-limited rapid reaction of superoxide with nitric oxide forms peroxynitrite, a potent oxidant and cytotoxic reactive nitrogen species that promotes mitochondrial protein oxidation/nitration and initiation of cell death (Pacher et al., 2003). It has been also suggested that doxorubicin induces nonenzymatic, iron-dependent increase in reactive oxygen species production (Berthiaume and Wallace, 2007; Myers, 1998). Intramitochondrial accumulation of iron is detrimental, since doxorubicin-derived superoxide will be converted to hydrogen peroxide, forming the highly toxic hydroxyl radicals in the presence of iron, by a reaction described as the Haber–Weiss reaction. In addition, doxorubicin reacts with iron directly to form a complex, resulting in iron cycling between the ferro [Fe(II)] and ferri [Fe(III)] forms that leads to additional reactive oxygen species production (Ichikawa et al., 2014; Xu et al., 2005).

The increased oxidant load by doxorubicin is further complicated by the ineffectiveness and/or inhibition of antioxidant mechanisms in the presence of doxorubicin (Gustafson et al., 1993). In line with this, overexpression of antioxidant systems (e.g., thioredoxin-1 (Shioji et al., 2002), glutaredoxin 2 (Diotte et al., 2009), manganese superoxide dismutase (Yen et al., 1996), catalase (Kang et al., 1996), metallothionein (Guo et al., 2014; Kang et al., 1997)), or glutathione administration (Mohamed et al., 2000) has been shown to alleviate doxorubicin cardiotoxicity. Similarly, the recently developed mitochondria-targeted antioxidants MitoTEMPO and MitoQ have proven to be cardioprotective in rodent models of doxorubicin-induced cardiotoxicity, without interfering with its antitumor effect (Dickey et al., 2013). Activation of nuclear enzyme PARP due to doxorubicin-induced oxidative DNA injury is a key event in doxorubicin-induced cardiotoxicity (Pacher et al., 2002). Accordingly PARP inhibitors (e.g., olaparib) that are novel FDA-approved chemotherapeutic drugs (currently for specific forms of ovarian cancer) might be worthy to be combined with doxorubicin due to their potentially increased chemotherapeutic efficacy and decreased cardiotoxicity (Pacher et al., 2002).

In the last decades it was widely accepted that doxorubicin-induced cardiotoxicity is independent from its antineoplastic activity (Carvalho et al., 2009). Doxorubicin-dependent blockade of DNA transcription and replication is responsible for the therapeutic effect in cancerous cells. However, cardiac myocytes being

terminal differentiated and nondividing cells should not be sensitive to the blockade of cell division. In cancer cells the primary target of doxorubicin is topoisomerase II alpha (Petit et al., 2004). However, cardiomyocytes also express a topoisomerase, the isoenzyme 2, Top2 β (Capranico et al., 1992), and topoisomerase function is also necessary for the maintenance of normal mitochondrial function (Sobek and Boege, 2014). Therefore it is likely that doxorubicin-induced cardiotoxicity is not merely a result of doxorubicin-dependent reactive oxygen species generation. Now it is becoming clear that doxorubicin by interfering with Top2 β directly activates transcriptional pathways of apoptosis and initiates marked changes both in the mitochondria (Pointon et al., 2010) and in the nuclear transcriptome to influence oxidative phosphorylation and mitochondrial biogenesis (e.g., downregulation of PGC1 alpha and beta) in cardiomyocytes, leading to mitochondrial oxidative stress and metabolic failure (Hao et al., 2015; Zhang et al., 2012). This further explains how doxorubicin causes both structural and functional mitochondrial abnormalities.

7.2.1.2 Cisplatin-Induced Cardiotoxicity

Cisplatin is an alkylating, broad-spectrum chemotherapeutic drug used in the treatment of various types of tumors (sarcomas, carcinomas, lymphomas, and germ cell tumors). A significant limitation for its therapeutic use is acute and cumulative cardiotoxicity, ototoxicity, and nephrotoxicity (Dugbartey et al., 2016; Ma et al., 2010; Mukhopadhyay et al., 2012; Patane, 2014; Zsengeller et al., 2012). Cisplatin-related cardiac dysfunction is a result of mitochondrial membrane depolarization that is associated with mitochondrial ultrastructural abnormalities. Cardiomyocytes show signs of endoplasmic reticulum stress response activation following cisplatin treatment, with increased caspase-3 activity and subsequent increased rate of apoptosis (Ma et al., 2010). It is now well documented that cisplatin promotes the development of tubular nephropathy by triggering mitochondrial reactive oxygen species generation (Zsengeller et al., 2012), and it is very likely that cisplatin induces cardiotoxicity in a similar manner. In line with these, antioxidants targeting specifically mitochondrial reactive oxygen species (MitoQ, MitoTEMPO) might be a promising way to alleviate both cardiac and kidney complications of cisplatin chemotherapy (Mukhopadhyay et al., 2012).

7.2.1.3 Trastuzumab-Induced Cardiotoxicity

Trastuzumab is a monoclonal antibody developed for the treatment of metastatic breast cancer. It inhibits the homo-/heterodimerization of the Her2/neu receptor, thereby interfering with activation of downstream

signaling pathways that lead to the growth/metastasis of breast cancer cells (Nemeth et al., 2016). Her2 possesses an important role in embryonic heart development, and in the adult heart it is involved in cardiac protection (Negro et al., 2004). HER2 signaling is essential for the growth, survival, and inhibition of cardiomyocyte apoptosis; therefore in cases where the heart is subjected to biomechanical stress (e.g., hypoxia, myocardial injury, or concomitant anthracycline use), neuregulin binds to Her2/Her4 heterodimers, thereby promoting cardiomyocyte survival via the activation of so-called survival kinases, the PI3K and MAPK pathways (Negro et al., 2006; Zeglinski et al., 2011). Decrease in Her2 and Her4 protein expression has been shown to be associated with the pathomechanism of transition from compensated cardiac hypertrophy to heart failure (Rohrbach et al., 1999). In addition, trastuzumab triggers oxidative stress and induces the expression and activation of proapoptotic proteins. These events eventually lead to mitochondrial dysfunction, opening of the mitochondrial permeability transition pore, and activation of mitochondrial cell death pathways (Baines et al., 2005; Zorov et al., 2000). ERRs are critical determinants of the regulation of mitochondrial biogenesis. Recently, Her2 and IGF-1R pathways have been identified as upstream regulators of ERR activity (Chang et al., 2011), thereby linking Her2 inhibition by trastuzumab to a parallel inhibition of mitochondrial biogenesis via ERR. Although the parallel inhibition of both pathways (Her2 and ERR) might be beneficial in breast cancer cells, this synergism is likely a major contributor of trastuzumab-induced cardiotoxicity.

Anthracyclines (e.g., doxorubicin) are often used in the treatment of metastatic breast cancer. Even when administered at low and safe doses, doxorubicin still induces subclinical myocardial damage. This subclinical cardiomyocyte injury is associated with the parallel activation of Her2-dependent pathways in the cardiomyocytes to prevent further cardiomyocyte injury and the development of cardiomyopathy. Several lines of evidence (*in vitro*, *in vivo*, and clinical) clearly show that trastuzumab significantly aggravates doxorubicin-induced cardiac injury, resulting in a high incidence of heart failure (reaching 27% of treated patients) (Slamon et al., 2001). The incidence of heart failure in trastuzumab-treated patients alone is significantly lower (around 4%) (Untch et al., 2004). Although trastuzumab monotherapy exhibits lower rate of cardiac toxicity, its application is still associated with significant alterations in the expression of genes essential for DNA repair and mitochondrial function in the mouse heart (ElZarrad et al., 2013). Nevertheless, the debate on the cardiac safety of trastuzumab is still ongoing, and further studies are required to address this important issue (Nemeth et al., 2016).

7.2.1.4 Arsenic Trioxide-Induced Cardiotoxicity

Arsenic trioxide is an antineoplastic drug, highly effective in the therapy of acute promyelocytic leukemia by inducing apoptotic signaling in cancer cells. The clinical efficacy of arsenic trioxide is burdened by serious cardiac toxicity (prolongation of the QT interval, torsade de pointes arrhythmia, and sudden death) (Naito et al., 2006; Unnikrishnan et al., 2001). It also appears that arsenic trioxide directly inhibits the promyelocytic leukemia protein–retinoic acid receptor (PML-RAR) alpha fusion protein that is often detected in acute promyelocytic leukemia. In the experimental setting, it has been shown that exposure of a cardiomyoblast cell line (H9c2) to arsenic trioxide results in loss of mitochondrial membrane potential (Varghese et al., 2017), induction of apoptosis, increased reactive oxygen species formation, intracellular calcium overload, and caspase-3 activation (Zhao et al., 2008). The negative molecular effects lead to apparent functional contractile deficit. Arsenic trioxide-exposed hearts show impaired response to beta-adrenergic stimulation, showing impaired contractile reserve capacity (Li et al., 2002). The proapoptotic effect of arsenic trioxide was further confirmed by Watanabe et al. in the HL-1 mouse atrial cardiomyocyte line. They found Parkin-dependent ubiquitin proteasome activation to be associated with arsenic trioxide-induced loss of mitochondrial membrane potential (Watanabe et al., 2014).

7.2.1.5 Mitoxantrone-Induced Cardiotoxicity

Mitoxantrone is a commonly used non-anthracycline antineoplastic agent that inhibits the DNA topoisomerase II. It is used for the treatment of various forms of cancers (e.g., metastatic breast cancer, non-Hodgkin's lymphoma, leukemia in children, and metastatic prostate cancer). In addition to its use in cancer chemotherapy, it is therapeutically utilized in multiple sclerosis to slow down the progression and prevent the relapses of the disease. Cardiomyopathy is a quite common and severe irreversible side effect of long-term mitoxantrone therapy that is in many cases unpredictable and develops only years after the treatment received (Schell et al., 1982; Stuart-Harris et al., 1984). Mitoxantrone induces cardiac energetic imbalance characterized by low ATP levels, loss of mitochondrial membrane potential, and significant rise in the intracellular calcium levels *in vitro*. This is in part a result of the inhibition of ATP synthase expression and activity, with concomitant increase in reactive oxygen species formation (Rossato et al., 2013). It is also confirmed that cardiac functional alterations develop due to malfunctioning mitochondria that show changes in mitochondrial complex IV and V activities, leading to low cardiac ATP levels (Rossato et al., 2014). There is further evidence that mitoxantrone as a DNA

topoisomerase inhibitor interacts with the mitochondria-related topoisomerase enzyme (i.e., topoisomerase II β) that maintains mitochondrial biogenesis and mitochondrial DNA integrity and therefore cellular energy homeostasis (Douarre et al., 2012). The existence of mitochondrial off-target effect is further supported by reports showing lack of cardiotoxicity of structurally related but more selective compounds (like pixantrone) that are selectively targeting topoisomerase II α over topoisomerase II β (Hasinoff et al., 2016).

7.2.1.6 Imatinib Mesylate-Induced Cardiotoxicity

Imatinib mesylate was one of the first drugs developed by rational drug design for tyrosine kinase inhibition (inhibitor of the breakpoint cluster region-Abelson (BCR/Abl) fusion protein kinase). In spite of its recorded cardiotoxic effect, imatinib mesylate still represents a revolution in the management of chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (Deininger et al., 2005; Kantarjian et al., 2003). The first reports dealing with cardiotoxicity were published 5 years later of its FDA approval (Kerkela et al., 2006). In group of patients, sudden development of New York Heart Association (NYHA) class 3–4 heart failure has been reported after a few months (7.2 ± 5.4) of imatinib therapy. The ultrastructural analysis revealed prominent membrane whorls in myocytes, with increased number of pleomorphic mitochondria with effaced cristae, indicative of increased compensatory mitochondrial biogenesis. *In vitro* studies on isolated cardiomyocytes revealed that imatinib produces a dose-dependent harmful effect on mitochondrial membrane potential. The use of imatinib resulted in increased endoplasmic reticulum stress (increased protein kinase R-like endoplasmic reticulum kinase (PERK) activation and phosphorylation of eukaryotic translation initiation factor 2A (eIF2 α)). Since translocase of the inner membrane (TIM23), a major component of the inner membrane of the mitochondrial protein import machinery, is degraded after eIF2 α phosphorylation, unfolded proteins that are otherwise targeted to the inner mitochondrial membrane accumulate in the intermembrane space, leading to mitochondrial proteostasis and inducing mitochondrial death pathways (Rainbolt et al., 2014).

7.2.1.7 Cardiotoxicity of Antiangiogenic Drugs

Antiangiogenic drugs either directly inhibit vascular endothelial growth factor (e.g., bevacizumab, a recombinant humanized monoclonal antibody against vascular endothelial growth factor A (VEGF-A)) or interfere with downstream signaling pathways involving many kinases (e.g., by inhibiting the VEGF, platelet-derived growth factor, stem cell growth factor receptor (c-kit), RET proto-oncogene, fms-like tyrosine kinase 3 (FLT3)

signaling) (Kerbel, 2006). During their clinical use, several cardiovascular side effects have been reported (Bordun et al., 2015; Chu et al., 2007; Schmidinger et al., 2008) involving left ventricular dysfunction and subsequent heart failure that develop due to inadequate angiogenesis. However, in the case of the small-molecule multitargeted receptor tyrosine kinase inhibitors, other mitochondria-related signaling pathways play important roles in the observed cardiotoxicity (Will et al., 2008).

Sunitinib and sorafenib are the most widely used receptor tyrosine kinase inhibitors with potent antiangiogenic activity. Sunitinib is highly cardiotoxic, deteriorating myocardial contractility in up to 28% of treated patients (Chu et al., 2007). The mechanism of sunitinib cardiotoxicity can be explained by impairment of autophagy (Jacob et al., 2016) and inhibition of mitochondria-related off-target pathways, like the ribosomal S6 kinase and AMP-activated protein kinase (Hasinoff et al., 2008; Kerkela et al., 2009) that are both involved in mitochondrial energy homeostasis and mitochondrial biogenesis (Zhao et al., 2010).

7.2.2 Cardiotoxicity of Antiviral Drugs

Multiple antiviral drug combinations have been developed recently to control the replication of HIV and prevent the development of AIDS. Highly active antiretroviral therapy regimes involve nucleoside analogues that inhibit the reverse transcriptase of HIV. Zidovudine (azidothymidine (AZT)) approved in the United States in 1986 was the first compound successfully used in the management of HIV infection. Later it was recognized that long-term treatment with AZT may cause cardiomyopathy, as a result of mitochondria-related toxicity. AZT triphosphate inhibits the mitochondrial DNA polymerase γ , which is the enzyme responsible for mitochondrial DNA replication (Lewis et al., 1994). AZT may directly inhibit important mitochondrial transport mechanisms such as the mitochondrial ADP/ATP translocator (Barile et al., 1997), leading to loss of effective ATP production, or the mitochondrial deoxynucleotide carrier (Dolce et al., 2001), leading to inhibition of the synthesis of DNA in mitochondria, inhibiting mitochondrial replication and biogenesis (Liu et al., 2012). Although energy depletion by both mechanisms may contribute to cardiac dysfunction, it is now also evident that AZT induces increased mitochondrial reactive oxygen species production as well (Gao et al., 2011). This is further supported by the fact that AZT-induced cardiomyopathy is prevented in mitochondrial superoxide dismutase transgenic mice (Kohler et al., 2009). Similarly mitochondria-targeted catalase also prevents AZT-induced oxidative stress and cardiomyopathy (Kohler et al., 2009). In line with these results, a sudden increase

in mitochondrial reactive oxygen species production in AZT-treated human cardiomyocytes has been reported that was associated with activation of mitochondrial cell death pathways (caspase-3 and caspase-7, as well as PARP) (Gao et al., 2011).

7.2.3 Cardiotoxicity of Addictive Drugs

7.2.3.1 Cardiotoxicity in Chronic Alcohol Use Disorder

Chronic alcohol abuse is an increasing problem worldwide; 7.2% (17 million) of adults in the United States had alcohol use disorder in 2012 according to the recent Alcohol Facts and Statistics of the National Institute on Alcohol Abuse and Alcoholism. Chronic and acute alcohol abuse impairs myocardial contractility that eventually leads to systolic dysfunction and later to dilation of the chambers of the heart, known as alcoholic cardiomyopathy (El Hajj et al., 2014; Gardner and Mouton, 2015; Matyas et al., 2016).

High alcohol levels may exert direct cardiotoxicity since alcohol dehydrogenase, responsible for alcohol detoxification, is almost absent from cardiomyocytes (Awtry and Philippides, 2010). In addition, acetaldehyde, the metabolic by-product formed in the liver during oxidative alcohol catabolism, further triggers cardiomyocyte injury by reducing the synthesis of myocardial proteins (Lang and Korzick, 2014; Schreiber et al., 1975), triggering oxidative stress (Brandt et al., 2016), and thereby disturbing calcium homeostasis and inducing endoplasmic reticulum stress (Guo and Ren, 2006; Li and Ren, 2008). There are reports showing production of fatty acid ethyl esters (by nonenzymatic reactions) in the heart after alcohol exposure. Accumulation of this by-product is able to induce mitochondrial dysfunction by uncoupling mitochondrial oxidative phosphorylation (Lange and Sobel, 1983). In addition, alcohol-induced cardiac toxicity further involves inhibition of the mitochondrial respiratory chain by impairing the function of TCA enzymes (Mihailovic et al., 1999). Alcohol induces oxidative stress due to its metabolism by cytochrome p4502E1 isoenzyme (Hoek et al., 2002; Zhang et al., 2013), responsible for microsomal ethanol metabolism. Oxidative tissue injury in ethanol-treated animals also contributes to subsequent myocardial fibrotic changes (Brandt et al., 2016; Vendemiale et al., 2001). Alcohol-induced oxidative stress is further enhanced by increased angiotensin II signaling (enhanced angiotensin 2 type 1 receptor expressions), resulting in upregulation of NADPH oxidase subunits, and increased inflammation and fibrosis (Tan et al., 2012), which are known contributors of myocardial dysfunction (Varga et al., 2013). Ethanol also may activate inducible nitric oxide synthase via Toll-like receptor-mediated signaling

(partially due to increased endotoxin load from the gastrointestinal tract (Bala et al., 2014)) to produce NO, which may in turn react with superoxide anion to form the highly reactive substance peroxynitrite, which can further deteriorate mitochondrial function by posttranslational protein modifications (Pacher et al., 2007; Varga et al., 2015). There is also compelling evidence from animal models of chronic alcohol abuse for decreased mitochondrial number that may result from the decreased expression of master regulator of mitochondrial biogenesis, PGC-1 α (Hu et al., 2013; Marin-Garcia and Goldenthal, 2004). The combination of these events of toxicity contributes to the pathology observed and potentially leads to the decrease of myocardial contractile function.

7.2.3.2 Cardiotoxicity in Cocaine Abuse

Cocaine abuse causes structural and functional abnormalities in the heart, resulting in irreversible reduction of left ventricular contractility and increased incidence of arrhythmias. Additionally, due to acute toxicity, coronary vasoconstriction develops, which makes cocaine users more susceptible to myocardial infarction (Riezzo et al., 2012).

The currently recognized major cause of cardiac cocaine-related effect is the overstimulation of the adrenergic system. Toxic effects of cocaine are mediated by increased oxidative stress or mitochondrial dysfunction caused by metabolism of the excess of catecholamines (Liaudet et al., 2014). Excess of catecholamines is metabolized into aminochromes (e.g., to adrenochrome or to adrenolutin), which may undergo redox cycling after entering the mitochondria, leading to the generation of significant amounts of reactive oxygen species (Behonick et al., 2001). This is supported by strong experimental evidence from human cocaine-related cardiomyopathy samples (Frustaci et al., 2015). There is also evidence for inhibition of mitochondrial complex I by cocaine (Yuan and Acosta, 2000) and for xanthine oxidase-dependent increase in reactive oxygen species production after cocaine exposure that negatively impacts mitochondrial function (Vergeade et al., 2012). As a consequence, calcium overload and oxidative stress promote mitochondrial permeability transition pore opening, apoptosis initiation, and cardiomyocyte cell death, both via parallel activation of the apoptotic and necrotic pathways (Lattanzio et al., 2005). The central role of mitochondrial oxidative stress in cocaine-induced cardiotoxicity is also supported by the results of Vergeade et al., showing that cocaine-related cardiotoxicity can be mitigated by MitoQ treatment (Vergeade et al., 2010). There is further indirect human evidence for the involvement of mitochondrial dysfunction in cocaine-induced cardiac injury. In a human study (Lai et al., 2015), involving 180 participants

(80 were chronic cocaine users and 100 were nonusers), a significant correlation was found in myocardial fat accumulation in chronic cocaine users that was independently associated with duration of cocaine use, leptin, and visceral fat in all subjects, indicating an effect on cocaine on myocardial lipid metabolism (e.g., on mitochondrial fatty acid oxidation).

7.2.3.3 Cardiotoxicity in Methamphetamine and Ecstasy Abuse

Methamphetamine abuse is a significant health problem with a steeply increasing frequency of use in the United States and worldwide (Neeki et al., 2016). Chronic methamphetamine use is recognized to be associated with congestive heart failure (Sliman et al., 2016) due to myocardial structural alterations like focal contraction band necrosis, subsequent cellular degeneration, vacuolization, and myocytolysis. After chronic methamphetamine administration, cardiac hypertrophy, intracellular vacuolization, and fibrosis can be also observed (Yi et al., 2008). The direct cardiotoxic mechanisms of methamphetamine are not known in detail. Increased mitochondrial superoxide production has been reported (Mashayekhi et al., 2014) along with increased mitochondrial protein tyrosine residue nitration (Lord et al., 2010) and induction of Fas- and mitochondria-dependent apoptosis (Liou et al., 2014). Methamphetamine exposure increases DNA damage-inducible transcript 4 expression and decreases phosphorylation of mTOR that is accompanied with increased cardiomyocyte autophagy and apoptosis, linking the effect of methamphetamine to mitochondrial quality control pathways (Chen et al., 2016).

Ecstasy (MDMA; 3,4-methylenedioxymethamphetamine) is a substituted amphetamine, producing structural and functional alterations in the myocardium, which are associated with increased oxidative/nitrosative stress. There are reports showing that ecstasy significantly increases nitrotyrosine content in the heart, associated with nitration of contractile proteins (troponin T, tropomyosin alpha-1 chain, myosin light polypeptide, and myosin regulatory light chain), mitochondrial proteins (ubiquinone–cytochrome *c* reductase (complex III) and ATP synthase (complex V)), and sarcoplasmic reticulum calcium ATPase (Shenouda et al., 2008). Ecstasy also causes lysosomal destabilization following activation of

the autophagy–lysosomal pathway, through which released lysosomal proteases damage myofibrils and potentially mitochondria and therefore induce cardiac dysfunction (Shintani-Ishida et al., 2014).

7.2.3.4 Cardiotoxicity of Synthetic Cannabinoids

Synthetic cannabinoids (aka spice, K2, etc.) are getting popular especially among young people (Adams et al., 2017). These illicit drugs are among the most frequently used ones, since these mixtures still can be easily purchased due to the lack of legal restrictions (manufacturers are constantly changing, redesigning, and substituting different chemicals in their mixtures). Several case reports were published recently, showing occurrence of life-threatening complications induced by synthetic cannabinoids, involving cases of cardiotoxicity (Young et al., 2012), myocardial infarction (Hamilton et al., 2017), cardiac arrest (Davis and Boddington, 2015), acute kidney injury (Buser et al., 2014), or cerebral ischemia (Takematsu et al., 2014). The majority of studies suggest cannabinoid receptor-1 (CB1)-dependent toxicity (Tomiyama and Funada, 2014; Wiebelhaus et al., 2012), which is supported by the data showing increased endocannabinoid levels (Mukhopadhyay et al., 2007a, 2011) and overactivated CB-1 signaling (Mukhopadhyay et al., 2010) in various heart failure models, including doxorubicin-induced heart failure (Pacher and Kunos, 2013).

7.3 Conclusions

Vast majority of experimental evidence implies that the cardiotoxicity of multiple commonly used chemotherapeutic/anticancer drugs, antiretroviral compounds, or illicit drugs of abuse involves direct or indirect mitochondria-related toxicity. More thorough understanding of the common mechanisms of mitochondrial cardiac toxicity is needed to develop sensitive and high-throughput mitochondrial toxicity screening methods. There is further need for *in vivo* animal models to find new diagnostic markers of cardiotoxicity and to better predict the unforeseen cardiotoxicity issues of novel drug candidates. Development of novel cardioprotective strategies selectively targeting mitochondrial toxicity events is of high clinical importance.

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8

Skeletal Muscle Mitochondrial Toxicity

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8.1 Introduction

Skeletal muscle accounts for approximately 45% of the human body mass, and not surprisingly the blood supply is rich and metabolic rate is high in this tissue. As a result, depending upon uptake into the tissue, skeletal muscle is exposed to circulating drugs and their metabolites and is therefore a target for some toxins. Myopathy is generally a nonfatal disease of muscles that manifests as pain, weakness, and cramps, sometimes accompanied by a raised serum creatine kinase (CK) level. By definition, myopathy refers to an intrinsic defect within the muscle fibers rather a problem with associated nerves, for example, the neuropathies. There are many types of genetic myopathies including muscular dystrophies, metabolic myopathies, and mitochondrial myopathies (Jefferies et al. 2016). Acquired myopathies include not only those induced by drugs, alcohol, and other toxic agents but also a number of other conditions such as polymyositis, dermatomyositis, and rhabdomyolysis. The focus of this chapter is the two major drug groups, statins and nucleoside analogue reverse transcriptase inhibitors (NRTIs), that act or may act via mitochondria to mediate skeletal muscle toxicity. We review the history of myopathy by these

drugs and the major recent findings and critique the currently proposed mechanisms of action.

8.1.1 Type 1 and Type 2 Skeletal Muscle Fibers

In adult mammals there are four major skeletal muscle fiber types: types 1, 2A, 2B, and 2X (Schiaffino & Reggiani 2011). Individual skeletal muscles are highly heterogeneous in terms of content of fiber type. Muscles are usually classified according to the relative content of different fiber types. Type 1 fibers are defined by their myosin heavy chain (MHC) content as a marker; type 1 fibers contain MHC 1/β, whereas type 2 contain MHC 2A, 2X, and 2B. Type 1 fibers are generally slow to contract and highly oxidative with a high mitochondrial content; type 2 fibers contract rapidly and are highly glycolytic. The different fiber type composition defines the properties of a particular muscle in mammals. A further degree of complexity is apparent due to species differences, for example, diaphragm is a fast-acting muscle in rodents but a slow muscle in larger animals (Schiaffino & Reggiani 2011). Moreover, in humans no MHC IIB (type 2B fiber) is detected in any of the fiber types (Smerdu et al. 1994).

8.1.2 Drug-Induced Myopathy

Terminology to describe skeletal muscle pathology related to drug use is sometimes confusing. The definitions described by the American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute Clinical Advisory on the “Use and Safety of Statins” are adopted (Pasternak et al. 2002). Myopathy is defined as any pathology of muscle; myalgia is muscle pain or weakness without an elevation in serum CK; myositis is muscle symptoms with increased CK; rhabdomyolysis is a serious condition with muscle symptoms and a marked CK elevation (>10 times upper limit of the normal range) with elevated urinary creatinine, usually with brown urine and urinary myoglobin. At least four classification systems (Pasternak et al. 2002; Rosenson et al. 2014; Mancini et al. 2016) have been described to account for the adverse effects of drugs on skeletal muscle in man (recently reviewed by Thompson et al. (2016)). Some of the classifications provide utility in clinical practice, whereas others are more applicable in clinical research. Some terminology is often used interchangeably, for example, the usage of the terms “myopathy” and “myositis” is sometimes unclear. Generally, myopathy is reserved for the spectrum of muscle symptoms, whereas myositis depends on an associated rise in serum CK. It is generally recognized that severity of muscle injury by drugs is indicated by the presence of elevated serum CK levels, suggesting myonecrosis; however, CK can also arise from muscle via leakage through sarcolemma rather than cell death. Moreover, vigorous exercise can result in elevated CK levels (Baird et al. 2012).

Rhabdomyolysis is the breakdown of skeletal muscle cells to release myoglobin and may precede CK elevation in serum. It is often accompanied by increased serum K^+ , hyperuricosuria, acidosis, and the appearance of myoglobin in urine. The kidneys are a target for the effects of myoglobin with potentially fatal renal failure and other complications such as arrhythmias and disseminated intravascular coagulation ensuing (Slater & Mullins 1998; David 2000; Hohenegger 2012). Some drugs (rarely) cause rhabdomyolysis, and this is often preceded by myalgia and muscle weakness. Rhabdomyolysis is a rare complication of statin toxicity that caused the withdrawal of cerivastatin from the market in 2001 (Staffa et al. 2002).

8.2 Statin Myopathy

Statins are a widely and successfully prescribed class of drugs used to lower levels of circulating low-density lipoprotein (LDL) cholesterol. They achieve this by

competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase that is responsible for converting HMG-CoA into mevalonate; this is the rate-limiting step in the synthesis of cholesterol (Figure 8.1). Mevalonate is the precursor of other biologically important molecules such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FP), that are involved in protein prenylation, and dolichol (Figure 8.1). GGPP is also a precursor of ubiquinone, an active component of the mitochondrial electron transport chain, discussed in the following text with respect to statin myopathy. Statins have a polar head group that binds to the enzyme in the same way as the natural substrate, HMG-CoA. The hydrophobic moiety of the statin structure enables additional interactions.

The first statin to be approved by the FDA and marketed was lovastatin (Mevacor) in 1987, which originally had been extracted from *Aspergillus terreus* (Alberts et al. 1980). Simvastatin is a semisynthetic analogue of lovastatin and was introduced a year later. These are referred to as “type 1” statins and often identified by their decalin ring. Type 2 statins (atorvastatin, cerivastatin) are synthetic molecules with a larger hydrophobic moiety. Statins are the most frequently prescribed drugs in developed countries and are well tolerated. These act on the liver as hepatocytes are the main site of cholesterol synthesis. Hepatocytes take up statins using the organic anion transporter OATP1B1. It is believed that statins that possess a lower hydrophobic character will cause fewer side effects due to greater difficulty in crossing cell membranes.

By decreasing levels of LDL cholesterol, statins reduce the development of atherosclerotic plaques, which can lead to ischemic heart and cerebrovascular diseases. They are therefore of major importance in patients who are at risk of hypercholesterolemia and cardiovascular disease. The 4S trial (“Scandinavian Simvastatin Survival Study”), undertaken in the 1990s in patients with known coronary heart disease, showed a 30% reduction in death in the group taking simvastatin compared with the placebo group (Scandinavian Simvastatin Survival Study 1994). A very recent review on the efficacy and safety of statins in man concludes that the beneficial use of statins has been understated and the reported harmful side effect has been overstated (Collins et al. 2016).

Some of the negative “noise” around the use of statins and the potential myopathy is due to the case of cerivastatin. If cerivastatin is removed from the analysis, the incidence of rhabdomyolysis is around <3 per 100,000 person-years estimated from a wide range of cohort and randomized control trials (Law & Rudnicka 2006).

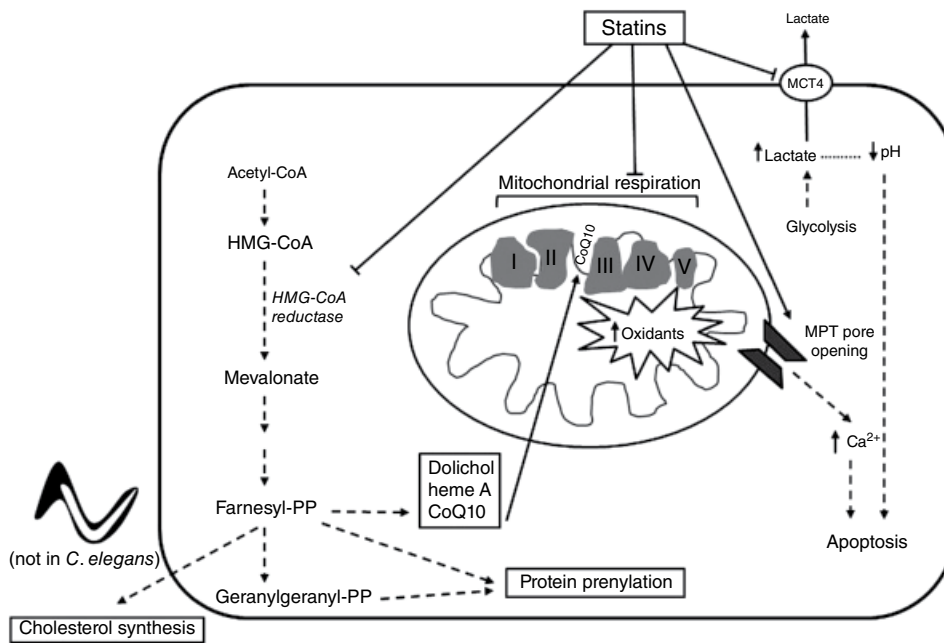


Figure 8.1 Proposed mechanisms of myopathy by statins. Statins inhibit HMG-CoA reductase and therefore the biosynthesis of cholesterol and other important biomolecules from acetyl-CoA. The sterol pathway is absent in *C. elegans*. In addition to cholesterol, statins inhibit the synthesis of other important molecules such as CoQ10, dolichol, and farnesyl pyrophosphate (farnesyl-PP). The latter along with geranylgeranyl pyrophosphate mediates protein prenylation, which is important in the membrane association of some key biochemical signaling molecules, such as small GTPases. Statins are also reported to inhibit the mitochondrial electron transport chain, promote mitochondrial oxidant formation, and stimulate mitochondrial pore transition (MPT) pore opening, leading to apoptosis. Furthermore, inhibition of lactate efflux from cells by statins may occur at the level of MCT4 inhibition and lead to apoptosis via a rise in intracellular pH.

Myopathy is related to the dose of a statin and presumably its concentration in the bloodstream. For example, 80 mg/day simvastatin resulted in a 10-fold higher rate of myopathy than 20 mg, and so the former is not usually recommended (Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine (SEARCH) Collaborative Group et al. 2010).

There is a well-recognized potential for drug interactions, for example, between cerivastatin and gemfibrozil that resulted in reduction in mitochondrial complex IV activity (Arenas et al. 2003). Similarly, a decrease in complex IV activity and in CoQ10 content has been reported in skeletal muscle in patients experiencing myopathy when treated with a combination of simvastatin, cyclosporine, and itraconazole (Duncan et al. 2009).

There are several suggestions in the literature that patients with underlying subclinical mitochondrial disorders and/or have a condition exacerbated by exercise are more likely to present with myopathy following statins (Antons et al. 2006; Eckel 2010; Golomb et al. 2012; Parker & Thompson 2012).

A strong association between common genetic variants in the *SLC01B1* gene and myopathy induced by statins has been described (SEARCH Collaborative

Group et al. 2008). This gene encodes the organic anion transporter protein OATP1B1, and variants affect the uptake of simvastatin into the liver. As a consequence, plasma levels rise with concomitant effects on the occurrence of myopathy.

8.2.1 Observations on Skeletal Muscle Fiber Type Selectivity During Statin Myotoxicity in Rodents

There have been numerous reports, and it is quite well accepted that statins cause necrosis primarily of type 2 glycolytic skeletal muscle fibers and have lesser effects on type 1 fibers. This has been observed for lovastatin, simvastatin, and pravastatin myopathy in rats, particularly for type 2B muscle fibers (Smith et al. 1991) and for *gastrocnemius* (glycolytic fast twitch) and not *soleus* (slow twitch and oxidative) in rats exposed to lovastatin (Waclawik et al. 1993). Three-week-old male Wistar rats treated for up to 3 weeks with lovastatin or simvastatin showed severe myopathy in the *extensor digitorum longus* (mainly type 2 glycolytic fibers) with only relatively minor damage in *soleus* muscle (Reijneveld et al. 1996); this study also illustrated the greatly increased sensitivity

of young animals to statin myopathy. Westwood et al. (2005, 2008) showed differential fiber sensitivity in rats, with type 2 fibers most sensitive to myopathic effects of simvastatin, cerivastatin, and rosuvastatin. In contrast to the majority of studies indicating that statins preferentially target type 2 muscle fibers in experimental animals, Obayashi et al. (2011) reported myopathy predominantly in type 1 fibers in young male F344 rats fed cerivastatin in the diet for 10 days. No satisfactory explanation of this finding has emerged.

Based on the data within these rodent *in vivo* studies, it would seem counterintuitive that statins might target mitochondria in skeletal muscle. Those fibers with the highest mitochondrial content were not the subject of myotoxicity. However, it has been suggested that mitochondria are protective against injury (Thompson et al. 2016). One possibility is that all mitochondria are affected but those in type 1 fibers are required, in some way, to maintain skeletal muscle fiber integrity. The concept that mitochondria in type 1 glycolytic fibers fulfill a vital redox balance function (e.g., NAD^+ regeneration (Ponsot et al. 2005)) is discussed in the following text. An alternate explanation for the observed fiber selectivity has been suggested based on monocarboxylate transporter 4 (MCT4), which is predominantly expressed in glycolytic type 2 fibers (Pilegaard et al. 1999) and involved in homeostatic lactate efflux from these cells. In the human embryonal rhabdomyosarcoma cell line RD, statin cytotoxicity was inhibited by either MCT4 knockdown (Kikutani et al. 2016) or chemical inhibition (Kobayashi et al. 2012). These findings support earlier observations in rat skeletal muscle fibers where MCT4 inhibition blocked the perturbation in calcium homeostasis induced by simvastatin (Sirvent et al. 2005a). Importantly, transport and accumulation of statins by MCT4 appears not to be involved in myotoxicity (Mosshammer et al. 2014; Kikutani et al. 2016). Instead, it has been proposed that inhibition of mitochondrial function by statins (see following sections) causes a reduction in ATP concentration and therefore an increase in ADP (Slade et al. 2006), which results in activation of AMP-activated protein kinase (AMPK). In skeletal muscle, MCT4 expression is upregulated by AMPK (Furugen et al. 2011). In the RD cell model, MCT4 mediates lactate efflux (Kobayashi et al. 2005), and so inhibition by statins is hypothesized to result in intracellular lactate accumulation, a raised pH, and apoptosis.

It is recognized that there are likely to be multiple pathological mechanisms contributing toward the adverse effects of statins on skeletal muscle (Tomaszewski et al. 2011; Banach et al. 2015; Taylor & Thompson 2015; Hargreaves et al. 2016), and this has been recently reviewed by Apostolopoulou et al. (2015). An excellent

clinically focused review by Golomb and Evans (2008) successfully highlights the evidence from human studies that statins cause pathology in skeletal muscle, and possible to other tissues, via effects on mitochondrial function (Golomb & Evans 2008). Mechanisms for statin effects are depicted in Figure 8.1 and include effects on membranes due to inhibition of cholesterol synthesis and decreased synthesis of GGPP and FP intermediates involved in posttranslational modification of small GTPases. Loss of this prenylation results in apoptosis in rat and human myocytes (Johnson et al. 2004). Although statins do not cause muscle atrophy, an increase in atrogen-1, which also increases during early muscle atrophy, is noted on statin exposure (Cao et al. 2009); this effect is believed to be due to loss of prenylation by GGPP. Myopathy may also be induced by lack of dolichols, other intermediates in the mevalonate pathway, which are involved in N-linked glycosylation and loss of which, therefore, may inhibit proper receptor function (Siddals et al. 2004).

8.2.2 Evidence for the Direct and Indirect Impact of Statins on Mitochondrial Function

8.2.2.1 Statins and the Biosynthesis of CoQ10

CoQ10 (also known as ubiquinone), an important electron carrier in the mitochondrial electron transport chain, is a product of the mevalonate pathway (Figure 8.1), and so synthesis would be expected to be decreased by statins.

8.2.2.1.1 Genetic Polymorphisms of COQ2 and Statin Myopathy

A number of studies have suggested that polymorphisms within the *COQ2* gene, which codes for coenzyme Q2 polyprenyltransferase, are associated with increased statin intolerance. This enzyme is involved in the final steps of CoQ10 synthesis. Muscle symptoms (muscle weakness, tenderness, and/or pain) were first associated with two polymorphisms in *COQ2* in patients taking statins (Oh et al. 2007).

“Muscular intolerance” to rosuvastatin or atorvastatin has also been associated with *COQ2* gene polymorphism (Puccetti et al. 2010). The *COQ2* polymorphism rs4693570 was confirmed to be associated with an increased risk of myalgia for patients receiving statins (Ruano et al. 2011). These studies provide a basis for the suggestion that variation in CoQ10 synthesis might play a role in susceptibility for statin-induced myopathy and their variants might be important predictive biomarkers for this adverse effect in patients. Moreover, although causation has not been proven, these data add support to the hypothesis that CoQ10 depletion plays a mechanistic role in the development of statin myopathy.

8.2.2.1.2 Depletion of Skeletal Muscle CoQ10 by Statins

Studies in hypercholesterolemic patients, with or without myopathy, showed that statins can increase the serum lactate/pyruvate ratio, a measure of the overall *in vivo* mitochondrial redox state, and suggested that mitochondrial inhibition might be induced in some patients receiving statins (De Pinieux et al. 1996; Goli et al. 2002). Often these symptoms resolve on drug withdrawal. In the study by De Pinieux et al. (1996), serum CoQ10 levels were lowered in patients receiving statins compared with untreated patients. However, no correlation was found between the lactate/pyruvate ratio and serum CoQ10 levels (De Pinieux et al. 1996). In rabbits, Nakahara et al. (1998) found myopathy following lipophilic simvastatin or hydrophilic pravastatin by gavage. Muscle necrosis and myopathy were evident for simvastatin-treated animals and at the higher dose of pravastatin. Mitochondrial swelling and a significant reduction in the CoQ10 content (49–72% in the high-dose pravastatin group) were observed, yet activities of NADH–cytochrome *c* reductase, succinate cytochrome *c* reductase, cytochrome *c* oxidase, and citrate synthase were unchanged in both the *soleus* and *extensor digitorum longus* muscles. The authors therefore concluded that myopathy was not a consequence of disturbed mitochondrial respiration in muscles and that a robust reduction in CoQ10 levels did not result in mitochondrial dysfunction. The reserves of CoQ10 within muscle cells appeared to be sufficient to maintain mitochondrial electron transport at effective levels. In support of these conclusions, a 15-day repeat dose study in rats (Schaefer et al. 2004) showed that cerivastatin caused myopathy but that ubiquinone levels were not significantly affected. These data indicate that decreases in ubiquinone were not the primary cause of the skeletal pathology induced by cerivastatin.

Subsequently, there has been much discussion in the literature on the role of inhibition of CoQ10 synthesis in statin-induced myopathy, dietary supplementation with CoQ10 being a plausible treatment strategy. In a systematic review on this subject, Marcoff and Thompson (2007) evaluated 18 studies on statins in humans and concluded that a reduction in serum CoQ10 by statins was probably due to the overall reduction in LDL cholesterol and therefore in serum transport of CoQ10 by LDL. It is estimated that half of the body's requirements for CoQ10 is from dietary intake and subsequent transport via LDL. Nevertheless, the changes to circulating CoQ10 levels do not appear necessarily to be reflected in muscle levels of CoQ10. A randomized double-blind, placebo-controlled study of statin-naïve, hypercholesterolemic patients on a high statin dose (80 mg/day simvastatin for 8 weeks) showed reductions in skeletal muscle CoQ10 by about one-third although these remained in the low normal range defined within the study (Paiva et al. 2005).

CoQ10 changes were not observed for the group receiving 40 mg atorvastatin despite an equivalent lipid-lowering effect to the 80 mg dose. In those patients whose skeletal muscle CoQ10 levels were most affected by simvastatin ($n=6$ compared with matched controls), there was evidence of reduced activities of mitochondrial complexes II, III, and IV and citrate synthase (Paiva et al. 2005). Indeed, in a subsequent report by the same group (Schick et al. 2007) and using the same CoQ10 data, it was observed that the change in CoQ10 levels over the 8-week study correlated well with decreased mitochondrial DNA (mtDNA) content across the combined control, simvastatin, and atorvastatin groups. As a marker of mitochondrial mass, mtDNA content by qPCR indicates that the reduced CoQ10 in muscles from simvastatin-treated patients reflects a reduced mitochondrial content due to the effects of statins on muscle cells or on their mitochondria.

Other studies do not report similar changes in CoQ10 when usual doses of statins are investigated (Laaksonen et al. 1995, 1996; Lamperti et al. 2005). Laaksonen et al. (1996) compared 19 hypercholesterolemic but otherwise healthy patients receiving 20 mg/day simvastatin with a group of untreated healthy controls and observed no changes in skeletal muscle CoQ10 or ATP/creatine phosphate levels following a 6-month statin treatment; this was in contrast to a significant fall in serum CoQ10 following statin treatment. This study supported their earlier work where a fall in serum CoQ10 following 4 weeks of simvastatin treatment was not mirrored by a reduction in skeletal muscle CoQ10; in fact levels were increased (Laaksonen et al. 1995). In 18 patients with statin-related myopathy (cramps, myalgia, proximal weakness), Lamperti et al. (2005) reported normal muscle CoQ10 levels compared with an unspecified control group (Lamperti et al. 2005); a wide range of skeletal muscle CoQ10 levels was reported for the patient group, and a minority of individuals did show values $>2SD$ from the mean for the control group in either direction (i.e., high or low levels). Once again, a pathogenic role for low skeletal muscle CoQ10 was not supported by these data, although compared with the study by Paiva et al. (2005) the patients studied here received lower statin doses (5–20 mg/day).

Subsequent to the systematic review by Marcoff and Thompson (2007), the work of Larsen et al. (2013), despite applying some elegant analytical procedures to measure mitochondrial function (discussed in more detail in the next section), does not add significantly to the debate on skeletal muscle CoQ10 levels and statin myopathy. These authors claimed a decrease in CoQ10 in skeletal muscle from male hypercholesterolemic patients treated with simvastatin (10–40 mg/day). However, the analysis of muscle CoQ10 was performed

using a non-validated methodology that certainly does not measure CoQ10 directly (Navas 2013); moreover comparison of statin-treated hypercholesterolemic patients with healthy controls was not optimal. We are left with the conclusion that the role of CoQ10 lowering in skeletal muscle as a mechanism for statin myopathy is unproven and possibly only relevant, if at all, at high statin doses. Given all of this evidence, we remain unconvinced, yet open-minded, of the need for CoQ10 supplementation as a therapy for statin myopathy because CoQ10 is well tolerated. Despite this conclusion, there have been several trials of CoQ10 supplementation in patients addressing the mitigation of skeletal muscle effects by high-dose statins (e.g., see Thibault et al. 1996; Kim et al. 2001).

8.2.2.2 Evidence for Direct Effects of Statins on Mitochondrial Function

8.2.2.2.1 In Vitro and Ex Vivo Studies

Sirvent et al. (2005a, b) showed that application of a relatively high dose of simvastatin on human skeletal muscle fibers *in vitro* caused mitochondrial membrane depolarization and Ca^{2+} release into the cytoplasm, followed by a larger sarcoplasmic reticulum Ca^{2+} release that precipitated the myotoxic effects of simvastatin. This chain reaction, therefore, appeared to be due to inhibition of mitochondrial function by simvastatin. Subsequently, the same research group identified inhibition of complex I of the mitochondrial respiratory chain as being responsible for initiating these effects in human and rat skinned skeletal muscle samples (Sirvent et al. 2005b). The described mitochondria/ Ca^{2+} mechanism of acute *in vitro* toxicity of statins observed by this group was supported by subsequent studies in both rat and human skeletal muscle (Sacher et al. 2005; Liantonio et al. 2007; Nadanaciva et al. 2007). Using isolated rat liver mitochondria, Nadanaciva et al. (2007) investigated oxygen consumption as an index of respiration for a group of statins albeit using concentrations of statins in excess of plasma C_{max} . These data, therefore, reflect potential effects of statins following intracellular accumulation. Uncoupling of state 2 respiration (using glutamate/malate as substrates) was observed for simvastatin, lovastatin, and fluvastatin at 100 nmol/mg mt protein; atorvastatin and cerivastatin were less potent and pravastatin did not inhibit. At this level, state 3 respiration was affected only by cerivastatin. Subsequently, using a high-throughput assay involving immunocapture of mitochondrial electron transport chain complexes from bovine heart mitochondria, several new pieces of information were reported for the particular statins investigated (Nadanaciva et al. 2007). Notably, simvastatin and lovastatin inhibited complexes I + II (combined) and complexes IV and V; simvastatin also inhibited complex

I independently. Fluvastatin and cerivastatin inhibited complex V, and atorvastatin and pravastatin did not inhibit the respiratory complexes under the conditions employed. Complex II appeared not to be inhibited independently by any of the statins even at these relatively high concentrations.

The effects of several statins on mitochondrial parameters have also been investigated using a rat skeletal muscle cell line (L6). Lipophilic cerivastatin, fluvastatin, and atorvastatin (100 μM) decreased mitochondrial membrane potential by about a half, whereas another lipophilic drug, simvastatin, and hydrophilic pravastatin were much less toxic (Kaufmann et al. 2006). Mitochondrial swelling, cytochrome *c* release, and DNA fragmentation were induced by lipophilic statins in the L6 cells. These statins generally impaired mitochondrial function in isolated mitochondria also; parameters measured included state 3 respiration, respiratory control ratio, and β -oxidation of fatty acids. The authors acknowledged that the concentrations of statins used were relatively high, leading to some uncertainty about the translation of these findings to humans taking therapeutic doses of statins. Additionally, the effect of lipophilic statins on mitochondrial function is unlikely to be the major reason for the myopathy associated with statins, in general, since hydrophilic pravastatin is also capable of inducing myopathy.

Rhabdomyosarcoma cells are a human immortalized cell line that preferentially metabolize using glycolysis and therefore provide an interesting model for investigations of statin myopathy. Basal mitochondrial oxygen consumption, ATP levels, mtDNA content, and cell viability were reduced when these cells were exposed to pharmacologically relevant concentrations of simvastatin (5 μM (Vaughan et al. 2013)). All endpoints were rescued by supplementation with CoQ10. Nevertheless, because of the cell type used, the relevance of the results derived from these experiments to human myopathy is unclear.

Decreased cell ATP levels were observed following fluvastatin, lovastatin, and simvastatin treatment of C2C12 murine myotubes (Wagner et al. 2008). Subsequently, a decrease of the mitochondrial membrane potential and inhibition of basal oxygen consumption in myotubes isolated from mice and mouse C2C12 myotubes was observed for simvastatin (10 μM ; 6–24 h duration) measured in whole myotubes (Mullen et al. 2011). This effect was not observed in liverlike HepG2 cells. Moreover, inhibition of maximum respiration induced by an electron transport chain uncoupler, FCCP, was observed in myotubes derived from mouse muscle but not in HepG2 cells. Data on isolated mitochondria from simvastatin-treated C2C12 myotubes indicated that succinate-driven state 2 and state 3

respiration was inhibited by simvastatin (Mullen et al. 2011). The difference in responses to simvastatin of myotubes versus HepG2 cells was attributed to Akt phosphorylation; in C2C12 cells Akt phosphorylation was reduced by simvastatin (10 μ M) but not in HepG2 cells. Moreover, in HepG2 cells inhibition of Akt revealed a susceptibility to simvastatin cytotoxicity and inhibited basal mitochondrial oxygen consumption. These data suggest the IGF-1/Akt signaling axis is an important determinant of statin-induced cell toxicity. Presumably HepG2 cells are able to maintain Akt signaling, and this affords some protection against statin toxicity, although exactly how statins affect Akt signaling is unknown.

The intermediates in the cholesterol synthetic pathway, for example, GGPP, FP, and dolichol, impact upon many biochemical pathways including cell signaling proteins such as the small GTPase Rap1, which shows reduced prenylation after simvastatin treatment (Mullen et al. 2010). In C2C12 myotubes, oxygen consumption rate was partly rescued by transfection with Rap1 although Akt phosphorylation was not, suggesting multiple pathways may be involved in (simva)statin-induced mitochondrial inhibition. Recent data, albeit in an entirely different cell model, has shown that Rap1 is able to reduce high glucose-induced mitochondrial oxidant production and release of apoptotic cytochrome *c* and the presence of proapoptotic caspases in the cytosol of HK cells (Xiao et al. 2014), an immortalized proximal tubule epithelial cell line from normal adult human kidney. One possible mechanism for the deleterious actions of statins on mitochondrial function is, therefore, their effects on posttranslational modification of cell signaling pathways such as Akt. The protection of mitochondrial function is, therefore, one possible mechanism for the observations of Mullen et al. (2011).

In human permeabilized primary skeletal muscle myotubes, simvastatin did not affect basal state 2 respiration. However, maximal ADP-stimulated state 3 respiration supported by complex I substrates, palmitoylcarnitine + malate and glutamate + malate, was reduced by around one-third by 5 μ M simvastatin (Kwak et al. 2012). This was associated with increased simvastatin-mediated mitochondrial superoxide production and increased hydrogen peroxide levels arising from mitochondrial respiration in human skeletal muscle myotubes, although no causative role for these oxidants was demonstrated. In this study, simvastatin was used at a concentration of 5 μ M, which reflects peak serum levels for doses in man equivalent to approximately 40–80 mg/day. This is significant as concentrations of simvastatin in many previous *in vitro* studies were much higher with questionable relevance to peak pharmacological concentrations in man (Sirvent et al. 2005b; Kaufmann et al. 2006).

8.2.2.2.2 Studies in Rodents and Humans

Early observations on effects of statins suggested a possible effect of pravastatin on the age-related decline in mitochondrial function in rats (Sugiyama 1998). Specifically, pravastatin was shown to accelerate the age-related decline in complex I activity in mitochondria isolated from diaphragm muscle but did not inhibit respiratory function in cardiac or hepatic mitochondria. A 15-day repeat dose study of cerivastatin toxicity in rats detected significant myopathy associated with raised serum CK levels, necrosis, and inflammation (Schaefer et al. 2004). Mitochondrial dysfunction, for example, reduced state 3 respiration rate, was only apparent where damaged myofibers were present and did not precede the appearance of muscle pathology. This study also reported a lack of inhibition of function by cerivastatin (<10 μ M) in isolated skeletal muscle mitochondria from control rats. The authors concluded that mitochondrial injury was not a primary cause of skeletal muscle pathology in cerivastatin-treated rats.

Liver mitochondria from hypercholesterolemic LDL receptor knockout (*ldlr*^{-/-}) mice treated with lovastatin for 15 days showed a greater susceptibility to membrane permeability transition (MPT); MPT is associated with opening of a pore in the inner mitochondrial membrane with oxidation of membrane protein thiols and is classically inhibited by cyclosporine A (Velho et al. 2006). *In vitro*, lovastatin decreased total protein thiols in liver and skeletal muscle mitochondria and induced MPT; the mechanism involved Ca-dependent oxidation of protein thiols within the mitochondria. Such processes may lead to alterations in mitochondrial function and provide a mechanism for induction of apoptosis in skeletal muscle exposed to statins.

Young male F344 rats were treated with cerivastatin in their diet for 10 days, resulting in muscle pathology and elevated serum CK concentrations (Obayashi et al. 2011). Myopathy was predominantly confined to type 1 muscle fibers, for example, in the *soleus* muscle where type 1 fibers predominate. This was preceded by mitochondrial swelling and appearance of autophagic vacuoles as the earliest observed pathological changes leading to type 1 muscle fiber necrosis. Although it was suggested that mitochondrial toxicity was a causative factor in cerivastatin-induced type 1 skeletal muscle pathology, this was not proven.

Observations in four patients experiencing skeletal muscle symptoms due to statin therapy, yet without elevated serum CK, provided some impetus for this area of research (Phillips et al. 2002). These individuals experienced statin-related pain that reversed during placebo. Mitochondrial dysfunction was suggested from histopathological changes in skeletal biopsies from all four patients with confirmed statin-induced myopathy

and muscle weakness. Abnormal changes included increased lipid stores (oil red O staining), ragged red fibers, and cytochrome oxidase negative fibers (Phillips et al. 2002). These features are characteristic of mitochondrial diseases, specifically mitochondrial electron transport chain dysfunction. Ragged red fibers in sections of muscle stained with Gomori trichrome are observed due to accumulation of damaged mitochondria in subsarcolemmal regions of fibers.

Subsequently, mitochondrial function was reported in a small trial in humans with and without muscle symptoms following chronic (>2 years) therapeutic dosage with statins (Sirvent et al. 2012). Oxygen consumption in skinned preparations of muscle fibers from the *vastus lateralis* was measured using a Clark electrode. Respiration rate with pyruvate as substrate was lower in patients taking statins versus the control group. The respiration rate based on octanoate oxidation (dependent on β -oxidation) was decreased in the symptomatic patient group versus the asymptomatic and control groups. Further analysis revealed that maximal ADP-stimulated respiration, when electrons were supplied into complex I (glutamate + malate), was inhibited in patients taking statins. Respiration with electron donation into complex II (succinate) was not affected; however, there was some evidence that symptomatic patients had reduced complex IV activity compared with asymptomatics and control groups. The authors concluded that complex I of the respiratory chain was the main target for the adverse action of statins, supporting their proposed mechanism for statin effects elucidated *in vitro* (Sirvent et al. 2008) and suggesting a pathway for dysregulation of calcium ion homeostasis that might explain statin symptomology. Limitations of this study include the low number of patients studied and the lack of matching for age and BMI of the patient group compared with the control group; as in many studies in this field, the controls were healthy and the statin-treated groups had hypercholesterolemia, potentially with other symptoms. As a result, we cannot be certain that the observed differences were due to statin effects alone or in part due to phenotypic differences between patients that are inherent in the pathological process(es).

In order to address these possible confounding effects of pathology and concomitant treatments, the same research group has studied the effects of high-dose simvastatin (80 mg/day for 8 weeks) on mitochondrial respiration and calcium homeostasis in a randomized double-blind placebo-controlled parallel groups study in healthy men (Galtier et al. 2012). Two groups of 12 men were studied in parallel, and mitochondrial respiration was measured in fibers isolated from *vastus lateralis* muscle. The men were well matched for age and BMI, were without medication, and did not exercise excessively.

Overall, there were no global effects on mitochondrial respiration when using pyruvate or succinate (+rotenone) as substrates for mitochondrial respiration in 12 men randomized to simvastatin compared with controls. CK levels in serum were increased by simvastatin in these volunteers. In the subgroup consisting of six men with above median CK levels, respiration using complex II substrates (succinate in the presence of rotenone) was decreased compared with the subgroup with lower CK levels and compared with the placebo group; this subgroup also displayed evidence of altered calcium ion homeostasis. These data raise the possibility that complex I is not the only site that may be targeted by statins. The authors conclude that the data support the hypothesis that early changes in mitochondrial function by high-dose simvastatin alter calcium ion homeostasis, which leads to disturbed skeletal muscle physiology and eventually myopathy. This work also raises the possibility that different statins might affect mitochondrial function in different ways; their previous study reported data for patients taking a range of statins (Sirvent et al. 2012). Moreover, the patients from the latter study had received statin therapy for >2 years prior to the investigation of mitochondrial function. The arguments around differences in healthy controls versus patients with hypercholesterolemia again apply to inter-study interpretation of data.

Complex III of the mitochondrial electron transport chain has been suggested as a likely target for statin lactones (Schirris et al. 2015), which are metabolites of glucuronosyltransferase enzyme activity *in vivo* (Prueksaritanont et al. 2002). Basal oxygen consumption in C2C12 myoblasts, treated acutely, was observed for lactones of atorvastatin, cerivastatin, and pitavastatin (Schirris et al. 2015). Specifically, 5/8 statin lactones reversibly inhibited mitochondrial complex III in isolated mitochondria albeit at relatively high concentrations (100 μ M). *In silico* docking analysis supported the possibility that statin lactones might inhibit the Q_0 site of complex III of the mitochondrial electron transport chain. Inhibition of respiration by statin lactones was attenuated by simultaneous convergent electron flow, which competed for binding at the Q_0 site, providing a possible therapeutic option for mitigation of statin myopathy. In muscle biopsies from patients with statin-induced myopathy, complex III activity and mitochondrial ATP synthesis capacity were reduced compared with healthy controls (Schirris et al. 2015). Although age and sex corrected, these groups differed significantly with respect to phenotype, for example, they presumably suffered from an underlying hyperlipidemia, some having hypertension and diabetes, and there were possibly several other confounding factors present including a difference in obesity profiles between groups.

Intriguingly, however, primary skin fibroblasts from individuals with complex III deficiency (28–46% of control) were more sensitive to statin lactone- (but not statin acid-) induced cytotoxicity *in vitro* (Schirris et al. 2015) (note: this study was performed using $n = 3$ patients and $n = 2$ controls).

Bouitbir et al. (2012) proposed an intriguing mitochondrial mechanism to explain how statins might result in cardioprotection (via a mechanism termed “mito-hormesis”) yet cause adverse events in skeletal muscle. Observations in a small number of humans revealed decreased atrial appendage oxidant levels and increased mRNA expression of the cytoplasmic and mitochondrial isoforms of superoxide dismutase in patients taking statins. This was associated with an increase in mRNA expression of peroxisome proliferator-activated receptor gamma coactivator (PGC-1) family members. PGC-1 members are transcription factors that promote mitochondrial biogenesis (Kang & Li Ji 2012). In contrast, in patients with statin-associated myopathy, oxidant levels were increased, and superoxide dismutase expression was greatly decreased in deltoid muscle biopsies compared with untreated healthy controls. Expression of PGC-1 family members in the skeletal muscle biopsies was also substantially attenuated in the statin myopathy group. Maximal mitochondrial respiration was increased in atrial appendages but decreased in deltoid muscle in statin groups.

The apparent cytoprotection by statins in heart muscle in humans was further investigated in rats administered with atorvastatin. Atorvastatin was shown to activate mitochondrial biogenesis (possibly via PGC-1 β pathways and mediated by increases in oxidant production by the statin) and increased mtDNA content in rat heart. In response to increased oxidants, mitochondrial SOD (SOD2) was increased, which subsequently resulted in lower oxidant detection in cardiac muscle. In glycolytic skeletal muscle, with poor endogenous antioxidant protection, atorvastatin enhanced oxidants as detected by electron paramagnetic resonance and dihydroethidium staining. This resulted in a state of oxidative stress in the skeletal muscle cells and decreased PGC-1 expression, glutathione depletion, and SOD2 decreases (via PGC-1 decreases) (Bouitbir et al. 2012). Furthermore, the reduced PGC-1 expression was associated with a decrease in the maximum mitochondrial respiration in skeletal muscle. Taken together, these effects, mediated by enhanced oxidant production by atorvastatin, were proposed to account for myalgia and general myopathy observed for some statin-treated individuals.

Based on these data, one might expect statins to result in *in vivo* oxidative stress. However, a recent study by Rasmussen et al. (2016) in healthy male volunteers reported no effect of short-term treatment with

simvastatin on *in vivo* oxidative stress measured using a panel of six markers including well-validated urinary markers of DNA and RNA oxidation.

Mitochondrial function was also studied in patients with hypercholesterolemia who had been treated with simvastatin (10–40 mg/day) for an average of 5 years (Larsen et al. 2013). Mitochondrial respiration was measured using high-resolution respirometry in permeabilized muscle fibers prepared from *vastus lateralis* muscle. Maximal oxidative phosphorylation with complex I and II linked substrates was not different between patients and controls; however, complex I linked substrate (glutamate) sensitivity was higher in simvastatin-treated patients than controls. Maximal oxidative phosphorylation capacity with maximized *ex vivo* input of electron donation into complexes I and II was higher in patients treated with simvastatin compared with controls. This is significant given that maximal fluxes *in vitro* are still much lower than those observed *in vivo* (Tonkonogi & Sahlin 1997). The authors claimed that CoQ10 content of muscles was reduced in the simvastatin group of patients but that mitochondrial content (assessed by measurement of citrate synthase, VDAC, and cardiolipin) was similar between the two groups. However, as reported previously, the methodology used in this study provided at best an indirect measure of ubiquinone synthesis (Navas 2013), so any conclusions about the role of CoQ10 depletion by simvastatin in myopathy are yet to be justified.

In this study (Larsen et al. 2013), comparison was made between patients taking long-term simvastatin and healthy controls. The two groups under study were well matched for age, sex, and other parameters but differed in that patients were treated with simvastatin because of hypercholesterolemia and also were at risk of developing other pathologies such as diabetes, hypertension, and metabolic syndrome. Therefore, we do not know whether the changes observed, particularly to oxidative phosphorylation capacity, are due to the disease itself rather than the statin exposure. A more challenging, yet informative, study would involve a crossover design. One of the problems with this type of study would be that treatments with statins would need to be for a shorter period; we do not know if the changes observed in oxidative phosphorylation and CoQ10 would manifest during acute exposure to statins. Interestingly, the authors reported a quite different profile of skeletal muscle (*vastus lateralis*) fiber content between patients and controls. Patients had a lower content of type 1 fibers (42%) compared with healthy controls (58%), which may affect mitochondrial substrate utilization (Ponsot et al. 2005) and contribute to the observed increase in mitochondrial substrate sensitivity with substrates that donate electrons into complex I of the electron transport chain. For example,

mitochondria from glycolytic muscles are well disposed via metabolic adaptation to regenerate NAD^+ in the cytosolic compartment, thereby enabling proper glycolytic function (Ponsot et al. 2005). The healthy control group used in this study does not allow us to fully interpret the potential “changes” in fiber content and whether this might be mediated by simvastatin exposure. If simvastatin does reduce the content of type 1 fibers in patients, then this will be very interesting given that in rats a variety of statins result in toxicity primarily to type 2 rather than type 1 fibers.

8.2.2.2.3 Studies in Other Species

While the effects of statins have been investigated mainly in humans and animal *in vivo* models, other notable models have been used to study the influences of these drugs on muscle mitochondria. A good example is the use of *Caenorhabditis elegans*, which lacks the pathway leading from mevalonate to cholesterol (see Figure 8.1) and can be used to study mechanisms not associated with sterol synthesis. Exposure of wild-type worms to statins causes phenotypic effects that are rescued by mevalonate (Morck et al. 2009) and independent of inhibition of CoQ synthesis by statins (Rauthan et al. 2013). Forced activation of the mitochondrial unfolded protein response (UPR^{mt}) results in the resistance of *C. elegans* to the effects of statins (Rauthan et al. 2013), suggesting that mitochondria are an important target in the toxic pathways from statins, at least in this model. This fits with the hypothesis that statins interfere with a mitochondrial surveillance pathway, that is, the process of sensing of mitochondrial damage in *C. elegans* and the subsequent activation of a protective response to the insult (Liu et al. 2014). Subsequently, the importance of HMG-CoA reductase was highlighted in a *C. elegans* mutant (*hmgr-1(tm4368)*) that lacks the reductase gene and therefore this enzymic activity (Ranji et al. 2014). This mutation recapitulated the actions of statins in wild-type worms, which included effects on the organization of mitochondria in the muscle. These muscle defects were rescued by the addition of mevalonate to culture media and by UPR^{mt} activation; subsequently this group and others (Liu et al. 2014) demonstrated that the mevalonate pathway is required for UPR^{mt} activation. Moreover, the resistance to statin cytotoxicity could be induced by activation of the UPR^{mt} in cells from other species, namely, *S. pombe*, and the NIH 3T3 fibroblast cell line (Rauthan et al. 2013). Although full details of the mechanism are not yet described, it seems that the mevalonate pathway provides FP and GGPP, which are essential for prenylation of proteins, including small GTPases, which is required for the proper activity of these proteins via membrane association. Inhibition of HMG-CoA reductase prevents the activation of UPR^{mt} ,

and this possibly occurs through inhibition of the function of a key GTPase that requires prenylation for activity.

8.3 AZT and Mitochondrial Myopathy

NRTIs are one of six classes of antiretroviral drugs used to treat patients infected with human immunodeficiency virus (HIV). Seven NRTI drugs are currently approved by the FDA for the treatment of HIV in the United States. For treatment-naïve patients, current recommendations suggest a combination of two NRTIs plus another drug from a different class of antiretroviral agents. In many treatment regimens, NRTIs are prominent components in the highly active retroviral treatment (HAART) or antiretroviral treatment (ART) of HIV infection to delay or prevent disease progression. As discussed in the following text, zidovudine (AZT) has been most commonly associated with NRTI myopathy and still may be included in one of the current NRTI combinations that are part of lifelong treatment in HIV-positive people. It remains the preferred drug for use in pregnant women and newborns and for prophylaxis following either occupational or nonoccupational exposure to the virus. Overall, since treatment regimens moved away from monotherapy with AZT and following a reduction in doses of AZT administered to patients, a decrease in the myopathic effects attributed to AZT has been observed.

NRTI are deoxynucleoside analogues that counteract HIV via their high affinity for and hence inhibition of the viral reverse transcriptase enzyme. The inhibition of the reverse transcriptase DNA polymerase activity, which converts viral RNA into DNA, aims to prevent the HIV virus from replicating. Zidovudine (3-azido-3'-deoxythymidine; known as “AZT” or “ZDV”) was the first NRTI to be marketed in 1987 for HIV treatment. AZT can also be used for prevention of HIV transmission from mother to fetus during pregnancy. There are six other current drugs in this class that are approved for treating HIV by the FDA, but AZT is by far the most commonly associated with myopathy. The evidence for its proposed myotoxic mechanism(s) of action is therefore discussed in some detail later on.

Usually, DNA polymerase enzymes utilize thymine, adenine, cytosine, and guanine deoxynucleoside triphosphates (dNTP) to build a DNA polymer. Elongation requires reaction between a 3' OH group on the deoxyribose moiety of the growing polymer and the 5'-phosphate residue of the incoming dNTP. The majority of NRTIs—for example, AZT, dideoxyinosine (ddI), dideoxycytidine (ddC) (withdrawn from clinical use in

the United States), lamivudine (3TC), stavudine (d4T), abacavir, and emtricitabine—are dideoxynucleotide analogues and contain no 3' hydroxyl group on the ribose sugar, and therefore their incorporation into DNA causes chain termination of the nascent polynucleotide. Importantly, the dideoxynucleotide analogues require conversion into their triphosphate forms (ddNTP) by cellular kinases in order to elicit an effect.

As an off-target toxicity effect, NRTIs inhibit DNA polymerase of mammalian origin with polymerases β and γ most sensitive *in vitro* (see review by Brinkman et al. (1998)). DNA polymerase β is mostly involved in DNA repair, and there is little evidence that inhibition by NRTI has a significant effect on this process in cells. However, inhibition of polymerase γ by NRTI (Lewis & Dalakas 1995), which is the sole DNA polymerase involved in mtDNA synthesis and repair, is significant. The decreased mtDNA synthesis and the associated reduced expression of mtDNA-encoded gene products are believed to lead to mitochondrial dysfunction and interference with ATP production by oxidative phosphorylation. Mitochondrial respiratory chain complexes I, III, IV, and V consist of protein components encoded by mtDNA, and so the expected pattern of mitochondrial protein deficiency mirrors that arising from inherited defects in mtDNA-encoded genes. All organ systems are potentially affected, but those with high ATP demand such as skeletal muscle are most susceptible. Clinical manifestation of disease in different organs and the susceptibility of different tissues have been reviewed (Carr 2003). Originally the clinical expression of the disease was believed to be primarily determined by the level of mtDNA depletion in post-mitotic tissues such as muscle showing evidence of greatest effect due to lack of mtDNA replication. Tissues with low turnover of mtDNA would also accumulate most mtDNA defects.

However, this is the classical, and somewhat simplistic, view of NRTI-mediated mitochondrial dysfunction leading to adverse events. Beyond the inhibition of polymerase γ by NRTI, several other theories propose alternate or complementary mechanisms for mitochondrial inhibition, in part to account for some of the unanswered questions and discrepancies related to observations on the effects of NRTI. For example, there is a lack of correlation between the extent of mtDNA depletion and mitochondrial toxicity. AZT is more toxic to mitochondria than 3TC, yet causes less inhibition of DNA polymerase γ (Apostolova et al. 2011). Apostolova et al. (2011) and Smith et al. (2013) have recently summarized and speculated on these alternate mechanisms for mitochondrial inhibition, many of which may apply in skeletal muscle. More research is needed, but electron transport chain and mitochondrial enzyme inhibition, inhibition of purine/pyrimidine nucleoside transport,

disturbance of deoxyribonucleotide pools (Selvaraj et al. 2014), NRTI phosphorylation, and oxidant generation are all hypothesized to play roles. One major issue is that both HIV itself and NRTI drugs appear to modulate mitochondrial function directly, making it difficult to assign the cause of mitochondrial toxicity *in vivo*.

The majority of side effects of NRTIs are attributed to mitochondrial toxicity and are similar to the spectrum of effects causing mitochondrial diseases (Brinkman et al. 1998). NRTI mitochondrial toxicity results in a range of effects including peripheral neuropathy, lactic acidosis, cardiovascular risk, myopathy, hepatotoxicity, pancreatitis, dyslipidemia, and insulin resistance. Moreover, NRTI drugs have differing pathological effects. AZT can result in myopathy (Dalakas et al. 1990), whereas ddI, ddC, and 3TC often cause neuropathy; d4T may cause both myopathy and neuropathy (Dalakas 2001). Which tissue is affected by a particular NRTI is probably determined by the distribution of phosphorylation activities for the specific drug (Lewis & Dalakas 1995).

Effectiveness of AZT therapy is limited by the toxic effects at high doses for prolonged duration, which include myopathy and cardiomyopathy. In a 1-year follow-up study of HIV patients taking relatively high doses of AZT (1–1.2 g/day) for more than 270 days, 17% of patients showed signs of myopathy (Peters et al. 1993). Elevated CK levels preceded the clinical signs of muscle problems. Myopathy was not observed in short-term therapy, and all signs and symptoms resolved on drug withdrawal.

Generally, symptoms of AZT myopathy include muscle pain, weakness, and fatigue and often muscle atrophy, and a raised serum CK is observed (Owczarek et al. 2005). Alongside mtDNA depletion (Dalakas et al. 1990; Arnaudo et al. 1991; Masanes et al. 1998), these findings indicate mitochondrial disease induction by AZT, although the exact mechanism is still not certain. mtDNA depletion is hypothesized to result in reduced ATP synthesis due to problems with the synthesis and construction of mitochondrial electron transport complexes. Glycolytic metabolism may compensate to some extent, but this also leads to increased lactic acid production. Overall a dysfunction of muscle ensues due to lack of ATP synthesis.

Histopathologically, ragged red fibers and abnormal mitochondrial morphology are often observed in skeletal muscle biopsies from patients receiving AZT (Owczarek et al. 2005). These fibers are often seen in some inherited mitochondrial diseases and result from an increase in subsarcolemmal proliferation of aberrant mitochondria. AZT is also associated with muscle fibers lacking cytochrome *c* oxidase due to complex IV deficiency and fat accumulation in cells (Dalakas et al. 1990).

The exact mechanism(s) of mtDNA depletion via DNA polymerase γ inhibition is uncertain. The original hypothesis was that AZT is phosphorylated in tissues by thymidine kinase (TK) to form AZT triphosphate, which subsequently inhibits DNA polymerase γ (Arnaudo et al. 1991; Lewis et al. 1992, 1994). Long-term inhibition would result in mtDNA depletion, which has been observed in skeletal muscle from patients and rats treated with AZT (Arnaudo et al. 1991; Lewis et al. 1992). In heart muscle, cytoplasmic thymidine kinase 1 (TK1) is virtually absent, and nucleosides such as thymidine and AZT are transported to the mitochondrial matrix for phosphorylation by TK2. McKee et al. (2004) showed that thymidine was rapidly transported into rat heart mitochondria and phosphorylated to TTP in the matrix, presumably by TK2. AZT was metabolized as far as AZT monophosphate (AZT-MP) only, resulting in sequestration within the matrix. The lack of AZT triphosphate synthesis suggests lack of inhibition of polymerase γ . Similarly, AZT triphosphate is not detected in the matrix of liver mitochondria treated with AZT (Lynx et al. 2006); therefore, the concentration is insufficient to inhibit polymerase γ . However, AZT is a potent inhibitor of thymidine phosphorylation in heart mitochondria (McKee et al. 2004) presumably by inhibition of TK2 by AZT-MP and could therefore disrupt the supply of TTP for mtDNA synthesis. A disruption in thymidine salvage by mutations in the thymidine phosphorylase gene has been observed in mitochondrial neurogastrointestinal encephalopathy (MNGIE) (Nishino et al. 1999). The resultant phenotype shares many similar features with mitochondrial toxicity by AZT, albeit by different molecular mechanisms.

In mice treated with AZT, dietary uridine supplementation overcomes all aspects of AZT-induced myopathy including mtDNA depletion, likely acting via restoration of the pyrimidine metabolism (Lebrecht et al. 2008). A related mechanism has been proposed for inhibition of mtDNA synthesis by AZT and ddI (Sun et al. 2014a, b) with downregulation in TK2 expression following ddI administration in U2OS cell (human osteosarcoma cell line). These authors suggest that intramitochondrial degradation of TK2 protein following oxidant damage due to AZT might be responsible, although this was not proven. Previously, it was reported that changes in mitochondrial protein expression mediated by AZT were correlated with decreases in mtDNA levels (Lewis et al. 1992). Exposure of rats to AZT caused decreased mRNA expression levels of mtDNA-encoded genes in skeletal muscle fibers, yet mRNA expression of two nuclear-encoded genes was not affected (Lewis et al. 1992). *TK2* is a nuclear gene, and changes to its transcriptional, translational, and mitochondrial import by AZT treatment have not been investigated, to our knowledge.

Although previous circumstantial data point to accelerated intramitochondrial TK2 protein degradation due to AZT being likely (Sun et al. 2014a, b), these other effects on TK2 biology cannot be ruled out.

Alternatively, the deleterious effects of AZT on mitochondrial activity may not be due to the inhibition of DNA polymerase γ (reviewed in Scruggs and Dirks Naylor 2008). For example, AZT inhibited respiration in rat skeletal muscle mitochondria, particularly at electron transport chain complexes I and II (Modica-Napolitano 1993). In cultures of human muscle cells, AZT, ddI, and ddC all decreased proliferation, caused lipid droplet accumulation in cytoplasm, and increased lactic acid production (Benbrik et al. 1997). All three drugs impaired mitochondrial respiratory chain function, but the high potency of ddC, in particular, casts doubt upon the hypothesis that AZT acts via a simple direct mitochondrial toxic effect since a toxic myopathy is seen with this drug but not with ddC or ddI.

Prior to changes being observed in mtDNA content, AZT has been shown to be able to affect ATP synthesis and inhibit rat myocyte contraction (Cazzalini et al. 2001). Studies in cells also demonstrated a reduction in glutathione levels in response to AZT, implicating oxidative stress as a mechanism. In mice, AZT resulted in increases in 8-oxo-deoxyguanosine, a marker of oxidative damage, in mtDNA (de la Asuncion et al. 1998). One hypothesis is that disruption of the mitochondrial electron transport system results in increased oxidant production and oxidative stress (Yamaguchi et al. 2002) and that this precipitates subsequent mtDNA deletion.

L-Carnitine as a Target for NRTI: L-carnitine is important as it facilitates long-chain fatty acid transport into mitochondria, which is key for muscle ATP synthesis. AZT is also reported to reduce L-carnitine levels in patient muscle biopsies (Dalakas et al. 1994) and in myoblasts (Georges et al. 2003), resulting in accumulation of cytoplasmic lipid droplets. In human muscle cells, these effects are mitigated by L-carnitine supplementation (Semino-Mora et al. 1994), suggesting that it is the AZT-induced L-carnitine depletion rather than mtDNA depletion, which causes the defect in fatty acid transport into mitochondria. At least in a mouse myoblast cell line, it appears that AZT acts to reduce the transport of L-carnitine into cells via direct inhibition of the transporter for L-carnitine (Georges et al. 2003).

Apoptosis as a Mechanism for AZT Myopathy: Studies on induction of apoptosis by AZT in skeletal muscle are uncommon. Desai et al. (2008) noted modest dose-dependent effects by AZT on expression of genes associated with apoptosis (and mtDNA maintenance or mitochondrial transport) in skeletal muscle of neonatal B6C3F₁ mice. Animals were treated with AZT from postnatal day 1 for 8 days to model the postnatal NRTI

antiretroviral prophylaxis regimen for babies born to HIV-positive mothers; this treatment is administered in order to prevent mother-to-child transmission of the virus. Recently Adebiyi et al. (2016) suggested that AZT caused an increase in markers of skeletal muscle apoptosis in rats, that is, AZT increased Bax/Bcl-2 ratios.

8.4 Do Other Nucleoside Analogue Drugs Cause Myopathy?

In a retrospective study of 69 patients with probable or possible HIV-associated neuromuscular weakness syndrome, an association with NRTI exposure and raised serum lactic acid suggested a mechanism involving mitochondrial toxicity in muscle; however, causation was not proven (HIV Neuromuscular Syndrome Study Group 2004). Moreover, this was a retrospective analysis, and adequate control group(s), for example, an NRTI-naïve HIV patient group, was not included. d4T was the main NRTI ART (89% of patients), and serum lactate levels tended to be raised in the d4T group compared with the small number of patients not taking d4T. It was clear that muscle pathology was present in patients receiving NRTI apart from AZT.

Therapy with d4T (as part of HAART) has also been implicated in hyperlactatemia, skeletal muscle mtDNA depletion, and decreased whole-body oxidative capacity when compared with healthy controls (Haugaard et al. 2005). However, muscle mtDNA content was also decreased in antiretroviral-naïve patients, suggesting HIV effects independent of treatment and that caution must be used when using mtDNA as a potential surrogate measure of myotoxicity (Haugaard et al. 2005). Similarly, HIV infection is reported to result in lymphocyte mtDNA depletion independent of NRTI administration (Maagaard et al. 2008).

Fialuridine was used as an antiviral nucleoside analogue for the treatment of chronic hepatitis B infection. Preclinical studies had indicated no adverse effects. Unfortunately, fialuridine resulted in liver failure and death in 5 out of 15 patients treated experimentally (McKenzie et al. 1995). Myopathy was observed in several patients, and biopsies showed ragged red fibers and pathology similar to, but less severe than, AZT myopathy. Fialuridine was shown to be toxic to human skeletal muscle myotubes *in vitro*. Effects included accumulation of lipid droplets and with early, major, and irreversible alterations in mitochondrial structure shown by electron microscopy (Semino-Mora et al. 1997). Effects of fialuridine on mtDNA of human skeletal muscle cells are pronounced and progressive with duration of exposure (Birkus et al. 2003).

Unfortunately, adverse events following therapy for hepatitis B did not end with the fialuridine tragedy. Long-term treatment with the nucleoside analogue clevudine has been associated with myopathy and is well reviewed by Fleischer and Lok (2009). Myopathy occurred between 8 and 13 months of treatment in two clinical trials and was reversible following drug cessation (Kim et al. 2009; Seok et al. 2009). Severe myonecrosis, elevated CK levels, and raised serum lactate were variously observed. Mitochondrial changes included decreased mtDNA in skeletal muscle biopsies (Seok et al. 2009). These effects were not predicted by preclinical studies, and clevudine was not incorporated into mtDNA and did not inhibit/was not a DNA polymerase γ substrate (Chu et al. 1998). It was subsequently highlighted that clevudine was preferentially phosphorylated by TK2 in mitochondria (Wang & Eriksson 1996; Tehrani et al. 2008), drawing a parallel with the mechanisms proposed for AZT myopathy as discussed previously.

8.5 Other Drugs Possibly Associated with Myopathy Due to Mitochondrial Toxicity

Leflunomide is a disease-modifying antirheumatic drug that acts on dihydroorotate dehydrogenase, a key component of pyrimidine biosynthesis and a mitochondrial enzyme. There are several side effects of leflunomide treatment including at least three reports of patients suffering from myalgia, polymyositis, and rhabdomyolysis (Rivarola de Gutierrez & Abaca 2004; Ochi et al. 2009; Adamski et al. 2011). To our knowledge, no mechanism has been elucidated.

Propofol is a general anesthetic and sedative for intensive care. In rare cases, propofol toxicity, which results in severe life-threatening “propofol infusion syndrome” (PRIS), has been associated with myopathy, elevated serum CK levels, and even rhabdomyolysis (Stelow et al. 2000). Initial reports suggested reduced complex IV activity in the mitochondrial electron transport chain of skeletal muscle from children with PRIS (Cray et al. 1998; Mehta et al. 1999). Subsequent reports suggested that propofol reduces oxygen utilization and inhibits mitochondrial electron transport chain in guinea pig hearts (Schenkman & Yan 2000). Impaired fatty acid oxidation in a child given propofol has been reported as a mechanism for PRIS (Wolf et al. 2001). The biochemical findings were consistent with both reduced entry of long-chain acylcarnitine esters into mitochondria and inhibition of mitochondrial electron transport at complex II. A rise in malonylcarnitine was proposed as a mechanism for reduced transport of long-chain fatty

acids into the mitochondria via inhibition of carnitine palmitoyltransferase 1. Recent studies in rats suggest that propofol inhibits mitochondrial complex (II + III) activity in skeletal muscle, possibly at the level of CoQ10 (Vanlander et al. 2015).

Multitargeted tyrosine kinase inhibitors are useful anticancer drugs but have been associated with cardiac and skeletal muscle side effects in man. For example, sunitinib has been reported to cause raised serum CK and severe rhabdomyolysis, resulting in renal failure in two patients with renal cell cancer (Ruggeri et al. 2010) but without evidence for mitochondrial liability. Myositis was also reported for sorafenib in patients being treated for hepatocellular carcinoma (Diaz-Sanchez et al. 2011) but without elevated muscle enzymes. Subsequently, severe rhabdomyolysis was observed in a patient receiving sorafenib that occurred within 10 days and resolved on cessation of the drug (Tsuji et al. 2013). There is some evidence that sorafenib is able to directly inhibit mitochondrial function, at complex (II + III) and complex IV, in H9c2 cells, which are an *in vitro* model of both cardiac and skeletal myocytes (Will et al. 2008). These results are supported by recent observations in isolated mitochondria (Zhang et al. 2017).

8.6 Concluding Remarks

Despite 30 years of statin use and millions of prescriptions, we still do not fully understand the exact mitochondrial mechanism(s) resulting in skeletal muscle pathology in some individuals. Depletion of muscle CoQ10 observed in some studies does not appear to account for myopathy in man, and, seeking a mechanism, we are forced to address direct inhibition of mitochondrial function. Recent studies highlighting targeting complex III of the mitochondrial electron

transport chain by statin lactones (Schirris et al. 2015) are intriguing, but further confirmation is required. Studies in *C. elegans* have elegantly pointed toward effects on protein prenylation by statins as potential cell signaling mechanisms that require investigation in man.

Off-target effects of relative high doses of AZT imply inhibition of DNA polymerase γ in human skeletal muscle to account for observed myopathy, but the lack of AZT triphosphate synthesis in mitochondria argues against this. Instead, it is more likely that AZT-MP inhibits TTP synthesis via TK inhibition in the mitochondrial matrix. Proposals that AZT leads to TK2 protein degradation are worthy of further investigation as are the observations that AZT directly inhibits other aspects of mitochondrial function. While the drive to find an exact mechanism for AZT-induced mitochondrial myopathy appears to be no longer an imperative due to reduced usage and lower doses, it is sobering to note the adverse skeletal muscle events with other nucleoside analogues within the last decade.

Mitochondrial toxicity remains a major consideration for drug development with evidence of myopathy for sorafenib, propofol, and leflunomide involving a mitochondrial mechanism. Further research is required on the possible mitochondrial effects leading to skeletal muscle pathology by these drugs. Moreover, since it appears that mitochondria are a key target for many adverse events of a wide array of chemical entities, we need to maintain and further develop *in vitro* assays to sensitively predict, at an early stage, potential mitochondrial liabilities (Will & Dykens 2014). Where possible these should reflect mechanisms of action and better mirror the clinical effects in humans. To this end application of skeletal muscle cells under conditions that sensitize them to mitochondrial toxicity is a potential area for development (Will et al. 2008; Dott et al. 2014; Dott et al. 2018).

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9

Manifestations of Drug Toxicity on Mitochondria in the Nervous System

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CHAPTER MENU

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9.1 Introduction: Mitochondria in the Nervous System

Although mitochondria are critical for the function of most tissues, they are particularly important for the maintenance and integrity of the nervous system. Neurons are responsible for disproportionate oxygen consumption at rest in humans. The brain uses approximately 20% of the total oxygen consumed at rest but represents only 2% of body mass (Mink et al., 1981). In addition, neurons are critically and almost exclusively dependent on mitochondrial oxidative phosphorylation (OXPHOS) as a major source of adenosine triphosphate (ATP) and have a limited capacity to upregulate energy supply through glycolysis when OXPHOS is compromised (Herrero-Mendez et al., 2009).

Moreover, it has been known for many years that cells match the rate of ATP production and utilization with little or no measurable change in metabolic intermediates. The maintenance of cellular metabolites during alterations in workload has been termed metabolic homeostasis and is probably most thoroughly studied in cardiac and skeletal muscle (Balaban, 2002, 2006; Glancy et al., 2013). However, neurons are also subject to changes in workload, and it is known that most of the energy in the brain is consumed by the

process of synaptic transmission (Attwell & Laughlin, 2001; Hall et al., 2012; Harris et al., 2012). Increased synaptic transmission is inevitably associated with a disturbance of ionic gradients across the plasma membrane due to increased Na^+ and Ca^{2+} influx, which subsequently require increased ATP consumption as the consequence of increased Na^+/K^+ ATPase pump activity to restore basal ion homeostasis (Erecinska & Silver, 1994). In high energy-demanding tissues such as the brain or the skeletal and cardiac muscle, the major sustainable source of energy is ATP generated by OXPHOS.

The potential for injury as a consequence of inhibition of mitochondrial function in neurons is illustrated by a number of different examples of disease states that can be modeled using electron transport chain inhibitors, including Parkinson's disease (PD) and Huntington's disease (HD) (Beal et al., 1993; Betarbet et al., 2000). The links between impaired mitochondrial function and central nervous system (CNS) diseases are further emphasized by the prominently neurological phenotype of syndromes associated with mtDNA mutations such as Leber's hereditary optic neuropathy (LHON) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Wallace, 1992). Another important concept that can be observed after

administration of mitochondrial respiratory chain inhibitors such as rotenone is the phenomenon of “selective” vulnerability. For example, administration of rotenone produces a parkinsonism-like disorder (Betarbet et al., 2000). While rotenone inhibits complex I in the entire brain, the development of the disease is mainly associated with the loss of dopaminergic neurons (reviewed in Surmeier & Schumacker, 2013). The concept of selective vulnerability remains poorly understood. It is important to note in this context however that mitochondria differ across tissues, cell types, and even subcellular compartments within neurons (Dubinsky, 2009; Kuznetsov et al., 2009). Indeed, inherent differences in mitochondrial function, or in the susceptibility to mitochondrial dysfunction, are proposed to mediate the differential neuronal vulnerability in several neurodegenerative disorders, including HD (Brustovetsky et al., 2003; Dubinsky, 2009; Oliveira & Goncalves, 2009; Pickrell et al., 2011).

Mitochondrial dysfunction and impairments in ATP generation also represent a primary cause of epilepsy. Moreover, seizures by themselves create a substantial challenge to energy metabolism (Kovacs et al., 2002), which triggers mitochondrial damage and secondary dysfunction (reviewed in Zsurka & Kunz, 2015). Although mitochondrial dysfunction is now considered an important factor in patients with epilepsy, until recently it was almost unrecognized (Arnold, 2012; Lee, Kim, Choung, Kim, & Yu, 2008; Zsurka & Kunz, 2010). Another study concluded that energy failure associated with mitochondrial dysfunction contributes to the severity of the epileptic disorders (El Sabbagh et al., 2010). Furthermore, certain antiepileptic therapeutics such as enzyme-inducing antiepileptic drugs (AEDs), for example, carbamazepine, phenytoin, and phenobarbital, may have protective effects on mitochondria, and it has been reported (Miles et al., 2012) that those actions are associated with promoting mitochondrial proliferation and enhanced ETC complex IV (COX) activity. It is also important to consider that some harmful consequences of AEDs such as valproic acid or the classical aromatic AEDs have been related to drug-induced mitochondrial toxicities (see discussion later on).

Calcium ions play a crucial role in the modulation of cellular respiration (reviewed in Llorente-Folch et al., 2015; Rueda et al., 2014). In brief, Ca^{2+} is a versatile and ubiquitous intracellular messenger, acting as a mediator of almost all energy-demanding processes in mammalian cells. The capacity of mitochondria to take up large quantities of Ca^{2+} in a membrane potential-dependent manner has been known for decades (Deluca & Engstrom, 1961; Harris, 1977; Nicholls, 1978). Mitochondrial Ca^{2+} accumulation serves not only as a Ca^{2+} buffering system in the cell but also as a pathway to modulate energy

metabolism. Ca^{2+} -dependent regulation of OXPHOS involves two principal mechanisms—(i) Ca^{2+} entry into mitochondria through the Ca^{2+} uniporter (mitochondrial calcium uniporter (MCU)) and (ii) Ca^{2+} -dependent activation of mitochondrial metabolite transporters (Ca^{2+} -regulated mitochondrial carriers)—where Ca^{2+} acts on the external surface of the inner mitochondrial membrane. Thus, even though cytosolic and mitochondrial Ca^{2+} signals are usually tightly coupled, they can have distinct effects on mitochondrial metabolism, ensuring differential regulation under physiological and pathophysiological conditions.

The physiological importance of mitochondrial Ca^{2+} handling has been highlighted by research on the first human disease associated with dysfunctional mitochondrial Ca^{2+} uptake, individuals harboring loss-of-function MICU1 mutations (Logan et al., 2014). MICU1 physically interacts with MCU and sets the threshold for mitochondrial Ca^{2+} uptake (Csordas et al., 2013; de la Fuente et al., 2014; Mallilankaraman et al., 2012). Patients harboring these mutations display a muscular and brain disorder that develops progressively, leading to learning difficulties, muscle weakness, and locomotor dysfunction, among other symptoms (Bhosale et al., 2015; Logan et al., 2014). Moreover, the first case of Aralar/AGC1 deficiency has been recently described (Wibom et al., 2009). Aralar/AGC1 is the mitochondrial transporter of aspartate–glutamate present in the brain and a component of the malate–aspartate NADH shuttle (MAS) (del Arco et al., 2016; del Arco & Satrustegui, 1998; Satrustegui et al., 2007). It plays a crucial role in extramitochondrial Ca^{2+} -mediated activation of neuronal respiration in basal state and upon stimulation (Llorente-Folch et al., 2016; Llorente-Folch et al., 2013; Rueda et al., 2016). The patient presented with global hypomyelination, hypotonia, delayed psychomotor development, seizures, and hyperreflexia (Wibom et al., 2009). Therefore, any drug that could potentially interfere not only with mitochondrial-mediated Ca^{2+} homeostasis but also with Ca^{2+} -mediated signaling in mitochondria could be a hazard for physiological brain function.

Mitochondrial functions also comprise the production of reactive oxygen species (ROS). The highest ROS production capacity in brain mitochondria has been proven for complex I and complex III of the respiratory chain and for the enzymes dihydrolipoamide dehydrogenase and monoamine oxidases (MAOs) (references in Adam-Vizi & Chinopoulos, 2006). ROS function as second messengers in signal transduction but are also mediators of oxidative damage and inflammation. Oxidative stress is believed to be a central feature in neurodegeneration, a finding that is supported by clinical evidences reporting lipid and protein peroxidation in postmortem brains of patients diagnosed with neurodegenerative CNS

disorders (Lin & Beal, 2006; Musiek et al., 2006; Sultana et al., 2006). As ROS often originate in mitochondria, mitochondria are particularly vulnerable to oxidative damage. This damage may play a critical role in controlling neuronal excitability and subsequent seizure susceptibility associated with acquired epilepsy (Waldbaum & Patel, 2010).

Neurons are particularly vulnerable to mitochondrial dysfunction not only because of their aforementioned energetic dependence on OXPHOS (Bolanos et al., 2010; Oliveira, 2011) but also because of their polarized morphology. Mitochondria need to be efficiently distributed to distant neurites, axons, and synapses to provide energy support for local disruptions in ion homeostasis and Ca^{2+} handling (Bolanos et al., 2010; Leitao-Rocha et al., 2015; Oliveira, 2011; Schwarz, 2013). The unique morphology and post-mitotic nature of neurons demands effective mitochondrial quality control mechanisms (Amadoro et al., 2014). Mitochondrial transport, fusion and fission, and the removal of defective mitochondria are complex and ATP-dependent processes that also rely on the integrity of the cytoskeleton (reviewed in Matic et al., 2015; Ni et al., 2015; Oliveira et al., 2016). Trafficking also depends on other signaling molecules including growth factors and neurotoxins that by themselves do not impair ATP synthesis (Chada & Hollenbeck, 2004; Malaiyandi et al., 2005; Reynolds et al., 2004). The relevance of these processes is highlighted by the fact that defects in mitochondrial trafficking and mitophagy have been described in neurodegenerative diseases (reviewed in Matic et al., 2015; Oliveira et al., 2016). It is therefore reasonable to assume that drugs targeting these processes will also disrupt neuronal function.

Even if we increase our understanding of the mechanisms of neurotoxic drug, it remains difficult to predict the manifestations of that toxicity in the nervous system. Typically, adverse effects of drugs that are toxic to nervous system mitochondria are reported as alterations in the function of a sensory or motor modality. Some of the best established examples of mitochondrial toxicity are manifested as peripheral neuropathy as discussed in the following subchapter. However, other manifestations include retinal toxicity, ototoxicity, and CNS toxicity. As discussed earlier, it is not always clear why selectivity exists for a particular subset of neurons or whether differential toxicity results from differential exposure, distinct properties of mitochondria within different types of neurons, or increased vulnerability of neurons arising from a specific feature of the interaction between mitochondria and their cellular environment. For this reason, the strategy taken in this chapter will be to review toxicities based on phenotype and target tissues and then to assess the evidence for a mitochondrial toxicity

mechanism. Given the considerable range of CNS-active compounds for which mitochondrial activity has been documented (Chan et al., 2005; Hargreaves et al., 2016), the potential for nervous system injury induced by drugs and toxic agents is substantial. The chapter will therefore conclude with a discussion of potential CNS injury mechanisms.

9.2 Mitochondrial Mechanisms of Peripheral Neuropathy

Peripheral neuropathy is a well-known condition most commonly caused by diabetes, alcohol abuse, paraneoplastic syndromes, or toxins. In this chapter we will focus on “drug-induced peripheral neuropathy,” a term defined as damage to nerves of the peripheral nervous system caused by a chemical substance used in the treatment, cure, prevention, or diagnosis of a disease.

Drug-induced peripheral neuropathy is a condition that requires the physician’s knowledge and awareness to be recognized and subsequently treated. It frequently presents to the physician as a sensory polyneuropathy with paresthesia that might be accompanied by pain with a distal, symmetric sock- and glove-shaped neuropathy caused by affection of the myelin sheath of the peripheral nerve (demyelination) with or without affection of the nerve axon itself (axonal degeneration). Motor symptoms and signs are generally minor. Although there are different clinical symptoms and signs, the drugs discussed later on may induce neurological impairments severe and persistent enough to restrict the patient’s daily activities (Vilholm et al., 2014). The key distinguishing features of neuropathy associated with drug treatment rather than the disease itself are a more abrupt onset of neuropathy. Drug-induced peripheral neuropathy can begin weeks to months after initiation of treatment with a particular drug and reach a peak at, or after, the end of treatment. In most cases, the onset is associated with the initiation of therapy (Moyle & Sadler, 1998), and generally the pain and paresthesia completely resolve after cessation of treatment. However, in some cases, drug-induced peripheral neuropathy is only partially reversible and can be permanent (Windebank & Grisold, 2008). Risk factors related to the specific treatment are reviewed in Vilholm et al. (2014, table 1). In the peripheral nervous system, it was demonstrated that more than 90% of mitochondria are localized in the axons; since axonal mitochondria are fundamental for energy generation in axons, a defect in mitochondrial energy metabolism can cause axonal transport degeneration and nerve failure (Park et al., 2008).

9.2.1 Reverse Transcriptase Inhibitors

Nucleoside and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are effective antiviral agents that are used in the treatment of herpes virus, hepatitis B virus, and, importantly, human immunodeficiency virus (HIV). While significant health advances have been achieved with the use of these drugs, the necessity for lifelong treatment to control HIV infection is limited by NRTI-associated toxicities that arise from virus-versus-host polymerase selectivity wherein NRTIs also serve as substrates for host polymerases (Brown et al., 2011; Johnson et al., 2001). Many mechanisms have been proposed for the origins of nucleoside analogue toxicity, but the most obvious sites of action are the enzymes responsible for host cell DNA replication and repair: DNA polymerases α , δ , and ϵ , which are responsible for chromosomal DNA replication; DNA polymerase β , which is involved in DNA repair; and DNA polymerase γ (pol γ), which carries out mitochondrial DNA (mtDNA) synthesis (Frick et al., 1988; Hall et al., 1994; Kewn et al., 1997). Interestingly, the toxicity manifested in many nonproliferating tissues is thought to result more specifically from the effects of inhibition of DNA pol γ by nucleoside analogues (Barile et al., 1998; Colacino, 1996; Honkoop et al., 1997; Horn et al., 1997; Perry et al., 1994).

mtDNA is necessary for many OXPHOS complex I proteins. mtDNA depletion causes a deficiency in complex I and an overutilization of complex II, resulting in elevated superoxide levels (Lewis et al., 2003). Depletion in mtDNA and increased mtDNA mutations may reduce synthesis of mtDNA-encoded protein subunits required for OXPHOS.

The potential for NNRTIs to cause neuropathy was first encountered with vidarabine in the treatment of hepatitis B and has subsequently been associated with the use of zalcitabine, didanosine, and stavudine (reviewed by Kakuda, 2000; Moyle & Sadler, 1998).

These drugs can induce mtDNA depletion, leading to mitochondrial respiratory chain and OXPHOS deficits. NRTIs exert rapid toxicity by directly inhibiting mitochondrial bioenergetics function (Yamaguchi et al., 2002). The resulting reduction in ATP and increased ROS production have the potential for further mtDNA damage (Lewis et al., 2001, 2006). The involvement of Ca^{2+} has been also described since the mitochondrial dysfunction induced by zalcitabine alters Ca^{2+} homeostasis in cultured DRG neurons (Werth et al., 1994) and in a model of zalcitabine-associated painful peripheral neuropathy (Joseph et al., 2004). In addition, NRTIs cause direct mitochondrial toxicity through inhibition of the mitochondrial transmembrane potential in neurons but not in Schwann cells (Keswani et al., 2003). *In vivo*, however, Anderson and colleagues (1994) showed that zalcitabine

administration to rabbits produced a neuropathy that was associated with dysfunctional mitochondria in Schwann cells but not neurons. This might suggest that disruption of neuronal support cells could be also critical to the neuropathy produced by NNRTIs. Prolonged exposure of the cell to nucleoside analogues can promote mitochondrial ultrastructural abnormalities in peripheral nerves and subcutaneous tissue (Cherry et al., 2002; Dalakas et al., 2001; Lewis & Dalakas, 1995).

The fact that toxicity of different drugs is cell, tissue, and organ specific (Benbrik et al., 1997) indicates that the differences in subcellular bioavailability and pharmacokinetic profile involving the activation and deactivation of these drugs may contribute to their specific clinical toxicity. Moreover, the situation in patients can be complicated by the presence of other factors that predispose to the development of neuropathy, including alcohol intake, diabetes, dietary deficiencies, and other pharmacological agents (Moyle & Sadler, 1998). The presence of these other risk factors may effectively amplify the burdens imposed by NNRTIs, although it is not known whether they share a common mechanism of action.

9.2.2 Chemotherapy-Induced Peripheral Neuropathies (CIPN)

Millions of patients are treated with chemotherapeutic agents for the treatment of solid and hematological cancers in the neoadjuvant, adjuvant, or palliative setting. Among these chemotherapeutic drugs, DNA-alkylating agents (platinum derivatives such as cisplatin and oxaliplatin), antitubulin compounds (taxanes such as paclitaxel and vinca alkaloids such as vincristine), and proteasome inhibitors (bortezomib) are frequently used. All these compounds are different in nature and chemical structures and also in their mechanism and site of action, but they have a common and relevant side effect that is the onset of a peripheral neurotoxicity that is frequently associated with the development of neuropathic pain (recently reviewed by Canta et al., 2015; Cavaletti & Marmiroli, 2015; Grisold et al., 2012; Travis et al., 2014; Windebank & Grisold, 2008).

The observation of the similar clinical presentations of peripheral neuropathies associated with chemotherapeutic agents of diverse chemical classes gave rise to the mitotoxicity hypothesis, which posits that the fundamental cause of this form of chronic peripheral neuropathy is a toxic effect on mitochondria in primary afferent somatosensory neurons (Flatters & Bennett, 2006; Xiao & Bennett, 2012; Zheng et al., 2011, 2012), aimed at resolving the striking paradox that similar neurological symptoms are observed despite diverse anticancer mechanisms of action. In fact, the results of mitochondrial toxicity

are more evident in regions with high metabolic request, such as primary afferent sensory neurons, where an elevated mitochondrial content is present (Heppelmann et al., 2001).

Cells of the peripheral nervous system are vulnerable because of several unique characteristics, namely, (i) primary sensory and autonomic neurons are contained in ganglia that lie outside the blood–brain barrier and are supplied by capillaries with fenestrated walls that allow free passage of molecules between the circulation and the extracellular fluid in the ganglia; (ii) long peripheral nerve axons are susceptible to any agents that interfere with the energy metabolism or the structural basis of axonal transport; (iii) drugs that act by disrupting microtubules of the mitotic spindle also disrupt microtubule-based axonal transport; (iv) agents targeting the increased mitochondrial activity of cancer cells may impair axonal transport; and (v) neurons have apoptotic cell death pathways that are particularly sensitive to DNA damage induced by many chemotherapeutic agents. Specific mechanisms of susceptibility will be discussed with individual classes of agents.

9.2.2.1 Microtubule-Modifying Agents and Mitochondria: Paclitaxel and Vincristine

Tubulin is a valuable target for anticancer chemotherapy as disruption of the mitotic spindle prevents cell replication. However, microtubules are critical for proper nerve function. They serve as tracks along which proteins, RNA, mitochondria, and other organelles that are synthesized in the cell bodies are transported by motor proteins to the nerve terminals, where synaptic vesicles carry out nerve actions requiring high ATP levels. Several agents with different antitubulin effects are neurotoxic on the peripheral nervous system, but newer compounds with less neurotoxic properties are under development (Klute et al., 2014).

There are two major subtypes of microtubule-targeted drugs, the taxanes and epothilones, that bind initially along microtubule surfaces or sites before becoming internalized into microtubules (Grisold et al., 2012; Ng et al., 2015; Park et al., 2013). These are referred to as microtubule stabilizers because, by binding along the microtubule lattice, they stabilize microtubule dynamic instability and inhibit microtubule depolymerization. On the other hand, vinca alkaloids and halichondrins bind only to microtubule ends at their lowest effective concentrations (Bennett et al., 2014; Ferrier et al., 2013; Park et al., 2013) and are referred to as microtubule destabilizers because, at high concentrations, they block the addition of new tubulin subunits to microtubule ends, thus leading to microtubule depolymerization. Despite sharing the ability to suppress microtubule dynamic instability, it has been found that these two

classes of drugs exert different effects on microtubule-supported transport (reviewed in Smith et al., 2016).

9.2.2.1.1 Paclitaxel

Paclitaxel, belonging to the taxane group, is a microtubule-binding compound that specifically and reversibly interacts with microtubules to enhance microtubule polymerization and decrease microtubule depolymerization, promoting a polymeric structure (Derry et al., 1995; Dumontet & Jordan, 2010; Jordan & Wilson, 2004; Kumar, 1981). The result is the arrest of cellular mitosis at the metaphase–anaphase transition (G2/M phase), inducing cancer cells death by apoptosis (Jordan et al., 1996; Jordan & Wilson, 2004). Paclitaxel is active against proliferating cells or cells having a reduced apoptosis threshold such as cancer cells. However neurons are susceptible to paclitaxel treatment: both *in vitro* and *in vivo* studies demonstrated that chronic paclitaxel treatment induces axonal degeneration followed by secondary demyelination and nerve fiber loss in the presence of a severe peripheral neuropathy (Sahenk et al., 1994). It has been proposed that the severity and incidence of CIPN depend on the cumulative dose of paclitaxel (Postma et al., 1995). The use of an albumin-bound paclitaxel formulation (abraxane) seems to be less neurotoxic (Conlin et al., 2010).

Although the exact mechanism of action is still unclear, it has been proposed that paclitaxel promotes mitochondrial damage and a chronic sensory axonal energy defect, in agreement with the mitotoxicity hypothesis. Several *in vitro* studies have reported paclitaxel-induced Ca^{2+} dysregulation, mitochondrial permeability transition pore (mPTP) opening, and alterations in the activity of Ca^{2+} -dependent mitochondrial enzymes such as dehydrogenases involved in the TCA cycle, probably affecting ATP production (Canta et al., 2015, reference herein). Moreover, it has been reported that changes in mitochondrial structure relate to a drug-induced disturbance of Ca^{2+} homeostasis (Jaggi & Singh, 2012). This finding was also confirmed by *in vivo* analyses where it has been observed that numerous swollen and vacuolated mitochondria accumulate in the axons of peripheral nerves of treated rats, supporting the concept of functional damage to peripheral nerve mitochondria (Flatters & Bennett, 2006). *Ex vivo* studies demonstrated cyclosporine A-sensitive cytochrome *c* release and apoptotic pathway activation (Andre et al., 2000; Carre et al., 2002) in mitochondria isolated from human neuroblastoma cells treated with paclitaxel.

9.2.2.1.2 Vincristine

Vincristine is the most severely neurotoxic compound among vinca alkaloids, predominantly used for the treatment of hematologic and lymphatic malignancies. They

are also used as adjunctive treatments in combination with other drugs for rhabdomyosarcomas, neuroblastomas, and breast ovarian, testicular, lung, and colorectal cancer.

Patients in treatment develop disturbances in both motor and sensory functions (Postma et al., 1993) with early numbness and tingling in hands and feet as well as ankle jerks (Balayssac et al., 2011). Moreover, neuropathic and muscle pain is frequently present, as well as the loss of temperature sensation. The severity of symptoms is related to the duration and the therapeutic doses received, and unfortunately sometimes, after discontinuation, the symptoms worsen instead of improving (Verstappen et al., 2005).

Apart from alteration in mitochondrial ultrastructure (Flatters & Bennett, 2006), it has been shown that vincristine-caused peripheral neuropathy is related to a dysregulation of mitochondrial-mediated Ca^{2+} buffering (Tari et al., 1986), modifying spatiotemporal Ca^{2+} concentration, Ca^{2+} signaling (Boitier et al., 1999), and Ca^{2+} -dependent neurotransmitter release (Montero et al., 2000) as well as OXPHOS in mitochondria. Moreover, inhibitors of the electron transport chain have been reported to attenuate vincristine-related neuropathic pain in rats (Joseph & Levine, 2006).

Disruption of microtubules in long axons of sensory and motor nerves ought to impact on mitochondrial trafficking and neuronal function. The studies reported here document a number of different effects on mitochondria altering their structure and function. However, neither altered morphology nor differences in Ca^{2+} handling are obvious results of a selective effect on inhibition of mitochondrial trafficking. Whether altered trafficking contributes to the neuropathy associated with microtubule disrupting drugs requires further analysis.

9.2.2.2 Platinum Compounds and Mitochondria: Oxaliplatin

Platinum compounds belong to a family of platinum (Pt)-based anticancer drugs that includes several generations of compounds generated to reduce toxicity and enhance their anticancer activity at the same time. Three members of the drug family are currently used: cisplatin, carboplatin (mainly in lung, breast, and ovarian cancer), and oxaliplatin (used for the treatment of colon cancer and other gastrointestinal malignancies). These compounds are alkylating agents that bind the DNA double strand by crosslinks, creating Pt-DNA adducts. If the amount of DNA damage exceeds the ability to affect repair, the cell undergoes apoptotic cell death.

Since 1978, when cisplatin was first approved in the United States to treat cancer, CIPN emerged as a major side effect (Avan et al., 2015). Distal, symmetrical sensory impairment and ataxia are the typical clinical features

observed in platinum drug-treated patients, and they are both intensity and cumulative dose related. A major issue in the use of platinum drugs is represented by CIPN worsening even after treatment withdrawal (the “coasting” effect) (Cavaletti et al., 2011), reviewed in Canta et al. (2015) and Grisold et al. (2012).

Understanding the oxaliplatin effects on mitochondrial function requires further investigation, but the principal known events are excellently summarized in previous reviews (Canta et al., 2015). In brief, it has been demonstrated that oxaliplatin activates the intrinsic apoptotic pathway in colon carcinoma cells (Arango et al., 2004) and requires the proapoptotic BCL-2 family proteins Bax and Bak during this process (Gourdiere et al., 2004). Moreover, it has been shown that oxaliplatin induced a deficit in respiration rate in both complexes I and II of mitochondrial electron transport chain followed by a decrease of ATP production in drug-treated rats (Zheng et al., 2011). These respiratory deficits could be aggravated using respiratory chain inhibitors (Xiao et al., 2012). Furthermore, increases in the mitochondrial ROS levels in neurons exposed to high concentrations of oxaliplatin have been reported (Kelley et al., 2014), and substantial mitochondrial morphological changes (Xiao & Bennett, 2012) could also play an important role in the pathogenesis of oxaliplatin-induced peripheral neuropathy.

9.2.2.3 Protease Inhibitor Bortezomib and Mitochondria

The ubiquitin–proteasome system is the major intracellular protein degradation pathway in eukaryotic cells, playing an important role in transcriptional regulation of key transcription factor such as nuclear factor- κB (NF- κB). The 26S proteasome, an ATP-dependent protease, is fundamental for the ubiquitin–proteasome pathway (Lenz, 2003).

Bortezomib, a boronic acid dipeptide, acts by inhibiting the proteasome–ubiquitin pathway (Adams, 2004) by specifically and reversibly binding to the 26S subunit of the proteasome (Curran & McKeage, 2009), leading to cell cycle inhibition and apoptosis (Shahshahan et al., 2011). One of the proposed explanations for this mechanism of action is the decreased activation of NF- κB that leads to cell death (Traenckner et al., 1994) and increased transcription and stabilization of the proapoptotic Bcl-2 protein PUMA (Concannon et al., 2007). In addition, bortezomib has been shown to affect polymerization of α -tubulin and result in microtubule stabilization, similar to those effects seen with taxanes (Meregalli et al., 2015; Poruchynsky et al., 2008; Staff et al., 2013), altering axonal transport in rat DRG neurons (LaPointe et al., 2013; Meregalli et al., 2014; Staff et al., 2013). Bortezomib monotherapy was approved by the US Food and Drug

Administration in 2003 and a year later by the European Medicines Agency for the treatment of refractory multiple myeloma and mantle cell patients (Adams & Kauffman, 2004; Altun et al., 2005). In addition to refractory multiple myeloma, the use of bortezomib has rationale for the treatment of non-hematologic malignancies (Roussel et al., 2010). Painful peripheral neuropathy is a significant dose-limiting toxicity of bortezomib (Cavaletti & Nobile-Orazio, 2007; Meregalli et al., 2012) with severe symptoms. Paresthesia, burning sensations, dysesthesias, numbness, sensory loss, reduced proprioception, and vibratory sensation (Argyriou et al., 2008) are some of the most common symptoms referred by patients, which typically occur within the first courses of bortezomib treatment, reaching a plateau typically after several cycles of treatments, and thereafter do not appear to increase (Richardson et al., 2006; Windebank & Grisold, 2008). Concerning the mechanism of bortezomib-induced peripheral neuropathy, a multifactorial cause seems likely, and various bortezomib toxicity mechanisms have been presented till now (extensively reviewed in Meregalli et al., 2015), but the results are not completely conclusive, so further investigations will be essential.

Interestingly, it was demonstrated that bortezomib induces a dysregulation of Ca^{2+} homeostasis that ultimately leads to apoptotic cell death. Bortezomib alters the levels of many apoptosis-related proteins, and a role for caspase-dependent, PUMA-dependent, and PUMA-independent pathways in cell death execution has been proposed (Landowski et al., 2005; Tuffy et al., 2010). Moreover, it has been published that bortezomib-treated rats exhibited defects in mitochondrial function, measured as a significant deficit in ATP production rate following stimulation of the electron transport system (Zheng et al., 2012), being prevented by a prophylactic treatment with acetyl-L-carnitine, which also inhibited the neuropathic pain observed in rats (Zheng et al., 2012). Finally, it has been described that bortezomib induces sensory-dominant axonal depolarization likely due to an impairment of the Na^+/K^+ -ATPase-dependent pump and consequently Na^+ axonal accumulation (Han et al., 2008; Kiernan & Bostock, 2000). Bortezomib-induced mitochondrial impairment would compromise mitochondrial ATP production, which would lead to continuous influx of Na^+ ions due to an overload of the Na^+/K^+ -ATPase-dependent pump, resulting in mitochondrial energy failure (Nasu et al., 2014).

9.2.3 Statins

HMG-CoA reductase inhibitors (statins) are perceived to have a favorable safety profile (Bernini et al., 2001; Davidson, 2001; Smith, 2000) and have well-documented

benefits for the treatment of cardiovascular diseases. The beneficial effects of statins have been objectively shown to reduce risks for both total mortality and total morbidity (indexed by serious adverse events), specifically in clinical trial equivalent middle-aged men who are at high cardiovascular risk (Criqui & Golomb, 2004; Long-Term Intervention with Pravastatin in Ischaemic Disease, LIPID Study Group, 1998; Pedersen et al., 2004).

Statins inhibit the enzyme HMG-CoA reductase at a stage early in the mevalonate pathway (Buhaescu & Izzedine, 2007). This pathway generates a range of other products in addition to cholesterol, such as coenzyme Q10 (CoQ10), heme A, and isoprenylated proteins (Buhaescu & Izzedine, 2007), which have pivotal roles in cell biology and human physiology. Additionally, cholesterol itself is also an intermediate in the synthesis of sex steroids, corticosteroids, bile acids, and vitamin D, several of which have been shown to be affected with statin administration (Hyyppa et al., 2003; Mol et al., 1989). The biochemical influences of statins include effects on nitric oxide (NO) and inflammatory markers (Cimino et al., 2007) or on polyunsaturated fatty acids (PUFA) (Harris et al., 2004), among many others.

Despite their efficacy and that statins are generally well tolerated, many patients are obliged to discontinue statin therapies due to adverse events (reviewed in Gluba-Brzozka et al., 2016). Approximately 0.5% of patients present with myopathic side effects, the most commonly reported adverse effects associated with statin therapy (reviewed in Golomb & Evans, 2008). The induction of skeletal muscle fiber apoptosis, mitochondrial dysfunction, and terpenoid depletion may mediate statin-associated myopathy (Dirks & Jones, 2006; Jimenez-Santos et al., 2014; Johnson et al., 2004).

As statin therapy is extended to a larger population and an earlier onset on the primary prevention of high-risk individuals is advised, the milder nonspecific secondary effects of statins gain relevance and must be addressed. Second-generation statins were developed shortly after their initial development in the 1970s. They include simvastatin, atorvastatin, cerivastatin, rosuvastatin, and pitavastatin (Tobert, 2003). Today, all the approved statins had already lost patent protection in 2016. This has prompted the pharmaceutical industries to seek for new chemical entities or statin derivatives capable of offering new ways to inhibit HMG-R (Pfefferkorn, 2011).

Brain tissue shares with muscle tissue a high mitochondrial vulnerability as both are post-mitotic tissues with high metabolic demand (De Flora et al., 1996; Kopsidas et al., 2000; Linnane et al., 2002; Ozawa, 1995; Sastre et al., 2003). Separate from the effects on skeletal muscle, there is clearly evidence of neuropathy induced by statins (Backes & Howard, 2003; De Vivo & DiMauro,

1990; Peltier & Russell, 2006). Furthermore, mitochondrial defects predispose to problems on statins. Additionally, statins predispose to mitochondrial defects in all users and, to a greater degree, in vulnerable individuals (Golomb & Evans, 2008).

The pathogenesis of the adverse myopathic side effects associated with statin therapy is yet to be fully elucidated; however interesting links with mitochondrial function have been documented. Lactic acidosis (Keaney et al., 2014) and elevated lactate/pyruvate ratios (Goli et al., 2002) have been reported in patients following statin therapy, indicating mitochondrial respiratory chain impairment. Statins reduce serum CoQ10 levels (De Pinieux et al., 1996; Mortensen et al., 1997; Rundek et al., 2004). Some studies have also shown deficit in CoQ10 status directly assessing skeletal muscle following statin treatment (Laaksonen et al., 1996; Lamperti et al., 2005; Larsen et al., 2013; Paiva et al., 2005). Statin-induced depletion of CoQ10 may be associated with enhanced production of free radicals and subsequent damage of mtDNA (Deichmann et al., 2010) and reduced mitochondrial OXPHOS capacity, resulting in mitochondrial dysfunction (Larsen et al., 2013) in skeletal muscle. Importantly, changes in tissue mitochondrial and respiratory function clearly occur also in the brain (Barbiroli et al., 1997; 1999). Interestingly, Duberley et al. (2013) reported that a 24% decrease in neuroblastoma cell CoQ10 status was sufficient to perturb OXPHOS as indicated by the decrease in cellular ATP status commensurate with a loss of mitochondrial membrane potential. Moreover, it has been shown that CoQ10 supplementation is beneficial in PD (Shults et al., 2002). The results of other studies have suggested that statins may induce myopathy through the impairment of calcium signaling (reviewed in Gluba-Brzozka et al., 2016). The mevalonate pathway, which statins inhibit, also produces heme A, which has its own central involvement in mitochondrial electron transport (Manoukian et al., 1990). Lovastatin and simvastatin inhibit mitochondrial respiration in isolated liver mitochondria by a direct effect on electron transport at complex II/III, complex IV, and/or complex V (Nadanaciva et al., 2007). Evidence for a loss in complex IV activity following statin treatment has been reported in patients treated with the statin cerivastatin together with gemfibrozil (Arenas et al., 2003) and simvastatin in conjunction with cyclosporine and itraconazole, respectively (Duncan et al., 2009). Furthermore, a recent study that investigated the pharmacological effect of statins in their acid and lactone forms found that mitochondrial respiratory complex III inhibition was a potential off-target mechanism associated with statin-induced myopathies (Schirris et al., 2015). *In vivo* effect of statins on platelet energy metabolism can be attributed to drug

effects on complex I of the electron transport system (Vevera et al., 2016).

It is important to bear in mind that although statin-induced CoQ10 deficiency and mitochondrial respiratory chain impairment may be a contributory factor to the myopathic adverse effects associated with statin therapy, multiple pathophysiological mechanisms are thought to contribute to statin myotoxicity (Apostolopoulou et al., 2015; Banach et al., 2015; Taylor & Thompson, 2015; Tomaszewski et al., 2011), and further studies are required to elucidate mitochondrial dysfunctions that contribute to statin-induced neuropathy.

9.3 Mitochondria and Retinal Drug Toxicity

Humans obtain approximately 80% of external information from vision. Since loss of vision markedly decreases quality of life, it is extremely important to assay the risk of drugs and chemicals for visual toxicity. The focus of this section will be on injury to the retina and optic nerve.

Optic neuropathies are an important cause of chronic visual impairment and have in common the degeneration of retinal ganglion cells (RGCs), whose axons form the optic nerve (Pascolini & Mariotti, 2012). Optic nerve dysfunction can present as the only manifestation of disease or can be part of other systemic or neurological disorders; it can result from ischemic insults, damage by shock, toxins, radiation, trauma, or hereditary factors (Miller & Newman, 2004).

The optic nerve is susceptible to damage from toxins, including drugs, metals (e.g., lead, mercury, and thallium), organic solvents (ethylene glycol, toluene, styrene, and perchloroethylene), methanol, carbon dioxide, and tobacco. This group of disorders is named toxic optic neuropathy (TON) and is characterized by bilateral visual loss, papillomacular bundle damage, central or cecentral scotoma, and reduction of color vision. In most of the cases of TON, the primary lesion is not limited to the optic nerve and may possibly originate in the retina or chiasm, among other origins. It is generally accepted that the common pathway, for at least some of the toxins, is mitochondrial injury and imbalance of intracellular and extracellular-free radical homeostasis (Wang & Sadun, 2013).

The optic nerve is an extension of the CNS and has some unique structural features that potentially make it vulnerable to disease processes. At an ultrastructural level, unmyelinated nerve fibers exit the eye via the lamina cribrosa, becoming myelinated at its posterior border. Studies of human retina and optic nerve specimens using electron microscopy and histoenzymatic stains for

the mitochondrial respiratory complexes showed an inverse relationship between the mitochondrial activities and myelination (Andrews et al., 1999; Bristow et al., 2002; Minckler et al., 1976). The distribution of mitochondria in the optic nerve head reflects functional energy requirements to maintain conduction in the unmyelinated axon. It is abundantly clear that RGCs comprising the optic nerve axons are exquisitely sensitive to even minor disturbances in mitochondrial biogenesis or dynamics, with an innate susceptibility to undergo apoptosis under conditions of heightened cellular stress and elevated ROS levels (Yu-Wai-Man et al., 2011, references herein). This is more remarkable if we consider that the brain itself has tremendous energy needs. Furthermore, the relative oxygen consumption of the retina is higher than that of the brain, being one of the highest oxygen-consuming tissues of the body (Yu & Cringle, 2001). The link between drug-induced mitochondrial dysfunction and retinal damage is anticipated by findings of inherited diseases associated with mitochondrial genes and optic neuropathy.

The two classical mitochondrial optic neuropathies are LHON and autosomal dominant optic atrophy (DOA), which share overlapping clinical and pathological features, despite being caused by mtDNA point mutations and a growing list of nuclear genetic defects, respectively. The defining neuropathological feature of LHON and DOA is the early loss of RGCs within the papillomacular bundle, resulting in an expanding field defect, known as a scotoma, within the patient's central vision (Gorman et al., 2015). Interestingly, a subgroup of patients can develop a syndromic form of LHON and DOA that is characterized by prominent neurological deficits in addition to optic atrophy.

The unique susceptibility of RGCs in mitochondrial optic neuropathies still remains a puzzling mystery, and although several hypotheses have been put forward, none has been conclusively demonstrated (Carelli et al., 2004; Yu-Wai-Man et al., 2011). The highly specialized anatomical and physiological properties of RGCs are likely to be relevant, namely, their relatively high metabolic requirements due to sustained spiking activity; the constant exposure to potentially damaging light; the sharp 90° bend as their axons exit the eye at the lamina cribrosa; and the relatively long unmyelinated intraocular portion that necessitates a high density of packed mitochondria for efficient signal conduction (Andrews et al., 1999; Bristow et al., 2002).

There is still an ongoing debate whether the determining factor that drives RGC loss in mitochondrial optic neuropathies is a bioenergetics crisis or toxic ROS levels, or possibly both factors (Levin, 2015). Other cellular mechanisms that have gained increasing attention in the context of mitochondrial optic neuropathies are mtDNA

maintenance and mitophagy and the cross-talk with the endoplasmic reticulum at mitochondria-associated membrane (MAM) interfaces (Burte et al., 2015; Tatsuta & Langer, 2008).

In vitro approaches have been employed, and the effort of several research groups worldwide has led to the generation of faithful animal models for both LHON and DOA (Burte et al., 2015; Lin et al., 2012). These observations from inherited diseases validate the concept that retinal injury can result from impairment of mitochondrial function and provide a framework for understanding the effects of xenobiotics. In this section, the actions of three well-known retinal toxins will be discussed, along with the evidence for a mitochondrial target for the toxicity.

9.3.1 Chloramphenicol

Chloramphenicol inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. The toxicity associated with chloramphenicol arises from inhibition of mitochondrial protein synthesis (Gilman et al., 1985) because of the similarity in structure of bacterial and mitochondrial ribosomes (Holt et al., 1993; Yunis, 1988). Systemic chloramphenicol is now rarely used for treating severe disseminated infections; however, until the late 1970s, it was frequently used in the management of patients with cystic fibrosis, and a serious complication noted in this group of patients was bilateral optic neuropathy (Godel et al., 1980; Venegas-Francke et al., 2000; Yunis et al., 1970). Transmission electron microscopy of bone marrow cells of patients taking chloramphenicol has shown swollen mitochondria with disrupted cristae and an abnormally high level of intramitochondrial iron deposits, confirming the toxic effect of the drug (Smith et al., 1970). These findings strongly suggest that the pathological changes in chloramphenicol optic neuropathy are due to a similar mitochondrial toxic effect.

In experimental models involving the study of neuronal function, additional evidence suggesting an interaction between chloramphenicol and mitochondrial function also emerges. For example, injury to the auditory nerve induced by high noise levels or gentamicin produces an injury that is associated with (and perhaps ameliorated by) increased biogenesis of mitochondria. Treatment of animals with chloramphenicol exacerbates this form of injury (Hartlage-Rubsamen & Rubel, 1996; Hyde & Durham, 1994; Hyde & Rubel, 1995). Chloramphenicol also decreases glucose utilization and inhibits NADH oxidation in rat brain (Mottin et al., 2003; Moulin-Sallanon et al., 2005). Considering the special energetic requirements and mitochondrial distribution of RGCs previously mentioned, the presumption that the

chloramphenicol-induced optical neuropathy is due to mitochondrial dysfunction is reasonable.

9.3.2 Ethambutol

Ethambutol is a commonly used first-line antituberculous drug; however the course of its ocular toxicity remains unpredictable (Chan & Kwok, 2006). Besides its optic nerve toxicity, ethambutol could also be toxic to the neural retina, which can result in field defects, color vision abnormalities, loss of visual acuity, or even permanent visual impairment (Ezer et al., 2013; Garg et al., 2015; Koul, 2015). It is generally considered that optic neuropathy is the dose-limiting toxicity with ethambutol treatment (Barron et al., 1974; Fraunfelder et al., 2006). Peripheral neuropathy is a rare side effect that can occur also at a lower dose and is duration and dose dependent (Lee et al., 2008; Melamud et al., 2003).

The mechanism by which ethambutol produces a selective effect on optic nerve function is not clear, but studies have suggested a specific involvement of mitochondria and various pathophysiological mechanisms have been proposed including disruption of mitochondrial complex IV activity through a copper-chelating action, zinc-mediated lysosomal membrane permeabilization, and glutamate excitotoxicity.

Ethambutol's therapeutic action is hypothesized to act as a chelating agent for zinc and copper. Ethambutol is a metal chelator, destroying bacteria by inhibiting arabinosyltransferase, an important enzyme in mycobacterial cell wall synthesis. Due to the similarity between mammalian mtDNA and bacterial ribosomes, ethambutol also disrupts OXPHOS and mitochondrial function by interfering with iron-containing complex I and copper-containing complex IV (Kozak et al., 1998). Copper is a required cofactor for cytochrome *c* oxidase, an essential component in the electron transport chain. Ethambutol may reduce the level of copper, thereby interfering with OXPHOS. Replacing copper leads to improved RGC survivability in *in vivo* models of ethambutol optic neuropathy (Yoon et al., 2000). It is interesting that copper deficiency due to malabsorption from bariatric surgery also has been associated with visual loss from optic neuropathy (Becker et al., 2012; Pineles et al., 2010). Other studies suggest that zinc also might play a role in ethambutol toxicity (Leopold, 1978; Yoon et al., 2000) and individuals with reduced serum zinc level may be more susceptible to ethambutol ocular toxicity (De Palma et al., 1989; Delacoux et al., 1978). A study using cell cultures demonstrated that the chelating effect of ethambutol may inhibit lysosomal activation, resulting in accumulation of zinc in lysosomes with increased lysosomal membrane permeability and cell death (Chung et al., 2009), perhaps

indicating a mechanism of facilitated zinc entry into neurons as is observed with agents such as pyriethione (Kandel, et al., 2012).

Although the exact mechanism of the ethambutol ocular toxicity is not yet identified (Koul, 2015), it has been suggested (Heng et al., 1999) that glutamate-mediated excitotoxicity may be the mechanism by which ethambutol is specifically toxic to RGCs. Excitotoxicity may mediate this effect through the generation of ROS, resulting in injury and death of the affected cells (Ezer et al., 2013; Wang & Sadun, 2013). It is well documented that NMDA receptor-induced cell death is associated with excess calcium influx that is followed by mitochondrial dysfunction, free radical formation, and oxidative stress (Ganapathy et al., 2011; Lipton, 2007). In this context, the (partial) NMDA receptor antagonist memantine has been reported to provide protection against ethambutol-induced retinal injury, involving normalization of oxidative stress and Müller cell stress responses (Abdel-Hamid et al., 2016). Memantine is a selective blocker of the excessive NMDA receptor activity without affecting their physiological function (Lipton, 2007; Seki & Lipton, 2008).

Thus, the proposed effects of ethambutol on mitochondrial function recognize several different mechanisms that may contribute to neuronal injury.

9.3.3 Methanol

Methanol is a natural chemical that is used in many aspects of daily life, including its use as an industrial solvent, as a fuel source, or as a cheap adulterant of illicit liquors, and hence poses danger to human health.

Methanol has been found to induce CNS dysfunction (Murray et al., 1991; Sweeting et al., 2010) and neurodegeneration (Henzi, 1984; Manuchehri et al., 2015; Zakharov et al., 2015). Interestingly, methanol metabolism and mechanisms responsible for its toxic actions in primates have been extensively investigated in the periphery; however the mechanisms underlying its toxicity to the brain remain inexplicit in primates. Many studies have reported that methanol toxicity to primates is mainly associated with its metabolites, formaldehyde (FA) and formic acid. For example, formic acid has been found to be responsible for the metabolic acidosis witnessed in methanol-intoxicated humans (Jacobsen & McMartin, 1986; McMartin et al., 1980) and nonhuman primates (Eells et al., 2003; McMartin et al., 1977) and the ocular toxicity observed in methanol-poisoned humans (Jacobsen & McMartin, 1986; Sharpe et al., 1982) and nonhuman primates (Martin-Amat et al., 1978; Martin-Amat et al., 1977). Only recently that the toxicity of methanol has been linked to its metabolites, FA and/or formic acid, in the brain (Zhai et al., 2016).

It is believed that visual symptoms are related to mitochondrial damage and suppression of oxidative metabolism by inhibiting cytochrome oxidase activity (Eells et al., 2000; Sadun, 1998) so that toxicity is associated with acute metabolic failure (Nicholls, 1976). Formate is not a particularly potent inhibitor of cytochrome oxidase, with K_i values reported between 1 and 30 mM, depending on the oxidation state of the cytochrome (Nicholls, 1976). However, millimolar concentrations of alcohols in plasma are typical following normal consumption. As a result of the methanol intoxication hyperemia, edema and optic nerve atrophy occur (Sadun, 1998).

The failure to oxidize formic acid is a critical element in the toxicity of methanol in humans. As most animal species have the ability to detoxify formic acid, they do not normally suffer methanol toxicity. However, it is possible to model methanol toxicity in rodents by inhibiting formic acid oxidation, and formate-induced mitochondrial dysfunction and retinal photoreceptor toxicity have been documented in this animal model (Eells et al., 2000; Murray et al., 1991; Seme et al., 1999; 2001).

Interestingly, photobiomodulation, also known as low-level laser therapy or far-red to near-infrared (FR/NIR) light therapy, has been used as an innovative and noninvasive therapeutic approach for retinal conditions including methanol-induced retinal damage. Since the stimulation of cytochrome *c* oxidase is the suspected primary effect of FR/NIR phototherapy, it is not surprising that treatment with 670 nm light after methanol poisoning was able to ameliorate the retinal damage in a rat model of methanol toxicity (Eells et al., 2003) (see Geneva, 2016).

9.4 Mitochondria and Ototoxicity

Ototoxicity is the functional impairment and cellular degeneration of the tissues of the inner ear caused by therapeutic agents or chemicals. While some ototoxic agents have a propensity to affect the auditory system (cochleotoxic) and others the vestibular system (vestibulotoxic), there is an overlap between the two. There are a number of compounds currently in clinical use with known ototoxic properties, including aminoglycoside antibiotics, salicylates, antineoplastic platinum-based chemotherapy agents, antimalarial drugs, heavy metals, and loop diuretics.

Despite differences in chemical composition and function between the relevant drug groups, the symptomatic hearing loss is typically a high-frequency sensorineural hearing loss, progressing from higher to lower frequencies with prolonged treatment using aminoglycosides (Brummett & Fox, 1989), platinum-based chemotherapy

(Blakley & Myers, 1993), salicylates (Jung et al., 1993), antimalarial (Taylor & White, 2004), heavy metals (Rybak, 1992), and loop diuretics (Rybak, 1993). There are however exceptions; aspirin, for example, can give a flat audiogram (Jung et al., 1993). Ototoxic hearing loss is typically symmetrical; however unilateral and asymmetrical cases have been reported (Einarsson et al., 2010). Despite known risks of ototoxicity, certain medications continue to be used but require a close monitoring of plasma levels.

In general, although the associations between the use of these drugs and ototoxic adverse effects are well documented, the mechanisms underlying damage to the ear are not well understood. This section will focus on reviewing the ototoxicity of aminoglycoside antibiotics and cisplatin, because both groups of drugs can cause the most severe and irreversible hearing loss.

Aminoglycoside antibiotics include streptomycin, isolated from *Streptomyces griseus*; natural products such as neomycin, kanamycin, gentamicin, and tobramycin; and semisynthetic products, such as netilmicin and amikacin. These agents are used clinically for treating infectious diseases, such as tuberculosis, and bacterial infections produced by aerobic Gram-negative bacteria (such as bacterial endocarditis, urinary tract infections, and pneumonia). The incidence of reported hearing loss ranges from a few percent to up to 33%, and vestibular toxicity occurs in about 15% of patients who are administered with aminoglycosides (Chen et al., 2007). The hearing impairment is a consequence of damage first to the outer hair cells, which appear to be most sensitive, followed by the inner hair cells. Selectivity of injury to the inner ear over other tissues may arise from the accumulation and slow clearance of these drugs from the ear after extended treatment (Bates, 2003). Aminoglycoside antibiotics exert their antibacterial effects at the level of the prokaryotic ribosome, inducing errors in protein synthesis (Benveniste & Davies, 1973; Gale et al., 1981). The basis for the selective bactericidal effects of the aminoglycosides is presumably their preferential binding to the bacterial ribosome (Alberts et al., 1989). Since mitochondrial ribosomes are structurally more similar to their prokaryotic ancestors than either ribosome is to the eukaryotic ribosome, aminoglycosides might be expected to interfere with mitochondrial protein synthesis, which could be in the basis of their ototoxicity.

Work in various eukaryotic systems has shown that aminoglycosides are capable of forming complexes with membrane lipids (Au et al., 1987; Forge et al., 1989; Lipsky & Lietman, 1980; Sastrasinh et al., 1982; Schacht, 1978) and free iron (Priuska & Schacht, 1995). Ternary complexes between these molecules are capable of propagating highly reactive ROS and reactive nitrogen species (RNS) from H_2O_2 (Lesniak et al., 2003; 2005).

Moreover, there is ample evidence of swollen mitochondria in hair cells exposed to aminoglycosides (Bagger-Sjoberg & Wersall, 1978; Dehne et al., 2002; Fermin & Igarashi, 1983; Lundquist & Wersall, 1966; Mangiardi et al., 2004; Owens et al., 2007). This type of morphology is consistent with mitochondrial Ca^{2+} overload (Giorgi et al., 2012; Lemasters et al., 2009). Indeed, it has been shown that mitochondrial Ca^{2+} -driven increases in $\Delta\psi$ are both a necessary and a sufficient component of aminoglycoside-induced hair cell death (Esterberg et al., 2014). Recently, it has been described that a functional consequence of this event is the oxidation of mitochondria and production of ROS (Esterberg et al., 2016). Thus, while many pathways from distinct cellular compartments intersect to govern redox homeostasis (Daiber, 2010), it is proposed that mitochondria are perhaps the largest contributor to the oxidative changes observed during aminoglycoside-induced hair cell death (Esterberg et al., 2016). These findings are in agreement with those of Chen, Zheng, Schacht, and Sha (2013), who demonstrated the important role of the mitochondrial superoxide anion radical scavenger peroxiredoxin 3; when rendered inactive, intracellular ROS levels are elevated and murine cochlear hair cells are sensitized to aminoglycoside toxicity (Chen et al., 2013). An alternative hypothesis suggests that aminoglycosides promote activation of the NMDA receptor via stimulation of the polyamine site, a proposal supported by the observation that concurrent treatment with NMDA receptor antagonists limits aminoglycoside ototoxicity (Basile et al., 1996). Notably, this mechanism would also involve the excess production of oxidants following toxic NMDA receptor activation and mitochondrial Ca^{2+} accumulation (Stout et al., 1998). Antioxidant therapies have been proposed for the attenuation of aminoglycoside toxicity (Huth et al., 2011; Tadros et al., 2014; Xie et al., 2011) using compounds such as reduced glutathione, *N*-acetylcysteine, ubiquinone, and vitamin E in order to retain an appropriate redox balance (Kowaltowski et al., 1998; Mari et al., 2009; 2013; Nordberg & Arner, 2001).

9.4.1 Cisplatin

As mentioned earlier, cisplatin is a widely used chemotherapeutic agent for the treatment of various malignancies, including testicular, ovarian, bladder, cervical, head and neck, and non-small cell lung cancers. The mechanism of antitumor action of cisplatin involves uptake by the cancer cell and aquation within the tumor cell, which makes the drug more reactive for cellular targets. Then, the platinum atom of cisplatin forms covalent bonds with DNA at the N7 positions of purine bases to form intrastrand and interstrand crosslinks. A number of

downstream signaling pathways can then be activated to cause DNA damage. Cisplatin-induced ototoxicity is associated with cumulative dose and dose intensity (Brydoy et al., 2009; Oldenburg et al., 2007b; Rademaker-Lakhai et al., 2006; Rybak, 2007), although considerable interindividual variability exists (Oldenburg et al., 2007a,b; Ross et al., 2009; Rybak, 2007). Studies of long-term cisplatin ototoxicity that include audiometry (median follow-up = 4–6 years; range = 1–13 years) (Bokemeyer et al., 1998; Osanto et al., 1992) show altered hearing thresholds in 28–77% of patients. Cisplatin-related hearing loss is usually bilateral and, although it initially involves higher frequencies, eventually affects a broader range, particularly those critical for speech perception (Biro et al., 2006). Long-term tinnitus after cisplatin affects 19–42% of patients (Brydoy et al., 2009). Both elderly and pediatric patients are reportedly more sensitive to cisplatin ototoxicity. A recent study thoroughly reviews the cochleotoxicity classification systems that have been published to date (Crundwell et al., 2016).

Cisplatin ototoxicity can result from the overproduction of ROS in the cochlea (Rybak, 2007), causing irreversible free radical-related apoptosis of outer hair cells, spiral ganglion cells, and the stria vascularis (Rybak, 2007; van Ruijven et al., 2005).

The superoxides generated by the various cochlear tissues can (i) interact with NO and form peroxynitrites, which nitrosylate and inactivate proteins (Lee et al., 2004a, b); (ii) form free hydroxyl radicals, which on interaction with iron (Fe) react with PUFA in the lipid bilayer of the cell membranes to generate highly toxic aldehyde 4-hydroxynonenal (4-HNE), leading to cell death (Lee et al., 2004a, b) (this increase in 4-HNE has been associated with increased Ca^{2+} influx into the outer hair cell and apoptosis) (Clerici et al., 1995; Ikeda et al., 1993); (iii) inactivate antioxidant enzymes (Pigeolet et al., 1990); and (iv) cause translocation of Bax from the cytosol to mitochondria, leading to release of cytochrome *c* and subsequent activation of caspase-3 and caspase-9. Caspase-activated deoxyribonuclease (CAD) is then activated, causing DNA breakdown (Watanabe et al., 2003) and cleavage of fodrin in the cuticular plates of injured hair cells (Wang et al., 2004).

In the presence of cisplatin, ROS have been suggested to be produced via mitochondria (Kruidering et al., 1997; Young et al., 2002). Another study has indicated the involvement of NADPH oxidase since a unique isoform of nicotinamide adenine dinucleotide phosphate oxidase, NOX 3, has been demonstrated in the rat cochlea and is upregulated following ototoxic doses of cisplatin (Mukherjee et al., 2006).

Ideal otoprotectant to be used along with cisplatin should be easy to administer and should not interfere

with antitumoral effects of cisplatin. Data from animal models suggest that upregulation of antioxidant pathway activity, such as glutathione *S*-transferases (GST) that are expressed in the mammalian cochlea (van Ruijven et al., 2004), may help protect against ototoxicity (el Barbary et al., 1993). Interestingly it has been shown that siRNA against NOX3 blocked this high ROS generation in the organ of Corti hair cell cultures (UB/OC-1 cells) (Mukherjea et al., 2008).

Cisplatin-induced cell death with increased lipid peroxidation and altered mitochondrial permeability transition (So et al., 2005) was also inhibited by a calcium channel blocker, flunarizine. Flunarizine is an antagonist of T-type specific calcium channels that has been widely used to treat vertigo, migraine, epilepsy, and tinnitus. The protective mechanism of flunarizine on cisplatin-induced cytotoxicity is associated with direct inhibition of lipid peroxidation and mitochondrial permeability transition activation (Elimadi et al., 1998). Oral flunarizine is a novel approach to decrease the irreversible assault of cisplatin and its metabolites on auditory cells. Flunarizine is well tolerated and has minimal side effects with oral mode of administration, thus making flunarizine a potential otoprotective agent to be coadministered with cisplatin.

9.4.2 Mitochondrial Disorders, Hearing Loss, and Ototoxicity

Mitochondrial disorders are usually associated with pathologies affecting the central and peripheral nervous systems (Carelli & Chan, 2014). The mtDNA is particularly prone to damage, with a mutation rate 10 times higher than that of nDNA (Jeppesen et al., 2011). Moreover, it replicates more often than the nDNA, and the efficiency of repair mechanisms appears to be less efficient than the one for nDNA (Kazak et al., 2012). Point mutations are, in general, maternally inherited and heteroplasmic, with an estimated incidence of 1:5000 (Chinnery et al., 2012). They can affect mtDNA-encoded proteins, tRNA or rRNA, and eventually ATP production by promoting OXPHOS deficiency and ROS excess, which may foster cell apoptosis (Carelli & Chan, 2014; Chinnery et al., 2012; Copeland, 2014).

In fact, the function of the inner ear is particularly sensitive to impairment of mitochondrial function, and this is reflected by the relatively frequent association of maternally inherited mitochondrial dysfunction with hearing impairments.

Almost 600 pathogenic point mutations have been identified in the last years (see review Pinto & Moraes, 2014), involving most of the mtDNA molecule (according to MITOMAP, 299 point mutation involving tRNA–

rRNA and control regions and 274 involving OXPHOS proteins). For example, about 80% of MELAS symptoms cases are caused by a very common A3243G mutation in the mitochondrial tRNA^{Leu}(UUR) gene, whereas 10% of cases carry the T3271C mutation in the same tRNA^{Leu}(UUR). Another well-known multi-systemic disease and hearing loss disease is the one produced by large mtDNA deletion in Kearns–Sayre syndrome (KSS) (Spector & Johanson, 2010).

The first identified mutation was the A1555G mutation in the mitochondrial 12S rRNA gene causing the non-syndromic hypersensitivity to ototoxic effects of aminoglycosides by increased binding of aminoglycosides to mitochondrial ribosomes, leading to the disruption of mitochondrial protein synthesis (Harpur, 1982; Noller, 1991). Another recently identified mutation in the mitochondrial 12S rRNA gene is the T1494C in the conserved stem structure of 12S rRNA (Zhao et al., 2004). Other nucleotide changes at position 961 in the 12S rRNA gene have been found to be associated with hearing loss. However, further studies are required to confirm whether and how often these changes would predispose carriers to aminoglycoside toxicity (Bacino et al., 1995; del Castillo et al., 2003). Several mutations in the mitochondrial tRNA^{Ser}(AGY) gene are also known to cause maternally inherited non-syndromic hearing loss (MIHL) by disturbing the tRNA structure and function (Guan et al., 1998).

MtDNA changes can be a consequence of mutations in nDNA-encoded genes involved in the maintenance of mtDNA integrity and mtDNA copy number. The most common mutations affect POLG, the gene encoding for the catalytic subunit of the mtDNA polymerase gamma, and PEO1, which encodes for the DNA helicase Twinkle (Milenkovic et al., 2013; Spelbrink et al., 2001). Over 200 mutations in POLG associated with mitochondrial diseases have been identified, causing a plethora of heterogeneous disorders involving different tissues time of onset and severity. Sensorineural hearing loss is an important pathological feature in a range of mitochondrial diseases including patients with POLG mutations (Kullar et al., 2016). Two different groups created the “mutator mouse” by knocking in PolgA gene carrying two different mutations: the D257A mutation (Trifunovic et al., 2004) or the AC → CT substitution in positions 1054 and 1055 (Kujoth et al., 2005). These mutations provoked an accumulation of mtDNA point mutations in different tissues (Williams et al., 2010), causing two very similar clinical phenotypes, which included hearing loss.

As previously described, Twinkle has a helicase activity essential for mtDNA replication and maintenance (McKinney & Oliveira, 2013). The Twinkle A360T

mutation and aa 353–365 duplication are typical of PEO so that Twinkle mice have been generated knocking in the mutated gene (Tyynismaa et al., 2005), resulting in an accumulation of multiple large mtDNA deletions mostly in brain and muscle. Recently it has been described that a 13-year-old patient present a novel biallelic mutation in C10orf2, encoding the Twinkle mtDNA helicase, with features of infantile onset spinocerebellar ataxia (IOSCA), including a severe hearing loss (Pierce et al., 2016).

Finally, mutations in proteins involved in regulation of mitochondrial functions can also promote hearing dysfunction, and one example is OPA1. OPA1 protein (Alexander et al., 2000; Delettre et al., 2000) is a mitochondria-targeted dynamin-related GTPase that localizes to the inner mitochondrial membrane (Delettre et al., 2000; Olichon et al., 2006). It promotes fusion of the inner mitochondrial membrane (Olichon et al., 2006), maintains the integrity and structure of mitochondrial cristae (Frezza et al., 2006), and is also implicated in maintenance of membrane potential and OXPHOS (Lodi et al., 2004). Interestingly, hearing dysfunction in OPA1 patients is underlain by auditory neuropathy due to degeneration of auditory nerve fibers. Santarelli et al. (2015) and Kullar's results (2016) suggest a complex pathophysiology for sensorineural deafness in mitochondrial diseases, ranging from prevalent auditory neuropathy in OPA1 missense mutations to prominent cochlear or mixed cochlear/neural dysfunction in other genetic defects, particularly in mtDNA-related diseases.

9.5 Mitochondrial Mechanisms of Central Nervous System injury

The preceding sections have highlighted well-established examples of drugs that produce characteristic adverse effects in the nervous system that are most likely the result of impairment of mitochondrial function. Attribution of the adverse effects to mitochondrial impairment is facilitated by the ability to associate specific and, typically, sensory deficits with drug exposure and then ultimately to link pathophysiological sequelae to one or more mitochondrial targets. This association proves to be much harder to establish in the context of potential toxic effects in the CNS that arise from mitochondrial inhibition. Although there are numerous drugs that act on the nervous system that also affect mitochondrial function (reviewed by Chan et al., 2005; Hargreaves et al., 2016), it proves to be much more difficult to establish explicit pathological consequences for the CNS that arise from mitochondrial inhibition. This is because the injury phenotypes would be expected to develop relatively slowly and would be manifested by altered behaviors or perhaps subtle neurological symptoms rather

than alterations in sensory modalities that are easier to detect and isolate. Rather than attempting to create speculative links between drugs and potential CNS toxicity, it is perhaps more useful to review mitochondrial mechanisms underlying neuronal injury based on known mechanisms derived from established neurotoxins. This will provide the context for understanding the potential manifestations of drug-induced injury mediated by mitochondria and will provide an appreciation of the nature of the challenge of studying this kind of toxicity.

9.5.1 Mitochondrial Mechanisms of Neuronal Injury

Mitochondrial dysfunction and neurotoxicity have been extensively studied along the years and also recently reviewed (Ayala-Pena, 2013; Bates, 2003; Brand & Nicholls, 2011; Chaturvedi & Flint Beal, 2013; Duchen, 2012; Herrero-Mendez et al., 2009; Llorente-Folch et al., 2015; Moncada & Bolanos, 2006; Nicholls, Brand, & Gerencser, 2015; Siddiqui et al., 2012).

There are several well-established mechanisms by which mitochondrial mechanisms mediate neuronal injury. Elevated intracellular calcium, increased ROS, and energy depletion commonly represent the major known pathophysiologic mechanisms impacting neurons. There is also emerging evidence linking effects on mitochondrial morphology and trafficking with neuronal viability that will be briefly discussed. Mitochondria also serve as a focal point for the intrinsic pathways of apoptosis where they release cell death-activating factors. However, it would be unusual for a drug to directly trigger release of proapoptotic factors from mitochondria, and this is considered beyond the scope of the present discussion.

Glutamate is the main excitatory neurotransmitter in the brain, and it is involved in synaptic transmission and neuronal plasticity, neuronal development, outgrowth, and survival and in learning and memory. Under physiological conditions, neuronal mitochondria respond to glutamate by activating mitochondrial respiration (Clerc et al., 2013; Gleichmann et al., 2009; Jekabsons & Nicholls, 2004; Johnson-Cadwell et al., 2007; Llorente-Folch et al., 2016; Rueda et al., 2015; Yadava & Nicholls, 2007). This respiratory stimulation responds to the increased workload imposed by the rise in cytosolic Na^+ and Ca^{2+} that flow through NMDA, AMPA, and kainate receptors (KR) and voltage-gated Ca^{2+} channels, which rapidly activates Na^+ and Ca^{2+} extrusion mechanisms that consume ATP (Rueda et al., 2015; Yadava & Nicholls, 2007). In such a way, the increased workload is matched by an increase in respiration and ATP production to cope with the increased energy demand (Llorente-Folch et al., 2016). In physiological glutamate neurotransmission,

glutamate release is rapidly terminated, and glutamate removal mechanisms work efficiently to clear glutamate up from the synaptic cleft. The NMDA receptor-mediated Ca^{2+} influx is closely coupled to the generation of NO through the neuronal nitric oxide synthase (nNOS) activity, which acts as a secondary messenger involved in the control of physiological functions (Moncada & Bolanos, 2006).

In contrast to the aforementioned scenarios, the persistent presence of glutamate at synaptic and extrasynaptic sites leads to tonic activation of NMDAR, a situation in which mitochondria fail to counteract the stress induced by glutamate, resulting in a form of neuronal death termed excitotoxicity (Duchen, 2012; Mattson, 2003; Olney & Sharpe, 1969). Excitotoxic neuronal death is important in conditions such as epilepsy or stroke and even in neurodegenerative diseases such as Alzheimer's disease and PD (Brennan et al., 2009; Dong, Wang, & Qin, 2009; Hardingham & Bading, 2010; Mattson, 2003, 2008; Nicholls, 2008; Parsons & Raymond, 2014). The survival mechanisms of the cells are overridden by others governed by excessive Ca^{2+} load, ROS production, disruption of transmembrane ionic gradients, and limitation in energy substrates and ATP production.

It has long been appreciated that neurons are injured by large elevations in intracellular Ca^{2+} . There is now clear evidence that the entry and persistence of Ca^{2+} in mitochondria play a major role in glutamate excitotoxicity. Initially, this was inferred from the protective effects of uncouplers and other pharmacological approaches preventing mitochondrial Ca^{2+} uptake that protected neurons from death caused by glutamate (Stout et al., 1998). More recently the finding that silencing of MCU protects against glutamate excitotoxicity (Qiu et al., 2013) has given further support to the role of matrix Ca^{2+} in glutamate excitotoxicity. There is also evidence that zinc released from intracellular pools can also actively modulate the excitotoxic cascade (Sensi et al., 2009).

On the other side, ROS production at the onset of glutamate exposure is also involved in excitotoxicity. Activation of NMDARs is closely coupled to the generation of NO through nNOS, which is associated with the receptor through the postsynaptic density protein PSD-95, promoting RNS and downstream ROS production. Glutamate activation of NMDAR leads to a PI3K-mediated rise in NADPH oxidase 2 (NOX2) activity, with a consequent increase in ROS production (Baxter et al., 2014; Brennan-Minnella et al., 2015). Mitochondria are also involved in excitotoxic superoxide production (Duan et al., 2007), and there is evidence showing that ROS scavengers decrease excitotoxic neuronal death (Brennan-Minnella et al., 2013; Duan et al., 2007; Keelan

et al., 1999). Additionally nNOS inhibitors (Pramila et al., 2015) and PSD-95-interacting peptides that block its recruitment to NMDAR (Chen et al., 2015) and NOX2 inhibitors (Brennan-Minnella et al., 2013) all block excitotoxic neuronal death (for recent reviews see Brennan-Minnella et al., 2015; Lai et al., 2008). NO production has been suggested to play a role in respiratory inhibition at short times after glutamate stimulation by directly inhibiting cytochrome oxidase through competition of NO with O_2 (Laranjinha et al., 2012; Li et al., 2009). In this regard, the relation between ROS/RNS generation and Ca^{2+} overload in excitotoxicity remains unresolved.

In cortical and cerebellar granule neurons (CGNs), a glutamate-induced transient increase in cytosolic Ca^{2+} is followed by a delayed irreversible rise in cytosolic Ca^{2+} named delayed calcium deregulation (DCD) (Tymianski et al., 1993), which precedes neuronal death. DCD occurs at the time of a collapse in the mitochondrial membrane potential (Abramov & Duchen, 2008; Duchen, 2012) and is followed by an acidification of the mitochondrial matrix (Bolshakov et al., 2008; Li et al., 2009), suggesting the involvement of the PTP. Mitochondrial oxidative stress, or more precisely an increased level of superoxide, is an effect, rather than a cause, of DCD (Vesce et al., 2004), and it is presumably due to the leakage of electrons from complex I and III in the respiratory chain.

In the context of drug toxicity to neurons, the inhibition of electron transport and subsequent production of ROS may be one of the more pathophysiologically relevant events. Interestingly, concentrations of rotenone sufficient to stimulate ROS production by brain mitochondria are similar to those associated with Parkinson-like lesions in animal models, suggesting that mitochondrial ROS generation could be associated with the emergence of specific neurological disease phenotypes (Chaturvedi & Flint Beal, 2013). Moreover, mitochondrial Ca^{2+} overload and dysfunction, due to excessive Ca^{2+} entry through overactivated glutamate receptors, is a crucial early event in the excitotoxic cascade that follows ischemic or traumatic brain injury (for review see Murphy et al., 1999). From a therapeutic point of view of ischemia/stroke, interfering with the group of receptor-operated cation channels opened by glutamate might be interesting. However, clinical trials with agents targeting these receptors have been disappointing (Davis et al., 2000; Kalia et al., 2008; Lees et al., 2000). Thus, it is necessary to consider other routes of Ca^{2+} entry into cells in order to planning new therapies. Combination therapy, using blockers of several routes of Ca^{2+} influx, may be necessary to obtain neuroprotective effects (Sattler & Tymianski, 2001; Sattler et al., 1996).

The third component of the toxic triumvirate is energy depletion. As noted earlier, the brain is highly dependent on OXPHOS to support ion transport to maintain

normal neuronal activity. At a gross level, ischemic brain injury is the consequence of the interruption of supply of key substrates and mitochondrial ATP production.

Nicholls' and Mattson's groups (Gleichmann et al., 2009; Jekabsons & Nicholls, 2004) along with others (Clerc et al., 2013; Rueda et al., 2015) have consistently reported that in cultured brain neurons, the early glutamate/NMDA-induced increase in mitochondrial respiration is transient and drops rapidly in the continuous presence of glutamate/NMDA. The drop in respiration is highly sensitive to inhibitors of PARP-1, and in their presence glutamate/NMDA-induced increase in respiration is more sustained (Rueda et al., 2015), pointing at PARP-1 as cause of the drop in respiration. PARP-1 uses NAD^+ as substrate, and the regeneration of NAD^+ uses ATP; it was concluded that the cause of PARP-1 toxic effects was related to the drop in NAD^+ and ATP, resulting in energetic failure (Alano et al., 2010; Eliasson et al., 1997; Ying et al., 2001). Interestingly, a recent study demonstrated that exogenous NAD^+ can cross the plasma membrane and elevate mitochondrial NAD^+ levels in mammalian cells, causing significant enhancement in mitochondrial oxygen consumption and ATP production (Pittelli et al., 2011). Furthermore, recently, two different groups have reported a direct action of PAR polymers in mitochondria-associated hexokinase 1 (HK1), leading to an impairment of glycolysis and substrate supply to mitochondria with subsequent decrease in NAD^+ and ATP after PARP-1 activation (Andrabi et al., 2014; Fouquerel et al., 2014). Limitation in substrate supply caused by PAR polymers may be prevented by the provision of alternative substrates to mitochondria, such as pyruvate (Andrabi et al., 2014), which explains the partially protective effect of pyruvate on glutamate excitotoxicity (Ruiz et al., 1998). The effects of PARP-1 activation have been thoroughly discussed recently (Rueda et al., 2016).

As discussed earlier, in addition to the limitation in substrate supply to mitochondria caused by PARP-1 activation, excitotoxicity causes an irreversible dysfunction of mitochondria involving the PTP (Duchen, 2012; Nicholls, 2008). Involvement of the PTP in mitochondrial dysfunction due to glutamate excitotoxicity has considerable experimental support, but still leaves many open questions. Regarding the main topic of this chapter, one of these questions is the mechanism of the irreversible drop in mitochondrial respiration and mitochondrial dysfunction. It seems reasonable to assign it to the opening of the PTP, since that process, by allowing the release of metabolites and cofactors to the cytosol, would compromise respiration. On the other side, the results of Veas-Perez de Tudela et al. (2015) suggest that it could be due to an irreversible inhibition of mitochondrial complex I by ROS produced as a consequence of

ATP synthase inhibition through phosphorylation by Bcl-xL before PTP opening.

On the other hand, glutamate-induced energy depletion is associated with AMP protein kinase (AMPK) activation, a critical regulator of cellular function in response to energy stress within the cells (Hawley et al., 2003; Woods et al., 2003). AMPK activation may have a dual function in neurons promoting (i) cell survival during transient energy depletions (Culmsee et al., 2001; Weisova et al., 2009) or (ii) cell injury after prolonged AMPK activation (Li et al., 2007; McCullough et al., 2005), which implies that the duration and the level of AMPK activity within neurons after energetic stress is a pivotal factor in the decision between cell death and cell survival (reviewed in Weisova et al., 2011).

It has been shown that transient glutamate excitation in CGNs results in an AMPK-dependent increase in GLUT3 translocation to the cell surface and uptake of glucose, which alters mitochondrial bioenergetics in a Ca^{2+} -independent manner and enhances neuronal survival (Weisova et al., 2009).

On the contrary, prolonged AMPK activation due to cellular ATP depletion can lead to neuronal apoptosis via the transcriptional activation of a proapoptotic Bcl-2 family member, Bim (Concannon et al., 2010). Though regulation of bim expression is highly complex (Biswas et al., 2007), it has been demonstrated that bim activation and cell death induction requires a two-step FOXO3 activation by both AMPK-dependent FOXO3 nuclear translocation and direct FOXO3 phosphorylation by AMPK, representing a key step in excitotoxic neuronal death (Davila et al., 2012).

Within the past decade, studies have highlighted the impetus of mitochondrial dynamics in maintaining integral cell and animal physiological processes, influencing function and differentiation, and ultimately affecting survival (Chen & Chan, 2005, 2009). More specifically dysregulated mitochondrial fission and fusion dynamics have been shown to increase ROS, decrease ATP production, and alter apoptosis (Liesa et al. 2009) and mitophagy (Rambold et al., 2011; Shen et al., 2014). These deficits are also associated with numerous neurodegenerative disorders including PD, Alzheimer's disease, Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis, and HD (Aloboia et al., 2013; Korobova et al., 2013; Matic et al., 2015; Press & Milbrandt, 2008; Vohra et al., 2010; Wang et al., 2005; Wen et al. 2011).

More recent mechanisms to emerge from studies of mitochondrially mediated neuronal injury include alterations in mitochondrial trafficking and morphology. For example, agents that inhibit mitochondrial energy production including glutamate, oligomycin, and NO also acutely impair mitochondrial movement in neurons

(Brown, 1999; Rintoul et al., 2003, 2006; Zanelli et al., 2006). Mitochondrial trafficking is also impaired by toxic proteins including expanded polyQ huntingtin (Chang & Reynolds, 2006; Trushina et al., 2004) and by low but toxic concentrations of intracellular zinc (Malaiyandi et al., 2005).

Mitochondrial morphology might also be an important regulator of neuronal viability, given that mitochondrial fission appears to be associated with a greater vulnerability to cellular injury (Bossy-Wetzel et al., 2003; Brocard et al., 2003; Frank et al., 2001) and that toxins like glutamate decrease mitochondrial size (Rintoul et al., 2003).

However, there are also signals that regulate mitochondrial movement that are clearly not toxic to neurons, including growth factors (Chada & Hollenbeck, 2004), and signals that stop mitochondrial movement in the vicinity of synapses may be important to ensure correct localization of mitochondria at sites of high energy demand (reviewed by Chang & Reynolds, 2006; Schwarz, 2013). Nevertheless, the direct impact of impeding mitochondrial trafficking on neuronal health and mitochondrial physiology warrants further investigations.

9.5.2 Potential Manifestations of Drug-Induced Mitochondrial Dysfunction in the CNS

The previous section detailed some of the neuronal injury mechanisms that are associated with mitochondrial dysfunction. In many cases, these mechanisms have been established experimentally by the acute application of high concentrations of toxins in short-term experiments *in vitro*. It can be challenging to extrapolate from these findings to account for potential effects of mitochondrial toxins that are the result of long-term exposures of low concentrations of toxins *in vivo*. However, assuming that drug-mediated neuronal injury will be the consequence of relatively modest and potentially long-term exposures, it is possible to identify scenarios that could be indicative of the effects of a mitochondrial toxin in the CNS.

Effects on a selectively vulnerable neuronal population: the experiments that expose rats to systemic low doses of rotenone and produce a Parkinson-like syndrome (Betarbet et al., 2000) raise the possibility that toxin exposure at relatively low concentrations at a mitochondrial target within a selectively vulnerable neuronal population could promote neuronal loss and trigger a disease like PD. The basis for selective cell vulnerability is rarely understood, although selective vulnerability is implied in most of the established examples of drug toxicity described in this chapter. Understanding the primary target for this form of injury and the dose and time

relationships between drug exposure and injury would allow the prediction of toxicity in this condition.

Additivity with other toxic burdens: as already documented, relatively modest inhibition of mitochondrial function will amplify neuronal injury triggered by other mechanisms. Perhaps the most obvious scenario would be the addition of a low-level drug-mediated impairment of mitochondrial function with a second form of injury such as a stroke. The consequence in this case would be a worse outcome ("double hit"). This would be a more difficult form of drug effect to study, because the conclusions would also be dependent on the choice of injury mechanism. It would also be difficult to detect following drug exposure in humans due to the limited number of comparable injury-producing events in drug-taking patients.

Slowly accumulated injury: a surprising observation is the time that is sometimes required to see expression of injury following mitochondrial impairment. Examples of slowly developing injury that is unequivocally attributable to altered mitochondrial function include the mitochondrial late-onset degeneration (MILON) mouse that requires several months following interruption of mtDNA replication and gene expression in the forebrain prior to manifestations of injury (Sorensen et al., 2001) and the MitoPark mouse in which dopaminergic neurons die over a year after the elimination of mitochondrial transcription factor A (Ekstrand et al., 2007). Injury that requires long periods (perhaps several years) of low-level drug exposure prior to development of a phenotype is very difficult to detect in preclinical models and typically requires extrapolation from the consequences of substantially higher drug doses.

These mechanisms are clearly not mutually exclusive, and an anticipated paradigm would reasonably reflect both the slow accumulation of injury and selective tissue vulnerability. Individual pharmacogenomic variation could also be important through an impact on drug absorption, metabolism, or excretion that changes either the drug exposure levels or the formation of active metabolites. The consequence of this combination of variables could plausibly be CNS injury that can only be detected with careful retrospective analysis of data from very large databases of reported adverse reactions, such as a recent report of an amyotrophic lateral sclerosis-like syndrome in patients taking statins (Edwards, Star, & Kiuru, 2007).

9.6 Conclusion

This chapter has documented several relatively well-established cases where drug adverse effects are likely to be caused by toxic effects on mitochondrial function.

The manifestations of these established toxic effects are largely impairments of sensory or motor function and arise from inhibition of mtDNA replication or transcription and translation of the mitochondrial genome. While the mechanisms that link drug action to injury are compelling, it is rare that the basis for the selectivity of the injury is completely understood. In addition, there are numerous cases where drugs have measurable effects on

electron transport and ATP generation. Several inhibitors of this type are known to produce selective neuronal injury. A further understanding of the consequences of modest mitochondrial impairment as well as the mechanisms underlying the injury produced by known neurotoxins should provide important insights that will refine predictive approaches to understanding drug toxicity in the nervous system.

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10

Nephrotoxicity: Increasing Evidence for a Key Role of Mitochondrial Injury and Dysfunction and Therapeutic Implications

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CHAPTER MENU

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10.1 Scope of the Problem

Drug- and toxin-induced nephropathies are common, numerous, and frequently underdiagnosed. Drugs may cause hemodynamic actions, leading to renal ischemia, or may be directly toxic to renal cells, leading to lethal or sublethal injury, mainly of tubular and endothelial cells. Several nephrotoxic drugs have been reported to damage mitochondria. A common manifestation of nephrotoxicity is acute kidney injury (AKI). It is estimated that a quarter of all episodes of AKI result from drug-induced nephrotoxicity (Fillastre and Godin, 1998). AKI may fully recover upon withdrawal of the drug. However, it may evolve into chronic kidney disease requiring renal replacement therapy, either as a result of continuing exposure to the drug or as a result of a severe acute insult that interferes with recovery mechanisms.

Drug-induced tubular cell death (Ortiz et al., 2003) may cause AKI of the acute tubular necrosis type. However, the term acute tubular necrosis precedes the coinage of the term apoptosis, and it does not indicate a role for a specific form of cell death (Ortiz et al., 2003).

Tubular cell death can proceed through apoptosis, necrosis, or diverse forms of regulated necrosis. However, in humans it is often difficult to determine the

predominant mode of cell death, because histological sections can only recognize the absence of tubular cells. The relative contribution of the different cell death mechanisms to the initial tubular cell loss depends on the cause and severity of the insult.

Additional mechanisms of drug-induced renal injury include idiosyncratic immune-mediated renal injury, leading to acute tubulointerstitial nephritis, and intratubular precipitation of the drug or its metabolites, leading to crystalluria, nephrolithiasis, and obstructive renal disease. Nephrotoxic drugs may directly cause mitochondrial injury or inflammation, which the latter can further damage mitochondria (Ruiz-Andres et al., 2015; Tang and Dong, 2016).

10.2 Peculiarities of Tubular Cells

Daily function of the kidneys implies the glomerular production of 150–200 L of a plasma ultrafiltrate essentially free of proteins. Tubular cells modify the composition of ultrafiltrate by reabsorbing the bulk of it and by secreting certain molecules, such as organic anions and numerous drugs. Proximal tubular cells are the key contributors to transport processes and are rich in mitochondria, which

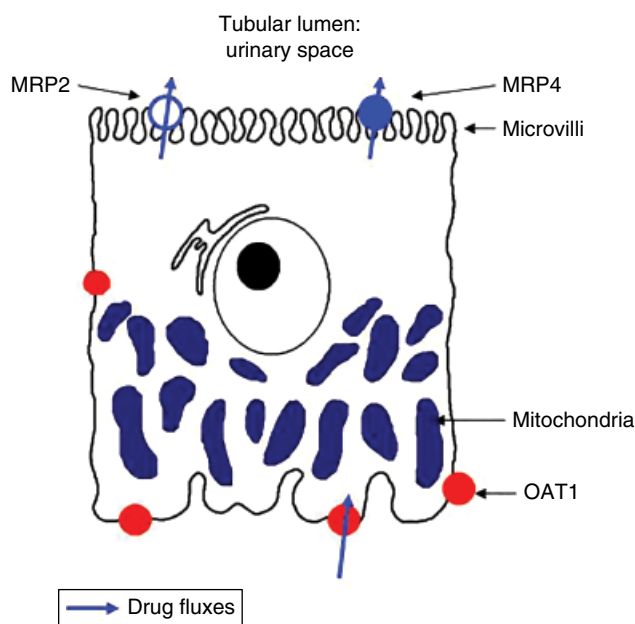


Figure 10.1 Schematic representation of the proximal tubular cell. Note the increased cell surface both at the luminal (apical, created by microvilli) and basolateral (membrane invaginations) aspects in order to increase the transport capacity. Different transporter molecules that facilitate antiviral drug fluxes and nephrotoxicity are indicated. A great number of mitochondria provide the energy required for such an intense transmembrane transport. MRP, multidrug-resistant protein; OAT, organic acid transporter. Courtesy of Alejandro Ortiz.

provide the energy source for transport, and in cell membrane transporter molecules (Figure 10.1). The key ATP-consuming transporter is the Na^+/K^+ ATPase in the basolateral membrane that creates an ionic gradient that is then used to drive transport of very diverse molecules in the luminal membrane. Cell membrane transporters contributing to active drug secretion into urine result in higher intracellular concentrations of potentially cytotoxic drugs in proximal tubular cells than in other organs, as it is the case for certain antiviral drugs. The combination of a high number of mitochondria, high cellular energy requirements, and high intracellular concentrations of potentially nephrotoxic drugs and compounds facilitates proximal tubular cell-specific toxicity.

10.3 Nephrotoxicity and Mitochondria

Renal mitochondria are involved in substrate oxidation, ATP generation, cellular iron homeostasis, and cellular calcium detoxification. In addition, specific parts of the nephron have specific mitochondrial functions. As an example, 1α hydroxylase in proximal tubule mitochondria activates 25 dihydroxycholecalciferol to calcitriol, the active metabolite of vitamin D. These mitochondria

also release the ammonia required by distal segments to secrete protons into the urine. This cornerstone position of mitochondria in the intermediate renal metabolism explains their contribution to nephrotoxicity induction, mediation, or protection. Mitochondrial biogenesis and dynamics require the cooperation of nuclear and mitochondrial genes. Mitochondrial fission and fusion are tightly regulated by dynamin-related protein (Drp1) and mitochondrial fission-1 (Fis-1) for fission and mitofusin 1 and 2 (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) for fusion.

10.3.1 Respiratory Chain: Reactive Oxygen Species (ROS) Formation

Small amounts of electrons leak from the respiratory chain at the NADH ubiquinone reductase and the ubiquinone cytochrome *c* reductase levels are trapped by oxygen and form soluble oxygen radicals (www.cyberlipid.org/perox/oxid0003.htm, access January 20, 2007). The radical superoxide produced will be reduced to form H_2O_2 . The physiological production of H_2O_2 is quite important, leading to a cellular concentration of 10^{-9} to 10^{-7} M. The activity of the respiratory chain is the rate-limiting factor for the generation of free radicals. It is usually regulated by mitochondrial ADP availability, which in turn depends on cellular ATP consumption: increased ATP consumption increases ROS production. Under certain circumstances, ADP availability does not regulate mitochondrial oxidation. This circumstance, known as mitochondrial uncoupling, can be observed in the presence of certain nephrotoxins such as nitrophenols or in the presence of very high intracellular calcium levels. When mitochondrial uncoupling takes place, oxidation increases, but ATP production will decrease.

The oxidative chain is also regulated by local production of nitric oxide (NO). The mitochondrial isoform of nNOS (mtNOS) (Kanai et al., 2004) has specific post-translational modifications. Under physiological conditions and with low NO concentrations (<100 nM), it inhibits cytochrome *c* oxidase activity, decreasing the respiratory chain rate (Poderoso et al., 1996). Under those circumstances, the NOS inhibitor L-NAME increases mitochondrial ROS production. At higher concentrations of NO, free radical production increases. NO reaction with ROS generates nitrogen radicals, that is, peroxynitrites that damage mitochondria and may trigger apoptosis (Navarro-Antolín et al., 2007).

10.3.2 ATP Generation

Respiratory chain redox reactions pump protons outside the mitochondrial matrix, generating a resulting chemiosmotic gradient that allows ATP synthesis by the ATP synthase when this enzyme pumps protons back into the

relatively alkaline mitochondrial matrix. Synthesis of ATP requires ADP availability. ADP enters the mitochondria by a nucleotide translocase (ANT), which exchanges neo-synthesized mitochondrial ATP for cytosolic ADP. The translocase oscillates between two conformations: c (cytoplasmic) and m (mitochondrial). Complete and systemic inhibition of ATP synthesis generally causes acute hepatic and renal necrosis and is often lethal. Under these circumstances, the cell cannot maintain its sodium and calcium gradients and necrosis occurs. We must emphasize that apoptosis is an active process that requires a supply of energy. When this supply is not enough or is diverted to other more demanding processes during toxic aggression, ATP cell levels fall and morphological signs of necrosis usually appear.

10.3.3 Cellular Iron Homeostasis

Mitochondria are key regulators of iron metabolism. Iron is a cofactor for enzymes involved in many metabolic processes but may be harmful by enhancing the production of ROS and contributes to ferroptosis, a recently described form of cell death (original description in (Dixon et al., 2012)). Iron is transformed into its biologically available form in the mitochondria by the iron-sulfur (Fe/S) cluster and heme synthesis pathways (Levi and Rovida, 2009). The need for iron-containing proteins and the key role of mitochondria in iron homeostasis may sensitize tubular cells to nephrotoxicity by iron chelators such as deferasirox, discussed in the following.

10.3.4 Calcium Detoxification by Mitochondria: Role of Mitochondrial Permeability Transition Pore (MPT)

The chemiosmotic gradient created by the respiratory chain may also be used by mitochondria to import calcium, as long as its cytosolic concentration is high enough. Calcium enters mitochondria through a voltage-dependent uniporter (VDAC). The elevation of intramitochondrial-free calcium (mtCa^{++}) must be quickly reduced, since elevated mtCa^{++} may irreversibly combine with ATP. Although mitochondria may exchange mtCa^{++} for 2Na^+ or 2H^+ , this does not occur when mitochondria are acting as a cytosolic calcium scavenger. Increased levels of mtCa^{++} induce the assembly and transient opening of the MPT by interaction of the “c” form of the nucleotide translocase (cANT) with the calcium transporter (VDAC). Cyclophilin D (CyD) interacts with cANT, facilitating pore assembly (Vyssokikh et al., 2001).

The formation of the MPT pore implies the creation of an anchoring point between the inner and the outer mitochondrial membrane, allowing the entry of

water and other components in favor of the osmotic mitochondrial gradient.

In the absence of ADP, opening of the pore increases the mitochondrial volume. In the presence of an alkaline pH, phosphate entry through the pore will chelate mtCa^{++} . Reduction in free mtCa^{++} will disassemble the MPT, permeability return to normal values, and VDAC and ANT transporters to their normal function.

Under excess of ADP, ANT works as a translocase and it is not possible to assemble the MPT pore. However, once the pore is formed, neither VDAC nor ANT transports Ca^{++} or nucleotide, respectively, unless excess ADP becomes available.

ROS may modify MPT pore function. ROS increase VDAC permeability and may allow early leaks of cytochrome *c* even before nonreversible opening of MPT pore. However, superoxide anion may open the MPT pore independently of mtCa^{++} . MPT pore opening must be transient. While the MPT pore is opened, membrane potential is clamped. Thus, it is critical that mtCa^{++} concentration is not excessive. High mtCa results in long-lasting opening of the MPT pore and turns the physiologic mechanism for temporary detoxification of Ca^{++} into a lethal agent, causing mitochondria depolarization and leak of cytochrome *c* and other mediators of apoptosis.

Besides excess calcium, other agents able to open the MPT pore in an irreversible way include nephrotoxic agents such as high concentrations of NO, cisplatin, CsA, tacrolimus, and vancomycin.

10.4 Evidence of Mitochondrial Injury in Nephrotoxicity

Evidence of mitochondrial injury in nephrotoxicity derives from the observation of morphological changes, altered mitochondrial function and gene expression, and functional intervention studies in preclinical models.

10.4.1 Morphological Changes

These are usually more prominent in proximal tubules and have been observed in AKI induced by cisplatin, gentamicin, aristolochic acid (an herbal nephrotoxin linked to Balkan nephropathy and medicinal herbs nephropathy), and the antiretroviral drugs tenofovir and adefovir (Hall et al., 2013; Jiang et al., 2013; Mukhopadhyay et al., 2012). Morphological abnormalities included swelling and dysmorphia, with loss and disorientation of cristae, an apparent decrease in mitochondrial quantity, mitochondrial fragmentation, and high variability in mitochondria size and shape (Brooks et al., 2009; Herlitz et al., 2010; Jiang et al., 2013; Tanji et al., 2001; Zsengellér et al., 2012).

10.4.2 Mitochondrial Dysfunction

Mitochondrial dysfunction has been observed in AKI induced by nephrotoxic drugs (Abraham et al., 2013; Hall et al., 2013; Kohler et al., 2009). Changes include reduced kidney ATP, mitochondrial membrane potential, COX enzyme activity, and respiratory complex I activity (Brooks et al., 2009; Hall et al., 2013; Jiang et al., 2013; Kohler et al., 2009; Tanji et al., 2001; Zsengellér et al., 2012).

10.4.3 Mitochondrial Gene Expression

Peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) associates with nuclear respiratory factors (NRFs) (Wu et al., 1999) and mitochondrial transcription factor A (Tfam) to promote transcription of nuclear genes encoding mitochondrial proteins (reviewed in Ruiz-Andres et al. (2016b)). Downregulation of PGC-1 α and/or mitochondria-related target genes has been observed in preclinical models of nephrotoxicity, including AKI induced by folic acid or cisplatin (Portilla et al., 2002; Ruiz-Andres et al., 2015; Stallons et al., 2014). While nephrotoxic drugs may directly decrease PGC-1 α expression in cultured tubular cells, as exemplified by cisplatin (Portilla et al., 2002), *in vivo* recruitment of inflammation by injured tubular cells may further compromise PGC-1 α expression and mitochondrial biogenesis through NF- κ B activation and epigenetic mechanisms (Ruiz-Andres et al., 2015, 2016a). Expression of genes regulating mitochondrial dynamics may also be altered. Thus, mitochondrial fission protein Drp1 is upregulated in both animals and cell culture models of cisplatin nephrotoxicity, while Mfn1- or Mfn2-null cells are more sensitive to cisplatin-induced apoptosis (Brooks et al., 2011).

10.5 Calcineurin Inhibitor Nephrotoxicity

CsA and tacrolimus (FK506) are the two calcineurin inhibitors currently used as immunosuppressants. Nephrotoxicity is dose limiting (Caramelo et al., 2004; Shihab, 1996). Tubular and vascular renal damage and hypertension are among the most relevant side effects. Long-term calcineurin inhibitor therapy was associated with a 10- to 5-fold increase in mean mitochondrial injury grade in endothelial cells and proximal tubular cells, respectively, obtained from human renal biopsies (Singh et al., 2015).

CsA causes acute and chronic kidney disease. The occurrence of end-stage renal disease (ESRD) requiring chronic dialysis has been reported in up to 20% of

nonrenal transplant recipients. The use of calcineurin inhibitors is a risk factor (Stratta et al., 2005). Acutely, CsA induces reversible renal vasoconstriction (Caramelo et al., 2004; Shihab, 1996). The acute hemodynamic effect of CsA is mediated by an imbalance of vasoconstrictors and vasodilators. Chronic nephrotoxicity is characterized by tubular atrophy, loss of tubular cells, obliterative arteriolopathy, and interstitial fibrosis (reviewed in Mihatsch et al. (1988)). Histopathological studies have suggested a toxic effect of the drug on afferent arterioles and tubular epithelial cells. In vessels, vascular smooth muscle cells were initially thought to be the main CsA target, but endothelial cell toxicity appears to be more relevant (Alvarez-Arroyo et al., 2002; Gallego et al., 1994; Navarro-Antolín et al., 2001, 2007). Widespread damage to the peritubular capillaries is deemed to be critical in both acute and chronic CsA nephropathy. CsA also causes tubular epithelial cell death, leading to tubular atrophy. CsA may also cause epithelial–mesenchymal transition in cultured cells (Berzal et al., 2012) (Figure 10.2). This could be relevant for the conspicuous interstitial fibrosis characteristic of CsA nephrotoxicity.

In lymphocytes, CsA and tacrolimus bind the cytosolic peptidylprolyl *cis/trans* isomerases (PPIases), designated as cyclophilin and FK-BP, respectively. The resulting CsA–cyclophilin—or tacrolimus–FK-BP—complex binds to and inhibits a calcium/calmodulin-dependent type 2B protein phosphatase, calcineurin (Caramelo et al., 2004). Calcineurin regulates the dephosphorylation and nuclear import of the transcription nuclear factor of activated T cells (NFAT). This mechanism may be active in other cells types, since CsA inhibits NFAT–DNA binding in endothelial cells (Alvarez-Arroyo et al., 2002). Even though most of the toxic effects appear to be mediated by calcineurin inhibition, CsA binds to several cyclophilins.

The precise downstream sequence of toxic signaling has not yet been completely clarified, but the role of ROS and peroxynitrite appears to be critical (Caramelo et al., 2004; Navarro-Antolín et al., 2007). Other intracellular targets of CsA include mitochondrial respiration, cellular calcium signaling, protein kinase C, and protein synthesis. However, the significance of these intracellular effects for CsA nephrotoxicity remains to be demonstrated.

10.5.1 Calcineurin Inhibitors: Mitochondrial Dysfunction

Early magnetic resonance spectroscopy studies indicated that the renal toxicity of calcineurin inhibitors implies mitochondrial failure (Ruiz-Cabello et al., 1994). This technique also showed that renal mitochondria are more

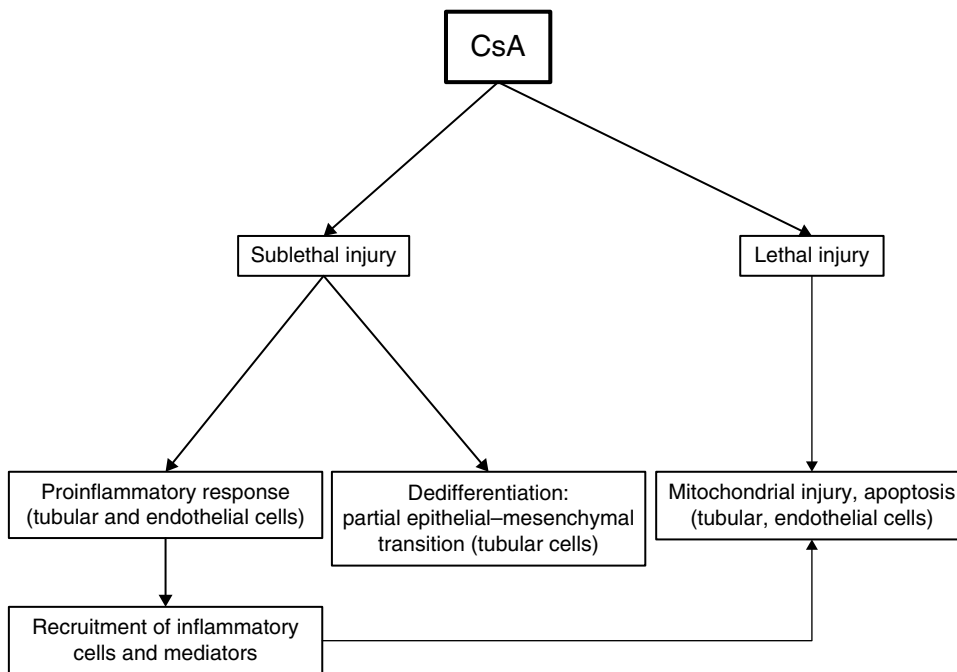


Figure 10.2 Anti-calcineurin nephrotoxicity: the example of cyclosporine A (CsA).

sensitive to injury by CsA than those from other organs in the living animal (Serkova et al., 2003). CsA causes direct, acute, and dose-dependent toxicity on proximal tubule mitochondria. CsA decreases constitutive NO synthesis as well as induced NO synthesis by proximal tubule cells, resulting in increased H_2O_2 production (Hortelano et al., 2000).

The main effect of CsA on proximal tubule mitochondria is the inhibition of cyclophilin D (CyD) Ca^{++} activation by the formation of a CsA–CyD complex. CyD like ANT and VDAC is part of the MPT pore that is responsible for the loss of mitochondrial membrane potential. CsA binding to CyD prevents its binding to the ANT and MPT pore activation (Nakagawa et al., 2005). Thus, in the presence of CsA, mtCa^{++} cannot open the MPT pore. In short-term experiments and at low doses, this effect may protect against other nephrotoxic agents whose toxicity is mediated by a direct and long-lasting opening of the MPT pore and explains some of the contradictory observations about the protective effect of CsA on different cell types and under different circumstances (Alvarez Arroyo et al., 2002; Hortelano et al., 2000). In long-term experiments and at higher doses, the lack of MPT pore assembly leads to higher levels of mtCa^{++} while maintaining oxidative phosphorylation. The progressive increase in mtCa^{++} levels finally overwhelms CsA binding to CyD. Under these circumstances, MPT pore opening lasts longer and no reversion is observed (Hortelano et al., 2000), mitochondrial potential is clamped, and cytochrome *c* and other proactivators of apoptosis leak

to the cytosol. The initial inhibition of the MPT pore assembly induced by CsA is also observed with CsA analogues able to bind cyclophilin but with no anti-calcineurin activity, such as MeVal-4-CsA and Melle-4-CsA (Alvarez-Arroyo et al., 2002).

10.5.2 Apoptosis in CsA Nephrotoxicity

An increased rate of tubular cell apoptosis was observed in humans and animals with CsA-induced nephrotoxicity (Hortelano et al., 1999; Shihab, 1996). In addition, CsA and tacrolimus induce apoptosis in cultured tubular cells in a dose- and time-dependent manner (Amore et al., 2000; Healy et al., 1998; Hortelano et al., 2000; Justo et al., 2003; Ortiz et al., 1998). Initially, it was suggested that death receptors might be involved in apoptosis induced by CsA. CsA increases Fas expression in cultured tubular cells, and increased FasL and Fas expression has been reported in chronic CsA nephrotoxicity (Justo et al., 2003; Yang et al., 2002). However, neutralizing anti-FasL antibodies did not decrease apoptosis induced by CsA, and CsA did not sensitize cells to death induced by FasL (Justo et al., 2003). In addition, no activation of caspase-8 was detected, and the caspase-8 inhibitor IETD did not prevent CsA-induced apoptosis (Justo et al., 2003). Caspase-12 was not processed, arguing against involvement of the endoplasmic reticulum (Justo et al., 2003). By contrast, caspase-12 was processed in tubular cells exposed to another nephrotoxin and acetaminophen, indicating heterogeneity in the

mechanisms of activation of lethal pathways in nephrotoxicity (Lorz et al., 2004).

Mitochondrial injury is a key event in CsA-induced tubular cell apoptosis (Hortelano et al., 2000; Justo et al., 2003). CsA-induced Bax translocation and oligomerization lead to outer mitochondrial membrane permeabilization and release of cytochrome *c* and Smac/Diablo (Justo et al., 2003), which results in the loss of the mitochondrial membrane potential. Bax is a critical mediator of mitochondrial injury induced by CsA in tubular cells and Bax antisense oligodeoxynucleotides protected from CsA-induced apoptosis and cell death (Justo et al., 2003).

Caspases also participate in CsA nephrotoxicity. Caspase-2, caspase-9, and caspase-3 are activated (Justo et al., 2003). Initiator caspase-2 and caspase-9 are activated upstream of caspase-3, but the hierarchy between them in CsA-induced apoptosis is unclear. Since caspase-2 neither processes nor activates executioner caspases directly (Guo et al., 2002), caspase-2 is likely the first step starting the molecular cascade. The pan-caspase inhibitor zVAD did not prevent Bax translocation or cytochrome *c* release, thus placing these events upstream of activation of most caspases. As zVAD does not inhibit caspase-2 (Lassus et al., 2002), a role for caspase-2 in promoting cytochrome *c* release was not explored (Justo et al., 2003). In other models of cell death, caspase-2 may induce apoptosis via cleavage of Bid and the subsequent engagement of the mitochondrial pathway (Bonzon et al., 2006). The pan-caspase inhibitor zVAD did not prevent the release of cytochrome *c* in response to CsA, but did prevent the loss of mitochondrial membrane potential (Justo et al., 2003).

Specific inhibition of any of the three caspases prevented apoptosis and prolonged cell survival in long-term (7 days) *in vitro* assays (Justo et al., 2003). The increase in long-term cell survival indicates that caspase inhibitors rescue cells from apoptosis and other forms of cell death. In this regard, certain forms of apoptotic tubular cell death are not prevented by caspase inhibition; rather, caspase inhibition induces a shift from apoptosis to a form of necrosis recently characterized as necroptosis (Justo et al., 2006; Linkermann et al., 2013; Lorz et al., 2005).

In summary, CsA causes MPT-independent mitochondrial outer membrane permeabilization mediated by Bax (with the role of caspase-2 not fully characterized), leading to cytochrome *c* release, which, in turn, activates caspases that further damage the mitochondria and lead to the loss of mitochondrial transmembrane potential (Figure 10.3). The feedback loop is essential for apoptosis and cell death to proceed. This is one of several models for the participation of mitochondrial injury in apoptosis (Green and Kroemer, 2004). This model is consistent with the known effects of CsA on mitochondria via binding to

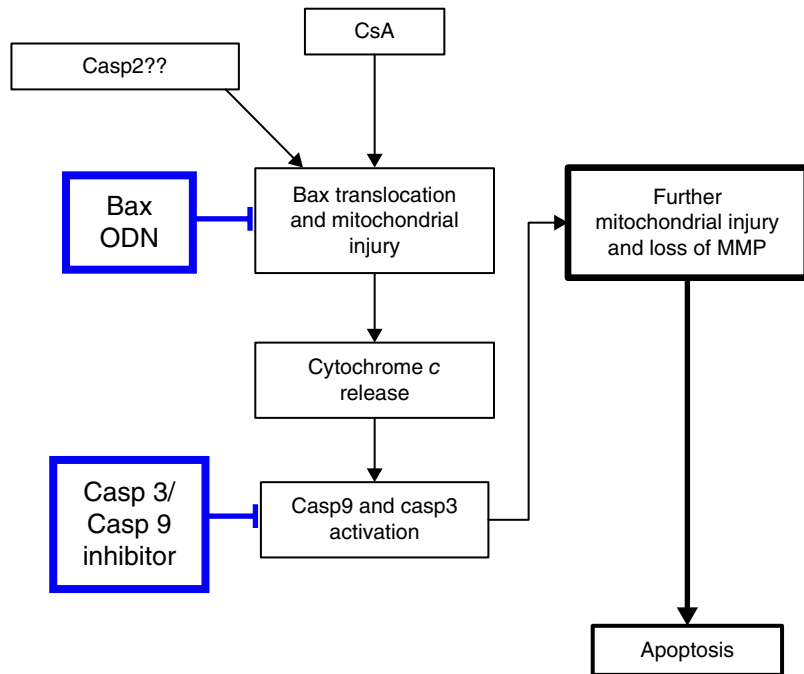
CyD and is not shared by other nephrotoxins, such as acetaminophen. Although not specifically studied in CsA-induced apoptosis, caspases may cause mitochondrial respiratory dysfunction. Caspase-3 cleaves components of the electron transport chain, leading to loss of mitochondrial transmembrane potential and increased production of ROS (Ricci et al., 2004). This results in lipid peroxidation and organelle swelling that might then further facilitate the dissociation and release of apoptosis-inducing factor (AIF) and others from the intermitochondrial membrane space (Breckenridge and Xue, 2004).

In cultured tubular cells, toxic concentrations of CsA increased the expression of Drp1 and decreased Mfn2 and Opa1 (de Arriba et al., 2013). Furthermore, in proximal tubular cells, CsA decreased the electron transfer flavoprotein- α subunit, which accepts electrons from dehydrogenases and transfers them to the respiratory chain and also of prohibitin, an IMM protein involved in the assembly of mitochondrial respiratory chain enzymes (Puigmulé et al., 2009).

Induction of apoptosis in tubular cells contrasts with the fact that CsA is a potent inhibitor of various forms of apoptosis. Several potential mechanisms of action may account for this effect. CsA binds several cyclophilin family members and other proteins, which could be involved in its pro- and antiapoptotic actions. Indeed, CsA and FK506 protect macrophages against apoptosis through the inhibition of the expression of iNOS (Alvarez Arroyo et al., 2002). By contrast to macrophages, CsA/FK506 and NO exert a synergistic proapoptotic action on tubular epithelial cells, probably through an increased release of mitochondrial apoptotic mediators (Hortelano et al., 2000). This would be deleterious under conditions of renal inflammation such as the immediate posttransplant period.

In cultured endothelial cells, CsA was cytotoxic/proapoptotic or cytoprotective/antiapoptotic at high or low concentrations, respectively (Caramelo et al., 2004). The response to CsA in endothelial cells depends on the interaction of CsA with cyclophilin rather than with calcineurin. Proximal tubules and endothelial cells adapt to the presence of CsA, minimizing the expected damage. Chronic CsA treatment increases mitochondrial concentration of CyD, as well as synthesis of chaperon molecules involved in CyD exportation to mitochondria (HsP70). Inhibition of HsP70 with geldanamycin increases endothelial toxicity of CsA. VEGF is also a critical factor in the cytoprotective effect of CsA (Alvarez-Arroyo et al., 2002). Endogenous VEGF protects renal tubular cells against CsA toxicity in cell culture and *in vivo* (Slattery et al., 2005). Cilastatin protected against CsA-induced apoptosis by inhibiting CsA transport across membranes and reducing CsA in mitochondria

Figure 10.3 Intracellular pathways for CsA-induced renal tubular cell apoptosis.



(Pérez et al., 2004). Cilastatin is an inhibitor of brush border dipeptidases in clinical use, which decreases the renal degradation, transport, and toxicity of imipenem in proximal tubules. Clinical and experimental studies have reported that administration of the antibiotic imipenem/cilastatin reduces CsA-associated nephrotoxicity as well as nephrotoxicity of vancomycin, cisplatin, and FK-506 (reviewed in Pérez et al. (2004)). The basis for such broad protection was related to decreased intracellular accumulation of nephrotoxic drugs (Camano et al., 2010; Moreno-Gordaliza et al., 2011; Pérez et al., 2004).

Recent attention has also been drawn to the recruitment of inflammatory cells and mediators in anti-calcineurin nephrotoxicity target cells, such as endothelial and tubular cells exposed to toxic concentration of CsA and tacrolimus (González-Guerrero et al., 2013, 2016; Rodríguez-Díez et al., 2016). Inflammation can promote mitochondrial dysfunction by downregulating PGC-1 α expression and expression of its downstream targets in mitochondria (Ruiz-Andres et al., 2015, 2016a, b). This effect appears to be mediated at least in part by epigenetic mechanism such as histone crotonylation and acetylation (Ruiz-Andres et al., 2015, 2016a).

10.6 HAART and Nephrotoxicity

Highly active antiretroviral therapy (HAART) for the treatment of HIV infection was introduced two decades ago. It targets multiple critical steps in the life cycle of

HIV. It usually includes two nucleoside analogue HIV reverse transcriptase inhibitors and at least one HIV protease inhibitor or non-nucleoside inhibitor of HIV reverse transcriptase. The reverse transcriptase inhibitor prevents transcription of viral RNA to proviral DNA, and the protease inhibitor prevents the cleavage of viral precursor polypeptides. Renal damage caused by antiretroviral drugs can result in a variety of toxic effects presenting as acute renal failure, tubular dysfunction, kidney stones, or chronic renal disease (Campos et al., 2016). An incidence of 5.9 cases of acute renal failure per 100 patient-years has been reported (Campos et al., 2016). Long-term survival favors an increase in HAART-induced metabolic alterations, including elevations in serum lipid levels, diabetes, and hypertension, which are likely to be associated with an increase in secondary renal damage, such as hypertensive nephrosclerosis and diabetic glomerulopathy.

Acyclic nucleotide phosphonates (adefovir, cidofovir, and tenofovir) are eliminated predominantly into the urine. Renal failure is their dose-limiting toxicity. They induce proximal tubular cell toxicity that may lead to Fanconi syndrome (glycosuria, tubular proteinuria, inappropriate phosphaturia, aminoaciduria, and bicarbonaturia, alone or in combination) and acute renal failure with a histological substrate of acute tubular necrosis (Lalezari et al., 1997; Ortiz et al., 2005; Tanji et al., 2001; Vandercam et al., 1999; Verhelst et al., 2002). The high incidence of toxicity prompted the discontinuation of clinical trials for adefovir in the treatment of HIV infection.

Tenofovir was initially reported to be non-nephrotoxic in clinical trials (Schooley et al., 2002) and in cultured proximal tubular epithelial cells (Cihlar et al., 2002). However, cases of tenofovir-induced acute renal failure have been published (Verhelst et al., 2002), and, as recently reviewed (Fernandez-Fernandez et al., 2011), nephrotoxicity has emerged as the main adverse effect of tenofovir. These patients frequently presented risk factors such as preexisting chronic renal disease, cirrhosis, or the use of potentially nephrotoxic drugs. Renal function usually recovers following drug withdrawal (Verhelst et al., 2002). The combination of ritonavir and tenofovir may potentiate nephrotoxicity.

Cidofovir has activity against a wide array of DNA viruses, including poxvirus (smallpox) and cytomegalovirus (CMV). HIV patients may use this latter indication. Nephrotoxicity is a dose-limiting side effect (Lalezari et al., 1997). Cidofovir nephrotoxicity is mainly observed in proximal tubular epithelium and leads to Fanconi syndrome and acute renal failure, which may be irreversible (Ortiz et al., 2005). Treatment with cidofovir requires the routine use of prophylactic measures to prevent nephrotoxicity. These include hydration, the use of probenecid, and the avoidance of other nephrotoxic agents. Renal dysfunction peaks one week following cidofovir administration (Vandercam et al., 1999). Renal toxicity associated with the use of nucleoside reverse transcriptase inhibitors is uncommon, although they may potentiate the toxicity of nucleotides (Murphy et al., 2003). However, two protease inhibitors, indinavir and ritonavir, have been associated with nephrotoxicity (Röling et al., 2006; Valle and Haragsim, 2006). Indinavir nephrotoxicity is related to crystalluria (Röling et al., 2006; Tashima et al., 1997). Case reports have linked ritonavir use to reversible renal failure (Chugh et al., 1997). The majority of reported patients had received concomitant medication with potentially nephrotoxic drugs (such as tenofovir or indinavir) or had other underlying renal pathology. Ritonavir nephrotoxicity usually occurs early, following introduction of the drug (3–21 days) (Table 10.1).

In addition to different antiretroviral drugs, which may interact, HIV patients are frequently exposed to other potentially nephrotoxic drugs that include acyclovir, aminoglycosides, amphotericin, foscarnet, pentamidine, sulfadiazine, and trimethoprim–sulfamethoxazole (de Sequera et al., 1996). In this regard, incubation of cells with associations of tenofovir with other antiretroviral drugs increased toxicity (Vidal et al., 2006). As a concluding remark, in addition to the putative toxic potentiality of each individual drug, the complex array of interactions between the multiple drugs involved creates new sources of toxicity in the fast-changing scenario of HIV therapy.

Table 10.1 HAART drugs and nephrotoxicity.

Drug family	Drug	Nephrotoxic effect
Acyclic nucleotide phosphonates (transcriptase inhibitors)	Adefovir ^a	Proximal tubular cell toxicity:
	Tenofovir ^b	Fanconi syndrome, acute
	Cidofovir ^c	renal failure (acute tubular necrosis)
Protease inhibitors	Indinavir	Crystalluria and nephrolithiasis may lead to acute renal failure and chronic kidney disease
	Ritonavir ^b	Case reports of acute renal failure (acute tubular necrosis)

Nucleoside transcriptase inhibitors are generally safe, although there are case reports of tubular dysfunction with didanosine and lamivudine–stavudine. Non-nucleoside transcriptase inhibitors and fusion inhibitors are generally safe.

^a No longer used because of nephrotoxicity.

^b Displays nephrotoxicity mainly when in combination with other potentially nephrotoxic drugs.

^c Used for treatment of CMV infection in HIV patients.

10.6.1 The Transporters

A critical aspect of proximal tubular epithelial cell toxicity is drug fluxes in and out of the cell (Figure 10.1). Intracellular drug accumulation as a consequence of such fluxes is involved in the specific cytotoxicity of certain drugs against these cells. Tubular secretion of the drugs requires first uptake into the cell and then secretion to the tubular lumen. The renal organic anion transporter 1 (OAT1) in the basolateral membrane mediates the uptake of tenofovir and cidofovir from blood into proximal tubule cells, leading to selective accumulation and toxicity (Cihlar et al., 1999; Ho et al., 2000). OAT3 also contributes to this transport. Probenecid inhibits OAT1, prevents the uptake of cidofovir by proximal tubular epithelium, and is used to decrease the incidence of clinical nephrotoxicity (Cihlar et al., 1999). The clinical usefulness of other drugs should be explored, since 56% of patients have side effects of probenecid that are dose limiting in 7% of all patients (Lalezari et al., 1997). Nonsteroidal anti-inflammatory drugs (NSAIDs) efficiently inhibit hOAT1-specific transport of adefovir at clinically relevant concentrations and reduced adefovir cytotoxicity without interfering with its anti-HIV activity (Mulato et al., 2000). However, NSAIDs may promote acute renal failure in patients with risk factors.

MRP2 and MRP4 are the only members of the multidrug-resistant protein family expressed at the apical membrane of kidney proximal tubules. MRP4 extracts both adefovir and tenofovir from the proximal tubule cells and secretes them into the tubular lumen, but it makes only a limited contribution to the urinary excretion of cidofovir

(Imaoka et al., 2007). The luminal efflux mechanism of cidofovir remains unclear. Probenecid, dipyridamole, NSAIDs, and nucleoside drugs, such as acyclovir, are potent MRP4 inhibitors (El-Sheikh et al., 2007; Smeets et al., 2004). MRP2 efficiently transport ritonavir and indinavir, the former behaving as an inhibitor (Huisman et al., 2002). This effect may facilitate interaction with other nephrotoxic drugs.

10.6.2 HAART and Mitochondrial Dysfunction

HAART nephrotoxicity may result from damage to mitochondrial DNA (mtDNA). Damage to mtDNA has been well studied in cases of adefovir nephrotoxicity (Tanji et al., 2001). Adefovir serves as substrate for DNA polymerase γ , which is responsible for the replication of mtDNA, and inhibits mtDNA replication. A similar mechanism of cellular toxicity has been implicated in the myopathy caused by a related nucleoside analogue, zidovudine. Renal biopsies from patients with acute renal failure induced by adefovir revealed proximal tubule necrosis with dysmorphic and enlarged mitochondria. Enzymatic studies revealed deficiency of mtDNA-encoded enzymes. Single-tubule PCR showed that the amount of mtDNA in the affected tubules was 35–64% of that measured in unaffected tubules and tubules of normal kidney samples. However, there were no abnormalities in nuclear DNA-encoded proteins. Mitochondrial membrane potential, oxidative phosphorylation coupling, and ATP synthesis are not modified by HAART (Nerurkar et al., 2003). Biochemical consequences of mtDNA depletion are fatty acid accumulation, dicarboxylic acid accumulation, lactic acidosis, and ROS damage (Kakuda, 2000; Moyle, 2005). Mitochondrial dysfunction is also involved in hepatic steatosis, lipodystrophy, myopathy, and peripheral neuropathy observed in patients on HAART therapy (Hörschele, 2006). However, the *in vitro* toxicity index based on the relative rates of mtDNA and nuclear DNA replication underpredicts the toxicity of some drugs, such as tenofovir, suggesting that factors other than inhibition of DNA polymerase γ be responsible for nephrotoxicity (Lee et al., 2003). Mitochondrial nephrotoxicity may involve more than one drug and/or pathogenesis. Indeed, kidney mtDNA depletion was associated with HIV infection and concurrent tenofovir/didanosine therapy but not tenofovir use alone, while kidney ultrastructural mitochondrial abnormalities were seen with tenofovir use (Côté et al., 2006). In this regard, tenofovir did not produce significant changes in mtDNA levels in cultured human renal proximal tubular cells, contrary to observations with less nephrotoxic reverse transcriptase inhibitors (Birkus et al., 2002).

10.6.3 Nucleotide Antiviral Drugs and Tubular Cell Apoptosis

While the cytotoxicity of nucleotides in a variety of cell lines including proximal renal tubular cells is well established, the molecular mechanisms of cell death have only been evaluated in the case of cidofovir. Cidofovir was found to specifically induce apoptosis in human proximal tubular epithelial cells in an OAT1-dependent, probenecid-sensitive manner (Ortiz et al., 2005). Apoptosis was evident in the human proximal tubular cell line HK-2, as well as in primary tubular cell cultures. In contrast, human renal fibroblasts, devoid of OAT-1, were resistant to cidofovir toxicity. The time course (5–7 days of exposure to reach the peak apoptotic effect) was consistent with that observed in the clinical situation. The lethal concentration was within the therapeutic range. Cell death was caspase dependent and was inhibited by caspase-3 inhibitors and by survival cytokines, such as IGF-1 and HGF. The protective action of survival factors that increase BclxL expression and caspase-3 inhibitors points to a mitochondrial mechanism causing toxicity similar to what has been reported for CsA. However, the involvement of mitochondria was not specifically studied. The exquisite selectivity of cidofovir for proximal tubular epithelium and the fact that it is administered in a programmed fashion every 2 to 3 weeks make it an ideal candidate for proof-of-concept studies of tubular cell apoptosis manipulation in the clinical setting.

10.7 Other Nephrotoxic Drugs and Mitochondria

Mitochondrial injury has been implicated in nephrotoxicity induced by other drugs, including anticancer drugs, antibiotics, iron chelators, and environmental and endogenous nephrotoxins.

10.7.1 Anticancer Drugs: Cisplatin

Cisplatin causes acute renal failure by acute tubular necrosis and may lead to chronic renal failure requiring long-term renal replacement therapy (Arany and Safirstein, 2003). Proximal tubular epithelial cells accumulate cisplatin to a higher degree than other cells, explaining the high susceptibility to cisplatin-induced apoptosis. Activation of mitochondrial pathways is important in apoptosis induced by cisplatin. In cultured tubular cells, cisplatin caused Drp1 translocation to mitochondria and mitochondrial fragmentation prior to cytochrome *c* release and apoptosis (Brooks et al., 2009). Cilastatin limits entry into proximal tubular cells of a

number of nephrotoxic compounds and prevented cisplatin-induced mitochondrial injury and apoptosis and prevented cisplatin-induced DNA fragmentation in tubular cells without compromising antitumor efficacy (Camano et al., 2010; Humanes et al., 2012; Moreno-Gordaliza et al., 2011).

10.7.2 Antibiotics: Aminoglycosides and Polymyxins

Aminoglycoside nephrotoxicity results in nonoliguric renal failure with a slow rise in serum creatinine after several days of treatment (Mingeot-Leclercq and Tulkens, 1999). The time course reflects the need for the drugs to accumulate in tubular cells, especially in lysosomes. It has been hypothesized that excessive intralysosomal accumulation of the drug leads to permeabilization of lysosomes and release of aminoglycosides and lysosomal enzymes. Localization of aminoglycosides in the cytosol would lead to mitochondrial injury, with loss of mitochondrial membrane potential, release of cytochrome *c*, caspase activation, and apoptosis (Servais et al., 2006, 2008). Polymyxin is a last-resort antibiotic for multiresistant bacteria that is extremely nephrotoxic. Polymyxin B dose-dependently decreased the proportion of cells with filamentous mitochondria (regular morphology) and decreased mitochondrial membrane potential and increased mitochondrial superoxide production and apoptosis of cultured renal tubular cells (Azad et al., 2015).

10.7.3 Iron Chelators: Deferasirox

Deferasirox is an oral iron chelator used to treat blood transfusion-related iron overload. Nephrotoxicity is the most serious and common adverse effect and may present as an acute or chronic kidney disease. Kidney proximal tubular cells are the target cells and presentation may be as isolated generalized proximal tubular dysfunction (Fanconi syndrome) (Díaz-García et al., 2014). Deferasirox induced dose-dependent tubular cell death with features of apoptosis or necroptosis in culture proximal tubular cells (Martin-Sanchez et al., 2017). However, deferasirox-induced cell death was not caspase or necroptosis protein dependent. A striking feature was mitochondrial injury identified by electron microscopy, associated with loss of mitochondrial membrane potential and TOM22-Bax clumping at mitochondria and cytochrome *c* release to cytosol. These features were dependent on iron deficiency and BclxL downregulation (Martin-Sanchez et al., 2017). It was hypothesized that proximal tubular cells may be especially sensitive to toxicity given that they fail to accumulate iron in transfusion-dependent anemia states, but are rich in mitochondria that need adequate iron stores for correct function.

10.7.4 Environmental Nephrotoxins: Aristolochic Acid

Aristolochic acid is a key environmental nephrotoxin. It induced proximal tubular mitochondria dysmorphia, reduced the respiratory control ratio and ATP content, impaired the activity of respiratory complex I more than II, and reduced mtDNA *in vivo* (Jiang et al., 2013).

10.7.5 Endogenous Nephrotoxins: Glucose, Glucose Degradation Products, and Heme

In some cases, endogenous products are nephrotoxic. High concentrations of glucose promote tubular epithelial cell death (Ortiz et al., 1997). This may account for the increased susceptibility of diabetic kidneys to acute tubular necrosis in diverse clinical situations (Mangano et al., 1998). High glucose-induced apoptosis is Bax dependent, suggesting a role for mitochondrial injury (Moley et al., 1998). More recently, glucose degradation products, such as 3,4-dideoxyglucon-3-ene (3,4-DGE), have been shown to promote Bax-dependent mitochondrial injury and apoptosis in tubular epithelial cells and podocytes (Justo et al., 2005; Sanchez-Niño et al., 2014). This is in part dependent on the downregulation of the mitochondrial protective factors HSP27/HSPB1 (Sanchez-Niño et al., 2014). Interestingly, intravenous glucose-containing solutions are rich sources of 3,4-DGE (Chen et al., 2015). Heme-containing pigments are additional endogenous nephrotoxic compounds that are released in the course of hemolysis or rhabdomyolysis. These may result from adverse effects of drugs (e.g., statin-associated rhabdomyolysis). Evidence of mitochondrial injury has also been observed in pigment-induced AKI (Moreno et al., 2012; Tábara et al., 2014).

10.8 Therapeutic Implications and Future Lines of Research

Tubular epithelial cells and especially proximal tubular epithelial cells are targets for nephrotoxic drugs that damage mitochondria. The abundance of mitochondria and the existence of transport mechanisms that facilitate drug accumulation inside these cells are responsible for cytotoxicity. A great deal has been learned about cell damage mechanisms, as well as about the intracellular molecular pathways of cell death following injury. The challenge for the future is to design new prophylactic and therapeutic strategies that limit renal injury. The first opportunity relates to the use of drugs that decrease the renal accumulation of nephrotoxins. Probenecid is already used for this purpose in the prevention of cidofovir nephrotoxicity. However, the drug has frequent side

effects, and alternatives should be developed. Drug combinations that limit the entry of toxic compounds into the cell and facilitate their efflux should be studied. Cilastatin remains an enigmatic drug that merits further studies, but evidence so far suggests that it decreases drug entry to proximal tubular cells. A second opportunity relates to interfering with mechanisms of injury. The acute use of renal survival factors is feasible for drugs such as cidofovir, which is administered once every 2 to 3 weeks. However, specific delivery of survival factors to renal cells may be required if they are going to be used chronically in order not to interfere with other physiological processes. Taking advantage of the existence of transporters at the cell membrane to increase the local tubular cell concentration of such drugs is a possibility. An alternative would be to administer tubular localizing drugs that modulate the local expression of intracellular or extracellular survival factors.

Finally, mitochondria-targeted drugs that protect mitochondria are the subject of intense investigation in the kidney injury setting, even at the clinical trial stage for renal ischemia (Tábara et al., 2014). Mitochondrial division inhibitor-1 (mdivi-1) inhibits Drp1 and prevented mitochondrial fragmentation and tubular cell apoptosis and partially preserved renal function during AKI induced by cisplatin or rhabdomyolysis (Brooks et al., 2009). Mitochondria-specific antioxidants include elamipretide (Bendavia), quinine analogues, and SOD

mimetics. Elamipretide is in clinical development and binds to cardiolipin on the IMM (Hall, 2013). The elamipretide/cardiolipin complex protects cardiolipin from peroxidation by cytochrome *c*, thus protecting mitochondrial cristae. A beneficial effect has been reported in several nephropathies, but there is little experience in nephrotoxicity (Szeto et al., 2011). MitoQ was the first antioxidant mitochondria-targeted drug to undergo clinical trials, and the antioxidant component of MitoQ is the same ubiquinone as found in coenzyme Q10 (Kelso et al., 2001). Mito-CP is an SOD mimetic that accumulates in mitochondria because of the negative transmembrane potential (Dilip et al., 2013). Both Mito-CP and MitoQ prevented cisplatin-induced renal oxidative stress and AKI (Mukhopadhyay et al., 2012).

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11

Mammalian Sperm Mitochondrial Function as Affected by Environmental Toxicants, Substances of Abuse, and Other Chemical Compounds

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11.1 Introduction

Infertility is a relevant clinical condition affecting nearly 15% of couples of reproductive age, with male factor accounting for nearly half of all cases, either as a contributor or as the single responsible factor (Skakkebaek et al., 2006; ASRM, 2012; Jungwirth et al., 2012). Interestingly, the success rates of assisted reproduction treatments have remained remarkably constant for decades (around 30–40% in most cases), which reflects both the lack of investment in the field at a basic level (when compared with cancer, metabolic and neurodegenerative disorders, regenerative medicine, aging, etc.) and the intrinsic difficulty of experimenting directly in the human system for both ethical and practical reasons. Given that some retrospective studies have indicated a progressive worldwide increase in male infertility, as well as a decrease in sperm quality (Skakkebaek et al., 2006; ASRM, 2012; Jungwirth et al., 2012), researchers tend to focus more on this gender, although the fact that high quantity/quality samples of the male gamete are much easier to obtain than their female counterparts certainly also plays a role at this level. The causes of the decline in

male fertility are likely multifactorial, involving congenital and/or acquired conditions including occupational, lifestyle, and environment-associated changes, genetic defects, endocrine disturbances, testicular pathologies (Nieschlag, 2000; Wiser et al., 2012), and systemic diseases that can affect the male reproductive system (Wiser et al., 2012). Importantly, male infertility of unknown origin continues to represent about 50% of all cases (WHO, 2010; Hamada et al., 2011; Jungwirth et al., 2012).

Given the aforementioned availability of the human male gamete, it is technically possible (although not trivial, given the need for persistent patient recruitment and cooperation throughout) to obtain multiple samples and thus to evaluate the effects of different types of compounds, both in *in vitro* assays, consumed voluntarily, or to which patients may be exposed chronically. For example, dietary interventions and different supplements, notably antioxidants, have received much attention in terms of how they may influence sperm quality (Agarwal and Sekhon, 2010; Carlomagno et al., 2011). Studies have also focused on the negative effects of drugs linked to substance abuse or on environmental toxicants, especially those with hormone-disrupting effects (endocrine disruptors).



Figure 11.1 Structure of the human spermatozoon. The human sperm cell is composed of a sperm head, mostly occupied by the haploid condensed nucleus, which is partially overlaid by a secretory vesicle (the acrosome) in the anterior portion. The full length of the tail includes the midpiece, where mitochondria are clustered.

As a terminally differentiated cell type, mature sperm present multiple challenges in terms of routine evaluation, including in terms of mitochondrial function (Amaral and Ramalho-Santos, 2010; Sousa et al., 2014). They have a unique hydrodynamic shape and are composed of three distinctive subcellular compartments: the head, the midpiece, and the principal piece (Figure 11.1). Importantly, the much reduced sperm cytoplasm lacks most of the typical somatic cell organelles (endoplasmic reticulum, Golgi complex, lysosomes, peroxisomes), and there is a high compaction of the haploid nuclear chromatin (characterized by the replacement of most histones with protamines) that is associated with a lack of transcription (Sutovsky and Manandhar, 2006; Barratt et al., 2009). This implies that sperm proteins are limited to those formed during spermatogenesis or that can be acquired via contact with both the male and female reproductive tracts (i.e., imported to the surface of the male gamete from the outside media, including epididymis and oviduct secretions). Thus, many of the assays normally used to probe the functions of somatic cells (such as gene silencing) are not possible to carry out in sperm, and even the delivery and behavior of probes may prove problematic due to the lack of an extended cytoplasm or to the way sperm-specific structures are formed. For example, probes normally used to assess mitochondrial membrane potential (MMP) are clearly less dynamic in sperm than in other cells (Amaral and Ramalho-Santos, 2010), possibly due to the organization of these organelles in the midpiece. Therefore, the transitioning of established protocols to sperm cells must be accompanied by appropriate controls.

The sperm plasma membrane has a high level of polyunsaturated fatty acids (PUFAs), and metabolically the mature sperm seems to be a very versatile cell. In fact, in order to obtain energy, sperm are able to use pathways as varied as glycolysis, mitochondrial oxidative phosphorylation, and fatty acid β -oxidation, purportedly depending on substrate availability (Ruiz-Pesini et al., 2007; Amaral et al., 2013a, b, 2014a, b; Paiva et al., 2015). Energy in sperm is used for several processes intimately linked to reproductive success, the most obvious of which is rapid motility (Sutovsky and Manandhar, 2006; Barratt et al., 2009). Other energy consumers include

the acrosome reaction, the Ca^{2+} -dependent controlled release of enzymatic contents from the acrosome, a specialized secretory vesicle anterior to the nucleus (Florman et al., 2008). However, prior to the acrosome reaction, several events must take place in the female reproductive tract, globally termed “sperm capacitation,” which include increased plasma membrane permeability and fluidity due to cholesterol efflux, intracellular Ca^{2+} increase, cytoplasm alkalization, and protein phosphorylation, characteristically on tyrosine residues. Sperm capacitation is also associated with the triggering of hyperactivated motility, which will further allow sperm progression through cervical mucus and oocyte penetration following the acrosome reaction (Abou-haila and Tulsiani, 2009).

Mitochondria are localized in the sperm midpiece, forming a helicoidal sheath consisting of 22–75 mitochondria in total (in humans), but the number of mitochondria and midpiece length vary with species (Sutovsky and Manandhar, 2006; Turner, 2006). The putative importance of mitochondria in sperm function was initially studied strictly in terms of ATP production for motility. However, it has been suggested that, at least in some species, mitochondrial ATP may be irrelevant for sperm motility and that other metabolic pathways, functioning at the full length of the tail (notably glycolytic), are the main providers of ATP for motility, the rationale being that transporting ATP from the midpiece to all relevant force-producing dyneins in the tail would be problematic (see Figure 11.1). However, this remains a controversial issue (Ramalho-Santos et al., 2009). Nonetheless, several studies have associated different aspects of sperm functionality to mitochondrial activity, and, regardless of its exact role in sperm, different aspects of mitochondrial activity provide extensive predictive insights into the potential of a given sperm sample and how it may be affected by different factors and substances. Indeed, from an evolutionary point of view, the maintenance of these organelles after a complex differentiation process and in such a precise region stresses this putative importance (Ramalho-Santos and Amaral, 2013).

For example, it has been shown that the activity of several complexes in the electron transfer chain (ETC) of the inner mitochondrial membrane as well as MMP is

correlated with sperm motility (Ruiz-Pesini et al., 1998; Troiano et al., 1998; Donnelly et al., 2000; Barroso et al., 2006; Paoli et al., 2011; Wang et al., 2012a). Furthermore, sperm MMP correlates with the acrosome reaction and with fertilization success (Kasai et al., 2002; Gallon et al., 2006; Marchetti et al., 2002, 2004, 2012), and an association between mitochondrial function and sperm quality has also been reported (Gallon et al., 2006; Sousa et al., 2011). Moreover, sperm mitochondria have the ability to accumulate calcium, suggesting a role in Ca^{2+} signaling pathways (Publicover et al., 2007, 2008). From a clinical standpoint impaired sperm mitochondrial function has been described in some infertile individuals (Wang et al., 2003; Mayorga-Torres et al., 2013), and changes in morphology are present in sperm mitochondria of patients with gametes with abnormally low motility, a condition designated as asthenozoospermia (Mundy et al., 1995; Pelliccione et al., 2011). Changes in mitochondrial DNA (mtDNA) also correlate with compromised sperm quality, motility, and function and have been reported in infertile men (St John et al., 2001; Jensen et al., 2004; Nakada et al., 2006; Amaral et al., 2007).

The production of reactive oxygen species (ROS) is also relevant in this context, as mitochondria are the major generators (and targets) of ROS (Orrenius et al., 2007). ROS are produced in semen, where the leukocytes and spermatozoa are the major endogenous sources (Agarwal et al., 2014a, b), and sperm-produced ROS play a functional role in capacitation, the acquisition of hyperactivated motility, the acrosome reaction, and sperm–oocyte interactions, although this is only the case when ROS are produced at controlled levels. In contrast, increased ROS production can have detrimental effects (Tremellen, 2008; Agarwal et al., 2014a). Increased ROS have been associated with compromised sperm membranes and sperm DNA damage, thus reducing fertilization potential and introducing the possibility of transmission of genetic alterations to the embryo (Agarwal et al., 2005, 2006, 2014b). At the membrane level, ROS-induced lipid peroxidation disturbs fluidity, leading to motility loss and impaired membrane fusion events, including the acrosome reaction. In addition, ROS might also directly damage both the mitochondrial and nuclear genomes of human spermatozoa, causing base modifications, deletions, single- and double-strand breaks, chromatin crosslinking, and chromosomal rearrangements (Tremellen, 2008). While the sperm tail enters the oocyte at fertilization, sperm mitochondria are subsequently destroyed, and paternal mtDNA is therefore not inherited by the embryo (Ramalho-Santos et al., 2009). However, defective sperm mitochondria may be less efficient in ATP production and may also generate more ROS, which may consequently damage mitochondria and

mtDNA (and other cellular components), in a vicious cycle of damage, leading to a state of energy crisis and a global decline of sperm function (Kao et al., 1998). This issue is also very important in sperm cryopreservation protocols, as the freezing–thawing cycles required also promote increased ROS formation and sperm death (for a recent review see Amidi et al., 2016).

However, although the relation between sperm ROS levels and seminal quality has been established, the source of ROS is not always clear and is somewhat controversial. In spermatozoa, there are two main ROS producers, the cell membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the mitochondria (Agarwal et al., 2014a). Recently, the purported development of specific probes for different types of mitochondria-produced ROS (mROS) has provided some insights, and the importance of mitochondrial-originating sperm ROS has been demonstrated in several studies involving spermatozoa. It should be stressed that these results must always be taken with care and appropriate controls included for probe specificity, as probes in sperm may behave in a slightly distinct manner than in other cells (Amaral and Ramalho-Santos, 2010). Sperm membrane peroxidation, loss of motility (Koppers et al., 2008; Aitken et al., 2012), and nuclear DNA damage (Koppers et al., 2008) were reported as a result of excessive mROS production. Our group has also isolated different sperm populations based on their mROS levels, showing that the subpopulation producing the lowest amount of mROS represented the most functional subset of male gametes within an ejaculate. Concomitantly, this subpopulation was observed to increase in samples with better quality, and that gave rise to pregnancies following assisted reproduction protocols (Marques et al., 2014).

In this chapter we will focus on the possible effects of different classes of chemical compounds on sperm mitochondria as a surrogate for fertilization potential.

11.2 Pesticides, Herbicides, and Other Endocrine-Disrupting Chemicals (EDCs)

Endocrine-disrupting chemicals (EDCs), commonly known as endocrine disruptors, are exogenous substances that interfere with hormonal homeostasis, mimicking endogenous hormones and affecting their production, binding, and/or action. The fact that some of them can mimic hormones crucial for spermatogenesis (particularly testosterone and estradiol), potentially affecting male reproductive health in the exposed

organism and/or its progeny, suggests that they may play a role in the reduction of male fertility worldwide described previously. Given that these substances are widespread and resistant to degradation, they constitute a threat to human, animal, and wildlife populations that are continuously exposed through air, soil, water, and food contamination (Tavares et al., 2016). Recently the direct effects of some of these substances on sperm (i.e., not via a hormone-related pathway) have also begun to be investigated (Schiffer et al., 2014).

EDCs are divided into distinct categories and include man-made substances (e.g., bisphenol A (BPA), alkylphenols, phthalates, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides) and naturally existing compounds such as phytoestrogens and mycotoxins. Importantly, significant concentrations of EDCs have been reported in reproductive fluids, namely, seminal and follicular fluids, and in secretions of the female reproductive tract (Tsutsumi et al., 1998; Younglai et al., 2002; Drbohlav et al., 2005). Despite their action at the gene expression level, EDCs also interfere with preexisting signaling pathways, possibly disturbing spermatozoa functionality and ultimately threaten sperm fertilizing ability (Tavares et al., 2016). Indeed, many studies have shown altered sperm parameters, including mitochondrial function, after *in vitro* and *in vivo* exposure to EDCs.

11.3 In Vivo Studies

Adult exposure to 25 mg/kg/day body weight of BPA has been shown to be enough to decrease rat sperm mitochondrial activity determined by cytochrome *c* oxidase activity (Wisniewski et al., 2015). Concomitantly, a significant reduction in the number of morphologically normal spermatozoa, acrosomal integrity, and sperm viability was observed at 5 and 25 mg/kg/day BPA, along with an important decrease in sperm production, epididymal sperm reserves, and altered serum levels of FSH, LH, testosterone, and estradiol (Wisniewski et al., 2015). Since the reported no observable adverse effect level (NOAEL) for rat reproductive and developmental toxicity was previously established to be 50 mg/kg/day BPA (Schwetz and Harris, 1993; WHO, 2010), these findings are rather troubling as male reproductive impairment was detected with concentrations thought to be safe (Wisniewski et al., 2015). Others have also observed altered sperm mitochondrial integrity upon *in vivo* BPA treatment in other mammalian species. In an *in vitro* study, using human sperm BPA (10^{-8} and 10^{-6} M) altered MMP (Skibińska et al., 2016), while *in utero* exposure (200 µg/kg body weight; Vilela et al., 2014) decreased sperm mitochondrial function paralleled by reduced

sperm motility, morphology, membrane integrity, and sperm penetration rates in the vesper mouse.

Although human exposure to diesters of phthalic acid (commonly termed phthalates) may occur mainly through diet, these compounds are also usually used as plasticizers in several polyvinyl chloride products and personal care goods, coatings, and polishes (Tavares et al., 2016), representing further sources of phthalate exposure. Seminal (di(2-ethylhexyl) phthalate (DEHP) levels (mean: 0.13–0.77 µg/mL) found in men from rural and urban areas (but not levels of other phthalates such as diethyl phthalate, dimethyl phthalate, di-*n*-octyl phthalate, and di-*n*-butyl phthalate) have been negatively correlated with both sperm motility and mitochondrial integrity and positively associated with sperm ROS production, lipid peroxidation, and nDNA fragmentation (Pant et al., 2008). Such findings suggest that both mitochondrial impairment and elevated ROS production resulted in oxidative stress upon exposure, leading to peroxidation of sperm membrane PUFAs and nDNA damage, rendering spermatozoa nonfunctional (Pant et al., 2008).

Other studies have shown similar results when spermatozoa from different species were exposed *in vivo* to extensively applied compounds mainly used to obtain better crop yields (Grizard et al., 2007; Aly and Khafagy, 2014; Tavares et al., 2015).

In *in vitro* assays lindane and the major and most stable compound of the pesticide dichlorodiphenyltrichloroethane (DDT)—*p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE)—have been shown to positively correlate with the number of human sperm with depolarized mitochondria, high ROS levels, lipoperoxidation, and DNA fragmentation (mean lindane: 3.1–18.64 µg/L; mean *p,p'*-DDE: 8.4–22.60 µg/L; Pant et al., 2014). In addition, the cyclodiene broad-spectrum insecticide endosulfan also induces sperm mitochondrial membrane depolarization and increases sperm ROS generation in adult rats dosed with 5 mg/kg/day for 2 weeks, possibly due to increased H₂O₂ production and decreased activities of the antioxidant enzymes SOD, CAT, and GPx as well as to GSH content in the testis (Aly and Khafagy, 2014). Some of the effects reported have also been studied in terms of possibly involving apoptosis. For example, following a decrease in MMP and cytochrome *c* release from the mitochondria, increased activation of caspase-8 was also detected, initiating a cascade process that may have led to the observed rise in caspase-9 activity that in turn activated caspase-3, thus suggesting apoptosis in testicular cells (Aly and Khafagy, 2014). Both rat sperm motility and viability declined upon endosulfan treatment (Aly and Khafagy, 2014), and the same was shown by Wang and colleagues after a 3-week exposure of adult mice to a concentration of 0.8 mg/kg/day (Wang et al., 2012b).

Finally, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most potent toxicant ever made by man and often used as the prototype for a wide group of halogenated aromatic hydrocarbons such as PCBs and PAHs. Using TCDD a dose-dependent reduction in sperm MMP from C57BL/6 male mice exposed *in vivo* for 24 h to concentrations ranging from 1 to 50 µg/kg body weight was observed, although interestingly in this case the *in vitro* results were contradictory (see next section).

11.4 In Vitro Studies

Nonylphenol (NP), a short side-chain derivative of alkylphenolpolyethoxylates (APEs) commonly used in pesticides, plastics, cosmetics, detergents, emulsifiers, and intravaginal spermicides, also affects sperm mitochondria. Increasing NP doses (100, 250, and 500 µg/mL) have been associated with a sharp decline in rat sperm MMP *in vitro* (Uguz et al., 2009). Indeed, it has been suggested that NP may act as an oxidative phosphorylation uncoupler (Uguz et al., 2015), as has been reported for other *in vitro* studies with NP doses ranging from 1.8 to 8.2 mg/mL (Argese et al., 1994; Bragadin et al., 1999). It is therefore not surprising that NP is able to reduce sperm motility. Other compounds such as PCBs (Aly, 2013) and pesticides (Grizard et al., 2007; Wang et al., 2012b; Aly and Khafagy, 2014; Tavares et al., 2015) also affect sperm motility *in vitro* possibly via mitochondrial impairment. The PCB mixture Aroclor 1254 decreased not only rat sperm motility and viability but also MMP and acrosome reaction at all concentrations tested (10^{-7} , 10^{-8} , 10^{-9} M; Aly, 2013), in addition to inducing sperm nDNA damage at 10^{-7} and 10^{-8} M. Moreover, Aroclor 1254 induced mitochondrial cytochrome *c* release and caspase-3 activation and decreased ATP levels in a concentration-related manner with additional oxidative stress and increased lipid peroxidation being detected (Aly, 2013).

The herbicide alachlor, generally used in the production of corn, peanuts, rice, and soybeans, has also been found to promote decreased human sperm motility, viability, and MMP, which was time and/or concentration related and accompanied by an increase in sperm ROS production, nDNA fragmentation, and externalization of phosphatidylserine to the outer leaflet of the plasma membrane, an early sign of apoptosis (Grizard et al., 2007). As found *in vivo*, *p,p'*-DDE also promotes sperm mitochondrial depolarization *in vitro* at 25–100 µM. In fact, the same authors reported that *p,p'*-DDE is capable of promoting a non-regulated Ca^{2+} influx *in vitro* even at concentrations as low as 1 pM and 1 nM through the activation of the sperm-specific plasma membrane channel Catsper, and induces acrosome reaction,

before impairing human sperm motility (Tavares et al., 2013, 2015). Since mitochondria play an important role in regulating Ca^{2+} homeostasis, elevated cytosolic Ca^{2+} levels may lead to a mitochondrial Ca^{2+} overload that would culminate in general mitochondrial dysfunction and ATP depletion, consequently affecting sperm motility and viability (Tavares et al., 2015). In fact, many other (but not all) EDCs have been described as modulators of sperm intracellular Ca^{2+} levels *in vitro* (Silvestroni and Pallesch, 1999; Luconi et al., 2001; Mota et al., 2012; Schiffer et al., 2014).

Finally, results with TCDD are somewhat inconclusive. Following 24 h of *in vitro* exposure, 1 nM and 1 µM TCDD fail to affect not only human sperm MMP but also other sperm functional parameters (Mota et al., 2012). However, a previously noted study that detected a dose-dependent reduction in sperm MMP collected from C57BL/6 male mice exposed to TCDD also observed effects when epididymal sperm cells from the same mouse strain were exposed to 1 and 5 nM of the compound *in vitro* (Fisher et al., 2005). Despite this puzzling result, overall the literature suggests that the large majority of EDCs affect sperm mitochondrial status from diverse mammalian species and produce undesirable effects, possibly contributing to a decrease in male fertility.

11.5 Drugs of Abuse

Substance abuse is prevalent in Western society, with a higher consumption by males than females, and it has been increasingly implicated as a factor for couple infertility. Common drugs of abuse include opioid narcotics, methamphetamines, anabolic-androgenic steroids, marijuana, cocaine, and tobacco, although the latter has the clear distinction of being almost universally legal under controlled circumstances (Fronczak et al., 2012; Alvarez, 2015). All these substances exert negative effects on male fertility, which include hormonal secretion suppression, spermatogenesis impairment, and changes in sperm morphology, viability, and motility (Fronczak et al., 2012).

11.5.1 Marijuana

According to the National Survey of Drug Use and Health (NSDUH), marijuana has the highest rate of consumption among reproductive age males in the United States (Du Plessis et al., 2015). The marijuana plant (*Cannabis sativa*) is enriched in cannabinoids that when ingested promote paranoia, illusions, hallucinations, delusions, depersonalization, confusion, restlessness, and excitement (D'Souza et al., 2004) with additional effects including changes in cognition and memory,

sedation, analgesia, appetite stimulation, relief of muscle spasticity, and antitumoral activity (Pertwee, 2000). The presence of cannabinoids in human seminal plasma, mid-cycle oviductal fluid, and follicular fluid has been reported (Schuel et al., 2002), and cannabinoid consumption has been linked not only to male infertility and reduced sperm count and function but also to decrease libido, cause gynecomastia, and increase the chances for erectile dysfunction (for review see Park et al., 2004).

Tetrahydrocannabinol (THC), the major psychoactive cannabinoid compound in marijuana, is known to block the release of gonadotropin-releasing hormone (GnRH), disrupting the hypothalamic–pituitary–testicular axis, which leads to a suppression of LH release and a subsequent decline in testosterone levels and spermatogenesis (Vescovi et al., 1992), and to affect several aspects of sperm function (Fronczak et al., 2012). The human body also produces endogenous cannabinoids or *endocannabinoids* from unsaturated fatty acids (Devane et al., 1992; Felder et al., 1992), with anandamide (arachidonylethanolamine) and 2-arachidonoylglycerol being the two most studied endocannabinoids. Both cannabinoids and endocannabinoids are known to exert their effects through the binding or activation of specific cannabinoid receptors (CB-R), which are present, among many other places (Howlett et al., 2002) in the testis, specifically in the sperm head and midpiece (Rossato et al., 2005). In fact, *in vitro* studies using anandamide showed that binding of cannabinoids to these sperm receptors was reported to affect sperm motility, viability, calcium signaling, mitochondrial function, and the acrosome reaction (Schuel et al., 2002; Rossato et al., 2005; Badawy et al., 2009). It should be noted that in these *in vitro* studies, the concentrations used varied widely, with motility, calcium signaling, and the acrosome reaction affected at nanomolar to 1 μ M range of anandamide (Schuel et al., 2002; Rossato et al., 2005), while mitochondrial respiration was affected at much higher (30–240 μ M) concentrations of THC (Badawy et al., 2009). A dose-dependent decrease in the number of functional mitochondria, indicating a decline in mitochondrial activity, was also monitored, resulting in a decreased energy supply and thus affecting sperm motility. In accordance with these results, studies have shown that cannabinoids exert negative effects on mitochondrial function by interfering with the electron transport, depleting NADH and ATP levels, and affecting the mitochondrial permeability transition pore complex with effects on several parameters being detected after 1–2 h exposures to concentrations ranging from 0.5 to 10 mg/mL of THC (Sarafian et al., 2003). A similar study with delta-8-THC and delta-9-THC (the two main active cannabinoids in the marijuana plant) confirmed these direct negative effects

on sperm mitochondrial function, notably describing a decrease in oxygen consumption by human spermatozoa, probably by interfering with the ETC chain, at 120–240 μ M of THC (Badawy et al., 2009).

11.5.2 Cocaine

Cocaine is also one of the most commonly and consistently consumed drugs by both males and females during reproductive years (Fronczak, et al., 2012). Some of the psychotic symptoms include delusions (especially paranoid delusions), hallucinations with varying levels of seriousness, and stereotypical behavior (Tang et al., 2009). Similarly to marijuana components, cocaine has also been found in seminal plasma (Cone et al., 1996), and one of the early studies showed that prolonged exposure to cocaine led to low levels of sperm motility as well as concentration and to abnormal morphology in humans (smoked, intranasally, or intravenously) (Bracken et al., 1990). Interestingly, however, there were no significant effects detected in *in vitro* assays of cocaine exposure (1 μ M to 1 mM) using human sperm (Yelian et al., 1994). Studies have shown that a reduction in pregnancy rates was associated with male cocaine exposure (using daily or weekend injections of 15 mg/kg; George et al., 1996), causing spermatogenesis impairment associated with abnormal tubular morphology and testicular cell degeneration (using hourly injections of moderate doses of 0.5 and 10 mg/kg body weight of cocaine hydrochloride over 5 h; Rodriguez et al., 1992). This resulted in an increase in apoptosis (Li et al., 1999) via a mitochondria-associated pathway (Li et al., 2003), thus suggesting that cocaine induces mitochondria-dependent germ cell apoptosis. In the last two studies, the protocol involved 30-day-old male Sprague–Dawley rats being given cocaine hydrochloride (15 mg/kg body weight) subcutaneously daily for 90 days, and this is the only data linking mitochondrial effects to cocaine-induced male gametogenesis, but there are no published effects on mature sperm, possibly due to the previously mentioned negative data (Yelian et al., 1994).

11.5.3 Nicotine

Although it is obviously legal, albeit controlled in different circumstances/countries, nicotine from tobacco smoking is a pharmacologically active and addictive alkaloid (Jana et al., 2010), highly accumulated in the liver, lungs, kidneys, and brain. In the brain, nicotine binds to nicotine acetylcholine receptors (AChR) in dopaminergic neurons, with the consequent release of dopamine causing the side effects of pleasure and reduced stress and anxiety. In the liver, nicotine is

metabolized into different metabolites, including cotinine, 3-hydroxycotinine, and nicotine isomethonium (Benowitz, 2009). Both nicotine and cotinine adversely affect spermatogenesis, epididymal sperm count, motility, and the fertilizing potential of rodent sperm in rats injected with nicotine at a dose of 0.4 mg/100 g body weight daily for 3 months (Aydos et al., 2001). Early *in vivo* studies on human spermatozoa carried out by performing spermograms on smokers and nonsmokers revealed the presence of nicotine, cotinine, and hydroxycotinine in the seminal plasma of smokers (Pacifici et al., 1993) and reported a decrease in sperm concentration and motility, as well as a high prevalence in the number of abnormal cells (Kulikauskas et al., 1985). Furthermore, an increase in sperm nDNA fragmentation (Sepaniak et al., 2006) and in the concentration of leukocytes present in the semen of smokers has also been shown (Taszarek et al., 2005). However, there is still some controversy in the literature as other studies show no differences in testicular volume, FSH and testosterone levels, or sperm concentration, motility, and morphology in a population of fertile smoker patients (Pasqualotto et al., 2004; Chohan and Badawy, 2010). Nonetheless, given that sperm cells also express the nicotine AChR (Bray et al., 2005), *in vitro* studies on the influence of nicotine in sperm function (incubations with 200–300 µg/mL of cotinine) reported reduced sperm motility, specifically progressive motility, while viability was unaltered (Jorsaraei et al., 2008). Although the actual molecular events leading to nicotine-induced reproductive toxicity are still unclear, the adverse effects are, to some extent, due to increased ROS production (Bandyopadhyaya et al., 2008) in which mitochondria might be involved. Indeed, *in vitro* human sperm (from healthy nonsmokers) exposure to cigarette smoke extract showed a time- and concentration-dependent decrease in sperm MMP and motility, paralleled by an increase in sperm apoptosis, supported by an increase in the number of spermatozoa with phosphatidylserine externalization and nDNA fragmentation (Calogero et al., 2009).

11.5.4 Anabolic–Androgenic Steroids

The use of anabolic–androgenic steroids by athletes and body builders started in the 1950s and has significantly increased particularly in males (Sagoe et al., 2014). Although there are few studies published, anabolic steroids are proven to suppress the hypothalamic–pituitary–gonadal axis via feedback inhibition (Thompson, 1994). There are also reports of erectile dysfunction due to a decrease in the production of testosterone as well as a detriment in several sperm parameters, such as density (with reports of no or decreased sperm production),

motility, and morphology. These issues include defects in the head and midpiece, indirectly suggesting a possible mitochondrial effect (Torres-Calleja et al., 2001; Bonetti et al., 2008). Fortunately, and contrary to other illicit substances, spermatogenesis is likely to recover once steroid use is ceased (Knuth et al., 1989; Thompson, 1994).

11.6 Nutritional Elements: Vitamins and Supplements

The correlation of increased oxidative stress with infertility suggests that dietary antioxidants could serve as a possible treatment for male infertility, and several studies exist in this field, either in a clinical context or using *in vitro* approaches. It is important to keep in mind that seminal ROS should only be lowered to non-damaging levels, assuring that the physiological processes that depend on their presence are unaffected (Comhaire and Mahmoud, 2003; Zini and Al-Hathal, 2011). Although many studies focus on dietary supplements and their effects on human (in)fertility and particularly sperm function, these studies are somewhat weakened by the varying types and doses of supplements used (alone or in combinations), often lacking placebo controls and the use of very diverse number of patients and endpoints. Although there is some evidence that dietary supplementation, in particular with antioxidants, may be beneficial to sperm function (Agarwal and Sekhon, 2010; Zini and Al-Hathal, 2011), there have been no systematic reports regarding the effects of specific male infertility-related supplements (e.g., Gametix-M, Spermax, and Fertilix) on sperm function. To avoid confusing interpretations, we will focus on studies in which only one substance (not in combination with others) has been used. All the compounds discussed later have been described as influencing mitochondrial function in other contexts.

11.6.1 Coenzyme Q10

Coenzyme Q10 (CQ10) is a lipid-soluble carrier that shuttles electrons from complexes I and II to complex III during oxidative phosphorylation, therefore playing a strategic role in mitochondrial bioenergetics. There are therefore two forms of CQ10: reduced (ubiquinol) and oxidized (ubiquinone) (Arroyo et al., 2000; Turunen et al., 2004). Additionally, not only does CQ10 have antioxidant properties, but it can also regenerate other antioxidants, such as vitamins C and E (Arroyo et al., 2000; Turunen et al., 2004).

In accordance with these findings, clinical trials involving oral CQ10 supplementation for 12 months improved

seminal parameters (concentration, motility, and morphology) and increased seminal CQ10 levels and the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), in the seminal plasma of infertile men while simultaneously improving pregnancy rates (Safarinejad, 2012). In a later study supplementation with ubiquinol (the reduced form of CQ10) was shown to be more effective than the oxidized form (Safarinejad et al., 2012). Recently, through an *in vitro* approach, Liu and collaborators reported that mitoquinone (MitoQ), a ubiquinone specifically targeted to the mitochondria, was able to improve some sperm parameters, including sperm MMP, and to decrease oxidative damage in sperm. These positive effects were also extended to post-thawed human sperm cryopreserved treated with MitoQ (Liu et al., 2016). However, other studies reported no beneficial effects of CQ10 on sperm parameters, but a better antioxidant capacity in the seminal plasma of infertile men (Nadjarzadeh et al., 2011). The duration of treatment, different doses of supplementation, and study population heterogeneity/size might explain differences between studies.

11.6.2 L-Carnitine

L-Carnitine (β -hydroxy- γ -trimethylammonium butyrate) is a vitamin-like water-soluble compound (Pekala et al., 2011) popular in the context of exercise supplements. A correlation between L-carnitine seminal concentration and several human seminal parameters, such as sperm concentration, motility, and morphology, has already been established in several studies, and infertile patients are reported to have lower seminal plasma levels of L-acetyl-carnitine than controls (Comhaire and Mahmoud, 2003). L-Carnitine supplementation decreased the prevalence of several sperm defects observed in subfertile patients, including head and midpiece anomalies, which mainly involved abnormal (defected or degenerated) mitochondria (Abd El-Baset et al., 2010). Similar results were also obtained by Nada and collaborators (Nada et al., 2015). Therefore, oral carnitine supplementation improved sperm morphology and consequently sperm quality, suggesting a beneficial role on sperm mitochondria, through either antioxidant or metabolic properties.

11.6.3 Melatonin

Melatonin is a lipophilic hormone secreted by the pineal gland that was initially shown to have a role in male reproductive function from the observation that a pineal gland tumor altered pubertal development. Later on, levels of melatonin in seminal plasma, as well as melatonin

receptors in the testis and spermatozoa, have also been described (Carlomagno et al., 2011). Melatonin may act by scavenging both ROS and reactive nitrogen species (Reiter et al., 2010; Galano et al., 2011) or by stimulating the activities of antioxidant enzymes (Shang et al., 2004). Indeed, in rat models, both *in vivo* administration (1 mg/kg/day, subcutaneously; Sönmez et al., 2007) and *in vitro* incubation (100 μ M; Rao and Gangadharan, 2008) with melatonin protect spermatozoa against oxidative agents, such as homocysteine (0.71 mg/kg/day; Sönmez et al., 2007) and mercury (1–100 μ M; Rao and Gangadharan, 2008), and it is likely that these effects are promoted or facilitated by the existence of melatonin receptors in sperm cells (Van Vuuren et al., 1992). In *in vitro* settings exogenous melatonin (consistently used in the 1–2 mM range in all cases) improved human sperm motility and viability and reduced endogenous NO levels (Du Plessis et al., 2010) and, in a different study, improved sperm parameters (Ortiz et al., 2011), suggesting that the increase in motility is due to a melatonin-mediated stimulation of mitochondrial ATP production, together with a better organelle protection due to the antioxidant properties of the compound. In fact, following melatonin treatment *in vitro*, both succinate dehydrogenase activity and sperm MMP were improved in spermatozoa exposed to oxidative conditions (Shang et al., 2004). Furthermore, human sperm pretreatment with melatonin before incubation with oxidative stress promoters (such as H₂O₂) decreased cell death, the activation of apoptotic markers such as caspase-3 and caspase-9, and phosphatidylserine externalization, as well as sperm mitochondrial ROS production (Espino et al., 2010). Importantly, these effects, as well as oxidative-induced sperm nDNA fragmentation, were melatonin receptor mediated (Espino et al., 2011).

11.6.4 Vitamins E and C

There are several isomers of vitamin E, a lipid-soluble molecule not synthesized in mammalian cells, such as tocopherols and tocotrienols, with α -tocopherol being the most active form. The role of vitamin E in preventing the propagation of ROS-mediated reactions, avoiding lipid peroxidation, and boosting the activity of several antioxidants is well established (Rizvi et al., 2014). It is due to these attributes that it is considered one of the main agents protecting sperm membranes against ROS-induced damage (Akiyama, 1999; Wang et al., 2007). However, vitamin C (ascorbic acid) is a very potent water-soluble antioxidant that works as a cofactor for several enzymes. Besides its importance for the maintenance of testicular function (Chinoy et al., 1986; Sönmez et al., 2005), high seminal plasma concentrations protect

spermatozoa from oxidative damage, neutralizing ROS (Agarwal and Sekhon, 2010), and studies in humans reported that fertile individuals have a higher vitamin C seminal plasma content than infertile ones (Colagar and Marzony, 2009). In both cases few studies are available in the literature that specifically focus on the effects of vitamins E and C on sperm mitochondria and in general studies rather infer a possible role due to the putative management of mitochondria-produced ROS.

Vitamin E supplementation has been observed to improve several aspects of male reproductive function, such as seminal quality in rabbits (1 g/L of drinking water; Yousef et al., 2003), likely due to its antioxidant properties that include an increase in the activity of several antioxidant enzymes, such as SOD or GPX (Agarwal and Sekhon, 2010; Yue et al., 2010; Majid et al., 2015). In humans, oral intake of vitamin E can improve sperm parameters (Kessopoulou et al., 1995), including motility (Suleiman et al., 1996), DNA integrity (Schmid et al., 2012), and pregnancy rates, and can lead to a decrease in sperm lipid peroxidation in infertile men linked to improved clinical pregnancy outcomes (Suleiman et al., 1996). Other studies have shown that oral vitamin E supplementation is capable of reducing the oxidative damage induced by pollutants such as lead (in mice, 100 mg vitamin E/kg body weight; Fahim et al., 2013) or pesticides such as lambda-cyhalothrin, improving sperm motility, count, and viability as well as seminal plasma oxidative status (in rabbits, 2 mg/kg of vitamin E; Yousef, 2010).

Fewer studies have been carried out with vitamin C, although dietary administration has been shown to improve seminal quality in heavy smokers (Dawson et al., 1992) and also in rabbits (1.5 g/L in drinking water; Yousef et al., 2003) and guinea pigs exposed to ethanol toxicity (25 mg/100 g body weight; Harikrishnan et al., 2013). During cryopreservation, ascorbate added *in vitro* (300–600 μ M) was able to protect human sperm, improving parameters such as viability, motility, and MMP, decreasing ROS production, and inhibiting DNA damage and early apoptotic events in frozen–thawed sperm (Li et al., 2010).

11.6.5 Lycopene and Fatty Acids

Different studies investigated the potential of lycopene, a lipophilic reddish carotenoid family member frequently found in tomatoes and several red fruits, and, similarly to vitamin E, to attenuate the toxicity of other compounds such as the bacterial lipopolysaccharide (LPS)-induced toxicity (Aly et al., 2012). In this instance pre-administration of lycopene *in vivo* (4 mg/kg/day by oral gavage) prevented the LPS-induced (0.1 mg/kg/day for 7 days i.p.)

decrease in rat sperm count and motility. Additionally, positive effects were also observed in isolated testicular mitochondrial function from the same animals, mainly in terms of lipid peroxidation, the activity of tricarboxylic acid (TCA) cycle and antioxidant enzymes, and levels of antioxidants. The amelioration of these mitochondrial function indicators was associated with improved sperm function (Aly et al., 2012) in rats. In similar *in vivo* experimental settings using rodent models, lycopene administration also seems to protect from the toxic effects of other substances that may act, at least in part, by increasing oxidative stress, such as cisplatin, a chemotherapy drug that has several side effects such as testicular toxicity (Salem et al., 2012; in rats), or zearalenone (ZEA), a worldwide mycotoxin food contaminant shown to adversely affect reproduction in farm animals and humans (Boeira et al., 2015; in mice).

11.7 Natural Plant Products

The medicinal properties of several plants and plant products have been recognized for many years, mainly owing to the knowledge from indigenous tribes. Since these populations have no access to modern healthcare, they usually rely on readily available and low-cost biological materials, identifying the suitable products for different pathologies and passing this knowledge onward through generations. The use of these biological compounds as an alternative medicine to treat distinct conditions and diseases, including male infertility, is therefore common mainly in developing and developed countries.

Evidence suggests that tea plant (*Camellia sinensis*) components have antioxidant, anti-obesity, and antiaging properties, with tea being one of the most consumed beverages in the world. According to the degree of leaf processing, there are three types of tea (Dias et al., 2013): unfermented (green and white teas), partially fermented (oolong tea), and completely fermented (black tea), with unfermented teas possessing the most powerful antioxidant capacity given their higher polyphenol content (Higdon and Frei, 2003).

As noted previously for other types of compounds with antioxidant properties, orally administered green tea extract (GTE) also has beneficial effects on alterations induced by several toxic agents in rodent models, such as doxorubicin (i.p. in mice; 0.15 mg/kg/twice weekly/5 weeks w/GTE as diet supplementation, 200 and 500 mg/kg/day/14 weeks; Sato et al., 2010), nicotine (i.p. in rats; 1 mg/kg/day/2 months w/GTE as the sole beverage, 2% w/v for /2 months; Mosbah et al., 2015), and, to a less extent, *t*-butyl hydroperoxide (in rats, i.p.; 300 μ M/kg/2

Table 11.1 Effects of different types of compounds on the mitochondrial function of mammalian sperm.

Category	Compound/mixture	Main outcome in sperm	Species	References
Dietary supplements and natural plant products	Coenzyme Q 10 (mitoquinone)	Improve sperm MMP Decreased sperm ROS levels Decreased sperm lipid peroxidation Improved sperm motility	Human	Liu et al. (2016)
	L-Carnitine	Decreased sperm midpiece defects	Human	Abd El-baset et al. (2010)
	Melatonin	Decreased sperm apoptosis via the mitochondrial pathway Decreased sperm mitochondrial ROS production Improved sperm viability	Human	Espino et al. (2010, 2011)
	Vitamin C (ascorbate)	Improved Sperm MMP Improved sperm viability, and motility and decreased DNA damage (after freeze-thawing)	Human	Li et al. (2010)
	<i>Mucuna pruriens</i>	Restored sperm MMP Restored sperm parameters (reversion of treatment effects)	EE-treated rats	Singh et al. (2013)
		Restored sperm MMP and mitochondrial functional status (cytochrome c complex presence)	STZ-treated rats	Suresh et al. (2013)
		Restored sperm parameters (reversion of treatment effects)		
Drugs of abuse	Anabolic androgenic steroids	Defects in the midpiece Impaired sperm parameters	Human	Bonetti et al. (2008) and Torres-Calleja et al. (2001)
	Anandamide	Inhibition of sperm mitochondrial activity Decreased sperm parameters	Human	Rossato et al. (2005)
	THC	Inhibited sperm mitochondrial oxygen consumption	Human	Badawy et al. (2009)
	Nicotine	Lower MMP Decreased sperm motility	Human	Calogero et al. (2009)
Endocrine disruptors	Bisphenol A	Decreased mitochondrial activity (cytochrome c oxidase measurement)	Rat	Wisniewski et al. (2015)
		Decreased sperm parameters	Mouse	Vilela et al. (2014)
		Decreased MMP	Human	Skibińska et al. (2016)
		Decreased sperm parameters		
	Nonylphenol	Altered MMP Increased mitochondrial superoxide anion production		
		Decreased MMP	Rat	Uguz et al. (2009)
		Decreased sperm parameters		
	DEHP	Decreased MMP Impaired sperm parameters	Human	Pant et al. (2008)
	Aroclor 1254	Decreased MMP	Rat	Aly (2013)
		Impaired sperm parameters Increased apoptosis via the mitochondrial pathway		
	Endosulfan	Decreased MMP Impaired sperm parameters	Rat	Aly and Khafagy (2014)
		Increased apoptosis via the mitochondrial pathway		
	Alachlor	Decreased MMP Impaired sperm parameters	Human	Grizard et al. (2007)
	<i>p,p'</i> -DDE	Decreased MMP	Human	Tavares et al. (2015)
		Decreased sperm parameters		
	TCDD	Decreased MMP	Mouse	Fisher et al. (2005)

DEHP, di(2-ethyl-hexyl) phthalate; EE, Ethinyl estradiol; MMP, mitochondrial membrane potential; *p,p'*-DDE, dichlorodiphenyldichloroethylene; ROS, reactive oxygen species; STZ, Streptozotocin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; THC, Tetrahydrocannabinol.

weeks w/GTE as the sole beverage, 2% w/v for 10 weeks; Awoniyi et al., 2012) and on oxidative stress-induced reproductive toxicity, improving sperm quality in all cases, although none of these studies monitored sperm mitochondrial function directly. White and rooibos tea (*Aspalathus linearis*) also have high antioxidant properties and are described to improve sperm parameters and antioxidant status in rat sperm maintained *in vitro* (white tea aqueous extract, 0.5 and 1 mg/mL for 24, 48, and 72 h; Dias et al., 2014, 2016) and following *in vivo* administration (rooibos tea aqueous extract as the sole beverage, 2 g/100 mL/10 weeks (Awoniyi et al, 2012) and 2 and 5% for 52 days (Opuwari and Monsees, 2014); white tea aqueous extract as the sole beverage, 1 g/100 mL/2 months; Oliveira et al., 2015). However, none of these studies reference effects on sperm mitochondrial function.

Withania somnifera is a medicinal plant with free radical scavenging and antioxidant properties (Umadevi, 1996, Misra et al., 2000), and several *in vivo* studies in humans have reported many reproductive health benefits of *W. somnifera* treatment in male infertility, including an improvement in sperm count (Ahmad et al., 2010), concentration and motility (Ahmad et al., 2010; Mahdi et al, 2011; Ambiye et al., 2013), seminal plasma antioxidant status (Ahmad et al., 2010; Mahdi et al., 2011), decreased lipid peroxidation (Ahmad et al., 2010; Mahdi et al., 2011), increased sperm ROS production, and decreased apoptosis (Shukla et al., 2011) in infertile patients (Ahmad et al., 2010; Shukla et al., 2011; Ambiye et al., 2013) and in normozoospermic infertile patients (patients with normal semen parameters; Mahdi et al., 2011). Furthermore, the *W. somnifera* root extract (oral gavage, 500 mg/kg/15 days) decreases lipid peroxidation and ROS production in the cytosol and mitochondria of testicular cells and restores antioxidant defenses in streptozotocin (single i.p.; 90 mg/kg)-induced diabetic rats (Kyathanahalli et al., 2014).

Mucuna pruriens, a leguminous plant whose seed extracts are rich in levo-3,4-dihydroxyphenylalanine (L-DOPA) and include a variety of alkaloids and nutritional elements (Prakash et al., 2001), has been recognized to control diabetes, atherosclerosis, rheumatoid arthritis, nervous disorder, and also male infertility (Rajeshwar et al., 2005) owing to the hypoglycemic, hypolipidemic, and antioxidant properties of its extracts (Sharma et al., 1978). The beneficial effect of compounds derived from *M. pruriens* spermatozoa is evident in both human (*M. pruriens* seed powder, 5 g/day/3 months; Ahmad et al., 2008; Shukla et al., 2009, 2010; Gupta et al., 2011) and rat (*M. pruriens* seed extract, 200 mg/kg/day/2 months; Suresh et al., 2010, 2013) *in vivo* models. The extracts

specifically improve mitochondrial function (MMP; Suresh et al., 2010, 2013), as well as sperm concentration, viability, mobility, morphology (Ahmad et al., 2008; Shukla et al., 2009, 2010; Suresh et al., 2010, 2013; Gupta et al., 2011), and enzymatic and nonenzymatic antioxidants levels (Ahmad et al., 2008; Shukla et al., 2010; Suresh et al., 2010, 2013). In addition, decreases in lipid peroxidation and ROS production were also observed. Using ethinyl estradiol-treated animals (3 mg/kg/day orally for 14 days), which present compromised spermatogenesis, it was also shown that both *M. pruriens* (300 mg/kg/day orally for 8 weeks) and L-DOPA (20 mg/kg/day orally for 8 weeks) improved sperm count and motility, decreased sperm ROS production, and increased sperm MMP (Singh et al., 2013).

11.8 Conclusions and Perspectives

Sperm mitochondrial functionality assessment is recognized as important, being more frequently monitored in recent studies, and used as an indicator for sperm functionality, in context of male infertility and reproductive toxicity. As summarized in Table 11.1, in some cases direct effects of chemical substances on sperm mitochondria are very clear, as in the case of the dietary supplements L-carnitine, melatonin, and vitamin E and natural antioxidants from *C. sinensis* and *M. pruriens*; of all the endocrine disruptors mentioned; and of drugs of abuse such as cannabinoids and nicotine. In contrast to other cases, this can only be hypothesized using parallels with other cell types or via the known association of oxidative stress and mitochondrial dysfunction. This was noted for the dietary supplements vitamin C and fatty acids; the natural antioxidants from *A. linearis*; and cocaine. Finally, in others cases alterations were reported at the testicular mitochondria level, as is the case of vitamin E, lycopene, *W. somnifera*, and cocaine, allowing for tentative extrapolations to the mature gamete.

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Part 3

Methods to Detect Mitochondrial Toxicity

In Vitro, Ex Vivo, In Vivo, Using Cells, Animal Tissues, and Alternative Models

12

Biological and Computational Techniques to Identify Mitochondrial Toxicants

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CHAPTER MENU

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Of the drugs identified in preclinical testing, only 11% succeed in clinical trials, with failures often being due to toxicity and adverse drug events (ADEs) (Kola and Landis 2004). A frequent culprit of ADEs is the interaction with mitochondrial proteins, particularly in cases that lead to mitochondrial toxicity (Varga et al. 2015; Hargreaves et al. 2016). The cost of failed drugs due to mitochondrial toxicity necessitates a better understanding of the chemical causes of mitochondrial toxicity. Such studies require biological methods to identify mitochondrial toxicants and computational methods to identify the structural features common to these toxicants.

12.1 Identifying Mitochondrial Toxicants

The identification of mitochondrial toxicants requires a reliable and cost-effective means of measuring mitochondrial health. Several of these methods, along with their advantages and disadvantages, are outlined in the following text.

The expression of mitochondrial markers is frequently reduced at both the mRNA and protein level following

mitochondrial toxicity. Exposure of renal cells to toxicants such as *tert*-butyl hydrogen peroxide suppressed the mRNA and protein expression of oxidative phosphorylation proteins such as ND6, cytochrome c oxidase, and ATP synthase β (Rasbach and Schnellmann 2007; Wilmes et al. 2015). Kidney injury also involves oxidant damage to mitochondria, causing comparable losses in mitochondrial markers in animal models and human patients (Funk and Schnellmann 2012; Emma et al. 2016).

Suppression of mitochondrial biogenesis and associated mitochondrial damage and loss have been observed following toxicant exposure in multiple cell types, including hepatocytes (Guo et al. 2014; Sheik Abdul et al. 2016), cardiomyocytes (Liu et al. 2012b; Jiang et al. 2015), and neurons (An et al. 2015; Peng et al. 2016). Other toxicants, such as the nucleoside reverse transcriptase inhibitors, disrupt the replication of the mitochondrial genome to cause mitochondrial dysfunction (Bienstock and Copeland 2004). Despite the correlation of injury with the suppression of mitochondrial markers, measuring such endpoints is low-throughput, particularly for the measurement of protein expression. Toxicity may also modulate the activity rather than the expression of mitochondrial markers, particularly at low doses or early time points (Hargreaves et al. 2016). Although these markers

are useful for the confirmation of potential mitochondrial toxicity and for the identification of its mechanism, their lack of sensitivity for mitochondrial damage and the low-throughput nature of their measurement limit their utility for mitochondrial toxicity screening.

The development of mitochondria-selective dyes has enabled investigators to gather a wealth of information regarding the mitochondria. These dyes have positively charged functional groups that lead to their accumulation within the mitochondria due to its negative membrane potential, allowing for the fluorescent detection of mitochondrial content and morphology within cells (Terasaki and Jaffe 2004). Potentiometric dyes such as JC-1 and TMRM allow the visualization of mitochondria as well as a determination of their functional status vis-à-vis mitochondrial membrane potential (Cottet-Rousselle et al. 2011). These dyes have identified unexpected mitochondrial toxicities of pollutants (Padmini and Usha Rani 2011), chemotherapeutic agents (Santofimia-Castano et al. 2014), and antiretroviral drugs (Apostolova et al. 2013). Other groups have used machine learning algorithms to develop high content imaging techniques that enable the quantification of the morphology of thousands of mitochondria as well as their membrane potential and calcium content (Leonard et al. 2015). This technique identified concentration-dependent changes in mitochondrial morphology and membrane potential following exposure to known inhibitors of mitochondrial respiration such as oligomycin, 4-(trifluoromethoxy)phenylhydrazine (FCCP), rotenone, and antimycin A. While these imaging techniques have promise for the identification of the effects of potential toxicants on mitochondrial morphology, these parameters do not necessarily correlate to mitochondrial function. Additionally, partial loss of mitochondrial membrane potential can be an insensitive marker of mitochondrial dysfunction.

The fundamental metric of mitochondrial toxicity is a decline in mitochondrial function, and the best recognized function of the mitochondria is the NADH-mediated reduction of oxygen, leading to ATP production. ATP itself can be measured to assess mitochondrial toxicity, but the numerous pathways capable of generating ATP prevent it from being a specific marker for mitochondrial dysfunction. In contrast, specificity is obtained via measurement of the oxygen consumption rate (OCR) in the presence of various compounds that interrogate mitochondrial function (Figure 12.1). For example, the difference between the basal OCR and the OCR following the addition of ATP synthase inhibitors (e.g., oligomycin), mitochondrial uncouplers (e.g., FCCP), and electron transport chain inhibitors (e.g., antimycin and/or rotenone) enables the determination of ATP-coupled oxygen consumption, maximal cellular respiration and electron transport chain activity, and spare respiratory capacity. The cells and tissues that

are most susceptible to mitochondrial toxicants often have high mitochondrial reserve capacity, making basal OCR paradoxically insensitive to mitochondrial toxicants. For example, in renal proximal tubule cells, toxicants such as cisplatin, HgCl_2 , and gentamicin did not significantly affect basal OCR values, but they did decrease FCCP-uncoupled OCR in a dose-dependent manner (Beeson et al. 2010). These data show that even in the absence of diminished basal OCR or cytotoxicity, decreased FCCP-uncoupled OCR can be a sensitive readout for mitochondrial toxicity.

Measurement of oxygen consumption has been used by multiple groups to assess mitochondrial function using several techniques. The earliest means of assessing mitochondrial function was the Clark electrode (Wolfbeis 2015). Clark electrodes measure the current generated by the reduction of oxygen to water at a platinum electrode. This current is proportional to the oxygen available to the system. More recently, fluorescent and phosphorescent dyes for oxygen have been developed (Ferrick et al. 2008). These fluorescent or phosphorescent probes are generally dissolved and incorporated into cells or are fixed to a sensor. Oxygen quenches the excited state of the probe, allowing for the detection of oxygen levels via changes in quantum yield or excited-state lifetime. Unlike Clark electrodes, these optical probes can be readily adapted to high-throughput screening.

12.2 Models to Identify Mitochondrial Toxicants

One advantage of measuring changes in OCR is the ability to use either whole cells or isolated mitochondria. Due to their intact transport and metabolic pathways, whole cells can give a more accurate depiction of the effects of bioaccumulation and biotransformation on a compound's toxicity (Westwood et al. 2005; Dykens et al. 2008; Will and Dykens 2014). Similarly, whole cells allow for the detection of molecules that indirectly impact the mitochondria through cytosolic and nuclear signaling processes (e.g., suppression of mitochondrial biogenesis, increased autophagic flux, proteotoxic stress) (Kerkela et al. 2006; Zhao et al. 2010; Zhang et al. 2012). Immortalized cells can be passaged almost indefinitely, and even primary cells allow for significant yield from a single animal, particularly for cell types that can be repeatedly harvested (e.g., leukocytes, spermatozoa) (Vicente-Carrillo et al. 2015; Das et al. 2016). However, the advantages of immortalized cells for determining toxicity have caveats. First, different organs can have different susceptibilities to toxicants (Magnasco et al. 2008; Lin and Will 2012), so the use of a single cell type yields limited data in terms of *in vivo* toxicity.

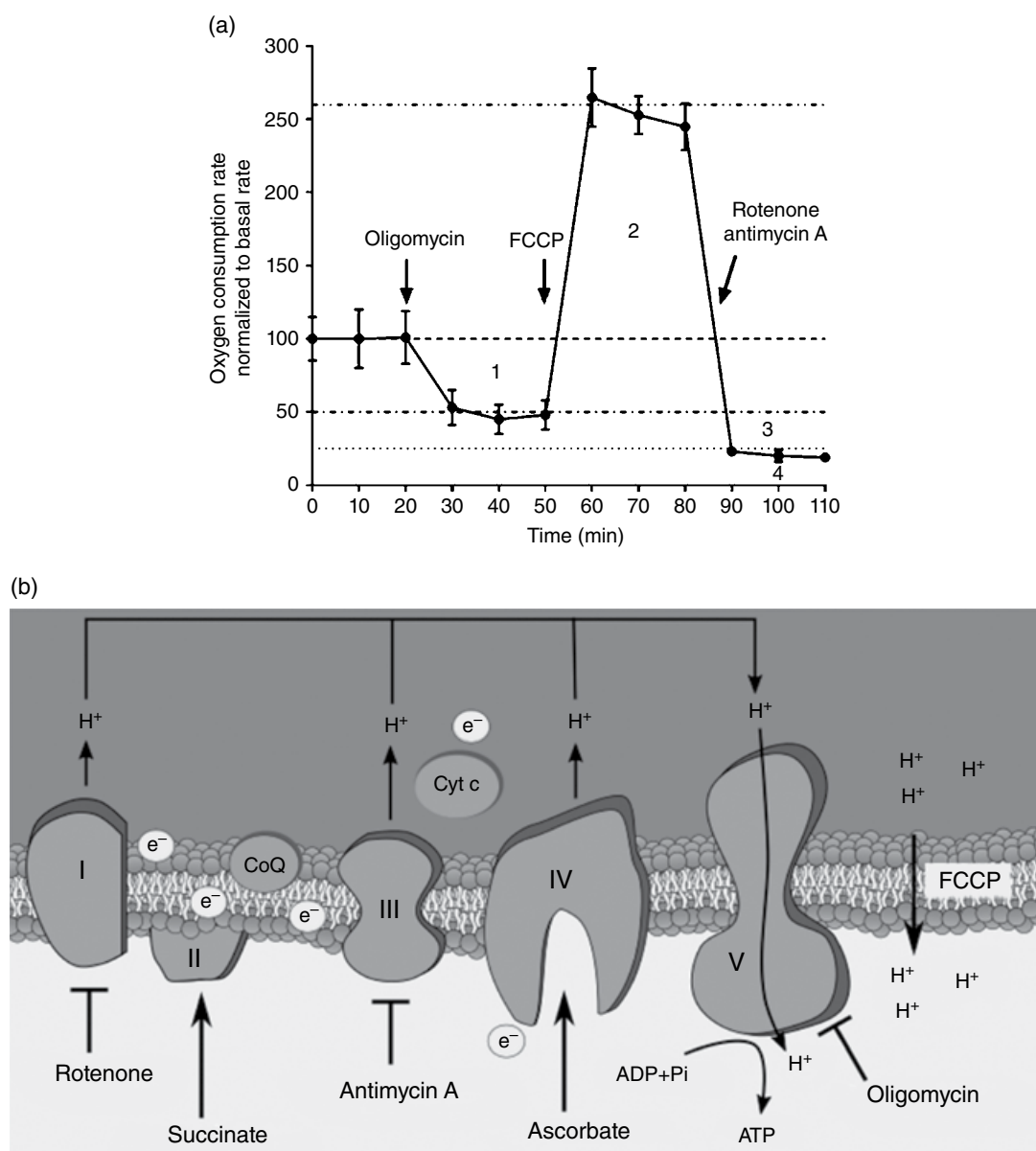


Figure 12.1 Probing mitochondria with small molecules. (a) A representative oxygen consumption rate (OCR) following the addition of the ATP synthase inhibitor oligomycin, the uncoupler FCCP, and the electron transport chain inhibitors rotenone and antimycin A. Using these compounds allows for the identification of oxygen consumption required for ATP production (1), maximal oxygen consumption and electron transport chain activity (2), proton leak (3), and non-mitochondrial oxygen consumption (4). (b) Inhibitors (rotenone, antimycin A, and oligomycin) and activators (succinate, ascorbate) of electron transport chain complexes and uncouplers of the mitochondrial proton gradient (FCCP) can be used to selectively monitor electron flow. These compounds can be used to determine the mechanism of mitochondrial toxicants.

Just as different tissues contain different metabolizing enzymes, different cells within a single tissue can have different effects on the biotransformation and bioaccumulation of compounds, thereby leading to different toxicity profiles for compounds (Schmid et al. 1993; Liu et al. 2012a). This phenomenon is particularly evident in hepatocytes, which can be characterized into zones based on their metabolic profiles. As a result, static cell

culture may not sufficiently identify a compound's toxicity; however, work with microfluidic cell culture has been able to mimic these *in vivo* effects in cell culture systems (Prill et al. 2016). Another concern in using cells is the specificity of a compound on the mitochondria. An increase or decrease in OCR may be due to the direct effects of a compound on the mitochondria or may be a result of modulation of distant signaling pathways that

affect oxygen consumption by the mitochondria or by other oxygen-consuming enzymes, such as NADH oxidoreductases (Herst and Berridge 2007).

Isolated mitochondria provide a means of specifically identifying direct mitochondrial toxicity (Rogers et al. 2011; Wills et al. 2013; Lu et al. 2016; Seydi et al. 2016; Salimi et al. 2017). This is of particular importance for identifying the mitochondrial targets of toxicants, as the mitochondrial proteome is much smaller than the cellular proteome. By employing inhibitors of various portions of the electron transport chain, specific targets of mitochondrial toxicants can be identified (Figure 12.1) (Schnellmann and Mandel 1986; Schnellmann et al. 1987a, b; Rogers et al. 2011). Additionally, isolation of mitochondria from transgenic cells or animals allows for the identification of genetic determinants of mitochondrial toxicity (Rogers et al. 2011). On the other hand, testing compounds on isolated mitochondria may not properly account for bioaccumulation or metabolites that modulate toxicity *in vivo* or even in whole cells (Dykens et al. 2008). Also, unlike cells, mitochondria cannot be cultured, so their use requires fresh isolation from metabolically competent tissues for each assay.

To reliably detect mitochondrial toxicants in cells, several criteria should be met. First, the cells should exhibit oxidative capacity to generate sufficient signal to noise to identify decreases in FCCP-uncoupled OCR. Because many immortalized cell lines rely on glycolysis rather than oxidative phosphorylation to generate ATP, cells in primary culture are an ideal system (Beeson et al. 2010; Wills et al. 2015; Liu et al. 2016). However, even primary cells require substantial optimization to maintain their basal OCR and therefore increase sensitivity while maintaining cellular viability. This may be achieved through the use of glucose-free or galactose-supplemented media, the addition of non-glycolytic substrates (such as lactate), supplementation with antioxidant cofactors (such as selenium and vitamin C), and mechanical means of increasing OCR (such as orbital shaking) (Nowak and Schnellmann 1995, 1996; Marroquin et al. 2007). Many of these requirements are also necessary for the use of mitochondria isolated from cultured cells, as the mitochondria isolated must be oxidatively competent to provide sufficient signal to detect toxicity (Bogucka et al. 1995). Alternatively, mitochondria may be directly isolated from freshly harvested tissue (Wills et al. 2013), thereby ensuring *in vivo*-like mitochondrial function.

Proper timing of treatment is also imperative, as mitochondrial toxicants can demonstrate effects on FCCP-uncoupled OCR at different times. For example, in a screen of a chemical library at 1, 6, and 24 h following treatment, different compounds decreased FCCP-uncoupled OCR at each time point (Wills et al. 2013).

These compounds were classified as fast-, moderate-, and slow-acting toxicants. The temporal distinction of these compounds' toxicities may relate to their capacity to act directly on the mitochondria as opposed to effects on other cellular processes, such as protein translation. Additionally, compounds that cause mitochondrial toxicity at early time points may have opposite effects at later ones. The isoflavone genistein decreases FCCP-uncoupled OCR at early time points in HepG2 cells (Attene-Ramos et al. 2013), but at later time points in primary cultures of kidney cells, this compound induces mitochondrial biogenesis to increase FCCP-uncoupled OCR (Rasbach and Schnellmann 2008).

12.3 Computational Models for the Identification and Development of Mitochondrial Toxicants

The development of techniques that readily identify mitochondrial toxicants has facilitated early preclinical screening for compounds with mitochondrial toxicities. However, such screening requires that the compound be synthesized and tested. Techniques that identify potentially toxic functional groups during the initial design of compounds can reduce the time and cost required for the development of new drugs. Both quantitative structure–activity relationship (QSAR) and toxicophore models have been widely used to predict cytotoxicity (Mercer et al. 2011; Beck et al. 2015; Onlu and Turker Sacan 2017), carcinogenicity (Hammond et al. 2003; Ford et al. 2016; Papamokos and Silins 2016), and specific organ toxicities (Bhavani et al. 2006; Graham et al. 2008; Frid and Matthews 2010; Singh et al. 2016). Preliminary work to apply these techniques to mitochondrial endpoints has proven that they hold great predictive value for identifying mitochondrial toxicants (Naven et al. 2013; Wills et al. 2013).

QSAR models identify common structural features and chemical descriptors from a testing set of compounds with known activities. Using statistical techniques, these models can be refined by pruning compounds from the training set and descriptors from initially generated models. The final model is used to predict the activities of unknown compounds. In choosing a training set of compounds, a balance must be struck between chemical similarity and diversity. An overly homogeneous training set diminishes predictive capacity by overemphasizing common but unimportant features (Christoph and Jeroen 2006). However, an overly diverse training set reduces the likelihood of common chemical features from which descriptors may be derived. Similar caution must be taken in identifying descriptors for the final

model (Eriksson et al. 2005). Clearly, a model with too few descriptors will ignore potentially important chemical information. On the other hand, too many descriptors can prevent accurate prediction of unknown compounds with chemical features not represented in the training set. Furthermore, related features (such as log *P* and number of aliphatic carbons) can overemphasize the importance of certain chemical properties. Perhaps the greatest limitation in the development of QSAR models is the need for adequate experimental data from which to generate a training set (Singh et al. 2016). This is particularly problematic for specific toxicological activities, such as mitochondrial uncoupling or inhibition of the ETC, where extensive testing of a training set is required before models can be developed.

Biophore models are another important computational method for the identification of toxicity. Biophores are generated by the superposition of multiple compounds with a similar activity (Kar and Roy 2013). Common chemical features (e.g., aliphatic groups, aromatic groups, hydrogen bond donors or acceptors) are identified based on this superposition, and their three-dimensional positions are used to create a generalized biophore. Depending on the desired activity and the chemical properties of the training set, biophores can comprise as few as three features or as many as tens of features. In the setting of toxicology screening, these toxicologic biophores (or toxicophores) are generally smaller, representing fragments of the compounds that relate to their unwanted toxicity but not their desired activity.

Like QSAR models, toxicophores can be used to screen chemical libraries to identify compounds that meet the criteria set forth by the toxicophore model (Kar and Roy 2013; Naven et al. 2013; Wills et al. 2013). As screening tools, toxicophores have several advantages over QSAR models. First, because they are generated from a set of chemical features, toxicophores are not bound to a chemical scaffold or even particular atoms and can be applied to chemical libraries as general models. They are also easier to develop and interpret than QSAR models, particularly with software that can perform conformational analyses and flexible alignments of component compounds. However, unlike QSAR models, biophores are not based on the potency of compounds (Wills et al. 2013), so toxicophores can predict whether a toxic event may occur but not the concentration at which it does so. This is particularly important in drug development, as derivatization can limit the effects of a toxicant by preventing bioaccumulation and enhancing the selectivity for its desired target (Nadanaciva et al. 2007). By combining toxicophores with QSAR models, compounds can be profiled for potency and efficacy of toxic effects.

One of the greatest problems in developing toxicophores and QSAR models is identifying the specific

activity that is being predicted. For small compound sets with well-defined targets, this is fairly simple to achieve. On the other hand, the development of these models from large chemical libraries can pose substantial challenges due to their chemical diversity, especially with functional readouts for mitochondrial toxicity. A screen of compounds for mitochondrial uncoupling was able to identify two main classes of toxicants (protonophores and redox cyclers) from which QSAR models and toxicophore fragments were derived (Naven et al. 2013). These models were able to identify two-thirds of uncouplers in a test library, but they were unable to accurately identify chemically dissimilar uncouplers. This work emphasizes the need for larger screens of compounds for mitochondrial toxicity to generate sufficient toxicophore models for early prediction of potential toxicants.

The problem of overly diverse chemical libraries can be resolved by clustering compounds by chemical similarity. A high-throughput respirometric screen of a large chemical library identified 31 mitochondrial toxicants that were sorted into two clusters by Tanimoto coefficient (Wills et al. 2013). From each cluster, compounds were aligned in chemical space to generate toxicophore models (Figure 12.2). One toxicophore, generated from 1,3-dimethyl-8-phenylxanthine, 3-isobutyl-1-methylxanthine, 1,3-dimethyl-7-[3-(3-methyl-1-piperidinyl)-3-oxopropyl]-xanthine, and calcimycin, was an eight-point model representing a xanthine-like core composed of two aromatic features, two hydrogen bond acceptors, a hydrogen bond donor or acceptor, a hydrophobic feature, a hydrogen bond donor or hydrophobic feature, and a hydrogen bond acceptor or bioisostere of CO₂ (Figure 12.2a). The other toxicophore based on *N*-(4-[4-(2-methoxyphenyl)-piperazin-1-yl]-butyl-5-(dimethylamino)-naphthalene-1-sulfonamide, 1-[(5-iodonaphthalen-1-yl)sulfonyl]-1,4-diazepane, and 1-(4-phenylpiperazin-1-yl)-2-tosylethanone was a seven-point model composed of two hydrophobic features, two hydrogen bond acceptors, a cation and hydrogen bond donor, and two aromatic or hydrophobic features (Figure 12.2b). Screening a 50,000 compound library using the second toxicophore identified 27 compounds, five of which decreased FCCP-uncoupled OCR in whole cells and three of which were toxic to isolated mitochondria.

Although no mechanistic studies were performed on these compounds, the authors were able to make inferences of their actions on mitochondria based on the known activities of compounds in the same chemical cluster. For example, the compounds used to generate the first toxicophore model are known to decrease mitochondrial respiration by disrupting the mitochondrial membrane potential. As more compounds are screened and more models are generated, toxicophores can be developed for specific targets to aid in drug development.

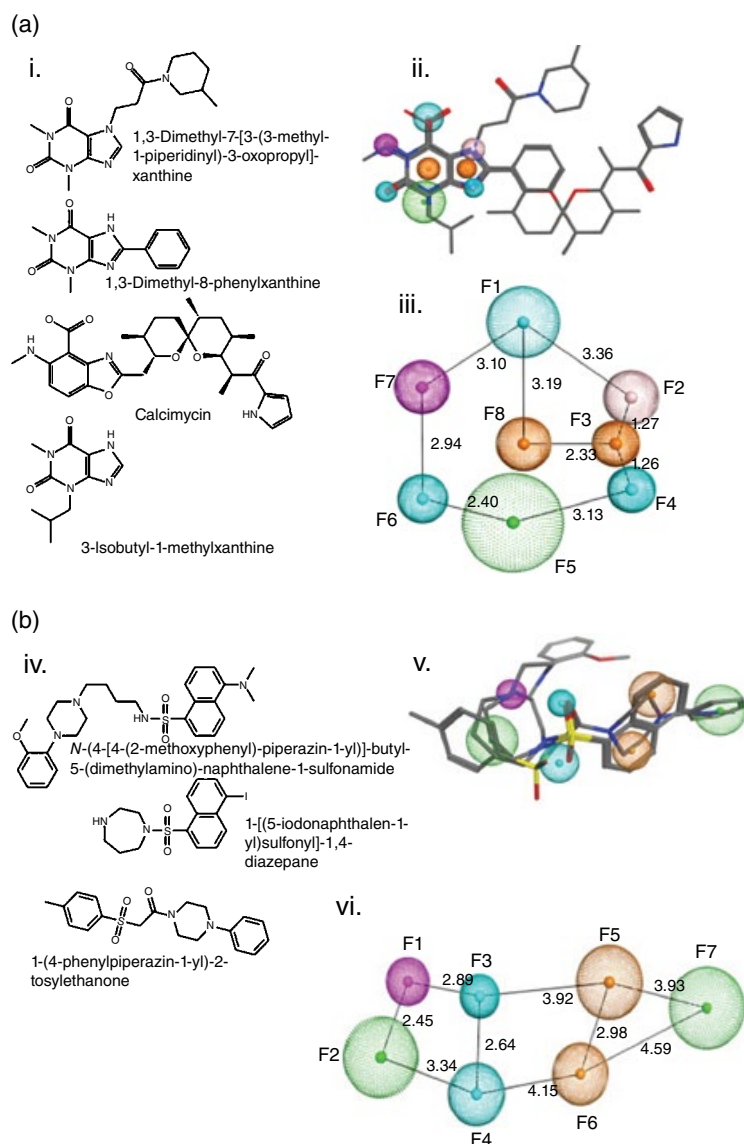


Figure 12.2 Developing toxicophore models from known toxicants. (a) Highly similar compounds with a shared chemical scaffold (i) are rigidly aligned (ii) to develop a toxicophore model (iii). This strategy is particularly useful for identifying functional groups to exclude from consideration during the optimization of lead compounds. (b) Compounds with moderate structural similarity but a dissimilar chemical scaffold (iv) are flexibly aligned (v) to generate a toxicophore model (vi). This strategy is particularly useful for the identification of potential toxicants during initial screening of large chemical libraries. Adapted from Wills et al. (2013). Reproduced with Permission of Elsevier. (See insert for color representation of the figure.)

12.4 Concluding Remarks

The development of high-throughput respirometric assays has facilitated the screening of large chemical libraries to identify mitochondrial toxicants. By measuring OCR, investigators can determine the effects of new compounds on mitochondrial function in whole cells and isolated mitochondria. Despite the numerous considerations required to optimize a system for the identification of mitochondrial toxicants, the number

of compounds withdrawn due to mitochondrial toxicities necessitates sensitive preclinical screens for mitochondrial function. As more functional screens are performed, computational techniques such as QSAR and toxicophore models can identify chemical features responsible for various aspects of mitochondrial toxicity. As these features are identified, compounds can be developed to minimize mitochondrial toxicity and ADEs and better streamline the drug development pipeline.

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13

The Parallel Testing of Isolated Rat Liver and Kidney Mitochondria Reveals a Calcium-Dependent Sensitivity to Diclofenac and Ibuprofen

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CHAPTER MENU

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13.1 Introduction

The integrity of the mitochondrial inner and outer membranes is a prerequisite for the viability of higher eukaryotic cells, and disruption of the outer mitochondrial membrane (MOMP) is considered as “point of no return” that irreversibly seals the cell’s fate to die (Kroemer et al., 2007). Cellular damaging insults may proceed toward MOMP indirectly, for example, via signaling events that activate so-called BH3-only proteins that may subsequently cause MOMP (Kuwana et al., 2002) or via elevation of the universal cellular stress signal calcium that may provoke mitochondrial permeability transition (MPT) with subsequent outer membrane rupture (Bernardi, 2013; Feldmann et al., 2000; Halestrap, 2010). Mechanistically, such indirect mitochondrial impairments may be distinguished from toxic conditions or chemicals that directly target molecular structures in mitochondria, for example, their capacity to produce cellular energy or their capability to sustain their inner membrane potential (mitochondrial membrane potential (MMP)). Thus, although the final outcome may be cell death for many, if not most, of these

insults, mechanistically, mitochondrial damage is not a uniform stereotypic process. Especially with respect to potential (therapeutic) countermeasures, it is a prerequisite to understand the specific mode of action of diverse mitochondrio-toxic conditions. This is one main focus of “mitochondrial toxicology,” an emerging research field that aims at unraveling the mechanisms of mitochondrio-toxic insults and their cellular and systemic consequences and at finding potential remedies.

Due to their organ-specific tasks, mitochondria from diverse tissues vary considerably in their molecular composition (Fleischer et al., 1967; Mootha et al., 2003) and may consequently respond with organ-specific vulnerabilities toward toxic insults. Therefore, one key issue of mitochondrial toxicology is to determine such—potentially tissue-specific—toxic modes of action.

A tissue-specific mode of mitochondrial impairments may appear as unintended side effect of diverse pharmaceuticals. For example, the widely used nonsteroidal anti-inflammatory drugs (NSAIDs) are considered well-tolerated drugs. However, gastrointestinal symptoms as well as mild or severe liver and kidney toxicity have been reported (Boelsterli, 2003; Lim et al., 2006; Ng et al., 2006; Szewczyk and Wojtczak, 2002). Here, the incidence of renal adverse effects is dramatically higher

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(1–5%) (Whelton, 1999) compared with NSAID-related liver injury (ranging from 3 to 23 per 100,000 patients) (Aithal and Day, 2007). Especially the NSAID diclofenac has been discussed as a potential “mitotoxin,” exerting its toxic effect via an increase of intracellular calcium and mitochondrial ROS production (Boelsterli and Lim, 2007). Such mitochondrial impairment may subsequently lead to energetic crises in tissues already damaged or predisposed by mitochondrial alterations (e.g., silent mutations in the mitochondrial DNA or oxidative stress) (Boelsterli, 2003).

At present, however, tissue-specific sensitivities toward mitotoxins are poorly predictable by theoretical considerations alone and require empirical examination. Accordingly, we have developed a protocol for the parallel isolation of mitochondria from diverse rat tissues, for example, liver and kidney, that allows simultaneous analyses of tissue-specific mitochondrial responses toward a variety of toxicants (Schulz et al., 2015). This mitochondrial damage can be followed in parallel in a dose-dependent fashion by a multitude of parameters, for example, by their ATP production capacity or by their oxygen radical (ROS) production, enzymatic activities or respiratory measurements, and others more (Lichtmanegger et al., 2016; Schulz et al., 2013). Here, we have chosen the eliciting of the MPT (assessed by $OD_{540\text{ nm}}$ changes in mitochondrial suspensions) and the stability of the MMP ($\Delta\psi_m$, assessed by the fluorophore Rh123) as two readouts of potential organelle damage. Whereas the MPT is a severe irreversible mitochondrial damage that is associated with cell death, a change in MMP is a very sensitive marker for an initial mitochondrial impairment that might even be reversible in several cases. Both readouts are also linked together as the MPT process starts with an increased permeability of the inner membrane and thus with an MMP depletion.

Using these readouts, we have comparatively analyzed the influence of increasing ibuprofen and diclofenac concentrations on rat liver and kidney mitochondria in parallel. We report here that these NSAIDs are directly toxic to these mitochondria if additional low levels of calcium are present. This mitochondrial damage may be overcome by MPT-blocking compounds in liver mitochondria but to a much lesser extent in kidney mitochondria.

13.2 Methods

13.2.1 Parallel Isolation of Mitochondria from Rat Tissues

Mitochondria from rat liver and kidney were isolated as recently reported (Schulz et al., 2015). An outline of the

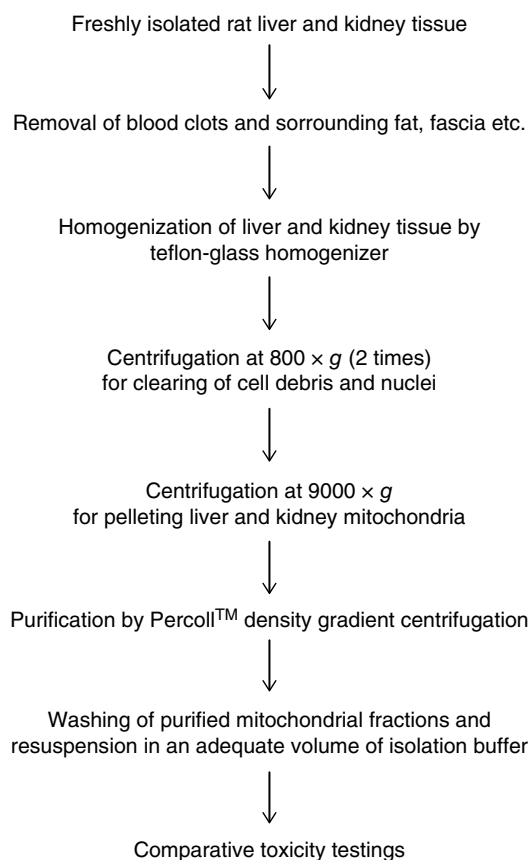


Figure 13.1 Flow chart for the simultaneous isolation of mitochondria from rat liver and kidney for further structural and functional analysis.

isolation procedure is given in Figure 13.1. Briefly, rat organs were rinsed with ice-cold 0.9% NaCl, placed in ice-cold isolation buffer containing BSA (0.3 M sucrose, 5 mM TES, 0.2 mM EGTA, 0.1% BSA (w/v), pH 7.2), and cleared from surrounding tissue and blood. Liver and kidney were homogenized using a Teflon-glass homogenizer with 5–10 strokes at 600 rpm. Both tissue homogenates were centrifuged twice for 10 min at $800 \times g$ and 4°C for clearance of cell debris and nuclei. From the supernatant, liver and kidney mitochondria were pelleted by centrifugation at $9000 \times g$ and 4°C for 10 min. The resulting crude mitochondrial fractions were further purified by density gradient centrifugation at $9000 \times g$ using an 18/30/60% Percoll™ gradient system. The purified organelles were washed and resuspended in isolation buffer without BSA (0.3 M sucrose, 5 mM TES, 0.2 mM EGTA, pH 7.2). Ideally, the isolation procedure can be performed by one person in less than 2 h.

Structural integrity of isolated mitochondria was verified by electron microscopy (Figure 13.2a) and functional integrity by measurement of the MPT as well as the MMP. Mitochondria from both organs revealed a stable optical density (Figure 13.2b, c) and membrane potential over

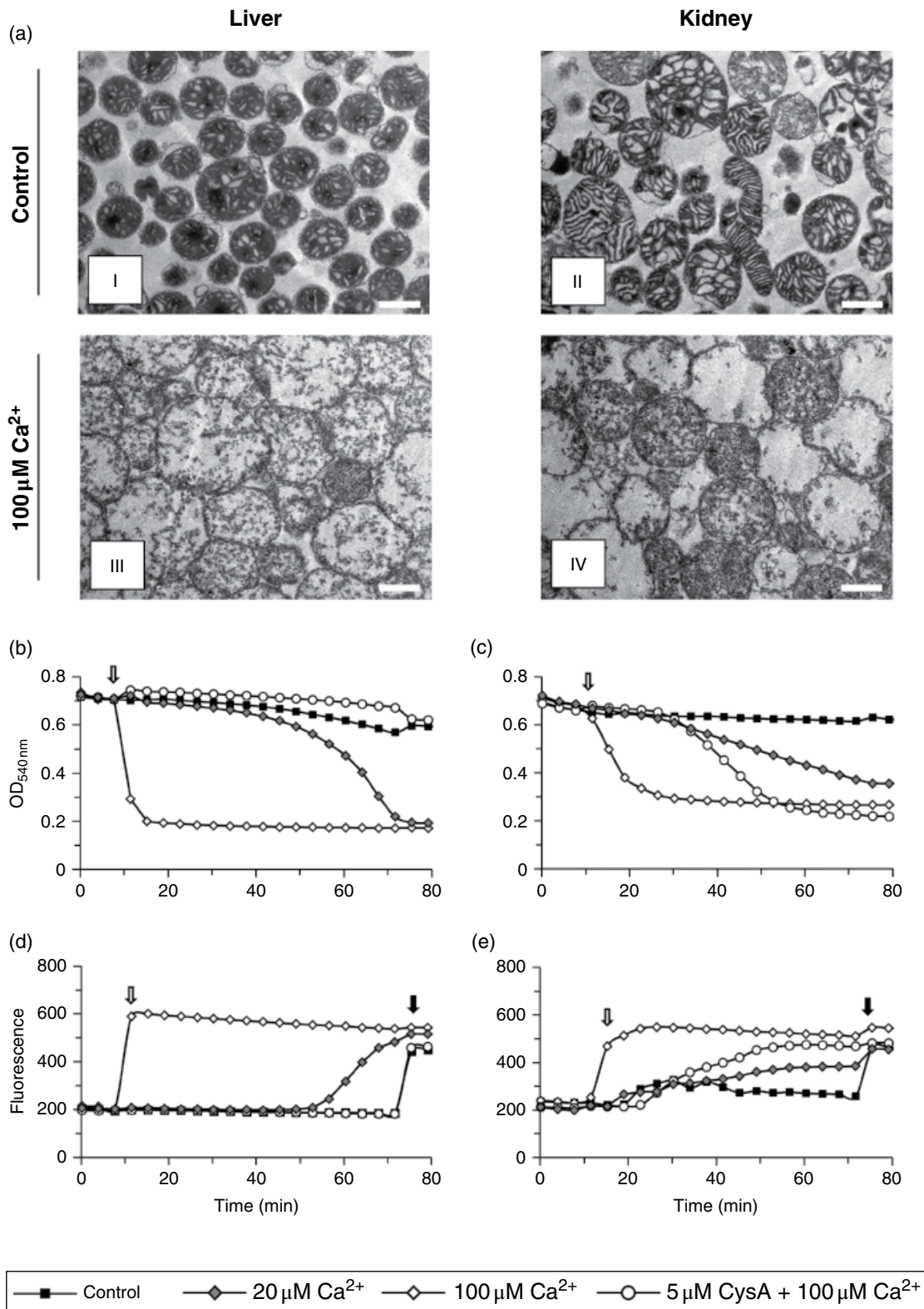


Figure 13.2 Isolated mitochondria from rat liver and kidney respond differently to calcium challenge. (a) Electron micrographs of mitochondria isolated in parallel from rat liver and kidney tissues show comparable high purities and intact inner and outer membranes. A 100 μM Ca^{2+} challenge results in mitoplast formation in mitochondria isolated from both tissues, that is, enlarged inner membrane vesicles with highly electron-transmissive matrices and disrupted or depleted outer membranes. Scale bars equal 1 μm . (b, c) In rat liver and kidney mitochondria, the presence of 20 μM Ca^{2+} leads to a late loss of OD_{540nm}, whereas 100 μM Ca^{2+} rapidly induce mitochondrial permeability transition (MPT) as assessed by a massive OD_{540nm} decrease in comparison with control conditions. The preincubation of rat liver mitochondria with 5 μM cyclosporine A (CysA) completely prevents Ca^{2+} -induced MPT. In contrast, CysA only slows down Ca^{2+} -induced MPT in kidney mitochondria. (d, e) In liver and kidney mitochondria, 100 μM Ca^{2+} results in an immediate mitochondrial membrane potential (MMP) depletion, which can be fully prevented by 5 μM CysA in liver, but only delayed in kidney mitochondria. White arrows indicate the Ca^{2+} addition, and black arrows indicate the addition of the protonophore FCCP as internal positive control. Shown are exemplary curves ($N=3$).

75 min (Figure 13.2d, e) under control conditions (i.e., in the presence of succinate as substrate and complex I inhibitor rotenone to prevent reverse electron flow).

13.2.2 Electron Microscopy

Electron microscopy of isolated mitochondria was done as previously described (Schulz et al., 2013; Zischka et al., 2008) on a 1200EX electron microscope (JEOL, Tokyo, Japan) at 80 kV. Pictures were taken with a KeenView II digital camera (Olympus, Germany) and processed by the iTEM software package (analySIS FIVE, Olympus, Germany).

13.2.3 Assessment of the Mitochondrial Membrane Potential (MMP)

MMP was monitored by Rh123 fluorescence for 80 min (Schulz et al., 2015; Zamzami et al., 2000). Addition of the protonophore FCCP after 75 min served as internal control (Kessler et al., 1976).

13.2.4 Analyses of the Mitochondrial Permeability Transition (MPT)

The MPT (“swelling”) was monitored in mitochondrial suspensions (75 µg mitochondrial protein in a total volume of 200 µL) at OD_{540nm} in the presence of succinate/rotenone for 80 min (Bernardi et al., 1992; Zischka et al., 2008).

13.2.5 Miscellaneous

Protein concentrations were determined by the Bradford assay (Bradford, 1976). Stock solutions of ibuprofen and diclofenac (Sigma-Aldrich, Munich, Germany) were prepared with ethanol or double-distilled water, respectively.

13.3 Results and Discussion

13.3.1 Parallel Isolation of Intact Mitochondria from Various Rat Tissues

The parallel isolation of mitochondria from rat liver and kidney tissues as outlined in Figure 13.1 (Schulz et al., 2015) resulted in highly homogeneous mitochondrial populations as evidenced by electron microscopy (Figure 13.2a, panels I and II). These populations appeared with high functional stability as demonstrated by a constant optical density of mitochondrial suspensions (OD_{540nm}) and a time-stable MMP over 75 min under control conditions (Figure 13.2b–e). This robustness is a

prerequisite for the direct comparison of mitochondrial sensitivities to mitotoxic agents in a dose-dependent fashion.

Figure 13.2a (panels I and II) depicts the comparable structural integrity of the mitochondrial populations isolated using the present protocol. Typically, isolated mitochondria appear in the “condensed” state, that is, with well-defined cristae and electron-dense matrices, as originally reported by Charles Hackenbrock (1966). Profound differences were, however, apparent between these two mitochondrial populations despite their overall structural similarity. Whereas liver mitochondria appeared with a prominent electron-dense matrix, kidney mitochondria showed a high cristae abundance, frequently in tubular orientation (Figure 13.2a, panel I vs. II). While these structural peculiarities may be the visible manifestation of the differing molecular compositions of these mitochondrial populations (Forner et al., 2006; Mootha et al., 2003), they also indicate that organelles with different structures may respond differently to mitotoxic insults.

Subjecting isolated liver and kidney mitochondria to a challenge of 100 µM Ca²⁺ elicits the MPT. This results in mitochondrial volume expansion, termed “swelling,” and mitoplast formation. The latter are enlarged inner membrane vesicles with electron-transmissive matrices and disrupted or depleted outer membranes (Figure 13.2a, panels III, IV). The MPT with consequent mitoplast formation is typically assessed by a decline in the optical density at 540 nm of mitochondrial suspensions (Zischka et al., 2008). While both populations of mitochondria underwent MPT at 100 µM Ca²⁺, lower Ca²⁺ challenges caused differential responses. At 20 µM Ca²⁺, liver mitochondria responded with a delayed but rapid OD_{540nm} loss, whereas in kidney mitochondria a sustained long-lasting OD_{540nm} loss was apparent (Figure 13.2b vs. c). In addition, over the tested time period, Ca²⁺-induced MPT (100 µM) was fully inhibited in liver mitochondria by the MPT inhibitor cyclosporine A (Halestrap, 2010) (5 µM CysA; Figure 13.2b, c). On the contrary, the eliciting of the MPT in rat kidney mitochondria was delayed but not abolished by CysA (Figure 13.2b vs. c). This demonstrates molecular differences with respect to MPT induction and progression in these two mitochondrial populations.

While both mitochondrial populations revealed a time-stable MMP over the tested 75 min under control conditions, Ca²⁺ (100 µM)-induced MPT was accompanied by a simultaneous MMP loss (Figure 13.2d, e; i.e., an increased Rh123 fluorescence, FCCP served as internal control, respectively). However, a gradual MMP loss occurred in kidney mitochondria upon 20 µM Ca²⁺ challenge, which was comparatively delayed and abrupt in liver mitochondria. This different responsiveness to

calcium was even more pronounced upon a $100\mu\text{M}$ Ca^{2+} challenge in the presence of CysA. While kidney mitochondria responded with an early gradual MMP loss, liver mitochondria were fully protected over the tested time period (Figure 13.2d, e). Our findings confirm earlier reports concerning differences of liver and kidney mitochondria. For example, calcium uptake of liver mitochondria was found to already occur at low intracellular calcium concentrations ($0.5\text{--}1\mu\text{M}$), whereas calcium uptake of kidney mitochondria was present only when the free cytosolic Ca^{2+} was raised to $1.5\text{--}2\mu\text{M}$ (Favaron and Bernardi, 1985). In agreement, Zazueta et al. (2010) described a higher sensitivity of liver versus kidney mitochondria to calcium-induced swelling. Therefore, the current protocol for comparative mitochondrial testing provides important information whether a given toxic condition directly affects mitochondria, whether such mitochondrial sensitivity is tissue specific, and/or whether the resulting mitochondrial damage varies in different tissues. The latter issue is of key interest with respect to potentially different consequences in the affected tissues, ranging from repair of the injury to cell death.

13.3.2 Ibuprofen and Diclofenac Differently Impair the MMP of Mitochondria from Rat Liver and Kidney

The nonsteroidal anti-inflammatory drugs ibuprofen and diclofenac are mostly well-tolerated drugs. Nevertheless, unintended side effects have been described for both (Lim et al., 2006; Pérez-Gutthann et al., 1999; Sarzi-Puttini et al., 2014; Szewczyk and Wojtczak, 2002). As Boelsterli and Lim (2007) have pointed out, mitochondria are key organelles in cellular homeostasis and thus are potential targets of drug-induced toxicity. These authors have linked mitochondrial impairments as potential matter of concern to several drugs (Kashimshetty et al., 2009; Neustadt and Pieczenik, 2008; Pessayre et al., 1999). One hypothesis, which is relevant to the present study, is that otherwise pre-damaged mitochondria may present with an increased susceptibility toward pharmaceutical intervention (Chang et al., 2010; Geng et al., 2012; Gupta and Lewis, 2008; Irurzun et al., 1995; Lajdova et al., 2009). This would provide an explanation why such side effects may occur in some cases but not in others. To test this hypothesis, we isolated mitochondria from rat liver and kidney and treated both with increasing concentrations ($100\text{--}400\mu\text{M}$) of ibuprofen or diclofenac, respectively. These tests were done either in the absence or presence of nontoxic calcium challenges in order to mimic an “intracellular stress milieu,” which typically coincides

with intracellular calcium elevations (Kroemer and Reed, 2000). The effects of both NSAIDs on mitochondrial function were assessed in parallel using the eliciting of the MPT and the highly sensitive MMP ($\Delta\psi_{\text{m}}$) as readouts of potential organelle damage. Each MMP measurement was terminated by the addition of the MMP-depleting protonophore FCCP, which served as internal MMP control. The ratio of the MMP-dependent Rh123 fluorescence before and after FCCP addition served as additional semiquantitative parameter of mitochondrial functional integrity.

In the tested concentration range and in the absence of additional calcium, ibuprofen showed no direct toxic effects on rat liver mitochondria, on MPT eliciting (Figure 13.3a), or on MMP (Figure 13.3c). Similarly, ibuprofen did not elicit the MPT in rat kidney mitochondria as demonstrated by unaltered optical density (Figure 13.3b). However, slight MMP depletions were noted in kidney mitochondria upon an ibuprofen challenge (especially at $400\mu\text{M}$; Figure 13.3d) as indicated by slightly decreased $\text{FCCP}_{\text{before/after}}$ ratios ($100\mu\text{M}$, 1.65 ± 0.02 ; $200\mu\text{M}$, 1.64 ± 0.04 ; $400\mu\text{M}$, 1.63 ± 0.15 vs. control: 1.87 ± 0.06 , $N=3$). It therefore appears that, under the chosen experimental conditions, MMP is slightly affected in kidney but not in liver mitochondria by higher ibuprofen concentrations.

Diclofenac did not elicit the MPT in liver mitochondria (Figure 13.3e) but higher concentrations ($400\mu\text{M}$) apparently affect the MMP (Figure 13.3g) ($\text{FCCP}_{\text{before/after}}$ ratio: $400\mu\text{M}$: 1.34 ± 0.04 vs. control: 2.24 ± 0.13 , $N=3$). This latter finding is in excellent agreement with previously published data of diclofenac-associated cell toxicity. Li et al. (2009) and Masubuchi et al. (2002) described a diclofenac-dependent MMP reduction in primary rat hepatocytes at concentrations ranging from 250 to $800\mu\text{M}$. Furthermore, such diclofenac concentrations may cause significant decreases in cellular proliferation as evidenced in immortalized human hepatocytes (Lim et al., 2006).

Similarly, to the observations in liver mitochondria, in the absence of additional calcium, diclofenac did not elicit MPT in kidney mitochondria (Figure 13.3f). However, the MMP of kidney mitochondria was impaired by diclofenac in a dose-dependent manner (Figure 13.3h). The $\text{FCCP}_{\text{before/after}}$ ratios were markedly decreased compared with control conditions (1.87 ± 0.06), ranging from 1.58 ± 0.14 ($100\mu\text{M}$ diclofenac) down to 1.26 ± 0.04 ($400\mu\text{M}$ diclofenac). This MMP-depleting effect of diclofenac may be of importance as diclofenac-induced (rare) cases of acute kidney failure have been linked to alterations of MMP (Ng et al., 2006). In fact, these authors have demonstrated concentration-dependent diclofenac ($5\text{--}100\mu\text{M}$)-induced MMP depletion in

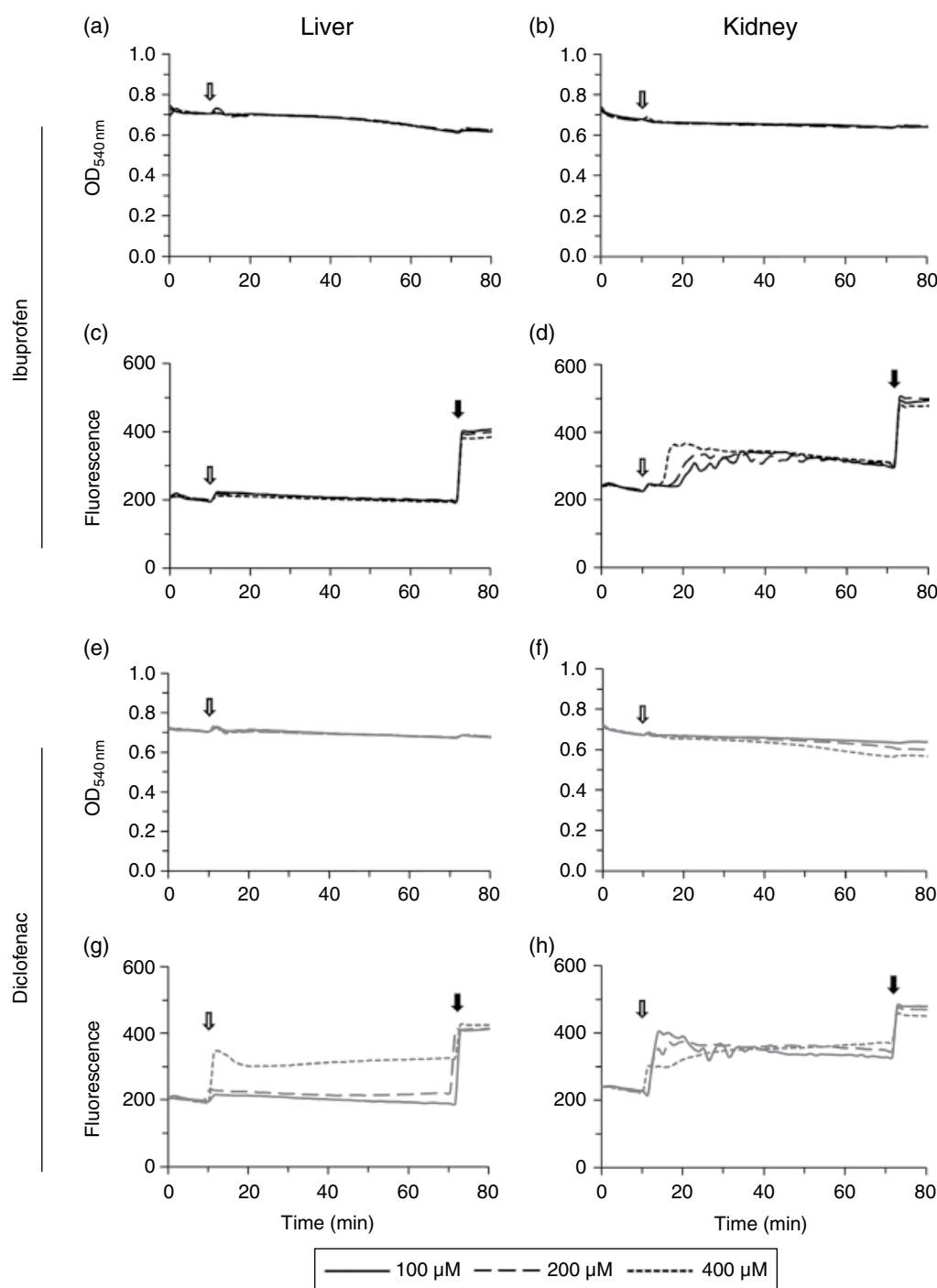


Figure 13.3 Ibuprofen and diclofenac do not induce the mitochondrial permeability transition, but high concentrations may impair the mitochondrial membrane potential. (a, b) Ibuprofen does not induce MPT in liver and kidney mitochondria. (c, d) In contrast to liver mitochondria, kidney mitochondria reveal a higher sensitivity to ibuprofen as shown by partial MMP losses. (e, f) MPT is not induced in rat liver mitochondria by diclofenac. However, a slight loss of OD_{540nm} is detectable in kidney mitochondria, indicating a higher susceptibility compared with liver mitochondria. (g, h) Diclofenac impairs the mitochondrial membrane potential in liver and especially in kidney mitochondria in a dose-dependent manner. White arrows indicate the NSAID addition, and black arrows indicate the addition of the protonophore FCCP as internal positive control. Shown are exemplary curves ($N=3$).

kidney mitochondria, which may subsequently cause impaired levels of ATP production (Ng et al., 2006).

Despite this latter finding, the herein reported mitochondrial testing demonstrated a relative robustness of liver and kidney mitochondria to ibuprofen and diclofenac, which reflects the fact that both NSAIDs typically present with a low risk of unintended side effects. The plasma concentration of ibuprofen usually does not exceed 400 μM (Bessone, 2010), although ibuprofen tissue accumulations up to 900 μM have been reported (Baggott et al., 1992). Thus, the absence of direct damaging of ibuprofen on mitochondria with the concentration range tested here (100–400 μM) is of pharmacological relevance. In contrast to these concentrations, typical diclofenac plasma concentrations are markedly lower (around 5 μM (Assandri et al., 1992; Brühlmann et al., 2006)). Thus, the herein observed MMP-depleting effect of 100–400 μM diclofenac may be considered as a “theoretical risk,” that is, what might happen at the very upper end of the concentration scale. This estimation surely is valid for intact “healthy” mitochondria. In cellular stress situations, however, mitochondrial vulnerabilities might be significantly different. This is due to the fact that stress responses like emerging ROS or elevated intracellular calcium levels directly impact on mitochondria (Cichoż-Lach and Michalak, 2014; Duchon, 2000). We therefore tested the NSAIDs in the presence of elevated (nontoxic) calcium challenges in order to mimic such an intracellular stress milieu.

13.3.3 Ibuprofen and Diclofenac Toxicity on Isolated Mitochondria Is Markedly Increased by Calcium

The preincubation of liver (Figure 13.4a, c) and kidney (Figure 13.4b, d) mitochondria with 20 μM calcium, a concentration that per se caused a late occurring MPT (Figure 13.2b, c), induced a massively pronounced sensitivity to ibuprofen and diclofenac in a dose-dependent manner.

In the presence of calcium, already low concentrations of ibuprofen and diclofenac rapidly elicited the MPT paralleled by a complete MMP loss within minutes (Figure 13.4). These findings are in agreement with previous studies (Li et al., 2009; Masubuchi et al., 2002) that demonstrated the impairment of the MMP by 50–100 μM diclofenac in the presence of calcium in isolated rat liver mitochondria as well as primary hepatocytes.

Taken together, these results indicate that such NSAIDs should be handled with care in comorbidity cases, especially in patients with pre-damaged livers (Laffi et al., 1997; Riley and Smith, 1998; Rossi et al., 2008). Interestingly, diclofenac was previously described to alter the intracellular calcium concentrations in

hepatocytes itself (Lim et al., 2006), and Pigoso et al. (1998) reported the release of calcium from isolated rat kidney mitochondria upon diclofenac treatment. A cytosolic calcium peak was observed about 8 h after the intake of 1 mM diclofenac, which preceded cellular toxicity detected after greater than 48 h (Lim et al., 2006). Whether such calcium pulses might contribute to mitochondrial pre-damaging, thereby increasing the sensitivity to diclofenac, is an intriguing issue that remains, however, for future studies.

13.3.4 Cyclosporine A (CysA) Provides Mitochondrial Protection to Ibuprofen/ Ca^{2+} -Induced Damage

In rat liver mitochondria, CysA completely inhibited ibuprofen/ Ca^{2+} -induced MPT (Figure 13.5a), and the MMP remained stable over 75 min until the termination of the measurement by FCCP addition (Figure 13.5c). This finding indicates that MPT-blocking compounds might be plausible interventions in cases of ibuprofen-caused accelerated liver damage of otherwise pre-damaged livers. In rat kidney mitochondria, however, the ability of CysA to block ibuprofen/ Ca^{2+} -induced MPT was limited to lower ibuprofen concentrations (Figure 13.5b, d).

In contrast to the situation with ibuprofen, the $\text{OD}_{540\text{nm}}$ decrease and MMP loss of rat liver mitochondria treated with diclofenac/ Ca^{2+} was only prevented by CysA at lower diclofenac concentrations (Figure 13.5e, g). Importantly, CysA did not protect kidney mitochondria from MPT damage and MMP loss upon diclofenac/ Ca^{2+} challenges (Figure 13.5f, h). Here, already the lowest tested diclofenac concentration decreased the MMP dramatically (Figure 13.5h). Thus, from a “mitochondrial perspective,” a kidney with elevated intracellular calcium levels is at risk of organ damage by diclofenac, which can hardly be decreased by MPT-blocking compounds (Rossi et al., 1985; Shohaib, 2000).

13.4 Conclusions

As mitochondrial integrity and functionality are key issues for cellular homeostasis, toxicity on these organelles has to be assessed upon exposure to chemicals, toxins, and pharmaceutical interventions. In this respect, it is of importance to test such compounds at the level of isolated organelles, either to establish or to rule out directly damaging effects on mitochondria. In addition, this approach enables the straightforward, parallel, and dose-dependent testing of mitochondrial sensitivities to combinations of potentially damaging compounds. The parallel isolation of intact organelles with similar purity from various tissues (here liver and

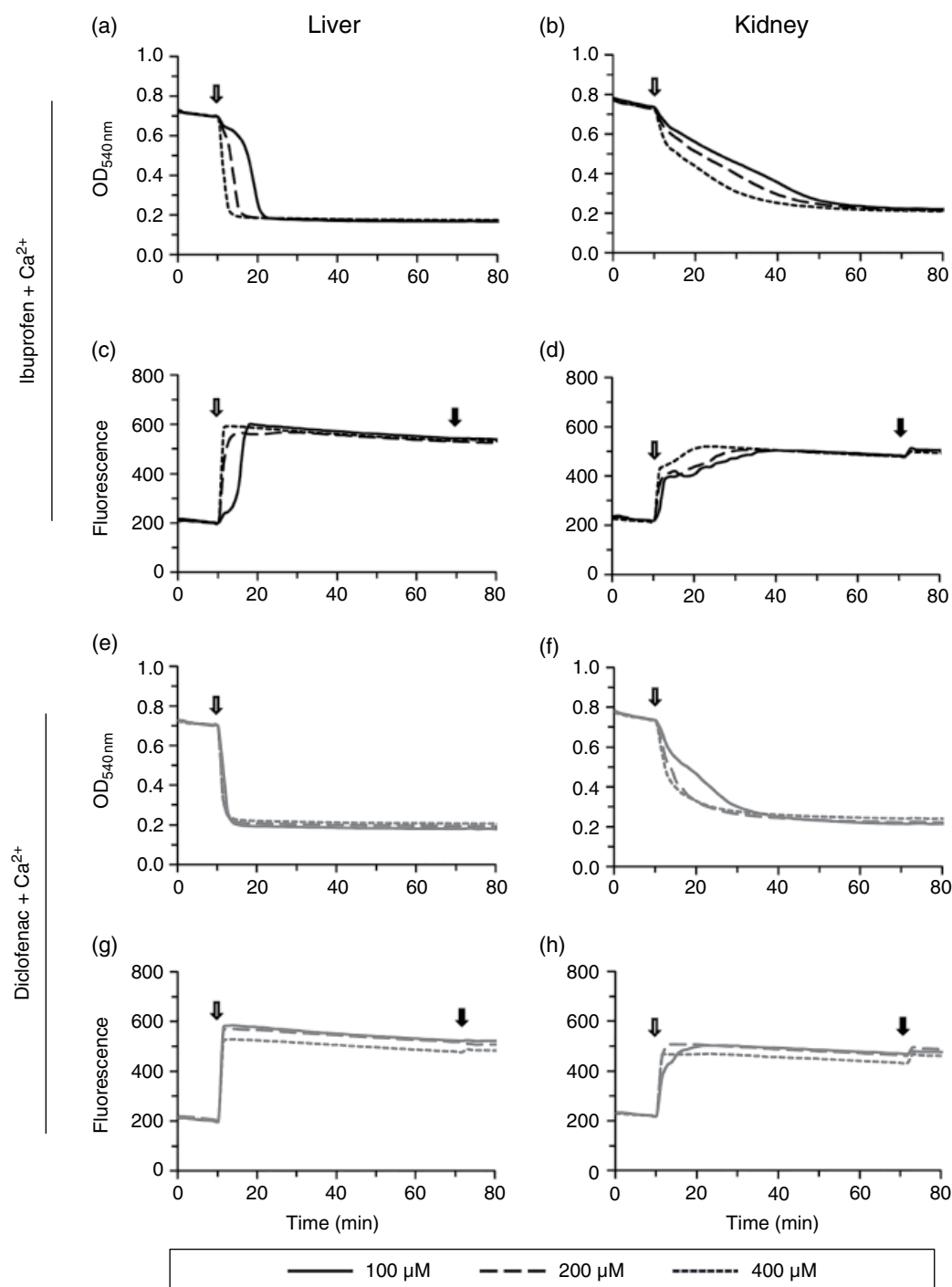


Figure 13.4 Ibuprofen and diclofenac cause the induction of the MPT and the loss of MMP in calcium-pretreated liver and kidney mitochondria. (a–d) In the presence of 20 μM Ca^{2+} , ibuprofen causes an immediate dose-dependent MPT induction and MMP loss in rat liver and kidney mitochondria. (e–h) In the presence of 20 μM Ca^{2+} , diclofenac immediately induces MPT and MMP loss in liver and kidney mitochondria, whereas kidney mitochondria are slightly less sensitive. White arrows indicate the NSAID/ Ca^{2+} addition, and black arrows indicate the addition of the protonophore FCCP as internal positive control. Shown are exemplary curves ($N=3$).

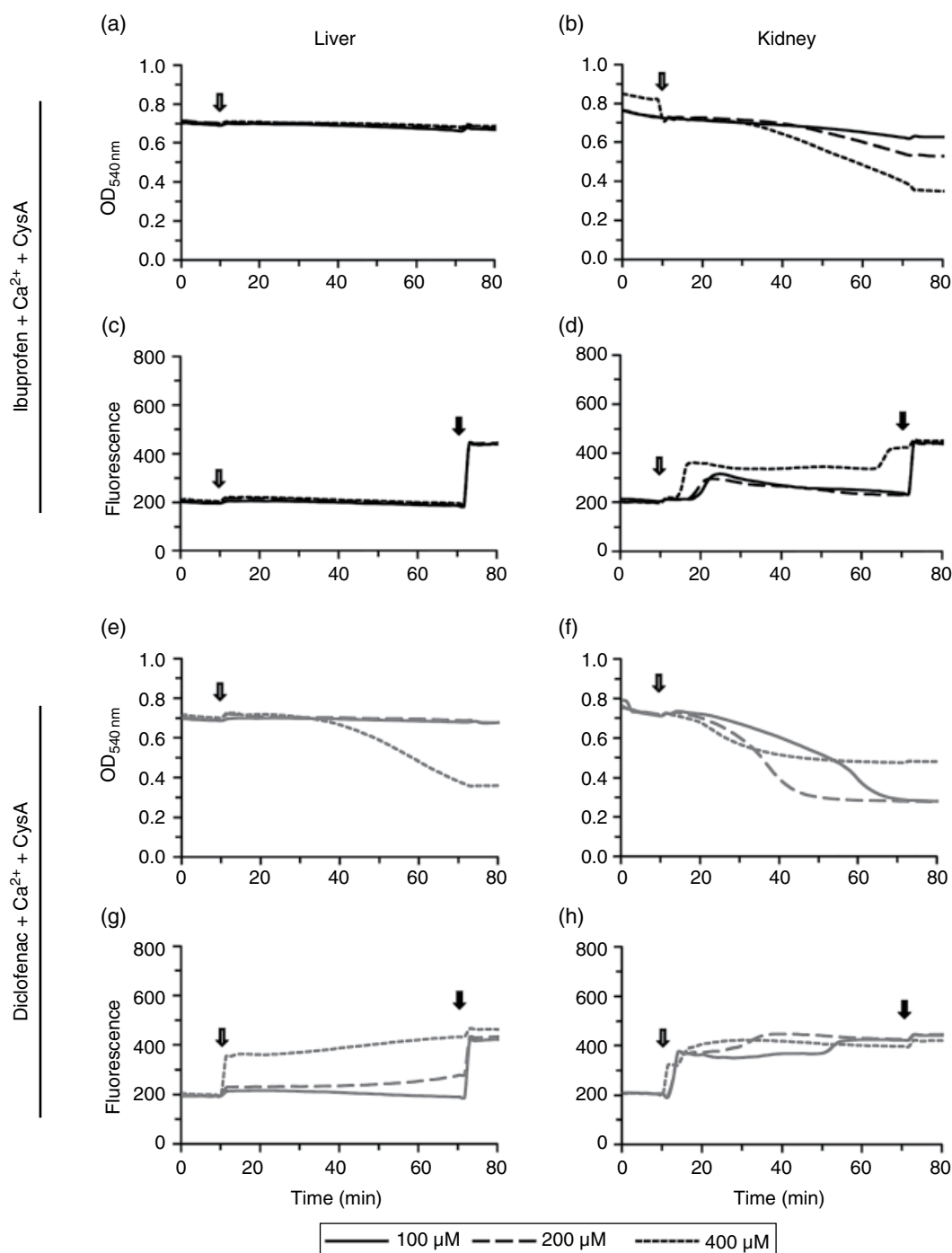


Figure 13.5 Cyclosporine A can protect against the calcium-dependent toxicity of ibuprofen in rat liver mitochondria. (a–d) CysA is protective against ibuprofen/Ca²⁺ MPT induction and MMP loss in rat liver mitochondria but to a markedly lesser extent in kidney mitochondria. (e–h) The diclofenac/Ca²⁺ induced MPT and MMP loss is largely prevented by CysA in liver but not in kidney mitochondria. CysA was present at the beginning of the measurement. White arrows indicate the NSAID/Ca²⁺ addition, and black arrows indicate the addition of the protonophore FCCP as internal positive control. Shown are exemplary curves ($N=3$).

kidney, elsewhere also heart or brain (Schulz et al., 2015)) allows for the comparative assessment of tissue-specific potentially increased mitochondrial vulnerabilities. In fact, such isolated mitochondrial populations may be tested for a plethora of parameters, for example, structural integrity by electron microscopy, functionality by biochemical or biophysical assays, and molecular composition by proteomics or lipidomics. Finally, this approach may not only provide important information on compounds that directly

damage mitochondria in a tissue-specific manner but also create testable hypotheses under which conditions or pathological settings such compounds may cause clinically apparent tissue damage.

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14

***In Vitro* Methodologies to Investigate Drug-Induced Toxicities**

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CHAPTER MENU

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14.1 Mitochondria as a Biosensor to Measure Drug-Induced Toxicities: Is It Relevant?

Mitochondria are cellular organelles responsible for the production of energy and coordination of cell survival and death, being also important targets for many toxic compounds (Hargreaves et al. 2016). Besides energy production and regulation of cell fate, mitochondria are also involved in critical cellular functions such as calcium signaling, regulation of cellular proliferation and metabolism, and biosynthetic pathways, including heme or steroid syntheses (Miller 2013; Zorov et al. 1997). Since mitochondria are uttermost important for cellular physiology, large fluctuations in their function resulting from toxic compounds may lead to collapse of cellular energetic processes, which may affect the function of the respective tissue. Recognized drug-induced mitochondrial dysfunction mechanisms include disruption of mitochondrial transmembrane potential (e.g., amphetamines, cocaine) (Cunha-Oliveira et al. 2006); inhibition of enzymatic activities, including those of oxidative phosphorylation (OXPHOS) complexes (e.g., nefazodone, troglitazone, tamoxifen); TCA cycle and fatty acid metabolism (e.g., valproic acid, ibuprofen); uncoupling of electron transport from ATP synthesis (tolcapone, nimesulide, tamoxifen); inhibition of mitochondrial DNA (mtDNA) replication

(e.g., antiretrovirals including zalcitabine); inhibition of ATP synthesis (e.g., antibiotics including chloramphenicol and tetracyclines); oxidative stress (e.g., doxorubicin, acetaminophen/paracetamol); and the mitochondrial permeability transition pore (MPTP) (e.g., nimesulide, troglitazone, acetaminophen/paracetamol) (Nadanaciva and Will 2011).

Mitochondria regulate apoptotic signaling pathways, integrating cell signals, which skews the balance between cell survival and cell death (Vega-Naredo et al. 2015). Severe drug-induced mitochondrial dysfunction may in fact result in apoptotic cell loss. *In vitro* screening tests for several parameters related to mitochondrial function can decrease ethical issues related to the use of laboratory animals by reducing the number and suffering in later drug developmental stages. The mechanisms of mitochondrial chemical toxicity may be evaluated using different *in vitro* models, ranging from mitochondrial fractions isolated from different organs (Pereira et al. 2009b) to human cell lines (Swiss and Will 2011) or 3D tissues (An et al. 2014). The diversity of biological models, together with adequate instrumentation, can help in the designing and testing of multiple chemical entities so that a mitochondrial-safe drug candidate can be produced. Databases linking mitochondrial toxicity to chemical structure (structure–activity relationships (SAR)) may help predict which chemical modifications

contribute to mitochondrial liabilities and help design safer drug candidates (Naven et al. 2013).

The development of molecules that are relevant for human exposure, including drugs and cosmetics, or even used in livestock and veterinary industries, demands a low or null intrinsic toxicity. The process of drug discovery and development is highly regulated and has several critical points that determine the viability of a molecule to be a promising drug. In fact, more than 80% of drug candidates in clinical trials are halted from further development due to unexpected safety issues or lack of efficacy (Shah 2006). Drug toxicity developed during treatment is detrimental to the patients and must be avoided. If drug toxicity is observed, the drug must be withdrawn from the market, with a tremendous cost not only to the patients but also to the pharmaceutical companies. Even after drug approval for clinical use, the US Food and Drug Administration (FDA) may issue black box warnings if reports of severe adverse effects in some patients are noticed, which limits the clinical utilization of those drugs. Interestingly, a high percentage of drugs with black box warnings present mitochondrial liabilities in different target organs (Dyken and Will 2007), suggesting that the evaluation of mitochondrial toxicity should be used to predict and prevent future drug-induced mitochondrial liabilities. Perturbation of mitochondrial function can contribute to drug-induced organ injury, originating different phenotypes that contribute to organ failure and, in some cases, even death.

The toxicological evaluation of new compounds in early developmental phases may allow ascertaining their safety before proceeding to more expensive protocols, increasing the probability of success of the compounds in later stages. If needed, it may also allow for adjustments in the chemical structure or formulation at an early stage, which are easier and cheaper than at later stages. Predicting mitochondrial toxicity in the early stages of drug development is critical to avoid later-stage attritions and reduce unnecessary financial costs to the industry. The preclinical phase is thus the best opportunity to test and prevent drug-induced organ injury. In fact, the early screening of mitochondrial toxicity may save millions of dollars in the development of drugs, or other compounds, which would later present severe adverse effects (Dyken and Will 2007).

As the avoidance of mitochondrial liabilities is important in the context of drug discovery, novel molecules should always be evaluated for mitochondrial toxicity. Mitochondrial toxicity can be measured in terms of alterations in specific endpoints, including respiration (oxygen consumption), transmembrane potential, calcium-induced damage, or the activity of individual complexes in the respiratory chain, preferably using high-throughput methodologies, as described in the following sections.

14.2 Drug-Induced Cellular Bioenergetic Changes: What Does It Mean and How Can We Measure It?

Cellular bioenergetics describes the flow and transformation of energy that occurs in living systems through a set of biochemical reactions. Cellular respiration, glycolysis, and several other metabolic pathways are used in cells with the ultimate objective of producing energy for cell anabolism, integrating multiple pathways that regulate how cells survive or die. In living systems, this energy is stored in adenosine triphosphate (ATP) whose hydrolysis is coupled to the formation of adenosine diphosphate (ADP) and inorganic phosphate (Pi), releasing energy in the process. However, ADP can also be dephosphorylated to adenosine monophosphate (AMP) and Pi, but with half of the energy of ATP reactions (Atkinson and Walton 1967). In living systems, AMP, ADP, and ATP are constantly turning over, responding to cellular metabolic needs. When cells are exposed to xenobiotics, changes in cellular bioenergetics may be observed, which can undermine homeostasis. Some methodologies that are used to investigate xenobiotics-induced alterations in cellular bioenergetics will be presented in the next sections. Basic principles of those methods, as well as their advantages and disadvantages, will be described.

14.2.1 Pinpointing Mitochondrial Toxicity: Manipulation of Culture Media Fuels

In order to assess mitochondrial liabilities in cell cultures, it is important to choose the correct culture conditions to detect mitochondrial toxicity. Proliferative cell cultures are generally grown in high-glucose media to conveniently increase the rate of cell proliferation, but these culture conditions relegate mitochondria to a lower stage in terms of energy production (Marroquin et al. 2007) and may cover up mitochondrial toxicities that would be relevant under more physiological conditions. For example, HepG2 cells cultured in high-glucose medium are highly resistant to mitochondrial toxicants (Marroquin et al. 2007). A convenient assay relies on the replacement of glucose as an energy source by glutamine and pyruvate, which enter the Krebs cycle, supplying electrons to the OXPHOS system, complementing with galactose as carbon source. By comparing the effects of different xenobiotics in cells cultured in high-glucose or in glucose-deprived OXPHOS-stimulating media, it is possible to determine direct toxicity effects of different molecules in mitochondria, as already described for human hepatoma HepG2 cells (Marroquin et al. 2007). This assay has been successfully used for the prediction of drug-induced liver injury (Dragovic et al. 2016; Kamalian et al. 2015).

14.2.2 Oxygen Consumption

Most of the oxygen consumed by cells is reduced to water in mitochondria during OXPHOS, which generates most of the cellular ATP. Mitochondrial function and mitochondrial metabolism have been well described elsewhere (Nicholls and Ferguson 2013) and will not be reiterated here. However, due to the importance of mitochondrial function for cellular survival, alterations in oxygen consumption rate (OCR), measured in tissues, cultured cells, or isolated mitochondria, can be indicative of a compromised mitochondrial function and can be used as a fingerprint to evaluate mitochondrial performance. Mitochondrial respiration can be followed *in vitro* by measuring the dissolved oxygen concentration in an aqueous solution that serves as a matrix for biological samples by using an oxygen-sensitive electrode. The first oxygen electrode was developed by Leland C. Clark (1953), and its basic principle is still used in more advanced systems. The Clark-type electrode is composed by a platinum or gold cathode, and by a silver anode immersed in an electrolyte, normally potassium chloride (KCl) solution. The electrodes are separated from the reaction chamber by a Teflon membrane, permeable to the oxygen present in the aqueous solution, which also separates the sample and chemical agents from the electrodes. When an electric potential difference is applied, the metallic silver in the anode is oxidized to silver chloride (AgCl) according with the reaction $4 \text{Ag} + 4 \text{Cl}^- \leftrightarrow 4 \text{AgCl} + 4 \text{e}^-$, where e^- represents one electron. However, oxygen is reduced to 2OH^- , according to the reaction $4 \text{e}^- + \text{O}_2 + 2\text{H}_2\text{O} \leftrightarrow 4 \text{OH}^-$ in the platinum or gold cathode. Electron production at the anode and electron consumption at the cathode results in a current flow of approximately $1 \mu\text{A}$, which is proportional to the oxygen concentration dissolved in the liquid support of the biological samples. The current that is produced is converted into a signal that can be registered on a computer or recorder. The simple oxygen electrode developed by Clark was improved by some companies, namely, by Hansatech and Oroboros. Oxygen consumption-measuring systems from both companies improved the sensitivity and resolution, especially the latter. Also, what was initially shown as traces on a paper recorder, later analyzed by hand by the researcher, is now digitally transformed and analyzed through dedicated software. All the devices can be used to measure oxygen consumption in isolated mitochondrial fractions (Makrecka-Kuka et al. 2015), permeabilized or homogenized tissues (Burtscher et al. 2015; Dias et al. 2014; Duicu et al. 2013), or intact/permeabilized cells (Divakaruni et al. 2014). Of importance is the fact that the evolution of the Clark-type oxygen electrode allowed the use of smaller samples. The use of stirrer bars and temperature control allows

for a constant mixing of the oxygen in solution and maintains optimal temperatures. The original Clark-type oxygen electrode and its later incarnations, including the Oroboros Oxygraph-2k, can also be adapted to simultaneously measure oxygen consumption with other parameters, including H_2O_2 levels, by using the dye Amplex Red (Makrecka-Kuka et al. 2015) or mitochondrial membrane potential by using the Safranin O dye (Krumshnabel et al. 2014), all based on fluorimetric detection. This simultaneous measurement of different parameters is an enormous advantage not only for the basic understanding of mitochondrial physiology but also to investigate chemical-induced mitochondrial toxicity.

High-resolution respirometry using the Oroboros instruments allowed to identify alterations in mitochondrial respiration in human samples with a precision not obtained using conventional methodology (Burtscher et al. 2015; Dechand et al. 2016). Interestingly, this technology was used to identify alterations in bioenergetic behavior between cultured prostate cancer cells and tissue samples, which warranted a word of caution on the need to take data obtained from 2D cell models carefully (Schopf et al. 2016).

Classically, mitochondrial respiration is evaluated *in vitro* by the analysis of different respiration states, which provides information on the site of potential hazards from chemical agents. This can be easily performed on isolated mitochondrial fractions from different origins, or in permeabilized cells and tissues, although the latter process introduces confounding factors. Based on the pioneer work of Chance and Williams (1956), later modified by different authors (Brand and Nicholls 2011; Nicholls and Ferguson 2013), different respiratory states can be considered depending on substrate and oxygen availability: State 1 refers to oxygen consumption in mitochondria to which no substrates have been added. In well-coupled mitochondria, this is a very low oxygen consumption. State 2 refers to increased oxygen consumption when respiratory system is activated by the addition of one or more substrates. This can be accomplished by adding substrates that result in the production of NADH in mitochondria, reducing complex I (e.g., glutamate/malate or pyruvate/malate) or by directly adding succinate, which reduces complex II. In some cases, ascorbate and TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) are used to reduce complex IV. State 2 respiration is low and serves to compensate the decrease in membrane potential caused by passive proton flux through the inner mitochondrial membrane (IMM), both at the lipid or protein moieties, as well to compensate the depolarization resulting from cation influx. The addition of ADP in the presence of phosphate triggers state 3, which is the “phosphorylative” state of mitochondria. The enzyme ATP synthase is activated,

and the mitochondrial membrane potential is used to produce ATP. The drop of membrane potential is compensated by an increase in respiration. When all added ADP is converted into ATP, respiration decreases to a value equivalent to state 2, the so-called state 4. The ratio between state 3 and state 4 is defined as the respiratory control ratio (RCR) and is an important endpoint of mitochondrial dysfunction, reflecting the integrity of the OXPHOS process. The amount of oxygen needed to phosphorylate the added ADP is also known as the ADP/O ratio and is a measurement of the efficacy of the phosphorylative system. Often, oligomycin, which inhibits the ATP synthase (complex V), can be used during state 3 or after mitochondrial respiration reaches state 4. Because proton influx through the ATP synthase is inhibited, a small decrease in respiration is expected. The difference between state 4 and state 4+oligomycin respirations represents the component of oxygen consumption needed to compensate for proton influx through complex V, which is not converted into ATP production. Finally, an uncoupler such as 2,4-dinitrophenol (2,4-DNP or DNP), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine (FCCP), or carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) can be also used. These molecules dissipate the mitochondrial proton gradient by shuttling protons from the intermembrane back to the matrix, bypassing complex V. The dissipation of the proton motive force allows respiration to accelerate to its maximal capacity. Some also consider state 5 when all the oxygen is consumed from the reaction chamber (Nicholls and Ferguson 2013). When succinate is used as the electron donor, rotenone, a complex I inhibitor, is often used to avoid electron backflow from complex II to I. Compounds to be tested in terms of their toxicity can be added in multiple time points although often incorporating a brief preincubation exposure before the measurements begin. Different effects at the multiple respiration states can give important information on the target of the interaction with mitochondrial systems (Pereira et al. 2007, 2009a).

One possible limitation with the Clark-type electrode and its more recent optimizations is the low-throughput nature of the measurements. However, technically multiple devices can be connected to a single computer, but due to spatial and handling limitations, only 3–4 reaction chambers can be used at the same time. Although this option increases the number of the assays that can be performed at the same time, it is very limiting for pharmaceutical companies that aim to test dozens or more compounds a day. Because of this, high-throughput methodologies have been developed, which measure oxygen consumption in multiplate-based systems. One of such examples involves the phosphorescent dye MitoXPress from Luxcel. The extramitochondrial/extracellular probe can be used with cells or isolated mitochondria and has two intense

absorbance bands with maxima at 380 and 535 nm, emitting in the red channel (around 650 nm). MitoXPress has a long decay emission in the microsecond range, which facilitates the use of time-resolved fluorescence detection, improving the sensitivity and avoiding interference from different factors, including sample scattering (Will et al. 2006). Most plate readers can be used for this, and a full 96-well plate is normally utilized with cells. A standard protocol is to investigate the chemical toxicity of a compound when isolated mitochondria are under state 2 or state 3 respiration (i.e., adding excess ADP to the respective wells) (Will et al. 2006). One important concern is that each well must be covered by a thin layer of mineral oil to block oxygen diffusing into the media. This means that no additions to the system can be made during the measurements. Despite this limitation, the method is extremely valuable for rapid screening in a high-throughput manner. Luxcel also developed intracellular oxygen dyes that can be imaged by fluorescence microscopes or time-resolved fluorescence plate readers (Fercher et al. 2011; Hynes and Carey 2015).

Another high-throughput methodology involves the use of multi-well plates, which already incorporate oxygen-sensitive dyes (Mousavi Shaegh et al. 2016). One example are the OxoPlates from PreSens, which incorporate a system with two different dyes at the bottom of flat or round multi-well plates (Arain et al. 2005). One dye serves as the oxygen sensor, while the other serves as reference, requiring one reader calibration before use. Another example is BD BioSensor plates, which use the same principles (Rolo et al. 2009). Standard multi-well plates can be used, although operating in a dual kinetic mode only. As with the previous dye, there is a requirement to include a layer of mineral oil on top of each well to avoid oxygen back-diffusion, which limits the manipulation of the samples after the assay starts.

Finally, another technology can be used for high-throughput measurement of oxygen consumption in cells and isolated mitochondria, allowing also for manipulation of the system during measurement. The Seahorse XF Extracellular Flux Analyzer (from Agilent) works with dedicated 8-, 24-, or 96-well plates and allows for the manipulation of fuels and inhibitors that are added to the reaction chamber. This occurs because there are four injector ports (or 3 in older versions) that allow titration of the metabolic state of the cell or isolated mitochondria. With this methodology, the OCR can be measured simultaneously with the extracellular acidification rate (ECAR), the latter providing information of the glycolytic rate, although there are caveats. The developments in the plates available increased the type of biological models used. From isolated mitochondria (Bharadwaj et al. 2015) and cells in monolayer (Deus et al. 2016), the researcher can now use 3D cellular cultures (Fan et al.

2015) or intact organisms (Sakata-Kato and Wirth 2016; Shim et al. 2016). By using this technology, the bioenergetic health index (BHI) has been proposed as a single value that would characterize the energetic viability of cells, with potential application in human samples from patients (Chacko et al. 2016). This parameter is calculated from a series of endpoints, which are determined after titrating OCR with different inhibitors. Such endpoints include basal respiration, ATP synthesis-associated respiration, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration.

The choice between different methodologies depends on multiple factors, including the type and amount of sample, the objective of the work, and the cost. For fast toxicity screening, dye-based methods are preferred, since preliminary data can be obtained in a high-throughput manner (e.g., in a 96-well format) with a relatively low cost. For high-precision respirometry, these methodologies are not adequate, especially when there is the need to incorporate additions of different substrates/inhibitors during the assay. In that case, technologies such as the Oroboros or the novel reincarnations of the Clark-type electrode are preferred. When a high-throughput evaluation of cellular/mitochondrial metabolism is needed, combining versatility and real-time calculation of the measured parameters, then the Seahorse XF technology is preferred, although this is a relatively expensive technology. Regardless of the technology/methodology used, one important take-home message is that one should always know what is being measured and the principles and limitations of each technique. For example, data normalization is not always straightforward, and there are many aspects that are overlooked by many researchers. One interesting question is whether cellular respiration depends on cellular volume and protein amount and/or activity. This has been considered previously by Wagner et al. (2011) who compared rates of oxygen consumption in different cell lines in standard culture conditions.

14.2.3 ATP, ADP, and AMP Measurements

Since ATP is the main cellular fuel, the bioenergetic status of the cell depends on the levels of the adenine nucleotides. On one hand, measuring more ATP in a cell, compared with ADP and AMP, can signify more energy available to the biological processes, and on the other hand, lower amount of ATP available may signify lower energy available to cell metabolism (Khlyntseva et al. 2009).

One of the most common parameters used to measure the bioenergetic status of the cell is the ATP/ADP ratio (Maldonado and Lemasters 2014). The reason for the existence of this parameter is the fact that many biological

reactions are energetically unfavorable and are coupled to the dephosphorylation of ATP into ADP and Pi, thereby consuming ATP and increasing ADP levels (Nicholls and Ferguson 2013).

Another parameter also used to measure the bioenergetic status of the living being is the energy charge (EC), defined by Atkinson and Walton (1967). In this parameter, ADP is also considered as a source of energy, since it can also be dephosphorylated, forming AMP and Pi. The equation to calculate EC is the following:

$$EC = \frac{[ATP] + 1/2[ADP]}{[ATP] + [ADP] + [AMP]} \quad (14.1)$$

Changes in the levels of adenine nucleotides can be used as a measure of mitochondrial toxicity. Decreases in ATP/ADP and in EC are considered a signal of mitochondrial dysfunction, and since these measurements are relatively cheap and fast to perform, this parameter is widely used. Also, since the effect of a drug on adenine nucleotide levels can allow to detect mitochondrial toxicity without causing cell death, measuring these parameters can be advantageous (Swiss et al. 2013).

There are several alternative methodologies to evaluate ATP, including chromatographic, luminescent, and fluorescence-based methods, among others. High-performance liquid chromatography (HPLC) is a chromatographic technique that allows the separation, identification, and quantification of different components of a mixture. After separation, components pass through a detector that measures a parameter over time, which allows their identification and quantification (Holman et al. 1993). The components that interact strongly, or have more affinity, with the column suffer a longer delay in the migration, which results in a later arrival to the detector. ATP analysis is generally made by reverse-phase HPLC. This technique makes use of a polar mobile phase and a nonpolar stationary phase. Therefore, most polar components suffer shorter delays (Dong 2006). Since ATP, ADP, and AMP have different polarities, it is possible to quantify these components in a biological extract, by integration of the absorbance over time at 254 nm, using an UV detector (Holman et al. 1993). HPLC has many advantages: (i) it can be automated by using automatic injectors; (ii) it can be applied to the analysis of many types of samples, allowing a fast and accurate analysis; and (iii) it provides a very sensitive detection. However, depending on the samples used, there may be some limitations, because complex mixtures can be difficult to analyze and also because some nucleotides can be degraded during the extraction process, if not performed properly (Roda et al. 1999).

Other methods to assess cellular ATP levels take advantage of luciferase, an enzyme that converts

chemical energy into light. Basically, luciferase oxidizes luciferin with the usage of ATP (chemical energy) and O_2 , generating an electronically excited oxyluciferin that releases light when returning to the non-excited state, proportional to the amount of ATP present (Baldwin 1996). Thus, by measuring the luminescence at 562 nm, it is possible to quantify ATP in a biological extract (Fan and Wood 2007). Luciferin/luciferase assays can be performed in low-throughput or high-throughput systems. Some luciferases can only be used in low-throughput assays due to the generation of an unstable luminescent signal that decays shortly after generation of oxyluciferin. This precludes the performance of assays in multiple wells at the same time, since the luminescence must be read shortly after starting the reaction (DeLuca and McElroy 1974). Other luciferase enzymes allow for high-throughput assessments, but only if the luminescent signal is stable over time, allowing the performance of assays in multiple wells at the same time (Roda et al. 1999). By using enzymes that convert ADP and AMP into ATP, it is also possible to quantify ADP and AMP using this assay and, therefore, to calculate the EC and ATP/ADP (Brovko et al. 1994). These assays are easy to perform and have a high sensitivity. However, once again, during the extraction process, some nucleotides can be degraded, which may hinder a proper use of the luciferin/luciferase assay (Khlyntseva et al. 2009).

In 2009, Imamura et al. developed a fluorescence resonance energy transfer (FRET)-based indicator for ATP that allows ATP quantification by FRET (Imamura et al. 2009). FRET is a technique in which energy is transferred directly from a donor fluorophore to an acceptor molecule when donor and acceptor are spatially close and when the donor's emission spectrum overlaps with the acceptor's absorption spectrum. Therefore, after receiving energy from the donor, the proper acceptor can emit light in a different wavelength (Periasamy 2001). This indicator was named ATeam and consists of a protein composed by a variant of cyan fluorescent protein (msecFP) and a variant of yellow fluorescent protein (cp173-mVenus) linked by ϵ subunit of a bacterial F_1F_0 -ATP synthase. This is important because this small ATP-binding protein has high specificity without hydrolyzing it. When ATP is not bound, the protein has low FRET efficiency, since the two fluorophores are distant. Upon ATP binding, this protein undergoes conformational changes that brings the fluorophores closer, thereby increasing FRET efficiency (Imamura et al. 2009). By transfecting this protein into cultured cells (Forkink et al. 2014), it is possible to quantify ATP by measuring the fluorescence intensity at 527 nm caused by the emission of light by the YFP variant, which increases in the presence of higher ATP concentrations. The specificity of the probe to ATP is both an advantage and a disadvantage, since it does not allow the quantification of ADP and

AMP (Imamura et al. 2009). This technique also allows to quantify ATP levels in the cytosol or in mitochondria and, therefore, clarify in which compartment ATP levels change in response to drug treatments. This is possible since ATeam can be specifically transfected to target only the cytoplasm or the mitochondria (Forkink et al. 2014). Another advantage of this technique is the possibility to perform real-time monitoring of ATP levels in living cells and, therefore, detect in real-time alterations in ATP levels and ATP production kinetics in response to drug treatments (Imamura et al. 2009).

14.2.4 Respiratory Chain and ATP Synthase Enzymatic Activities

Although measurements of cellular oxygen consumption are usually considered as indicative for mitochondrial toxicity, there is often a need to identify exactly where toxicity occurs in mitochondria. Among the multiple possible targets, including the lipid bilayer, mtDNA, and fusion and fission proteins, one important site for toxicity is the mitochondrial respiratory chain and the ATP synthase.

There are different methodologies to measure mitochondrial OXPHOS complex activity, mostly based in colorimetric or fluorimetric techniques, some coupled to blue gel native electrophoresis (Diaz et al. 2009). For example, it is possible to follow the enzyme activity by observing the decrease in 2,6-dichlorophenolindophenol (DCPIP) absorbance upon addition of NADH for complex I. We can also follow the increase in absorbance of oxidized cytochrome *c* upon addition of decylubiquinol for complex III. A decrease in absorbance of reduced cytochrome *c* for complex IV activity, measured by following oxygen consumption after adding ascorbate + TMPD, can also be used to measure mitochondrial OXPHOS complex activity (Birch-Machin and Turnbull 2001; Long et al. 2009; Luo et al. 2008; Rossignol et al. 2000). ATP synthase or ATPase activity can also be followed by pH alterations associated with ATP synthesis and hydrolysis, respectively (Moreno and Madeira 1991). It must be stressed that regardless of the methodology used, one critical and usually overlooked control is the use of specific inhibitors of the referred complex. The rate of reaction that is insensitive to the specific inhibitor should be subtracted from the total rate.

Although initially the methods described earlier were introduced to be performed in a low-throughput scale, usually in a single cuvette-based kinetic assay, adaptations can be made to a multi-well system, so that a higher number of samples can be analyzed at the same time and with much lower amount of sample (Pereira et al. 2016).

In addition to the methods described earlier, there are other alternatives for a high-throughput measurement of respiratory chain component activity. MitoSciences,

now part of Abcam company, developed a system in which target enzymes (i.e., the different complexes of the respiratory chain) are immunocaptured before subsequent toxicity analysis. After the enzyme has been immobilized in the well, a specific substrate is then added, and enzyme activity is measured by the change in absorbance of either the substrate or the product of the reaction. This methodology can allow for a fast and high-throughput screening of chemical toxicity against specific complexes of the respiratory chain or ATP synthase, providing that the chemical agent is preincubated with the immune-captured complexes (Kalgatgi et al. 2013; Lai et al. 2013; Nadanaciva et al. 2007; Zhao et al. 2014). This methodology has the advantage of precisely pinpointing the sites in the OXPHOS components that are affected by a particular chemical entity, which may help illuminate the total pathway flux, usually measured as oxygen consumption. One important limitation includes the fact that multiple effects in multiple targets are overlooked. The immunocapture assay has been further developed to be integrated into the dipstick concept, in which the capture antibodies are blotted into a membrane and the sample flows on top of the blotted antibodies (Vafai et al. 2016).

14.3 Evaluation of Mitochondrial Physiology

14.3.1 Measuring Reactive Oxygen Species (ROS) Production with Oxidant-sensitive Probes

In order to study mitochondria-associated oxidative stress, there is the need to first understand the meaning of oxidative stress. The much-reviewed subject of oxidative stress was thought to be based only on an imbalance between ROS production and its detoxification, broadly known as antioxidant capacity. Nowadays, it is also known that ROS can be involved in selective redox signaling mechanism that includes differential and compartmented effects (Jones and Go 2010). ROS form a highly reactive class of oxygen metabolites that can cause oxidative modification of cellular biomolecules including proteins, lipids, and DNA (Zhang and Gao 2015). Examples of ROS include superoxide anion ($O_2^{\cdot-}$), the hydroxyl ($\cdot OH$) radical, hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid ($HOCl$) (Dikalov and Harrison 2014; Zhang and Gao 2015). Although an over-excessive production of ROS has been linked to several pathologies including diabetes and cardiovascular and neurodegenerative diseases, biological production of some ROS in mitochondria have a pivotal role in cell and consequently in organism signaling and homeostasis. Mitochondrial ROS are formed in complexes I and III of the electron transport system and

through enzymatic activities such as α -ketoglutarate dehydrogenase, pyruvate dehydrogenase, and monoamine oxidase (Zhang and Gao 2015). Due to their reduced lifetime, ranging from nanoseconds to seconds depending on the level of reactivity and the cell antioxidant capacity, low concentrations *in vivo* (picomolar to low nanomolar), multiple sources, complex composition, and the special microenvironment compartmentalization, it is quite difficult to analyze and image ROS with high spatiotemporal resolution and specificity. In this way, a good ROS sensor must provide elevated specificity, capacity to detect changes in particular ROS levels, and identification of the local of production as well as allow quantification (Dikalov and Harrison 2014).

ROS sensors can be divided in different classes, namely, oxidant-sensitive probes (Zhang and Gao 2015), non-redox fluorescent probes (including fluorescent protein-based indicators), and spin traps, among others (Dikalov and Harrison 2014). These techniques have been successfully adapted to be used in a 96-well as well as in 384-well high-throughput format (Billis et al. 2014).

Most ROS-sensing molecules can penetrate the cell membrane, and, once inside the cell, they come into contact with the oxidant and become oxidized to their fluorescent product. Falling into this category are fluorescent dyes such as 2',7'-dichlorodihydrofluorescein ($DCFH_2$), dihydrorhodamine (DHR), and dihydroethidium (DHE) and its mitochondria-targeted form MitoSOX. $DCFH_2$ has been widely used to detect H_2O_2 (Hempel et al. 1999). This molecule is normally used in its diacetate form ($DCFH_2$ -DA), which confers it the capacity to penetrate the cell membrane, where it is hydrolyzed by esterases, yielding $DCFH_2$, which is retained in the cytosol. This molecule is oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) (Kalyanaraman et al. 2012). Despite being used to measure H_2O_2 , this probe has several limitations. Firstly $DCFH_2$ can also be oxidized by $\cdot OH$, nitrogen dioxide radical ($\cdot NO_2$), $ONOO^-$ -derived ROS, $HOCl$ (Kalyanaraman et al. 2012), cytochrome *c*, tyrosine, and some thiols (Wang et al. 2013). Moreover, even H_2O_2 is not directly detected and requires a peroxidase or other low molecular weight or protein-bound transition metal. Paradoxically the reaction of $DCFH_2$ to form DCF can itself generate H_2O_2 (Kalyanaraman et al. 2012). Other disadvantages include the rapid scavenging by added antioxidants and the fact that the dye can undergo light-induced oxidation (Winterbourn 2014).

DHE and its mitochondria-targeted form MitoSOX are normally used for cellular (Zhao et al. 2003) and mitochondrial detection of superoxide anion ($O_2^{\cdot-}$) (Dikalov et al. 2007), respectively. The principal disadvantage of this probe is that it can form two fluorescent products: 2-hydroxyethidium, which is a specific $O_2^{\cdot-}$ adduct, and ethidium, which is formed by nonspecific redox reactions (Zhao et al. 2005). Unfortunately, the

fluorescence spectra of these two products overlap compromising the measurement of $O_2^{\cdot-}$ formation alone (Robinson et al. 2006; Zhao et al. 2005). The use of HPLC or the careful use of precise wavelengths of excitation in confocal imaging allows the separation of signals and can be used to specifically detect $O_2^{\cdot-}$ (Dikalov and Harrison 2014). Additionally, it is important to note that high MitoSOX concentrations significantly interfere and even impair mitochondrial function (Robinson et al. 2006).

DHR is one of the dyes more often used for measuring $ONOO^-$ (Jourdeuil et al. 2001). The assay is based in the ability of this compound to suffer a two-electron oxidation with consequent formation of its fluorescent product rhodamine. DHR has a similar chemistry to DCFH₂, sharing its limitations (Kalyanaraman et al. 2012). DHR must not be used to specifically measure $ONOO^-$ because its oxidation to rhodamine is mediated by an intermediate DHR radical that can be reduced by ascorbic acid, thiols, and NO_2 . Therefore this probe should only be used as a nonspecific indicator of $ONOO^-$ and HOCl or other one-electron oxidant (Wardman 2008).

Trying to overcome some of the limitations and disadvantages of these dyes, new methods for ROS detection have been developed (reviewed by Pereira et al. 2012).

A very elegant method for detection of ROS is based on cellular transfection to produce proteins capable of sensing the redox status. Example of such proteins are HyPer (based on yellow fluorescent protein inserted into the regulatory domain of *Escherichia coli* protein OxyR) (Belousov et al. 2006) and Orp1-roGFP2 (redox-sensitive green fluorescent protein) (Morgan et al. 2011) that can detect H_2O_2 , and cpYFP (circularly permuted yellow fluorescent protein) that detects $O_2^{\cdot-}$. HyPer contains a H_2O_2 -regulated domain that triggers transcriptional responses to oxidative stress (Belousov et al. 2006). Upon oxidation by H_2O_2 , HyPer reaches its maximum fluorescence (Belousov et al. 2006). The main advantages of this protein are that its fluorescence is reversible, being able to follow the ROS fluxes over time, and that it is able to be successfully targeted to various organelles (including mitochondria, nucleus, and cytoplasm) (Dikalov and Harrison 2014). Regarding the disadvantages, HyPer is pH sensitive (needing pH measurement as control) and lacks specificity since it can be reduced by the glutathione system (Belousov et al. 2006). On the other hand, roGFP proteins have two different excitation peaks (400 and 490 nm). When oxidized, roGFP protein forms a disulfide bond that promotes protonation, resulting in increased fluorescence intensity at 400 nm, at the expense of the fluorescent intensity at 490 nm (Hanson et al. 2004; Morgan et al. 2011). Orp1(yeast peroxidase)-roGFP2 responds to low micromolar concentrations of H_2O_2 and has the advantage of being pH insensitive (Hanson

et al. 2004; Morgan et al. 2011). Regarding the detection of $O_2^{\cdot-}$ by cpYFP, this has the advantage of being selective, sensitive, and reversible, despite being highly pH sensitive (Wang et al. 2008).

Cyclic hydroxylamine spin probes can be used to measure the production of $O_2^{\cdot-}$ in cultured cells, tissues, and *in vivo* (Dikalov et al. 2002). These molecules should not be confused with spin traps as they do not trap the radical; instead they are oxidized by $O_2^{\cdot-}$. When this happens, the electron paramagnetic resonance signal is stable for several hours after nitroxides are formed (Dikalov and Harrison 2014). Due to their high rates of reaction with $O_2^{\cdot-}$, this enhances the efficiency for the detection of this radical. This reaction occurs only in one step, and these probes do not redox cycle, which decreases the possibility of potential artifacts. A wide range of cationic, anionic, and neutral cyclic hydroxylamine spin probes exist, having different properties regarding lipophilicity and cell permeability (Dikalov and Harrison 2014). For example, 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium (CAT1H) only detects extracellular $O_2^{\cdot-}$, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) is highly cell permeable and can detect both intracellular and extracellular ROS, and the phosphate-containing negatively charged 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine (PPH) accumulates inside cells, presumably via active transport (Dikalov and Harrison 2014). When using this kind of probe, there is a special need to eliminate artificial signals induced by transition metals.

Another group of ROS-sensitive fluorescent dyes are boronate-based fluorescent dyes. These molecules can react with H_2O_2 and $ONOO^-$ having different mechanisms of action (Dikalov and Harrison 2014). Peroxyfluor-1 dye molecule is protected by boronate. When exposed to H_2O_2 , the molecule undergoes nucleophilic attack, leading to the removal of the boronate from the fluorophore, which causes light emission (Miller et al. 2005). However, the dye coumarin-7-boronic acid reacts with $ONOO^-$, yielding the fluorescent phenol 7-hydroxycoumarin (Zielonka et al. 2012). Boronate also reacts with $ONOO^-$, leading to a site-specific nitration of an aromatic moiety that can be followed either by mass spectrometry or HPLC. It is accepted that the use of boronate-based fluorescent dyes is a more consistent technique to assess $ONOO^-$ than, for instance, DHR (Kalyanaraman 2011). However, it is also important to highlight that these dyes are not totally specific to this ROS.

14.3.2 Monitoring Mitochondrial Transmembrane Electric Potential

As described earlier, besides being the major responsible for ATP production, mitochondria also control or are

involved in the regulation of ion homeostasis (especially calcium), cell redox state (controlling ROS production), transport of metabolites (including proteins synthesized in the cytosol), lipid and amino acid metabolism, and cell death (Solaini et al. 2007). In order to cope with all these processes, mitochondrial function is dependent on the development of an electrochemical transmembrane potential, which contributes to the proton motive force (Δp) (Mitchell and Moyle 1969). ATP production relies heavily on this characteristic. This Δp is a combination of the mitochondrial pH gradient (ΔpH_m) and transmembrane electric potential ($\Delta \psi_m$). The simplified Nernst equation, calculated at 37°C, integrates these different components $\Delta p(\text{mV}) = \Delta \psi_m - 60 \Delta \text{pH}_m$ (Nicholls and Ward 2000; Reid et al. 1966). It is not easy to quantify Δp in cultured cells because it requires the quantification of both $\Delta \psi_m$ and ΔpH_m individually. However, because $\Delta \psi_m$ is the main constituent of Δp and because ΔpH_m can be neutralized using an electroneutral K^+/H^+ ionophore such as nigericin (Nicholls and Ferguson 2002), most methodologies are based on the detection and quantification of $\Delta \psi_m$ (Solaini et al. 2007).

In this regard, fluorescent lipophilic cationic dyes have been used for decades as indicators of $\Delta \psi_m$. These molecules accumulate in the mitochondrial matrix relying on the potential-dependent partitioning of charged lipophilic cationic dyes across biological membranes. These dyes will accumulate in mitochondria in an inverse relation to the $\Delta \psi_m$ (according to the Nernst equation). The more polarized (negatively charged in the matrix side) mitochondria will accumulate more dye and vice versa (Lemasters et al. 1995; Perry et al. 2011).

Mitochondrial $\Delta \psi_m$ can be monitored in two distinct ways, namely, in a “quenching mode” and in a “non-quenching mode” (Duchen 2004; Nicholls and Ward 2000). Higher concentration of dye is used in the former, so that the dye forms aggregates within the mitochondria, which quenches fluorescence. In this way, mitochondrial depolarization leads to the release of the dye to the cytosol, increasing its fluorescence as it unquenches. However, mitochondrial hyperpolarization will result in higher mitochondrial dye accumulation, causing quenching and consequently decreasing fluorescence. In quenching mode, after exposure to the test compounds, cells are loaded with the dye, which becomes retained inside mitochondria. Depolarization induced by FCCP (to dissipate the H^+ gradient) + oligomycin (to avoid ATPase-mediated repolarization) can be used to reveal the fluorescence of the retained dye, which is proportional to the $\Delta \psi_m$ in the cell population. Regarding the use in a non-quenching mode, lower probe concentrations are normally used to avoid aggregation in mitochondria. In this case, depolarized mitochondria will accumulate lower amounts of dye, and consequently less fluorescence will occur, while

hyperpolarized mitochondria will retain more dye and have higher fluorescence (Perry et al. 2011).

The more popular and used fluorescent lipophilic cationic dyes are tetramethylrhodamine methyl (TMRM) (Floryk and Houstek 1999) and ethyl (TMRE) esters (Scaduto and Grotyohann 1999), rhodamine 123 (Rhod123) (Emaus et al. 1986), 3,3'-dihexyloxycarbocyanine iodide (DiOC_6) (Rottenberg and Wu 1998), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Rottenberg and Wu 1998). These dyes can be measured by microscopy (either confocal or epifluorescence), flow cytometry, and fluorescence spectroscopy (Solaini et al. 2007).

TMRM and TMRE are lipophilic cations that accumulate in mitochondria based on the $\Delta \psi_m$. Upon accumulation, both dyes exhibit a red fluorescence emission spectrum (Perry et al. 2011), with TMRM having the lowest mitochondrial binding and ETC inhibition capacity than TMRE. Having a fast equilibration rate makes them less appropriate for quenching studies than a more slowly permeant dye (e.g., Rhod123) (Scaduto and Grotyohann 1999).

Rhod123 is a cell-permeant, cationic, green fluorescent dye that is readily sequestered by active mitochondria without cytotoxic effects (Huang et al. 2007). This probe is often used in fast-resolving acute studies (normally in quenching mode). Depolarization of mitochondria causes unquenching and consequent increase in fluorescence (Perry et al. 2011). When compared with TMRM, Rhod123 does not bind to mitochondrial structures as strongly and presents a similarly low ability to inhibit the respiratory chain; when compared with TMRE, Rhod123 presents a lower ability to inhibit the electron transport chain (Scaduto and Grotyohann 1999).

The dye JC-1 accumulates in mitochondria in response to $\Delta \psi_m$, resulting in a fluorescence emission shift from green to red. Consequently, mitochondrial depolarization yields a decrease in the red/green fluorescence ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent aggregates (Cossarizza et al. 1993). However, H_2O_2 , for example, can change JC-1 fluorescence independent of $\Delta \psi_m$ (Perry et al. 2011), which means that redox modulation of JC-1 fluorescence is a disadvantage of this probe.

Lastly, DiOC_6 is a fluorescent dye used for the staining of endoplasmic reticulum (Terasaki 1989), vesicle membranes, and mitochondria (Koning et al. 1993). Although frequently used to measure $\Delta \psi_m$ by flow cytometry (Perry et al. 2011), DiOC_6 inhibits mitochondrial respiration (Nicholls and Ward 2000; Reid et al. 1966), which limits the use of this dyes.

In all referred cases, proper optimization may lead to the use of the dyes in multi-well formats, thus allowing for a progressive increase in the throughput of the assays.

When assessing $\Delta\psi_m$ it is important to have in consideration that many cell lines cultured in high-glucose media (25 mM) maintain $\Delta\psi_m$ despite the low OXPHOS activity, by hydrolyzing glycolytic ATP at complex V, which is common in cancer cells (Chevrollier et al. 2011). In glycolytic cells, oligomycin paradoxically dissipates $\Delta\psi_m$ rather than hyperpolarizing it (Wu et al. 2007).

14.3.3 Calcium Flux Measurements

As referred in the previous sections, mitochondria play distinct roles regarding Ca^{2+} regulation in cells (Carafoli 2003; Rizzuto et al. 2000). They have a pivotal role in Ca^{2+} kinetics and amplitude signal. However, they also have a key role in Ca^{2+} buffering (Pozzan and Rudolf 2009). Moreover, Ca^{2+} modulates key mitochondrial processes, including energy metabolism, hormone metabolism, and cell death (Ferri and Kroemer 2001; Pizzo and Pozzan 2007). Mitochondrial calcium can be measured in different systems including *in vitro* (mitochondrial suspensions and cell cultures), *in situ* (tissue slices), or *in vivo* (Pozzan and Rudolf 2009).

Given the significance of cellular Ca^{2+} homeostasis, the measurement of intracellular fluxes is important. Mitochondrial Ca^{2+} levels have been measured using different approaches that can be divided into two categories: fluorescent Ca^{2+} -sensitive dyes, such as rhod-2AM and its derivatives (X-rhod-1, rhod-FF, and others), and luminescent and fluorescent-targeted proteins, such as aequorin and green fluorescent protein-based Ca^{2+} indicators (GFP-based fluorescent CIs) (Fonteriz et al. 2010). Although other Ca^{2+} dyes exist, including Fura-2AM and Fluo-3 and Fluo-4, these are not specific to mitochondria, being useful to measure total cellular Ca^{2+} levels. These probes have been successfully used in medium- to high-throughput assays (Gunter and Gunter 2001).

Rhod-2AM is a cationic dye with the ability to enter electrophoretically into the mitochondria, being used as a selective indicator for mitochondrial Ca^{2+} (Tsien 1981), with the AM (acetoxymethyl) group conferring the ability for cell retention. Contrary to Fura-2AM, which responds to increasing Ca^{2+} levels by a change in the emission/excitation spectrum, rhod-2AM responds by increasing the fluorescent emission (Pozzan and Rudolf 2009). Disadvantages of rhod-2AM include incomplete targeting (Launikonis et al. 2005) and local mitochondrial phototoxicity caused by small concentrations of this dye (Fonteriz et al. 2010). In order to overcome these disadvantages, new strategies were designed to improve Ca^{2+} imaging. Luminescent and fluorescent-targeted proteins such as aequorin (Aeq) (Shimomura et al. 1962) and GFP-based CIs were created (Pendin et al. 2015).

Different classes of GFP-based CIs were designed with distinct selectivity and affinity for Ca^{2+} , dynamic range, and kinetics for Ca^{2+} binding, as well as the possibility of being targeted to specific subcellular compartments including mitochondria (Pendin et al. 2015).

Aequorin (Aeq) is a Ca^{2+} -sensitive photoprotein responsible for the bioluminescence of the marine hydrozoan jellyfish *Aequorea victoria* (Shimomura et al. 1962). This photoprotein has a hydrophobic core with coelenterazine as a prosthetic group and three EF-hand motifs that bind Ca^{2+} . When this cation binds to the protein, the covalent bond between the apoprotein and coelenterazine is broken, and the latter is oxidized to celenteramide, releasing a photon (Shimomura et al. 1962). An essential characteristic of this reaction is that the rate of proton emission is dependent on the ion concentration, which enables the light emitted to be directly converted into Ca^{2+} concentration (Pendin et al. 2015). This protein can be targeted to mitochondria when fused with organelle-specific proteins (e.g., glycerol phosphate dehydrogenase (Rizzuto et al. 1998) or mitochondrial pre-sequence of subunit VIII of cytochrome *c* oxidase (Rizzuto et al. 1992)). As with any other approach, there are strong points and concerns with this technique. Aequorin has a highly dynamic range, obtained either by modifying the native Aeq or by using different coelenterazine derivatives. Other advantages are the low pH sensitivity, low interference with endogenous Ca^{2+} binding partners, and a high signal-to-noise ratio (Pendin et al. 2015). However, there are several drawbacks including the technical need to reconstitute Aeq with coelenterazine, the low levels of emitted light, the irreversibility of its Ca^{2+} binding reaction, and the fact that photoluminescence measurements are difficult to calibrate in the subcellular compartments (Brini et al. 1995).

Green fluorescence protein-based calcium indicators consist of a fluorescent protein of the same color fused to a Ca^{2+} -binding domain. When the cation connects to the protein, its fluorescent properties are changed (Pendin et al. 2015). One family of these dyes contains one fluorescent protein in which Ca^{2+} acts to alter the chromophore environment, changing either the intensity or the wavelength of the fluorescence emitted. The other family has two photoproteins that exhibit FRET properties (Pendin et al. 2015), which are linked through a Ca^{2+} -sensitive peptide. In many cases, calmodulin (CaM) is used as the Ca^{2+} -binding protein (e.g., camgaroo). It is noteworthy that some camgaroo constructs can be targeted to mitochondria (Griesbeck et al. 2001). Other families (chameleons) are ratiometric GFP-based Ca^{2+} sensors that can also be targeted to this organelle (Whitaker 2010).

14.3.4 Measuring the Activity of the Mitochondrial Permeability Transition Pore (MPTP)

In certain conditions, namely, after exposure to some xenobiotics, the mitochondrial inner membrane (IMM) can lose its selective impermeability, resulting in uncoupling of respiration from ATP production. MPTP is thought to be a redox and calcium-sensitive protein complex located in the IMM (Kwong and Molkentin 2015). Being the subject of much debate since its discovery, the exact composition of the pore is still under much controversy. The first studies indicated that the pore was composed by the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), the adenine nucleotide translocator (ANT) in the IMM, and cyclophilin D (CypD), which was thought to act as a mitochondrial matrix regulator of the pore (Crompton et al. 1998). Other proteins were then proposed to participate in the pore structure including the translocator protein (TSPO), complement 1q binding protein (C1qbp), the mitochondrial phosphate carrier (PiC), the pro-death Bcl-2 family members Bax and Bak, and more recently the mitochondrial F_1F_0 ATP synthase (Kwong and Molkentin 2015). The most recent MPTP model involves the formation of F_1F_0 ATP synthase dimers (Giorgio et al. 2009), and ANT, PiC, and CypD are proposed to serve as pore regulators in the mitochondrial matrix (Kwong and Molkentin 2015).

It was also proposed that MPTP can exist in two different conductance modes. In the low-conductance state, the pore presents very restricted permeability, allowing the passage of solutes below 300 Da (normally small ions such as H^+ , Ca^{2+} , and K^+). It is important to note that in this low-conductance state, the mitochondrial matrix does not suffer swelling. However, if the pore operates under its high-conductance state, it shows permeability to solutes under 1.5 kDa, which can ultimately lead to mitochondrial matrix swelling due to water entry for osmotic compensation (Bonora et al. 2016).

Under physiological conditions, the rapid opening of the MPTP is related to Ca^{2+} and oxidative stress, being also directly involved in the fast discharge of Ca^{2+} to the cytoplasm (Halestrap et al. 2004). However, under pathological conditions, MPTP long-lasting opening is connected to mitochondrial dysfunction, namely, mitochondrial depolarization, pyridine nucleotide depletion, disruption of ATP synthesis, excess of Ca^{2+} release, uncoupling of mitochondrial respiration, and matrix swelling. This will ultimately lead to IMM and OMM collapse, which causes the release of apoptotic signaling molecules including cytochrome *c*, endonuclease G, and the apoptosis-inducing factor (Bernardi et al. 2006).

By regulating mitochondrial energy metabolism, Ca^{2+} and ROS homeostasis, as well as cell death, the MPTP is widely studied as a central toxicity sensor. In this way, different approaches have been used to investigate MPTP opening (Bonora et al. 2016). Three examples of such methodologies include the cobalt (Co^{2+})–calcein assay (Woollacott and Simpson 2001), mitochondrial membrane depolarization (Bonora et al. 2016), and the swelling technique (Kaasik et al. 2007). Multiple agents are described to induce the MPTP in different biological models and experimental protocols. Excessive accumulation of mitochondrial Ca^{2+} (Haworth and Hunter 1979), pro-oxidative agents (Kowaltowski et al. 2001), oxidized thiols (Petronilli et al. 1994), Pi (Bravo et al. 1997), long-chain free fatty acids (Wieckowski et al. 2000), atractyloside (Wieckowski et al. 2000), mastoparan (Pfeiffer et al. 1995), and 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195) (Li et al. 2007) activate the MPTP. Nonetheless, high $\Delta\psi_m$ (Isenberg and Klaunig 2000), cyclosporine A (CsA) (Bernardi 1996), sanglifehrin A (Clarke et al. 2002), bongkreikic acid (Clarke et al. 2002), antamanide (Azzolin et al. 2011), ubiquinone and decylubiquinone (Devun et al. 2010), and others (Vianello et al. 2012) have been described to inhibit the MPTP. Still, some clinically used drugs induce a Ca^{2+} -dependent MPTP, which is not desensitized by CsA (Pfeiffer et al. 1995).

The Co^{2+} –calcein assay has been used as a direct technique to measure MPTP activity in live cells. Firstly, cells are incubated with calcein–AM that is captured in subcellular compartments, including mitochondria. After cellular esterases cut the AM moiety, calcein is retained in the referred subcellular compartments (Woollacott and Simpson 2001). The rationale behind this method is that the addition of Co^{2+} quenches the calcein fluorescence in the cytosol but not in mitochondria, due to its inability to permeate the membranes in those organelles. This leads to mitochondrial calcein fluorescence only. When the MPTP opens, calcein exits from the mitochondrial matrix, accompanied by the entry of Co^{2+} in the same organelle. In this way, a decrease in the calcein fluorescence is observable by fluorescence microscopy. Under microscopy, the mitochondrial fluorescent signal is lost (Petronilli et al. 1999). Other adaptations to the same method exist, based on different calcein loading protocols and mitochondrial loading (Kim et al. 2006, 2012).

The second method is based on the loss of the proton gradient across the mitochondrial membranes when MPTP is opened. As described in the previous sections, mitochondrial membrane potential dyes such as TMRM(E), Rhod123, JC-1, or DiOC6 accumulate in mitochondria according to $\Delta\psi_m$. The opening of the pore causes a decrease in fluorescence intensity that

can be easily quantified by fluorescence microscopy or by fluorimetry (Bonora et al. 2016).

The third (and last) method is based on mitochondrial volume and is only used with isolated mitochondrial preparations. Normally, MPTP opening leads to mitochondrial matrix swelling (Hunter and Haworth 1979), which can be assessed by monitoring changes in the mitochondrial suspension pseudo-absorbance, measured by following 90° light scattering, and reflecting changes in mitochondrial volume (Beavis et al. 1985;

Cancherini et al. 2007). Decreases in light scattering correlate with increased matrix swelling and vice versa. The changes in light scattering are usually followed at 540 nm, which falls within the range of mitochondrial isosbestic point (Cancherini et al. 2007; Marroquin et al. 2014). A desensitizer such as CsA (Bernardi 1996) is usually used as a negative control, inhibiting MPTP opening. It is noteworthy to add that mitochondrial swelling protocol has been successfully adapted to high-throughput formats in 96-well plates (Marroquin et al. 2014).

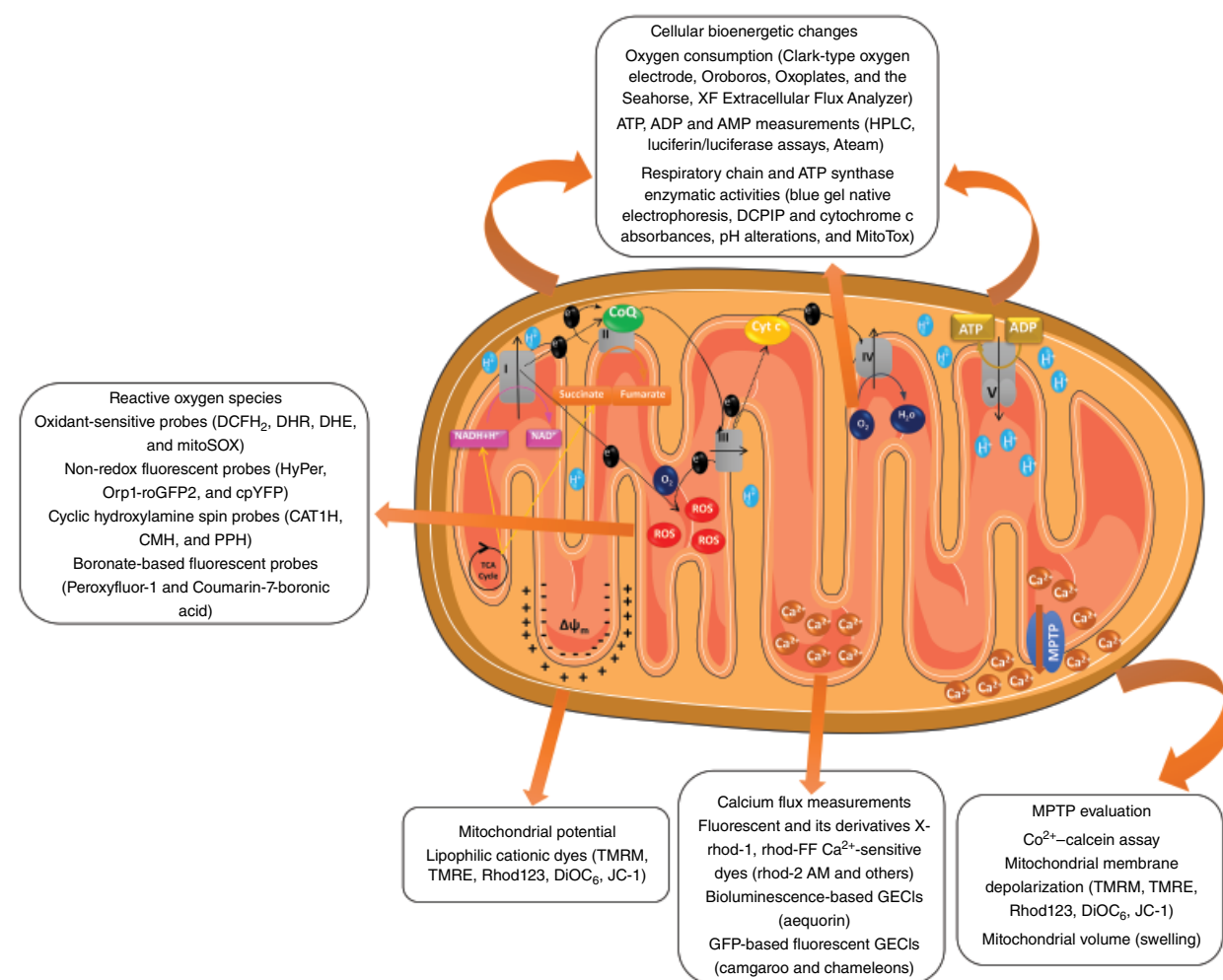


Figure 14.1 Mitochondria as bio-sensors for drug-induced toxicity. The different approaches described in this chapter that can measure mitochondrial alterations involving reactive oxygen species, mitochondrial membrane potential, calcium flux, adenine nucleotide concentrations and opening of the mitochondrial permeability transition pore. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; Ca²⁺, calcium; CAT1H, 1-hydroxy-2,2,6,6-tetramethylpiperidin-4-yl-trimethylammonium; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; Co²⁺, cobalt; DCFH₂, 2',7'-dichlorodihydrofluorescein; DCPIP, 2,6-dichlorophenolindophenol; DHE, dihydroethidium; DHR, dihydrorhodamine; DiOC₆, 3,3'-dihexyloxacarbocyanine iodide; GECI, genetically encoded calcium indicators; GFP, green fluorescent protein; HPLC, high-performance liquid chromatography; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; MPTP, mitochondrial permeability transition pore; PPH, 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine; Rhod123, rhodamine 123; Rhod-2 AM, rhodamine 2 acetoxymethyl; TMRE, tetramethylrhodamine ethyl ester; TMRM, tetramethylrhodamine methyl ester; YFP, yellow fluorescent protein.

14.4 Concluding Remarks

Figure 14.1 illustrates the methods described in this chapter and the choice of which to use depends on the type of toxic compound, equipment and reagents available, biological models, and available expertise. Method choice awareness will improve data collection and render the results more germane from a toxicological point of view.

The main objective of this chapter was to review some important techniques that can be used to evaluate drug-induced mitochondrial toxicities and make the reader aware of the importance of those studies during the development of new compounds.

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15

Combined Automated Measurement of Respiratory Chain Complexes and Oxidative Stress: A First Step to an Integrated View of Cell Bioenergetics

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15.1 Introduction

Mitochondria produce the vast majority of the ATP used by mammalian cells through oxidative phosphorylation (OXPHOS). They also play a central role in a number of crucial biochemical processes required for cellular homeostasis and act as the gatekeepers for apoptosis with the release of cytochrome *c* (Brookes et al., 2004). The mitochondrial respiratory chain complexes (mRCC), coenzyme Q, and cytochrome *c* catalyze energy transduction from respiratory substrates to a proton motive gradient for the synthesis of ATP. This pathway is made up of five multi-enzymatic complexes (complexes I–V) whose function is essential for ATP production. Complexes I and III of the electron transport chain (ETC) have been recognized as two main sites for the generation of superoxide radical and subsequently other reactive oxygen species (ROS) (Barja, 1999). The major cellular sources of ROS mitochondria are also the main target of their damaging effects (Endlicher et al., 2009). It is calculated that 1–4% of oxygen reacting with mRCC is incompletely reduced to ROS and the degree of free radical production directs mitochondrial metabolic efficiency (Chance et al., 1979; Richter, 1988). ROS are highly reactive, and when ROS exceed the antioxidant defense, biological systems suffer oxidative stress (OS)

with indiscriminate damages to proteins (Stadtman and Levine, 2000), lipids (Rubbo et al., 1994), polysaccharides (Kaur and Halliwell, 1994), and DNA (Richter, 1988; LeDoux et al., 1999), thus generating functional impairment, leading to global cellular dysfunction then cytotoxicity. Mitochondrial dysfunction with concomitant OS is evidenced in the brain and peripheral organs of many patients with neurodegenerative diseases (Halliwell, 2001) such as multiple sclerosis (MS) (Morris and Maes, 2014), Parkinson's disease (PD) (Ciccone et al., 2013), Alzheimer's disease (Wang et al., 2009), and autism (Rossignol and Frye, 2014). This intricate metabolism between ROS and mRCC in mitochondria explains why, whereas some mitochondrial diseases arise as a result of either germinal or somatic mutations in mitochondrial or nuclear DNA (Wallace, 1999), most of mitochondrial dysfunctions (a generic term that includes alteration of different metabolic pathways and damage to mitochondrial components) and impaired bioenergetics are implicated in the pathogenesis of many chronic illnesses. Nevertheless, as mitochondrial diseases are highly heterogeneous, the clinical presentation of patients remains largely heterogeneous. The age of onset is highly variable, and mitochondrial deficiency can result in either isolated organ defect or multi-visceral involvement (Rotig et al., 2004; Thorburn et al., 2004).

In most cases, there are only subtle metabolic or enzymatic variations that will be indicative of mitochondrial dysfunction.

As understanding mitochondrial behavior remains extremely complex and because mitochondrial insults can occur through still unknown mechanisms, it is not surprising that mitochondrial metabolism has often been found to be a target of drug-induced toxicity. Functional alterations of mitochondria can affect various organs such as the central and peripheral nervous system, skeletal and cardiac muscles, kidneys, intestines (Johns, 1995; Schon and DiMauro, 2003), and particularly liver (Chinnery and DiMauro, 2005). The latter, with its high content in biosynthetic and detoxifying enzymes, is highly ATP dependent. Hepatocytes contain a high density of mitochondria. Therefore, liver dysfunction behaves as a common feature when drug mitochondrial dysfunction is observed. During the last decade, in the field of pharmacotoxicology, mitochondrial dysfunctions that were not discovered during preclinical and clinical testing have been responsible for at least restriction of use as far as withdrawal of many drugs (Nadanaciva and Will, 2011). To minimize that risk, there is an increasing emphasis to early evidence the adverse role of new molecules on mitochondria to avoid late-stage attrition during drug development.

To evaluate mitotoxicity, several screens have been used in early compound assessment such as measurement of ATP production, membrane potential, apoptosis induction, O₂ consumption, and ROS production. Indeed, these assays are useful first-order screens to evaluate mitochondrial integrity, and some have been used in HTS strategies (Hynes et al., 2006; Swiss et al., 2013). Although they are able to identify acute toxic effects, unfortunately they remain largely inefficient to evaluate discreet and/or chronic effects that can lead to long-term mitochondrial dysfunction. They are also unable to decipher which mechanisms are involved in mitochondrial disruption, which OXPHOS complexes are disturbed, or more precisely which enzyme inhibitions are directly responsible for that toxicity. For these reasons, used either alone or in combination, these tests cannot be trustfully used to drive a binary decision regarding the safety and the future development of a drug.

Catalytic defects in mitochondrial and/or OS pathways have been reported in a large number of living species (from unicellular to multicellular eukaryotes, from vegetal to animal) (Campbell et al., 1994; Schwarzländer and Finkemeier, 2013). In mammals, whereas OS seems ubiquitously distributed, mRCC dysfunction is frequently observed in specialized tissues. As compound evaluation can be performed in either preclinical or clinical stage, the development of versatile diagnostic enzyme assays usable either on a large subset

of cells or tissues or on a variety of living species is strongly mandatory.

Traditional quantification methods for either mRCC or OS are usually labor intensive and time consuming because they are still largely manual. Because of this, they are also only available in a small number of very specialized laboratories able to extract with appropriate means mitochondria from whole cells. Most of the conventional techniques lack analytical performances, especially precision, with intra- and inter-CV % largely above 10%, impairing the detection of tiny variations that are common in chronic illnesses. In addition, the measurement of a large panel of markers necessitates the use of a big range of different techniques. It also requires to measure parameters successively and not in parallel, generating an unavoidable degradation of the starting material.

For all that reasons, we decide to develop automated robust tests on a random-access analyzer usually used daily in clinical biology laboratories. These apparatus allow to measure in parallel with the highest precision a large number of parameters in a few minutes without any degradation of the starting material. They do not require previous mitochondrial extraction as they work on whole cell extracts. As it is also possible to measure protein content in the same sample, aside from classical quantification, it becomes possible to compare the different results and to establish ratios (in enzyme activities, in substrate production, etc.). This allows a true comparison of cell metabolism pathways at a precise moment. Such automated measurement was not a new idea as the use of such equipment was initially described for combined cytochrome *c* oxidase (complex IV) and citrate synthase (CS) activity measurement (Williams et al., 1998). It was further extended for joint measurement of complexes I, II+III, and IV in cultured skin fibroblasts to detect congenital mRCC deficiencies (Kramer et al., 2005). Nevertheless, both the expensive price of such analyzers and the difficulties to transfer classical tests on them had restrained their development.

We report here the development and the validation of a panel of RCC and OS automated assays that allow for the first time the simultaneous determination of five mitochondrial OXPHOS complexes and antioxidant and glutathione pathway enzymes in various media.

15.2 Technology

Protein concentrations, respiratory chain, and OS enzyme activities were performed on a Roche Diagnostics/Hitachi Modular P analyzer. This apparatus, normally dedicated to routine clinical biochemistry, consists in an automated spectrophotometer that reads

the absorbance at a specified wavelength in a kinetic fashion. This instrument functions with semipermanent cuvettes and up to 300 specimen capacity, with continuous loading of five-position sample racks and a maximum capacity of 800 tests/h. It automatically transfers samples and reagents to a cuvette, incubates them at 37°C, and reads the absorbance at a specified wavelength. It can measure a maximum of 86 different photometric tests. Each programmed assay can use up to four different reagents. First reagents are dispensed into the cuvette as few as 10 s after the sample. Other reagents can be dispensed between 1 and 10 min, after the first one. Absorbance readings are taken every 20 s, allowing very precise follow-up of the reaction, and calculations can be programmed into the instrument to automatically transform reaction rate data into enzyme activities.

These technical specificities gives to these biochemical assays a very high reliability and small variation coefficients. CV is expressed as a percentage (standard deviation/mean $\times 100\%$), is unitless and dimensionless, and can be used easily to compare results over time between machines or even distant sites. With that kind of automated devices, very low intra- and inter-assays, some largely lower than 5%, can be observed for the different tests. The lower the CV is, the less variation there is and the highest the confidence in the results given by this precise test is. Thus, being confident, there is no need to iterate the test to ascertain the validity of results. Hence, in terms of power, high CVs have low power to detect small-scale differences (Figure 15.1), and only low CV tests display sufficient power to

significantly detect tiny variations. Generally, the only way to increase power with high CV techniques relies in increasing the number of replicates and the need of a large amount of starting material. Using tests with the least inherent variability will induce the least replication and thus will be the most cost-effective. Indeed, due to this high level of precision, the experiments can be performed only once for each sample like what is routinely done for markers in patients' plasma or serum (e.g., in all world clinical biochemistry laboratories, blood glucose measurement is only performed once as the CV% of the glucose oxidase-based test is largely below 1%). This procedure saves sample volumes and allows carrying out all the experiments on the same aliquot. With such specificities, and because we do not need to extract mitochondria, we can perform test panel on as few as $3 \cdot 10^6$ cultured cells, 5–10 mg biopsies, or nucleated blood cells or red blood cells extracted from a 5 mL blood vacutainer.

The automation allows launching one assay every 6 s on the analyzer. A panel of analysis like ours consisting of more than 20 assays may be therefore launched in 2 min time, limiting the biological evolution of the sample. Therefore, we can assume that all measurements are performed on a same sample at the same time. Moreover, as all the results are given in less than 30 min, it becomes possible with the same apparatus and the same reagents to verify samples, whose results seem unfitting, in a second analysis without time degradation between the series. It allows also the possibility to measure in the same series cell homogenates, biopsies or tissue

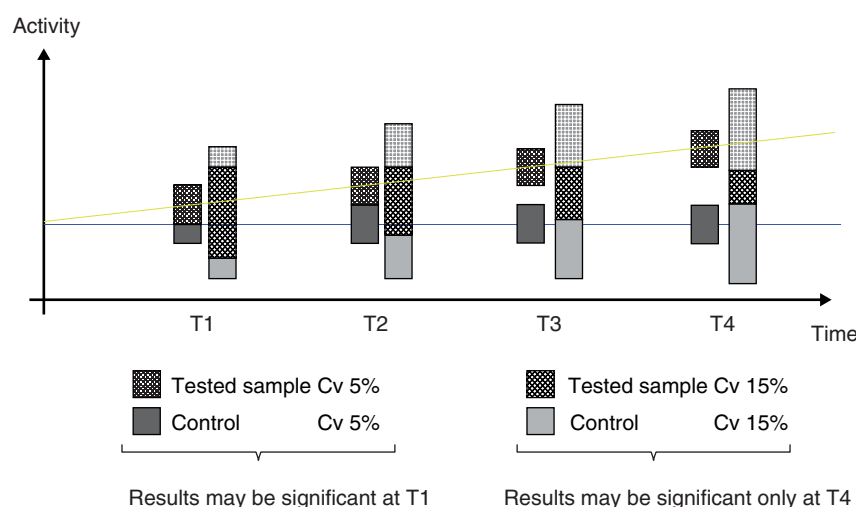


Figure 15.1 Low CVs' importance to detect tiny metabolic variations. Low CVs, as compared with high ones, allow detecting significant small variations. The figure here summarized the increase along the time of, for example, two enzymatic activities. With a low CV technique, we have to wait only for the second observation time T2 to consider without any doubt that the two observed enzymatic activities are significantly different (the two box plots do not show any overlap). In the case of the high CV technique, at T2, the important overlap between the observed results impairs any conclusion regarding the two results. They still must be considered as identical. It holds the same at T3 and even at T4 as there are still overlapping values between the two series.

Table 15.1 Advantages and key benefits of functional metabolism evaluation.

Technical advantages	Key benefits
Unmatched precision: inter- and intra-CV < 5%	Highest quality standards in the pharmaceutical and cosmetics industries
Reduced sample volume (5 mL) associated with multiparametric assay testing on single sample (up to 30 assays per sample)	Accurate and reliable results
High-throughput instruments: one test pipetted every 6 s	Eliminates bias due to inter-experiment variability
Minimal sample preparation	Eliminates bias due to sample degradation during experiment
Minimal sample needs	No mitochondrial extraction needed Allowing performing a full panel of 20 tests on as low as a Falcon T75 starting material

Decision-making tool supporting subsequent testing or development.

homogenates from different species, or human plasmas, nucleated cells, and red blood cell hemolysates. These characteristics give a valuable advantage as compared with individual or even plate-derived spectrophotometric determinations of the different activities and/or concentrations (Table 15.1).

15.2.1 OXPHOS Complex Measurements

All respiratory chain complex assays were based on methods described by Kramer and Nowak (1988) and Krähenbühl et al. (1994, 1996), both modified to match our apparatus requirements. The activities of all the complexes in each sample were normalized by the amount of protein or referred to CS activity to allow sample comparison.

- **Complex I Activity Measurement**
Briefly, reduced nicotinamide adenine dinucleotide phosphate (NADPH)–ubiquinone reductase activity (complex I) was measured by following the disappearance of NADPH using rotenone as a specific inhibitor to ensure the specificity of the assay.
- **Complex II Activity Measurement**
Complex II activity, succinate–ubiquinone reductase, was assayed through the reduction of 2,6-dichloro phenolindophenol, a final electron acceptor, after the addition of succinate.
- **Complex III Activity Measurement**
The activity of complex III, ubiquinone–cytochrome *c* reductase, was determined by assaying the rate of reduction of cytochrome *c*.

- **Complex IV Activity Measurement**
The cytochrome *c* oxidase (complex IV) activity was based on the same assay as for complex III using potassium cyanide to inhibit the activity of this enzyme.
- **Complex V Activity Measurement**
Complex V activity was measured according to a method coupling ADP production to NADH disappearance through the conversion of phosphoenolpyruvate into pyruvate and then into lactate (Rustin et al., 1993).
- **CS Activity Measurement**
The activity of CS was assayed as described previously (Itoh and Srere, 1970) with the reduction of DTNB caused by the deacetylation of acetyl-CoA.
- **Evaluation of the Energetic Capability**
ATP/ADP/AMP cellular levels were assessed by capillary electrophoresis after protein precipitation with 2% PCA (Uhrová et al., 1996; Markuszewski et al., 2003). Two calculations can then be derived:
 - **Adenylate energy charge (AEC)** is a measure of disposable energy at a given moment in the cell. $AEC = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP])$.
 - **Total adenine nucleotides (TAN)** evaluation gives an idea of the pool of adenine nucleotide available in the cell. $TAN = AMP + ADP + ATP$.
- **Assessment of Anaerobic Glycolysis**
When mitochondria cannot supply sufficient ATP for cell metabolism, anaerobic glycolysis is activated. We therefore see in the activation of anaerobic glycolysis an indirect proof of mitochondrial dysfunction. Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the production of ATP in anaerobic conditions using glucose as substrate and generating lactate. It was assayed as described by Beutler and Mitchell (1968).

15.2.2 OS Pathway Measurements

To study the reduction of O_2 into water, we measured the activities of each of the main antioxidant enzymes and antioxidant compounds in this pathway.

- **Assessment of Superoxide Radical Reduction**
To study $O_2^{\cdot -}$ reduction to H_2O_2 , cytosolic superoxide dismutase (SOD) (Cu/Zn-SOD, SOD1) and mitochondrial SOD (Mn-SOD, SOD2) activities were both measured. Using a slightly modified protocol based on the method developed by McCord and Fridovich (McCord and Fridovich, 1969; Huet et al., 2011), total SOD was measured at pH 7.8, and SOD1 was measured at pH 10.2. SOD2 activity was calculated as the difference between total SOD and SOD1 activities.
- **Assessment of H_2O_2 Reduction**
 H_2O_2 is reduced to water mainly by the glutathione antioxidant pathway. Glutathione peroxidase (GPX) activity was measured using a method based on the one developed by Paglia and Valentine (1967). Catalase

(CAT) activation is another mode of H_2O_2 removal. CAT activity was measured using a derived method from the one developed by Johansson and Borg (1988).

- **Assessment of the Glutathione Antioxidant Pathway**
This pathway includes the antioxidant enzymes GPX, glutathione reductase (GSR), and G6PD and nonenzymatic antioxidant compounds such as reduced and oxidized glutathione (GSH and GSSG).

GSR and G6PD activities were estimated as already described by Beutler and Mitchell (1968). Glutathione synthesis was estimated by measuring the activity of glutamate–cysteine ligase (GCLC) and glutathione synthetase (GSS) as described previously (Huang et al., 1993).

GSH and GSSG concentrations were assayed by capillary zone electrophoresis after protein precipitation with 2% PCA (Muscari et al., 1998; Serru et al., 2001). Total glutathione is the sum of both GSH and GSSG.

- **Lipid Peroxidation Assay**
The MDA concentration was measured by spectrofluorimetry as previously described (Conti et al., 1991). Briefly, the sample was treated with diethylthiobarbituric acid (DETBA), and the fluorescent compound was then extracted with butanol and determined by synchronous fluorescence spectroscopy.

MDA in each sample was also normalized with total proteins assayed by the method of Bradford (Bradford, 1976).

15.3 Applications of Functional OXPHOS and OS Measurements in Drug Evaluation

15.3.1 Combined OXPHOS and OS Measurements in Drug Toxicity Evaluation

The mitochondrial respiratory chain is the major site of ATP production, but it also plays an important role in apoptosis and cell death. Inhibition of OXPHOS complexes blocks the ability of some ETC components to change in a reversible manner from an oxidized state to a reduced one. It results in accumulation of reduced forms ahead and of oxidized ones downstream the inhibitor point. Since energy is not released, ATP synthesis stops.

To evaluate the capability, our technology has to detect drug-induced mitochondrial dysfunction, and we used a well-known complex I inhibitor, namely, rotenone. Aside from its effect on ATP synthesis, rotenone has been also shown to induce apoptosis via enhancing the global amount of mitochondrial ROS (Li et al., 2003; Sipos et al., 2003). Indeed, mitochondria-induced ROS are vital as, once produced, they can quickly influence mitochondrial functions and alter mitochondrial DNA structure (Murphy, 2009). Two sites of ROS production, complex I

and complex III, have been identified as a major source of mitochondrial ROS (Turrens et al., 1985; Cochemé and Murphy, 2008). Thus, evaluation of mitochondrial dysfunction must include not only ATP synthesis measurement but also ROS production evaluation.

OXPHOS and OS were measured *in vitro* on different cell lines (KM201, HCT116, HepG2, HepaRG) and on fresh extracted human lymphocytes, treated or not by rotenone. Results are presented in Table 15.2. As expected, 0.05 μ M rotenone has an important inhibitory effect on complex I activity (about 50% decreases for KM201 and HCT116, about 30% for other cells) and displays no effect on complexes II–V whose activities stay unchanged. Interestingly, some unexpected results are found. Whatever the analyzed cells, a constant drop in GPX activity is observed with a maximum in KM201 cells. This decrease in GPX activity will alter cell global antioxidant defense. While GSH synthesis is not modified, GPX activity reduction is associated with a slight but noticeable decrease in GR activity in every cell, generating a lack in glutathione recycling. This defect can be evidenced by the associated rise of oxidized GSSG. Altogether, cell rotenone-induced disability to correctly eliminate ROS mitochondrial production induces lipid peroxidation as shown by the rise of MDA levels.

The observation of rotenone-induced mitochondrial dysfunction of complex I has helped to demonstrate the potential relevance of complex I defects to several pathologies such as neurotoxicity (Betarbet et al., 2000). The rotenone model has been used to study PD pathogenesis (Sherer et al., 2003). More than rotenone-complex I dysfunction, OS induction by rotenone is likely to sustain its neurotoxicity as brains of PD patients show decreased GSH levels and oxidative modifications such as lipid peroxidation (Bashkatova et al., 2004). However, our results on our cell models differ from those observed on human neuroblastoma SH-SY5Y cells where rotenone induced a rise in GPX activity and no associated GR activity modifications (Molina-Jiménez et al., 2005). This discrepancy can be related to the dose of rotenone used in our study that is 100 times lower than the one that induces a significant rise in GPX activity. In acute OS induction, it is generally accepted that every antioxidant defense system is induced to detoxify the large amount of generated ROS, and once the system is overstepped, mitochondrion is destroyed and cell is induced to death through proapoptotic pathways. In lower OS induction, it is likely to be different as it will be in chronic diseases where low noise dysfunctions could appear as a result of long-term ROS accumulation.

This example shows that, using performing and precise means, it becomes possible to combine OXPHOS and OS analysis in a same sample and to evidence slight modifications of deep metabolism and enzyme activities that could lead with time to functional cell dysfunction.

Table 15.2 Rotenone effect on either OXPHOS or OS pathways.

	Prot	CS	CP1	CP2	CP3	CP4	CP5	GPX	G6PD	GR	GCS	GCG	GSH	GSSG	Total	GSSG/GSH	MDA
	g/L	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	U/mg Prot	μmol/mg Prot	μmol/g Prot	μmol/g Prot		μmol/g Prot
KM201 control	1.4	95.8	25.2	18.9	1.9	17.0	352.6	21.5	21.3	42.0	nd	nd	nd	nd	nd	nd	nd
KM201 rot 0.05 μM	1.2	111.2	12.0	19.9	1.9	20.8	361.5	4.3	27.8	27.8	nd	nd	nd	nd	nd	nd	nd
HCT116 control	1.9	80.0	32.0	15.3	1.7	18.5	442.1	458.0	68.5	123.1	nd	nd	nd	nd	nd	nd	nd
HCT116 rot 0.05 μM	2.2	75.2	17.1	14.3	1.3	11.0	429.3	156.2	68.0	34.0	nd	nd	nd	nd	nd	nd	nd
HepG2 control	1.0	680.3	150.1	21.7	0.9	138.5	540.0	83.1	138.5	60.5	178.9	141.9	17.2	2.9	23.0	0.2	1.0
HepG2 rot 0.005 μM	0.9	668.1	99.0	23.8	0.9	153.2	658.5	75.2	124.0	52.3	192.4	54.1	15.0	4.2	23.4	0.3	1.3
HepG2 rot 0.05 μM	1.1	727.7	87.7	21.0	0.8	122.3	560.4	72.8	145.5	56.8	172.8	137.6	12.7	4.9	22.4	0.4	1.4
HepaRG control	0.9	702.4	166.9	23.1	0.9	172.2	697.2	93.7	147.5	57.3	246.3	170.9	18.3	3.5	25.3	0.2	0.8
HepaRG rot 0.005 μM	0.8	730.1	132.0	27.4	1.0	218.3	768.1	71.3	153.0	52.2	272.8	198.5	18.0	3.5	25.0	0.2	1.0
HepaRG rot 0.05 μM	0.5	570.4	115.2	31.0	1.2	289.5	813.1	60.5	225.7	39.8	246.7	198.6	16.9	6.3	29.4	0.4	1.7
Fresh human lympho control	1.6	569.4	69.1	24.2	2.0	93.1	328.2	208.3	166.0	99.8	nd	nd	2.5	1.1	4.5	0.4	1.6
Fresh human lymphocytes rot 0.05 μM	1.5	522.0	39.0	23.9	2.1	105.0	352.0	162.3	182.0	87.8	nd	nd	2.1	2.7	4.1	1.3	2.1

Controls cells are cultured in a medium devoid of rotenone.
nd, not determined.

15.3.2 Glucose as an Underestimated OXPHOS and OS Metabolic Modifier in Cultured Cells

Understanding metabolic changes in cells is an important issue for drug development as the drug may work on cells or tissues in a particular condition and not in another. It is particularly relevant for cancer cells as most newly drugs are designed to address either all cancer cell subpopulations or a specific one in a given tumor tissue. Indeed, every tumor cell will not behave identically toward the same chemotherapeutic drug or radiation level.

To demonstrate that our technology is able to link specifically OXPHOS and OS in that context, two prostate cell lines (PNT2 SV40 immortalized cell line, derived from normal prostate, mimicking normal epithelial prostate cells and LNCaP cell line, derived from prostate cancer patient axillary lymph node, behaving as a classical androgen-sensitive prostate adenocarcinoma cell line) were cultured in regular RPMI medium using two different concentrations of a natural molecule that is necessary for cell growth but becomes toxic at high concentration, namely, glucose. Two concentrations of 1 and 5 g/L were chosen (1 g/L is the usual glucose concentration in regular RPMI and 5 g/L glucose is used in regular DMEM culture medium). The physiological plasmatic concentration of glucose is around 1 g/L (5 mmol/L) in humans (and certainly much lower in tissues). A concentration of 5 g/L (25 mmol/L) is considered as very high and generally found in the plasma of largely uncontrolled diabetic patients. This high glucose concentration can generate OS and induce mitochondrial dysfunction (Blake and Trounce, 2014; Liemburg-Apers et al., 2015; Asmat et al., 2016) that will be responsible of well-described diabetes-induced tissue oxidation particularly in the kidney, heart, vessels, eyes, and nervous system, generating either nephropathy, cardiomyopathy, arteritis, retinopathy, or peripheral neuropathy (for review see Forbes and Cooper, 2013; Lotfy et al., 2017). Aside from glucose-induced OS and its potential effect on mitochondrial function, analyzing glucose effect remains an interesting issue as it is a starter for energy production within the cell, and its function in the context of tumor cells is still a matter of debate (Kamarajugadda et al., 2012; Yin et al., 2012).

• Normal PNT2 versus Tumor LNCaP Cells Cultured under Normal Conditions (1 g/L Glucose)

At first glance, the simple observation of the two profiles of PNT2 and LNCaP cells (Figure 15.2) shows dramatic differences, the LNCaP cells showing large decrease in glutathione content and OXPHOS cascade and an important increase in several enzyme OS activities. When normalized with sample protein content,

the overall respiratory activity appears increased in LNCaP cell line when compared with normal PNT2 cell line (Figure 15.3a). While complexes I and II activities are moderately increased, there is a huge increase in complex V activity (1.5-fold). However, when normalized with CS activity, a constitutive enzyme of the mitochondrial matrix and a validated marker of the mitochondrial content (Vigelsø et al., 2014), all respiratory complex activities appear decreased (Figure 15.3b). This paradoxical feature fits with the Warburg theory that postulates the existence of a global metabolic reprogramming of tumor cells with a drop of respiratory chain activity and consequently an increase conversion of glucose to lactate even under normoxic conditions (Warburg, 1956; Goodwin et al., 2014). At the light of our results, we can postulate that, in response to OXPHOS decreased activity, LNCaP cells are likely to increase their mitochondrial number (large CS increase) to maintain a superior global respiratory activity (even better than the one of normal PNT2 cells), thus supporting the existence of a mitochondrial adaptation to cell energetic needs.

• PNT2 Cells in 1 g/L Glucose versus PNT2 Cells in 5 g/L

The overall respiratory activity seems decreased in PNT2 cell line at 5 g/L when compared with 1 g/L glucose, with a severe drop in complex III and IV activity. Nevertheless, complex V activity remains stable. CS is very slightly increased, suggesting stability in the number of mitochondria. Energetic charge as well as TAN is unchanged with higher glucose concentrations, although intracellular ATP is slightly increased at 5 g/L glucose when compared with 1 g/L glucose (Figure 15.4a).

Concerning OS, SOD1 activity is slightly increased, while SOD2 activity is significantly higher at 5 g/L glucose as compared with 1 g/L of glucose. This suggests the existence of an OS that is major in the mitochondrial compartment and minimal in the cytoplasmic one (Figure 15.4b). In the glutathione antioxidant pathway, a decrease of almost twofold in GSH and 1.6-fold in GSSG concentrations is noted at 5 g/L glucose when compared with 1 g/L glucose concentration in the media. The total glutathione level is twice lower at 5 g/L glucose than the one measured at 1 g/L glucose. On the contrary, a better GSH/GSSG ratio is observed, which is doubled at 5 g/L. The GSR enzyme activity is not affected by glucose concentration, and the synthesis of glutathione is slightly stimulated, with only a rise of the activity of GCLC of 1.2-fold. As for G6PD, its activity is decreased by 1.4-fold with the rise of glucose concentration.

The MDA levels remain constant.

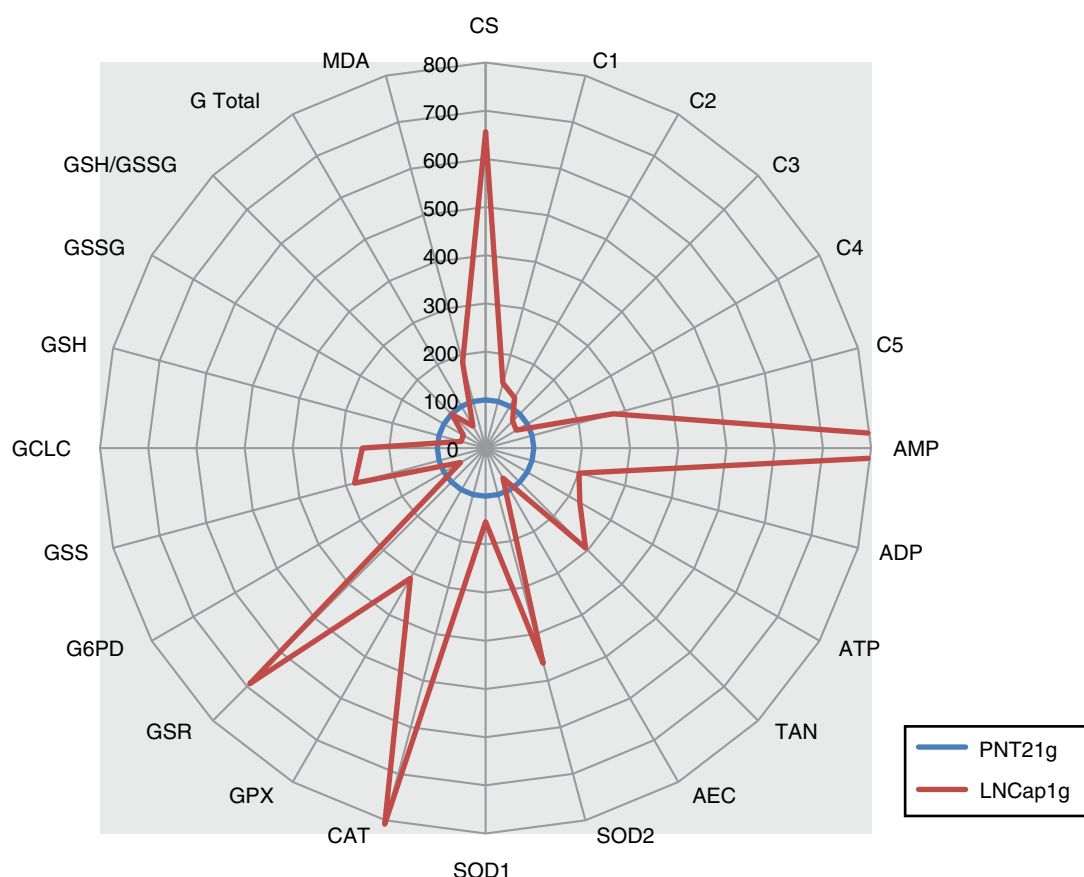


Figure 15.2 Spider representation of LNCaP versus PNT2 OXPHOS and OS metabolism (culture of both cells at glucose 1 g/L; PNT2 cells metabolism is used as reference and represents 100%).

• LNCaP Cells in 1 g/L Glucose versus LNCaP Cells in 5 g/L

The respiratory activity of LNCaP cells is globally unaffected by glucose concentrations, with the exception of complex IV activity, which is slightly decreased, and complex V whose activity is slightly enhanced at 5 g/L glucose (Figure 15.5a). Interestingly, when compared with PNT2 cells, the variation of the OXPHOS cascade follows a same drop in 5 g/L glucose as the one observed in 1 g/L, confirming that while glucose is needed to counterbalance OXPHOS dysfunction, the external glucose moiety cannot modify the Warburg phenotype of the LNCaP cells (Figure 15.3c, d).

Energetic charge as well as TAN is unchanged at all glucose concentrations, and the ATP level falls (1.3-fold) at 5 g/L glucose.

Higher concentrations of glucose affect neither the SOD1 activity nor the SOD2 one. The GPX activity is decreased, whereas CAT one is unchanged (Figure 15.5b). Concerning the glutathione redox system, a decrease in GSH level is observed at 5 g/L glucose, while the level of GSSG increases in the same range. However, total glutathione and GSH/GSSG ratio are both decreased with

higher glucose concentrations, respectively, 1.48-fold and 2.45-fold. The activities of the enzymes responsible of the recycling of the glutathione, that is, GSR and G6PD, are both unchanged by glucose concentration. A small increase (1.13-fold) in the activity of synthesis of glutathione is observed at 5 g/L glucose.

MDA levels are stable at 5 g/L glucose.

One fundamental modification that occurs during malignant transformation is the mode of glucose utilization. Normal cell physiology requires sufficient supply of reduced carbon source for the generation of ATP, building blocks, and reducing power (Eagle, 1955). These fundamental needs are first increased and then overstepped by the rapid proliferation of tumor cells. Nevertheless, blood supply is rapidly insufficient to feed that needs, and cancer cells have to rapidly adapt their metabolism to insufficient carbon and nitrogen sources as well as oxygen. Besides angiogenesis, glutaminolysis and the Warburg effect (aerobic increased glucose consumption and lactate production with a natural switch to glycolysis with OXPHOS arrest) are two of the main metabolic changes that occur in tumor cells (Warburg, 1956; Medina and Núñez de Castro, 1990).

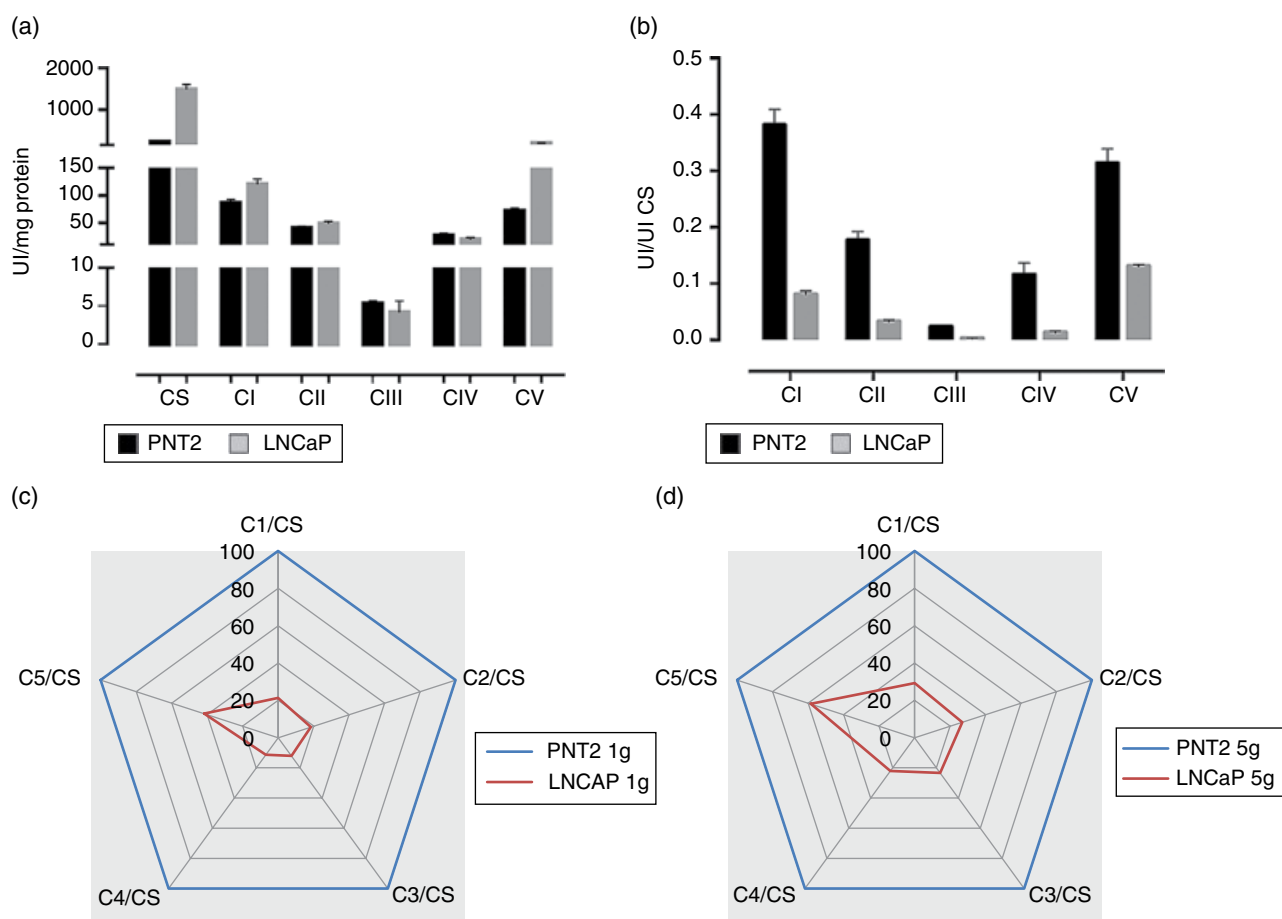


Figure 15.3 OXPHOS complex results in PNT2 and LNCaP cells. (a) OXPHOS complexes and CS results for both cells under normal conditions (glucose 1 g/L). (b) OXPHOS complex results after normalization with CS value. (c) Spider representation of OXPHOS/CS values for PNT2 and LNCaP cells cultured at 1 g/L. PNT2 values are given as reference (100%) and LNCaP values in % are calculated from the reference. (d) Spider representation of OXPHOS/CS values for PNT2 and LNCaP cells cultured at 5 g/L. PNT2 values are given as reference (100%) and LNCaP values in % are calculated from the reference. For Figure 15.3a and b, results values are given as mean $\pm$ SD of five different culture flasks for each cell line.

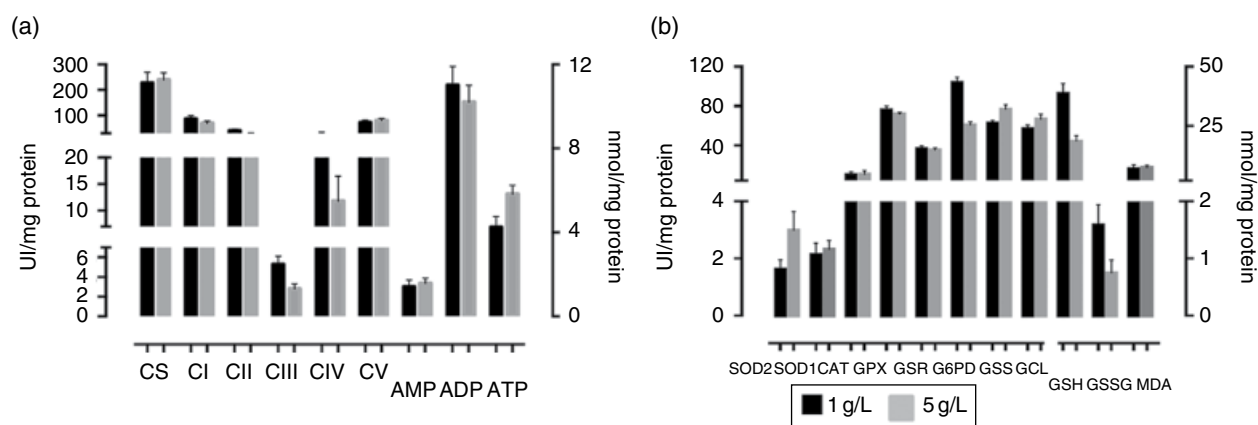


Figure 15.4 OXPHOS and OS metabolisms of PNT2 cells cultured at 1 and 5 g/L glucose. (a) OXPHOS metabolism. (b) OS metabolism. For Figure 15.4a and b, results values are given as mean $\pm$ SD of five different culture flasks for PNT2 cell line.

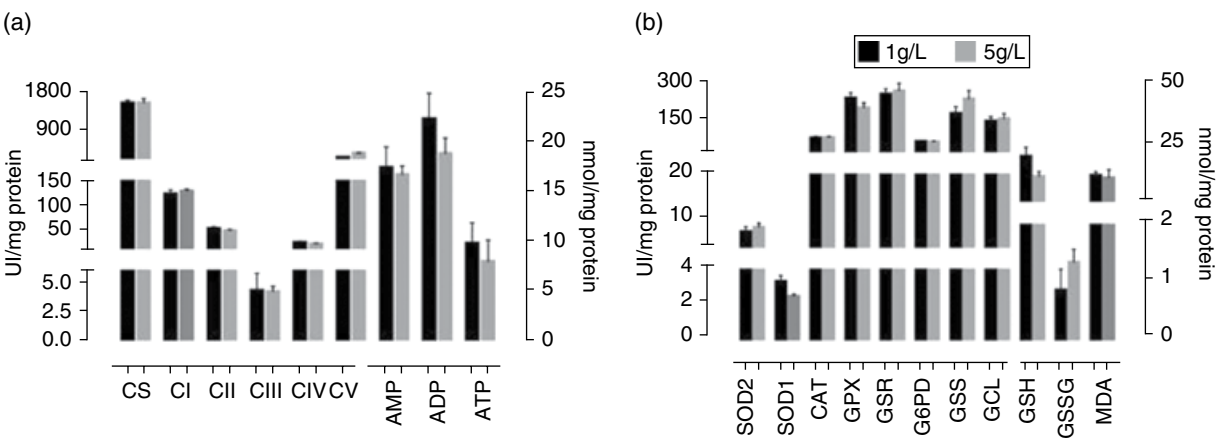


Figure 15.5 OXPHOS and OS metabolisms of LNCaP cells cultured at 1 and 5 g/L glucose. (a) OXPHOS metabolism. (b) OS metabolism. For Figure 15.5a and b, results values are given as mean $\pm$ SD of five different culture flasks for LNCaP cell line.

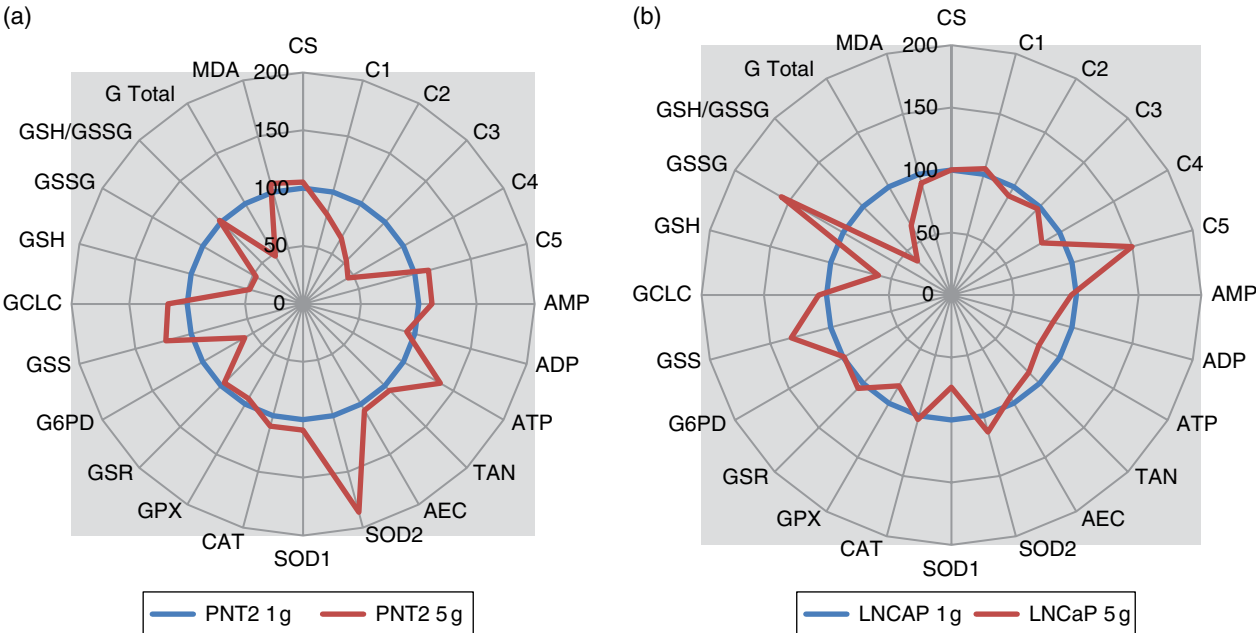


Figure 15.6 Spider representation of compared OXPHOS and OS metabolisms at 1 and 5 g/L glucose in culture. (a) PNT2 cells. (b) LNCaP cells. For both cell lines, 1 g/L glucose results are given as reference (100%) and 5 g/L values are calculated from the reference.

In this study, we explore the effect of glucose availability on the energetic metabolism of different prostate cancer cell lines using RPMI culture media enriched with different concentrations of glucose. The measurement of several antioxidant enzymes as well as important antioxidant and energy-containing substrates reveals the overall energetic metabolism of the cells.

Indeed, we show that PNT2 cells, which behave as normal prostate cells, are very sensitive to glucose excess in the environment (Figure 15.6a). When these cells grow in an environment of 5 g/L glucose, we observe an increase in both SOD activities—a slight one for SOD1

and a major one for SOD2—suggesting the occurrence of a minimal OS within the cytoplasmic compartment and a major one in the mitochondrial one. Detoxification of superoxide radical anions by superoxide dismutation generates hydrogen peroxide (H_2O_2) that is a more powerful pro-oxidative molecule than superoxide itself. The concomitant GPX decrease suggests a detrimental accumulation of H_2O_2 in the mitochondrial compartment that can explain the important decrease in complex III and IV activities that appear as the most affected. Mitochondrial number is slightly increased by glucose, but while each mitochondrion is less efficient, global

ATP synthesis is increased. Individual mitochondrial dysfunction is certainly counterbalanced by a global glycolysis increase that generates 2 ATP/glucose.

To overcome this OS, the cell used glutathione and we observed a fall in glutathione level both in its reduced and oxidized forms when PNT2 cells are grown in 5 g/L glucose media. As a response to this decrease, *de novo* glutathione-synthesizing enzymes show an increase in their activity. Nevertheless, stressed PNT2 cells use more glutathione than they are able to create. Interestingly, GSSG decreases, so GSH/GSSG ratio remains unchanged. A possible explanation could be GSSG secretion in the extracellular media due to an inefficient reduction with decreased G6PD activity associated with stable GR activity. The lack of NADPH is likely to reduce the global reducing power of PNT2 cells in glucose 5 g/L. This disruption in the antioxidant metabolism drives unavoidably PNT2 cells in an OS state. This OS mainly directed against mitochondria does not increase cellular MDA.

LNCaP cells do not react in the same manner to increasing glucose concentrations. Mitochondrial SOD2 activity appears unaffected, while a slight decrease in SOD1 activity is measured at 5 g/L glucose (Figure 15.6b). As GPX and CAT activities stay in the normal range, no H₂O₂ seems to be generated. As compared with PNT2 cells, no mitochondrial stress is induced by glucose and OXPHOS activity level stay unchanged. Nevertheless, the energetic charge of the LNCaP cells decreases, suggesting that cells do use more ATP than they synthesize.

LNCaP cells seem also using a lot of GSH that is oxidized. As either glutathione synthesis (normal GSS and GCLC activities) or reducing power (normal GSR and G6PD activities) is normal, glutathione is certainly used by a different pathway than ROS.

These experiments on both PNT2 and LNCaP cells show that our technology is able to detect differences in the different metabolic cell pathways within a cell. By its precision, it allows to detect very slight variations in enzyme or substrate activities that can explain a particular behavior of the cell under specific culture conditions. By its capability to explore several metabolic routes in parallel with the same level of accuracy, it can be of valuable help to decode the involved mechanisms supporting cell compartment.

PNT2 and LNCaP cells, while both of prostate origin, do not behave identically when glucose is added in the culture medium. This certainly holds also true for all the different cells in the body as both intrinsic genetic program and enzyme equipment are different and may generate dramatic changes in the induction of metabolic and regulatory pathways. Such cell variability will unavoidably have consequences on further experiments. When a drug has to be tested on a particular cell line, its effect will largely depends on the basal state of the cells at

the beginning of the experiment. If cells are stressed for known or unknown reasons, it is probable that the observed results would largely differ from those observed on an unstressed cell. To avoid variations from one lot to another that could explain results dissemination, the evaluation of cell metabolic state at the beginning of an experiment (i.e., creation of a kind of metabolic passport) is certainly mandatory.

15.4 Versatility of the Technology

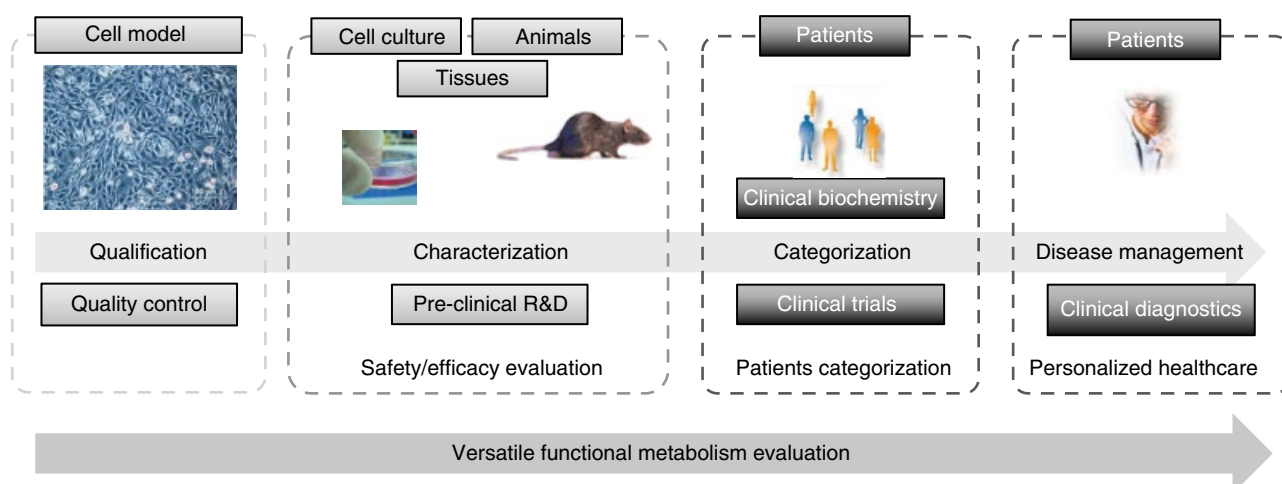
Of primary importance is the versatility of our approach. Indeed, one of the main interests of our technology relies in its capability to be used on various media with the same criteria of precision. These automated measurements can be performed on a large variety of cultured cells (Table 15.3), but they can also be used to measure both OXPHOS and OS in every extracted nucleated cell (blood white cells) or OS on non-nucleated cells (red blood cells). It is also possible to measure the different parameters on biopsies or tissue extracts or on liquids of diverse origins (urine, cerebrospinal fluid, articular liquid, peritoneal fluid, etc.).

Nature of species samples does not represent a barrier to our technology. It becomes possible to measure our parameters in every eukaryote, from all animals to plants, fungi, and protists. Indeed, measuring substrate concentrations and/or enzyme activities abolishes species specificity. There is no need to find very specific antibodies for each tested species, as it is the case when quantification of enzyme expression has to be performed at the protein level. For example, SOD2 or complex I activities can be detected not only in rat or human cells and tissues but also in *Caenorhabditis elegans* extracts or in *Arabidopsis thaliana* leaves. Table 15.3 shows the different species that have been yet tested for either OXPHOS or OS metabolism.

More interestingly, merging these two specific features, and because it represents an integrative approach consisting of a large panel of very precise assays, it becomes possible to use our technology during all the process, bringing a pharmaceutical drug to the market. First, and because it works well on cultured cells, it can be used in target discovery as finding new drugs targeting mitochondria is of utmost importance (Figure 15.7). Preclinical and clinical data have demonstrated the considerable potential of mitochondrial targeting approaches, and potential therapeutic applications span a broad range of pathological conditions (Sewczyk and Wojtczak, 2002; Frantz and Wipf, 2010). Our technology will work with the same precision from preclinical *in vitro* or *in vivo* studies on microorganisms and animals to clinical phase I–IV trials on humans. It can be

Table 15.3 Versatility of functional metabolism evaluation.

Species	Cells	Tissues	Media
Unicellular			
<i>Escherichia coli</i>	ES, IPS	Kidney	Plasma
<i>Trichomonas vaginalis</i>	Fibroblasts	heart	Urine
<i>Trypanosoma cruzi</i>	Keratinocytes	Liver	Cerebrospinal fluid
<i>Trypanosoma brucei</i>	Melanocytes	Spleen	Articular fluid
<i>Plasmodium falciparum</i>	Astrocytes	Lungs	Tears
<i>Leishmania donovani</i>	Cardiomyocytes	Skin	Seminal Fluid
<i>Leishmania infantum</i>	Motoneurons	Muscles	Cervical mucus
<i>Toxoplasma gondii</i>	Trophoblasts	Penis	Bronchoalveolar lavage
	Spermatozoa	Prostate	Bronchial condensate
	Oocytes	Bladder	Organ perfusion liquid
Pluricellular			
<i>Caenorhabditis elegans</i>	Monocytes	Colon	Organ preserving liquid
Trout	Lymphocytes	Brain	Cell culture medium
Mouse	Macrophages	Cerebellum	
Rat	Reticulocytes		
Guinea pig	Hepatocytes		
Chicken	Epithelial cells		
Dog	Endothelial cells		
Pig	Cell lines		
Horse	HUVEC, HepG2		
Human	PNT2, LNCaP, 22RV1		
	T24		
	HT29, CACO2, SW480		
	LMTK		
	HEK		

**Figure 15.7** Automated functional metabolic measurement can be performed with the same accuracy from early drug discovery experiments to late phase clinical studies.

used for toxicity evaluation of a compound and also be useful to drive drug safety conclusions. In addition, concerning disease models, several models can efficiently be addressed by our platform. These models will fit with all human medical applications where metabolism dysfunction is involved.

The increasing prevalence of age-related disorders calls for innovative solutions, and mitochondrial drugs clearly have the potential to emerge as a key platform technology for the next generation of medicines. In this context, our technology can be part of the concept of personalized medicine, allowing the precise evaluation of drug behavior at the cell or tissue level in a small group of patients or even in an individual.

15.5 Conclusions and Future Perspectives

Assessing global metabolic behavior earlier and more comprehensively in the drug development process will help avoid costly late-stage attrition and, more importantly, will improve drug safety. As more novel drugs, heading for restoring congruous cell, tissue, or organ equilibrium in every disease that is metabolic in nature, are supposed to be developed in the next future, the probability of concomitant mitochondrial and cytoplasmic metabolic pathway dysfunctions will largely increase. Because most of these defects are likely to be insidious, they will necessitate more sensitive and frequent evaluation.

The evaluation of the global state of any sick person coming in the ICU of an hospital must include a large number of blood markers, either general (electrolytes Na^+ , K^+ , Cl^- , HCO_3^- , total protein content, Ca^{2+} , CRP, etc.) or organ specific (heart (troponin, creatine kinase), liver (ASAT, ALAT, alkaline phosphatase, bilirubin), kidney (bun, creatinine, cystatin), pancreas (lipase, amylase), etc.), to offer, in the shortest time, the physician all the elements to best diagnose or follow his patient. It holds for us also true for testing a drug on cells or tissues. Understanding mitochondrial dysfunction must include general data on cell bioenergetics and on the metabolic routes that fuel mitochondrion. Thus, to better appreciate the impact of a drug on cell behavior, anyone needs to get reliable and very precise data in an integrated context. The use of multiparametric equipment able to measure several markers in parallel with a high precision, without any bias due to either inter-experiment variability or sample degradation, is certainly an interesting opportunity to evaluate the impact of a compound at different stages of its development. By its unique characteristics, our platform will help strategic decisions regarding subsequent drug testing or development.

OXPHOS and OS combined measurements represent a first step to metabolism integration. As our example have evidenced, it seems difficult to evaluate cell bioenergetics without having glycolysis and TCA data. We are actually working to add glycolysis and pentose phosphate pathway to generate a more global evaluation of drug effect. In the next future, we plan to implement TCA cycle and lipid degradation pathway to tend to a more global and indispensable exploration.

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16

Measurement of Mitochondrial Toxicity by Flow Cytometry

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CHAPTER MENU

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16.1 Introduction

Mitochondria are cellular organelles that play essential roles in cellular homeostasis by generating ATP, regulating programmed cell death, and balancing cellular redox state (Bratic and Larsson 2013; Mayer and Oberbauer 2003; Nunnari and Suomalainen 2012). In the past decade, increasing evidence suggests that xenobiotics, including environmental toxins and many drugs, can induce mitochondrial damage, which can contribute to various organ toxicities observed *in vivo* (DiMauro and Schon 2003; Dykens and Will 2007; Pereira et al. 2009; Will and Dykens 2014). To evaluate effects of these small molecule entities on mitochondrial function, a great variety of technologies, ranging from high-resolution respirometry to magnetic resonance, were applied (Papkovsky et al. 2006; Vickers 2009; Will et al. 2006). Among these technologies, multiparametric flow cytometry provided a quick and sensitive method to assess mitochondrial function in individual cells and had been widely used to assess mitochondrial health (Hu et al. 2015; Kluza et al. 2004; Li et al. 2014). In the current chapter, we review flow cytometry technologies commonly used for assessing xenobiotics-induced mitochondrial dysfunction, including alterations in mitochondrial membrane potential (MMP) and production of mitochondrial

reactive oxygen species (ROS). Application of various mitochondria-specific fluorescent probes in flow cytometry assays is also discussed in detail.

16.2 Evaluation of Mitochondrial Function by Flow Cytometry

Flow cytometry is a laser-based technology designed to detect fluorescence intensity in single cells. Measuring mitochondrial function *in vitro* by flow cytometry has been practiced for over 30 years. Many fluorescent probes and staining protocols have been developed to evaluate the mitochondrial function with high sensitivity and specificity. Among them, flow cytometry-based mitochondrial MMP and ROS measurement are the most well developed and widely used tools to assess chemical and xenobiotics-induced mitochondrial toxicity—the primary focus of this chapter.

16.2.1 Mitochondrial Membrane Potential (MMP) Measurement

One of the most important functions of mitochondria in eukaryotic cells is the synthesis of ATP via oxidative phosphorylation. Mitochondria can account for more

than 90% of energy produced in cells under aerobic condition. During oxidative phosphorylation, mitochondrial respiratory chain transfers protons from mitochondrial matrix to intermembrane space which establish a proton gradient across the inner mitochondrial membrane (Harris and Das 1991; Hatefi 1985; Jornayvaz and Shulman 2010). The energy stored in the proton gradient is then used to drive ATP synthesis (Chen 1988; Nicholls and Budd 2000). Therefore maintaining the proton gradient across the mitochondrial inner membrane represents a fundamental requirement for healthy mitochondria. Theoretically, the integrity of the proton gradient can be evaluated by measuring either MMP (electrical gradient) or mitochondrial pH (chemical gradient). However, fluorescent probes that can specifically measure the mitochondrial pH change in cells by flow cytometry are currently not available. Even though several recent studies reported the discovery of novel fluorescent probes for measuring mitochondrial pH change with other technologies (for details see Section 16.5), the performance of those probes in flow cytometry-based assays have yet to be evaluated (Lee et al. 2014). To date, proton gradient across the inner mitochondrial membrane has been assessed predominantly by measuring MMP using flow cytometry, and a variety of fluorescent probes have been developed for this purpose.

Some of the key features of these probes are summarized in Table 16.1. Many of these fluorescent probes belong to the cation family, which can penetrate the plasma membrane due to their lipophilic nature. Once inside the cell, these fluorescent probes can preferentially accumulate in the mitochondrial matrix because of their positive charge. The first generation of fluorescent probes for detecting MMP was derived from those used for detecting plasma membrane potential: rhodamine 123 (Rh123) and DiOC6(3) (3,3'-dihexyloxacarbocyanine iodide) (Chen 1988; Korchak et al. 1982). These probes exhibited ideal mitochondrial uptake and were sensitive to MMP change. However, several shortcomings such as poor cellular/mitochondrial retention over time, poor specificity (also sensitive to endoplasmic reticulum [ER] and plasma membrane potential), fluorescent quenching at high concentration, and mitochondrial toxicity due to high protein binding limited their use which triggered research and discovery of next-generation MMP probes (Rottenberg and Wu 1998; Salvioli et al. 1997).

Tetramethylrhodamine methyl ester (TMRM) and tetramethylrhodamine ethyl ester (TMRE), two fluorescent derivatives of Rh123, were developed by Ehrenberg et al. (1988) as improved mitochondrial probes in 1988. These probes were initially qualified in isolated mitochondrial assays and subsequently used to detect MMP

change in cells by flow cytometry (Ehrenberg et al. 1988; Scaduto and Grotyohann 1999). Similar to Rh123, TMRM and TMRE can freely penetrate the plasma membrane and preferentially accumulate in the mitochondria. These probes are more specific to polarized mitochondria because of their low partition coefficient rate (Scaduto and Grotyohann 1999). TMRM and TMRE are also bright fluorescent probes that can detect MMP fluctuation at low nanomolar concentrations. At such low concentrations, mitochondrial toxicity is usually not a concern (Metivier et al. 1998; Rasola and Geuna 2001). However, like Rh123, TMRM and TMRE are sensitive to xenobiotics-induced plasma membrane potential change, which can lead to fluctuation of fluorescent signals. Additionally, TMRM and TMRE can move relatively freely across plasma membrane, leading to leakage of these dyes over time, which can be a challenge when screening compounds in a high-throughput format (Gerencser et al. 2012; Ward et al. 2007).

Another set of Rh123 derivatives developed for detecting MMP change are MitoTracker probes that include MitoTracker Red (CMXRos), MitoTracker Orange (CMTMRos), and their reduced forms (reduced MitoTracker Red [CM-H2XRos] and reduced MitoTracker Orange [CM-H2TMRos]) (Bkaily et al. 1997; Poot et al. 1996). These probes are sensitive to MMP and can quickly accumulate in mitochondria. MitoTracker Red and Orange are fluorescent; however, their reduced forms are nonfluorescent and therefore should undergo intracellular oxidation to become fluorescent. One unique feature of those probes is that they contain thiol-reactive chloromethyl moieties that can bind to mitochondrial proteins, causing retention in mitochondria even after formaldehyde or paraformaldehyde fixation (Poot et al. 1996). This unique feature allows these probes to be used in cells that potentially contain infectious agents, for example, human immunodeficiency virus (HIV), and thus MitoTracker probes are ideal reagents for use in human blood cells (Macho et al. 1996). Additionally, withstanding fixation allowed MitoTracker probes to be compatible with the simultaneous assessment of other cellular parameters, for example, TUNEL technique for determination of DNA fragmentation in apoptosis (Macho et al. 1996).

Another fluorescent probe introduced to the flow cytometry field for the detection of MMP change is JC-1 (Cossarizza et al. 1993). Similar to other MMP probes, JC-1 penetrates plasma membrane and subsequently accumulates in mitochondria due to its cationic nature. In contrast to monochromatic rhodamine probes, JC-1 has a polychromatic fluorescence emission: a red fluorescence (peak emission 590 nm) from an aggregate state and a green fluorescence (peak emission 525) from a monomeric state. In cells with an intact MMP, JC-1

Table 16.1 Fluorescent probes for detecting mitochondrial membrane potential (MMP) change or mitochondrial reactive oxygen species (ROS) production by flow cytometry.

Fluorescent probe	Fluorescence spectrum	Sample ex/detector in flow cytometers	Parameter measured	Application and limitation
Rhodamine 123	Abs: 507 nm Em: 529 nm	Ex: 488 nm Detector: FITC channel	MMP	● Quick probe loading ● Also measures plasma membrane potential. Tends to leak out of cells
DiOC6(3)	Abs: 482 nm Em:504 nm	Ex: 488 nm Detector: FITC channel	MMP	● Quick probe loading ● Also measures plasma/ER membrane potential. Tends to leak out of cells ● Mitochondrial toxicity at high concentrations
TMRM	Abs: 549 nm Em: 575 nm	Ex: 488 nm Detector: PE channel	MMP	● Quick probe loading, specific to mitochondria ● Also measures plasma membrane potential. Tends to leak out of cells
TMRE	Abs: 549 nm Em: 574 nm	Ex: 488 nm Detector: PE channel	MMP	● Quick probe loading, specific to mitochondria ● Also measures plasma membrane potential. Tends to leak out of cells
JC-1	Monomer Abs: 514 nm Em: 529 nm Aggregate Abs: 585 nm Em: 590 nm	Monomer Ex: 488 nm Detector: FITC channel Aggregate Ex: 488 nm or 561 nm Detector: PE channel	MMP	● Ratiometric probe, specific to mitochondria ● Less impact from plasma membrane ● Poor water solubility
JC-10	Monomer Abs: 515 nm Em: 529 nm Aggregate Abs: ~490 – 510 nm Em: 590 nm	Monomer Ex: 488 nm Detector: FITC channel Aggregate Ex: 488 nm Detector: PE channel	MMP	● Ratiometric probe, specific to mitochondria ● Less impact from plasma membrane
MitoTracker Red	Abs: 579 nm Em: 599 nm	Ex: 488 nm Detector: PE channel	MMP	● Can tolerate fixation ● Irreversibly bind to mitochondrial protein
MitoTracker Orange	Abs: 554 nm Em: 576 nm	Ex: 488 nm Detector: PE channel	MMP	● Can tolerate fixation ● Irreversibly bind to mitochondrial protein
MitoSOX	Abs: 510 nm Em: 580 nm	Ex: 488 nm Detector: PE channel	Mitochondrial ROS	● Only commercially available probe specific to mitochondrial ROS ● Mitochondrial toxicity at high concentrations

accumulates in mitochondria and forms red fluorescent aggregates at high concentrations. During mitochondrial depolarization, JC-1 released from mitochondria is diluted in the cytosol, causing dissociation of aggregates into green fluorescent monomers. The ratiometric change from red to green fluorescence can then be used to measure MMP in cells (Cossarizza et al. 1993; Salvioli et al. 1997). The advantage of using JC-1 is that changes in plasma membrane potential, mitochondrial size, shape, and density have much less impact on the ratiometric change. Furthermore, JC-1 can be retained in cells relatively well (over 2h) and is, therefore, suitable for screening larger batches of compounds (Robinson et al. 2015). Although widely used, JC-1 has poor water solubility and tends to precipitate in aqueous solutions. A similar ratiometric probe, JC-10, was later developed with better solubility in water (Filippi et al. 2015; Reibetanz et al. 2016).

16.2.2 Mitochondrial Reactive Oxygen Species (ROS) Measurement

Besides MMP, flow cytometry has also been widely used to detect cytoplasmic and mitochondrial ROS production. Mitochondrial ROS are now known to be biologically important in a variety of physiological systems, including adaptation to hypoxia, regulation of autophagy, regulation of immunity, regulation of differentiation, and regulation of longevity (Sena and Chandel 2012). Under physiological conditions, a tonal level of ROS production in the mitochondria of eukaryotic cells (Green and Van Houten 2011; Wallace 1999) has a wide range of effects to maintain homeostasis (Sena and Chandel 2012). Following stress, this level of mitochondrial ROS may fluctuate to alter signaling pathways and can be further upregulated if the mitochondrial respiratory chain is disrupted (Lenaz 2001; Murphy 2009). It has been shown that mitochondrial ROS production contributes to xenobiotics-induced toxicities (Begriche et al. 2011; Pereira et al. 2012).

Many fluorescent probes sensitive to ROS, including fluorescein probes (dichlorofluorescein diacetate [H2DCFDA] and its carboxylated, diacetylated, fluorinated, or acetyl esterated analogues), dihydrorhodamine, and (di)hydroethidine, have been used to detect mitochondrial ROS by flow cytometry (Bulua et al. 2011; Corda et al. 2001). However, these probes are not specific to mitochondrial ROS. By conjugating a mitochondria-targeting cationic structure (triphenylphosphonium (TPP)) to hydroethidine, a new mitochondrial-specific ROS probe, MitoSOX, can accumulate in the mitochondrial matrix and become more specific to detect mitochondrial ROS production (Table 16.1) (Robinson et al. 2006). Similar to its parent molecule hydroethidine,

MitoSOX is oxidized by ROS to form ethidium, which binds to nucleic acids, thereby greatly enhancing its fluorescence when detected at approximately 610nm wavelength (excited by a blue argon laser at 488nm) (Robinson et al. 2006). Hydroethidine and MitoSOX were also reported to form superoxide-specific products, 2-OH-ethidium or 2-OH-Mito-ethidium, respectively. However, the emission wavelengths of 2-OH-ethidium and 2-OH-Mito-ethidium are highly overlapping with ethidium bromide fluorescence, which means using flow cytometry to detect the fluorescence of these oxidized molecules can be challenging (Robinson et al. 2006; Zielonka and Kalyanaraman 2010). Caution also needs to be taken in that ethidium is known to inhibit mitochondrial respiration; therefore prolonged incubation of cells with MitoSOX could also lead to mitochondrial respiratory chain inhibition (Miller et al. 1996; Roelofs et al. 2015).

16.3 Evaluation of Xenobiotics-Induced Mitochondrial Toxicity by Flow Cytometry

Emerging evidence in the past decade suggests that mitochondrial toxicity contributed to various xenobiotics-induced organ toxicities. There is an increasing interest in pharmaceutical companies to develop assays to identify compounds that can potentially induce mitochondrial toxicity *in vivo* (Dyken and Will 2007; Will and Dyken 2014). Development of more specific and reliable fluorescent probes for MMP (JC-1 or TMRM) and mitochondrial ROS (MitoSOX) has enabled researchers to establish mitochondrial function assays by flow cytometry. However, like other *in vitro* assays, careful design of the experiment and a good understanding of factors that can potentially affect assay need to be considered. Some of these are discussed in the following text.

16.3.1 Cell Culture Conditions: Glucose- versus Galactose-Containing Media

An important mechanism to allow identification of xenobiotics specifically affecting mitochondrial function *in vitro* was the Crabtree effect, which is an important mechanism for cells to survive via ATP generated by glycolysis when mitochondrial function is impaired (Marroquin et al. 2007; Rodriguez-Enriquez et al. 2001). One of the methods developed to detect xenobiotics-induced mitochondrial toxicity was growing cells in two types of media: one supplemented with glucose and the other with galactose (glucose-free culture)

(Marroquin et al. 2007). In glucose media, cells utilize glycolysis to generate ATP (Crabtree effect) and are resistant to mitochondrial toxicant-induced cell death. In contrast, cells cultured in galactose media mainly rely on ATP produced by mitochondria and therefore are sensitive to mitochondrial toxicants. The differential sensitivity to xenobiotics-induced cell death between cells grown in glucose media as opposed to those grown in galactose media could be used to identify mitochondrial toxicants, for example, a compound with an IC₅₀ value of cell viability in glucose media greater than threefold than that in galactose media was considered as a mitochondrial toxicant. Similarly, MMP measurement in cells grown in glucose media versus galactose media was also used to identify mitochondrial toxicants, for example, a compound with an IC₅₀ value of MMP in glucose media greater than threefold than that in galactose media was considered as a mitochondrial toxicant (Rana et al. 2011). When certain cell types are difficult to adapt to galactose culture, an alternative method to identify xenobiotics specifically disrupting mitochondrial function can be considered. In this scenario, cells can be cultured only in glucose media. Xenobiotics-induced MMP decrease at noncytotoxic concentrations was used as a marker for mitochondrial toxicants, that is, a compound with an IC₅₀ value of MMP decrease less than threefold than that of cell viability was considered as a mitochondrial toxicant (Li et al. 2014). The underlying mechanism for this method is also the Crabtree effect: mitochondrial toxicants can deplete MMP in the absence of cell death by replenishing cellular ATP level via glycolysis.

16.3.2 Loading Fluorescent Probes

Flow cytometric studies necessitate careful consideration and understanding of loading kinetics of appropriate fluorescent probes into the cell type of interest for detection of MMP or ROS changes. Under most circumstances, cells were preincubated with xenobiotics for a specified period and then loaded with fluorescent probes (Li et al. 2014). Fluorescent probes for MMP and ROS, such as JC-1, TMRM, or MitoSOX, are designed to quickly penetrate the plasma membranes and reach equilibrium in mitochondria (10–15 min). However, time to reach equilibrium for fluorescent probes can vary significantly among different cell types. For example, JC-1 reaches equilibration in HL-60 cells within less than 10 min but can take as long as 90 min in cardiomyocytes (Figure 16.1) (Li et al. 2014; Mathur et al. 2000). Therefore, optimizing the fluorescent probe loading protocol for each cell types is critical before testing compounds. Samples have to be analyzed immediately since JC-1 fluorescence is stable in the dark at room temperature for only about 2 h. Most flow cytometers today are

capable of reading samples in a 96/384-well plate reader format or are equipped with a loader carousel that can provide an ideal throughput to evaluate effects of many xenobiotics in one experiment.

16.4 Benefits and Limitations

Flow cytometry-based mitochondrial function assays can provide a robust platform to evaluate xenobiotics-induced mitochondrial toxicity by directly measuring MMP change and ROS production in individual cells, which are important mechanisms associated with xenobiotics-induced mitochondrial toxicity. By systematically evaluating approximately 70 drugs and chemicals, our results showed that flow cytometry-based mitochondrial function assays can identify compounds causing mitochondrial toxicity with high sensitivity and specificity (Li et al. 2014). Additionally, these flow cytometry-based methods can be easily adapted to a high-throughput sampling (HTS) mode, allowing screening of a large number of drug candidates within a short period incorporating measurement of other cellular function endpoints simultaneously (Luu et al. 2012; Robinson et al. 2015).

Given all the benefits provided, flow cytometry-based methods also have limitations, and precaution is needed during assay design and data interpretation. Many MMP and ROS probes accumulate in mitochondria and can be toxic to these organelles themselves when loaded at high concentrations. Additionally, the intracellular concentration of fluorescent probes can vary among cell types. Therefore, optimizing fluorescent probe loading time and concentrations is critical when using a new cell type. Flow cytometry is also limited to measuring fluorescent signals from cells in suspension. Adherent cells are required to be detached from culture plates before loading fluorescent probes. Like many other *in vitro* platforms, flow cytometry-based MMP and ROS assays are most commonly used as hazard identification platforms. Therefore, these results need to be interpreted in the context of animal or potentially human exposure (e.g., IC₅₀ of MMP decrease compared with xenobiotics serum C_{max}) and *in vivo* toxicological findings to further understand the risk of xenobiotics-induced mitochondrial toxicity.

16.5 Emerging New Fluorescent Probes and Technologies for Mitochondrial Function Assessment

Besides the fluorescent probes now in common use, many labs are developing new tools that can potentially provide improved sensitivity and specificity in detecting

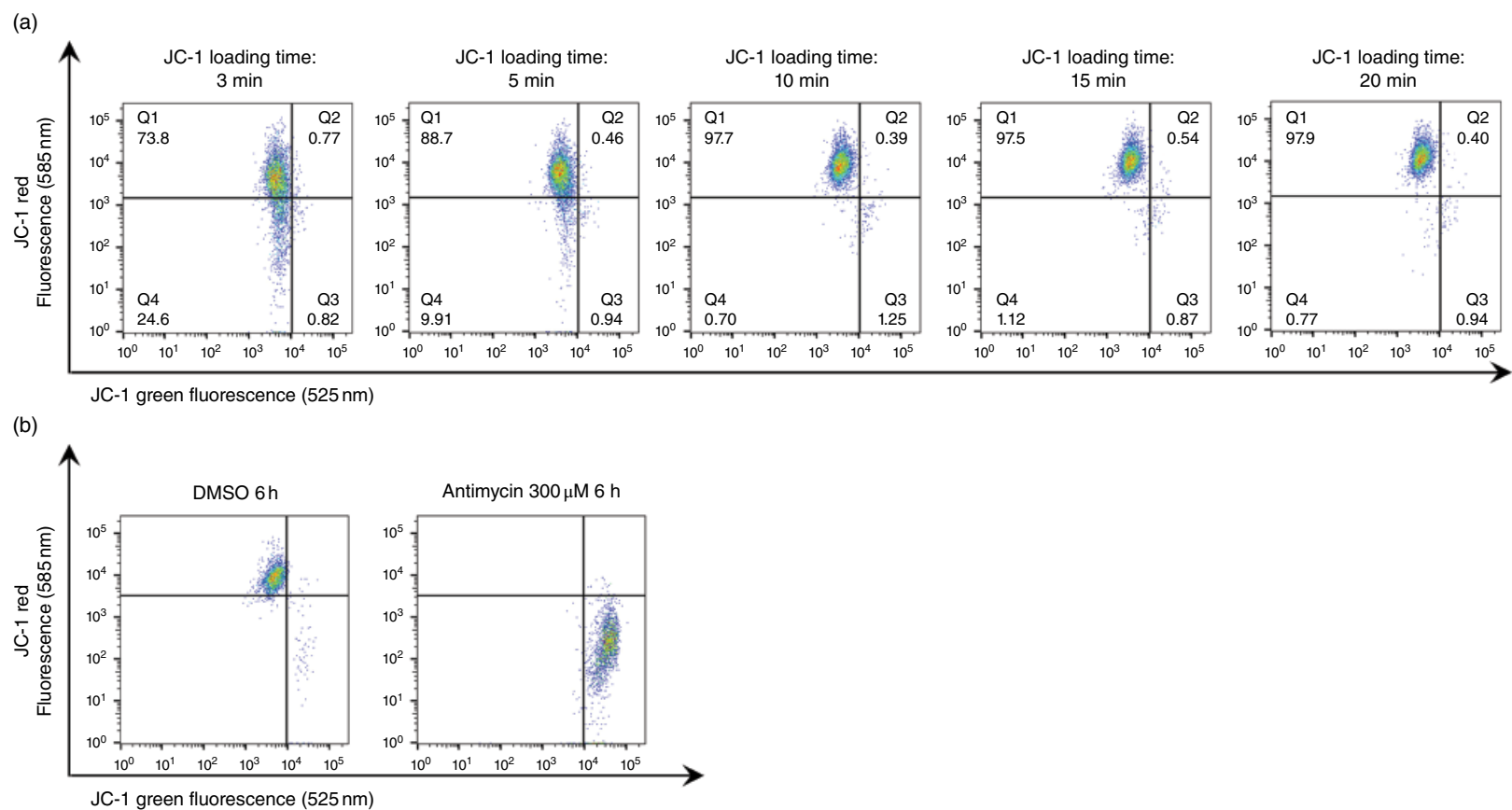


Figure 16.1 (a) Kinetics of JC-1 loading in HL-60 cells. Cells were pelleted by centrifugation at $250\times g$ for 2 min. Cells were then resuspended in 100 μ L PBS containing 5 μ M JC-1. Cells were incubated with JC-1 for 3, 5, 10, 15, and 20 min at 37°C. After incubation, cells were pelleted at $300\times g$, supernatant removed and resuspended in 100 μ L PBS. (b) In a separate experiment, sample data of MMP completely depleted by treating HL-60 cells with 300 μ M antimycin for 6 h is shown. JC-1 fluorescence was measured on a BD-LSR™ flow cytometer.

mitochondrial function changes. One of the fluorescent probes recently discovered for measuring MMP is tetraphenylethene-indolium (TPE-indo), which is a cationic probe selectively targeted to mitochondria (Zhang et al. 2015). TPE-indo appeared to be more sensitive to valinomycin-induced MMP decrease than JC-1, which could potentially enable the detection of drugs that weakly disrupt MMP (Zhang et al. 2015). Another study reported the discovery of a naphthalimide-derived mitochondrial-specific pH-sensitive fluorescent probe, which will potentially enable detection of the xenobiotics-induced mitochondrial pH change (Lee et al. 2014). More interestingly, a new fluorescent probe, MitoAP-1 (also called 3-2Zn(II)), is reported to detect mitochondrial ATP production in cells (Kurishita et al. 2012). The probe has been successfully used to evaluate mitochondrial ATP production in human neutrophils from sepsis patients (Sueyoshi et al. 2016).

In addition to the discovery of new probes, emerging new techniques can also potentially expand the endpoints measurable by flow cytometry. One example is the quantification of mitochondrial fusion by flow cytometry. Several recent studies suggested that dysfunction of mitochondrial fusion process could potentially contribute to xenobiotics-induced organ toxicity (Kang et al. 2016; Rosdah et al. 2016). One of the methods to measure mitochondrial fusion was to measure the rate of color mixing after fusing cells expressing distinct mitochondrial-targeted fluorescent proteins. Traditional flow cytometry measures total intracellular fluorescence

intensity in cells. Translocation of fluorescent signaling among subcellular structures tends to be challenging to detect. A recent report took advantage of the development of Amnis™ imaging flow cytometry system, which allowed acquisition of images of individual cells when passing through a flow cell and successfully quantified mitochondrial fusion (Nascimento et al. 2016). Such an assay will be useful to assess a compound that has an intrinsic property to induce mitochondrial fusion as one of its mechanisms to elicit cytotoxicity, that is, ethacrynic acid (EA). EA's ability, independent of causing cytoplasmic depletion of glutathione levels, to alkylate one or more specific cysteine residues in proteins that are involved in mitochondrial fusion/fission dynamics is known to contribute to its cytotoxicity (Bowes and Gupta 2005). Development of imaging flow cytometry technology can potentially be used to evaluate effects of xenobiotics-induced mitochondrial fusion dysfunction.

16.6 Summary

Flow cytometry-based MMP and mitochondrial ROS assessment has been developed and widely used to evaluate mitochondrial function. Many fluorescent probes currently in use, such as JC-1 or MitoSOX, can detect mitochondrial dysfunction with high sensitivity and specificity. Therefore flow cytometry-based mitochondrial function assays can be useful to evaluate for potential xenobiotic-induced mitochondrial toxicity *in vitro*.

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MitoChip: A Transcriptomics Tool for Elucidation of Mechanisms of Mitochondrial Toxicity

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CHAPTER MENU

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17.1 Development of Mitochondria-Specific Gene Expression Array (MitoChip)

Mitochondrial dysfunction is a hallmark of a wide range of drug toxicities, diseases, and disorders (Chan et al., 2005; Finsterer and Ohnsorge, 2013; Lee et al., 2016; Nadanaciva and Will, 2011; Sardao et al., 2008; Varga et al., 2015; Wada and Nakatsuka, 2016; Wallace, 2015; Walters et al., 2016). Mechanisms responsible for mitochondrial toxicity are diverse and can significantly disturb mitochondrial function. These include mutations in mitochondrial genes and/or nuclear genes encoding mitochondrial proteins, faulty transcription and translation of these genes into defective proteins, and improper assembly of the proteins, in particular proteins associated with multi-subunit complexes of oxidative phosphorylation in the mitochondrial inner membrane (Yadav and Chandra, 2013). Additionally, altered mitochondrial membrane potential, uncoupling of oxidative phosphorylation, impaired transport of ions and solutes due to inefficient mitochondrial membrane channels, and disturbance in mitochondrial calcium homeostasis also can result in mitochondrial dysfunction (Chan et al., 2005; Vercesi et al., 2006; Wallace, 2007). The consequence of

one or a combination of these factors is altered bioenergetics and other key metabolic pathways, leading to mitochondrial toxicity that may account for drug toxicities or pathologies in various tissues, in particular tissues that rely heavily on mitochondrial energy. Excessive production of reactive oxygen species (ROS) as a result of mitochondrial dysfunction is considered a key aspect underlying a number of drug toxicities and diseases. This can lead to increased mitochondrial and cellular oxidative stress, resulting in programmed cell death (apoptosis) and subsequent injury to a wide variety of organs (Lahoti et al., 2012; Lin et al., 2012; Pal et al., 2015).

While many studies investigate specific aspects of mitochondrial function in drug toxicity or pathophysiological conditions, knowledge of interactions among various important metabolic pathways within mitochondria during toxic insults or diseases is lacking. Such information is crucial to a more comprehensive understanding of the role of mitochondria as in drug toxicities or diseases. To address this, the first MitoChip was developed in a mouse model at the US Food and Drug Administration's (FDA's) National Center for Toxicological Research (NCTR) (Desai et al., 2007). This original mouse MitoChip consisted of 542 oligonucleotides, each representing individual genes associated with various

mitochondrial pathway/biological processes including oxidative phosphorylation, fatty acid β -oxidation, Krebs cycle, heme biosynthesis, steroid biosynthesis, mitochondrial biogenesis, mitochondrial fusion and fission, mitochondrial DNA (mtDNA) transcription, replication and repair, apoptosis, and various mitochondrial membrane transporters. In contrast to commercial high-density whole genome expression arrays, consisting only of nuclear genes encoding mitochondrial proteins, the MitoChip contains distinctive genes encoded by both mitochondrial and nuclear genomes. In most cells, mitochondria produce more than 90% of energy via oxidative phosphorylation required for cellular viability. Oxidative phosphorylation is a complex system of five multi-subunit protein complexes that are comprised of 13 proteins encoded by mtDNA and synthesized within the organelle, plus approximately 94 proteins encoded by nuclear DNA and synthesized using cytosolic ribosomes. Therefore, more comprehensive understanding of the cross-talk between mitochondrial and nuclear genomes is crucial to defining mechanisms of mitochondrial dysfunction in the context of drug toxicities and disease etiologies. This comprehensive genomics approach of simultaneously measuring transcriptional levels of hundreds of mitochondrial and nuclear genes provides a wealth of knowledge related to multiple facets of mitochondrial activity in a single experiment.

Design, development, and use of the mouse MitoChip and associated quality control measures for a specified microarray experiment require multiple steps, including tissue extraction of RNA, reverse transcription to cDNA, labeling with fluorescent dyes, hybridization of fluorescently labeled cDNA on microarrays, scanning, data acquisition, and analysis, all of which have been described previously (Desai et al., 2007, 2009). One of the major challenges in DNA microarray technology is the accurate analysis of large-scale complex gene expression data to provide meaningful significance to its biological interpretations. Biological pathways are sets of genes that work in concert to efficiently execute biological processes or actions. It is, therefore, essential to consider cumulative changes in the transcriptional levels of the family of genes associated with a given biological pathway, as opposed to interpreting transcriptional changes in individual genes that contribute to the pathway. To this end, using the Mouse Genome Informatics database (<http://www.informatics.jax.org>), genes on the MitoChip microarrays are categorized by diverse Gene Ontology (GO) terms that reflect specific molecular functions, pathways, or other biological processes. The overall significance of the treatment effect on each GO term is estimated by a modified meta-analysis method to combine *p*-values calculated for each gene within the

GO term while at the same time taking into account the inter-gene correlation structure in each GO term (Delongchamp et al., 2006; Lee et al., 2008).

Mouse MitoChip has proven to be an invaluable array-based platform for rapid and high-throughput analysis of differentially expressed genes associated with a host of mitochondrial pathways linked to drug toxicities. Such utility has been demonstrated via novel findings that have advanced our understanding of the mechanisms underlying mitochondrial dysfunction in different organ systems during exposures to therapeutic drugs, a weight-loss dietary supplement, and environmental toxins. For example, MitoChip studies illuminate mechanisms of mitochondrial toxicity induced by (i) anti-HIV drugs (e.g., nucleoside reverse transcriptase inhibitors zidovudine and lamivudine) in the liver of C3B6F₁*trp53* mice (Desai et al., 2008) and in skeletal muscle of B6C3F₁ mice (Desai et al., 2009), (ii) a weight-loss dietary supplement (e.g., usnic acid) in the liver of B6C3F₁ mice (Joseph et al., 2009), and (iii) environmental toxins such as acrylamide and glycidamide in the liver of transgenic Big Blue mice (Lee et al., 2012). Additionally, MitoChip has provided important insights into the selective transcriptional changes within various mitochondrial pathways during treatments with anticancer drugs such as flutamide and cisplatin in several mouse models (Kashimshetty et al., 2009; Li et al., 2009). Importantly, the mouse MitoChip reveals subtle transcriptional changes associated with mitochondrial function prior to overt drug toxicity (Desai et al., 2008; Joseph et al., 2009), thus providing potential for identifying early gene expression patterns that could serve as biomarkers for predicting early stages of mitochondrial toxicity. Such information may have value in assessing and predicting risks associated with drug toxicity and environmental exposures.

As additional mitochondrial proteins have been identified, the number of genes associated with mitochondrial structure and function on the MitoChip has correspondingly increased from 542 to 811 using the Mouse Genome Informatics (<http://www.informatics.jax.org>) and National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) databases. To further enhance the utility of MitoChip technology for toxicological evaluations, an additional 117 genes associated with phase I (e.g., cytochrome P450 oxidoreductases) and phase II (e.g., glutathione *S*-transferases, UDP-glucuronidases, sulfuryl transferases) reactions of drug metabolism, 71 genes associated with both intrinsic and extrinsic apoptotic pathways, and 20 housekeeping genes have all been added to the originally designed 811 genes, providing a total of 1019 unique genes to the most recent version of mouse MitoChip on a commercial platform (Agilent Technologies, Santa Clara, CA).

Utility of the MitoChip perspective and technology is discussed in Sections 17.2 and 17.3 using findings from animal studies of mitochondrial dysfunction and associated organ toxicity induced by the anticancer drugs flutamide, cisplatin, and doxorubicin in the liver, kidney, and heart, respectively.

17.2 Mouse MitoChip: Assessment of Altered Mitochondrial Function in Mouse Models

17.2.1 Flutamide-Induced Liver Toxicity in *Sod2*^{+/-} Mice

Flutamide is a nonsteroidal androgen receptor antagonist approved by the FDA for the treatment of metastatic prostate cancer. However, severe adverse effects observed in the liver of some flutamide-treated patients have become a major clinical concern (Brahm et al., 2011; Lin et al., 2003). Flutamide has been recognized as a mitochondrial toxicant (Ball et al., 2016). A variety of experimental models are being designed to improve insights into the role of mitochondrial toxicity in flutamide-induced idiosyncratic hepatotoxicity in humans. Although the contribution of mitochondria in flutamide-mediated hepatotoxicity is not yet well understood, it has been postulated that patients at risk of drug-induced mitochondrial dysfunction and liver injury may exhibit underlying mitochondrial defects that would predispose them to toxic effects of these drugs (Boelsterli and Lim, 2007). In view of this, the role of mitochondria in flutamide-induced liver toxicity was investigated in a mouse model exhibiting underlying mitochondrial oxidative stress due to presence of only one of two copies of mitochondrial superoxide dismutase (*Sod2*^{+/-}), an enzyme responsible for neutralization of highly reactive superoxide radicals (Kashimshetty et al., 2009).

To understand the role of mitochondria, the mouse MitoChip was used for transcriptional profiling of 542 mitochondria-related genes in the liver of *Sod2*^{+/-} heterozygous and wild-type *Sod2*^{+/+} mice treated with flutamide (0, 30, 100 mg/kg/day) for 28 days (Kashimshetty et al., 2009). The high flutamide dose used in these mice is equivalent to the commonly used therapeutic human dose (750 mg/day) when corrected for interspecies differences with the dose scaling factor (CDER, 2005). Data indicated no apparent differences in the expression levels of mitochondria-related genes between vehicle-treated mutant mice (*Sod2*^{+/-}) and vehicle-treated wild-type mice (*Sod2*^{+/+}). As anticipated, a statistically significant approximately 1.5-fold downregulation of *Sod2* transcript was observed in vehicle-treated *Sod2*^{+/-} mice, further

demonstrating the technical accuracy of the analysis. Further evaluation of gene expression data revealed that daily dosing of mice with 30 mg/kg for 28 days had no statistically significant effect on mitochondrial transcript levels in either genotype. In contrast, treatment of mice with 100 mg/kg/day for 28 days significantly altered transcript levels of a number of genes, the majority of which were downregulated in the liver of both genotypes compared with respective vehicle-treated controls. When comparing hepatic transcript levels between *Sod2*^{+/+} with *Sod2*^{+/-} mice, no statistically significant differences were observed at the 100 mg/kg/day dose treatment. This suggests that although lack of one copy of *Sod2* in mutant mice did not significantly influence the transcript levels of genes related to mitochondrial pathways evaluated by MitoChip, its influence on other cellular pathways cannot be ruled out. GO analysis of transcriptomics data showed a significant effect of 100 mg/kg/day flutamide on various mitochondrial functions/pathways including oxidative phosphorylation, DNA repair, fatty acid metabolism, and apoptosis (Table 17.1). A notable observation was statistically significant alterations in the transcript levels of genes related to complexes I and III of the electron transport chain. Many of these genes (16 of 37 genes [43%] of complex I and 3 of 8 genes [37%] of complex III) were downregulated including all seven mtDNA-encoded genes (*ND-1*, *ND-2*, *ND-3*, *ND-4*, *ND-4L*, *ND-5*, *ND-6*) of complex I and *Cyt-b* of complex III. Corroborating these findings are differentially expressed nuclear genes related to complexes I, III, IV, and V in mouse hepatocyte cell line TAMH (Coe et al., 2007) and inhibition of complexes I and III in association with the decline in ATP levels in rat hepatocytes (Fau et al., 1994) following flutamide exposure. Similar effects have been reported in the acute glucose or galactose-conditioned HepG2 cells treated with 2-hydroxyflutamide, a primary reactive metabolite of flutamide, that showed a significant decline in mitochondrial complex I-, II- and V-linked respiration (Ball et al., 2016).

Mitochondrial complex I of the electron transport chain is a major source of ROS (Kussmaul and Hirst, 2006). It is likely that complex I inhibition could potentially induce ROS production followed by further exacerbation of mitochondrial oxidative stress. ROS levels within mitochondria that exceed the antioxidant defense mechanisms may cause oxidative injury to macromolecules including mtDNA. Interestingly, 12 of 13 mtDNA-encoded genes (except *mt-Co1*) involved in oxidative phosphorylation and 7 of 22 mitochondria-specific tRNAs were significantly downregulated in mouse liver following 100 mg/kg/day flutamide dose (Kashimshetty et al., 2009). A significant decline in the expression of genes involved in DNA repair (*Apex2* and *Ung*) in these

Table 17.1 Genes significantly altered by flutamide in the liver of male *Sod2*^{+/-} mice.

GenBank Acc. ID	Gene description (<i>gene symbol</i>)	Fold change
<i>NADH-ubiquinone dehydrogenase (complex I) (37), p = 0.029</i>		
NM_023312	Complex I, alpha subcomplex, 13 (<i>Ndufa13</i>)	0.829
NM_010885	Complex I, alpha subcomplex, 2 (<i>Ndufa2</i>)	0.883
NM_023202	Complex I, alpha subcomplex, 7 (<i>Ndufa7</i>)	0.824
NM_027175	Complex I, alpha subcomplex, assembly factor 1 (<i>Ndufaf1</i>)	0.881
NM_026612	Complex I, beta subcomplex, 2 (<i>Ndufb2</i>)	0.889
NM_025316	Complex I, beta subcomplex, 5 (<i>Ndufb5</i>)	0.891
AK002501	Complex I, Fe-S protein 3 (<i>Ndufs3</i>)	0.850
NM_010887	Complex I, Fe-S protein 4 (<i>Ndufs4</i>)	0.898
NM_144870	Complex I, Fe-S protein 8 (<i>Ndufs8</i>)	1.133
AC084065	Complex I, flavoprotein 3 (<i>Ndufv3</i>)	0.701
AK018753	Complex I, subunit 1, mitochondrial (<i>mt-Nd1</i>)	0.824
AU018363	Complex I, subunit 2, mitochondrial (<i>mt-Nd2</i>)	0.785
J01420	Complex I, subunit 3, mitochondrial (<i>mt-Nd3</i>)	0.666
AB042809	Complex I, subunit 4, mitochondrial (<i>mt-Nd4</i>)	0.820
AA109866	Complex I, subunit 4L, mitochondrial (<i>mt-Nd4l</i>)	0.891
AU018713	Complex I, subunit 5, mitochondrial (<i>mt-Nd5</i>)	0.682
AA435281	Complex I, subunit 6, mitochondrial (<i>mt-Nd6</i>)	0.830
<i>Ubiquinol-cytochrome c reductase (complex III) (8), p = 0.009</i>		
AK075856	Complex III, core protein 2 (<i>Uqcrc2</i>)	1.294
NM_025641	Complex III, hinge protein (<i>Uqcrh</i>)	0.857
NM_025352	Complex III, complex III subunit VII (<i>Uqcrg</i>)	0.853
AU018811	Cytochrome b, mitochondrial (<i>mt-Cytb</i>)	0.802
<i>Mitochondrial tRNAs (19), p = 0.011</i>		
J01420	tRNA alanine (<i>mt-Ta</i>)	0.812
J01420	tRNA leucine 1 (<i>mt-Tl1</i>)	0.878
J01420	tRNA methionine (<i>mt-Tm</i>)	0.796
J01420	tRNA asparagine (<i>mt-Tn</i>)	0.864
J01420	tRNA glutamine (<i>mt-Tq</i>)	0.770
J01420	tRNA serine 1 (<i>mt-Ts1</i>)	0.834
J01420	tRNA tryptophan (<i>mt-Tw</i>)	0.823
<i>DNA repair (6), p = 0.002</i>		
NM_029943	AP endonuclease 2 (<i>Apex2</i>)	0.785
NM_008743	Nth (endonuclease III)-like 1 (<i>Nthl1</i>)	1.199
NM_011677	Uracil-DNA glycosylase (<i>Ung</i>)	0.778
<i>Apoptosis (19), p = 0.032</i>		
AF133279	Bcl2-like 1 (<i>Bcl2l1</i>)	0.851
NM_013479	Bcl2-like 10 (<i>Bcl2l10</i>)	0.884
AK082134	Cullin 7 (<i>Cul7</i>)	0.833
NM_023646	DnaJ (Hsp40) homolog, subfamily A, member 3 (<i>Dnaja3</i>)	0.753
NM_012019	Programmed cell death 8 (<i>Pdcd8</i>)	0.815
NM_022032	p53 apoptosis effector related to Pmp22 (<i>Perp</i>)	0.894
NM_030750	Sphingosine-1-phosphate phosphatase 1 (<i>Sgpp1</i>)	0.850

Table 17.1 (Continued)

GenBank Acc. ID	Gene description (<i>gene symbol</i>)	Fold change
<i>Fatty acid metabolism</i> (23), $p = 0.018$		
NM_177470	Acetyl-coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) (<i>Acaa2</i>)	0.805
NM_007382	Acetyl-Coenzyme A dehydrogenase, medium chain <i>Acadm</i>	0.760
NM_007760	Carnitine acetyltransferase (<i>Crat</i>)	0.853
NM_016772	Enoyl coenzyme A hydratase 1, peroxisomal (<i>Ech1</i>)	0.731
NM_023737	L-specific multifunctional beta-oxidation protein (<i>Ehhadh</i>)	0.435
NM_008149	Glycerol-3-phosphate acyltransferase <i>Gpam</i>	0.838
NM_178878	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), alpha subunit (<i>Hadha</i>)	0.790
NM_145558	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), beta subunit (<i>Hadhb</i>)	0.810

Number of genes evaluated for each gene ontology term (GO; molecular function/biological process) is given in parenthesis. p -values indicate a significant flutamide effect on the GO term. Fold changes in gene expression are significant at the level of $p \leq 0.05$.

flutamide-treated mice may suggest the potential for enhanced oxidative stress in liver mitochondria. However, a modest but significant upregulation of *Nth1* that is involved in base excision repair might be a compensatory response to downregulation of *Apex2* and *Ung* (Table 17.1).

In flutamide-treated mice, a prominent influence of drug on liver mitochondria was substantiated by significant declines in the transcript levels of genes involved in fatty acid β -oxidation (e.g., *Acaa2*, *Acadm*, *Crat*, *Ech1*, *Ehhadh*, *Hadha*, *Hadhb*) (Table 17.1). These findings are in concordance with a significant downregulation of *Acadm*, *Ehhadh*, *Hadha*, and *Hadhb* in mouse hepatocyte cell line TAMH following flutamide treatment (Coe et al., 2007). These transcriptional changes may have implications for perturbation in the flux of the fatty acid β -oxidation pathway. Also, it seems likely that flutamide-induced downregulation of genes associated with complex I may limit the availability of NAD^+ , a cofactor essential for fatty acid β -oxidation (Coe et al., 2007). Flutamide-treated mice also showed a significant decrease in transcript levels of antiapoptotic genes (*Bcl2l1*, *Bcl2l10*, *Cul7*) and proapoptotic genes (*Dnaja3*, *Pdcd8*, *Perp*, *Sgpp1*). Although expression of a few proapoptotic genes was also reduced, a critical balance and interaction between antiapoptotic and proapoptotic proteins determines the initiation and control of apoptosis in cells. Thus, these changes may have significance in promoting apoptotic changes in the liver of flutamide-treated mice. Interestingly, increased apoptosis and small areas of cellular necrosis were observed in the liver of 5 out of 10 (50%) mutant mice (*Sod2*^{+/-}) when treated

with 100 mg/kg dose (Kashimshetty et al., 2009). While MitoChip analysis showed similar flutamide-induced changes in transcript levels associated with various metabolic pathways in both mutant and wild-type mice, histopathological manifestations in the liver were identified only in mutant mice. It is suggested that the initial oxidative challenge, due to mitochondrial perturbations, may be similar in both genotypes; however, the mechanisms that combat this stress may determine the outcome of the drug insult (Kashimshetty et al., 2009).

Several *in vitro* studies provide substantial evidence of flutamide effects on mitochondria that indicate a decline in mitochondrial respiration and ATP levels (Fau et al., 1994) or altered transcript levels of genes associated with the complexes of mitochondrial electron transport chain, fatty acid metabolism, and apoptotic pathways (Coe et al., 2007; Legendre et al., 2014). Data generated using mouse MitoChip not only verified previously published results in the literature, but this transcriptomics tool also proved valuable in revealing novel information of flutamide effects on mtDNA and DNA repair genes in mouse liver (Kashimshetty et al., 2009). Such effects of flutamide may enhance oxidative stress in mitochondria, leading to hepatocellular injury.

17.2.2 Cisplatin-Induced Acute Kidney Toxicity in KAP2-PPAR α Transgenic Mice

Cisplatin is another anticancer drug that targets mitochondria. It is one of the most effective chemotherapeutic drugs used to treat a variety of solid tumors, either as a monotherapy or in combination with other

therapeutic agents (Arany and Safirstein, 2003; Sahni et al., 2009). However, the therapeutic use and efficacy of cisplatin are limited by dose-related severe toxicities, mainly observed in the kidney (Hanigan and Devarajan, 2003; Sanchez-Gonzalez et al., 2011). Several *in vitro* and *in vivo* studies provide substantial evidence of cisplatin-induced mitochondrial toxicity that demonstrated altered activities of the complexes of the electron transport chain (Kruidering et al., 1997), impaired mitochondrial respiration, depolarization of the mitochondrial membrane potential, calcium-dependent mitochondrial swelling (Custodio et al., 2009; Gordon and Gattone, 1986), binding to mtDNA (Garrido et al., 2008; Olivero et al., 1995), decreased mitochondrial mass, disruption of cristae (Zsengeller et al., 2012), and decline in ATP levels (Choi et al., 2015). It is suggested that ROS, primarily produced within mitochondria, play a major role in cisplatin-induced kidney toxicity (Matsushima et al., 1998). Attenuation of toxic effects of cisplatin in kidneys by various antioxidants, such as carvedilol, dimethylthiourea, and amifostine, further supports ROS involvement in cisplatin-induced renal toxicity (Carvalho et al., 2014; dos Santos et al., 2007; Rodrigues et al., 2011; Sastry and Kellie, 2005).

Dysregulation of fatty acid oxidation has also been implicated in cisplatin-induced renal toxicity. Oxidation of fatty acids by mitochondria and peroxisomes is a major source of energy in proximal renal tubular cells (Wirthensohn and Guder, 1983) and is regulated by peroxisome proliferator-activated receptor alpha (PPAR α), a transcription factor that belongs to the ligand-activated nuclear hormone receptor superfamily (Lopez-Hernandez and Lopez-Novoa, 2009). Inhibition of transcript levels and enzyme activity of carnitine palmitoyltransferase 1, the rate-limiting enzyme for mitochondrial fatty acid β -oxidation, was related to cisplatin-mediated reduced PPAR α transcriptional activity in kidneys of male SV/126 mice administered a single intraperitoneal dose of 20 mg/kg cisplatin (Li et al., 2004; Portilla et al., 2002). These studies also emphasized the importance of regulation of fatty acid β -oxidation by PPAR α in preservation of kidney morphology and function in this acute renal failure mouse model. Li and colleagues (2009) further developed a female transgenic mouse model expressing PPAR α under the control of the kidney androgen-induced protein 2 (KAP2) to explore the precise role of PPAR α during acute kidney injury induced by 20 mg/kg cisplatin. Additionally, cisplatin-treated transgenic mice were pretreated with testosterone to examine the benefits of PPAR α induction on cisplatin-mediated changes in kidneys. Evaluation of only a few genes involved in fatty acid β -oxidation (*Mcad*, *Lcad*, *Vlcad*, *L-Cpt-1*) by quantitative real-time RT-PCR revealed cisplatin-mediated significant decline in transcript levels in kidneys of transgenic mice

and their mitigation by pretreatment with testosterone (Li et al., 2009).

To further advance understanding of involvement of other key mitochondrial pathways in acute kidney injury in female KAP2-PPAR α transgenic mice, transcriptional profiling of 542 mitochondria-related genes was performed using MitoChip in kidneys of cisplatin-exposed mice in the absence and presence of testosterone (Li et al., 2009). Data revealed a profound effect of acute cisplatin treatment on mitochondria that showed a significant decline in the expression of 454 of the 542 genes (84%) in kidneys of transgenic mice. These included genes associated with various critical mitochondrial pathways including fatty acid β -oxidation (*Acaa2*, *Acadm*, *Acads*, *Acadsb*, *Acsl3*, *Acsl5*, *Acsl6*, *Cpt1a*, *Crat*, *Ech1*, *Ehhadh*, *Hadhb*) (Table 17.2). Also, transcript levels of 63 of 78 genes (81%) directly linked to oxidative phosphorylation were significantly downregulated in kidneys of cisplatin-exposed transgenic mice. Among these 63 altered genes, 12 genes were encoded by mtDNA that showed a statistically significant declines of greater than 1.3-fold when compared with respective saline-treated control mice (Table 17.2). One hypothesis to explain this decline in gene expression changes can be attributable to the potential of cisplatin accumulating in mitochondria and binding to mtDNA. This, in turn, could induce intra- and interstrand adduct formation, altering mtDNA expression (Garrido et al., 2008; Gordon and Gattone, 1986). MitoChip data also revealed a significant decline in transcript levels of nuclear genes involved in DNA replication (*Peo1*, *Polg*, *Ssbp1*, *TK2*) and DNA repair (*Apex2*, *Mre11a*, *Nth1*, *Ogg1*, *Ung*) in kidneys of cisplatin-treated transgenic mice that may further intensify cisplatin-induced renal mitochondrial toxicity in transgenic mice. Collectively, cisplatin-mediated altered transcript levels of genes involved in oxidative phosphorylation and fatty acid β -oxidation suggest severely compromised energy production in acute kidney injury. These data support the inhibition of mitochondrial respiratory chain complexes and impaired respiration reported in aforementioned *in vitro* and *in vivo* studies. In addition, transcript levels of genes linked to a number of other key mitochondrial pathways/processes (e.g., Krebs cycle, apoptosis, steroid biosynthesis, mitochondrial fusion) were significantly altered by cisplatin in kidneys of transgenic mice (Li et al., 2009).

Most of these cisplatin-mediated effects on transcript levels were significantly reversed by pretreatment of mice with testosterone by inducing PPAR α activity in proximal tubules of the kidney, and such pretreatment also significantly improved kidney function with marked reduction in kidney damage. A volcano plot of relative fold change in transcript levels of 542 genes against a false discovery rate for each gene influenced by cisplatin

Table 17.2 Genes altered by cisplatin alone or in presence of testosterone in kidney of female KAP2-PPAR α transgenic mice.

		Fold change	
GenBank Acc. ID	Gene description (<i>gene symbol</i>)	Cisplatin	Testosterone + cisplatin
<i>NADH-ubiquinone dehydrogenase (complex I) (33)</i>		<i>p</i> = 0.0005	<i>p</i> = 0.020
NM_019443	Complex I, alpha subcomplex, 1 (<i>Ndufa1</i>)	0.753	1.068
NM_024197	Complex I, alpha subcomplex 10 (<i>Ndufa10</i>)	0.835	1.012
NM_023312	Complex I, alpha subcomplex, 13 (<i>Ndufa13</i>)	0.566	1.152
NM_010885	Complex I, alpha subcomplex, 2 (<i>Ndufa2</i>)	0.864	1.129
NM_010886	Complex I, alpha subcomplex, 4 (<i>Ndufa4</i>)	0.771	1.079
NM_026614	Complex I, alpha subcomplex, 5 (<i>Ndufa5</i>)	0.840	1.176
NM_025987	Complex I, alpha subcomplex, 6 (<i>Ndufa6</i>)	0.755	1.185
NM_023202	Complex I, alpha subcomplex, 7 (<i>Ndufa7</i>)	0.502	1.331
NM_027175	Complex I, alpha subcomplex, assembly factor 1 (<i>Ndufaf1</i>)	0.793	1.066
AK002517	Complex I, beta subcomplex, 10 (<i>Ndufb10</i>)	0.814	1.175
NM_026612	Complex I, beta subcomplex, 2 (<i>Ndufb2</i>)	0.817	1.251
NM_025597	Complex I, beta subcomplex 3 (<i>Ndufb3</i>)	0.741	1.247
NM_025316	Complex I, beta subcomplex, 5 (<i>Ndufb5</i>)	0.824	1.015
NM_025843	Complex I, beta subcomplex, 7 (<i>Ndufb7</i>)	0.881	1.199
NM_025523	Complex I, subcomplex unknown, 1 (<i>Ndufc1</i>)	0.805	1.144
NM_145518	Complex I, Fe-S protein 1 (<i>Ndufs1</i>)	0.642	1.158
NM_153064	Complex I, Fe-S protein 2 (<i>Ndufs2</i>)	0.818	1.058
NM_010887	Complex I, Fe-S protein 4 (<i>Ndufs4</i>)	0.785	1.182
NM_029272	Complex I, Fe-S protein 7 (<i>Ndufs7</i>)	0.823	1.155
NM_133666	Complex I, flavoprotein 1 (<i>Ndufv1</i>)	0.793	1.087
AK013511	Complex I, flavoprotein 2 (<i>Ndufv2</i>)	0.829	1.277
AU018363	Complex I, subunit 2, mitochondrial (<i>mt-Nd2</i>)	0.747	1.097
J01420	Complex I, subunit 3, mitochondrial (<i>mt-Nd3</i>)	0.526	1.388
AB042809	Complex I, subunit 4, mitochondrial (<i>mt-Nd4</i>)	0.483	1.184
AA109866	Complex I, subunit 4l, mitochondrial (<i>mt-Nd4l</i>)	0.369	1.513
AU018713	Complex I, subunit 5, mitochondrial (<i>mt-Nd5</i>)	0.474	1.432
AA435281	Complex I, subunit 6, mitochondrial (<i>mt-Nd6</i>)	0.673	1.119
<i>Succinate-ubiquinone dehydrogenase (complex II) (3)</i>		<i>p</i> = 0.044	NS
NM_023374	Complex II, Ip subunit (<i>Sdhb</i>)	0.775	1.059
NM_025321	Complex II, integral membrane protein (<i>Sdhc</i>)	0.850	1.066
<i>Ubiquinol-cytochrome c reductase (complex III) (8)</i>		<i>p</i> = 0.006	NS
NM_025650	Ubiquinol-cytochrome c reductase subunit (<i>Uqcrc</i>)	0.806	1.072
NM_025407	Complex III, core protein 1 (<i>Uqcrc1</i>)	0.833	1.157
NM_025710	Complex III, Rieske iron-sulfur polypeptide 1 (<i>Uqcrcfs1</i>)	0.877	0.958
NM_025641	Complex III, hinge protein (<i>Uqcrcrh</i>)	0.749	1.211
NM_025352	Complex III, subunit VII (<i>Uqcrcq</i>)	0.808	1.135
AU018811	Complex III, cytb, mitochondrial (<i>mt-Cytb</i>)	0.411	1.298

(Continued)

Table 17.2 (Continued)

GenBank Acc. ID	Gene description (<i>gene symbol</i>)	Fold change	
		Cisplatin	Testosterone + cisplatin
<i>Cytochrome c oxidase (complex IV) (19)</i>		<i>p</i> = 0.002	<i>p</i> = 0.033
NM_009941	Complex IV, subunit IV isoform 1 (<i>Cox4i1</i>)	0.787	1.107
NM_053091	Complex IV, subunit IV isoform 2 precursor (<i>Cox4i2</i>)	0.833	1.215
NM_007747	Complex IV, subunit Va (<i>Cox5a</i>)	0.832	1.125
NM_009942	Complex IV, subunit Vb (<i>Cox5b</i>)	0.596	1.295
NM_009943	Complex IV, subunit VI a, polypeptide 2 (<i>Cox6a2</i>)	0.726	1.114
NM_009945	Complex IV, subunit VIIa 2 (<i>Cox7a2</i>)	0.808	1.139
NM_009187	Complex IV, subunit VIIa polypeptide 2-like (<i>Cox7a2l</i>)	0.865	1.189
NM_025379	Complex IV, subunit VIIb (<i>Cox7b</i>)	0.687	1.321
NM_007750	Complex IV, subunit VIIa (<i>Cox8a</i>)	0.820	1.095
NM_007751	Complex IV, subunit VIIb (<i>Cox8b</i>)	0.451	1.306
NM_013677	Surfeit gene 1 (<i>Surf1</i>)	0.677	1.203
AU018394	Complex IV, subunit I, mitochondrial (<i>mt-Co1</i>)	0.774	0.988
AU019143	Complex IV, subunit II, mitochondrial (<i>mt-Co2</i>)	0.683	1.176
AB042432	Complex IV, subunit III, mitochondrial (<i>mt-Co3</i>)	0.534	1.381
<i>ATP synthase, H⁺ transporting, mitochondrial (complex V) (15)</i>		<i>p</i> = 0.0002	<i>p</i> = 0.006
NM_007505	Complex V, F ₁ complex, alpha subunit, isoform 1 (<i>Atp5a1</i>)	0.686	1.158
NM_016774	Complex V, F ₁ complex, beta subunit (<i>Atp5b</i>)	0.867	1.101
NM_020615	Complex V, F ₁ complex, gamma polypeptide 1 (<i>Atp5c1</i>)	0.779	1.119
NM_025983	Complex V, F ₁ complex, epsilon subunit (<i>Atp5e</i>)	0.706	1.217
NM_009725	Complex V, F ₀ complex, subunit b, isoform 1 (<i>Atp5f1</i>)	0.858	1.101
NM_007506	Complex V, F ₀ complex, subunit c (subunit 9), isoform 1 (<i>Atp5g1</i>)	0.821	1.133
NM_026468	Complex V, F ₀ complex, subunit c (subunit 9), isoform 2 (<i>Atp5g2</i>)	0.757	1.272
NM_175015	Complex V, F ₀ complex, subunit c (subunit 9), isoform 3 (<i>Atp5g3</i>)	0.684	1.357
NM_027862	Complex V, F ₀ complex, subunit d (<i>Atp5h</i>)	0.785	1.161
NM_016755	Complex V, F ₀ complex, subunit F (<i>Atp5j</i>)	0.620	1.347
NM_007507	Complex V, F ₁ F ₀ complex, subunit e (<i>Atp5k</i>)	0.839	1.108
NM_138597	Complex V, F ₁ complex, O subunit (<i>Atp5o</i>)	0.812	1.158
AF093677	ATP synthase, subunit 6, mitochondrial (<i>mt-Atp6</i>)	0.466	1.376
J01420	ATP synthase, subunit 8, mitochondrial (<i>mt-Atp8</i>)	0.474	1.186
<i>Fatty acid metabolism (23)</i>		<i>p</i> = 0.00003	<i>p</i> = 0.024
NM_177470	Acetyl-coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-coenzyme A thiolase) (<i>Acaa2</i>)	0.792	0.996
NM_007382	Acetyl-coenzyme A dehydrogenase, medium chain (<i>Acadm</i>)	0.667	0.967
NM_007383	Acyl-coenzyme A dehydrogenase, short chain (<i>Acads</i>)	0.667	1.131
NM_025826	Acyl-coenzyme A dehydrogenase, short/branched chain (<i>Acadsb</i>)	0.839	1.076
AK012088	Acyl-CoA synthetase long-chain family member 3 (<i>Acs13</i>)	0.802	1.139
AK006541	Acyl-CoA synthetase long-chain family member 5 (<i>Acs15</i>)	0.805	1.229
AF179975	Acyl-CoA synthetase long-chain family member 6 (<i>Acs16</i>)	0.839	1.091
NM_013495	Carnitine palmitoyltransferase 1a, liver (<i>Cpt1a</i>)	0.647	1.184

Table 17.2 (Continued)

GenBank Acc. ID	Gene description (<i>gene symbol</i>)	Fold change	
		Cisplatin	Testosterone + cisplatin
NM_009949	Carnitine palmitoyltransferase 2 (<i>Cpt2</i>)	0.923	1.195
NM_007760	Carnitine acetyltransferase (<i>Crat</i>)	0.705	1.146
NM_025797	Cytochrome b-5 (<i>Cyb5</i>)	0.646	1.075
NM_016772	Enoyl coenzyme A hydratase 1, peroxisomal (<i>Ech1</i>)	0.861	1.047
NM_023737	L-specific multifunctional beta-oxidation protein (<i>Ehhadh</i>)	0.733	1.001
NM_008149	Glycerol-3-phosphate acyltransferase, mitochondrial (<i>Gpam</i>)	0.519	1.288
NM_178878	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), alpha subunit (<i>Hadha</i>)	0.879	1.179
NM_145558	Hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase (trifunctional protein), beta subunit (<i>Hadhb</i>)	0.742	1.084
NM_009464	Uncoupling protein 3, mitochondrial (<i>Ucp3</i>)	0.785	1.180
<i>DNA repair</i> (6)		<i>p</i> = 0.00001	<i>p</i> = 0.00009
NM_029943	AP endonuclease 2 (<i>Apex2</i>)	0.730	1.077
NM_018736	Meiotic recombination 11 homolog A (<i>Mre11a</i>)	0.837	1.089
NM_008743	<i>nth</i> (endonuclease III)-like 1 (<i>Nthl1</i>)	0.868	1.159
NM_010957	8-oxoguanine DNA-glycosylase 1 (<i>Ogg1</i>)	0.873	1.096
NM_015810	Polymerase (DNA directed), gamma 2, accessory subunit (<i>Polg2</i>)	1.078	0.878
NM_011677	Uracil-DNA glycosylase (<i>Ung</i>)	0.686	1.084
<i>DNA replication</i> (5)		<i>p</i> = 0.00001	<i>p</i> = 0.00003
NM_153796	Twinkle (<i>Peo1</i>)	0.728	0.993
NM_017462	Polymerase (DNA directed), gamma (<i>Polg</i>)	0.766	1.221
NM_015810	Polymerase (DNA directed), gamma 2, accessory subunit (<i>Polg2</i>)	1.078	0.878
NM_212468	Single-stranded DNA binding protein 1 isoform 1 (<i>Ssbp1</i>)	0.904	1.039
NM_021028	Thymidine kinase 2, mitochondrial (<i>Tk2</i>)	0.856	1.109

Number of genes evaluated for each gene ontology term (GO; molecular function/biological process) is given in parenthesis. *p*-values indicate a significant treatment effect on the GO term. Fold change in gene expression highlighted in gray indicates a significant ($p \leq 0.05$) treatment effect. NS, not significant.

in the absence or presence of testosterone treatments clearly demonstrates cisplatin-induced changes in expression of mitochondria-related genes and their attenuation by testosterone (Figure 17.1).

Through transcriptional profiling of mitochondria-related genes, several key mitochondrial pathways that are targeted by cisplatin during acute kidney injury were revealed. This genomics approach continues to show promise for unraveling novel gene expression changes by revealing mechanistic pathways and the role mitochondrial genes play in cellular toxicity.

17.2.3 Doxorubicin-Induced Cardiotoxicity in B6C3F₁ Mice

Doxorubicin (DOX) remains one of the most potent anticancer drugs prescribed to patients as either monotherapy or in combination with other chemotherapeutics in the treatment of solid tumors and hematological malignancies in both children and adults (Carvalho et al., 2014; Octavia et al., 2012). However, its clinical use is restricted by the risk of severe cardiotoxicity that is dose dependent and cumulative and for some patients may

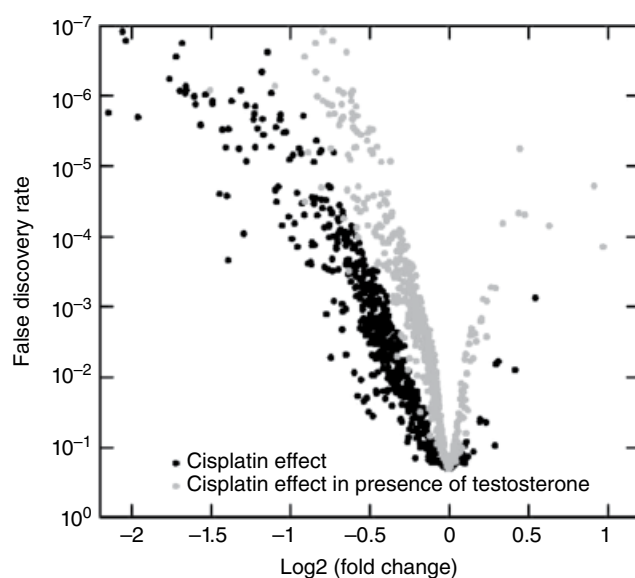


Figure 17.1 Representation of cisplatin effect on transcript levels of mitochondria-related genes in kidney of female KAP2-PPAR α transgenic mice and attenuation by pretreatment of mice with testosterone. Volcano plot presented as relative fold changes in the transcript level of 542 mitochondria-related genes against a false discovery rate for each gene influenced by cisplatin in the absence or presence of testosterone treatments. Pretreatment with testosterone modulated the expression level of mitochondria-related genes that were downregulated by cisplatin in kidneys of female KAP2-PPAR α transgenic mice. Black solid circles indicate effect of cisplatin in absence of testosterone in KAP2-PPAR α transgenic mice and gray solid circles indicate effect of cisplatin in presence of testosterone in KAP2-PPAR α transgenic mice.

irreversibly progress to life-threatening congestive heart failure (Finsterer and Ohnsorge, 2013; Lipshultz and Adams, 2010; Varga et al., 2015). Mechanisms underlying pathogenesis of DOX-induced cardiotoxicity are highly complex and multifaceted. The prevailing hypothesis suggests excessive ROS production, resulting in increased cardiac oxidative stress through (i) enzymatic-mediated mechanisms catalyzed by a number of oxidoreductases including mitochondrial NADH dehydrogenase (complex I) and (ii) nonenzymatic mechanisms relating to formation of a DOX–iron complex (Mordente et al., 2012). Additionally, a greater susceptibility of cardiac tissue to oxidative stress (i.e., via ROS), compared with other tissues, can be related to the heart's higher metabolic activity (Goffart et al., 2004) and relatively weaker antioxidant defense mechanisms (Chen et al., 1994). It has been established that mitochondria are a major target of DOX during development of cardiotoxicity. Mitochondria make up approximately 40–50% of the cell volume in the myocardium compared with only 8–35% cell volume in other major organs (e.g., liver, skeletal muscle, lungs, and kidney), which could partly explain the increased vulnerability of the heart to toxic effects of DOX (Else and Hulbert, 1985; Torres and Simic, 2012). The exogenous NADH dehydrogenase in heart mitochondria is capable of univalent reduction of DOX to a semiquinone radical that can undergo autoxidation with the formation of superoxide radicals. The presence of this form only in heart mitochondria further provides a more parsimonious explanation of selective cardiotoxicity of DOX (Nohl et al., 1998).

Mechanisms of mitochondrial dysfunction responsible for DOX-induced cardiotoxicity are diverse. These include impaired activities of complexes of the electron transport chain (Nicolay and de Kruijff, 1987) as well as

ROS-mediated oxidative damage to cardiolipin located in mitochondrial inner membranes (Goormaghtigh et al., 1990), multi-subunit complexes of the respiratory chain, and the mtDNA (Adachi et al., 1993; Serrano et al., 1999). The consequence of these effects in mitochondria is decreased ATP production, adversely affecting myocardial function. It is also suggested that altered β -oxidation of long-chain fatty acids and/or a decline in its substrate, L-carnitine, can lead to defects in heart function during DOX exposure (Abdel-aleem et al., 1997; Carvalho et al., 2014). The mitochondrial role in DOX cardiotoxicity has also been attributed to activation of mitochondria-dependent apoptotic signaling pathways by increased oxidative stress during drug treatment that may lead to loss of cardiomyocytes and diminished cardiac function (Liu et al., 2004; Papadopoulou et al., 1999).

Although the studies described earlier provide insights into DOX effects on specific aspects of mitochondrial function, knowledge of complex interactions between various mitochondrial pathways at the transcriptional level during DOX cardiotoxicity has been rudimentary. This issue was addressed by utilizing an updated mouse MitoChip of 1019 oligonucleotides in DOX-induced chronic cardiotoxicity mouse model (Vijay et al., 2016). This model was developed in male B6C3F₁ mice that were administered an intravenous dose of 3 mg/kg DOX once a week for 2, 3, 4, 6, and 8 weeks, resulting in 6, 9, 12, 18, and 24 mg/kg cumulative DOX doses, followed by sacrifice a week after the last dose. These cumulative doses correspond to 17.8, 26.6, 35.5, 53.3, and 71 mg/m² body surface area, respectively, in human using the formula described in the Guidance for Industry document (CDER, 2005). This chronic cardiotoxicity mouse model revealed the occurrence of various cardiac events,

ranging from a non-cardiotoxic phase (absence of injury or pathology at 6, 9, or 12 mg/kg), myocardial injury (elevated plasma level of myocardial injury marker, cardiac troponin T, at 18 and 24 mg/kg), and cardiac pathology (cytoplasmic vacuolization in cardiomyocytes at 24 mg/kg) during 8-week DOX treatment. These cardiac events were associated with significant transcriptional changes related to a number of crucial mitochondrial pathways represented by sets of genes (i.e., GO terms) (Table 17.3). A significant DOX effect on oxidative phosphorylation, a key function of mitochondria, was evident at the lowest cumulative dose (6 mg/kg) that significantly reduced expression of 16 genes (i.e., 15 nuclear genes and a mitochondrial gene, *mt-Atp8* of complex V) in mouse heart. Similar but more pronounced drug effect on oxidative phosphorylation was observed at the highest cumulative DOX dose (24 mg/kg) that downregulated 33 genes (i.e., 32 nuclear genes and *mt-Atp8*) with a greater decline in transcript levels than at 6 mg/kg dose (Vijay et al., 2016). Interestingly, there was lack of significant drug effect at 9–18 mg/kg cumulative DOX doses, which may suggest a compensatory response to the compromised bioenergetics as a result of altered transcript levels at 6 mg/kg dose. Further evaluation of transcript changes related to individual complexes of oxidative phosphorylation indicated that the lowest cumulative dose also significantly reduced transcript levels of complexes I and II, with complex I being more prominently affected by DOX among all five complexes (Vijay et al., 2016). It is suggested that complex I is more vulnerable to DOX effects when compared with other complexes in the respiratory

chain (Tokarska-Schlattner et al., 2006) and is a prime site of ROS production by redox cycling of DOX (Davies and Doroshov, 1986). The altered expression of genes associated with multi-subunit complex I in mouse heart might have implications for formation of a dysfunctional protein that can impair energy production and enhance ROS generation at initial stages of DOX treatment. Early impairment of complex I activity prior to the occurrence of congestive heart failure has been reported in DOX-treated rats (Ohkura et al., 2003). At the highest cumulative DOX dose (24 mg/kg) that manifested into cardiac pathology, all five complexes had a significant drug effect in heart (Table 17.3).

Transcriptomics analysis further indicated a significant downregulation of many genes related to other key pathways involved in cardiac energetics, such as fatty acid β -oxidation (e.g., *Acaa2*, *Acadl*, *Acs1*, *Acsc1*, *Echs1*, *Hadh*, *Hadhb*, *Hmgcl*, *Pcca*, *Peci*) and the Krebs cycle (e.g., *Cs*, *Idh3a*, *Idh3b*, *Ogdh*, *Sucla2*, *Sugla1*) at 6 mg/kg cumulative dose (Vijay et al., 2016). Importantly, a dose-related persistent decline in the expression level of genes related to these two pathways was observed with continued DOX treatment in mice. Both fatty acid β -oxidation and the Krebs cycle showed a significant drug effect in mouse heart throughout 8-week exposure, with the most prominent drug effect at 24 mg/kg cumulative dose (Table 17.3). Altogether, it can be assumed that downregulation of genes encoding proteins crucial for cardiomyocyte energy production may manifest into energy deficit that may provoke early cardiotoxic activity during DOX exposure, even at the lowest cumulative dose.

Table 17.3 Doxorubicin effect on selected gene ontology terms.

Gene ontology term	N	DOX exposure in weeks (cumulative dose in mg/kg)				
		2 (6)	3 (9)	4 (12)	6 (18)	8 (24)
		p	p	p	p	p
Oxidative phosphorylation	107	0.028	0.083	0.068	0.070	0.002
NADH–ubiquinone dehydrogenase (complex I)	44	0.031	0.094	0.081	0.185	0.002
Succinate–ubiquinone dehydrogenase (complex II)	4	0.027	0.215	0.836	0.770	0.002
Ubiquinol–cytochrome c reductase (complex III)	10	0.229	0.247	0.011	0.652	0.006
Cytochrome c oxidase (complex IV)	28	0.139	0.080	0.013	0.002	0.001
ATP synthase, H ⁺ transporting, mitochondrial (complex V)	21	0.053	0.120	0.689	0.187	0.005
Fatty acid metabolism	63	0.0001	0.0005	0.001	0.000001	0.000001
Krebs cycle	15	0.001	0.0002	0.009	0.001	0.000001
Apoptosis	71	0.003	0.030	0.171	0.001	0.000001
Steroid biosynthesis	12	0.126	0.399	0.208	0.001	0.000001

N represents number of genes evaluated for each gene ontology term (GO; molecular function/biological process). Effect of DOX was estimated compared to corresponding SAL treatment group. *p*-values < 0.05 are considered a significant DOX effect on the GO term and are highlighted in gray. DOX, doxorubicin; SAL, saline.

It is suggested that DOX-induced altered bioenergetics can lead to loss of cardiomyocytes (Carvalho et al., 2014; Ferreira et al., 2008). In our DOX-induced chronic cardiotoxicity mouse model, a modest but statistically significant greater than 1.2-fold increase in transcript levels of proapoptotic genes *Bax*, *Bcl3*, *Casp12*, *Casp4* at 6 mg/kg cumulative dose and *Bnip3l*, *Bok*, *Dapl1* at 12 mg/kg cumulative dose may likely provoke cardiomyocyte death by apoptosis at early stages of cardiotoxicity before occurrence of myocardial injury at 18 mg/kg cumulative dose (Vijay et al., 2016). A dose-related increase in expression of a proapoptotic gene *Bax*, involved in promoting mitochondria-mediated intrinsic apoptotic pathway, further highlights a key role of mitochondria in genesis of cardiotoxicity in initial stages of DOX treatment. The DOX effect on transcript levels of genes related to apoptosis was more intense at 24 mg/kg cumulative dose (Table 17.3).

It has also been suggested that cardiomyocyte death is one of the events involved in cardiac remodeling during transition from compensated hypertrophy to heart failure (Oka and Komuro, 2008). Changes in cardiac steroidogenesis have been indicated to be important in the development of hypertrophy with the progression to heart failure in a rodent model (Ohtani et al., 2009). In our chronic cardiotoxicity mouse model, a majority of the genes (7 of 11 genes; 64%) evaluated for the steroid biosynthesis pathway had higher expression compared with saline-treated controls throughout 8-week treatment, with a statistically significant effect on this pathway at the two highest cumulative DOX doses (18 and 24 mg/kg) used in the study (Table 17.3). Interestingly, an early upregulation of the gene encoding steroidogenic acute regulatory protein (*Star*), a rate-limiting factor in steroid biosynthesis, was observed in mouse heart at 6 mg/kg cumulative dose with persistent increase in the expression at 18 and 24 mg/kg doses (Vijay et al., 2016). These transcriptional changes related to steroid biosynthesis in the absence of enlarged heart and lack of pathological evidence of hypertrophy may suggest an impending hypertrophic event at DOX doses capable of provoking myocardial damage.

Collectively, transcriptomics using the mouse MitoChip in our chronic cardiotoxicity mouse model revealed a series of cardiac events progressing toward cardiotoxicity during DOX treatment. These cardiac events might have been initiated with an energy deficit, leading to apoptotic changes at the lowest cumulative dose (6 mg/kg) followed by compensatory hypertrophy and myocardial injury at higher cumulative doses (12 and 18 mg/kg) that may have subsequently manifested into cardiac pathology at 24 mg/kg cumulative dose (Figure 17.2). Findings from this chronic cardiotoxicity mouse model also revealed that DOX-mediated transcriptional changes for many

genes that occurred prior to myocardial injury also persisted after cardiac pathology. These included genes associated with energy metabolism (e.g., *Ndufs4*, *Cox7b*, *Atp5j*, *Hadh*), apoptosis (e.g., *Bax*), and hypertrophy (e.g., *Star*), suggesting these genes as candidate early genomics markers of DOX cardiotoxicity (Vijay et al., 2016). Genes with early transcriptional changes affecting highly vulnerable subcellular organelles, such as mitochondria, may serve as potential targets for novel therapeutics or cardioprotective interventions to help prevent or attenuate the severity of cardiotoxicity or delay progression toward life-threatening cardiomyopathy. Interestingly, many of these DOX-induced transcriptional changes related to mitochondria were significantly mitigated by pretreatment of mice with a cardioprotective drug, dexrazoxane, 30 min prior to a weekly administration of DOX, resulting in a significant reduction in plasma cardiac troponin T level and a complete amelioration of cardiac pathology (Vijay et al., 2016).

Several other DOX cardiotoxicity studies have performed global transcriptional profiling using commercial high-density microarrays. These studies showed differentially expressed genes associated with oxidative stress, signal transduction, apoptotic pathways (Yi et al., 2006; Zhao et al., 2002), and upregulation of mitochondria-related genes in response to stress through Nrf2 or HIF-1 pathways related to oxidative stress following DOX exposure (Thompson et al., 2010). These studies lack comprehensive analysis of DOX effects on cardiac mitochondria and likely interactions among mitochondrial pathways. The application of the mouse MitoChip that is primarily focused on mitochondrial biology not only generated novel information from our chronic cardiotoxicity mouse model but also provided better understanding on mechanisms of mitochondrial toxicity during progression toward cardiomyopathy in our mouse model.

17.3 Rat MitoChip: Assessment of Altered Mitochondrial Function in a Rat Model

In view of the usefulness of mouse MitoChip in providing important insights into the mechanisms of mitochondrial dysfunction during drug toxicities, a next-generation MitoChip was designed for the rat model. This custom-designed rat MitoChip (Agilent Technologies) was developed using Rat Genome Database (Shimoyama et al., 2015) and contains 918 unique genes; 754 associated with mitochondrial structure and function, 70 involved in apoptosis (both intrinsic and extrinsic pathways), and 94 involved in phase I (e.g., cytochrome P450 oxidoreductases) and phase II (e.g., glutathione

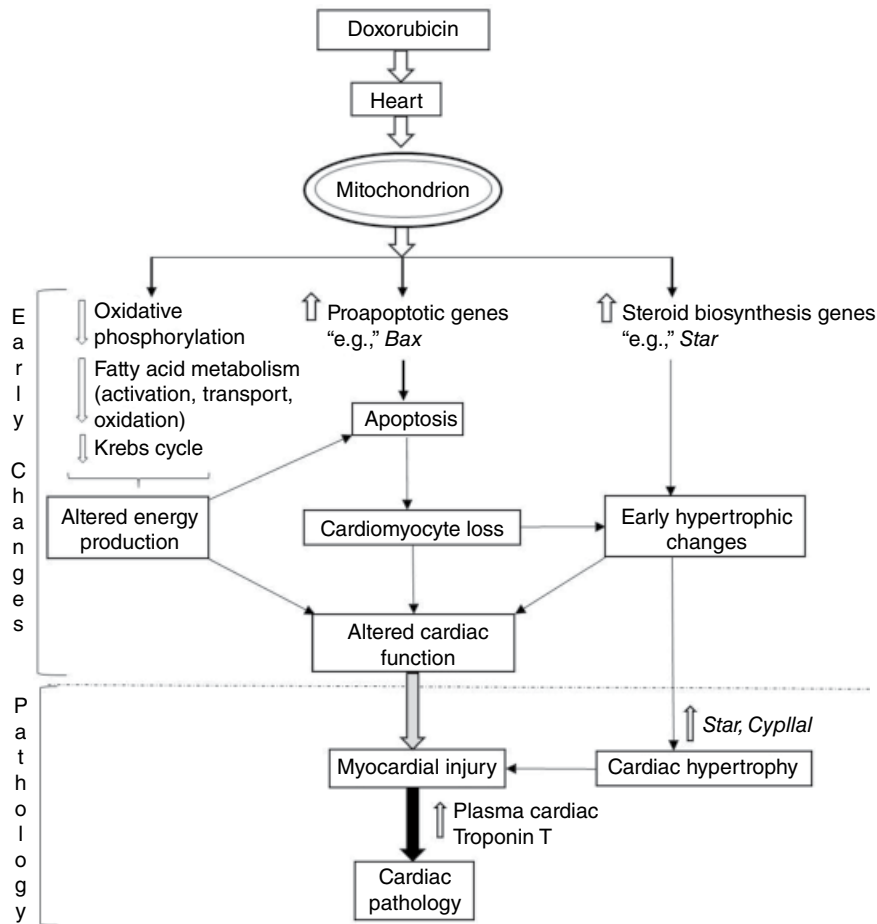


Figure 17.2 Illustration of role of mitochondria in various cardiac events leading to doxorubicin-induced cardiotoxicity in B6C3F₁ mice. Downregulation of cardiac genes associated with oxidative phosphorylation, fatty acid metabolism, and the Krebs cycle during early stages of doxorubicin treatment can impair cardiac energy metabolism. This can induce expression of apoptotic genes that may promote cardiomyocyte loss, leading to early hypertrophy-related changes in the heart. These doxorubicin-mediated early transcriptional changes together can alter cardiac function. Persistent doxorubicin treatment can severely alter transcript levels that ultimately can manifest into myocardial injury, resulting in elevated cardiac troponin T levels in plasma followed by overt cardiac pathology. Up arrow indicates increased activity of the gene/pathway/biological process, and down arrow indicates decreased activity of the gene/pathway/molecular function.

S-transferases, UDP-glucuronidases, sulfuryl transferases) reactions of drug metabolism. Moreover, it also contains 16 rat housekeeping genes for a total of 934 genes on the rat MitoChip. Similar to the mouse MitoChip, rat MitoChip microarrays were designed using a web-based eArray application and manufactured using inkjet technology for in situ synthesis of 60-base-long probes on a glass slide (Agilent Technologies). A detailed description of development of the rat MitoChip and quality control measures at various steps of microarray experiment have been presented previously (Gonzalez et al., 2015).

17.3.1 Doxorubicin-Induced Cardiotoxicity in SHR/SST-2 Rat Model

Sex is considered as one of the risk factors that can significantly influence development of DOX cardiotoxicity (Lipshultz et al., 1995). Young females and adult males appear to be more susceptible to DOX and are at increased risk for development of DOX-induced chronic cardiotoxicity (Belham et al., 2007; Trachtenberg et al., 2011). It has been reported that prepubertal females

show severe decline in left ventricular contractility compared with males of the same age after DOX therapy, with a greater difference in contractility between the sexes at higher cumulative doses (Krischer et al., 1997; Lipshultz et al., 1995). Sex-based differences in DOX cardiotoxicity have also been demonstrated in rat models where adult males were more susceptible to toxic effects of DOX than females (Gonzalez et al., 2015; Moulin et al., 2015; Zhang et al., 2014). Mitochondrial dysfunction has been suggested to underlie differential cardio-sensitivity to DOX between the sexes as indicated by reduced expression of genes involved in mitochondrial biogenesis and cardiolipin contents in hearts of DOX-treated male Wistar rats, but not in DOX-treated females (Moulin et al., 2015). This group also showed cardiac atrophy and reduced left ventricular ejection fraction in conjunction with a significant decline in mtDNA content and mitochondrial respiration after DOX exposure in adult males only, further associating impaired mitochondrial activity to DOX cardiotoxicity.

Mitochondrial role in sex-based differential DOX cardiotoxicity was also evaluated in hearts of male and female tumor-bearing spontaneously hypertensive adult

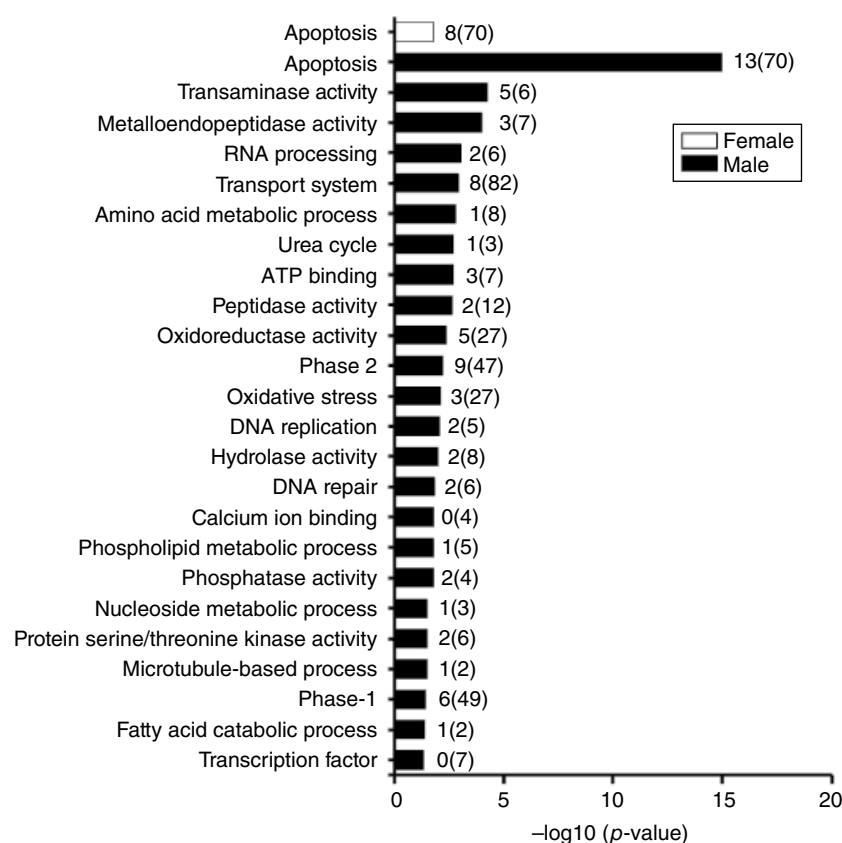


Figure 17.3 Effect of doxorubicin on various mitochondria-related gene ontologies in SHR/SST-2 rat heart. Male and female SHR/SST-2 heart tissues were analyzed for doxorubicin effect on mitochondria-related gene ontologies (pathways/molecular function). A greater drug effect in male SHR/SST-2 heart was indicated by a significant doxorubicin effect on 24 gene ontologies (black solid bars), whereas drug effect was observed only in one gene ontology (black open bar) in female SHR/SST-2 heart. Each bar represents the $-\log_{10} p$ -value of each statistically significant gene ontology. The total number of genes in each gene ontology is presented in parentheses to the right of each bar, and the number of statistically significant genes within that gene ontology is to the left of the parentheses. Significance was determined at an alpha of 0.05. Black solid bar represents male and black open bar represents female.

rats (SHR/SST-2s) treated with a single intravenous dose of 10 mg/kg DOX using rat MitoChip (Gonzalez et al., 2015). At 14 days after acute DOX exposure, this model showed an average cardiomyopathy score of 2.9 in males and 2.2 in females and an average serum cardiac troponin T level 22 times higher than saline controls in males whereas less than 4 times the saline controls in females, indicating a greater cardiotoxicity in male SHR/SST-2s compared with females. Transcriptomics analyses of 918 genes on the rat MitoChip also revealed a prominent effect of DOX in the hearts of male SHR/SST-2s. In male rats, 109 genes were differentially expressed, while in female rats the expression of only 42 genes was significantly altered compared with respective saline controls. A greater DOX toxicity in male heart was further substantiated by a significant drug effect on 24 GO terms representing various molecular functions/biological processes, whereas only a single set of genes related to apoptosis had a significant DOX effect in female heart (Figure 17.3). Among these 24 GO terms, a set of 27 oxidative stress-related genes showed a significant DOX effect with a significant upregulation of *Gpx5* and *Txn2* and downregulation of *Hba-a2* compared with saline controls in male SHR/SST-2 heart. These results suggest that a greater oxidant-mediated damage could, in part, be responsible for higher vulnerability of heart in

adult male SHR/SST-2s than females (Gonzalez et al., 2015). Supporting this notion is a differential cardiac oxidative stress proposed as one of the mechanisms underlying variations in susceptibility of heart to DOX toxic effects between the sexes (Lipshultz et al., 1995).

Another noteworthy finding in SHR/SST-2 rats was a drug effect on a set of 70 genes related to apoptosis that was more prominent in male hearts compared with females (Figure 17.3). Of these 70 genes, 13 apoptotic genes were differentially expressed in male SHR/SST-2 hearts with 10 genes (*Bad*, *Bax*, *Bcl2l1*, *Bik*, *Bnip3*, *Dapk3*, *Daxx*, *Pdcd1*, *Pdcd5*, *Sgpp1*) showing higher expression and three genes (*Bnip3l*, *Cidea*, *Dffb*) with lower expression compared with saline controls. In female SHR/SST-2 hearts, only eight apoptotic genes were differentially expressed; three genes were upregulated (*Bbc3*, *Bok*, *Dapl1*) and five genes were downregulated (*Bad*, *Casp7*, *Faf1*, *Pdcd11*, *Pmaip1*). This differential DOX effect on apoptotic genes that was observed between the sexes may be due to differential oxidative stress or protective mechanisms toward the development of cardiotoxicity between the sexes. Altogether, these findings identify a role for oxidative stress- and apoptosis-mediated mechanisms that may, in part, contribute to a greater cardio-sensitivity of male SHR/SST-2 hearts to DOX toxic effects.

DOX-induced significant alterations in the abundance of transcripts related to energy metabolism as measured by commercial rat whole genome microarray chip has been reported in hearts of male Sprague-Dawley rats after chronic treatment with 12 mg/kg cumulative dose (Berthiaume and Wallace, 2007) and in our chronic cardiotoxicity mouse model (Section 17.2.3; Vijay et al., 2016). However, there was no evidence of a significant influence of DOX on sets of genes related to energy metabolism (e.g., oxidative phosphorylation and fatty acid metabolism) in hearts of both male and female SHR/SST-2 rats. These discrepancies in DOX effects on cardiac energetics might be due to different animal models and/or DOX dosing regimen. DOX effects in SHR/SST-2 rats were in response to a single acute DOX dose of 10 mg/kg, whereas male Sprague-Dawley rats and male B6C3F₁ mice were chronically treated with DOX. Also, hearts of SHR/SST-2 rats were examined 14-day posttreatment, whereas male Sprague-Dawley rats and male B6C3F₁ mice were investigated 5- and 1-week posttreatment, respectively. Use of the rat MitoChip has been valuable in shedding light on the mechanisms that might be underlying sex-based differential DOX cardiotoxicity. Future studies investigating mechanisms of mitochondrial toxicity in various rat models are essential to confirm the usefulness of the rat MitoChip in toxicology.

17.4 Concluding Remarks

Information generated by both mouse and rat MitoChips demonstrated their potential in understanding mechanisms of mitochondrial dysfunction during different organ toxicities induced by potent anticancer drugs in rodent models. This transcriptomics tool not only verified the results already described in the literature but also generated novel findings. For example, drug effects were observed on the expression of mtDNA-encoded genes, which had not been feasible using commercial whole genome expression arrays because of lack of mitochondrial DNA-encoded genes on these arrays. This highlights the distinctive feature of the MitoChip that is capable of providing crucial information relating to the cross-talk between two genomes that regulate the mitochondrial function. Additionally, MitoChip has proved capable of detecting subtle changes in transcript levels prior to overt organ toxicity and facilitating identification of early genomics markers of toxicity that could serve as potential targets for novel therapeutics or interventions for alleviation of toxicity. In summary, this transcriptomics tool can prove valuable in (i) understanding mechanistic pathways underlying mitochondrial dysfunction, (ii) identifying

early molecular markers, (iii) recognizing molecular targets within mitochondria for development of effective treatment strategies, and (iv) designing novel approaches for preventing or minimizing the severity of toxicities or delaying the progression of degenerative diseases associated with impaired mitochondrial function.

17.5 Future Direction

Mitochondria represent a crucial component in toxicology studies because these organelles are a prime source of energy vital for the cell survival and also a major site of ROS production, which are the central to the etiology of number of drug-related toxicities and degenerative diseases. Interestingly, species-specific differences exist in the rate of mitochondrial respiration and ROS generation within mitochondria (Panov et al., 2007), suggesting a likely mitochondrial involvement in species-related differences in toxicities induced by drugs targeting mitochondria or disease susceptibilities/outcomes associated with mitochondrial dysfunction. Because of the usefulness of the MitoChip for understanding the toxicology in mice and rats, mitochondria-specific gene expression arrays for nonhuman primates (*Macaca mulatta* and *Macaca fascicularis*) plus human (*Homo sapiens*) are currently being designed and developed in our laboratory. Use of this transcriptomics approach in various species will advance our understanding of mitochondria-related pathways or gene expression profiles that might be responsible for interspecies differences in drug-induced toxicities or disease outcomes. Such knowledge will aid in designing effective treatment strategies and/or novel interventions to minimize the severity of drug-related toxicities or delay disease progression in humans.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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18

Using 3D Microtissues for Identifying Mitochondrial Liabilities

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CHAPTER MENU

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18.1 Significance of Metabolic Profiling in Drug Development: Current Tools and New Technologies

The role of metabolism in cellular and physiological processes is well established with many diseases now linked to metabolic dysfunction or reprogramming. Drug-induced mitochondrial toxicity has been studied for many decades in both academic and industrial settings. Recently, research on mitochondrial function has gained more momentum as it has become evident that mitochondria are major contributors to drug-induced organ injury. The blockbuster drugs troglitazone and cerivastatin, for example, were withdrawn from the market in 1997 and 2001, respectively, because of severe liver toxicity that at least in part was caused by mitochondrial toxicity (Will and Dykens, 2014). This example combined with the economic impact of late-stage attrition has prompted industry-wide focus on the development of better mechanistic-based cell and animal models for testing of mitochondrial liabilities *in vitro*.

The available tools and biological models to study drug-induced mitochondrial toxicity have evolved over time. Mitochondrial status has classically been monitored via oxygen consumption using low-throughput polarographic electrodes (Dyken and Will, 2007). While

the technique is compatible with intact cells, high quantities of isolated mitochondria are required to attain requisite measurement sensitivity. In addition, the use of isolated organelles potentially overrepresents drug effects because compounds have unrestricted access and are not subject to metabolism by neither activation nor degradation (Dyken and Will, 2007).

A common method working with intact cells instead of relying on isolated organelles is the so-called “glucose–galactose” assay (Marroquin et al., 2007). It has been documented that cancer cells have a propensity to use glycolysis to produce ATP and biomass, referred to as the “Warburg effect.” In this assay, glucose is replaced by galactose in the assay media, forcing the cells to shift to an oxidative phenotype. Cells using the OXPHOS complex due to absence of glycolysis were shown to be more susceptible to mitochondrial insult, thereby revealing toxicity that is primarily mediated by mitochondrial impairment. The glucose–galactose assay only detects about 2–5% of all mitotoxicants, which suggests that most toxicants cause organ toxicities via multiple off-target mechanisms (Hynes et al., 2013; Will and Dyken, 2014).

Several “high-throughput” assays for mitochondrial activity have been developed using soluble oxygen sensors and time-resolved fluorescence imaging (Hynes et al., 2012). The so-called respiratory screening technology

(Luxcel Bioscience, Ireland) distinguishes between inhibition and uncoupling of compounds. Its throughput allows to look at hundreds of compounds per day (Will and Dykens, 2014). The assay was able, for example, to discriminate troglitazone from the less mitochondrial toxicant pioglitazone (Will and Dykens, 2014).

The Seahorse Biosciences metabolic flux analyzer XF[®]96 platform (Agilent Technologies, CA, USA) enables direct measurement of mitochondrial activity in whole cells by measuring oxygen flux (OCR) and extracellular acidification (ECAR) using a pair of fluorescent sensors. The Seahorse XF Cell Mito Stress Test (MST) Kit uses modulators of cellular respiration that specifically target components of the electron transport chain (ETC) to reveal key parameters of metabolic function. Compounds, such as oligomycin, FCCP, and a mix of rotenone and antimycin A, are serially injected to measure ATP-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. In addition, the media acidification rate is measured, which is indicative for the glycolytic lactate efflux (Wu et al., 2007). The system allows measurements of mitochondrial functions in response to drugs on a wide variety of cell types and does not require artificial levels of galactose in the culture media. The platform provides higher throughput and in-depth information regarding which complex of the ETC is affected.

Whereas the glucose–galactose assay has become industry standard for early assessment of compounds, the Seahorse platform is preferred for follow-up investigations for compounds that are more advanced in the drug development process based on an anticipated favorable safety margin (Will and Dykens, 2014).

18.2 Use of 3D Microtissues to Detect Mitochondrial Liabilities

18.2.1 Limitations of Currently Used *In Vitro* Cell Models

The previously described methods have provided many insights to mitochondrial functions and enabled researchers to assess potential mitochondrial toxicants. However, it became apparent that these models relying on isolated organelles, cell lines, or primary cells in 2D culture are not able to reflect long-term exposures due to their limited usage time. Thus, long-term testing would be of advantage when using slow-acting mitochondrial toxicants. For example, drugs, which inhibit mitochondrial protein synthesis (some antibiotics) or impede mtDNA replication (some antivirals), undermine mitochondrial viability over longer time frames (Will and Dykens, 2014). In addition, active metabolism of drugs by cytochromes has shown to contribute to mitochondrial toxicity.

For example, CYP2C-mediated ring hydroxylation of diclofenac to 4'-hydroxydiclofenac and the phenolic metabolites of amiodarone cause mitochondrial injury (Chan et al., 2005). The frequently used HepG2 cells have very little CYP activity and are therefore not a suited tool to mimic physiologically relevant metabolism of drugs (Berger et al., 2016). Primary human hepatocytes (PHH) in 2D culture quickly lose their CYP expression due to dedifferentiation processes.

Despite the need, metabolic competent models with stable metabolic activity over several weeks in culture were so far not implemented into mitochondrial toxicity testing paradigms.

18.2.2 General Characteristics of 3D Microtissues

3D microtissues are spherical, scaffold-free, self-assembled tissues from single dispersed cell suspensions of up to 400 µm in diameter (Kelm and Fussenegger, 2004). They consist either of a single cell type or multiple cell types reflecting the cell composition in the corresponding native tissue. An important advantage of microtissue spheroids is that the same production processes can be applied for a broad range of tissue and organ types. A wide variety of microtissue models have been described in the literature such as liver microtissues (Messner et al., 2012; Kijanska and Kelm, 2016), cardiac microtissues (Beauchamp et al., 2015), tumor microtissues (Kelm et al., 2003), neuronal microtissues (Lancaster et al., 2013), or pancreatic islets (Zuellig et al., 2017). Hence, similar analytical protocols to measure mitochondrial impairment can be established for different types of microtissues as the model format is always the same, enabling a high degree of standardization. Two key organs were selected to exemplify analysis of mitochondrial impairment using advanced 3D models: (i) primary human liver microtissues and (ii) human cardiac microtissues.

Creating a physiologically more relevant 3D cell-based *in vitro* model necessitates the use of mature, fully differentiated cell sources reflecting organ functionality as close as possible to produce the corresponding tissue. In that sense, PHHs reflect closer the native liver metabolism as compared with classical cell sources such as HepG2 or HepaRG monolayer models (Berger et al., 2016). Coaxing the PHHs in a 3D environment even further improved metabolism (Messner et al., 2012; Bell et al., 2016; Berger et al., 2016). A gene expression analysis comparing gene expression of the major cytochromes of 2D HepG2, 2D HepaRG, 2D PHH, and 3D PHH cell culture models demonstrated an at least fourfold higher expression for all CYPs in 3D-PHH compared with the other cell systems (Berger et al., 2016). In addition, applying the same phenotyping probes, the 3D liver

microtissues displayed similar CYP inducibility as compared with *in vivo* inducibility (Berger et al., 2016). 3D liver microtissues have also shown to maintain a differentiated state and stable phenotype as well as metabolic activity for up to 4 weeks in culture, as verified by ATP content (viability), albumin secretion, and CYP3A4 activity. 3D liver microtissues also retain glycogen storage capacity and form functional bile canaliculi networks (Messner et al., 2012; Bell et al., 2016).

For myocardial tissue engineering the use of primary cell sources is rather limited by the availability of donor material as well as fully differentiated adult cardiomyocytes that are very difficult to handle *in vitro*. However, over the past years, human induced pluripotent stem cell (iPS)-derived cardiomyocytes have become the gold standard for evaluating *in vitro* cardiac-related adverse drug effects (Anson et al., 2011). Cardiac microtissues display well-developed myofibrils extending throughout the whole spheroid without a necrotic core (Beauchamp et al., 2015). Cardiac MTs exhibit spontaneous contractions over at least 4 weeks monitored by optical motion tracking and are responsive to electrical pacing. Response to contractile pharmacology was verified with several agents known to modulate cardiac rate and viability (Beauchamp et al., 2015). Calcium transients underlay the contractile activity and were also responsive to electrical stimulation, caffeine-induced Ca^{2+} release, and extracellular calcium levels. After a maturation phase, which depends on the iPS batch, microtissues exhibit a basal contraction rate of approximately 60 beats per minute (Beauchamp et al., 2015). With their spherical format, the cardiac microtissues fit into the Seahorse platform; however, due to continuous contraction, oxygen consumption rate (OCR) measurements are more challenging.

18.2.3 3D Microtissue-Based Assessment of Mitochondrial Activity

Metabolic profiling of 3D microtissue spheroids within the Seahorse platform has become possible by using a specifically designed spheroid microplate for the XF^e96 flux analyzer. Here, the assay is described using an advanced 3D *in vitro* human liver model (3D InSight™ Human Liver Microtissues, InSphero AG) and an iPS-derived human cardiac model (3D InSight™ Human Cardiac Microtissues, InSphero AG). Scaffold-free 3D InSight™ microtissues were produced by cellular self-assembly. Human liver microtissues were produced from cryopreserved PHHs (Messner et al., 2012), and human cardiac microtissues were produced by coculturing iPS-derived human cardiomyocytes and cardiac fibroblasts. After formation and maturation, the microtissues were maintained in the GravityTRAP™ culture platform.

Groups of six microtissues were treated with up to seven concentrations for different durations (from 2 up to 14 days) to achieve time-resolved dose–response curves. An important feature of the 3D microtissue format is that treatment and measurement can be fully decoupled from each other as the non-adhering spherical microtissues can be transferred from the GravityTRAP plate to the Seahorse assay plate (Figure 18.1a).

Incubation time and concentration of the electron chain uncoupler were optimized to compensate for the increased penetration times of the compounds into the tissue. Comparing the metabolic profile of human hepatocytes in a monolayer and microtissue format highlights the metabolic differences, which can occur between these culture formats (Figure 18.1b). Whereas the basal respiration is similar, the maximal and spare respiratory capacity (SRC) is significantly higher in the microtissue format as compared with the monolayer counterparts. This leads to a significant higher spare (reserve) respiratory capacity in the 3D model (Figure 18.1b and c).

Four independent measurements of the SRC between different PHH lots demonstrate that the SRC is robust and reproducible over different measurements and donors (Figure 18.2). The variations observed between measurements of individual microtissues were between 13.6 ± 2.7 pmol/min SRC in donor 1, 22.8 ± 1.8 pmol/min and 19.4 ± 2.6 pmol/min SRC in donor 2 for two different measurements as well as 14.6 ± 3.8 pmol/min SRC for microtissues derived from a multi-donor hepatocyte lot. This equaled an average standard deviation of 17% between microtissues within a measurement.

18.2.4 Difference of Spare Respiratory Capacity in 2D versus 3D Cultures

Mitochondria are bioenergetic organelles that are important in stress sensing to allow for cellular adaptation to the environment (Pfleger et al., 2015). Under normal homeostatic conditions, cells within a tissue require only a fraction of the energy produced by oxidative phosphorylation, which is the prime mechanism to generate cellular energy. In proliferating cells (e.g., cancer cells), however, aerobic glycolysis is the major energy source (Warburg effect) (Vander Heiden et al., 2009). The difference between the maximum respiratory capacity and basal respiratory capacity is referred to as reserve respiratory capacity or spare respiratory capacity (Pfleger et al., 2015). Depending on environmental stimuli, such as entering from a quiescent into a proliferative status or as stress response upon substance exposure, the energetic status of the cells changes and, consequently, changes the SRC. The SRC will decrease if either ATP demand increases or if proton leak increases—either of these mechanisms can be the result of increased

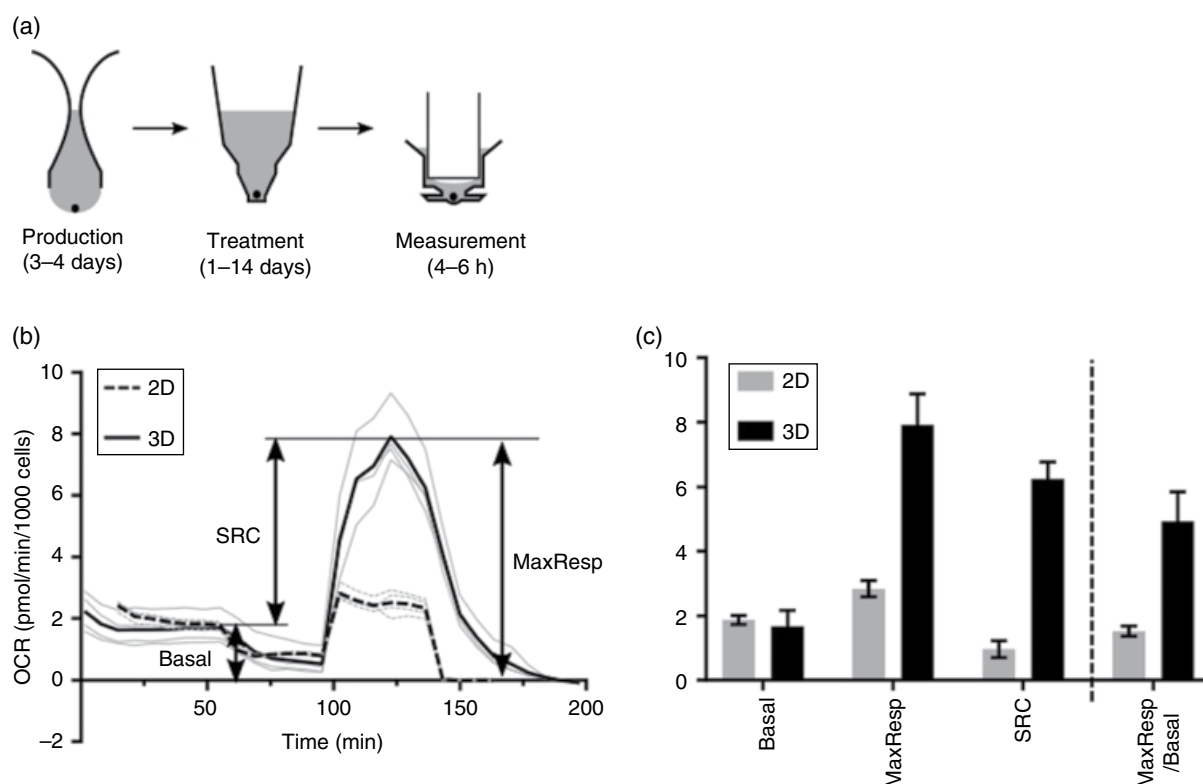


Figure 18.1 (a) Schematic representation of handling steps. 3D microtissues are formed in hanging drops and transferred in to GravityTRAP plates for compound treatment. For analysis, 3D microtissues are transferred to Seahorse assay plates. (b) OCR trace over time is recorded using the Seahorse MST assay for primary human hepatocytes cultured either in 2D or as 3D microtissues. (c) Comparison of basal respiration, maximal respiration, and spare respiratory capacity (SRC) between 2D and 3D.

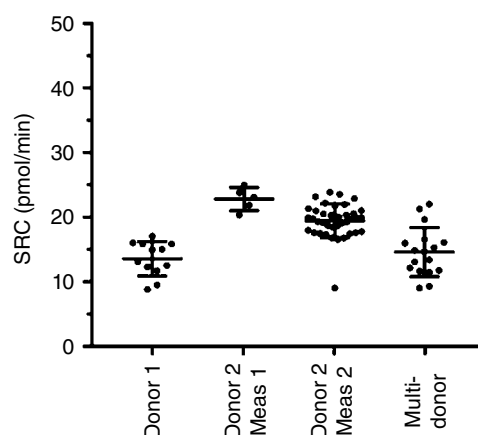


Figure 18.2 Spare respiratory capacity (SRC (pmol/min)) of 3D human liver microtissues derived from two single donor batches and one multi-donor batch (10-donor).

mitochondrial damage or stress (Nobes et al., 1990). In addition, Hill et al. examined the response to 4-HNE, a known mitochondrial damaging reactive lipid. The cells responded to 4-HNE by increasing ATP flux, effectively consuming SRC (Hill et al., 2009).

In our hands, we have observed that primary hepatocytes show significant differences in the SRC depending on the culture format. Cultured as monolayer only a marginal SRC could be measured (0.97 ± 0.26 pmol/min for 1000 hepatocytes). In contrast, significant higher SRC values were observed in the microtissue format (6.24 ± 0.52 pmol/min for 1000 hepatocytes). Of note, primary hepatocytes are not proliferating neither in 2D nor in 3D culture. One might postulate that the decrease in SRC results from a stress response caused by changes in cell shape and tension, which both are known to impact the differentiation and proliferation status of cells (McBeath et al., 2004).

The significantly higher SRC measured in the microtissue format makes it a valuable parameter to evaluate the impact of drugs on tissues. We present the use of SRC readout for efficacy and safety studies for liver and cardiac microtissue models. The high level of SRC observed in 3D InSight™ Human Liver Microtissues makes them an ideal model system for use as a primary readout to assess mitochondrial function, because a drop in the cellular energy reserve was shown to be the first reporting mechanism in mitochondrial assessment for many drugs such as perhexiline, rosiglitazone, troglitazone, and amiodarone (Eakins et al., 2016).

18.3 SRC-Based Detection of Mitochondrial Liabilities in 3D Human Liver Microtissues

The *in vitro* longevity of 3D liver microtissues allows mitotoxicity assessment to be divided into two distinct steps: (i) extended culture and flexible drug exposure times (up to 14 days) with repeated dosing and (ii) discrete transfer of the spheroids to the Seahorse XF⁹⁶ platform for subsequent OCR analysis (Figure 18.1a).

We showed that 3D human liver microtissues showed a sixfold higher SRC than the corresponding 2D culture, in which an increase in SRC was barely detectable. The increased SRC in 3D culture is important because it defines the extra capacity that is available in cells to produce energy in response to increased stress or work and as such is associated with cellular survival (van der Windt

et al., 2012). Furthermore, assessing dose response of the SRC in combination with tissue viability enables discrimination whether mitochondrial impairment is the primary toxicological mechanism or a secondary effect. Resulting IC₅₀ values for reducing the SRC (IC₅₀SRC) were therefore compared with cell viability IC₅₀ values (IC₅₀ATP, CellTiter-Glo[®] Assay, Promega Corp.).

The ratio of IC₅₀SRC to IC₅₀ATP can be used to determine mitochondrial liabilities, as exemplified for amiodarone using 3D InSight[™] PHH and HepaRG human liver microtissues (Figure 18.3). Comparing the IC₅₀ values of the total ATP content and the IC₅₀ values derived from the SRC showed clear difference in mitochondrial sensitivity between the two models. Liver microtissues composed of PHHs were more sensitive toward amiodarone than HepaRG microtissues, in both ATP-based viability assessment and decrease of SRC. Importantly, it was observed

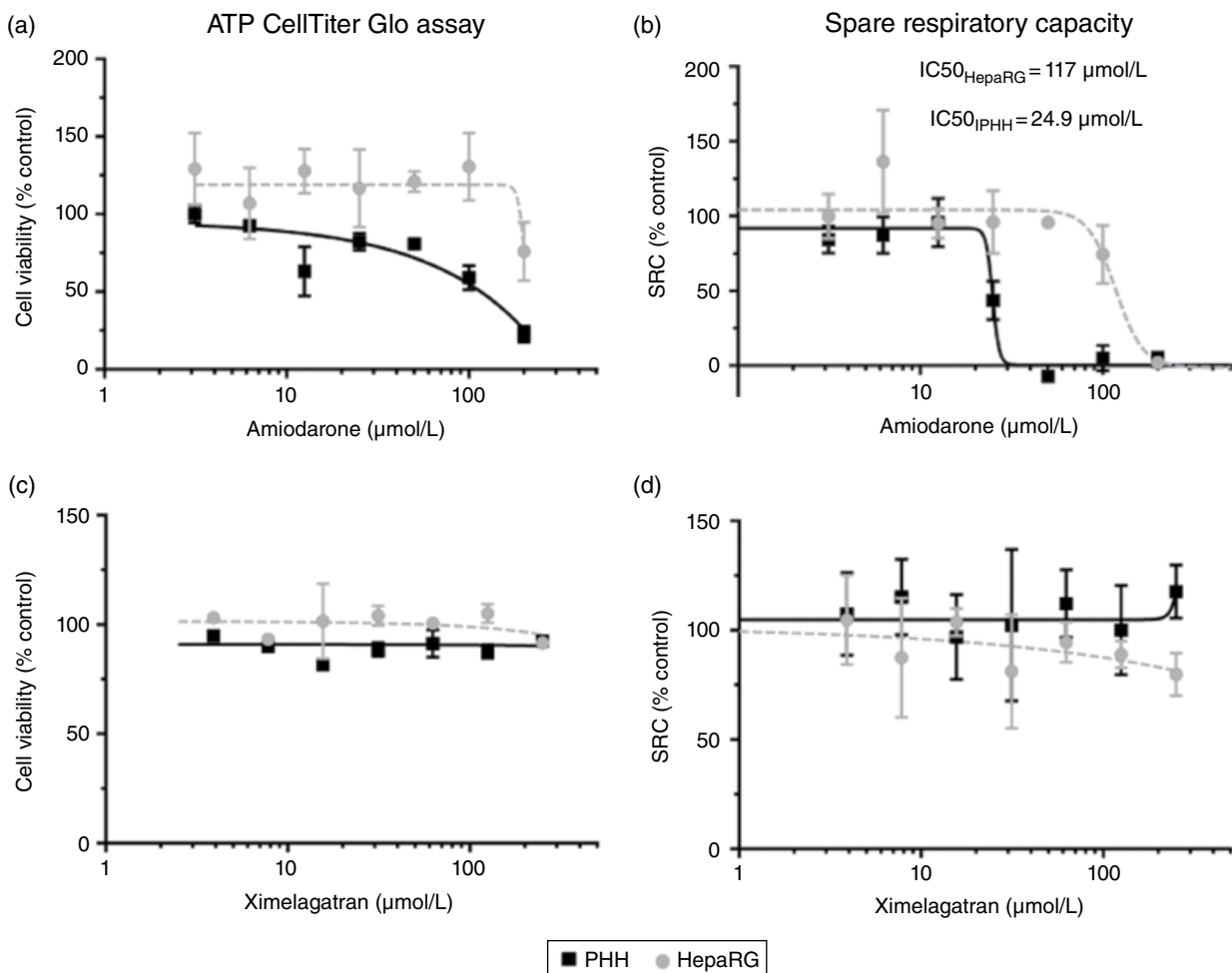


Figure 18.3 Toxicity assessment of 3D microtissues derived from HepaRG cells and primary human hepatocytes (PHH) using total ATP content (a, c) and SRC (b, d) for amiodarone (a, b, positive control) and ximelagatran (c, d, negative control). Microtissues have been exposed for 2 days to the compounds. Error bars indicate standard deviations of at least three replicates.

Table 18.1 Assessment of mitochondrial liabilities with 13 reference drugs comparing the effect on cellular viability ($IC_{50}ATP$ (μM)) and spare respiratory capacity ($IC_{50}SRC$ (μM)).

Compound class	Compound name	Known association with mitotoxicity	C_{max} (μM)	Results		Classification	
				$IC_{50}ATP$ (μM)	$IC_{50}SRC$ (μM)	DILI	Mitochondrial liability
Cardiovascular drugs	Amiodarone	Y	5.27	>100	~24.9	–	+
	Bosentan	N	7.43	>250	257.1	–	–
	Perhexiline maleate	Y	2.16	10.07	~7.6	+	+
	Ximelagatran	N	0.45	>250	>250	–	–
Antidiabetic drugs	Metformin HCl	N	7.74	>1000	774.2	–	–
	Troglitazone	Y	6.39	41.49	19.7	+	+
CNS drugs	Buspirone	N	0.01	>500	>500	–	–
	Entacapone	N	3.28	249.7	334.0	–	–
	Nefazodone	Y	4.26	~31.8	18.6	+	+
	Tolcapone	Y	47.58	57.01	45.3	+	+
Analgetic drugs	Acetaminophen	Y	165.38	6948	3801	+	+
Anti-inflammatory drugs	Diclofenac	Y	13.13	>250	177.0	–	+
Antiviral drugs	Fialuridine	Y	1	>100	>100	–	–

that the IC_{50} value for SRC was smaller than the IC_{50} value from the viability assay. This indicates that the mitotoxic drug amiodarone causes mitochondrial injury before cellular viability is affected. The non-mitotoxic drug ximelagatran was used as a negative control compound.

A panel of 13 drugs of different compound classes was tested using this method to assess their mitochondrial liabilities. Positive controls were chosen based on clinical data and known association with mitochondrial toxicity; as negative controls, drugs not previously associated with mitochondrial toxicity were used. For this study, 3D InSight™ Human Liver Microtissues were exposed to serially diluted compounds for 48 h, and the $IC_{50}SRC$ (Seahorse XF[®]96) and $IC_{50}ATP$ (cell viability, CellTiter-Glo) were determined. Results are summarized in Table 18.1.

These data indicate that the 3D human liver microtissues correctly detected the tested mitochondrial toxicants, apart from fialuridine, a drug that is known to affect metabolism only after longer exposures (Bell et al., 2016). Additionally, the classification method allowed categorization of drugs into different risk categories. A drug was only classified as positive (for DILI or mitochondrial liability) if the obtained IC_{50} value was lower than 30 times the treatment concentrations in human plasma (C_{max}).

This allows considering only clinically relevant concentrations of the tested drugs. For example, entacapone, a drug without clinical risk for DILI, resulted in a decrease of cellular viability ($IC_{50}ATP$: 249.7 μM), but this corresponds to 76-fold the C_{max} concentration in human plasma (3.28 μM) and is therefore not counted as DILI positive.

With this dataset, the sensitivity for detecting mitochondrial liability was 78% (7 out of 9 positive drugs correctly predicted) with a specificity of 100% (4 out of 4 negative drugs correctly predicted). Although employing a limited set of positive and negative control compounds, this dataset already indicates the suitability of the testing strategy to discriminate non-mitotoxic compounds from potential hazardous compounds with mitochondrial liabilities.

One of the wrongly classified drugs was fialuridine after a 2-day exposure. Therefore, the incubation time was prolonged to assess the subchronic effect. With increasing exposure time (7 and 14 days), there is a clear increase in the toxicological response that is more pronounced after 14 days ($IC_{50}ATP$: 8.5 μM ; $IC_{50}SRC$: 22.16 μM) as compared with 7 days ($IC_{50}ATP$: 98.6 μM ; $IC_{50}SRC$: 74.5 μM) (Figure 18.4). Both cellular viability and SRC decreased simultaneously over time, indicating that the SRC is not preferentially targeted by fialuridine.

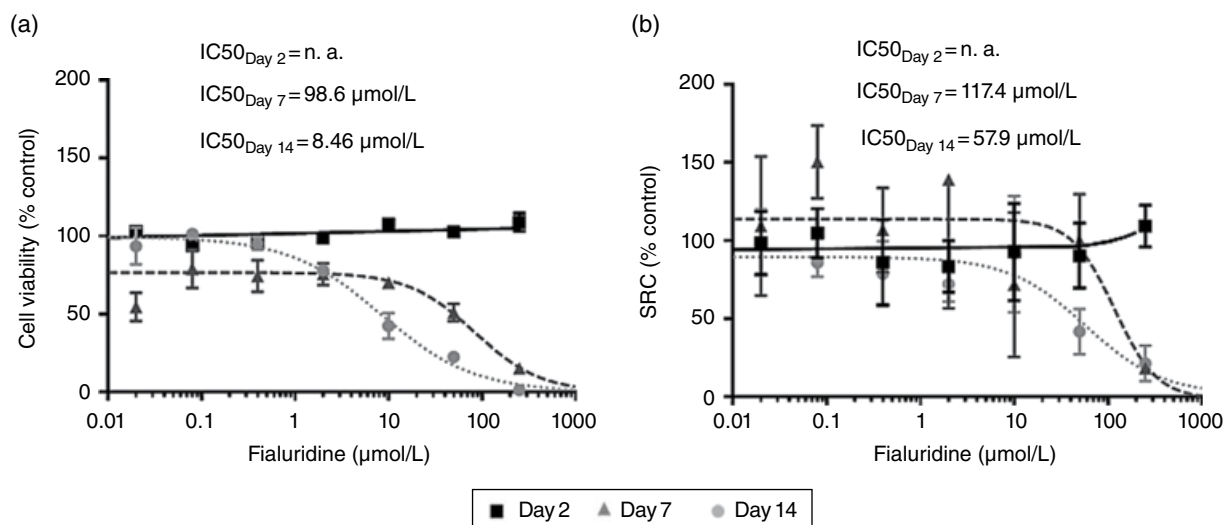


Figure 18.4 Long-term toxicity assessment (2, 7, 14 days) of 3D InSight™ Human Liver Microtissues using total ATP content (a) and SRC (b). Error bars indicate standard deviations of at least three replicates.

18.4 SRC-Based Detection of Mitochondrial Liabilities in Human Cardiac Microtissues

Most of the drugs used in cardiology impact mitochondrial activity. However, the degree of impairment depends on the type of drug. Toxicological responses induced by cardiac drugs include (i) impairment of respiratory chain functions resulting in reduced ATP production, (ii) increased production of reactive oxygen species with increased oxidation of proteins or lipids, (iii) reduction of the mitochondrial membrane potential, and (iv) apoptosis. Cardiac drugs that have exhibited mitochondrial toxicity include amiodarone, phenytoin, lidocaine, quinidine, isoproterenol, clopidogrel, acetylsalicylic acid, and molsidomine (Finsterer and Zarrouk-Mahjoub, 2015). Anthracyclines, such as doxorubicin and epirubicin, are potent cytotoxic drugs, but their clinical use is often limited by their cardiotoxic side effects. Other cytotoxic drugs that have reported cardiotoxicity include 5-fluorouracil, cisplatin, the taxoids paclitaxel and docetaxel, and newer drugs such as the monoclonal antibody trastuzumab (Cwikiel et al., 1995; Lieutaud et al., 1996; Gennari et al., 1999; Schimmel et al., 2004).

Here, amiodarone and docetaxel were tested whether mitochondrial liabilities could be observed using 3D InSight™ Human Cardiac Microtissues. A similar analytical setup was applied as with the human liver microtissues. In accordance with previous results, amiodarone

treatment led to a lower IC₅₀SRC (94.1 μM) than IC₅₀ATP (139 μM) (Figure 18.5), indicating mitochondrial impairment as primary toxicological pathway. Even more pronounced was the shift with docetaxel. Whereas there was no response observed in total ATP content after 2-day exposure time, there is a clear dose response observed looking at the SRC. Docetaxel has been associated with increased ROS production and hence mitochondrial impairment (Gorrini et al., 2013).

18.5 Conclusion

Mitochondrial impairment can currently be assessed with biochemical (isolated mitochondria) and cell-based assays (Glu-Gal and OCR measurements). The use of microtissues to evaluate the impact of drugs on mitochondrial function represents a next level of an *in vitro* tissue model. Especially for the liver, a metabolically relevant environment allows to test not only short-term effects but also effects of mitochondrial active metabolites and long-term effects. The 3D mitotoxicity assay described herein combines 3D microtissues with the Seahorse XF^e96 analyzer providing a novel platform for assessment of mitochondrial impairment. The high SRC of microtissues allows it to be used as an early marker for mitochondrial stress. As described in this chapter, the SRC has shown to be a sensitive and robust endpoint for mitochondrial impairment across three different tissue types. Moreover, setting the IC₅₀SRC in relation to the IC₅₀ATP, compound-mediated mitochondrial liabilities

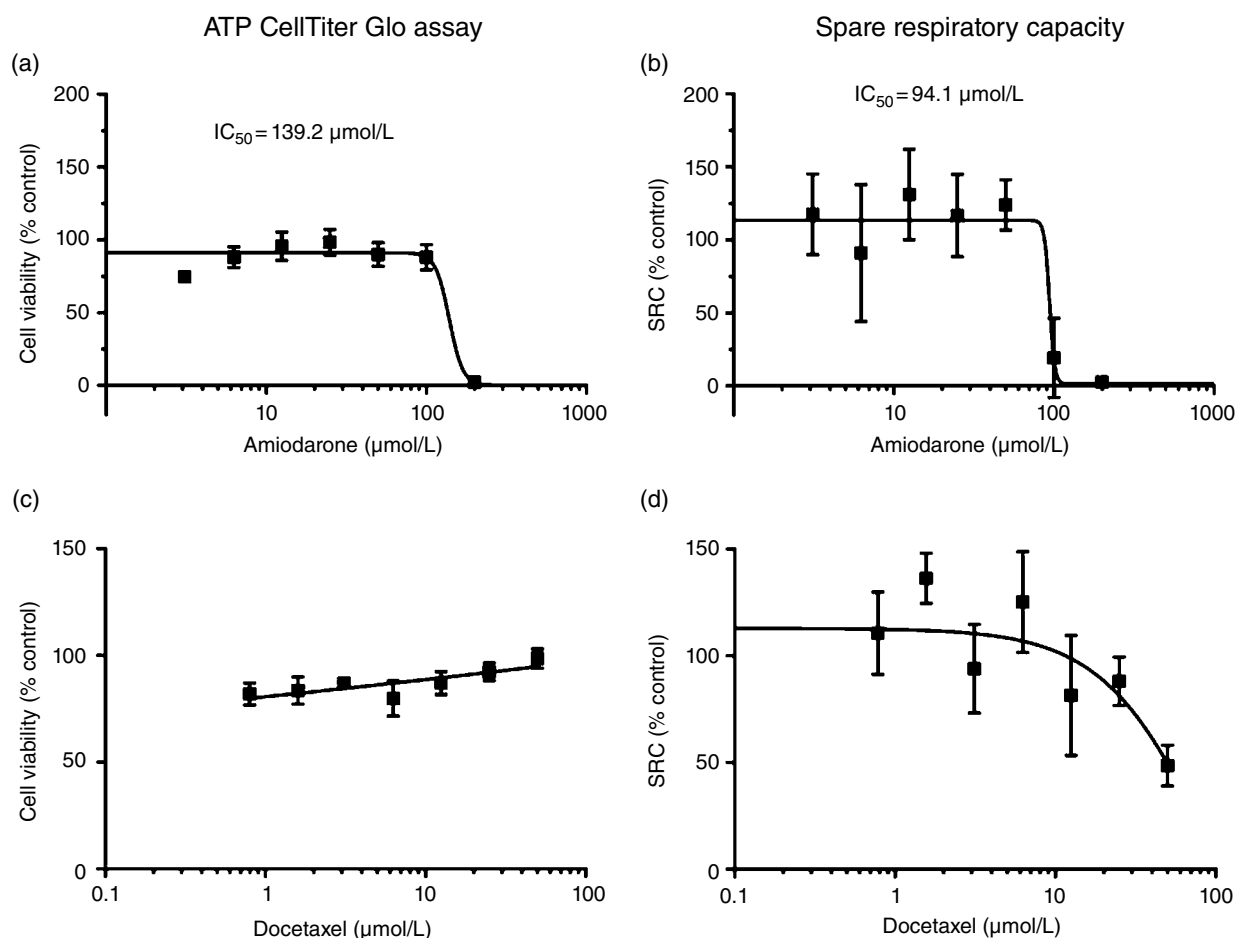


Figure 18.5 Toxicity assessment of 3D InSight™ Human Cardiac Microtissues using total ATP content (a, c) and SRC (b, d) for amiodarone (a, b) and docetaxel (c, d). Microtissues have been exposed for 2 days to the compounds. Error bars indicate standard deviations of at least three replicates.

can be discriminated from other toxicological pathways. Overall, the integration of 3D microtissues within the Seahorse technology generates a highly versatile platform for investigating mitochondrial response upon exposure of compounds and compound combinations.

Acknowledgments

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Toward Mitochondrial Medicine: Challenges in Rodent Modeling of Human Mitochondrial Dysfunction

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19.1 Introduction

Efficient mitochondrial function is essential to numerous cellular processes, and mitochondrial dysfunction is understood to be involved in pathologic mechanisms of a growing list of clinical disorders (Irwin et al., 2016; Moos and Dykens, 2015; Moos et al., 2016, 2017; Murphy et al., 2016; Parameshwaran et al., 2015; Steliou et al., 2015; Wallace, 2013). Lasting moderate mitochondrial dysfunction is a characteristic of numerous human disorders and can be readily produced in cell culture by various means (Matenia and Mandelkow, 2014; Park et al., 2016; Pickrell and Youle, 2015; Suliman and Piantadosi, 2016; Sun et al., 2016). Gaining insights into the mechanisms by which cellular events are translated into pathophysiological phenomena relies on laboratory animal modeling. Here, we highlight some mouse efforts at modeling mitochondrial dysfunction and the neurodegenerative and hepatotoxic hallmarks of human disease with mitochondrial involvement through multiple genetic and toxicological strategies.

19.2 Allotopic Expression of ATP6

All resident mitochondrial proteins, save the 13 encoded in the mitochondrial genome (mtDNA), are transcribed in the nucleus, translated in the cytoplasm, and transported to the various compartments of mitochondria. Numerous mtDNA mutations are known to result in human disease (Cannon et al., 2004; Dunn et al., 2012; Wallace, 2005, 2013). Nevertheless, one of the few strategies for recapitulating specific pathogenic human mtDNA mutations in cultured cells and the only way to create animal models to date has relied on a strategy of allotopic expression: recoding mtDNA-encoded genes, adding a mitochondrial transport signal, and expressing the transgene from the nucleus (Dunn and Pinkert, 2012, 2014, 2015; Gearing and Nagley, 1986; Guy et al., 2009; Manfredi et al., 2002; Zullo et al., 2005).

Subsequently, a line of transgenic mice was developed that allotopically expressed ATP6, a subunit of ATP synthase (Dunn and Pinkert, 2012, 2014, 2015). Like human patients with an mtDNA T8993G point mutation

(Holt et al., 1990), the transgenic protein expressed from the nuclear-coded ATP6 transgene contained a substituted arginine in place of a conserved leucine at residue 156. Severity of T8993G phenotypes ranges widely in human patients, partially in association with heteroplasmic load (Carelli et al., 2002; Mäkelä-Bengs et al., 1995; Uziel et al., 1997; White et al., 1999). Transgenic ATP6 mice expressed the mutated transgenic protein, which was transported into mitochondria, but the endogenous wild-type mtDNA-encoded ATP6 protein was also expressed. ATP6 is a very hydrophobic protein and has been postulated to cross mitochondrial membranes only with difficulty, but even a low level of normal protein would be expected to moderate deficits to some degree (Boominathan et al., 2016; Perales-Clemente et al., 2011). Therefore, transgenic ATP6 mice were hypothesized to display a phenotype resembling a mild form of human disease associated with T8993G mutation characterized by neuromuscular deficits (Mäkelä-Bengs et al., 1995). Rates of ATP production and oxygen consumption in heart, brain, and skeletal muscle tissues were not different between transgenic mice harboring mutant ATP6 and non-transgenic controls. Manganese superoxide dismutase (MnSOD, SOD2) protein levels and serum lactate concentrations also showed no difference. A battery of neuromuscular and motor tests was administered to experimental mice. Transgenic mutant mice exhibited lower measures of neuromuscular strength. Assessments of motor coordination yielded mixed results in which transgenic mutant mice displayed superior performance in some measures, but inferior in others.

19.3 Xenomitochondrial Mice

Respiratory complexes are very large multi-subunit enzymes made up of proteins encoded in both nuclear and mitochondrial genomes. The need for precise protein–protein interactions between subunits leads to coevolutionary pressures in both genomic compartments whenever residues involved in these interactions are changed via mutation. Therefore, as two species diverge through time, their mitochondrial and nuclear genomes would be expected to become increasingly less compatible with those of the other species.

This hypothesis was tested *in vitro* as multiple lines of xenomitochondrial cellular hybrids (cybrids) were produced containing nuclear DNA sequences from *Mus musculus* and mitochondrial genomes from a range of murid species (McKenzie et al., 2003; Pinkert and Trounce, 2002; Pinkert et al., 2014). Lactate production, as well as respiratory deficits, correlated with increasing evolutionary distance. Attempts at recapitulating these results *in vivo* followed (McKenzie et al., 2004; Pinkert

and Trounce, 2007; Trounce et al., 2004). Fusion of enucleated fibroblasts from related species (*Mus spretus*, *Mus terricolor*, *Mus caroli*, and *Mus pahari*) with Rho-0 embryonic stem (ES) cells from *M. musculus domesticus* allowed creation of cybrid ES cells that were then injected into mouse blastocysts and transferred to pseudopregnant female mice for gestation.

These efforts, following an exceptionally large number of blastocyst injections using several cybrid ES cell clones, led to production of over 60 founder chimeras, with five fertile germline females identified (McKenzie et al., 2004; Trounce et al., 2004, unpublished data). One of these lines of mice, which harbored mitochondria derived from *M. terricolor*, was expanded and characterized (Cannon et al., 2011; McKenzie et al., 2004). Respiratory oxygen consumption values were within the same range as those obtained from control mice. Behavioral and neuromuscular tests mostly displayed no differences between groups. One exception to this was rotarod analysis of neuromuscular coordination and endurance. Young xenomitochondrial mice (3–5 months of age) performed better than control mice, though this distinction was not seen in aged mice (9–14 months). Microarray analysis of gene expression in whole brain tissue samples of 3-week-old mice uncovered seven genes in the immediate early response gene family that were downregulated by at least twofold in comparison with control mice (Cannon et al., 2011).

19.4 Galactose Treatment

Galactose is a hexose sugar that is widely utilized in the production of glycolipids and glycoproteins and can be epimerized to glucose to be used as an energy source. However, it can be toxic as is seen in human patients with congenital mutations in genes encoding enzymes involved in galactose metabolism and in administration in high doses to rats (Lai et al., 2009). Cognitive decline, oxidative stress, and production of advanced glycation end products resulted from galactose treatment in rats (Chen et al., 2006; Lei et al., 2008; Zhang et al., 2005).

Accordingly, an effort was envisioned to create a mouse model of human aging characterized by mitochondrial dysfunction with associated cognitive and motor deficits (Parameshwaran et al., 2010). Eight-week-old mice received daily injections of D-galactose intraperitoneally over a period of 6 weeks at a dose of 100 mg/kg body weight. Galactose-treated and vehicle control mice were administered three experimental antioxidant compounds (L-carnitine, alpha-lipoic acid, and PMX-500FI) or no additional treatment. Locomotor coordination, anxiety, spatial memory, and serum lactate levels were measured. In this study, galactose-treated mice were not different from control animals in any of

the measures of cognitive ability and motor function. Mice receiving galactose also did not display increased serum lactate concentrations.

One potential factor in the lack of galactose-mediated toxicity/mitochondrial dysfunction lies in an important genetic difference between mice and humans. *Aplysia ras homology member 1* (ARHI, also known as *GTP-binding protein Di-Ras3* (DIRAS3)), a tumor suppressor gene that has been evolutionarily lost in mice, is hypothesized to play a central role in galactose toxicity in human patients with inborn errors in galactose metabolism (Lai et al., 2008). In human cells lacking galactose-1-phosphate uridylyltransferase (GALT), ARHI is upregulated when cultured in the presence of galactose. Overexpression of human ARHI in transgenic mice recapitulates many of the clinical signs of genetic galactose metabolism errors (Xu et al., 2000), and homozygous GALT knockout mice are fertile and symptom-free (Ning et al., 2000). The lack of any discernable phenotype described previously in mice injected with galactose makes sense in light of their genetic lack of ARHI, a putative mediator of galactose toxicity.

19.5 Rotenone Treatment

Rotenone, a natural isoflavonoid produced by plants (Lee et al., 2014), is commonly used as an insecticide and piscicide (Oberg, 1964). Its molecular mechanism of toxicity is as a specific inhibitor of NADH dehydrogenase (Chance et al., 1963; Giordano et al., 2012; Grivennikova et al., 1997; Lee et al., 2014; Palmer et al., 1968). Long-term occupational exposure to rotenone in pesticide applicators is associated with increased incidence of Parkinson's disease (Tanner et al., 2011). Rotenone treatment in laboratory animals is commonly used to model various aspects of Parkinson's disease (Arif and Khan, 2010; Friedrich, 1999; Lapointe et al., 2004; Panov et al., 2005; Parameshwaran et al., 2012, 2015).

With this in mind, rotenone administration was used to produce neurodegeneration in mice as a vehicle for testing the possible ameliorative effects of a synthetic carnitine–lipoic ester in mice (Parameshwaran et al., 2012, 2015). In the first trial (Parameshwaran et al., 2012), 2-month-old mice were given daily doses of 30 mg/kg body weight by oral gavage for 28 days. Behaviorally, mice were assessed for locomotor coordination and neuromuscular strength/endurance. Molecular analyses included reactive oxygen species (ROS) measurements (dichlorofluorescein diacetate (DCFDA) fluorescence) in lysates containing forebrain, midbrain, and cerebellum tissues as well as measurement of phosphorylated and total stress-activated protein kinase/c-Jun amino-terminal kinase (SAPK/JNK), mitogen-activated protein kinase 9 (MAPK9) as an assessment of generalized cellular stress (Nishina et al., 2004).

Rotarod analyses (locomotor coordination) displayed no differences between control and rotenone-treated groups. Grip strength/endurance measurements showed marked decreases in mice that had been treated with rotenone. DCFDA fluorescence was elevated, indicating increases in ROS production in forebrain and midbrain, but not in cerebellum tissues of rotenone-treated mice. Similarly, cellular stress (elevated pSAPK/JNK protein levels) was increased in the forebrain and midbrain, but not in the cerebellum. Here, rotenone-induced mitochondrial dysfunction was detected via behavioral and cellular analyses.

Subsequently, additional experiments were performed in which 3-month-old mice were treated daily for 7 days with a 400 mg/kg body weight oral dose of rotenone (Parameshwaran et al., 2015). Phenotypic measurements included electrophysiological recordings of long-term potentiation (LTP), potentiation during theta burst stimulation (TBS), and measurements of presynaptic neurotransmitter release following TBS. Rotenone-induced cell death in the hippocampus was assessed by measuring the mitochondrial fraction of proapoptotic effector protein Bcl-2-like protein 4 (BAX) as well as the phosphorylated fraction of proapoptotic protein Bcl-2-associated death promoter (BAD). Phosphorylation state of extracellular signal-related kinases 1/2 (ERK1/2) was also measured.

LTP recordings were reduced in hippocampal slices of mice treated with rotenone; no difference between groups was seen in potentiation during TBS, and rotenone treatment resulted in a lower level of presynaptic neurotransmitter release compared with controls. Proapoptotic signaling was enhanced by rotenone. Mitochondrial translocation of BAX from cytoplasmic stores was increased in rotenone-treated mice, while phosphorylation of the proapoptotic BH3-only protein BAD declined. Taken together, data from these two B-cell lymphoma 2 (Bcl-2) family proteins illustrate apoptotic neurodegeneration in hippocampi of rotenone-treated mice. The percentage of phosphorylated ERK, which becomes activated by signals that promote cell survival and proliferation (Mebratu and Tesfagzi, 2009; Roskoski, 2012), was decreased in the hippocampus tissue of rotenone-treated mice.

19.6 Hepatotoxicity with Mitochondrial Dysfunction

Troglitazone, an antidiabetic PPAR γ agonist, was found to display idiosyncratic drug-induced liver injury (idiosyncratic DILI or IDILI) in human patients, leading to the drug's market withdrawal (Chojkier, 2005). Mitochondrial dysfunction was identified in conjunction

with troglitazone hepatotoxicity. Isolated rat liver mitochondria exhibited impaired oxidative phosphorylation in the presence of troglitazone (Nadanaciva et al., 2007). In cultured human hepatoma cells, troglitazone induced numerous features of mitochondrial dysfunction including increased production of ROS and decreases in mtDNA content and mitochondrial membrane potential (Dan et al., 2015). Yet despite clear mitochondrial involvement in troglitazone-induced toxicity *in vitro*, liver damage is not found in troglitazone treatment of healthy mice (Ong et al., 2007). Indeed, a lesson learned from the failure of troglitazone is the lack of correlation between mitochondrial toxicity phenotypes in preclinical animal models and human patients. This growing realization that mitochondrial toxicology profiles in mice do not follow human clinical experience in a number of experimental compounds is a motivating factor in using transgenic mouse models designed to inhibit some aspect of mitochondrial function.

SOD2-deficient mice constitute one such line of transgenic rodents used to model hepatic toxicity. Mice homozygous for a targeted inactivation of SOD2 die within the first three postnatal weeks with severe cardiac myopathy and neurological degeneration (Lebovitz et al., 1996). Heterozygous mice display no abnormal phenotype (Lee et al., 2008; Ong et al., 2007). However, when heterozygous SOD2^{+/-} mice are treated with troglitazone (Fujimoto et al., 2009; Lee et al., 2008; Ong et al., 2007), flutamide (Kashimshetty et al., 2009), acetaminophen (Fujimoto et al., 2009), or trovafloxacin (Hsiao et al., 2010), hepatic injury occurs to varying degrees (Zhang et al., 2011).

Juvenile visceral steatosis (JVS) mice harbor a mutation in the organic cation/carnitine transporter 2 (OCTN2), leading to systemic carnitine deficiency. Homozygous mice display fatty changes in numerous organ systems and with carnitine supplementation can live up to a year (Kaido et al., 1997). Heterozygous mice display lower than normal levels of carnitine uptake but few pathological signs (Lahjouji et al., 2002). When heterozygous jvs^{+/-} mice were treated with valproic acid (Knapp et al., 2008) or with dronedarone (Felser et al., 2014), minor hepatocellular steatosis and activation of capase-3 in a subset of hepatocytes was observed in both models.

19.7 Hyperactivity of the Mitochondrial Stress Response in Mice

In general, the aforementioned results indicate that producing enduring mitochondrial impairment in animal models is more difficult than originally envisioned from initial observations in cell culture. From our work in allotopically expressing ATP6, the lack of pathologic phenotype might be partially due to inefficiency in mitochon-

drial transport of the cytosolic protein. Another potential factor could be the presence of endogenous mtATP6. On the other hand, if hydrophobic nuclear-expressed ATP6 is coating the surface of mitochondria, they might be expected to interfere with normal mitochondrial protein transport and thus cause some measure of mitochondrial dysfunction. Nevertheless, these transgenic mice did not express biochemical or neuromuscular phenotypes consistent with defects in mitochondrial function.

Xenomitochondrial experimentation produced results in mice that conflicted with those observed in cell culture. While transmitochondrial cybrid cell lines produced high levels of lactate and displayed diminished respiratory capacity, xenomitochondrial mice did not. In those models in which toxic agents were administered, similar phenomena materialized. Galactose treatment yielded no discernable pathology. As noted previously, lack of the ARHI gene in mice but its presence in humans might be involved in the different levels of toxicity seen in hypergalactosemia (Lai et al., 2009; Xu et al., 2000).

Unlike the three models discussed previously, rotenone administration did produce reliable neurodegenerative effects. The phenotypes produced in these studies were useful for their primary intended purpose of testing the potential protective effects of experimental compounds. However, the acute mitochondrial dysfunction produced in these efforts does not mimic the long-lasting chronic mitochondrial defects seen in human aging and other neurodegenerative disorders. Arriving at the rotenone dosage required to produce the desired phenotypes necessitated substantial titration. Generally, the window between high mortality and lack of dysfunction was narrow (unpublished data) with dosages given close to the reported oral LD₅₀ in mice of 350 mg/kg (Ellenhorn and Barceloux, 1988).

The compounds studied in models of DILI cited here induced little to no phenotype in normal mice but produced pathological effects of varying strength in mice with mutations affecting mitochondrial function. Similarly, two genetic mouse models with homozygous mutations in genes involved in mitochondrial homeostasis (ROS detoxification and carnitine transport) displayed severe pathologies, but heterozygotes were healthy and only exhibited a pronounced pathological phenotype in the presence of a secondary chemical insult.

Taken in the aggregate, these results suggest that in the face of slight to moderate stress, protective compensatory mechanisms minimize damage and prevent disease states previously hypothesized to result from mitochondrial dysfunction. Cellular mitochondrial turnover is largely modulated by biogenesis (Scott et al., 2014; Zhang et al., 2016), mitochondrial dynamics (Golpich et al., 2017; Samant et al., 2014; Zhang et al., 2016), mitophagy (a natural subform of autophagy (Boya et al., 2016; Towers and Thorburn, 2016) that cells evolved to remove their damaged mitochondria (Matenia and Mandelkow, 2014;

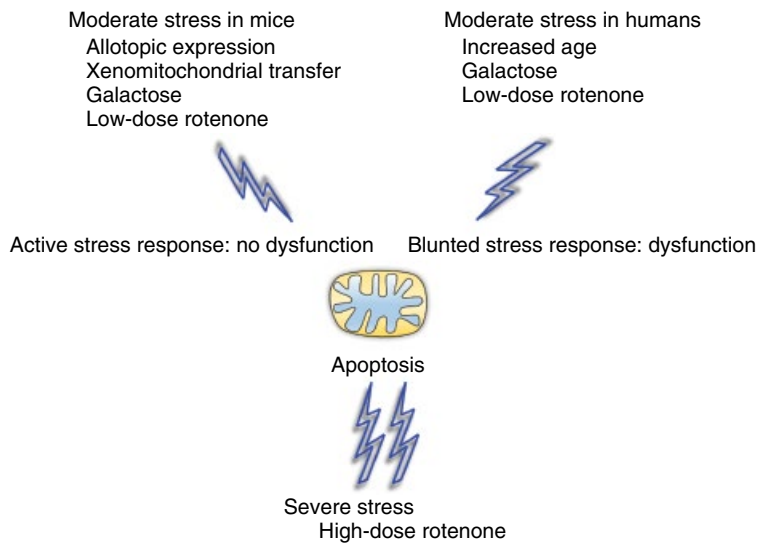


Figure 19.1 Mitochondrial stress response in mice and humans. Based on modeling efforts and a host of identified stressors, responsiveness in mice is more active. This in turn skews data interpretation regarding human mitochondrial dysfunction. Comparative studies characterizing regulatory mechanisms leading to human mitochondrial dysfunction and disease may lend support to our understanding of various pathways but are limited in their overall utility in developing solutions to human disease progression.

Pickrell and Youle, 2015; Wallace, 2013)), and a variety of stress responses (Golpich et al., 2017; Murphy et al., 2016; Zhang et al., 2016). Once the dysfunctional mitochondria are removed from the cell, the remaining healthy mitochondria undergo fission (divide) to replenish the numbers lost (Golpich et al., 2017; Pickrell and Youle, 2015; Samant et al., 2014; Scott et al., 2014; Zhang et al., 2016). Cellular nutrient levels and redox stress also contribute to the overall function of mitochondrial quality control and integrity (Diot et al., 2016; Gleyzer and Scarpulla, 2016; Jovaisaite et al., 2014; Murphy et al., 2016; Toyama et al., 2016; Zhang et al., 2016). Numerous signaling pathways interact to coordinate homeostasis. Varied evidence in our mouse models and those created by others (Fan et al., 2008; Irwin et al., 2013; Safdar et al., 2016; Trifunovic et al., 2004) are indicative of aspects of mitochondrial quality control signaling pathway activation.

The varied phenomena highlighted in this chapter describe a highly active mitochondrial quality control state in mice. In these varied models of mitochondrial dysfunction, mild or moderate pathological phenotypes are rare, but severe/lethal neurotoxic or hepatotoxic events occur as the degree of the insult increases. Thus as stress crosses a threshold and mitophagy reaches its limit, large-scale apoptosis ensues. This outcome is rem-

iniscent of the phenotypic threshold effect seen in diseases caused by mtDNA mutations (Rossignol et al., 2003), likely with the same underlying mechanisms.

19.8 Summary

Reproducing in animal models the types of mitochondrial dysfunction seen in human disease has proven to be a complex problem that lies at the junction of multiple homeostatic and signaling pathways. The way forward is in determining why the mitochondrial stress response seems more active in rodents than in humans (Figure 19.1), thereby making modeling of human mitochondrial dysfunction so difficult. Research efforts into identifying comparative species differences in the regulation of these pathways are required. Once these differences are discovered and optimal combinations of genetic and/or pharmacologic targets identified, creating “humanized” mice (or other species) using gene editing protocols (Cannon et al., 2015; Dunn and Pinkert, 2014; Pinkert et al., 2014; Seruggia and Montoliu, 2014; Singh et al., 2015) together with targeted delivery of appropriate chemical agents will allow creation of the animal models so desperately needed to more closely recapitulate pathways of human disease.

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20

Measurement of Oxygen Metabolism *In Vivo**M. P. J. van Diemen¹, R. Ubbink^{2,3}, F. M. Munker³, E. G. Mik², and G. J. Groeneveld¹¹ Centre for Human Drug Research, Leiden, The Netherlands² Department of Anesthesiology, Erasmus MC, Rotterdam, The Netherlands³ Photonics Healthcare B.V., Utrecht, The Netherlands**CHAPTER MENU**

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20.1 Introduction: The Importance of Measuring Mitochondrial Function in Drug Trials

Ever since the discovery of the causal involvement of defective mitochondria in Leber's hereditary optic neuropathy, the roles of mitochondria in cellular physiology have been expanded from merely being an energy producer to a potential driver of apoptosis (Wallace et al. 1988). Indeed, evidence is increasing that mitochondrial dysfunction plays important roles in many age-related disorders, in some measure because mitochondrial capacity declines with age.

Many different drug classes have toxic effects on mitochondria. Some of these medications are widely utilized so that it is of special interest to elucidate their mechanism of toxicity in various tissues, including muscle (Hargreaves et al. 2016). One of such drug class is the statins, with simvastatin being most commonly

prescribed (Diebold et al. 1994; Dai et al. 2010; Bouitbir et al. 2011). Statins induce mitochondrial dysfunction by at least three mechanisms. First, statins inhibit the mevalonate pathway, thereby repressing the biosynthesis of cholesterol and thus lowering plasma levels. However, the biosynthesis of coenzyme Q10 (Q10), an essential electron carrier in the electron transport system, is also repressed, which can correspondingly repress ATP production. A decrease in plasma and muscle Q10 levels after statin administration has been shown (Bleske et al. 2001; Lamperti et al. 2005; Paiva et al. 2005; Deichmann et al. 2010), although discussion persists as to whether such decreases erode oxidative phosphorylation (OXPHOS) (Smith et al. 1991). Second, simvastatin significantly inhibits respiratory complexes I, II, III, IV, and V using immunocaptured complexes (Nadanaciva et al. 2007; Schirris et al. 2015). Finally, among the statins evaluated, simvastatin most potently uncoupled respiration from phosphorylation, dissipating membrane potential and so forestalling OXPHOS.

Another class of medications that cause mitochondrial toxicity is the biguanides: phenformin, buformin, and metformin. The first two were withdrawn from the

*Drug-Induced Mitochondrial Toxicity—Continuing Insight Yields Progress toward the Clinic, Will et al., in press.

market because of fatal lactic acidosis, while metformin remains on the market and is associated with lactic acidosis as a persistent adverse event (Dykens et al. 2008). All three molecules inhibit complex I of the electron transport chain with IC₅₀s in accord with their systemic toxicity (El-Mir et al. 2000; Ota et al. 2009; Andrzejewski et al. 2014). Interestingly, metformin has been reported to exert its antidiabetic effect through this inhibition, which represses diminution of hepatic gluconeogenesis and enhancement of glucose utilization in peripheral tissue as glycolysis accelerates to compensate for reduced OXPHOS (Owen et al. 2000).

It is apparent that mitochondrial assessments are needed for development of novel drugs designed to enhance and/or maintain mitochondrial function. Given the growing number of drugs with deleterious effects on mitochondrial function, such assessments should be done early in the drug development program, while there is still chemical diversity of the lead screen hits. In addition to avoiding xenobiotic toxicity, the involvement of mitochondrial dysfunction in a wide range of diseases underscores the need for clinical mitochondrial assessments for diagnosis, progression, and prognosis. A number of *in vivo* techniques can assess mitochondria in their physiological environment, and we have been focusing on the development of noninvasive alternatives to more invasive procedures, such as a muscle biopsy.

20.2 Methods: *In Vivo* Methods to Measure Drug Effects on Mitochondrial Function in a Clinical Setting

The ideal method of measuring mitochondrial function would be inexpensive and noninvasive, yet able to show multiple parameters of mitochondrial function, such as both oxygen consumption and phosphorous metabolism. *In vivo* assessments reflect mitochondrial function *in situ*, which, because of the complexity of the interaction of mitochondria and intra- and extracellular signals, is arguably most accurate.

Several methods are currently available for both research and clinical settings. The gold standard of *in vivo* mitochondrial assessments relies on monitoring the stable isotope of ³¹P-phosphorus using magnetic resonance spectroscopy (³¹P-MRS) and has been used in the research lab and clinic. ³¹P-MRS is not a new technique, and its ability to measure phosphorus metabolism has been used clinically since the early 1980s to study mitochondrial diseases and myopathies (Gadian et al. 1981; Edwards et al. 1982; Radda et al. 1984). One of the parameters, best reflecting mitochondrial function, is the

phosphocreatine (PCr) recovery time (τ -PCr) (Bendahan et al. 2006; Lanza et al. 2011). When ATP is consumed, for instance, by muscle tissue during exercise, PCr serves as a “battery,” maintaining a constant level of ATP via PCr kinase. By focusing on the nuclear spins of phosphorus and hydrogen, the three phosphates of ATP and PCr are readily resolved. A typical experimental design entails some sort of stress such as restricting blood flow with a blood pressure cuff, or physical exertion, to deplete PCr and ATP and then monitor the rate of PCr recovery (τ -PCr), which has been well validated as directly reflecting mitochondrial status, a contention also corroborated by *in vitro* respirometry (Bendahan et al. 2006; Layec et al. 2009; Lanza et al. 2011). Although use of surface coils renders this technique noninvasive, dynamic ³¹P-MRS measurements are expensive and require specialized instrumentation and expertise, so it is not yet used for routine measurements in the clinic.

Mitochondrial function can be determined using several less burdensome and cheaper alternatives. For example, systemic mitochondrial dysfunction can be assessed *ex vivo* by monitoring the mitochondrial membrane potential (MMP) in peripheral blood mononuclear cells (PBMCs). The MMP has long served as a direct reflection of the integrity of the mitochondrial membrane (Perelman et al. 2012) and a direct index of mitochondrial health. It can be disrupted by blockade of the mitochondrial respiratory chain or interference with mitochondria-related death pathways (Chen 1988; Isenberg and Klaunig 2000; Kim and Blanco 2007; You and Park 2010; Choi and Lee 2011). Uncoupling the proton gradient from ATP production, via opening the mitochondrial permeability “transition pore” allows the protons to flow down the energy gradient bypassing ATP synthase (Moreno-Sanchez et al. 1999; Ding et al. 2005). Fluorescent dyes are typically used to examine the MMP. For example, JC-1 (Perelman et al. 2012) is attracted by the negative charge in the inner membrane space. JC-1 fluoresces green at low concentrations, but at high concentrations aggregates form that fluoresce red, thereby providing an index of MMP (Perelman et al. 2012). Measuring MMP as an *ex vivo* assessment can be performed in various types of intact cells or isolated mitochondria and is therefore a usable technique for various diseases and study designs. Perhaps most interestingly is measuring the MMP in PBMCs to assess systemic mitochondrial function. Several studies indeed show a decrease in MMP in circulating PBMCs of patients with neurodegenerative diseases, such as Alzheimer’s and Huntington’s disease (Panov et al. 2002; Lunnon et al. 2012).

Oxygen consumption rate can be determined in skeletal muscle tissue, using near-infrared spectroscopy (NIRS), and in skin, using a new technique called protoporphyrin IX–triplet state lifetime technique (PpIX-TSLT).

This novel technique makes use of the oxygen-dependent delayed fluorescence of protoporphyrin IX, a precursor protein in the heme synthesis, which occurs in the mitochondria.

20.3 Measuring Mitochondrial Oxygen Consumption with the Protoporphyrin IX–Triplet State Lifetime Technique

A novel technique is now available that enables real-time mitochondrial oxygen tension in mmHg *in vivo*. Mitochondrial oxygen tension (mitoPO₂) is measured by means of PpIX-TSLT, and it is used to assess mitochondrial function in human skin cells *in vivo* (Harms et al. 2016). This is possible because of an oxygen-dependent time of afterglow (triplet state lifetime) of protoporphyrin IX (PpIX), the final precursor of heme in the biosynthetic pathway located in the mitochondria (Poulson 1976). The human skin has been used to perform the measurements, because of easy access and non-invasive nature, but the technique is not limited to measurements in the skin. All PpIX-synthesizing cells are potential measurement locations, for example, the kidney and liver (Mik et al. 2008; Harms et al. 2012).

Overproduction of PpIX can be induced in active mitochondria by exogenously providing aminolevulinic acid (ALA), the precursor to PpIX. This bypasses the negative feedback controls in the heme biosynthetic pathway, so that PpIX builds up inside the mitochondria overwhelming the slower reactions, converting PpIX to heme (Malik et al. 1995; Fukuda et al. 2005). This is necessary because, under normal (non-sensitized) conditions, PpIX is present in human skin at very low concentrations and is not detected with the PpIX-TSLT. As a small molecule, ALA penetrates the stratum corneum, and topical administration of ALA not only increases PpIX to detectable levels but also ensures mitochondrial origin of the triplet state fluorescence signal (Kennedy and Pottier 1992; Mik et al. 2008; Mik et al. 2009). After topical administration of ALA on healthy skin, typically for 4 h or more, PpIX is synthesized. In healthy skin this limits the measurement location and signal origin of the skin sensor to the epidermis with a thickness of about 0.1 mm (Sandby-Møller et al. 2003). The recommended measurement location is the skin of the sternum. This provides a central measurement location less influenced by temperature changes, movement, and peripheral vasoconstriction (Campbell 2008).

The optical properties of PpIX make possible its use as a mitochondrial oxygen probe. After excitation with a short pulsed laser, both immediate and triplet state

delayed fluorescence can be detected. The delayed fluorescence lifetime is inversely proportional to the amount of oxygen according to the Stern–Volmer equation (Mik et al. 2002; Mik 2013). Local pressure is applied by gently pressing down the probe, which stops local blood flow, thereby allowing determination of cellular oxygen utilization. In this way, the oxygen disappearance rate (ODR) is determined, as is the recovery rate upon reperfusion (Harms et al. 2013). This may become an easily accessible technique to determine mitochondrial function in real time at the bed side. It has been evaluated in this capacity using healthy volunteers and to detect changes in mitochondrial function in rats and volunteers (Harms et al. 2011, 2012, 2013, 2015, 2016).

20.4 Features of a Novel COMET Measurement System: The First Bedside Monitor of Cellular Oxygen Metabolism

After introduction of the PpIX-TSLT as a new method to measure mitochondrial oxygen tension *in vivo*, the development of a clinical monitor was started. The prototype used in the volunteer trial has since been further developed by Photonics Healthcare B.V. (Utrecht, The Netherlands). This resulted in the COMET measurement system, an acronym for Cellular Oxygen METabolism. The COMET is a compact medical device approved for clinical use in Europe in 2016. It consists of a monitor and a skin sensor and uses a pulsed laser to illuminate the measurement site. The delayed fluorescent signal from PpIX accumulated in active mitochondria is projected on a gated red-sensitive photomultiplier tube. Lifetimes of the raw data are calculated on an embedded control board. The thumb-sized skin sensor holds optical fibers for excitation and detection. Sensor temperature, used as an approximation of skin temperature, is measured with an electrical resistive sensor.

20.5 Clinical Trial: Effect of Simvastatin on Mitochondrial Function *In Vivo* in Healthy Volunteers

The *in vivo* techniques discussed previously measure mitochondrial function in different ways and so are complementary. Combining several techniques in a single study could therefore give a more complete and corroborating understanding of how and to what extent a drug might undermine mitochondrial function. To test this notion, we performed a clinical study at the Centre for

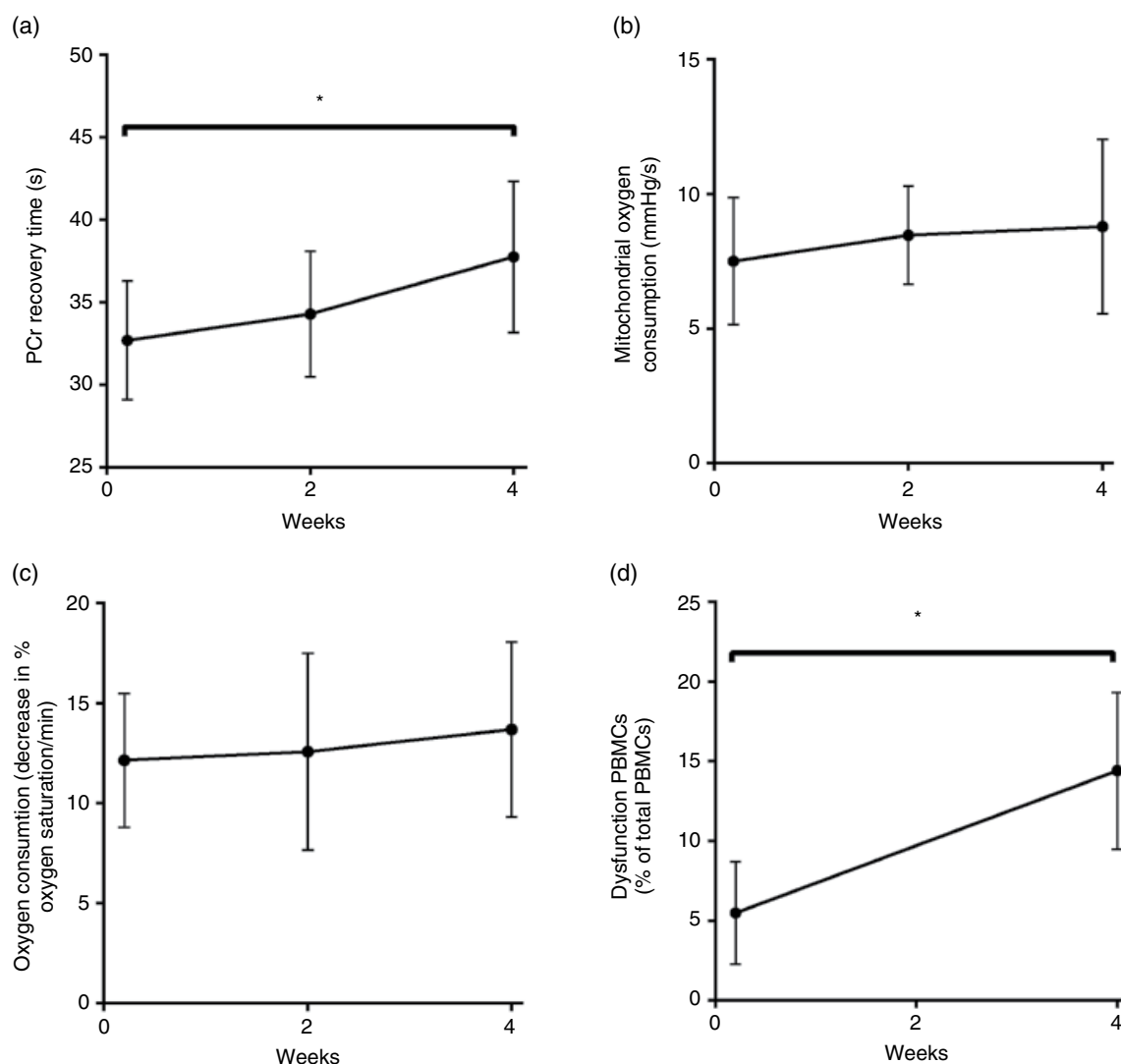


Figure 20.1 Different methods to measure mitochondrial function *in vivo* or *ex vivo*. (a) phosphorous MRS, (b) protoporphyrin IX–triplet state lifetime technique, (c) near-infrared spectroscopy, and (d) mitochondrial membrane potential. * $p < 0.05$.

Human Drug Research (CHDR, Leiden, The Netherlands), in which mitochondrial dysfunction was induced by administering a typical dose of simvastatin (40 mg once daily) for 4 weeks to healthy subjects. Mitochondrial function was assessed both *in vivo* and *ex vivo* using ^{31}P -MRS, NIRS, PpIX-TSLT, and MMP in PBMCs. After 4 weeks of simvastatin treatment, ^{31}P -MRS revealed that τ -PCr was significantly prolonged by 15.2% compared with baseline; this is pathognomonic for mitochondrial impairment (from 31.36 to 36.12 s, 95% CI, 2.0–30.0; $p < 0.05$) (Figure 20.1a). Over the same period, measurements by PpIX-TSLT revealed that oxygen consumption within mitochondria (mitoVO₂) increased 13% (95% CI, –0.014 to 2.716; $p = 0.052$) (Figure 20.1b). For all subjects, the mVO₂ showed a trend toward increasing (95% CI,

–0.25 to 3.418; $p = 0.089$) (Figure 20.1c), although the differences were not significant. Importantly, the percentage of dysfunctional PBMCs significantly increased from 5.20% at baseline to 14.43% after 4 weeks of simvastatin administration (95% CI, 2.416–16.056; $p = 0.016$) (see Figure 20.1d). The different techniques showed relative similar change, with the exception of the MMP in PBMCs, which showed a more drastic increase of dysfunction (see Figure 20.2).

Taken together, the data indicate that simvastatin induces detectable mitochondrial dysfunction in as little as 4 weeks. This is in accord with the ^{31}P -MRS results of Wu et al. (2011) who reported that PCr exercise recovery kinetic times doubled (from 28.1 to 55.4 s) after a 4-week regimen of statin therapy. They used a custom-built calf

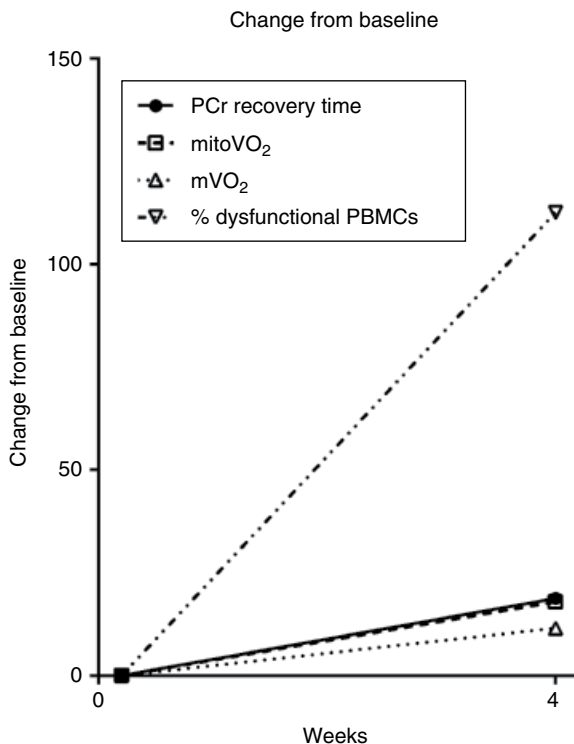


Figure 20.2 Change from baseline of different methods.

flexion pedal ergometer that could fit into the magnet and obtained spectra before, during, and after exercise (Wu et al. 2011).

Both using NIRS and PpIX-TSLT techniques, increased *in vivo* oxygen consumption was revealed, although, in the case of muscle oxygen consumption using NIRS, this effect was only apparent as a trend. However, the mitochondrial oxygen consumption, measured by PpIX-TSLT, shows to be close to significantly increased. Furthermore, the change in oxygen consumption follows the prolongation in τ -PCr, measured by ^{31}P -MRS.

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The effects of simvastatin on mitochondrial function and mitochondrial respiration can be distinguished between acute and chronic effects. The acute effect of simvastatin seems to be explained by a decrease of mitochondrial function only. Andreux et al. (2014) showed that simvastatin decreased the basal oxygen consumption rate in *Caenorhabditis elegans* after 30h of administration. The chronic effect of simvastatin, however, might point to an uncoupling effect on mitochondria, causing a decrease in mitochondrial function together with an increase of mitochondrial oxygen consumption. This idea is strengthened by the finding that the MMP in PBMCs decreased after 4 weeks of simvastatin administration.

It should be noted in this context that, in an acute exposure model using isolated rat liver mitochondria, most of the statins, including simvastatin, both inhibit respiration and uncouple OXPHOS. Using isolated organelles reveals what could happen in the cell, tissue, or patient, but the latter are the result of interactions between a host of variables, such as drug metabolism, uptake carriers that increase dose concentrations in a subset of cells, and MMP that can increase mitochondrial exposure. In this way, it is difficult to predict the phenotype of mitochondrial dysfunction. For example, simvastatin not only inhibits complexes I, II-III, IV, and V, which would eventually repress oxygen consumption and deplete MMP, but it also uncouples OXPHOS, which would increase oxygen consumption to the extent that ETS is inhibited. The data here imply that both inhibition (more dysfunctional PBMCs with lower MMPs and slower PCr recharge) and uncoupling (increases in oxygen consumption) are occurring. The approach of combining corroborating assays with different readouts, plus the development of the first bedside monitor of cellular oxygen utilization, provides a more robust assessment and clinically relevant of drug-induced mitochondrial dysfunction.

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21

Detection of Mitochondrial Toxicity Using Zebrafish

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21.1 Introduction

Zebrafish (*Danio rerio*) are ideal for toxicology and drug discovery studies, as they are small, fecund, and rapidly developing animals. This vertebrate model organism nicely fills the gap between traditional toxicological studies using cell culture and those that use *in vivo* rodent models. The zebrafish provide an inexpensive means to rapidly test the effects of drugs or chemicals on multiple animals like cells in a dish while gaining critical *in vivo* data, which could in later studies be used to evaluate whether to proceed with *in vivo* testing in the more expensive rodent system. Good concordance between complementary cell, mammalian, and zebrafish models has been reported; for example, for nonsteroidal anti-inflammatory drugs (NSAIDs), good concordance across the NSAID chemical classes was found for multiple toxicity endpoints in isolated mitochondria, rat hepatocytes, and zebrafish (Nadanaciva et al. 2013). Zebrafish are used to determine adverse outcome pathways for chemical risk assessment (Volz et al. 2011; Villeneuve et al. 2014) and have been used for analyzing the effects of the ToxCast chemicals (list of chemicals of most interest to the US EPA, which include industrial and consumer products) on developmental toxicity

(Judson et al. 2010; Padilla et al. 2012; Truong et al. 2014). The effects of these chemicals on mitochondrial function are another recent important endpoint (Attene-Ramos et al. 2013).

Researchers such as Dr Robert Tanguay and colleagues have also paved the way for high-throughput chemical screening, by developing new methods for measuring multiple *in vivo* endpoints in zebrafish, simultaneously (Truong et al. 2014). Others, such as Dr Leonard Zon, have used zebrafish for *in vivo* drug discovery and development. Dr Zon now has two novel therapeutics originally tested with zebrafish in clinical trials of patients with leukemia and melanoma (Zon and Peterson 2005; Bowman and Zon 2010; Santoriello and Zon 2012; Ablain and Zon 2013). Zebrafish not only allows for high-throughput *in vivo* screening of chemical agents but also for in-depth analyses of their mechanisms of action (Rihel et al. 2010). Transgenerational studies can also be performed, as zebrafish remain small up to adulthood and are relatively inexpensive to maintain over their lifespan (Baker et al. 2014a). They are easy to breed, and breeding pairs are able to produce hundreds of offspring up to once per week. Embryos are transparent, allowing researchers to monitor the development of organs with ease. Zebrafish are readily genetically

manipulated through new gene editing mechanisms, and imaging studies are aided by comprehensive transgenic methods. Many zebrafish lines are available within the zebrafish community and in resource centers such as the Zebrafish International Resource Center (ZFIN) in Oregon (Westerfield 2000), the European Zebrafish Resource Center (Geisler et al. 2016), and the National BioResource Project Zebrafish in Japan (Okamoto and Ishioka 2010). The resulting embryos rapidly develop to free-swimming larvae in just 5 days and can be used like cells in culture in multi-well plates. Zebrafish possess the requisite and genotypic and morphological traits to model human diseases. Although zebrafish are lower-order vertebrates, both zebrafish and rodents have approximately 82–84% of same genes known to be associated with human disease (Schröml et al. 2003; Howe et al. 2013). For mitochondrial toxicology, zebrafish are a particularly tractable model, as zebrafish, like humans and rodents, have the same-sized mitochondrial genome (mtDNA), mtDNA gene order and genes required for oxidative phosphorylation (OXPHOS), and mtDNA replication machinery and homologues of many mitochondrial genes (Broughton et al. 2001; Artuso et al. 2012). They can develop the same disease phenotypes that afflict humans with mitochondrial dysfunction, such as cardiovascular and neurological disorders (Steele et al. 2014). Many cellular-based mitochondrial assays have been translated to zebrafish, because they are small enough to be amenable to similar high-throughput methodologies to assay mitochondrial health, such as respiration measurements (Stackley et al. 2011; Jayasundara et al. 2015; Rahn et al. 2015). In this chapter, we outline the advantages of the zebrafish for mitochondrial toxicology studies and the tools, models, and methods that have been developed to study mitochondrial toxicology in zebrafish, as well as what has been learned from these studies.

21.2 Genetics and Manipulation of Zebrafish for Toxicological Studies

The use of zebrafish as a model for genetic studies stems from the work by Dr George Streisinger in the late 1960s to early 1970s (Grunwald and Eisen 2002). His pioneering work that allowed for the production of clonal lines of homozygous zebrafish that was later continued by several of his students spawned systematic large-scale screens for embryonic lethal mutations using forward genetic methods (Grunwald and Eisen 2002). These studies paved the way for early understanding of the genetic landscape of zebrafish. In 2001, the Wellcome Trust Sanger Institute began the zebrafish genome sequencing project, which

generated several genome assemblies of the Tuebingen strain, one of the widely used wild-type (WT) zebrafish strains (Howe et al. 2013). In 2017, the ZFIN took responsibility for updating and maintenance of these assemblies (<http://www.sanger.ac.uk/science/data/zebrafish-genome-project>).

As a result, by comparing the zebrafish and human reference genomes, approximately 70% of human genes have at least one clear zebrafish orthologue, and of the 3176 genes bearing morbidity descriptions listed in the Online Mendelian Inheritance in Man database, 2601 (82%) of human morbid genes have a zebrafish orthologue (Howe et al. 2013). Additionally, Gunnarsson et al. determined that zebrafish possessed orthologues for 86% of the 1318 human drug targets surveyed (Gunnarsson et al. 2008). Altogether, this high degree of similarity reinforces the importance of zebrafish in understanding how our genes work, to model human disease, and ultimately, to improve human health. One consideration when using zebrafish in genetic-based studies is that their genome was duplicated with a subsequent reduction at several loci (Howe et al. 2013). While duplication can add some complexity to analysis, it also provides an opportunity since redundancy can evolve into subfunctionalization or neofunctionalization, leading to tissue-specific control for some genes. For example, Venkatachalam et al. (2012) examined induction of fatty acid-binding protein genes by clofibrate (a lipid-lowering agent that can also cause mitochondrial toxicity (Qu et al. 2001)) and found a differential induction in several major tissues by different duplications of the genes. It appeared that there were tissue-specific mechanisms in place that can limit induction of these genes by clofibrate. Discoveries such as this one can be leveraged for experiments in zebrafish to inform of tissue-specific disease states in humans. Next-generation sequencing has further refined these initial genomic discoveries, enabling novel discoveries in understanding development and physiology that can be applied to human disease research (Qian et al. 2014).

Armed with this knowledge and advanced molecular techniques, researchers are able to produce specific mutations in genes with subsequent screening for phenotypes (reverse genetics), yielding extensive models for human diseases. Investigations of the effects of nuclear-encoded mitochondrial genes have been performed using many of these techniques (Steele et al. 2014). Initially, TILLING was used to generate loss-of-function mutations (Moens et al. 2008), though further technological advancements enabled researchers to use zinc-finger nucleases (ZFNs) and morpholinos to knockout and knockdown genes, respectively. ZFNs can be used to make targeted genetic mutations and germline

transmissions at a rate faster than TILLING but are limited by expense and off-target effects (Foley et al. 2009). Morpholinos can be used at a small scale and are a relatively inexpensive way to block specific molecular elements. They also require very little up-front predictive information but are limited by dilution over time (usefulness is ~4–5 days' post-fertilization (dpf)) and may require several controls and validation experiments due to potential off-target effects (Eisen and Smith 2008; Bill et al. 2009). Newer gene editing methods have been developed that can and are being used in zebrafish. Gene editing methods utilizing transcription activator-like effector nucleases (TALENs) allow for targeted genetic mutation with germline transmission that is faster than TILLING, has few off-target effects, is cheaper than ZFNs, can in combination with oligonucleotides and homologous recombination be used for knock-in mutations, and can be easily customized (Hwang et al. 2013). Their major limitation is that design and generation of TALENs as in-house generation can be laborious (Zu et al. 2013). The newest genetic method to find use in zebrafish uses engineered clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology, which adapts a process from bacteria to efficiently mutate specific sites and loci in zebrafish (Hwang et al. 2013). This method is somewhat easier for generating edited mutants than TALEN technology and is less expensive. Off-target effects can occur with this model but can be minimized (Hwang et al. 2013; Shah et al. 2015). CRISPR/Cas9 has been optimized for rapid genetic screening of new genes involved in biological processes in zebrafish; in a proof-of-concept study, two new electrical synapse genes were found using a combination of genetic and phenotypic screening (Shah et al. 2015). One advantage of using zebrafish is that embryos are amenable to quick transplantation experiments, as the location of the cells within the blastula and gastrula stage embryos have been fate-mapped (Kimmel et al. 1990, 1995). Transplantation of cells that are, for example, heart-fated between different embryos can allow one to determine the tissue-specific effects of a gene of interest, in a background of interest (Stainier et al. 1993). Transgenesis is another common genetic method for zebrafish studies, where a DNA construct is introduced into the genome of the organism (Felker and Mosimann 2016). Stable transmission of the transgene occurs when this DNA is integrated into germ cells, such that subsequent generations also inherit this transgene. For example, transgenesis using Tol2 and Cre/Lox methods and fluorescent reporter transgenes can be used to generate zebrafish lines, enabling mechanistic studies to determine the role of the gene of interest at different developmental time points and the role within a particular organelle, cell, and the organism as a whole.

21.3 Zebrafish Physiology

Zebrafish require an aquatic environment, and thus do not need lungs, but instead require a swim bladder and gills. As such, comparative studies for lung tissue are not advised; however, determining mitochondrial toxicity due to airborne compounds is reproducible in fish as these toxicants (e.g., cigarette smoke) can be bubbled into the water and will cause the same toxic effect in similar tissue types (Hammer et al. 2011; Ellis et al. 2014; Folkesson et al. 2016). Furthermore, because of its aquatic environs (which may have as low as 1/30th of the oxygen found in air), zebrafish is able to tolerate much greater levels of hypoxia than mammals (Rees et al. 2001; Puente et al. 2014). This particular trait has been used to great advantage for mechanistic studies regarding organ development in hypoxic environments, such as the fetal heart (Puente et al. 2014). Many tissues in the body are sensitive to oxygen levels and energy deficits and thus mitochondrial dysfunction. The brain, for example, consumes many times more oxygen than would be expected for the proportion of the body that it represents (Wallace 2013). We will thus expand on the organ systems that are most susceptible to mitochondrial dysfunction. We will also consider the mitochondrial factors associated with tissue regeneration, an unusual trait of the zebrafish.

21.3.1 Neuromuscular System

Neurotoxicity, in particular, is where the zebrafish model shines. As zebrafish embryos are transparent over the first few days of development, and because of the ease of transgenesis and microinjecting various constructs into the 1–4-cell stage embryo, fluorescent proteins targeted to different cell types, such as those of the nervous system, can be used to visualize the effects of toxicants on development in real time *in vivo* (see Section 21.4 and Figure 21.1). The zebrafish brain has similar regions to the mammalian brain, and for all vertebrates, axons projecting from the habenula relay thalamic and telencephalic inputs to the ventral brain (Butler and Hodos 1996). When the animals become larvae, they start to move, hunt, and feed. From this stage onward, researchers can now monitor the locomotor activity and behavior of these fish, which is important for monitoring neurotoxicity (Irons et al. 2010). Many pollutants commonly found in air are also water soluble as well as those found in aquatic environments. In zebrafish, embryonic DDT exposure enhanced later life susceptibility to seizures due to an exposure to domoic acid, a toxic compound produced by diatoms that impairs mitochondrial function (Tiedeken and Ramsdell 2009; Hiolski et al. 2014). Many antibiotics also affect mitochondria, due to the evolution of the mitochondrion from bacteria.

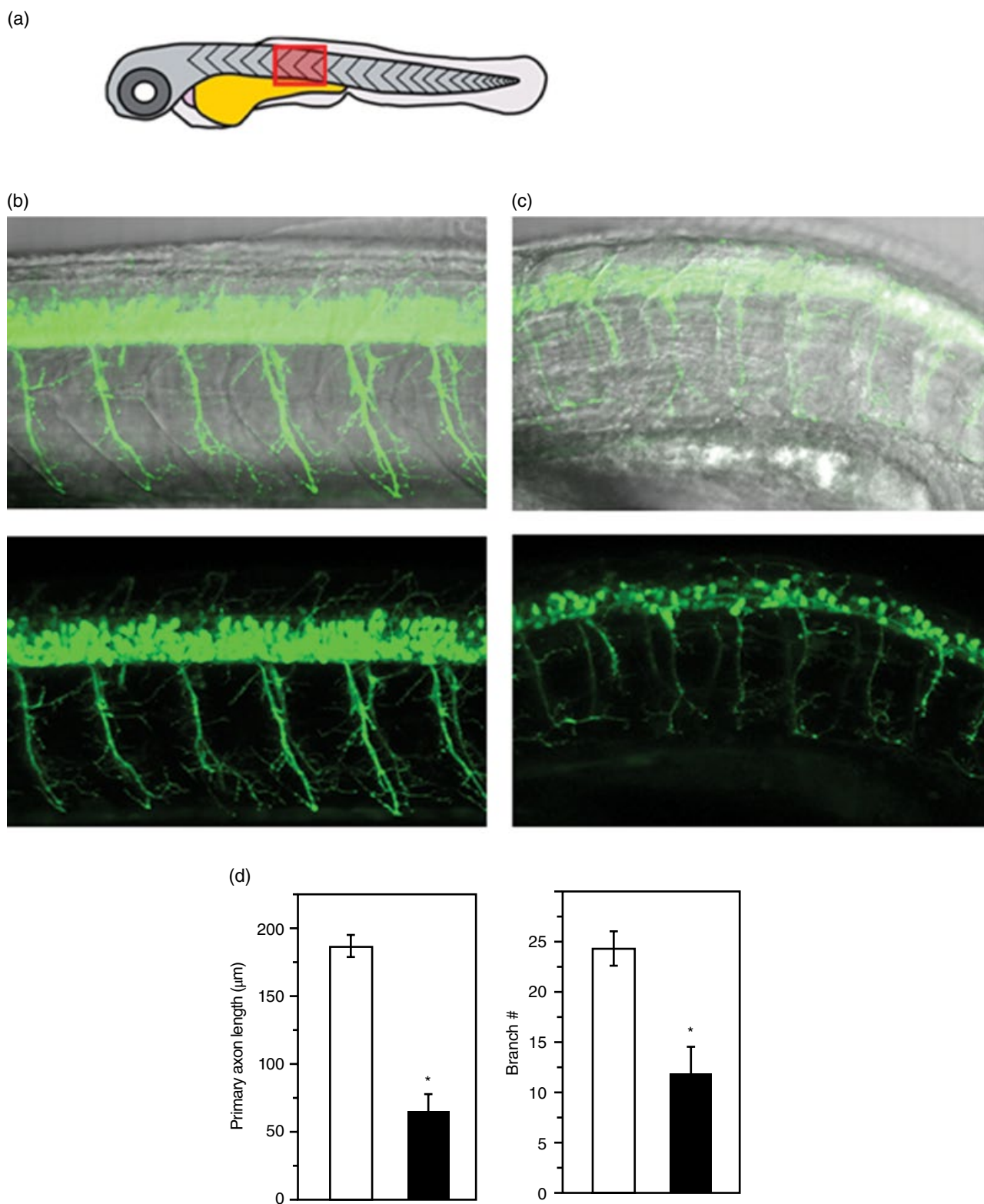


Figure 21.1 Motor neuron abnormalities are observed in Peo1 (mtDNA helicase) knockdown (KD) embryos. (a) Diagram of zebrafish embryo with red box indicating the approximate position of (b and c). Projections of 150 μm two-photon stacks of control (b) and Peo1 knockdown (c) embryos at 53 hpf. Peo1 KD embryos have altered caudal primary (CaP) motor neuron axonal branch patterns that fail to extend. Growth of CaP motor axon arbors is significantly inhibited in Peo1 KD embryos: total axon branch lengths were decreased as were the length of the primary axon, average sidebranch lengths, and branch numbers. * Indicates significant difference ($p < 0.05$) from control levels. (See insert for color representation of the figure.)

Aminoglycoside antibiotics (such as neomycin and gentamicin) are particularly toxic for people with underlying mitochondrial dysfunction (such as mutations in rRNA genes in mtDNA). Aminoglycosides preferentially kill inner ear sensory hair cells in mammals and in the hair cells of the lateral line organs of larval and adult zebrafish (Huth et al. 2011; Esterberg et al. 2016). In zebrafish studies, the mitochondrial response to aminoglycoside exposure was found to be quick—within 15 min mitochondrial swelling was detected, leading to hair cell damage and death (Owens et al. 2007). Because cells close to the surface of the fish were of interest, mitochondrial dyes (such as 2-(4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide (DASPEI)) along with cell vital dyes (such as the vital DNA dye YO-PRO-1) could be used to visualize mitochondrial structure, which were followed up with ultrastructural methods such as transmission electron microscopy.

The natural movement of zebrafish (swimming) also provides an excellent means of tracking neuromuscular toxicity. Toxicants that target mitochondria (both in the nerves and muscles that they are connected to) may disrupt bioenergetics that impact the ability of the zebrafish to move (Padilla et al. 2011; Faria et al. 2015; Dubinska-Magiera et al. 2016). A startle response to touch develops around 1 dpf (Ribera and Nusslein-Volhard 1998) and can be used to identify neuromuscular defects such as those associated with Charcot–Marie–Tooth type 2A neuropathy. Loss of MFN2 function, a mitochondrial protein involved with fusion of the outer mitochondrial membrane, results in several morphological defects with an impairment to motility. Vettori et al. used zebrafish to model the disease and used the startle response as a measure of severity of disease (Vettori et al. 2011). Interestingly, organophosphates, which include many insecticides, herbicides, and chemical warfare agents, have been linked to disrupting mitochondrial dynamics and impairment of motor proteins (Terry 2012). Simple tracking of movement using high speed cameras and software (e.g., Noldus Information Technology and ViewPoint Behavior Technology develop equipment and software for these types of zebrafish locomotor and behavioral studies) allows for the measurement of differential movement by zebrafish under different conditions. Researchers can use multi-well plates to track the movement over time under different conditions (varying light conditions, different chemicals, etc.) as early as 3 dpf (Padilla et al. 2011). In a study by Wang et al., zebrafish were exposed to bisphenol A (BPA), a known endocrine-disrupting chemical that also targets mitochondria (Agarwal et al. 2016), altering their movement (both response and speed) in a concentration-dependent manner (Wang et al. 2013). The use of larger tanks for larval, juvenile, and adult fish can also be used to track

diving behavior. The laboratories of Drs Edward Levin and Elwood Linney routinely use assays to measure diving ability, which reflect predatory avoidance behaviors (Levin et al. 2011; Roy et al. 2012), and three-chambered learning assays (Levin 2011) in their research that involves the influence of chemical exposures to development of the nervous system and subsequent behaviors. Researchers can also challenge the zebrafish to exercising and recovery using swim tunnels. These tunnels allow for the monitoring of not only swimming behavior but also physiological measurements, such as oxygen consumption. Several studies (e.g., Masse et al. 2013; Massarsky et al. 2015) have combined the use of these swim tunnels with chemical exposure to assess their effect on zebrafish. The type of movement (quick burst vs. continuous) would also help to define the type of muscle fibers being targeted. The quick burst movement is associated with “fast twitch” (white muscle), while continuous swimming movement is associated with “slow twitch” (red muscle). Different physiological properties of embryonic red and white muscles (Buss and Drapeau 2000) could be differentially affected by exposure to toxicants that target mitochondria, thus affecting their escape response or their continuous swimming. The correct organization of myotomes can also be easily visualized using a light microscope and two polarized light filters for birefringence. Shahid et al. used birefringence in conjunction with a transgenic green fluorescent protein (GFP) reporter to assess muscle fiber arrangement in response to known muscle toxicants that inhibit acetylcholinesterase (Shahid et al. 2016). They found that there was reduced muscle integrity with increased *hspb11* expression with a dose-dependent chemical exposure.

For ocular toxicity, photomotor response can be measured (Kokel and Peterson 2011), as well as optokinetic response, where animals from the 3 dpf stage up to adults can be immobilized in agar when they are young and small and with physical restraints when they are older (Mueller and Neuhauss 2010; Zou et al. 2010). A high-throughput, behavior-based approach to discover neuroactive small molecule discovery has been developed using the zebrafish model (Kokel et al. 2010). In this case, researchers used these methods to rapidly identify neuroactive small molecules that could be used as drugs. Conversely, these assays can also be used to determine the neurotoxic effects of chemicals (Olivares et al. 2016).

Neuronal activity can be noninvasively measured in an intact living zebrafish through the use of transgenic calcium sensors (Lin and Schnitzer 2016). The use of cell-type specific promoters allows for the identification and tracking of activity of individual neurons or a population of neurons that act within a tissue. The calcium

sensors used in zebrafish generally monitor flux of calcium ions that occur in response to depolarizing signals from upstream neurons into voltage-gated calcium channels and release from intracellular stores. Most often the GCaMP family of proteins is used, which are genetically encoded calcium indicators created from a circularly permuted GFP bound to calmodulin. When Ca^{2+} binds, a structural shift occurs, resulting in bright fluorescence, which can be imaged and recorded using confocal as well as conventional microscopy (Muto et al. 2011). These techniques can then be used to understand the mechanisms of action of toxicants and to monitor their adverse effects on neuronal activity, such as reported by Esterberg et al. (2014).

Healthy mitochondria are important for tissues that have large energy requirements for their function, such as the nervous system. In general, disruption of mitochondrial homeostasis results in impaired OXPHOS. The two major products of OXPHOS—energy in the form of adenosine triphosphate (ATP) and reactive oxygen species (ROS)—are altered, resulting in decreased cellular energy and increased oxidative stress, respectively. Oxidative stress resulting from high cellular ROS is strongly linked to many forms of neurodegeneration, including Parkinson's disease (Coyle and Puttfarcken 1993; Beal 1995; Schulz et al. 2000; Emerit et al. 2004; Lin and Beal 2006) and epilepsy (Patel 2002), as well as specific mitochondrial diseases (Hayashi and Cortopassi 2015). Alterations in ROS levels are easily visualized in real time in zebrafish by the use of a CM- H_2DCFDA (see Section 21.4.2). Many toxicants exacerbate ROS production (Mugoni et al. 2014); however exposure of zebrafish of some toxicants, such as 2,4-dinitrophenol (DNP), can decrease OXPHOS and ROS production (Bestman et al. 2015). DNP is a mitochondrial OXPHOS uncoupler that was originally used as a diet agent and is making a resurgence as an illicit drug that can be purchased online. Furthermore, DNP is 89 on the Agency of Toxic Substances and Disease Registry (ATSDR) priority list of hazardous substances. As ROS are important cell signaling molecules needed for development, it was not surprising to observe developmental toxicity, such as the development of the motor neurons (Bestman et al. 2015).

Mounting evidence points to mitochondrial dysfunction as a major underlying cause of many neurodegenerative diseases that can be caused by genetic defects in nuclear- and mitochondrial-encoded proteins required for mitochondrial function, as well as exposures to chemicals that target mitochondria (Schapira 2008; Schapira 2011; Exner et al. 2012; Federico et al. 2012; Yan et al. 2013; Burte et al. 2015). This suggests that exposures to mitochondria-targeted drugs and toxicants are important for modifying disease. For example, most of

the gene loci for Parkinson's disease encode mitochondrial proteins (e.g., PINK1, Parkin, POLG, LRRK2, DJ1) (Kitada et al. 1998; Lücking et al. 2000; Shimura et al. 2001; Valente et al. 2004; Morais et al. 2009; Schapira and Tolosa 2010), and many mitochondrial toxicants (such as rotenone, paraquat, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)) are associated with Parkinson's (Cookson 2005; Jackson-Lewis and Przedborski 2007; Tanner et al. 2011). Dr Marc Ekker's group has developed some excellent tools for identifying neurotoxicity in zebrafish models of Parkinson's disease, such as the MPTP mitochondrial toxicant model (Xi et al. 2011). MPTP is a prodrug of MPP^+ , which is a man-made neurotoxin that is transported to the dopaminergic (DA) neurons of the substantia nigra and inhibits complex I of the electron transport chain, causing Parkinson's disease (Goldman 2014). Bretaud et al. also exposed larval and adult zebrafish to MPTP, as well as rotenone and paraquat, two more inhibitors of complex I that can invoke Parkinson's disease to reveal developmental, behavioral, and DA sensitivity in larvae and decreased locomotor activity in adults (Bretaud et al. 2004). Dr Ekker's group also developed stable zebrafish lines containing fluorescent proteins targeted to mitochondria of distinct neuronal subtypes like the DA neurons (see Section 21.4) and that have been used to identify mitochondrial toxicity (Noble et al. 2012). Genetic models of Parkinson's disease such as the DJ-1 knockdown zebrafish were also utilized by Bretaud et al. to understand the effects of toxins on a Parkinson's background and the effects of DJ-1 in DA toxicity (Bretaud et al. 2007). Stable genetic models of Parkinson's disease in zebrafish include the knockout and dominant negative Polg (mtDNA polymerase) mutant models developed by our group through TALEN gene editing (Rahn et al. 2015). Polg is a locus for Parkinson's disease and shows rapid mtDNA depletion from 7 dpf. MtDNA is a new verified marker of human Parkinson's disease (Gui et al. 2015; Pyle et al. 2016). Altogether, these studies advocate zebrafish as a valuable model for dissecting the molecular mechanisms underlying the gene–environment interactions of Parkinson's disease.

21.3.2 Cardiovascular System

Since the heart is a highly energetic organ, mitochondria can constitute as much as 40% of the total volume of cardiomyocytes (Knaapen et al. 1997). Thus, the biomass and activity of mitochondria play an important role in proper heart development and repair of cardiovascular tissues, and exposure to mitochondrial drugs and toxicants can result in failure of mitochondria. The cardiovascular system in zebrafish features a two-chambered heart consisting of single atrium and a single ventricle

(Stainier et al. 1993). The atrium is fed by the sinus venosus and the bulbus arteriosus is the outflow tract fed from the ventricle that leads to the rest of the body. While zebrafish have a two-chambered heart, the genes and processes that drive the development of the tissues that constitute the heart are well conserved with those that form the human four-chambered heart (Stainier et al. 1993). The zebrafish heart can then act as a simplified model of cardiovascular processes that bear an effect on the human population. Additionally, the transparent nature of zebrafish embryos and the early completion of heart development within 72 hours' post-fertilization (hpf) enables measurements of developmental cardiovascular endpoints (Bakkers 2011). Zebrafish are also known for their ability to regenerate several tissues, including the heart, which has many implications for cardiovascular disease research and cell regeneration studies (Poss 2010). The health of mitochondria plays an important role in their ability to regenerate; thus toxicants that disrupt mitochondria will likely impact regeneration (Chung et al. 2007; Hom et al. 2011). Therefore, benefits of using zebrafish to study the impact of toxicants on mitochondria of the heart can be divided into two time periods with differing assays: early heart development and adult heart regeneration.

The use of the zebrafish for human cardiovascular research has been well discussed and reviewed by Drs Bakkers (Bakkers 2011) and Stainier (Stainier et al. 1993; Stainier 2001; Liu and Stainier 2012). In brief, the genetic tools available in the conserved zebrafish model present an opportunity for controlled manipulation and understanding of cardiovascular processes in a highly visible and robust model. These genetic techniques (discussed earlier in this and other chapters) have allowed for the direct control of genes that drive the major processes that are involved in the development of the heart and have produced distinct and highly reproducible phenotypes (Miura and Yelon 2011). For example, disruption of *gata5*, a gene that produces the transcription factor of the same name that is required for the correct development of the heart, will lead to cardia bifida and the reduction of ventricular tissues (Reiter et al. 1999). Thus, toxicants that disrupt mitochondria and directly interfere with important cardiac genes will produce a distinct, detectable phenotype that can be measured. An example of direct impact on mitochondrial function comes from Pinho et al. (2013). In their study, respiratory complex inhibitors were screened for their effects on heart development through visual screening of heart characteristics (e.g., edema, bleeding, looping) and their subsequent rescue using multiple quinones. Specifically, the quinones rescue through antioxidant mechanisms as well as electron transfer mechanisms to feed complex III for the production of ATP. This allows delay of heart

defects from exposure to complex I and II inhibitors. The authors reveal that these quinones do not appear to protect against complex III inhibition, as a large increase of ROS was observed. Instead, a link between the addition of these quinones and an increase in NAD(P)H-quinone reductase (NQO1) activity may better explain the cardioprotection observed. Pinho et al. also suggest that a reduced reliance on complex I/II in young zebrafish may explain the lack of disease phenotypes associated with exposures to inhibitors of those complexes and should be considered when interpreting data (Pinho et al. 2013).

The ability to use molecular techniques to directly tag mitochondria in particular tissues using specific promoters, such as *cmcl2* (Zhang and Gong 2013), for example, can enable real-time tracking of toxicant effects on the mitochondria in the heart (Figure 21.2). Use of dynamic proteins such as *kaede* (Bergamin et al. 2016) to visualize changes at different time periods (based on light exposure) can be used in the heart to track mitochondrial dynamics in response to exposures to the suspected toxicants. Quality and quantity of mitochondrial networks can be resolved through microscopy in the cardiovascular tissues using these techniques. Additionally, early heart tubes and early hearts can be removed using microdissection and mechanical separation from the developing fish for more direct measurements of the impacts of toxicants on the mitochondria and the heart (Lombardo et al. 2015). Granted the material needed to run protein level analysis is difficult to generate at this time scale due to the small size, however large clutch sizes make this possible. The large number of embryos coupled with the transparent nature of their development makes for excellent high-throughput screening of toxicological effects of mitochondria in the development of the heart (Padilla et al. 2012; Truong et al. 2014).

Researchers can also take advantage of transgenic lines that tag *fli1*, an ETS transcription factor that is involved with developing vasculature, in zebrafish. Zebrafish have a very distinct patterning for angiogenesis during development with the dorsal longitudinal anastomotic vessel branching ventrally and the aorta branching dorsally until these branches meet to form intersegmental vessels (Childs et al. 2002). In a study conducted by Delov et al. (2014), the intersegmental blood vessel was measured using the *fli1* EGF when the fish were exposed to increasing doses of chemicals. One of the chemicals in this study was triclosan (5-chloro-2'-(2,4-dichlorophenoxy)phenol), a common antibacterial that disrupts fatty acid biosynthesis in bacteria and is a mitochondrial toxicant causing the uncoupling of respiration (Weatherly et al. 2016). Triclosan exposure to zebrafish embryos caused vascular defects (Delov et al. 2014). Like Delov et al., we also

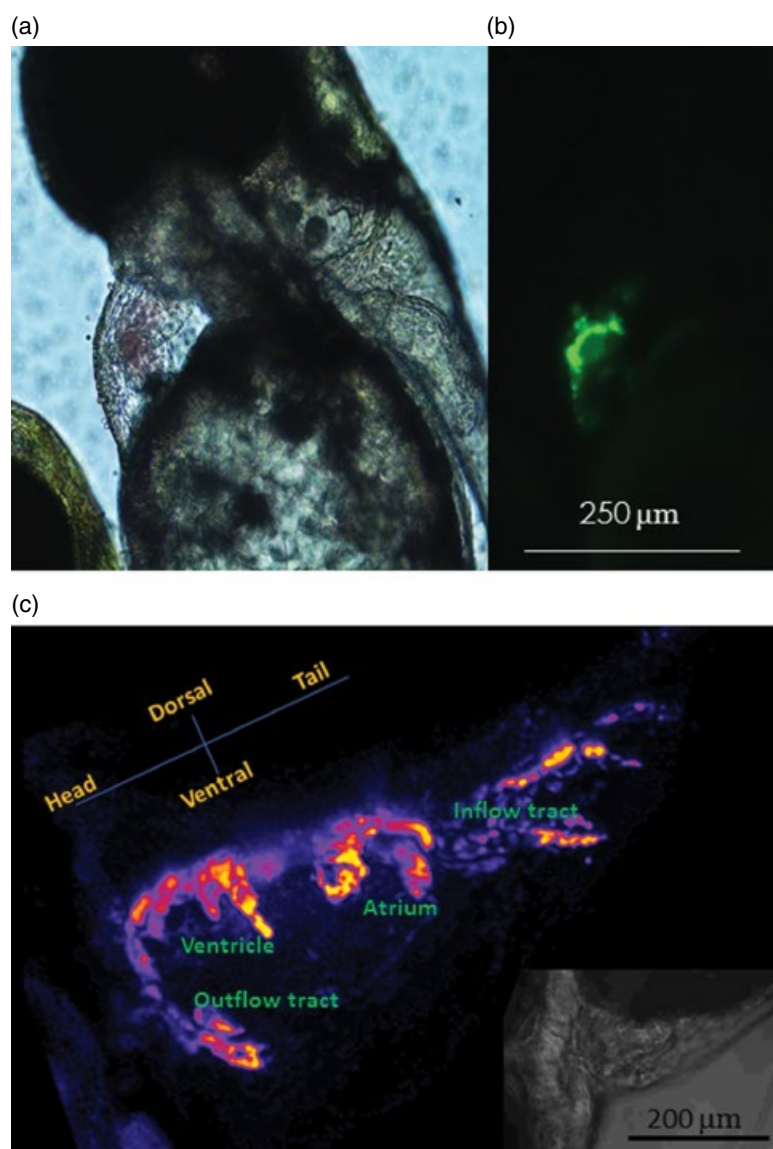


Figure 21.2 Mitochondrial GFP targeted to cardiac tissue in zebrafish. The plasmid construct used contains the *cmlc2* promoter, a mitochondrial leader sequence, and green fluorescent protein (GFP) sequence, flanked by Tol2 sites. Plasmid and Tol2 mRNA are injected into embryos at the 2-cell stage. (a) Bright-field image of a 72 hpf zebrafish at 100 $\times$ magnification. (b) Fluorescent mitochondria in the heart of the individual from A. Viewed using FITC filter on an inverted Zeiss microscope (excitation, 450–490 nm; emission, 515–545 nm). (c) 72 hpf WT zebrafish injected with the plasmid construct mentioned earlier. Zebrafish were fixed in 4% paraformaldehyde for 5 h at room temperature and washed three times for 10 min in PBS before mounting in agarose. This confocal image was taken on the Olympus FV1200 MPE intravital microscope at 30 $\times$ magnification and 1.5 μ m slice interval. Presented as a heat map of mitochondrial density in a 3D projection. (See insert for color representation of the figure.)

observed vascular defects in zebrafish when exposed with the mitochondrial uncoupler DNP (Figure 21.3). Other common deformities found in studies of chemicals and their effects on zebrafish vascular development include deflection that leads to no connections or looping back, reduction in vessel number, and reduction in size of these branching vessels (Chimote et al. 2014; Delov et al. 2014; Tal et al. 2016).

21.3.3 Liver

One of the primary targets for mitochondrial toxicants and toxicants in general is the liver. As a primary location for bioactivation and eventual detoxification, it is exposed to a litany of compounds on a regular basis. The importance of the liver for toxicant screens is reflected in several chapters of this volume. Its ability to

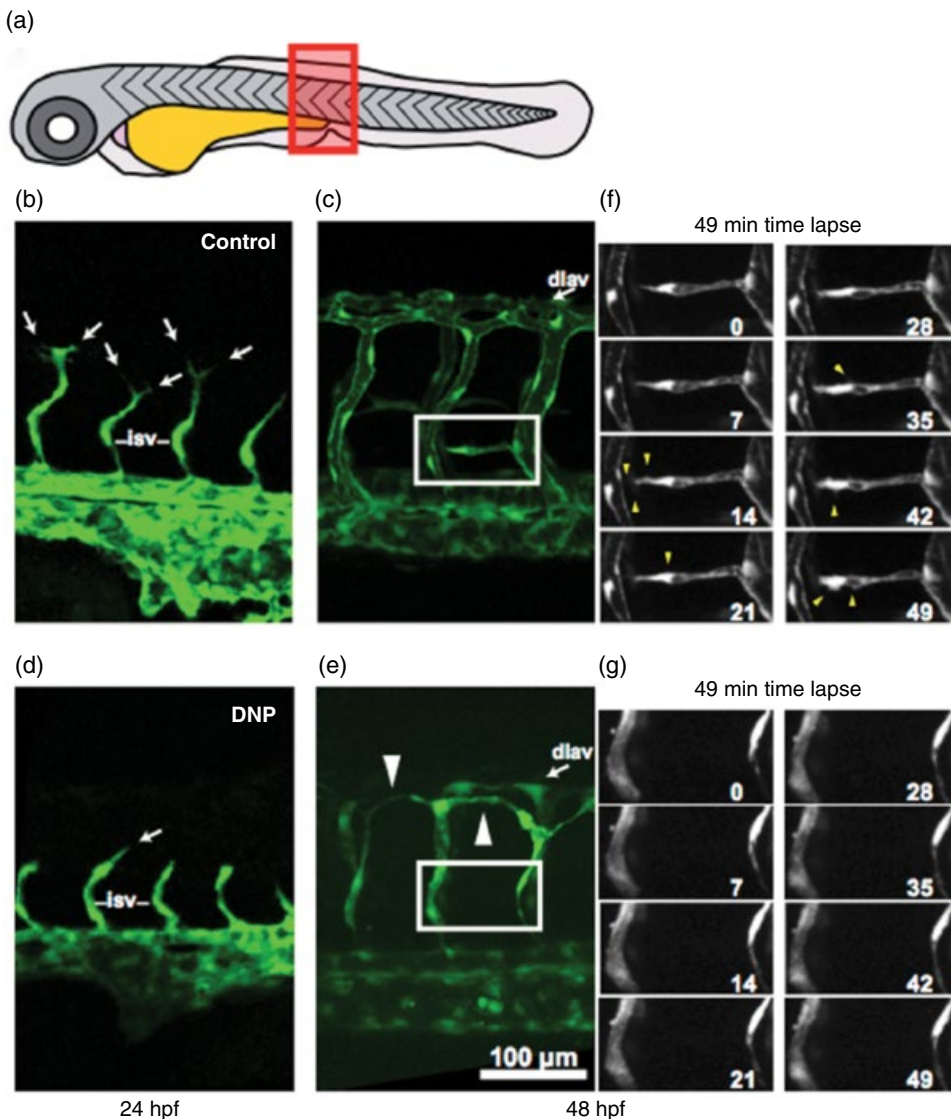


Figure 21.3 DNP exposure disrupts blood vessel development. *In vivo* 2-photon stacks acquired through the trunks of 24 and 48 hpf *fli1*:eGFP zebrafish reveal the developing blood and lymph vessels. (a) Diagram of zebrafish embryo with red box indicating the approximate position of (b–e). At 24 hpf, both control (b) and DNP-treated (d) zebrafish have dorsally growing intersomitic vessels (isv), but those of DNP-treated fish end in fewer filopodial projections (arrows, (b, d)), indicating that DNP inhibits angiogenesis. At 48 hpf, the dorsal longitudinal anastomotic vessel (dlav) of control fish has a fully formed lumen able to pass blood cells; it remains constricted in the DNP-treated fish (e) (arrowheads). Boxed regions in (c) and (e) are magnified and the time lapse frames reveal the filopodial projections (yellow arrowheads) on the growing tip of the developing branch of the parachordal chain in controls (f), which is absent in the DNP-treated animal (g). (See insert for color representation of the figure.)

deal with these toxins requires an extensive amount of energy and complex ROS signaling, which are linked to large numbers of mitochondria present (Sato 2007).

The liver of the zebrafish is organized slightly differently from a mammalian liver, but all the cell types of the liver, except for Kupffer cells, have been found in zebrafish performing many of the same functions as mammalian liver as early as 3 dpf (Goessling and Sadler 2015). While Kupffer cells, the specialized macrophages in the mammalian liver, are not present in zebrafish, they

do have macrophages that form aggregates present in the liver (Holden et al. 2012). Equally important, researchers have developed transgenic zebrafish lines where each of these different cell types of the liver express specific fluorescent proteins that can be visualized in live embryos and larvae. Thus, the development of these cells can be easily assessed (Goessling and Sadler 2015), and with additional assays, such as bilirubin and alanine aminotransferase (ALT) activity assays, liver function can also be determined (Li et al. 2016). Gross observation of

changes in color (darkening), texture (degradation), size, and yolk retention (yolk utilization is through the liver) can all be used as endpoints for liver toxicity in zebrafish (Hill et al. 2012).

Zebrafish show strong potential as a model to in the study of drug-induced liver injury (DILI), especially when used in conjunction with other complementary models. Nadanaciva et al. (2013) assessed two *in vitro* models alongside an *in vivo* zebrafish model for the effects of different chemical classes of NSAIDs on the liver. The authors reported good concordance between the different models in assessing liver toxicity, noting that the use of zebrafish allowed for larger drug screens for differential phenotypes across organ systems through the addition of extra assays or endpoints. They also noted one limitation with the use of zebrafish for the study of NSAID-related hepatic toxicity: certain phenotypes that are associated with liver toxicity can be masked by death of the organism at low concentrations. In the review by Vliegenthart et al. (2014), the authors point out that while the amount of drug being added to water that the zebrafish inhabit is known, the exact amount of the compound that is taken up by the fish is variable. However, it is possible to directly inject compounds into the yolk sac or use mass spectrometry to measure levels of compound from blood or tissue samples. Disruption of lipid metabolism can lead to adverse effects in liver, and mitochondria play a large role in the transport of lipids. For example, BNIP3 (encoded by *nip3a* in zebrafish (Feng et al. 2011)) has been shown to play an important role in the regulation of mitochondrial dynamics, mitophagy, and lipid metabolism (Glick et al. 2012). This protein is located on the outer membrane of the mitochondria. Disruption of BNIP3 altered lipid synthesis and mitochondrial mass. Despite an increase in mitochondrial mass, several mitochondrial parameters were impacted as shown by loss of membrane potential, abnormal mitochondrial structure, reduced oxygen consumption, and an increase in ROS. Interestingly, BNIP3 has been shown to be influenced by toxicants such as acute cyanide exposure (Zhang et al. 2011) or the heavy metal cadmium (Wang et al. 2014). While “liver toxicity” was not the primary focus of those papers, disruption of BNIP3 is expected to increase lipid accumulation in the liver. This disruption of lipid transport can be visualized using simple lipophilic dyes, such as oil red O, to image lipid droplets. An accumulation of these droplets would indicate dysfunction in the utilization of fatty acids (Kim et al. 2015). Different functional groups from the core of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (boron-dipyrromethene (BODIPY)) fluorescent dyes can be used to measure function of the liver due to differential uptake and breakdown of lipids (Otis and Farber 2016). This method for observing lipid utilization and

trafficking within the liver is highly effective when viewed with confocal microscopy.

Human mitochondrial diseases are complex and poorly understood, often resulting in misdiagnosis or a very-late-stage proper diagnosis. One of the mitochondrial disorders that results in liver disease is known as Alpers syndrome. This syndrome belongs to a group of mtDNA depletion disorders that are associated with mutations in POLG, the mtDNA polymerase (Nguyen et al. 2006). While this childhood-onset syndrome manifests with neurological developmental delay and seizures, it is often the hepatic failure that results in death (Narkewicz et al. 1991). As valproic acid is commonly prescribed for the treatment of epileptic seizures, this may include patients with as yet undiagnosed Alpers syndrome. Unfortunately for patients with POLG mutations and Alpers syndrome, valproic acid can cause fatal hepatotoxicity and should not be prescribed. Valproic acid has many cellular targets, including the inhibition of mitochondrial β -oxidation (Caldeira da Silva et al. 2008). However, fatty acid oxidation was not found to be the cause of toxicity; instead liver regeneration was affected by valproic acid exposure in this human population (Stewart et al. 2010).

21.3.4 Reproductive System and Gender

Zebrafish are rather fecund: under ideal conditions females starting from 3 to 4 months of age can produce 200–300 oocytes per week (Chhetri et al. 2014). The male fertilizes the oocytes released by females, and these fertilized eggs, embryos, are small in size, approximately 1 mm in diameter. This small size enables embryos and early larvae to be used in high-throughput screens (Vogt et al. 2009). Sperm and oocytes can be squeezed from adults for *in vitro* fertilization experiments. Furthermore, zebrafish sperm, but not oocytes, can be frozen down and stored in liquid nitrogen for the preservation of important lines (Draper et al. 2004; Draper and Moens 2009).

All zebrafish start to develop undifferentiated juvenile ovaries early in life until 30 days' post-hatching. Up to this point zebrafish are considered hermaphrodites. Past this period the juvenile ovary can mature into a proper ovary for females or transform into a testis for males (Liew and Orban 2014). As an addendum to this, gender was found to be flexible later in life, as females could transform into males by exposure to an aromatase inhibitor (Takatsu et al. 2013). In this study, ovaries of the female zebrafish retracted to give way to testes-like organs, which produced sperm that could be used to successfully fertilize eggs *in vitro*. BPA is an example of a well-established endocrine-disrupting compound affecting both male and female reproductive health. Zebrafish

testes show degradation at low concentrations (100 µg/L) of BPA (Lora et al. 2016). These authors demonstrate the ease at which morphological changes can be assessed for toxicological studies. At even lower levels of BPA exposure (0.228 µg/L), zebrafish show a gender bias toward females at both the F1 and F2 generations with decreased sperm density and quality (Chen et al. 2015). Specifically, mitochondrial membrane potential in the sperm is decreased (Singh et al. 2015). These environmentally relevant low concentrations also affect oocyte growth and maturation. Changes to chromatin structure through histone modifications and apoptotic signals in mature follicles when female zebrafish are exposed to 5 µg/L BPA constitute a detrimental effect on female reproduction (Santangeli et al. 2016).

There is good homology of the known sex differentiation genes and pathways between mammals and zebrafish (Marshall Graves and Peichel 2010). However, in addition to the differences stated earlier, gender specification is not as well understood in zebrafish as it is in mammals. Primarily, sex determination in zebrafish appears to be genetic through allelic combinations of several loci (Tong et al. 2010; Liew and Orban 2014). However, gender can be influenced by environmental factors, such as food consumption: zebrafish that grow faster are more likely to be female (Lawrence et al. 2008). Interestingly, dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)) is an aryl hydrocarbon receptor (AHR) agonist and an endocrine disruptor that can impact drug metabolism; exposure of zebrafish to dioxin can also lead to a greater female–male ratio, not just in the current generation but also in at least two later generations that had not been exposed (Baker et al. 2014b). Zebrafish are also an excellent model for determining the effects of chemicals found in wastewater, such as contraceptives. Hua et al. found that one such contraceptive agent, levonorgestrel, could significantly alter sex differentiation in zebrafish, with 100% males produced at environmentally relevant concentrations (Hua et al. 2015). Oxygen levels (upon which mitochondrial OXPHOS is also dependent) can also sway gender ratios, as hypoxia was shown to increase the rate of male offspring in zebrafish (Shang et al. 2006). Pluripotency also appears to be associated with oxygen and bioenergetic status. In mice, undifferentiated embryonic stem cells have high glycolytic metabolism and low oxygen consumption, which are needed to maintain their proliferative capacity (Kondoh et al. 2007; Facucho-Oliveira and St John 2009). We also observed in zebrafish that glycolysis is higher in early embryogenesis (Stackley et al. 2011). Thus chemicals that affect oxygen and energetic status not only are likely to interfere with the ability of the embryo to develop and differentiate properly but may also affect the final gender ratios in each clutch.

21.3.5 Regeneration

One of the most interesting abilities of the zebrafish is its ability to regenerate many of its tissues, including the heart, nervous tissue, liver, and fins (Gemberling et al. 2013). The regenerative abilities of the zebrafish have become an important area of interest especially for the studies in tissue repair and cardiovascular disease. In particular, Dr Ken Poss' research group has combined transgenic lines that allow of the visualization of specific cell types in heart with a cardiac resection technique that allows for the tracking of heart regeneration (Poss 2010; Kang et al. 2016). Mitochondria produce much of the energy needed to complete repair processes, but they also play an important role in cell signaling required to initiate repair processes (Marin-Garcia 2016). Our studies also show that zebrafish deficient in Polg (the only replicative DNA polymerase in vertebrate mitochondria essential for mtDNA replication and repair) mutant have severely decreased mtDNA copy number and surprisingly, an impaired ability to regenerate damage to their caudal fins (Rahn et al. 2015) (Figure 21.4). This suggests that chemicals that disrupt the ability of mitochondria to regulate mtDNA copy number (e.g., the antiviral nucleoside analogues used to treat HIV (Chan et al. 2007)) could, in turn, limit the ability of zebrafish to regenerate. Furthermore, as shown in the liver section, patients with POLG mutations and Alpers syndrome are susceptible to valproic acid hepatotoxicity, which is associated with defects in liver regeneration. As our Polg mutant zebrafish shows defects in regeneration, this model could be used to test toxicity of current and new epilepsy drugs on Alpers syndrome (Rahn et al. 2014).

21.4 Mitochondrial Biology and Methods

Zebrafish are an excellent model system for mitochondrial biology and diseases (Steele et al. 2014). Because zebrafish embryos and early-stage larvae are small and can fit easily into up to 384-well plates (Zon and Peterson 2005), many mitochondrial assays can be translated from cell culture. These studies have the added advantage (over cell culture) of monitoring the mitochondrial marker of interest across the whole body. For example, how specific mitochondria-targeted drugs (such as OXPHOS complex inhibitors) affect development and cardiovascular function has been carefully studied in the zebrafish (Pinho et al. 2013). *In vivo* studies such as these take into account the fact that within the organism there is communication and interaction between the different organ systems that is essential for understanding toxicity, particularly for mitochondrial toxicity where the

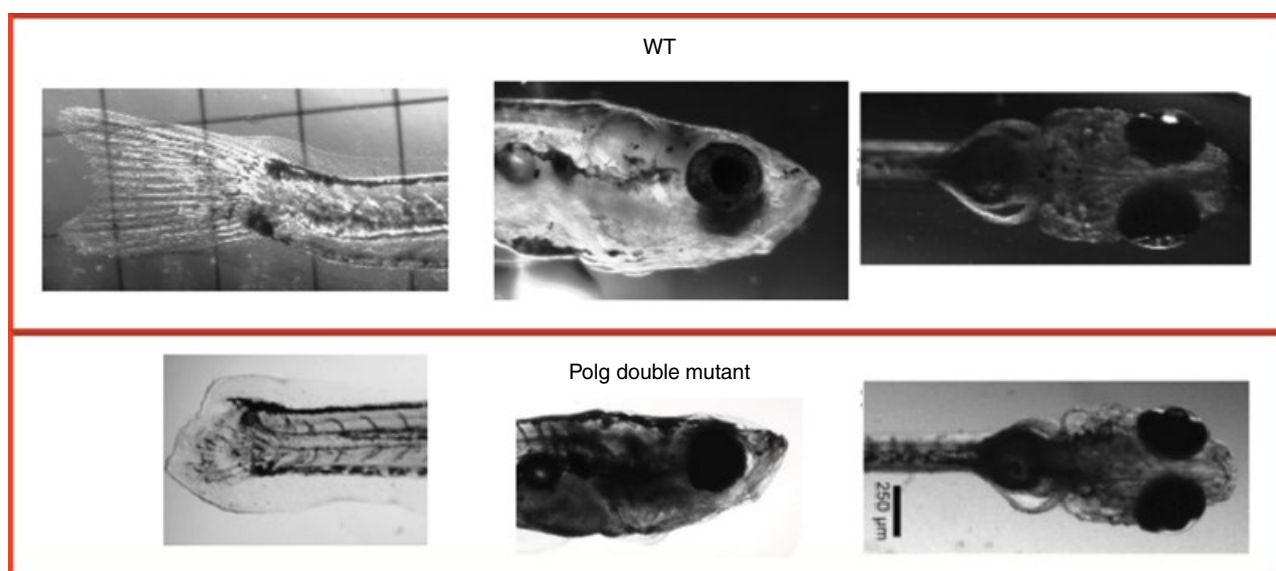


Figure 21.4 Zebrafish lacking functional mtDNA polymerase have regenerative defects. Compared with WT at 3 weeks and heterozygous mutants (not shown), zebrafish homozygous for the Polg polymerase mutation show defects in tail regeneration after amputation at 1 week old.

high-energy-requiring organs are generally more susceptible than organs that are less dependent on mitochondria (Wallace et al. 2010).

21.4.1 Energetics

As the mitochondrion's most important function (arguably) is to produce energy for the cell through OXPHOS, a major marker for mitochondrial health is respiration. The traditional method for measuring respiration is via the Clark electrode (Schnellmann 1994; Mendelsohn

et al. 2008). However, newer methods, such as those that use the Seahorse extracellular flux analyzer, enable measurements of respiration (oxygen consumption) in multi-well plates (24- and 96-well). We and others have developed new methods for utilizing this instrument to measure respiration in intact living zebrafish and larvae up to 10 dpf (Figure 21.5) (Stackley et al. 2011; Rahn et al. 2013), as well as in organs carefully dissected from larval and adult zebrafish (Jayasundara et al. 2015; Rahn et al. 2015). Additional use of inhibitors of the electron transport chain and the ATP synthase allows one to monitor

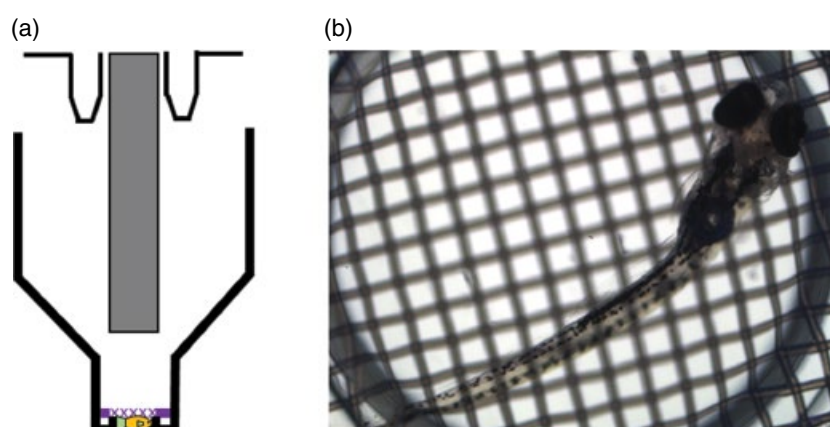


Figure 21.5 Zebrafish embryos and larvae up to 10 dpf can be assayed in the Seahorse Bioscience XF24 extracellular flux analyzer. (a) Cross section of a well within an XF24 plate. The embryo (bottom of well) is covered by an islet capture screen (crosses). The probe (gray) descends to form a microchamber with the plate and capture screen, and the consumption of oxygen by the embryo is measured through fluorophores on the probe head (the sensor) that monitor oxygen levels. Two of the four drug injection ports are shown next to the probe. (b) View from the top of a 10 dpf zebrafish embryo in an islet plate well prior to assay. The mesh shown is from the islet capture screen.

how oxygen is being consumed within the mitochondria and from non-mitochondrial sources. Furthermore, addition of a mitochondrial uncoupler such as carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) will provide a measurement of the maximal respiratory capacity of the mitochondria within the zebrafish. When the basal oxygen consumption rate is subtracted from the maximal FCCP-uncoupled oxygen consumption rate, this provides a measure of the mitochondrial spare respiratory capacity, which is an excellent marker of mitochondrial health and the ability of the mitochondria and the cell to respond to stressors. Another marker that is measured on this instrument is extracellular acidification, which in cells is a good marker for glycolysis (as lactic acid, the endpoint of glycolysis, is the major acid released from cells). In older animals, extracellular acidification may be a reasonable marker of glycolysis; however in embryos and larvae, we have found that media acidification in this case is reflective of carbonic acid production in the media due to hydration of CO₂ generated from aerobic respiration rather than lactic acid extrusion (Stackley et al. 2011), which can be measured using a modification of the traditional lactate assay used for cells (Brandt et al. 1980; Stackley et al. 2011). Total ATP levels can also be measured in whole zebrafish as in cells (Rahn et al. 2015). The ability to measure respiration in a high-throughput fashion has enabled researchers to determine how chemicals affect respiration in cells, and now zebrafish, to inhibit mitochondrial function (Stackley et al. 2011; Gibert et al. 2013; Rahn et al. 2013, 2015; Jayasundara et al. 2015; Massarsky et al. 2015; Felix et al. 2016; Jain et al. 2016). Conversely, this technique has also allowed researchers to identify chemical compounds that improve mitochondrial function (Beeson et al. 2010; Gohil et al. 2010; Rahn et al. 2014).

21.4.2 Reactive Oxygen Species

Disruption of mitochondrial homeostasis can result in impaired OXPHOS, leading to not only altered production ATP but also ROS, which is a major by-product of OXPHOS. These ROS are superoxide radicals that can cause damage to nucleic acids, proteins, and lipids and can be further converted to hydroxyl radical, which is extremely reactive, as well as to less reactive but longer-lasting hydrogen peroxide (via superoxide dismutase). Thus measurements of ROS are important for understanding the impact of toxicants on mitochondrial and overall organismal health. Unfortunately, the zebrafish embryo is not amenable to many of the mitochondrial stains that have been developed for cells, such as MitoSOX, which is used to monitor mitochondrial superoxide production (Josey et al. 2013). This is because the zebrafish does not take up many of these dyes,

although superficial cells, such as the neuromasts, can easily take up some of these dyes (Esterberg et al. 2014).

When we knock down *Peo1*, the mtDNA helicase needed for mtDNA replication, we observed an increase in ROS formation (Figure 21.6), which is in concordance with data in mice that showed that defective mtDNA replication can lead to oxidative stress (Lewis et al. 2007). Furthermore, exposures to a mitochondrial toxicant can give rise to increased ROS due to, for example, damage to the OXPHOS complexes such as complex I (Mugoni et al. 2014). However, shutdown of OXPHOS due to exposures to mitochondrial uncouplers such as DNP can shut down ROS production, as shown in Bestman et al. (2015), where we used CM-H₂DCFDA, a fluorescein derivative that is non-fluorescent until cleaved by intracellular esterases and then oxidized, to assess oxidation levels in living zebrafish embryos (Anichtchik et al. 2008; Walker et al. 2012). ROS are important second messengers required for developmental processes (Dennery 2007; Dumollard et al. 2009; Crespo et al. 2010; Gough and Cotter 2011; Hom et al. 2011). Thus, not surprisingly, we also observed a downstream impact on the developing DNP-exposed embryo. Oxidative DNA lesions such as 8OHdG can also be measured in zebrafish (Puente et al. 2014), but lack specificity in the origin of these lesions. More specific ROS methods are now being used for zebrafish, such as HyPer, which is a genetically encoded fluorescent sensor that is able to detect intracellular hydrogen peroxide (Belousov et al. 2006; Ermakova et al. 2014; Esterberg et al. 2016). Newer methods that will allow for multiple ROS measurements are also being developed that will greatly aid *in vivo* mitochondrial toxicity testing. For example, Dr Christopher Chang's group is developing new imaging probes that report on ROS as well as reactive sulfur and carbonyl species in living cells and animals (Lippert et al. 2011). These chemical tools will allow researchers to reveal *in vivo* the precise adverse actions of mitochondrial toxicants in terms of ROS.

21.4.3 Mitochondrial Dynamics and Mitochondrial Membrane Potential

One of the major drawbacks of cell culture studies is that they describe the effect in only that cell type, but the interactions of those cells within a tissue and further in the whole organism is lost. The zebrafish model bridges this gap, yet retains some of the inherent advantages of cells in culture due to its small size. The methods used for these types of studies are centered around various microscopy techniques and the ability to identify mitochondria and different cell types using targeted fluorescent proteins. Advancing technology in microscopy has made it easier to track mitochondria in specific cell types *in vivo*. The microscopy techniques in specific

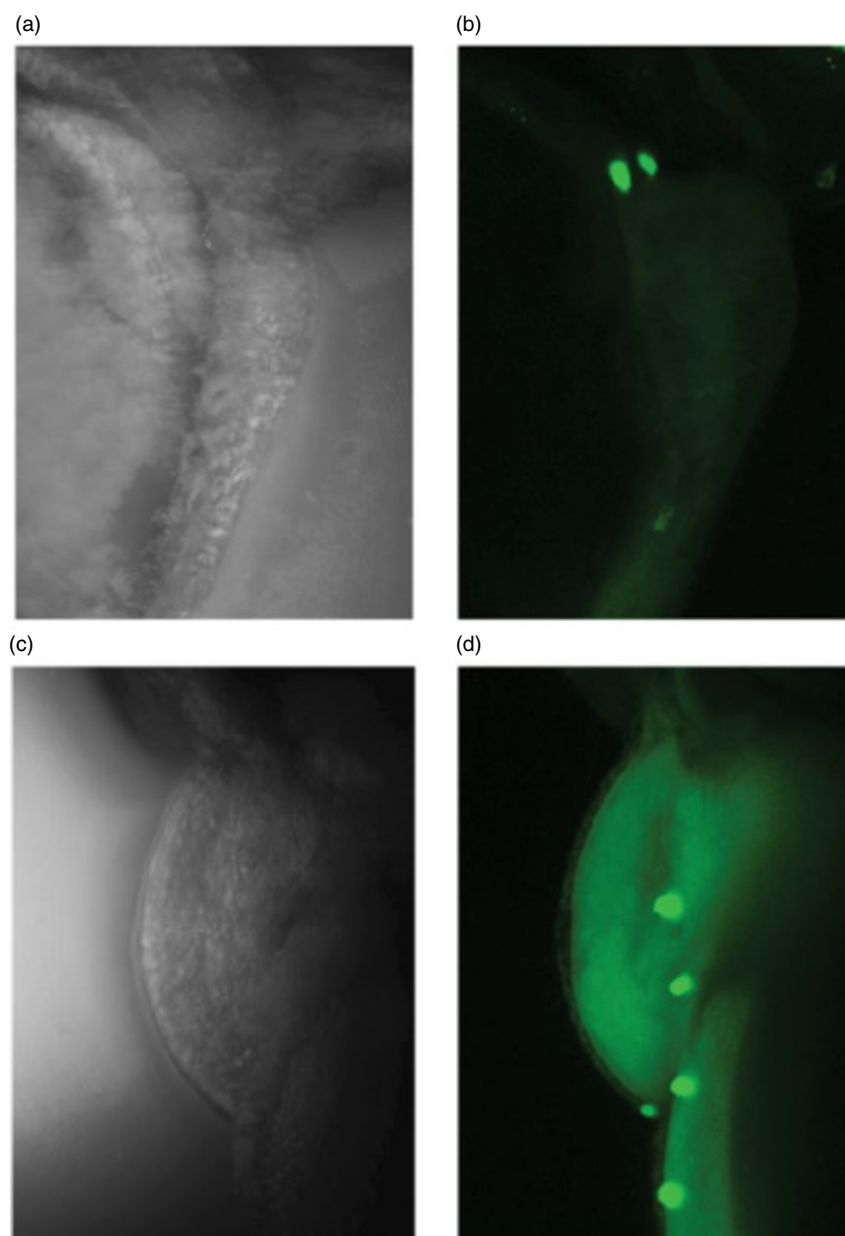


Figure 21.6 Increased reactive oxygen species observed in Peo1 knockdown embryos at 3 days' post-fertilization. Representative 2-photon image projections of H_2DCFDA fluorescence from mismatch control (a (brightfield) and b (fluorescence)) and Peo1 knockdown (c (brightfield) and d (fluorescence)) embryos. Peo1 knockdown hearts appears to have more H_2DCFDA fluorescent product than controls. The bright spots in both panels are epidermal cells (melanocytes) and are excluded from analyses. (See insert for color representation of the figure.)

organs are reviewed in this volume by Drs Andrew Hall and John Lemasters (see Chapters 34 and 35) and can be applied to zebrafish as a whole. For example, the use of spinning disk confocal microscopy can be used to capture exceedingly rapid dynamic events, such as transport within a nerve cell, which laser scanning confocal microscopy is unable to capture in real time (Godinho 2011). These events are easily visualized and can be measured for disruption by toxic agent exposures. The number of established transgenic fluorescent lines is

extensive, and availability of published lines coupled with relative ease of generating new lines makes the zebrafish model an attractive alternative in the study of toxic exposures to mitochondria. Dr Marc Ekker's research group, for example, has coupled eGFP targeted to DA neuron clusters with mCherry targeted to mitochondria to examine the effect of methylmercury on neuronal development (Noble et al. 2015). Their findings suggest that the function rather than number of DA neurons is affected due to changes in mitochondrial

dynamics (Noble et al. 2015). They have also been able to use these labeling techniques to examine neurotoxicity in different neuronal tissues such as the olfactory bulb, subpallium, pretectum, diencephalic clusters 2 and 3, caudal hypothalamus, and preoptic area using the DA neurotoxin MPTP, which differentially reduced mCherry labeling in these tissues. In our own studies, we have used fluorescent labeling to examine toxicity of DNP to different neuronal tissues in zebrafish (Bestman et al. 2015) and to target mitochondria to examine mitochondrial network stability in skeletal muscle with disruption of the mitochondrial dynamics protein Opa1 (Rahn et al. 2013). Other examples from the literature use a transgenic fluorescent reporter zebrafish line that could be photoactivated to observe mitochondrial dynamics (Bergamin et al. 2016; Dukes et al. 2016). Photoactivated mitochondria change from green to red, while newly formed mitochondria maintain a green color. A fission event would result in smaller green or red mitochondria. A fusion event between an activated mitochondrion and one that was not resulted in a yellow label. Through these color differences, the authors were able to report on mitochondrial dynamics in DA neuron transport.

The use of dyes to label mitochondria and aspects of the zebrafish is also a viable route for studies. One recent example uses the novel cyanine dye ZM214 to visualize membrane potential of mitochondria in neuromasts in zebrafish (Sasagawa et al. 2016). Using oligomycin and FCCP, known membrane potential disrupters as controls, they measured an increase (hyperpolarization) and a decrease (depolarization) in fluorescence, respectively, and concluded that the dye reflects membrane potential status of the mitochondria. They then tested for the mitochondrial toxicity of troglitazone, a thiazolidinedione developed to treat diabetes but was removed from the market due to hepatic toxicity, and tolcapone, a catechol-*O*-methyltransferase (COMT) inhibitor that was developed for Parkinson's disease but was also retracted due to off-target toxicity. In both cases, a decrease in mitochondrial membrane potential was observed. As mentioned earlier, DASPEI stains the mitochondria of live cells and is frequently used to stain the neuromasts, such as in studies to determine the cell death and regeneration response of neuromasts to exposures to aminoglycosides such as neomycin (Harris et al. 2003). While the aqueous environment allows for easy exposure to various dyes for uptake and visualization, efficacy can be limited with some dyes due to poor permeabilization into living tissue. Longer exposures or the use of fixed tissues can be used to increase permeabilization. The darkening of zebrafish from the growth of melanocytes as they mature from embryos to larva to adults increases the innate fluorescence and obscures transparency. Exposure to a low concentration of 1-phenyl-2-thiourea

(PTU) starting at 24 hpf impedes the darkening of the embryo as it grows for a limited time. Logistically, this method is most viable for the first week of life. Continuing to look at fluorescence in adult fish can be achieved through the generation of a stable line cross with the transparent *casper* zebrafish line and now the transparent *crystal* zebrafish line, which are superior to the *casper* line in terms of optical transparency (Antinucci and Hindges 2016).

21.4.4 Mitochondrial DNA Stability

Immature oocytes contain the starting mitochondria that will be passed onto the next generation. During maturation, oocytes undergo massive mtDNA replication. Mutations in mtDNA can be caused by genotoxins (Meyer et al. 2013), as well as via replication errors (Zheng et al. 2006). Heteroplasmy is the presence within a cell or individual of mtDNA, which have different sequences, whereas for homoplasmy every mitochondrial genome has the same sequence. The level of mtDNA heteroplasmy within a cell or tissue and the particular mutation(s) contained within mtDNA are important considerations for disease. Even 10% heteroplasmy can be pathogenic in certain cases (Suomalainen and Isohanni 2010; Wallace 2013), and 30–35% depletion of mtDNA can be lethal (Naviaux 2000). Wallace's group showed in an elegant study that in mice harboring deleterious mtDNA mutations, the ovary selectively eliminates the most deleterious mtDNA mutations, which should minimize their impact on population fitness. However, milder mtDNA mutations are not eliminated in the female germline and are introduced into the general population (Fan et al. 2008; Wallace 2010).

In zebrafish, many of the methods used to measure mtDNA stability in cells have been translated to zebrafish embryos, larvae, and organs. MtDNA copy number can be measured using real-time PCR amplification of a short mtDNA fragment and a short nuclear DNA fragment and determining the ratio of mtDNA/nDNA (Rahn et al. 2013; Rahn et al. 2015). When zebrafish were exposed to DNP, we observed an increase in mtDNA copy number that was likely due to induction of mitochondrial biogenesis (Bestman et al. 2015). MtDNA deletions can also be identified by another PCR-based method. An extra-long PCR amplifies mtDNA using primers that span a large portion of mtDNA, and all fragments are run on a gel to determine the percentage of smaller (deleted) fragments compared with the full-length product within a sample (Rahn et al. 2015). Real-time PCR methods have been developed for measuring mtDNA deletions in single cells and could also be used for zebrafish (He et al. 2002). We also developed an *in vivo* mtDNA polymerase activity assay utilizing

ethidium bromide (EtBr) to deplete mtDNA (Rahn et al. 2015). We used this assay to show that zebrafish with two copies of a mutated mtDNA polymerase (Polg) could not recover mtDNA content after mtDNA depletion by EtBr, revealing that the mutated mtDNA polymerase did not have sufficient DNA polymerase activity. DNA polymerase-blocking lesions can also be quantified using a PCR-based assay developed originally by the Van Houten group and translated to the zebrafish by the Meyer group (Santos et al. 2006; Hunter et al. 2010). This assay is described in detail in Dr Meyer's section within this book (Chapter 48).

21.5 Conclusions and Future Directions

In conclusion, there are many advantages to using zebrafish to study mitochondrial toxicology of drugs and other chemicals. Not only do they fill an important gap between cellular and rodent models, but they have great utility for high-throughput methodologies, an important consideration for chemical screens. New drugs have been developed stemming from zebrafish

screens, and new methods for genetic manipulation have contributed to the rise in use of zebrafish for disease models and for understanding gene–environment interactions. There is a greater awareness of the ability of many chemicals to affect mitochondrial function, and this can be a major mechanism for toxicity issues found for many drugs. Many mitochondrial assays have been developed for zebrafish that translate methodologies that have long been the domain of cell culture. Long-term studies for age-related diseases and epigenetic and transgenerational investigations are now being performed. Finally, zebrafish are an important model for tissue regeneration. As new studies reveal underlying and key roles of mitochondria in proper regeneration, it will be important to understand how exposures to chemicals that target mitochondria also affect tissue regeneration.

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22

MiRNA as Biomarkers of Mitochondrial Toxicity

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22.1 Introduction

The mitochondria are a major target of many drugs and xenobiotics. Loss of mitochondrial function and, more importantly, the number of mitochondria can have disastrous effects on cells (Dyken and Will, 2007). Because of the vital role of mitochondria in energy production, loss of mitochondria is extremely detrimental to target cells and tissues with the greatest energy demands. Mitochondrially rich/dependent tissues are often the most sensitive target of mitochondrial toxicants. Whereas mitochondrial toxicity can be assessed in tissues by functional assays or copy number assessments, reliable systemic biomarkers of mitochondrial toxicants are still needed to assess mitochondrial toxicity *in vivo*. This is particularly true in the clinical setting where humans are exposed to drugs, chemicals, or other xenobiotics that may be toxic to mitochondria. Detection of mitochondrial toxicity has been particularly difficult because the effects are often delayed until after depletion of mitochondria and/or mitochondrial function as most

cells can tolerate this condition until their minimal capacity or energy demand is reached (Picard et al., 2013). In addition, there are a number of diverse mechanisms that may be involved in mitochondrial toxicities including perturbations in lipid metabolism, oxidative phosphorylation, membrane integrity, proton gradient, and oxidative stress (Pereira et al., 2009a, b).

The aims of this chapter are to review the current work testing microRNA (miRNA) biomarkers of mitochondrial toxicity, to provide background information demonstrating the potential usefulness of mitochondrial miRNA (mitomiR) as biomarkers, to provide information guiding readers in assessing miRNA changes associated with mitochondrial toxicity in susceptible tissues, and to discuss current gaps in knowledge that need to be filled to use miRNA to their potential as biomarkers of mitochondrial toxicity. Presently, the science of mitomiR and their use as biomarkers of mitochondrial toxicity is in its infancy. Despite the description of many miRNAs that are associated with both toxicity and mitochondrial function, few efforts have focused on mitomiR changes

associated with mitochondrial toxicity and known mitochondrial toxicants. miRNAs have been isolated within mitochondria that are of both nuclear and mitochondrial in origin, and miRNA-targeting mitochondrial genes from both mitochondrial and nuclear genomes have been identified, demonstrating the importance and complexity of miRNA in the regulation of mitochondrial genes. Many tissue-specific or tissue-enriched miRNAs have also been found with links to mitochondrial function. This suggests the potential for miRNA markers of mitochondrial function that are tissue specific, ideally helping to monitor mitochondrial toxicity, in specific tissues of interest, or eluding to the target tissues where mitochondrial toxicity is occurring. There is still much to discover about miRNAs and their role in regulating mitochondrial function and compensatory actions due to mitochondrial toxicants. miRNAs have many chemical and physical properties that make them particularly appealing for use as biomarkers. These topics will be discussed in detail among others.

22.2 MicroRNAs: General

miRNAs are single-stranded, short noncoding RNAs that post-transcriptionally regulate the expression of target genes and that were first discovered in *Caenorhabditis elegans* by Lee et al. (1993). Biogenesis of the majority of miRNAs involves a standard canonical pathway that has been discussed in detail by several excellent reviews (Macfarlane and Murphy, 2010; Borralho et al., 2014; Ha and Kim, 2014; Makarova et al., 2016). In brief, miRNA biogenesis begins with transcription by RNA polymerase II to generate transcripts of varying lengths (generally 100 to 1000 nucleotides), forming hairpin-like structures with flanking ssRNA regions containing 5' cap and 3' poly A (adenosine) tails called primary miRNAs (pri-miRNAs) (Cai et al., 2004; Han et al., 2006). Pri-miRNAs (within the nucleus) are further processed by the microprocessor complex containing Drosha, RNase III endonuclease and its cofactor, and DiGeorge syndrome critical region gene 8 (DGCR8) to produce (cleaved at the stem) 60–70 bp hairpin-containing precursor miRNA (pre-miRNA) (Gregory et al., 2004; Borralho et al., 2015). Exportin-5 binds to the two nucleotide length 3' overhang in pre-miRNA and exports them from the nucleus to the cytoplasm in a Ran guanosine triphosphate (Ran-GTP)-dependent manner (Yi et al., 2003; Bohnsack et al., 2004). In the cytoplasm, an RNase III enzyme (Dicer), facilitated by RNA-binding proteins such as transactivation response RNA-binding protein (TRBP) or protein kinase R-activating protein (PACT), processes pre-miRNA further to produce double-stranded (hairpin-less) duplexes of miRNA (~18–25 nucleotides) that are

bound to AGO proteins (AGO1–4) with the help of chaperones (HSP70–90) to form the pre-RNA-induced silencing complex (pre-RISC) (Lee et al., 2006; Graves and Zeng, 2012; Srinivasan and Das, 2015; Makarova et al., 2016). In the pre-RISC complex, one strand of miRNA duplex (guide strand) is loaded with an AGO protein as mature miRNA and the other strand (passenger strand) is removed and degraded in the cytoplasm (Borralho et al., 2014) to form the mature RISC. In addition, alternative noncanonical pathways are also associated with the biogenesis of some miRNAs, which includes processing of de-branched introns to associate with the miRNA processing complex without cleavage by RNA III endonuclease DROSHA (mirtrons) or without other components of miRNA biogenesis (simtrons) (Meister, 2013; Borralho et al., 2014).

Mature miRNA, within the RISC complex, binds to a target mRNA sequence with partial complementarity (in most cases) base pairing to 3'UTR regions and induces post-transcriptional gene silencing by inhibiting translation or facilitating mRNA degradation via deadenylation and decapping (Lee et al., 2004; Carthew and Sontheimer, 2009; Fabian and Sonenberg, 2012; Li et al., 2012a; Bandiera et al., 2013; Bienertova-Vasku et al., 2013). AGO2 is an effector protein in the RISC complex, and GW182 is its key partner that mediates recruitment of deadenylating/decapping enzymes targeting mRNA to generate unprotected mRNA. The resulting unprotected mRNA gets cleaved by exonucleases or has inhibited translation due to binding of the cap-binding protein eIF4E or interfering with ribosomal mRNA scanning (Czech and Hannon, 2011). Recent studies also suggest that miRNA interference happens through the binding of miRNA to 5' UTR or open reading frames (ORFs) of their target mRNA sequence (Moretti et al., 2010). miRNA-mediated increases in gene translation have also been reported in certain instances (Vasudevan et al., 2007; Zhang et al., 2014).

Each miRNA may regulate multiple target genes (Duarte et al., 2014), and their roles are often associated with several key cellular processes including proliferation and/or apoptosis and differentiation (Tetreault and De Guire, 2013). miRNAs have often been estimated to regulate the expression of dozens of genes and in some cases may be as many as hundreds of genes (Qiao et al., 2013). Hence dysregulation or abnormal expression of miRNAs may contribute to cellular or organ dysfunction associated with toxicity or disease conditions (Nunez-Iglesias et al., 2010; Melman et al., 2014; Vliegenthart et al., 2015). In addition, miRNAs may play a compensatory role in affected cells to correct for lost or diminished function due to toxicity or disease conditions (Jacovetti et al., 2012; Plaisance et al., 2014). Understanding miRNA changes is likely a key to understanding the pathogenesis of many

Table 22.1 miRNA databases.

1	MicroRNA.org	For experimentally observed expression patterns as well as predicted microRNA targets and target down regulation scores
2	miRDB.org	For miRNA target prediction and functional annotations
4	miRGen 2.0	For microRNA genomic information and regulation
5	miRNAmap	Collection of experimentally verified microRNAs and miRNA target genes in multiple species
6	TargetScan	For prediction of miRNA biological targets by searching for the conserved 8mer, 7mer, and 6mer sites matching the seed region of miRNAs
7	miRWalk2.0	Comprehensive atlas of predicted and experimentally verified miRNA-target interactions

disease conditions such as cardiovascular diseases, obesity, diabetes, mental disorders, and cancers, in addition to mechanisms of various toxicities (Ali et al., 2011).

The mitochondrial genome is also a well-established target for miRNA in multiple tissues and species. Both computational modeling (Barrey et al., 2011) and experimental studies (Das et al., 2012) have demonstrated the presence of miRNA target sequences in the mitochondrial genome. Several publically available databases have also been established to identify the known functions of miRNA. They serve as an excellent resource for identifying the potential function of miRNA biomarkers, including those related to mitochondrial functions. Table 22.1 contains several online miRNA database resources.

22.3 Properties of miRNA: Useful Biomarkers

Traditional biomarkers of toxicity, such as those for cardiac and skeletal muscles, have limitations due to either lack of tissue specificity or short half-life (Calvano et al., 2016). Hence miRNAs with their long half-life, high stability, and higher tissue specificity may be ideal for use as potential sensitive biomarkers of these tissue toxicities. miRNAs are currently being used more frequently as translational biomarkers of tissue injury in cases where they are conserved between species (Pasquinelli et al., 2000).

In general, miRNAs are more stable and persist more readily in the systemic circulation than other amplifiable biomarkers such as mRNA (Seyhan, 2015). Evolutionarily, this may be partially due to their important regulatory

role in gene expression. Several studies have shown the presence of miRNA in a number of body fluid matrices including blood, urine, saliva, milk, tears, and bile (Turchinovich and Cho, 2014). Indeed, miRNAs are routinely found constitutively in the circulation in exosomes.

Tissues under the effects of drugs and xenobiotics undergo changes in gene expression and regulation that may be compensatory for deleterious effects in order to re-establish equilibrium (Jacovetti et al., 2012). These perturbations can be measured as changes in proteins, metabolites, or gene expression, which may all serve as biomarkers of toxic effects from drugs. miRNAs are also modulated in response to toxic insults, as key regulators of gene expression, and are often released in the systemic circulation or other body fluids (Nassirpour et al., 2015; Vliegenthart et al., 2015; Sharapova et al., 2016). Additionally miRNAs are released as a consequence of cell/tissue lysis due to necrosis. In these two ways, they can serve as systemic biomarkers of tissue dysfunction, damage, and loss.

Due to the unique sequence of each 18–25mer, miRNA can be easily and conclusively identified via RT-PCR, miRNA microarrays, or next-generation sequencing (RNA-Seq). The ability to identify and differentiate between individual miRNA affords strong specificity to their detection. As more miRNA functions are elucidated, the identification of specific mechanisms of toxicity/dysfunction associated with these will be more easily accomplished as well. This may also allow for the identification of specific tissues of injury in some cases. Alterations in miRNAs involved in mitochondrial function specifically are expected with drugs and xenobiotics that affect mitochondrial function or cause direct toxicity and loss of mitochondria. Changes in miRNA due to drug treatments and changes related to drug-mediated toxicities are well described (Cao et al., 2015; Harrill et al., 2016). Detection of miRNA is amenable to highly sensitive methodologies that involve the amplification of miRNA transcripts (Brustoloni et al., 2007; Muniesa et al., 2014). Because of this, the detection of very small amounts of miRNAs is possible. The sensitivity of miRNA changes may be, in some cases, sensitive enough to precede the detection of lesions by histology methods (Baumgart et al., 2016). This property makes miRNA particularly appealing as candidate biomarkers of mitochondrial toxicity. Typical PCR-based methods (as for miRNA detection) are highly quantitative (Brustoloni et al., 2007; Muniesa et al., 2014), giving researchers the opportunity to make accurate measurements of miRNA levels in tissues and body fluids. When these assessments are for safety biomarker use, accuracy is critical for confidence in the observed changes. Some methods such as RT-PCR or RNA-Seq can accurately estimate the precise copy number of transcripts of interest under some conditions.

22.4 Mitochondria and miRNAs

In the classical pathway of miRNA gene regulation, miRNA activity is located mainly in the cytosolic compartment within RNA-rich granules called processing bodies (P bodies) and chromatoid bodies (in male germ cells), which are the major sites of localization for miRNAs with RISC complex components (Hutvagner and Zamore, 2002; Kotaja et al., 2006). However, increased understanding of the role of miRNAs has suggested the involvement of several different organelles/intracellular components in miRNA localization including the nucleus (Jeffries et al., 2011; Chen et al., 2012a), exosomes (Mittelbrunn et al., 2011), endosomal membranes (Gibbings et al., 2009), and mitochondria (Dasgupta et al., 2015). An association between P bodies and mitochondrial membranes has been described and suggests a potential role of mitochondria in miRNA processing/activity as well (Huang et al., 2011). Interestingly, disruption of the RISC complex with release of AGO2 protein from P bodies is associated with increased mitochondrial membrane permeability and strongly supports an association of mitochondria with P bodies and miRNA activity (Huang et al., 2011). The presence of P body markers such as YB-1 in mitochondria further supports the idea that mitochondria are involved in miRNA signaling/processing (de Souza-Pinto et al., 2009).

Mitochondrial miRNAs (which are transcribed from either the nuclear or mitochondrial genomes) are

referred as mitomiRs. They have been identified within the mitochondria in multiple studies (Kren et al., 2009; Bian et al., 2010; Huang et al., 2011), and nuclear-encoded mitomiRs have been shown to be processed within the mitochondria to mediate post-transcriptional regulation of mitochondrial-encoded proteins as well. Interestingly, different cell types appear to harbor different sets of mitomiRs. Multiple miRNAs have been identified in purified mitochondrial fractions isolated from various tissues and cell types including the liver, skeletal muscle, heart, HEK 293 cells, and HeLa cells as listed in Table 22.2. Tissue-specific miRNA sequencing analyses are useful in understanding the differential expression of miRNAs in different tissue/cell types. For example, systematic analysis of small RNAs, including miRNA associated with mitochondria by subcellular fractionation and deep sequencing analyses, demonstrated differential expression of the majority of miRNAs in HEK293 and HeLa cells (Sripada et al., 2012). In the same study, a total of 28 miRNAs were aligned to positions of 16S rRNA and tRNA and subunits of complex I in mitochondrial genome. The results of other targeted approaches using tissue-specific microarray analyses also suggest the presence of cell- and tissue-specific mitomiRs. For example, miR-181c has been identified as a cardiac-specific mitomiR in rats (Das et al., 2012) in this way. In addition, a unique set of mitomiRs were identified in human skeletal muscle (Barrey et al., 2011) and HeLa cells (Bandiera et al., 2011).

Table 22.2 Mitochondria-associated miRNAs in cells/tissues.

Tissue/cell type	MiRNA	Reference
Myotubes, human	miR-720, miR-133b, miR-1974, miR-24, miR-133a, miR-125a-5p, miR-1979, miR-103, miR-125b, miR-103, miR-221, miR-23a, let-7b, miR-423-3p, miR-106a, miR-23b, miR-92a, miR-193b, miR-365, miR-93	Bandiera et al. (2011)
Liver, rats	miR-130a, miR-130b, miR-140*, miR-320, miR-494, miR-671, miR-202, miR-705, miR-709, miR-721, miR-761, miR-763, miR-198, miR-765	Kren et al. (2009)
Liver, mouse	miR-122, miR-805, miR-690, miR-689, miR-494, miR-705, miR-721, miR-720, miR-188-5p, miR-101, let-7f, miR-711, miR-432, miR-181b, miR-361-5p, miR-680, miR-181d, miR-29c, miR-29a, miR-762	Bian et al. (2010)
HeLa cells	miR-1973 miR-1275 miR-494 miR-513a-5p miR-1246 miR-328-5p miR-1908, miR-1972 miR-1977 miR-638 miR-1974 miR-1978 miR-1201	Bandiera et al. (2011)
HEK293 cells	hsa-miR-10a, hsa-miR-128, hsa-miR-1307, hsa-miR-140-3p, hsa-miR-185, hsamiR-196a, hsa-miR-25, hsa-miR-320a, hsa-miR-330-3p, hsamiR-340, hsa-miR-423-5p, hsa-miR-629, hsa-miR-744	Sripada et al. (2012)
HeLa cells	let-7i, hsa-miR-181b, hsa-miR-21, hsa-miR-23a, hsa-miR-29a, hsamiR-30a, hsa-miR-31, hsa-miR-452	Sripada et al. (2012)
Heart, mouse	21a-5p, 125b-5p, 128-3p, 151-3p, 203-3p, 212-3p, 22-3p, 23a-3p, 27a-3p, 27b-3p, 320-3p, 1932, 1199-5p, 5108, 375-3p, 3963, 341-3p, 342-3p, 148a-3p, 200c-3p, let7a-5p, 300-3p, let-7d-5p, 181b-5p, 5112, 203-3p, 423-3p, 378-5p, 2137, 5131, 26a-5p, 5119	Jagannathan et al. (2015)
Cardiomyocytes, rat	MiR-181c	Das et al. (2012)

The first evidence of an association of mitochondria with miRNA and RNA interference component proteins came from a study showing the specific association of mitochondrial tRNA(Met) with effector proteins of RNA interference such as AGO2 (Maniataki and Mourelatos, 2005). The identification of pre-miRNAs in mitochondria further suggests that mitochondria are involved in the processing of pre-miRNA sequences locally within the mitochondria to produce mature miRNA and regulate transcripts within the mitochondria (Barrey et al., 2011). In addition to miRNA and pre-miRNA, the detection of effector proteins of miRNA machinery such as Argonaute and Dicer confirms the presence of active miRNA processing and post-transcriptional gene silencing machinery inside mitochondria (Bandiera et al., 2011; Wang et al., 2015b). Zhang et al. (2014) provided direct evidence for the presence of AGO2 within the mitochondria using highly purified mitochondria and as well as mitoplasts (selectively removed outer mitochondrial membrane) by digitonin treatment. However GW182, a key partner of the AGO2 protein, appears to be absent in mitochondria (Zhang et al., 2014), suggesting that the RNA silencing machinery in mitochondria may be somewhat different from the cytoplasmic machinery.

The association of miRNA with mitochondria suggests several potential roles of mitochondria in regulating miRNA functions. Some miRNAs are highly enriched in the mitochondria compared with the cytoplasm, suggesting distinctive miRNAs are associated with the modulation of mitochondrial function (Bian et al., 2010). It has also been hypothesized that mitochondria may serve as storehouses for miRNA to supply them to the cytosol or as a vehicle to deliver miRNAs to other intracellular organelles such as the endoplasmic reticulum and P bodies on demand (Wang et al., 2015b). Mitochondria may also serve as a platform for the interaction of the RNAi components. The association of nuclear-encoded miRNAs and effector proteins of RNAi (AGO2) with the outer membrane of mitochondria is reported in multiple studies and suggests that mitochondria may serve as an anchor for miRNA signaling (Bandiera et al., 2011; Sripada et al., 2012). The mitochondrial uncoupler, carbonyl cyanide *p*-chlorophenylhydrazone (CCCP), induced mitochondrial morphology changes and significantly reduced RISC assembly and miRNA-mediated gene silencing, suggesting that mitochondrial toxicity/dysfunction may contribute for RNAi defects as well (Huang et al., 2011).

Substantial evidence also suggests that the mitochondrial genome is a source of mitomiRs (Sripada et al., 2012; Shinde et al., 2015). Some mitomiRs that originate from mtDNA (mitochondrial genome) appear to directly regulate mitochondrial transcripts (Bandiera

et al., 2013). In humans and mice, the production of small noncoding RNA sequences has been identified specifically from tRNA loci from within the mitochondrial genome (Mercer et al., 2011; Smalheiser et al., 2011). Pre-miRNA (pre-miR-let7b and pre-miR-302a) and miRNA (miR-1974, miR-1977, and miR-1978) sequences have also been shown to align within the human mitochondrial genome (Bandiera et al., 2011; Barrey et al., 2011). Bioinformatic analyses of the human mitochondrial genome in skeletal muscle predicted six novel miRNA sequences (hsa-miR-mit-1 to 6) (Shinde and Bhadra, 2015). Experimental studies further confirmed the localization of these six miRNAs within a highly purified human skeletal muscle mitochondrial fraction.

The influence of miRNAs on mitochondrial function is attributed mainly to three different routes of activity (Bandiera et al., 2013): (i) nuclear-encoded miRNAs may act in cytosol to cause posttranslational silencing of target mRNAs, which code proteins of mitochondrial function, (ii) nuclear miRNAs get translocated into mitochondria to regulate translation of genes within the mitochondria, and (iii) miRNAs encoded by mitochondrial genome act locally to regulate the translation of mitochondrial proteins inside mitochondria. In addition to translational repression, a primary effect of miRNAs (including presumably mitomiRs) is positive effects on translation in mitochondria (Lee and Vasudevan, 2013). For example, nuclear-encoded miR-1, which gets induced during muscle differentiation, is shown to enter mitochondria and enhance translation of specific mitochondrially encoded transcripts of the ND1-containing complex I and COX1-containing complex IV (Zhang et al., 2014). In the cytoplasm, however, miR-1 represses the expression of nuclear-encoded targets HDAC4 and elongation factor for RNA polymerase II 2 (ELL2), suggesting a different mechanism of regulation in the cytosol versus mitochondria (Chen et al., 2006; Zhang et al., 2014). Even though a clear mechanistic understanding of miRNA-enhanced translation in the mitochondria is not known, the lack of cap or typical long poly(A) tail in mitochondrial transcripts, and rearranged miRNA machinery without GW182 (a key partner of AGO2 protein in the mitochondria), has been proposed as a potential mechanism (Zhang et al., 2014).

22.5 miRNA Transport in the Mitochondria

The majority of mitomiRs are encoded in the nuclear genome and translocated into mitochondria. The first direct evidence that some miRNAs encoded in the

nuclear genome are translocated into the mitochondria came from a study showing miRNAs in mtDNA-less cell mitochondria (Dasgupta et al., 2015). 206p° cells are 143B-derived human osteosarcoma cells treated with ethidium bromide that contains no mitochondrial genome, and hence there is no transcription of mitochondrial RNAs in these cells. Next-generation sequencing analyses of the miRNA profile showed enrichment of multiple miRNAs including miR-181c-5p and miR-146a-5p in 206p° cells. Several other researchers have also confirmed the import of nuclear genome encoded miRNAs into the mitochondria (Bandiera et al., 2011; Mercer et al., 2011). In fact, some miRNA appear to localize preferentially in specific regions within the mitochondria as well. For example, miR-146a, miR-103, and miR-16 are reported to be preferentially localized within the intermembrane space of human mitochondria (Mercer et al., 2011). The biological significance of miRNA localization within the mitochondria is not currently well understood.

Das et al. (2012) reported the first evidence of mitochondrial transcripts serving as a target for nuclear-encoded miRNAs, demonstrating post-transcriptional regulation of mt-CO1 by miR-181c (Das et al., 2012). Import of miR-181c into the mitochondria, and post-transcriptional repression of its target gene COX1, was confirmed in mitochondria isolated from rat heart (Das et al., 2014). Exactly how these miRNAs get transported into the mitochondria is still under ongoing debate. Several hypotheses regarding mechanism have been proposed including the import of noncoding RNA via a protein import system. Passive transfer of miRNAs into mitochondria has been generally ruled out considering their size and charged nature (Geiger and Dalgaard, 2017). Translocation of nuclear-encoded miRNAs from the nucleus into the mitochondria appears to involve an ATP-dependent import mechanism(s), which may also vary in species-specific manner (Tarassov et al., 2007; Ballatore et al., 2013). The AGO2 protein may act as a carrier protein trafficking miRNA into mitochondria (Bandiera et al., 2011), or import may involve mitochondrial membrane channels, translocase of the outer membrane (TOM), or translocase of the inner membrane (TIM) complex(es) (Bandiera et al., 2011; Srinivasan and Das, 2015). In addition, AGO2-independent pathways possibly involving exoribonuclease polyribonucleotide nucleotidyl transferase (PNPT1/ PNPASE) of the mitochondrial intermembrane space (Wang et al., 2010), porins, and/or voltage-dependent anion-selective channel protein (VDAC) (Bandiera et al., 2013) have also been reported. It has also been hypothesized that there is a close association of mitochondria with RISC complex components such as P bodies and the endoplasmic reticulum, which may facilitate translocation of miRNA and

AGO2 protein into mitochondria (Huang et al., 2011; Makarova et al., 2016).

The results of an interesting study have shown localization of miRNA, AGO2/3 protein, and target mRNA in the outer mitochondrial membrane (Sripada et al., 2012). This finding suggests a possible role of the mitochondrial outer membrane as a platform for assembly of the RISC complex and site of regulation of intracellular protein levels (Sripada et al., 2012) but may also be related in some way to miRNA internalization.

Figure 22.1 outlines the biosynthesis, transport, and processing of miRNAs.

22.6 miRNA Secretion

Several active mechanisms have been proposed for the release/secretion of miRNAs from cells into the circulation, and miRNAs also appear to be released (leaked) passively from cells with damaged membrane integrity during the process of cell apoptosis or necrosis (Chen et al., 2012b). The active secretion of miRNAs has been reportedly associated with various extracellular vesicles (mainly exosomes and microvesicles), apoptotic bodies (Zernecke et al., 2009), or in association with RNA-binding proteins (microvesicle-free) such as high-density lipoproteins (HDL) (Vickers et al., 2011) and AGO proteins (Turchinovich et al., 2011). Among these secretion routes, the exosome pathway is the most studied and is generally considered to be the main pathway. The biological significance of secreted miRNAs is an interesting and growing focus of research. Extracellular miRNAs appear to be transferred between cells mainly through exosomes released from parental cells and are also functional in association with RNA interference machinery in recipient cells (Valadi et al., 2007; Chiba et al., 2012; Montecalvo et al., 2012). Hence, exosomes (with miRNAs) are getting increased attention as a mode of intercellular signaling/communication (Zhang et al., 2015).

miRNA profiling studies have revealed sequence-specific sorting mechanisms in parental cells to guide selected miRNAs into exosomes. For example, the preferential entry of miR-150, miR-142-3p, and miR-451 into exosomes has been shown in multiple cell lines (Guduric-Fuchs et al., 2012). The let-7 miRNA precursor is abundant in exosomes derived from the gastric cancer cell line AZ-P7a compared with exosomes derived from lung and colorectal cancer cell lines (Ohshima et al., 2010). In addition, significantly higher levels of miR-21 were reported in exosomes from serum samples of glioblastoma patients compared with healthy individuals (Skog et al., 2008). Interestingly, miR-21 is a mitomiR involved in mitochondrial redox regulation (Chau et al., 2012). Recently, a role for the hnRNPA2B1 protein, which

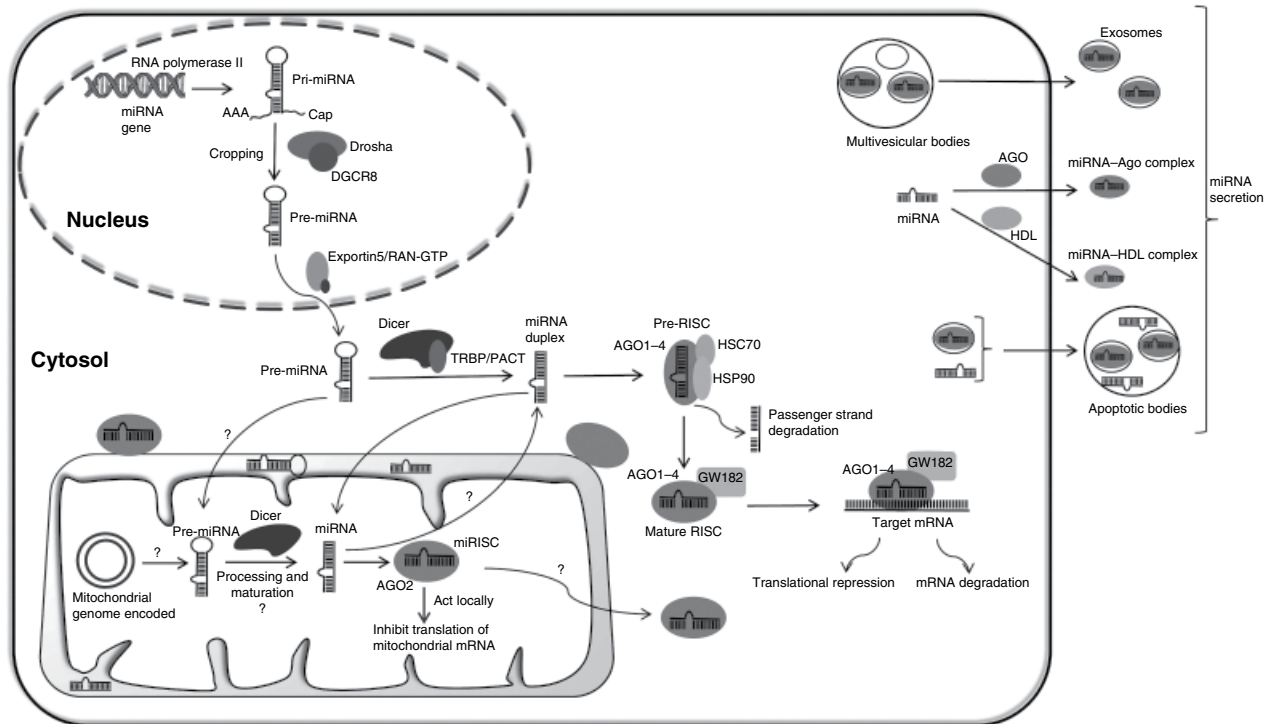


Figure 22.1 Biosynthesis of miRNAs: In the canonical pathway, miRNA biogenesis begins in the nucleus with transcription by RNA polymerase II into larger primary transcripts with hairpin-like structure called pri-miRNAs, which are further cleaved by a microprocessor complex (type III RNase, Drosha, and its cofactor DGCR8) into pre-miRNA. Exportin-5 binds to the 2 nucleotide length 3' overhang in pre-miRNAs and exports them from nucleus into the cytoplasm in a Ran guanosine triphosphate (Ran-GTP)-dependent manner for further processing. Once in the cytoplasm, pre-miRNAs get further cleaved by Dicer and processed into double-stranded miRNAs. These miRNA duplexes are bound to AGO proteins (AGO1–4) with the help of chaperones (HSP70–90) to form a multi-protein complex called the pre-RNA-induced silencing complex (pre-RISC). In the pre-RISC complex, one strand of the miRNA duplex (guide strand) is loaded onto AGO proteins as mature miRNA and other strand (passenger strand) gets removed. Mature miRNA (guide strand) within RNA-induced silencing complex (RISC) binds to target mRNA sequences and induces post-transcriptional gene silencing by inhibiting translation or facilitating mRNA degradation via deadenylation and decapping. Either pre-miRNA or miRNA (with AGO-2 protein or separately) is transported into the mitochondria (mechanisms not clearly understood). Once inside the mitochondria, nuclear-encoded pre-miRNA get further processed into mature miRNA (with the addition of an acetyl group), which acts locally inside mitochondria to regulate mRNA expression or for transport into cytosol to act on nuclear-encoded target mRNA. In addition to nuclear-encoded miRNA, miRNAs may also derive from mitochondrial genome and be processed similarly to act locally within the mitochondria or to get transported to cytosol. How mitochondrial-encoded miRNAs are transported into cytosol is still largely unknown. miRNAs also get secreted into circulation (extracellular fluids). Several pathways for miRNA secretion have been proposed. MiRNAs mainly get secreted in association with extracellular vesicles including exosomes or with apoptotic bodies in cells undergoing death. In addition, miRNA secretion also happens in association with RNA-binding proteins such as high-density lipoproteins (HDL) or AGO2 proteins. MiRNAs likely also get released (leaked) passively from cells with damaged membrane integrity during the process of cell apoptosis or necrosis.

specifically recognizes the GGAG sequence in miRNA, has also been associated with the transport of miRNAs into exosomes (Villarroya-Beltri et al., 2013). Conditions of stress, due to either disease or toxicity, affect the expression of miRNA as well as the localization of miRNA in mitochondria (Bian et al., 2010). However, how miRNAs may be released from the mitochondria into cytosol for excretion into circulation is not yet known. Changes in exosomal miRNA profiles with toxicity or disease, aligned with the ability to identify the origin of exosomes from specific tissues or cells using specific membrane proteins, will allow miRNA to be used more effectively as potential noninvasive

biomarkers for organ dysfunction (Gross et al., 2012; Zhang et al., 2015).

Even though the biological significance of circulating miRNAs is not completely defined, the recent discovery of sequence-specific sorting of miRNAs into extracellular vesicles such as exosomes (Villarroya-Beltri et al., 2013) clearly suggests a role of these circulating miRNAs in tissue fluids and lymph for intercellular communication. Several *in vitro* studies have reported the transfer of miRNA containing exosomes from donor cells and post-transcriptional regulation of genes in recipient cells (Collino et al., 2010; Penforis et al., 2016). There is also evidence suggesting that the RNA-binding protein,

AGO2, is involved in miRNA transport as a functional complex (independent of vesicles) in the circulation (Arroyo et al., 2011). Nucleophosmin, another RNA-binding protein, has also been shown to carry miRNA into serum to mediate intercellular communication (Wang et al., 2010). miRNAs associated with exosomes or RNA-binding proteins can enter recipient cells and regulate the genes of recipient cells just as endogenous miRNAs do. For example, miR-146A secreted from donor cells (COS-7 cells, a monkey kidney fibroblast-like cell line) is shown to be taken up by prostate cancer (PC-3M-luc) cells to post-transcriptionally suppress ROCK1 expression, a target gene for miR-146a (Kosaka et al., 2010). The mechanism of uptake of secreted miRNAs into recipient cells is also incompletely understood. Immature miRNAs also appear to be secreted into circulation. In cultured mesenchymal stem cells, for example, miRNA-mediated intercellular communication has been associated with the secretion of pre-miRNA-enriched microparticles and uptake by recipient cells (H9C2 cardiomyocytes) to form mature-miRNA/RISC complexes within the target cells (Chen et al., 2010). The significance of these *in vitro* findings has not yet been established *in vivo*, but the implications for intercellular communication via miRNA and pre-miRNA are exciting. Identification of extracellular circulating miRNAs in plasma (Lawrie et al., 2008), as well as in other mammalian body fluids (Weber et al., 2010), has spawned a great interest in the potential use of miRNAs as noninvasive biomarkers of diseases or toxicity.

22.7 miRNAs Associated with Mitochondrial Function

A dynamic relationship has been reported between critical mitochondrial functions and the activity of multiple miRNAs (Bian et al., 2010; Bandiera et al., 2011; Barrey et al., 2011; Mercer et al., 2011; Sripada et al., 2012; Shinde and Bhadra, 2015). Several miRNAs have been identified in mitochondria, and their dysregulation/alteration has been closely correlated with mitochondrial dysfunction. Indeed, miRNAs have been associated with a wide variety of dysfunctions in mitochondria including mitochondrial metabolism, apoptosis, fusion, fission, and mitophagy.

22.7.1 Energy Metabolism/Respiration

Mitochondrial energy metabolism, which involves oxidative phosphorylation and ATP production, is critical for providing the cell with the energy to perform biological processes. Impaired mitochondrial energy metabolism is associated with multiple neurodegenerative and

metabolic diseases (Sivitz and Yorek, 2010; Hroudova et al., 2014). The involvement of miRNAs in the regulation of mitochondrial oxidative phosphorylation (respiratory chain) genes is of special significance. Because tissue-specific differences exist in the composition of the oxidative phosphorylation system (Benard et al., 2006; Johnson et al., 2007), it is possible that the differential expression of mitomiRs accounts, at least partly, for tissue-specific differences in respiratory chain activity.

In obese mice fed with a high fat diet, mitochondrial dysfunction eventually results in skeletal muscle disorders. Mechanistically, decreased mitochondrial function and biogenesis in obese mice are associated with low levels of miR-149, high levels of the miR-149 target gene PARP-2, and decreased activation of the SIRT-1/PGC-1 α pathway (Mohamed et al., 2014). MiR-15b, miR-16, miR-195, and miR-424 are reported to target ADP-ribosylation factor-like 2 (ARL2), and the increased expression of these miRNAs is associated with decreased mitochondrial ATP production in rats (Nishi et al., 2010; Tomasetti et al., 2014). In addition, increased expression of both miR-15b and miR-195 is associated with ATP reductions and mitochondrial degeneration in rat cardiomyocytes (Duarte et al., 2014). Similarly, many other miRNAs are associated with effects on mitochondrial ATP production through effects on the electron transport system in various species and tissue/cell types. For example, miR-181c (with its target mt-COX1 gene) in rat cardiomyocytes (Das et al., 2012), miR-338 (with its target COX IV gene), has been associated with altered mitochondrial function in rat neurons (Aschrafi et al., 2012) and miR-210 (with its target COX 10 and succinate dehydrogenase subunit D (SDHD) genes; components of complexes IV and II, respectively) with altered mitochondrial function in human fibroblasts (Colleoni et al., 2013).

22.7.2 Mitochondrial Dynamics

Mitochondrial turnover and dynamics, which mainly involve fusion, fission, and mitophagy, are critical for maintaining cellular homeostasis and differentiation (Duarte et al., 2014). Mitochondrially rich cells such as cardiomyocytes are especially sensitive to changes in mitochondrial dynamics. Interestingly, changes in the expression of several miRNAs are associated with mitochondrial dynamics. For example, increased expression of miR-761 downregulates its target gene, mitochondrial fission factor (MFF), and suppresses the mitochondrial fission process (Long et al., 2013). MiR-30 is associated with apoptosis through modulation of the mitochondrial fission process and targeting of the p53 gene (Li et al., 2010).

MiR-484 targets the mitochondrial fission protein 1 (Fis1), and decreased levels of miR-484 are associated with increased Fis1 and mitochondrial fission and the

development of insulin resistance in diabetes mellitus (Yoon et al., 2011). MiR-140 promotes mitochondrial fission by targeting the mitochondrial fusion protein (Mitofusin 1) and contributing to apoptosis in cardiomyocytes (Li et al., 2014b). In Parkinson's disease, decreased expression of miR-34b/c is reported as an early event contributing to mitochondrial dysfunction and increased mitochondrial fission by targeting the DJ1 and Parkin proteins (Minones-Moyano et al., 2011).

Mitophagy is the removal of damaged mitochondria and is an essential intracellular quality control process for regulating mitochondrial abundance based on cellular metabolic or developmental stage demand (Kundu et al., 2008). MiR-101 (targeting STMN1, RAB5A, and ATG4D) (Frankel et al., 2011), miR-204 (targeting LC3-II protein) (Xiao et al., 2011), Mir-30a (targeting Beclin 1-protein) (Wang et al., 2014), and miR-137 (targeting FUNDC1 and NIX) (Li et al., 2014c) are all associated with inhibition of the mitophagy process.

22.7.3 Mitochondria-Mediated Apoptosis

Mitochondria play an important role in initiating the intrinsic apoptotic pathway. In response to apoptotic stimuli, proapoptotic molecules like Bax and Bad get translocated into mitochondria to increase mitochondrial membrane permeability and thereby promote the release of mitochondrial proteins (and possibly miRNAs) into the cytoplasm (Li et al., 2012b). Antiapoptotic proteins such as Bcl-2 and Bcl-xL get associated with proapoptotic Bax and Bad and prevent cell apoptosis (Cleland et al., 2011). miRNAs such as miR-15a and miR-16-1 promote apoptosis through regulating Bcl-2 and disrupting mitochondrial membrane potential (membrane permeability transition (MPT)) (Gao et al., 2010). Similarly, induction of the intrinsic apoptotic pathway through mitochondrial dysfunction is associated with miR-143 and miR-1 in human colorectal cancer DLD-1 cells (Nakagawa et al., 2007) and cardiomyocytes (Yu et al., 2008), respectively.

The following table (Table 22.3) contains miRNA associated with various mitochondrial functions, their corresponding target genes, and the models where they have been identified.

22.8 Mitochondrially Rich Tissues and Tissue-Specific miRNAs

Mitochondrial number generally varies from cell to cell and also between organisms. Tissues with high energy requirements such as the skeletal muscle, heart, kidney, and brain are richest in mitochondria (Bandiera et al., 2013).

A number of tissue-specific or -enriched miRNAs have been elucidated (Table 22.4). Several have been used successfully as biomarkers of toxicity of their host tissues. In addition, many of these have an association with mitochondrial function and could represent biomarkers of mitochondrial toxicity in specific organs with further study. Other tissue-specific miRNAs may be linked to mitochondrial regulation in the future. Our goal in this section is to outline many miRNAs that could qualify as future mitochondrial toxicity biomarkers. Assessing the expression of these miRNAs, within their respective tissues, in preclinical studies is likely to be the most sensitive approach to assessing toxicities, including mitochondrial toxicities. However, these may also offer the greatest potential for systemic biomarkers of mitochondrial toxicity in specific tissues. Some emphasis should be paid to the target tissues of defined mitochondrial toxicants as these can vary somewhat between toxicants. Tissue-specific or -enriched miRNA changes have great potential for use as novel safety biomarkers of acute or chronic injury in cell-free body fluids such as the plasma, serum, and urine as sensitive and tissue-specific biomarkers of multiple pathophysiological states (Salamin et al., 2016).

22.8.1 Heart

The heart (cardiac muscle) requires significantly higher energy than many other tissues and mainly depends on mitochondrial metabolism to generate this higher energy requirement (Lemieux and Hoppel, 2009). Hence cardiac tissue is highly enriched with mitochondria, and mitochondrial dysfunction is associated with multiple cardiovascular pathologies (Hatch, 2004). Gaining a better understanding of miRNA changes associated with dysfunction or toxicity of cardiac muscle is of great interest. MiR-208/miR208a, miR-1, miR-133a, and miR-133b have been reported to be heart-specific miRNA with possible utility as biomarkers (Nishimura et al., 2015). In rats, a comparison of serum levels of miRNA changes with conventional cardiac toxicity markers revealed that miR-208 levels increased in a cardiac lesion-specific manner and were detectable for a longer duration than conventional cardiac biomarkers such as cardiac troponin (cTn) and FABP3 (Calvano et al., 2016). In contrast to miR-208, which is reportedly cardiac muscle specific, MiR-133a/b is reported to be a sensitive biomarker for toxicity to both skeletal muscle and cardiac muscle (Calvano et al., 2016). Hence, the use of both these miRNAs in combination has been recommended for evaluation of toxicity to skeletal muscle and cardiac muscle. In a rat model of streptozotocin-induced diabetic cardiomyopathy, significant increases in miR-30d expression were reported. Increased miR-30d targets foxo3a

Table 22.3 List of miRNAs associated with mitochondrial functions.

miRNA	Function	Target	Model	Reference
miR-133a-1 and miR-133a-2	Mitochondrial metabolism	Dynamin 2	Mice	Liu et al. (2011)
miR-491-5p	Intrinsic mitochondria-mediated apoptosis pathways	TP53 and Bcl-XL	Human pancreatic adenocarcinoma cell lines	Guo et al. (2012)
miR-7	Regulates function of the mitochondrial permeability transition pore (MPTP)	VDAC1 (voltage-dependent anion channel 1)	Human neuroblastoma cells, C57BL/6J mice	Chaudhuri et al. (2016)
miR-15b	Mitochondrial apoptotic pathway	Bcl-2	Sprague Dawley (SD) rats, neonatal rat cardiomyocytes	Liu et al. (2014b)
miR-15b	Mitochondrial ATP production	ADP ribosylation factor-like 2 (Arl2)	Neonatal rat ventricular myocytes (NRVMs), Sprague Dawley rats	Nishi et al. (2010)
miR-21	Mitochondrial redox regulation, biogenesis	Mpv17-like	Mouse models of chronic kidney disease	Chau et al. (2012) and Gomez et al. (2016)
miR-22	Mitochondrial oxidative injury	Sirtuin-1 (Sirt1) and peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC1 α)	Sprague Dawley rats, H9c2 myocardial cell line	Du et al. (2016)
miR-27b	Mitochondrial biogenesis	miRNA-27b directly targets to Foxj3	Mouse C2C12 myoblasts (CRL-1772)	Shen et al. (2016)
miR-106b	Mitochondrial morphology, fusion	Mitofusin-2	Mouse C2C12 myoblasts	Zhang et al. (2013)
miR-122	Mitochondrial biogenesis/respiration	PPARGC1A, SDHA, and SDHB	Male C57BL/6 mice, human HCC patient samples	Burchard et al. (2010)
miR-125b	Mitochondrial apoptotic pathway	Multiple genes, including Bak1, Mcl1, and p53	Human cervical cancer HeLa cells, human prostate cancer PC3 cells	Zeng et al. (2012)
miR-532-3p	Mitochondrial fission	Apoptosis repressor with caspase recruitment domain (ARC)	Mice Cardiomyocytes	Wang et al. (2015b)
miR-125b-2	Mitochondrial apoptosis pathway	Undefined	Human glioblastoma tissues, primary glioblastoma cell cultures	Shi et al. (2012)
miR-145	Mitochondrial apoptotic pathway in heart challenged with oxidative stress	Bnip3	Mouse model of ischemia/reperfusion (I/R) injury, primary neonatal rat ventricular myocytes	Li et al. (2012b)
miR-149	Mitochondrial biogenesis	PARP-2	Male C57BL/6J mice C2C12 cells	Mohamed et al. (2014)
miR-181c	Mitochondrial morphology	Bcl2	Mouse myocardial cells	Wang et al. (2015a)
miR-210	Mitochondrial metabolism and ROS production	HIF-1 alpha	Human embryonic kidney (HEK293) cells, mouse embryonic fibroblasts	Mutharasan et al. (2011)
miR-210	Mitochondrial free radical response (metabolism) to hypoxia	Iron–sulfur cluster protein (ISCU), that is, HOXA1, HOXA9, and FGFR1	MCF7 and HCT116 cells	Favaro et al. (2010)
mir-214	Controlling Ca ²⁺ overload and cell death (activation of mitochondrial apoptosis)	Sodium/calcium exchanger 1 (Ncx1)	Mice	Aurora et al. (2012)
miR-494	Mitochondrial biogenesis	Mitochondrial transcription factor A and Forkhead box j3	C2C12 myoblasts, C57BL/6J mice	Yamamoto et al. (2012)

miR-761	Mitochondrial biogenesis	PGC-1a and the p38 MAPK signaling pathways	Mice, C2C12 myoblasts	Xu et al. (2015)
miR-378 and 378*	Mitochondrial metabolism and systemic energy homeostasis	MED13 and CRAT	Mice	Carrer et al. (2012)
miR-25	Mitochondrial Ca ²⁺ signaling	Mitochondrial calcium uniporter	HeLa, Hek293, HCT116, and RKO cells	Marchi et al. (2013)
miR-17*	Mitochondrial antioxidant enzymes	Manganese superoxide dismutase (MnSOD), glutathione peroxidase-2 (GPX2), thioredoxin reductase-2 (TrxR2)	Human prostate cancer cells	Xu et al. (2010)
miR-24	Mitochondrial apoptosis pathway	Bax	Primary cardiomyocytes from neonatal mouse hearts	Wang and Qian (2014)
miR-27	Mitochondrial fission (mitochondrial morphology changes)	Mitochondrial fission factor (MFF)	Human CHANG liver cells	Tak et al. (2014)
miR-30	Mitochondrial fission, apoptosis	p53, dynamin-related protein-1 pathway	Rat cardiac fibroblasts	Li et al. (2010)
miR-33	Mitochondrial respiration, energy metabolism	PGC-1 α , PDK4, and SLC25A25	Human macrophages	Karunakaran et al. (2015)
miR-34a	Mitochondrial function	Cytochrome <i>c</i>	Cerebrovascular endothelial cells	Bukeirat et al. (2016)
miR-141	Mitochondrial energy production	Mitochondrial phosphate carrier (Slc25a3) in the type 1 diabetic heart	Mice	Baseler et al. (2012)
miR-141-3p	Mitochondrial energy production	<i>PTEN</i>	HFD mice	Ji et al. (2015)
miR-151a-5p	Mitochondrial electron transport chain	<i>Cytb</i>	Humans	Zhou et al. (2015)
miR-181	Mitochondrial function, apoptosis	Bcl-2, Mcl-1, and Bim	Mouse primary astrocyte cultures	Ouyang et al. (2012)
miR-181c	Mitochondrial bioenergetics	COX1	Rats	Das et al. (2014)
miR-210	Mitochondrial respiration	Mitochondrial associated ISCU	Human placenta	Muralimanoharan et al. (2012)
miR-221/222	Mitochondrial apoptosis pathway	PUMA	Human glioblastoma cells	Chen et al. (2012b)
miR-326	Mitochondrial apoptosis pathway	Antiapoptotic Bcl-xL	Human platelets	Yu et al. (2015)
miR-335 and miR-34a	Mitochondrial antioxidative pathway	SOD2 and Txnrd2	Rats	Bai et al. (2011)
miR-361	Mitochondrial biogenesis and morphology	Prohibitin (PHB)	Mice, cardiomyocytes	Wang et al. (2015c)
miR-484	Mitochondrial fission and apoptosis	Fis1	Cardiomyocytes (mouse) and adrenocortical cancer cells	Wang et al. (2012a)
miR-497 and miR-302b	Mitochondrial apoptosis pathway	Bcl2 Protein and cyclin D2	SH-SY5Y cells	Yadav et al. (2011)
miR-499	Mitochondrial dynamics (fission)	Calcineurin and dynamin-related protein-1	Mice	Wang et al. (2011)
miR-761	Mitochondrial fission	Mitochondrial fission factor	Neonatal rat cardiomyocytes	Long et al. (2013)
miRNA-30c	Mitochondrial oxidative phosphorylation (OXPHOS)	Mitochondrial-encoded OXPHOS genes Cytb, COI, COII, and COIII	Mouse cardiomyocytes	Wijnen et al. (2014)

Table 22.4 Tissue-specific (enriched) miRNAs.

Tissue	miRNAs	References
Liver	miR-122, MiR-122a, miR-192, miR-101b, miR-148a, miR-15a, miR-193, -miR-194, miR-21, miR-720, miR-483, miR-92a	Baskerville and Bartel (2005), Liang et al. (2007), Wang et al. (2009), Jopling (2012), and Guo et al. (2014)
Brain	miR-9, miRNA-128, miR-125 a-b, miR-23, miR-132, miR-137, miR-139, miR-9, miR-124 a-b, miR-134, miR-135, miR-153, miR-219, miR-330, miR-199a, miR-199b, miR-214, miR-153, miR-137, miR-143, miR-99b, miR-125a, miR-125b, miR-31, miR-124, miR-129, miR-138, miR-218, miR-708, miR-128a, miR-128b, miR-186, miR-95, miR-149, miR-323, miR-330, miR-33a, miR-346, miR-93, miR-212, miR-128a/b	Baskerville and Bartel (2005), Landgraf et al. (2007), Liang et al. (2007), Choudhury et al. (2013), and Adlakha and Saini (2014)
Skeletal muscle	miR-206, miR-133b, MiR-1, MiR-133a, miR-134, miR-193a, miR-128a, miR-133b, miR-95, miR-208a	Beuvink et al. (2007), Liang et al. (2007), McCarthy (2008), Chen et al. (2009), Nielsen et al. (2010), and Guo et al. (2014)
Cardiac muscle/heart	miR-208, miR-302a, miR-302b, miR-302d, miR-302c, miR-367, miR-499, miR-1, miR-126, miR-208, miR-302d, miR-367, miR-133a, miR-133b, miR-133, miR-181c, miR-1192, and miR-883, miR-30	Liang et al. (2007), Chen et al. (2009), Li et al. (2010), Malizia and Wang (2011), Das et al. (2012), and Guo et al. (2014)
Kidney	miR-204, miR-215, miR-216, miR-200a, miR-196a, miR-196b, miR-10a, miR-10b, miR-146a, miR-30c, miR-204, miR-449c-5p and miR-449b-5p	Sun et al. (2004), Akkina and Becker (2011), Guo et al. (2014), Schena et al. (2014), and Ludwig et al. (2016)

expression and its downstream protein, apoptosis repressor with caspase recruitment domain (ARC), to promote pyroptosis (pro-inflammatory programmed cell death) (Li et al., 2014d).

Isoproterenol-induced cardiotoxicity is associated with mitochondrial dysfunction in cardiomyocytes (Mukherjee et al., 2015). In rats treated with isoproterenol, consistent elevations of plasma miR-208 levels were observed as early as 24 h post-dose, which is much earlier than significant increases in cTn concentrations, suggesting great promise as an early biomarker of cardiotoxicity. In addition, increased miR-208 was also observed, following repeated exposure of rats to isoproterenol, with chronic lesions also observed in rat hearts (fibrosis) (Nishimura et al., 2015). In a similar study, miR-208 was validated as a sensitive biomarker of isoproterenol-induced cardiac injury in superoxide dismutase-2 (*Sod2*(+/-) and C57BL/6J wild-type mice (Liu et al., 2014a).

Doxorubicin-induced cardiotoxicity has also been associated with mitochondrial toxicity (Ichikawa et al., 2014). Doxorubicin-induced cardiotoxicity (apoptosis of cardiomyocytes) is associated with upregulation of cardiac-specific miR-208, which targets GATA4. Therapeutic silencing of miR-208a restored GATA4 levels and also attenuated doxorubicin cardiotoxicity in Balb/C mice (Tony et al., 2015). Increased levels of miRNA-532-3p are also reported in doxorubicin-induced cardiotoxicity. MiRNA-532-3p targets apoptosis repressor with caspase recruitment domain (ARC) and regulates mitochondrial fission (Wang et al., 2015a). In addition, differential expression of several miRNAs with biological relevance

to cardiomyocyte function including miR-34a, miR-34b, miR-187, miR-199a, miR-199b, miR-146a, miR-15b, miR-130a, miR-214, and miR-424 is reported in doxorubicin-treated human cardiomyocytes (Holmgren et al., 2016). In a similar study, differential deregulation of multiple miRNAs including miR-187-3p, miR-182-5p, miR-486-3p, miR-486-5p, miR-34a-3p, miR-4423-3p, miR-34c-3p, miR-34c-5p, and miR-1303 was observed in human iPSC-derived cardiomyocytes during doxorubicin toxicity earlier than increases in other cytotoxicity markers such as lactate dehydrogenase (LDH) (Chaudhari et al., 2016). These results suggested great potential for the use of these miRNAs as sensitive early biomarkers of cardiotoxicity and in some cases the associated mitochondrial toxicity.

22.8.2 Kidney

The kidneys are a common target organ for xenobiotic-induced toxicities. miRNA changes associated with kidney injury are of great interest because urine can be easily analyzed for noninvasive biomarkers such as miRNAs. Kidney-specific miRNA changes are associated with renal dysfunction as well as toxicity. In rats, aristolochic acid I (AAI)-induced acute kidney injury (AKI) is associated with increases in plasma miR-21-3p preceding the increase in conventional kidney injury markers such as blood urea nitrogen (BUN) and creatinine (Pu et al., 2017). Hence, miR-21-3p has been suggested as a potential early biomarker for AKI in rats. Similarly, in a cisplatin-induced AKI rat model, significant increases in miR-146b were observed at a much earlier time point

than changes in creatinine and BUN levels. These results have been further confirmed under *in vitro* conditions with increased miR-146b following cisplatin treatment of kidney tubular epithelial cells (Zhu et al., 2016). In addition, enhanced cisplatin toxicity was observed in miR-155 deficient mice (Pellegrini et al., 2014).

In AKI, patient miRNA profiling in urine samples revealed significant increases in miR-21, miR-200c, and miR-423 levels and significant decreases in miR-4640 levels compared with non-AKI patients, suggesting potential application of these miRNAs as diagnostic as well as prognostic markers of AKI (Ramachandran et al., 2013). Increased miR-218 is associated with high glucose-related podocyte injury in a mouse diabetic nephropathy model. MiR-218 targets heme oxygenase to promote glucose-induced apoptosis of mouse podocytes (Yang et al., 2016). MiR-21 upregulation was reported in multiple chronic fibrotic renal disease and experimental rat and mouse models of diabetic nephropathy with a positive correlation between the severity of fibrosis and rate of decreased renal function (McClelland et al., 2015). MiR-21 targets PTEN and SMAD7 and promotes accumulation of extracellular matrix and renal fibrosis in diabetic nephropathy (McClelland et al., 2015). MiR-21 is considered a central regulator of metabolic activity in the kidneys. Increased expression of miR-21 is reported in both acute and chronic kidney diseases in animal models as well as in human kidney tissue samples (Zarjou et al., 2011; Kole et al., 2012). Upregulation of miR-21 suppresses multiple genes involved in mitochondrial biogenesis and promotes fibrosis and organ dysfunction in the kidneys (Gomez et al., 2016). MiR-155 and miR-146a levels are increased in diabetic nephropathy animal models as well as in diabetic nephropathy patients and correlated with inflammation-mediated injury to glomerular endothelial cells (Huang et al., 2014). High glucose-related increases in TNF- α , TGF- β 1, and NF- κ B, expression consequent to increased miR-155 and miR-146a levels, in human renal glomerular endothelial cells further support the *in vivo* findings (Huang et al., 2014).

22.8.3 Liver

MiR-122 has been reported to be highly tissue specific and is the most abundant miRNA in liver. MiR-122 plays a key role in the regulation of lipid and glucose metabolism and is considered to be a novel biomarker for metabolic diseases (Willeit et al., 2016). This miRNA has also been associated with mitochondrial function through its role in lipid metabolism (Jin et al., 2014) and has been considered to have ideal properties for use as a systemic biomarker of liver toxicity including good stability, tissue

specificity (Parkinson et al. 2013), and ease of detection in multiple species (Sharapova et al., 2016). Systemic release of miR-122 has been successfully used as a biomarker of general liver toxicity without regard to mitochondrial involvement (Laterza et al., 2009; Sharapova et al., 2016); however, alterations in tissue miR-122 may prove useful for detection of mitochondrial effects in the liver preclinically.

MiR199a-5p has an important role in mitochondrial activity and mitochondrial β -oxidation and lipid metabolism in liver. Increased expression of miR199a-5p has been observed in liver samples from nonalcoholic fatty liver disease (NAFLD) patients, as well as in obese db/db mice fed a high fat diet (Li et al., 2014a). Increased miR199a-5p was associated with decreases in caveolin (CAV1) and PPAR α , suggesting that miR199a-5p impairs FA β -oxidation in hepatocyte mitochondria that is mediated by CAV1 and the PPAR α .

22.8.4 Brain/Nerve

Increased levels of brain-specific miR-338 have been associated with mitochondrial dysfunction, reduced mitochondrial metabolic activity, and decreased ATP production in neuronal tissue (Wienholds et al., 2005; Aschrafi et al., 2008). MiR-338 targets the cytochrome c oxidase IV (COXIV) gene, which codes for a critical protein within the electron transport chain. Dysregulation of mitochondrial biogenesis (fission) is often associated with brain disorders, which implicate other miRNAs associated with mitochondrial fission as potential biomarkers of effects in brain as well (Yang et al., 2006; Edwards et al., 2010).

22.9 Work to Date Using MiRNA as Biomarkers of Mitochondrial Toxicity

Several studies mentioned previously have identified candidate tissue-specific miRNA biomarkers of organ toxicity that are, in some cases, associated with the regulation of mitochondrial function; however, only one study has been conducted to date using known mitochondrial toxicants specifically to identify miRNA biomarkers associated with mitochondrial toxicity and dysfunction (Baumgart et al., 2016). In this work, Sprague Dawley rats were dosed daily with the prototypical mitochondrial toxicants rotenone (respiratory complex I inhibitor) and 3-nitropropionic acid (3NP) (respiratory complex II inhibitor) for 1 week (Baumgart et al., 2016).

Changes in miRNAs associated with mitochondrial function were assessed in the kidney, skeletal muscle,

and serum. Interestingly, changes in the levels of identified miRNA preceded the formation of any histological lesions and generally correlated with decreases in mitochondrial copy number.

miRNA changes in the tissues of animal models, related to mitochondrial toxicity, may offer the greatest opportunity to detect very early biomarkers of mitochondrial dysfunction. In addition, as knowledge of mitomiR functions grows, the pattern of changes in mitochondrial miRNA may be interpretable for the specific mechanism(s) of dysfunction, just as patterns of gene (mRNA) expression change are often used to decipher mechanisms of toxicity in target tissues of xenobiotics. With the natural evolution of technology from microarray to RNA-Seq evaluations, this lays a foundation for the collection and management of miRNA-rich datasets and for interpretations in the context of histology and gene expression changes.

22.9.1 Kidney

MiR-338-5p is associated with mitochondrial dysfunction such as increased ROS production with loss of membrane potential and decreased ATP synthesis. In the Baumgart et al. (2016) study, dose-dependent induction of miR-338-5p was observed in the kidneys of rats treated with the mitochondrial toxicants rotenone and 3NP (Baumgart et al., 2016). The magnitude of increased miR-338-5p was higher in the kidneys of rotenone than 3NP-treated rats, and this change was also detectable in serum samples from those animals suggesting miR-338-5p as potential systemic biomarker of mitochondrial toxicity in kidneys (Baumgart et al., 2016).

Two other mitomiRs were altered in the kidneys of treated animals in this study. MiR-202-3p is associated with apoptotic cell death and inhibition of Bcl-2 (Zhao et al., 2013) and was shown to be significantly decreased in the kidneys of rats treated with rotenone as well (Baumgart et al., 2016). In addition, miR-546 appears to be another potential marker of mitochondrial toxicity in the kidney as it was dose-dependently downregulated in the kidneys of rats treated with rotenone (Baumgart et al., 2016). MiR546 has been linked to changes in membrane potential and levels of ROS in mitochondria (Wu et al., 1999; Fan et al., 2004).

22.9.2 Skeletal Muscle

MiR-122 has been repeatedly reported to be a liver-specific miRNA, as discussed previously in this chapter, with a role in controlling mitochondrial function (Lewis and Jopling, 2010; Filipowicz and Grosshans, 2011; Qiao et al., 2011). MiR-122 expression has also been strongly linked to mitochondrial damage (Burchard et al., 2010).

In the Baumgart et al. (2016) study, both rotenone and 3NP treatments of Sprague Dawley rats resulted in profound upregulation (up to 390-fold) of miR-122 in the skeletal muscle of treated rats (Baumgart et al., 2016). Induction of miR-122 was dose dependent for both mitochondrial toxicants, supporting the relationship of this induction with mitochondrial toxicity. In addition, miR-122 induction was greatest with 3NP treatments, in which mitochondrial copy number was also decreased to the greatest extent. Interestingly, despite the massive increases in miR-122 in skeletal muscle tissue, no significant changes were detected in serum, suggesting that this miRNA may not be readily released into the systemic circulation during miR-122 induction in skeletal muscle, as has been established in cases of liver toxicity where this miRNA is constitutively expressed. This may also be not only due to the early stage of toxicity, which preceded the observation of histologic changes, but also correlated with decreases in mitochondrial copy number. In extra-hepatic tissues, miR-122 induction within tissues would make a potentially outstanding biomarker of mitochondrial toxicity because of its lack of constitutive expression. It is plausible that it would be released as lesions form and may represent a valid biomarker for mitochondrial toxicity with the confounding complication of potential interference due to hepatotoxicity unrelated to mitochondrial toxicity. Evaluating tissue miRNA levels would obviously be best suited to a preclinical setting because of the invasiveness of sample collection.

MiR-546 levels were also significantly increased in the skeletal muscle of both rotenone- and 3NP-treated rats (Baumgart et al., 2016). Because this study reported miR-546 alterations in both the kidney and skeletal muscle, this may represent a mitochondrial toxicity biomarker that can be used across tissues.

22.9.3 Serum

The only serum miRNAs associated with mitochondrial function, in the Baumgart study, that were significantly altered by the treatment of rats with rotenone and 3NP were miR-34c and miR-338-5p (Baumgart et al., 2016). Interestingly, 3NP treatment significantly reduced and rotenone significantly increased serum levels of both miRNAs. This may be reflective of compensatory induction versus significant toxicity and loss of mitochondria. There were differences in the levels of mitochondrial toxicity between the two toxicants that are supportive of this conclusion. In addition, rotenone is a reversible inhibitor of mitochondrial respiration (Complex I), whereas 3NP is an irreversible respiratory inhibitor of Complex II. Fold changes in this study were substantial with decreases in 3NP-treated animals reaching ~ 7.7 -fold and increases in rotenone treated rats reaching

+21.1-fold. In concordance, 3NP-treated rats had greater and more consistent decreases in mitochondria numbers (copy number) in both the kidney and skeletal muscle. Compensatory changes in the case of rotenone treatment, with reversal related to mitochondrial losses with 3NP treatment, are the most likely explanation of the differences in direction of effects reported.

These have been the first evidence of serum miRNA biomarkers of mitochondrial toxicity reported. Both MiR-34c and MiR-338-5p have been linked to ROS production and loss of mitochondrial membrane potential (Aschrafi et al., 2008, 2012). These molecular responses are consistent with mitochondrial toxicants inhibiting oxidative phosphorylation such as rotenone and 3NP.

Clinically, serum biomarkers have the greatest potential for monitoring and identifying drugs that act as mitochondrial toxicants for reasons of invasiveness and ease of collection. If mitochondrial toxicants can be identified before significant depletion occurs, dosing can be stopped prior to lesion formation and irreversible tissue damage occurs in patients or healthy volunteers. MiRNA represent a plausible opportunity to find biomarkers of this serious toxicity.

22.10 Future Work Needed

The identification and characterization of miRNA biomarkers associated mitochondrial toxicity is still in its infancy as discussed throughout this chapter. To this point a single study has been reported attempting to identify some original candidate miRNA associated specifically with mitochondrial toxicity as described earlier (Baumgart et al., 2016). This work focused on miRNA changes in only two mitochondrially rich tissues (kidney and skeletal muscle) and serum. Additional work is needed to examine miRNA changes in other tissues, particularly mitochondrially rich tissues, such as the heart, liver, and brain, as well as other matrices such as urine, bile, lymph, saliva, and so on. Other tissues may also be appropriate for study of mitomiR biomarkers based on exposure levels, sensitivity, metabolite formation, or other factors.

Additional work is also needed to better understand the different miRNA biomarkers associated with various mechanisms of mitochondrial toxicity. With the identification of many tissue-specific or tissue-enhanced miRNAs, and many of those with ties to mitochondrial function, it may also be possible to identify miRNA biomarkers associated with mitochondrial toxicity from specific tissues in time. Based on the effects of known mitomiRs that have already been identified, miRNAs are involved in regulating the expression of mitochondrial proteins involved in a diverse array of functions. Likely,

others are yet to be elucidated, and continued efforts in this regard are clearly needed.

The identification of additional roles for miRNA is needed and will continue to shed light on the roles of miRNA on mitochondrial function. This information will likely lead to the discovery of additional potential miRNA biomarkers of mitochondrial toxicities. A role for the mitochondria in miRNA storage has been proposed, but additional work is needed to confirm this and define the role this might fulfill. With reports of miRNAs being deposited within the intermembrane space, it is possible that these miRNA play a role of some sort during apoptotic signaling as they would be released into the cytosol along with cytochrome *c* during MPT or pore formation via bax family members (Li et al., 2008). During MPT, the inner membrane swells to capacity after opening of the permeability transition pore and fluid influx. Because the outer membrane is smaller than the convoluted inner membrane, it lyses and releases the contents of the intermembrane space including cytochrome *c*, which binds the apoptosome and activates caspases 3, 7, and 9 causing apoptosis (Jiang and Wang, 2000).

One of the significant gaps in our current knowledge is how exactly miRNAs are transported into the mitochondria. Transport appears to be ATP related. Energy-dependent transporters of nucleosides have been identified and characterized in the mitochondria, and their role in the uptake of nucleosides and nucleoside analogues is well known (Govindarajan et al., 2009). Likely candidates for transport of miRNA into mitochondria include those already described for the transport of nuclear mRNA (Entelis et al., 2001). Others have been suggested previously in this chapter and include porins/VDAC (Bandiera et al., 2013), AGO2 (Bandiera et al., 2011), TIM and TOM complexes (Bandiera et al., 2011), and PNPT1/PNPASE (Wang et al., 2010). Ideally the mitomiR biomarker would be exported from the mitochondria and into the systemic circulation during injury, whether as a result of toxicity or as a compensatory reaction to functional deficit. Extracellular release would obviously be an earlier event than release due to cell death and lysis. Detecting effects on function, prior to the irreversible stage of cell death, provides opportunity to stop dosing (in the case of environmental exposures) or remove from exposure to protect affected tissues and allow for recovery.

Data analyses of mitochondrial RNA should take into account the proper normalizations for any gene expression analyses. These normalizations include corrections of RNA loading via a housekeeping gene and normalization to a control group or unexposed population. To avoid bias due to amplification efficiency, amplicons of a similar size to miRNA should be used and similar

amplification efficiencies confirmed between target and housekeeping gene/miRNA. A reasonable approach is to use the U6 snRNA, a commonly used reference transcript, as a housekeeping gene (Hu et al., 2012). Other housekeeping miRNAs may be identified in the future that are suitable for normalization. When a miRNA standard is available for generation of a standard curve, absolute quantitation is an option. If this approach is taken, care must be exercised to use excellent PCR hygiene practices to avoid carryover contamination of test samples (Aslanzadeh, 2004). If a control (predose) or untreated reference is available, relative quantitative analyses is an excellent option. By including the second normalization to a control, a relative quantity or fold-change value can be calculated relatively simply as follows (Baumgart et al., 2016):

$$\begin{aligned}\Delta\Delta Ct &= (Ct \text{ target mitomiR}_{\text{treated tissue}} \\ &\quad - Ct \text{ U6 snRNA}_{\text{treated tissue}}) \\ &\quad - (Ct \text{ target mitomiR}_{\text{control tissue}} \\ &\quad - Ct \text{ U6 snRNA}_{\text{control tissue}}) \\ \text{Relative quantity} &= 2^{-\Delta\Delta Ct}\end{aligned}$$

Considering species-to-species variation in miRNA expression and response to toxicants, including mitochondrial toxicants, the evidence seems to point to conservation of function for many miRNAs. It is important to consider that this is not universally the case and additional work is needed in this area. Bioinformatic predictions, in conjunction with microarray analysis and sequence-directed cloning, have identified several human miRNAs that are not conserved beyond primates (Bentwich et al., 2005). The extent to which tissue-specific miRNA abundance is conserved across species is another important question to understand when considering the translatability of miRNA biomarkers. Even though a conserved tissue-specific abundance pattern between human and rodents is shown for multiple miRNAs such as miR-133B, miR-124, and miR-9 (Ludwig et al., 2016), understanding differences for other miRNAs and in other species commonly used for toxicity evaluation is going to be critical for the further expansion of miRNA use as biomarkers, including biomarkers of mitochondrial toxicity. Higher mutation rates and accelerated evolution of the mitochondrial DNA in animal species may contribute to considerable differences in the mitochondrial DNA sequences between closely related species (Yang et al., 2014). Hence mitomiRs, specifically mitochondrial DNA encoded miRNA, may also vary between the species.

Sex-related variations in miRNA expression and/or tissue abundance patterns are another factor, which may influence interpretation of miRNA data in toxicity.

Investigation of intrinsic variability of circulating miRNA in healthy human males and females revealed differential expression with 63–95% higher levels of miRNAs such as hsa-miR-548-3p, hsa-miR-1323, and hsa-miR-940 in females compared with those in males (Duttagupta et al., 2011). Similarly, slightly higher serum levels of miR-130b and miR-18b have been reported in males compared with those in females (Wang et al., 2012b). Hence, a difference in the miRNA profiles between individuals due to sex is another important factor to consider. Sex-related differences in cellular metabolism are also well documented. Substantial gender-specific differences in mitochondrial function such as mitochondrial fusion/fission (Arnold et al., 2008), mitochondrial membrane potential and respiration (Weis et al., 2012; Demarest et al., 2016), and mitochondrial gene expression (Vijay et al., 2015) suggest that mitomiR profiles may also be influenced by gender. However, there is currently a gap in understanding the differential expression profile of mitochondrial function associated miRNAs in males and females and their response to toxicants.

22.11 Conclusions

Despite the early state of the science of using mitomiRs as biomarkers of mitochondrial dysfunction and toxicities, the key role of miRNA in mitochondrial function offers promise. The need for biomarkers of mitochondrial toxicants is clearly high, particularly for assessing mechanisms of toxicity for pharmaceuticals. Such markers would be invaluable as screens for compounds that could potentially impair mitochondrial function or result in direct mitochondrial toxicity. Because of the delayed nature of mitochondrial toxicant effects in humans consuming drugs with this adverse profile, these toxicities often go undetected until prolonged exposures have been reached (Julie et al., 2008). In some cases, delayed mitochondrial toxicities manifest clinically sometime after treatments have been discontinued (Julie et al., 2008).

The information presented here has given researchers an up-to-date summary of work in the area of miRNA use for assessing mitochondrial toxicants. More importantly, there has been considerable information collected to point researchers toward other potential miRNA biomarkers of mitochondrial toxicity that be explored. The gaps in knowledge needed to advance this science have also been presented to guide those interested in contributing to the realization of establishing biomarkers of mitochondrial toxicities.

It is our hope that this information will be of use in guiding efforts in the important area of biomarker

discovery. The applications of success may not be limited to screening patients in clinical trials or on medications. Translation of biomarkers to human industrial or envi-

ronmental exposures of chemicals or translations to other species in an environmental setting may eventually be possible as well.

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23

Biomarkers of Mitochondrial Injury After Acetaminophen Overdose: Glutamate Dehydrogenase and Beyond

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23.1 Introduction

In the drug discovery process, drug-induced liver injury is one of the most common reasons for failure in preclinical development and in clinical trials. In addition, idiosyncratic hepatotoxicity leads to black box warnings or even withdrawal of approved drugs from the market. Whereas currently used biomarkers for liver injury (alanine (ALT) and aspartate aminotransferases (AST)) and dysfunction (bilirubin) are sufficiently sensitive to detect dose-dependent hepatotoxins, there are no biomarkers available that could alert to a potential idiosyncratic toxicity. Clinically, acetaminophen (APAP) overdose remains the most common source of both drug-induced liver injury and acute liver failure (ALF) (Lee, 2013). Patients that develop ALF have a very poor outcome, with mortality up to 50% (Lee, 2013). Early identification of which patients will proceed to ALF is critical, as these patients can be treated more aggressively or listed for transplantation earlier. As such, biomarkers of patient outcome are of considerable clinical value for determining early during the patient's hospitalization which patients will proceed to ALF and will die or need a liver transplant and which patients will recover spontaneously.

The best biomarkers are those that are also informative of the mechanisms at play in the pathophysiology or valuable clinically due to prognostic capacity. Biomarkers

present in the serum or urine of patients are of the most interest and the greatest use. Many of these serum and urine biomarkers have a single point of origin in tissue and thus accurately reflect what is happening in these tissues, in a mechanistic fashion, without the need for biopsy. A number of these “mechanistic biomarkers” have recently been a source of focus in the literature, and considerable research has gone into fully investigating these compounds (Antoine et al., 2012; McGill et al., 2012; Luo et al., 2014; McGill and Jaeschke, 2014; Beger et al., 2015). Release of many of these mechanistic biomarkers can be traced back to damage of the mitochondria, and thus considerable progress has recently been made in the field of biomarkers of mitochondrial damage. The purpose of this chapter will be to define these markers and discuss their clinical viability and basic science relevance with regard to the murine APAP hepatotoxicity model and human patients with APAP overdose.

23.2 Acetaminophen Overdose as a Model for Biomarker Discovery

A number of mitochondrial biomarkers have been established for liver disease. Many of these were originally defined in the murine APAP overdose model (reviewed in McGill and Jaeschke, 2014). This model is convenient

for biomarker discovery for a number of reasons: (i) it is technically simple and highly repeatable, (ii) the mechanisms associated with the model are largely well delineated, and (iii) it is a clinically relevant murine model with high fidelity to the human condition (McGill et al., 2012; Jaeschke et al., 2014, Jaeschke, 2015). We will briefly discuss the APAP overdose model as it relates to mitochondrial dysfunction and subsequent cell death. This is not a complete overview of the understood mechanisms of APAP (for a more complete, updated overview: Ramachandran and Jaeschke, 2017; Woolbright and Jaeschke, 2017), but rather a version focused on the pathology associated with the mitochondria.

23.3 Acetaminophen Overdose: Mechanisms of Toxicity in Mice and Man

23.3.1 Drug Metabolism and Protein Adducts

APAP is an over-the-counter analgesic and antipyretic. Normally, greater than 85% of an APAP dose is conjugated to either UDP-glucuronide or sulfate and excreted via phase II metabolism (McGill and Jaeschke, 2013). Therapeutic doses are safe; however, an overdose of APAP partially overwhelms phase II metabolism and results in substantial oxidation of APAP to the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Dahlin et al., 1984), which is a reactive electrophile that covalently adducts cellular proteins causing oxidative stress in the cell (Dahlin et al., 1984) and is largely detoxified through a spontaneous reaction with the endogenous antioxidant glutathione (GSH) (Mitchell et al., 1973). This results in the depletion of cellular GSH levels in the liver. GSH depletion is currently used as a hallmark for measuring APAP metabolic activation experimentally (McGill and Jaeschke, 2013). The interaction between NAPQI and GSH is also the basis for the current gold-standard therapeutic, *N*-acetylcysteine (NAC), which is a precursor for GSH synthesis. The newly formed GSH can scavenge NAPQI (Corcoran and Wong, 1986) and later detoxify reactive oxygen and peroxynitrite (Knight et al., 2002). During this metabolism and GSH depletion, NAPQI begins to adduct sulfhydryl groups on proteins forming acetaminophen–cysteine (APAP–CYS) adducts (Pumford et al., 1989), which have been proposed as a diagnostic indicator of APAP overdose in patients (Roberts et al., 2017). Levels above 1 μ M of APAP–CYS in serum are associated with liver toxicity, although recent data indicate APAP–CYS adducts may be released even at therapeutic doses when patients do not have any liver toxicity (Heard et al., 2011;

McGill et al., 2013). In addition, while adduct formation and release into the blood occur very early in mice (<1 h after APAP treatment) (McGill et al., 2013), adduct formation is delayed in human hepatocytes (Xie et al., 2014). As a result, early-presenting patients (<8 h after APAP overdose) show very low adduct levels in serum compared with late-presenting patients despite that both groups took a massive overdose (Xie et al., 2015a). Thus, serum adduct levels can be important biomarkers to diagnose specifically APAP overdose, but the time of exposure needs to be considered when interpreting the data.

23.3.2 Critical Role of Mitochondria in APAP Hepatotoxicity

Mitochondria emerged as central players in the intracellular signaling events of APAP-induced cell death. First, it was recognized that while APAP and its meta-isomer *N*-acetyl-meta-aminophenol (AMAP) both form reactive metabolites and protein adducts, only APAP forms mitochondrial adducts and causes toxicity in mice (Tirmenstein and Nelson, 1989). However, AMAP forms mitochondrial adducts in human hepatocytes and causes toxicity (Xie et al., 2015b). Protein adduct formation impairs the mitochondrial respiratory chain (Meyers et al., 1988) and triggers a selective oxidant stress (Jaeschke, 1990) and peroxynitrite formation inside mitochondria (Cover et al., 2005). Some of these reactive oxygen species escape into the cytosol and trigger the activation of a mitogen-activated protein kinase cascade, which ultimately leads to c-Jun-*N*-terminal kinase (JNK) activation and translocation of phospho-JNK to the mitochondria where it amplifies the mitochondrial oxidant stress (Han et al., 2013; Du et al., 2015). The amplified oxidant stress and peroxynitrite formation leads to the opening of the mitochondrial membrane permeability transition pore (MPTP), which causes the collapse of the membrane potential and cessation of ATP synthesis (Kon et al., 2004; LoGuidice and Boelsterli, 2011; Ramachandran et al., 2011a). The MPTP opening also triggers matrix swelling and rupture of the outer mitochondrial membrane, which releases intermembrane proteins such as apoptosis-inducing factor (AIF) and endonuclease G both of which translocate to the nucleus and induce DNA fragmentation (Bajt et al., 2006). The mitochondrial dysfunction (MPTP) and resulting karyolysis cause necrotic cell death after APAP overdose (Gujral et al., 2002). The central role of mitochondria in APAP hepatotoxicity has been supported by many different experimental approaches. In addition to the direct evidence of a mitochondrial oxidant stress and peroxynitrite formation inside of mitochondria (Jaeschke, 1990; Cover et al., 2005), scavenging of

these oxidants by mitochondrial GSH is highly protective (Knight et al., 2002; Saito et al., 2010). In addition, preventing peroxynitrite formation by accelerated dismutation of superoxide through mito-TEMPO, a mitochondria-targeted SOD mimetic, effectively attenuated APAP-induced cell death (Du et al., 2017). Furthermore, animals with partial deficiency of mitochondrial SOD (SOD2) are much more susceptible to APAP toxicity (Fujimoto et al., 2009; Ramachandran et al., 2011b). In addition to these events, removal of damaged mitochondria by autophagy (mitophagy) limits APAP-induced cell death (Ni et al., 2012). Thus, there is little doubt that mitochondrial dysfunction and damage play a critical role in APAP-induced cell death. Importantly, these events can also be observed in primary human hepatocytes and in the metabolically competent human hepatoma cell line HepaRG (McGill et al., 2011; Xie et al., 2014).

These intracellular signaling events result in hepatocyte necrosis and leakage of cellular components into the serum. Detection of these biomarkers in serum has a number of potential uses including both understanding the mitochondria as a potential player in the injury, especially in the human pathophysiology where access to tissue is limited and the use of mitochondrial biomarkers as prognostic indicators of patient survival and recovery.

23.4 Biomarkers of Mitochondrial Injury

Currently understood biomarkers of mitochondrial injury are based largely off the mechanisms delineated in the experiments presented in the previous section. This section will be used to discuss the clinical and mechanistic utility of the associated biomarkers as well as focus on understanding how the above mechanisms are related to the release of these biomarkers.

23.4.1 Glutamate Dehydrogenase

Glutamate dehydrogenase (GDH) is located predominantly in the mitochondria, with minimal amounts being located in the nuclear fraction and other cellular locations (Lai et al., 1986). GDH converts glutamate to α -ketoglutarate in mammalian systems (Bunik et al., 2016). While it is also capable of interconverting α -ketoglutarate to glutamate, this reaction does not generally occur in mammals due to the high amount of ammonia necessary for the converse reaction (Bunik et al., 2016). GDH is present in the mitochondrial matrix where it completes its enzymatic activity. GDH proteins are released under

even healthy conditions into serum in a stable fashion due to normal hepatocyte turnover, and reference ranges have been established for patients (Van Waes and Lieber, 1977). However, GDH has been used for some time as marker for liver cell injury in animal models of necrosis (Gellert et al., 1980; Gopinath et al., 1980; Murayama et al., 2009) and in patients (Van Waes and Lieber, 1977). Though because of its predominant location in the mitochondria, it has become understood as a marker for mitochondrial damage during necrosis (McGill et al., 2012). Due to the very large size of the GDH complex, it is highly unlikely that GDH could reach the cytoplasm without mitochondrial damage (Li et al., 2012). During diseases with minimal necrosis, GDH levels typically remain low as there is no release of intracellular constituents. However, during diseases with considerable hepatic necrosis, which generally involves the opening of the mitochondrial permeability transition pore leading to extensive matrix swelling and rupture of the outer and even inner mitochondrial membranes, GDH can be released from the mitochondria into the cytoplasm and then into serum upon cell membrane leakage (Siegelman et al., 1962; McGill et al., 2012). As such, GDH has been proposed as a specific and injury-dependent biomarker of mitochondrial damage and dysfunction by our group and others (McGill et al., 2012, 2014a; Luo et al., 2014; McGill and Jaeschke, 2014).

Recent work from multiple laboratories has demonstrated high levels of GDH present in both human patients with APAP overdose and the mouse model of APAP overdose (McGill et al., 2012; Antoine et al., 2013; Schomaker et al., 2013). Others have shown that GDH is elevated in the rat model as well (Thulin et al., 2016), although this occurs in a delayed fashion, consistent with the attenuation of injury and reduced mitochondrial injury in the rat (McGill et al., 2012). The increase and subsequent decrease of serum GDH activities correlates with ALT levels in both populations, which is consistent with cellular release due to cell death. While human patients typically present distally from the point of their initial ingestion of APAP, studies in HepaRG cells, a metabolically competent hepatocyte-like cell line, and in primary human hepatocytes indicate the mitochondrial dysfunction associated with APAP overdose actually precedes cell death (McGill et al., 2011; Xie et al., 2014). Nevertheless, a critical question is whether GDH, like ALT or AST, is just another parameter of cell death or if it is actually a mechanistic biomarker that indicates mitochondrial damage. This issue was addressed in our study using furosemide overdose in mice. Previous investigations showed that the necrotic cell death caused by high doses of furosemide in mice does not involve mitochondrial dysfunction or injury (Wong et al., 2000). Interestingly, furosemide-induced liver injury showed

extensive necrosis accompanied by the release of high levels of ALT, but not GDH or mitochondrial DNA, into the serum (McGill et al., 2012). These findings suggested that GDH is not just a cell death biomarker but is indeed a biomarker for mitochondrial damage (McGill et al., 2012). Further support for the difference between ALT and GDH as biomarkers in drug-induced liver injury came from studying a larger cohort of APAP overdose patients. When ALT activities were measured in these patients at the time of hospital admission or at the peak of injury (peak ALT values), there was no significant difference in any of these parameters between surviving and non-surviving patients (McGill et al., 2014a). In contrast, there were significant differences in serum GDH activities with higher levels in non-surviving patients (McGill et al., 2014a). These observations suggest that based on the mitochondrial damage biomarker GDH and others, mitochondrial injury and dysfunction is a critical mechanism of cell death in patients and that a more severe mitochondrial injury correlates with a lower chance of survival. Of note, GDH can also serve as a component for a larger metric as its inclusion into the mitochondrial damage biomarker index (MDBI) improved the score and gave a greater sensitivity and specificity for predicting patient outcomes (McGill et al., 2014a). Importantly though, GDH may be even a more sensitive marker of liver injury than ALT or other traditional transaminases (Antoine et al., 2013). In patients that present to the hospital with ALT < 3x, the upper limit of normal, GDH levels rose before ALT levels and more accurately predicted which patients would progress to acute liver injury (Antoine et al., 2013). Since some overdose patients arrive at the hospital without increases in transaminases but develop severe liver injury at later time if not treated with NAC, GDH together with other biomarkers such as miR-122, high mobility group box-1 (HMGB1) protein, and cytokeratin-18 may have clinical value as an earlier determinant of injury that will provide clinicians insight into whether or not patients should be admitted for prolonged observation and treated with NAC.

Another recent study has indicated that GDH values rise substantially in patients with hypoxic hepatitis (Weemhoff et al., 2017). When compared with another population of APAP overdose patients, it was noted that GDH values in hypoxic hepatitis patients sometimes exceeded values in APAP patients, despite the fact that APAP overdose patients had consistently higher ALT values (Weemhoff et al., 2017). Whether this is due to increased mitochondrial injury, or another facet of the two populations is unknown, it argues that GDH-to-ALT ratios may have some value, both as a marker of mitochondrial damage and potentially as a diagnostic marker. These data need to be followed up in a larger cohort.

An experimental caveat of using GDH and other molecules as biomarkers of mitochondrial damage is the possibility that at least during severe necrosis, intact mitochondria may be released in the blood. If blood is centrifuged with *g* forces insufficient to sediment these intact mitochondria, the subsequent freeze–thaw cycle may liberate GDH from mitochondria and lead to elevated GDH levels. This has the potential to cause misinterpretations regarding the role of mitochondrial dysfunction in the pathophysiology (Jaeschke and McGill, 2013). Further studies are necessary to assess if this issue may be a relevant problem with the use of these biomarkers in clinical samples.

23.4.2 Mitochondrial DNA (mtDNA)

Mitochondria contain their own set of DNA specific to mitochondrial function. This DNA is restricted to the mitochondrial matrix under normal conditions. Similar to GDH, the presence of mtDNA in serum has been proposed as a marker of mitochondrial damage in APAP-induced liver injury (McGill et al., 2012). These transcripts are leaked into the cytoplasm during mitochondrial damage when the mitochondrial membrane breaks down. In addition to APAP, mtDNA has been found in serum in other disease states including shock and physical trauma-induced injury (Zhang et al., 2010a, b). Measurements in serum of specific mitochondrial transcripts for electron transport chain encoding sequences, including cytochrome *c* and NADH oxidase, indicate mtDNA levels are elevated in serum of APAP overdose patients (McGill et al., 2012) and hypoxic hepatitis patients (Weemhoff et al., 2017). Moreover, similar to GDH, mtDNA levels are capable of distinguishing non-surviving patients from surviving patients at their initial presentation (McGill et al., 2014a). A caveat is that given the substantial variation of the mtDNA levels in various patients, individual serum mtDNA values cannot be used to predict survival. Only the average levels in larger cohorts are higher in non-survivors and correlate with poor outcome (McGill et al., 2014a). However, the predictability of survival can be improved when mtDNA levels are included in an index that considers a battery of mitochondrial biomarkers (McGill et al., 2014a). MtDNA levels correlate well with serum ALT activities indicating their release is likely contingent upon cellular necrosis. As such, mtDNA is an excellent marker of mitochondrial damage in APAP overdose patients with potential to benefit clinical prognostic scoring systems. One important issue to consider is that the half-life of mtDNA in serum is considerably shorter than of ALT and GDH (McGill et al., 2012).

One more controversial aspect of mtDNA is its role in the innate immune response. MtDNA is understood to

be a damage-associated molecular pattern (DAMP) that can activate toll-like receptors (TLRs) such as TLR9 on immune cells (Imaeda et al., 2009; Zhang et al., 2010a, b). After an APAP overdose, the initial injury due to mitochondrial oxidant stress results in hepatic necrosis. Subsequently, these cells die and release mtDNA and other mitochondrial components such as formyl peptides (Marques et al., 2012; McGill et al., 2012). These molecules are recognized by TLRs expressed on Kupffer cells, which then respond with upregulation of pro-inflammatory genes and interleukins (IL) such as IL-1 β , IL-18, CXC chemokine ligand 1, CXC chemokine ligand 2, and more (Imaeda et al., 2009; Marques et al., 2012). These cytokines recruit neutrophils, which can exacerbate the initial injury (Imaeda et al., 2009). However, this hypothesis has been challenged (reviewed in Woolbright and Jaeschke, 2017). There is no question that APAP-induced necrosis causes the release of DAMPs, including mtDNA (McGill et al., 2012), which results in cytokine formation (Lawson et al., 2000; James et al., 2005) and hepatic neutrophil recruitment (Lawson et al., 2000; Cover et al., 2006). However, there is still no conclusive evidence that neutrophils, or any other inflammatory cell type, kill hepatocytes during APAP overdose (Woolbright and Jaeschke, 2017). Importantly, although mtDNA and other DAMPs are released during the injury phase (McGill et al., 2012), activation of neutrophils occurs mainly during the recovery phase (Williams et al., 2014). Thus, it is widely agreed upon that serum mtDNA levels are elevated during severe liver injury in APAP hepatotoxicity, ischemic hepatitis, and other disease states in animals and humans indicating mitochondrial damage during the mechanism of cell death. However, potential pathophysiological consequences of the release of these DAMPs into the circulation require further studies in the various disease states.

23.4.3 Nuclear DNA

Nuclear DNA fragmentation is noted in a number of different liver diseases including APAP overdose (Lawson et al., 1999; Gujral et al., 2002), alcohol-induced liver injury (Roychowdhury et al., 2013), obstructive cholestasis (Woolbright et al., 2013), nonalcoholic steatohepatitis (Feldstein et al., 2003), hepatic ischemia reperfusion injury (Yang et al., 2014), septic liver injury (Mignon et al., 1999), and more. In tissue, nuclear DNA fragmentation can be both detected and quantified through use of the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay (Grasl-Kraupp et al., 1995). While much of the literature discusses the use of the TUNEL assay in terms of measuring apoptosis, the TUNEL assay detects all forms of DNA damage that results in single-strand DNA (Grasl-Kraupp et al., 1995).

Nuclear DNA fragments are another DAMP released during APAP-induced liver injury that can also be measured in serum using an anti-histone ELISA, which makes it specific for nuclear DNA (McGill et al., 2012). Nuclear DNA is fragmented differently during different types of cellular injury (Jahr et al., 2001). During apoptosis, the active caspase-3 cleaves the inhibitor of caspase-activated DNase (ICAD) and liberates the active endonuclease CAD, which then cleaves DNA at the internucleosomal linker sites. This creates fragments consisting of individual nucleosomes (about 180 base pairs of DNA wrapped around a histone core) or multiples of these nucleosomes. Thus, apoptotic DNA fragments are generally smaller DNA fragments, which can be visualized on an agarose gel as DNA ladder (Jahr et al., 2001). In contrast, in a process of programmed necrosis such as APAP-induced cell death, mitochondrial dysfunction causes permeabilization of the outer membrane and release of intermembrane proteins such as AIF and endonuclease G, which then translocate to the nucleus and cause DNA fragmentation (Bajt et al., 2006). This results in fragments of variable length as the endonucleases responsible are less specific in their cleavage (Ray et al., 2001). When released into the cytosol, the larger DNA fragments will be detected by the TUNEL assay, leading to the characteristic staining of the entire necrotic cell (Gujral et al., 2002). In addition, both small and large DNA fragments will be released into the cytosol where nicked DNA strands can be detected using an anti-histone ELISA assay in both mice and patients (McGill et al., 2012). Nuclear DNA fragments correlate well with ALT in both APAP overdose (McGill et al., 2012, 2014a) and hypoxic hepatitis patients (Weemhoff et al., 2017), indicating they are closely linked to hepatic necrosis. Moreover, nuclear DNA fragments can predict patient outcomes after APAP overdose (McGill et al., 2014a). Nevertheless, since the anti-histone ELISA cannot distinguish between different sizes of nuclear DNA fragments, it is not specific for mitochondrial damage or necrosis. Thus, detection of nuclear DNA fragments in serum needs to be accompanied by measurements of caspase-3 enzyme activities and caspase-cleaved fragments of cytokeratin-18 to support apoptotic cell death (Antoine et al., 2012; McGill et al., 2012; Woolbright et al., 2013, 2015) or ALT, microRNA-122, full-length cytokeratin-18 and HMGB1 protein as indicator of necrosis (Antoine et al., 2012; Woolbright et al., 2013, 2015) and mtDNA and GDH as biomarkers for mitochondrial damage (McGill et al., 2012). Given this information, further effort should go toward evaluating nuclear DNA fragments in serum in other disease models as the focus has thus far largely been on tissue rather than the serum compartment.

23.4.4 Acylcarnitines

Long-chain fatty acids are largely incapable of entering the mitochondria for β -oxidation. These fatty acids must be conjugated to the amino acid derivative carnitine for transport into the mitochondria (Rinaldo et al., 2002). As such, acylcarnitines have been used as biomarkers of neonatal mitochondrial oxidation deficiency (Rinaldo et al., 2002). Impaired mitochondrial β -oxidation of fatty acids has been noted during APAP hepatotoxicity in mice, which also showed increased levels of long-chain acylcarnitines in blood (Chen et al., 2009). These findings in mice were confirmed by others (Bhattacharyya et al., 2013; McGill et al., 2014b). Importantly, acylcarnitine levels were not increased in serum of furosemide-treated mice (McGill et al., 2014b), which did not show evidence of mitochondrial dysfunction (McGill et al., 2012). Thus, long-chain acylcarnitines such as palmitoylcarnitine, linoleoylcarnitine, and oleoylcarnitine could be useful biomarkers of mitochondrial dysfunction or damage in drug hepatotoxicity. This is of particular importance as these biomarkers could be measured in serum before ALT activities increased, for example, before overt cellular necrosis (McGill et al., 2014b).

In contrast to the findings in mice, measurement in serum of various acylcarnitines in APAP overdose patients did not show any relevant increase of these biomarkers over baseline levels (McGill et al., 2014b). However, all patients were treated with the standard of care antidote NAC before the samples for acylcarnitine analysis were obtained (McGill et al., 2014b). Since the high clinical doses of NAC can improve mitochondrial energy metabolism and function (Saito et al., 2010), this was the likely cause of the lack of long-chain acylcarnitine levels being elevated in patients (McGill et al., 2014b). In fact, a study in children demonstrated that delayed NAC treatment resulted in higher acylcarnitine levels after APAP overdose (Bhattacharyya et al., 2014). However, the increase in acylcarnitine levels in blood of these pediatric patients was very modest (two- to fourfold over baseline) (Bhattacharyya et al., 2014) compared with 6-to-20-fold increases in mice with a high overdose of APAP (McGill et al., 2014b). While it is not currently well understood what causes the inhibition of β -oxidation, one possible scenario is that NAPQI directly adducts one or more of the enzymes responsible for β -oxidation in the mitochondria (McGill et al., 2014b). More investigations are necessary to better understand whether or not acylcarnitines have clinical value as biomarkers of mitochondrial damage in drug hepatotoxicity patients. Clearly, any intervention such as NAC that improves mitochondrial dysfunction will affect acylcarnitine release into the blood and thus affect their validity as a mechanistic biomarker.

23.4.5 Carbamoyl Phosphate Synthetase

A potentially useful recently discovered marker for mitochondrial dysfunction is carbamoyl phosphate synthetase (CPS-1), an enzyme that resides in the mitochondrial matrix (Weerasinghe et al., 2014). Markers such as ALT have extended half-lives up to 48 h. This long half-life can make understanding the point of liver injury difficult contextually as the primary injury phase might be over with falling ALT levels that reflect previous damage. As such, markers with short half-lives more accurately reflect the current state of injury. CPS-1 levels are elevated in mice with APAP-induced liver injury and in patients with APAP- or ischemia-induced ALE, but not during chronic viral hepatitis (Weerasinghe et al., 2014). However, CPS-1 levels fall far more precipitously than ALT levels, indicating that CPS-1 might be useful clinically for approximating degree of active liver injury (Weerasinghe et al., 2014). CPS-1 is an informative biomarker for mitochondrial damage and requires more direct comparison with more established markers such as mtDNA and GDH in patients and experimental models of liver injury with and without mitochondrial dysfunction.

23.4.6 Ornithine Carbamyl Transferase (OCT)

OCT is another hepatic enzyme that rises in value after APAP-induced liver injury (Lim et al., 1994). OCT is localized in mitochondria and catalyzes the reaction between carbamoyl phosphate and ornithine to form citrulline and phosphate. It has some specificity for liver injury as nephrotoxic agents fail to produce rises in serum OCT (Tegeris et al., 1969). Recent drug toxicity studies in rat hepatocytes demonstrated the release of OCT and ALT, with OCT-to-ALT ratios in the culture medium between 3 and 7 (Furihata et al., 2016). Because of the higher release of OCT compared to the traditional necrosis marker ALT, OCT may be a more sensitive biomarker for cell death. In addition, the OCT-to-ALT ratio appears to be drug specific (Furihata et al., 2016). Both alcoholic liver disease and primary sclerosing cholangitis patients with mild to no increase in other liver enzymes such as ALT and AST have elevations in OCT (Murayama et al., 2008, 2009; Matsushita et al., 2014). Patients with fibrosis have significantly higher OCT levels than patients without fibrosis. In contrast, patients with hepatitis B, hepatitis C, and autoimmune hepatitis showed very low OCT levels in serum (Matsushita et al., 2014). As such, OCT may be a super-sensitive marker of liver injury and dysfunction (Murayama et al., 2008). The interesting observation is that the mitochondria-derived OCT can be detected earlier than cytosolic enzymes such as ALT and AST, which suggests that the release of enzyme into the circulation appears to be dependent on the biomarker rather than its intracellular localization.

23.5 Conclusions

Mitochondrial markers of APAP and other drug hepatotoxicities in the clinic are still in their infancy, but the future is promising. Indices such as the MDBI have potential for predicting patient outcome, and early measurements of markers such as GDH, mtDNA, and others may more accurately predict which patients will progress

to acute liver injury, and thus help clinicians delineate patient needs. The use of these biomarkers to confirm the mitochondria as a central point in APAP toxicity should be expanded to other known diseases that are thought to have substantial mitochondrial involvement. However, more work is required in this area, both for validation of these markers in large cohorts and for the identification of potentially new, superior markers of injury.

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Acylcarnitines as Translational Biomarkers of Mitochondrial Dysfunction

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24.1 Introduction

Drug-induced liver injury (DILI) is a major reason drugs fail in clinical trials, are recalled after approval, or have black box warnings (Senior 2009). Mitochondrial injury has been reported as a primary factor in DILI (Kass 2006; Labbe, Pessayre, and Fromenty 2008; Begriche et al. 2011; Nadanaciva and Will 2011; Pessayre et al. 2012; Shi et al. 2015; Vuda and Kamath 2016). Acetaminophen (APAP) is responsible for 50% of acute liver failure and is associated with mitochondrial dysfunction (Coen et al. 2003; Kon et al. 2004; Chen et al. 2009). Mitochondrial dysfunction has been reported in drug-induced injuries to other organs, including renal toxicity (Stallons, Funk, and Schnellmann 2013; Yang et al. 2014), cardiotoxicity (Sardão, Pereira, and Oliveira 2008; Eirin, Lerman, and Lerman 2014; Varga et al. 2015), and neurotoxicity (Barbosa et al. 2015; Li, Yu, and Liang 2015). Therefore, identifying and validating translational biomarkers of mitochondrial injury is important to clinicians, the pharmaceutical industry, and regulatory agencies.

A number of biomarkers of mitochondrial injury have been associated with DILI and include the

mitochondrial enzymes alanine aminotransferase (ALT2), cytochrome *c*, glutamate dehydrogenase (GLDH), carbamoyl-phosphate synthase 1 (CPS1), mitochondrial DNA (mtDNA), and long-chain acylcarnitines, which undergo β -oxidation in mitochondria (Pessayre et al. 2012; Shi et al. 2015). This chapter will focus on acylcarnitines as potential translational biomarkers of mitochondrial dysfunction. Acylcarnitines are a form of fatty acid with an ester link to L-carnitine. Figure 24.1 shows the enzymes involved for moving short, medium, and long fatty acid (C2–C18) from the cytoplasm to the mitochondria for β -oxidation. Carnitine palmitoyltransferase 1 (CPT1) converts acyl-CoA to acylcarnitines that can be imported into the mitochondria. CPT1 is an integral outer mitochondrial protein (Rufer et al. 2007; Tonazzi et al. 2015). Carnitine and acylcarnitines cross the outer mitochondrial membrane by a voltage-dependent anion channel (VDAC) or porin (Stanley, Palmieri, and Bennett 2014). Carnitine/acylcarnitine translocase (CACT) imports acylcarnitines through the inner membrane of the mitochondria and exports carnitine out. Carnitine palmitoyltransferase 2 (CPT 2) converts the acylcarnitine back to acyl-CoA for

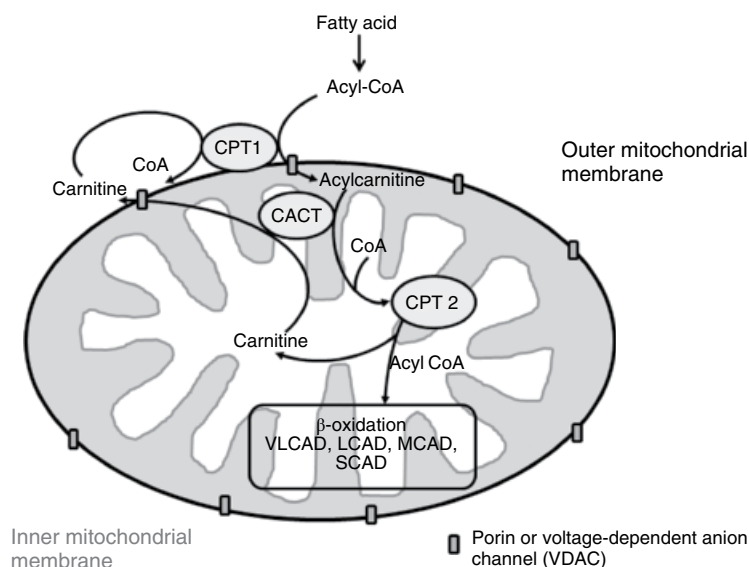


Figure 24.1 Cartoon of the acylcarnitine shuttle and β -oxidation of fatty acids in mitochondria. CACT, carnitine/acylcarnitine translocase; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; LCAD, long-chain acyl-CoA dehydrogenase deficiency; MCAD, medium-chain acyl-CoA dehydrogenase deficiency; SCAD, short-chain acyl-CoA dehydrogenase deficiency; VLCAD, very-long-chain acyl-CoA dehydrogenase deficiency.

β -oxidation. Increased blood levels of acylcarnitines have been observed in APAP toxicity (Chen et al. 2009; Bhattacharyya et al. 2013; Bhattacharyya et al. 2014; Beger et al. 2015) and in liver tissue in a study of APAP and green tea extract (GTE) interaction (Lu et al. 2013). In addition, increases in long-chain acylcarnitines have been reported in studies of other hepatotoxicants, including carbon tetrachloride in Sprague-Dawley rats (Sun et al. 2014a), dantrolene (DAN) in Sprague-Dawley rats (Sun et al. 2014b), and dronedarone in mice (Felser et al. 2014), and hepatocyte studies with valproic acid (Silva et al. 2001) and tert-butyl hydroperoxide (tBHP) (Cervinková et al. 2008).

24.2 Acylcarnitine Analysis

The principle of the 3Rs, or “refine, reduce, and replace,” advocates for the use of *in vitro* cell cultures to evaluate drug toxicity prior to initiating and planning animal studies. Acylcarnitines can be evaluated through *in vitro*, nonclinical, and clinical studies (Figure 24.2a). Metabolomics analysis of acylcarnitines has been conducted on blood samples of patients with APAP toxicity. Analysis (Bain et al. 2009) can be performed as open profiling using nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) methods to discover metabolites or patterns associated with an endpoint. Alternatively, focused metabolic profiling can be used for specific classes of metabolites, such as acylcarnitines. This section will focus on metabolic profiling methods of acylcarnitines and discuss instances where open profiling has detected changes in acylcarnitines in toxicity studies.

During *in vitro* toxicity studies, cells are collected at multiple time points before and after dosing with selected drugs. Metabolites in media samples represent those released by the cells. The optimal 3R design for nonclinical studies involves collection of blood samples before and at multiple time points after dosing in a single animal and limited collection of tissue samples (Figure 24.2b). Simultaneous detection of multiple acylcarnitines is complicated by low concentrations and the presence of isomers for some acylcarnitines. Uniform sample collection, storage, and processing are critical for accurate detection and comparison of data across studies. Consistent extraction techniques, typically involving protein precipitation of the blood sample followed by solid-phase extraction (SPE) (Minkler et al. 2008; Zuniga and Li 2011), are also important for analytical data quality. Quantitative profiling methods require the addition of isotope-labeled acylcarnitine(s) as internal standards to the samples to accurately determine percent recovery and concentration. In some methods an internal standard is added for every acylcarnitine being measured, while others only add a couple of internal standards. The next steps are choice of derivatization (if any), selection of column, and, finally, optimization of the ionization and detection parameters for specific acylcarnitines.

The use of MS-based methods to profile acylcarnitines was introduced in mid to late 1980s. Methods for measuring acylcarnitines by MS have evolved over the years as shown in Table 24.1. One of the first methods to measure multiple acylcarnitines in blood used liquid chromatography (LC) coupled to fast atom bombardment (FAB) tandem mass spectrometry (MS/MS) to monitor short, medium, and methyl esters of long-chain acylcarnitines in urine, blood, and tissue samples

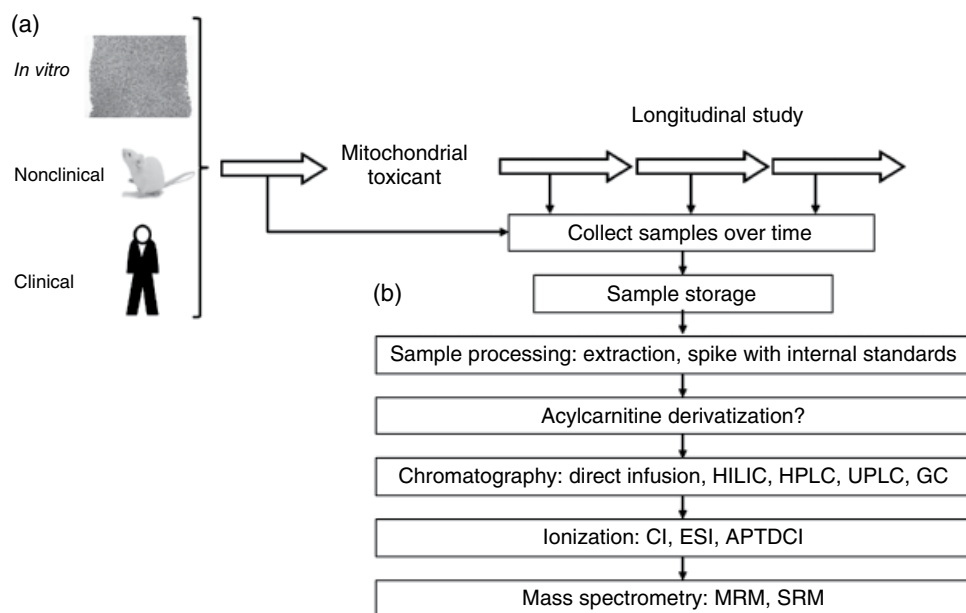


Figure 24.2 (a) Cartoon showing the steps involved in the collection of samples for the measurement of acylcarnitines during toxicity studies. Samples can be obtained from *in vitro* and nonclinical toxicity studies or in the clinic from patients with suspected drug-induced injury. (b) Flow chart showing the steps involved for the measurement of acylcarnitines during toxicity studies.

(Millington et al. 1989). This paper described the use of esterification of the carboxylic functional group to increase the effective surface area of acylcarnitines, which resulted in lower detection limits by MS. Other methods used N-demethylated ester derivatives to monitor medium-length acylcarnitines in urine samples by gas chromatography (GC)–mass spectrometry (GC/MS) (Huang et al. 1991) or converted the acylcarnitines to acyloxylactones for detection (Lowes et al. 1992). A method using high pressure liquid chromatography (HPLC) coupled to MS was developed to profile 47 pentafluorophenacyl ester derivatives of acylcarnitines (Minkler et al. 2008). Improvements in sensitivity, selectivity, and reproducibility of MS and chromatography have permitted analysis of acylcarnitines without chemical derivatization (Corso et al. 2011; Peng et al. 2013). Once the samples containing internal standards are prepared, they can be analyzed using chromatography and MS. More recently, LC/MS methods were developed that converted acylcarnitines to butyl esters to detect 48 acylcarnitines using dried blood spots and plasma to screen for inborn errors of metabolism (Gucciardi et al. 2012) or 56 acylcarnitines (from C2 to C18) in plasma and tissue samples (Giesbertz et al. 2015).

Over the years, many different types of chromatography have been used in profiling acylcarnitines. As mentioned earlier, in the 1990s, methods using GC/MS were developed to measure medium-length acylcarnitines (Huang et al. 1991; Lowes et al. 1992). Several years later, a GC/MS method was published to analyze

short-, medium-, and long-chain acylcarnitines (Costa et al. 1997). LC was used early on to measure short-chain acylcarnitines (Yergey, Liberato, and Millington 1984), and HPLC has been applied in many reported analytical methods of acylcarnitines (Hoppel et al. 1986; Bhuiyan et al. 1992; Minkler et al. 2008; Giesbertz et al. 2015). Ultra-high pressure liquid chromatography (UHPLC), which has narrower peaks and more reproducible retention times than standard LC, has been used in many recent methods to profile acylcarnitines (Zuniga and Li 2011; Gucciardi et al. 2012; Minkler et al. 2015). Minkler et al. (2015) were able to use isolation by SPE, derivatization with pentafluorophenacyl trifluoromethanesulfonate, reverse-phase UPLC, and MRMs to monitor carnitine and 65 acylcarnitines in a fourteen minute analysis. Other chromatography methods used to profile acylcarnitines include hydrophilic interaction liquid chromatography (HILIC) (Miller Iv, Poston, and Karnes 2012; Peng et al. 2013), capillary electrophoresis (Heinig and Henion 1999), and a direct infusion method with no chromatography using the Biocrates kits, which profile 150 lipids (Römisch-Margl et al. 2012). Electrospray ionization (ESI) is the ionization method most often used in profiling acylcarnitines by LC/MS. Other methods include chemical ionization (CI), electron ionization (EI), atmospheric pressure thermal desorption chemical ionization (APTDCI) to measure dry blood spots, and matrix-assisted laser desorption/ionization (MALDI) to visualize *in situ* acylcarnitines in tissue imaging (Chughtai et al. 2013). Most of the methods to profile

Table 24.1 MS methods for measuring acylcarnitines in biological samples.

First author and year	Derivation	Chromatography	Ionization	Internal standards	MS method	Number of acylcarnitines detected	Samples
Yergey, Liberato, and Millington (1984)		HPLC	Thermospray	2	MS	5	U, B
Millington et al. (1989)	Esterification of carboxylic acids	LC	FAB	1	Tandem MS	18	U, B, T
Huang et al. (1991)	<i>N</i> -Demethylated ester derivatives	GC	CI	1	Tandem MS	7	U
Lowes et al. (1992)	Acyloxylactones	GC	EI, CI	1	MS	11	U
Costa et al. (1997)	Acyloxylactones	GC	CI	11	MRM	22	U, P
Heinig and Henion (1999)		Capillary electrophoresis	Collision-induced dissociation	2	SRM	12	U, P
Minkler et al. (2008)	Pentafluorophenacyl esters	HPLC	ESI	42	MS/MS	47	U, B, T
Zuniga and Li (2011)		UHPLC	ESI	15	SRM	355	U
Gucciardi et al. (2012)	Butyl esters	UHPLC	ESI	13	MRM	48	DBS
Miller et al. (2012)		HILIC	ESI	8	MRM	12	DBS
Römisch-Margl et al. (2012)		Direct infusion	ESI	21	MRM	41	T
Peng et al. (2013)		HILIC	ESI	6	MRM	36	P
Giesbertz et al. (2015)	Butyl esters	LC	ESI	12	Tandem MS	56	P, T
Minkler et al. (2015)	Pentafluorophenacyl trifluoromethanesulfonate	UHPLC	ESI	12	MRM	65	U, B, T

B, blood; CE, capillary electrophoresis; CI, chemical ionization; CID, collision-induced dissociation; DBS, dry blood spots; EI, electron ionization; ESI, electrospray ionization; FAB, fast atom bombardment; GS, gas chromatography; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; P, plasma; SRM, single reaction monitoring; T, tissue; U, urine; UHPLC, ultra-high-performance liquid chromatography.

acylcarnitines use MS/MS to identify and quantify specific acylcarnitines (Millington et al. 1989; Minkler et al. 2008; Giesbertz et al. 2015). The exact MS/MS peaks used for analysis of each individual acylcarnitine depend on the type of derivatization performed. During the development of the LC/MS/MS method, the labeled standards are used to determine recovery and concentration accuracy. Generally, method development should follow the FDA's "Guidance for Industry: Analytical Procedures and Methods Validation for Drugs and Biologics." In general, methods should have $100 \pm 10\%$ of total recovery for internal standards, and concentration accuracy should be less than 20% for lower concentration metabolites and less than 15% for high-abundance metabolites.

In some cases, open profiling metabolomics approaches have detected changes in acylcarnitines in toxicity and disease studies (Bain et al. 2009; Lu et al. 2013; Sun et al. 2014a, b). Open profiling can detect changes in metabolites besides acylcarnitines and therefore can provide useful information about additional pathways, such as metabolites of the Krebs cycle, urea cycle, bile acid metabolism, and so forth. Metabolite identification in open profiling is usually semiquantitative and does not include internal standards for most of the metabolites measured, which lowers the certainty of the peak identification. Thus, it is best to confirm findings generated by open profiling studies through focused profiling methods that include internal standards.

24.3 Acylcarnitines in *In Vitro* and *In Vivo* Hepatotoxicity Studies

Relatively few studies have characterized acylcarnitine metabolism in the liver (Brass and Hoppel 1980). Carnitine and its acyl derivatives were studied in fasted rats (Brass and Hoppel 1978). Fasting increased hepatic concentration of carnitine, whereas urinary elimination of carnitine showed depression for 2–3 days with increases on days 5–6. Urinary elimination of acylcarnitine however showed depression for 4 days but was significantly increased after days 5 and 6 compared with controls (Brass and Hoppel 1978). Sandor and colleagues looked at the composition of [^3H]carnitine in the plasma after injection of [^3H]butyrobetaine and proposed that acylcarnitines in plasma originate from the liver (Sandor et al. 1990). Brass and Beyerinck (1987) showed that carnitine in rat hepatocytes can result in increases in short-chain acylcarnitines. This study goes on to show a major pool of the total carnitine may be present in the form of propionylcarnitine. The appearance of propionylcarnitine in the urine of patients with impaired propionyl-CoA metabolism (Roe et al. 1984) showed that

generated propionylcarnitine can move to extracellular compartments (Brass and Beyerinck 1987). This *in vitro* study provided further biochemical basis for the therapeutic use of carnitine in patients with propionic acidemia (Brass and Beyerinck 1987). Cobalamin (vitamin B12) deficiency is an important clinical disorder (Cooper and Rosenblatt 1987), and the effect of hydroxycobalamin (c-lactam) treatment on propionate and carnitine metabolism in the rat hepatocytes demonstrates that treatment causes a severe impairment in propionate metabolism and alterations in carnitine metabolism consistent with severe functional vitamin B12 deficiency (Brass and Stabler 1988).

Using NMR spectroscopy, Libert and colleagues (Libert et al. 1997) identified in urine *cis*-3,4-methylene-heptanoylcarnitine displaying a cyclopropane ring in their fatty acid moieties. Further studies showed that l-carnitine loading led to greater urinary excretion of *cis*-3,4-methylene-heptanoylcarnitine and was undetectable after treatment with antibiotic adriamycin in the urine (Libert et al. 2005). Using HPLC/MS, they were able to detect *cis*-3,4-methylene-heptanoylcarnitine in the human blood and plasma from a normal volunteer (Yang, Minkler, and Hoppel 2007). The results from a urine specimen spiked with synthesized C8:1 acylcarnitine standards further showed that the "C8:1" acylcarnitine in the urine specimen matches only *cis*-3,4-methylene-heptanoylcarnitine. Besides plasma, acylcarnitines can be found in the bile and urine (Mueller et al. 2003), suggesting that acylcarnitine efflux may serve as a detoxification process (Schooneman et al. 2015).

24.4 Acylcarnitines and Hepatotoxicants

García-Cañaveras and others (2016) performed metabolomics studies using HepG2 cells to develop predictive models that could be used to discriminate between non-toxic and hepatotoxic drugs and toxicity mechanisms (García-Cañaveras et al. 2016). Twelve drugs were examined and were classified by toxicity mechanism (oxidative stress, steatosis, or phospholipidosis). The metabolomics models had an R^2 of 0.83 and Q^2 of 0.69 for determining toxic versus nontoxic drugs and R^2 of 0.69 and Q^2 of 0.52 for delineating toxicity mechanisms (García-Cañaveras et al. 2016). Acylcarnitines and triglycerides were increased in cells treated with hepatotoxic drugs (oxidative stress, steatosis, and phospholipidosis), but the increase in acylcarnitines was only significant for drugs that caused oxidative stress and phospholipidosis (García-Cañaveras et al. 2016).

Several recent studies show the utility of long-chain acylcarnitines as preclinical biomarkers in drug or

compound toxicity evaluations. In a mouse model of APAP-induced hepatotoxicity, LC/MS analysis identified elevation of long-chain acylcarnitines in serum (Chen et al. 2009). These observations were confirmed by Bhattacharyya et al. (2013) and point to the involvement of fatty acid β -oxidation and mitochondrial dysfunction. GTE has been found to be hepatoprotective in murine models of liver injury for several compounds, including 2-nitropropane, galactosamine, carbon tetrachloride, pentachlorophenol, and APAP. The effects of GTE on APAP-induced hepatotoxicity were investigated using novel UPLC/MS- and NMR-based metabolomic profiling in mouse liver samples (Lu et al. 2013). Elevations of oleoylcarnitine and palmitoylcarnitine were observed in the liver samples of APAP-treated mice at 24 h compared with the control group. GTE treatment alone showed little effect on levels of oleoylcarnitine and palmitoylcarnitine in the livers of these mice. In a separate murine study, long-chain acylcarnitines were elevated as a result of co-exposure to a high-fat diet (HFD) and perfluorooctanoic acid (PFOA), a synthetic C8 perfluorinated carboxylic acid. Tan and others (2013) found that co-exposure to HFD and PFOA caused more severe liver damage in male mice compared with PFOA alone (Tan et al. 2013). HFD and PFOA had synergistic effects on hepatic fatty acid metabolites, especially the long-chain acylcarnitines, indicating a disorder of fatty acid oxidation (FAO) (Tan et al. 2013).

The idiosyncratic hepatotoxicant DAN was evaluated in a rat model of liver injury. Palmitoylcarnitine was increased in blood samples 6 h after DAN treatment and then fell to control levels after 24 h, while traditional biochemical indicators of liver injury (e.g., ALT, AST, and ALP) were unchanged (Sun et al. 2014a). Acylcarnitines were also increased in a rat model of carbon tetrachloride hepatotoxicity (Sun et al. 2014b). Liver samples had increased levels of hydroxybutyrylcarnitine and palmitoylcarnitine at both 6 and 24 h, and plasma samples had increased levels of oleoylcarnitine, hydroxybutyrylcarnitine, and palmitoylcarnitine at 6 and 24 h.

The antiarrhythmic and hepatotoxicant dronedarone was examined in a chronic dosing study in wild-type and heterozygous juvenile visceral steatosis (jvs) +/- mice. Jvs mice were discovered in C3H-H-2^o strain mice 5 days after birth, and there were swollen whitish fatty liver in homozygous mutants (jvs/jvs) (Horiuchi et al. 1993). Heterozygous mice jvs (+/-) were produced by mating carnitine-treated homozygous mutant males with heterozygous females (Horiuchi et al. 1993). Dronedarone (400 mg/kg/day for 14 days) led to decreased food consumption and body weight, impaired palmitate metabolism, and hepatotoxicity (Felser et al. 2014). *In vitro* studies showed that dronedarone (50–100 μ M) inhibited the conversion of palmitate to palmitoylcarnitine in mitochondria.

As mentioned earlier, the major role of L-carnitine (free carnitine) is to transport cytosolic long-chain fatty acids as acylcarnitines across the inner mitochondrial membrane (Figure 24.1), thereby delivering these substrates for β -oxidation and subsequent ATP production (Bremer 1983). There is evidence that long-chain fatty acylcarnitines activate proinflammatory signaling pathways in RAW 264.7 murine macrophages and in HCT-116 cells (Rutkowski et al. 2014). It is widely understood that long-chain acylcarnitine dysregulation may point to mitochondrial dysfunction, and many perturbations at the cell level may have a functional role in β -oxidation pathway pathophysiology. The industrial chemical tBHP is a powerful oxidant and causes oxidative stress, lipid peroxidation, and glutathione depletion in cellular models. There are reports showing peroxidative damage to liver mitochondria and hepatocytes with tBHP treatment. In a study using liver mitochondria to see the effect on respiration of rat mitochondria in the presence of palmitoylcarnitine and succinate, Cervinková and coworkers show that addition of ADP to the palmitoylcarnitine and malate reaction led to highly activated oxygen uptake (Cervinková et al. 2008), and this effect was reversed with the addition of tBHP to the reaction. These results further show that complex I was the most sensitive part of the mitochondrial respiratory chain to peroxidative damage. Valproate (VPA) and derivatives are used as antiepileptic agents and in certain cases can cause fatal hepatotoxicity. In a classical study using cell lines from control and FAO-deficient patients, Silva and colleagues (2001) used gas chromatography/chemical ionization mass spectrometry (GC-CI-MS) to evaluate the mechanisms by which VPA inhibits FAO. Control cell lines (skin fibroblasts) from individuals with normal FAO activities and mitochondrial FAO-deficient cell lines (mutant) were obtained from previously identified patients with very-long-chain acyl-CoA dehydrogenase (VLCAD), mitochondrial trifunctional protein (MTP), and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD). Fibroblasts from controls and mutants were cultured with and without VPA. The treatment of control cells with VPA decreased acylcarnitine (C2), whereas VPA induced an accumulation of long-chain acylcarnitines (C10–C16), both in controls and in different mutant cell lines that have established defect in long-chain fatty acid β -oxidation at the level of VLCAD, MTP, and LCHAD. This study established the effect of VPA on β -oxidation pathway and a possible cause for hepatotoxicity due to increase in long-chain acylcarnitines.

Excess free fatty acid is handled primarily by the liver (Rame 2016), whereas increased FAO can cause downstream pathways to further oxidize acetyl-CoA, resulting in a state of active hepatic ketogenesis and acylcarnitine efflux to the plasma compartment to prevent CoA

trapping and hepatic lipotoxicity. Palmitoylcarnitine has been shown to be a lipophilic modulator of protein kinase C (PKC) rather than a simple inhibitor (Nakadate and Blumberg 1987), and PKC family of serine/threonine kinases is involved in phosphorylation of target proteins that impact many cellular processes and the regulation of gene expression (Das, Ramani, and Suraju 2016).

Lipidomic profiling of liver and blood samples from C57BL mice dosed with 30 mg/kg cocaine for three consecutive days indicated that mitochondrial fatty acid β -oxidation was inhibited by the cocaine treatment with an associated increase in long-chain acylcarnitines in blood observed (Shi et al. 2012). Interestingly, lipidomic profiles of liver and blood from the cocaine study showed dramatic changes in other lipids besides long-chain acylcarnitines that included long-chain lysophosphatidylcholines (lysoPCs), phosphatidylcholines (PCs), lysophosphatidylethanolamines (lysoPEs), and phosphatidylethanolamines (PEs) (Shi et al. 2012).

24.5 Acylcarnitines in Cardiac Toxicity

To identify molecular markers of the early stages of cardiotoxicity, Schnackenberg and coworkers (2016) examined acylcarnitine profiles in plasma and cardiac tissue in B6C3F1 male mice treated with doxorubicin. Carnitine (C0), acetylcarnitine (C2), glutarylcarnitine (C5-DC), hexenoylcarnitine (C6:1), and pimelylcarnitine (C7-DC) were decreased in cardiac tissue, while 16 short-, medium-, or long-chain acylcarnitines were increased. Most notable in plasma were octadecanoylcarnitine (C18), hexadecanoylcarnitine (C16), tetradecanoylcarnitine (C14), propionylcarnitine (C3), and valerylcarnitine (C5). The metabolomics analysis suggests that these acylcarnitines may be candidate biomarkers of cardiotoxicity in mouse plasma and heart.

Yamada and coworkers (2000) showed that long-chain acylcarnitines, specifically palmitoylcarnitine (C16) and stearoylcarnitine (C18), enhance Ca^{2+} release in a concentration-dependent manner from cardiac SR-enriched membrane vesicles (Yamada, Kanter, and Newatia 2000). However, hypoxia-induced cardiac myocytes display rapid accumulations of long-chain acylcarnitines, and these molecules *in vitro* have been shown to inhibit excitatory Na^+ currents (DaTorre et al. 1991). Abnormal acylcarnitine concentrations have been observed in patients with diabetes, fatty acid disorders, and myocardial ischemia.

Besides liver and cardiac cells, other cell types also show acylcarnitine-induced pathophysiological effects. Most recently, Ferro et al. (2012) studied the effects of acylcarnitines on hERG channels in HEK293 cells using the patch clamp technique, and the results showed that

long-chain acylcarnitines (C16 and C18) have regulatory properties on the hERG channels. Furthermore, long-chain acylcarnitines, but not medium-chain or short-chain acylcarnitines, were shown to speed the deactivation of hERG channels in HEK293 cells (Ferro et al. 2012) and may trigger cardiac arrhythmias in pathological conditions. Skeletal muscle model C2C12 myotubes when treated with acylcarnitine (C16) show that long-chain acylcarnitines have the potential to rapidly increase intracellular calcium and induce membrane disruption to activate skeletal muscle inflammatory and cell stress pathways (McCoin, Knotts, and Adams 2015). The previously reviewed *in vitro* cell model studies show the importance acylcarnitines as potential biomarkers to regulate numerous signaling pathways in pathophysiology.

24.6 Clinical Hepatotoxicity

DILI is associated with mitochondrial dysfunction mediated through either direct or indirect disruption of β -oxidation (Pessayre et al. 2012) that results in perturbations in long-chain acylcarnitines in the blood. For example, valproic acid enters mitochondria without the carnitine shuttle and extensively forms valproyl-CoA, thus decreasing concentrations of free intramitochondrial CoA available to sustain fatty acyl-CoA formation inside the mitochondria. This mechanism inhibits β -oxidation of long-, medium-, or short-chain fatty acids. VPA can also inhibit CPT1 activity, preventing the entry of long-chain fatty acids into the mitochondria (Begrache et al., 2011) and subsequent alterations of blood acylcarnitine levels (Eyer et al. 2005). We and others have recently reported elevations of long-chain acylcarnitines (palmitoyl-, oleoyl- and myristoylcarnitines) in mice treated with toxic doses of APAP (Chen et al. 2009; Bhattacharyya et al. 2013). Figure 24.3 shows that palmitoylcarnitine in plasma from mice peaked at 4 h, which was before ALT peaked at 8 h, making it an early biomarker of APAP-induced liver injury. In order to examine the clinical relevance of data generated in animal models, we quantified acylcarnitines and other known indicators of APAP metabolism and toxicity in children with APAP poisoning (Bhattacharyya et al. 2014). The study included two APAP-exposed subject groups, one receiving therapeutic dose ($n = 187$) and the other with overdose or toxic ingestion ($n = 62$), that were compared with normal healthy controls with no APAP exposure ($n = 23$). Serum samples were used for measurement of APAP protein adducts, a biomarker of the oxidative metabolism of APAP, and for targeted metabolomics analysis of serum acylcarnitines using ultra-performance LC-triple-quadrupole MS. Significant increases in long-chain acylcarnitines (oleoyl- and palmitoylcarnitine) in

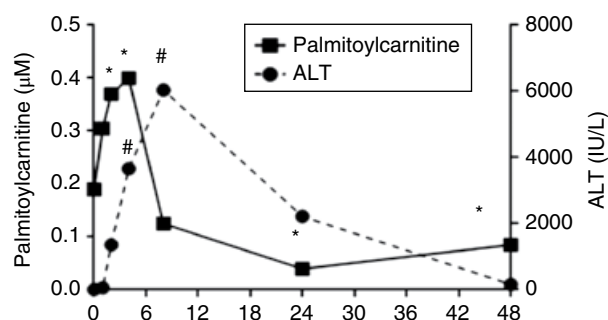


Figure 24.3 Time response of palmitoyl carnitine (solid line with solid squares) versus ALT (dashed line with solid circles) from mice dosed with 200 mg/kg APAP. * $p < 0.05$ for palmitoyl carnitine and # $p < 0.05$ for ALT. Adapted from Beger et al. (2015). <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4551536/>. Used under the license CC BY 4.0. <http://creativecommons.org/licenses/by/4.0/>.

the serum of children exposed to low and overdose of APAP were observed compared with normal healthy controls. Significant increases in oleoylcarnitine C(18:1) and palmitoylcarnitine (C16) in the serum of children exposed to low and overdose of APAP were observed compared with normal healthy controls. In addition, higher levels of serum ALT, APAP protein adducts, and acylcarnitines were observed in children that had delayed treatment with the antidote *N*-acetylcysteine (NAC), compared with those receiving NAC within 24 h of the overdose. The APAP-induced perturbations in serum acylcarnitines in children suggest that mitochondrial injury and associated impairment in the β -oxidation of

fatty acids are important mechanisms in APAP-induced hepatotoxicity. Comparable findings were reported by McGill and colleagues (McGill et al. 2014) in the mouse model.

24.7 Conclusions

Long-chain acylcarnitines are biomarkers of fatty acid β -oxidation dysfunction in liver and other organ toxicities. Quick and accurate LC/MS methods have been developed to profile many acylcarnitines, and these methods can be used in samples from *in vitro* and non-clinical toxicity studies. A number of studies have identified differences in acylcarnitine expression and their functional roles in disorders of inborn errors of metabolism and across a range of diseases/disorders involving obesity, diabetes, insulin resistance, hypertension, and heart and drug toxicity. Increases in long-chain acylcarnitines in plasma have been observed in APAP overdose patients. As many diseases and external factors can alter acylcarnitine profiles, further research is needed to validate their use in the clinical setting. In brief, the mechanisms by which acylcarnitines contribute to mitochondrial dysfunction have yet to be fully elucidated, although several hypotheses exist. One possibility is that acylcarnitines promote the signaling pathway, leading to necrosis and oxidative stress. Finally, this chapter has focused on the potential for acylcarnitines as biomarkers in disease and drug toxicity and their far-reaching impact on mitochondrial dysfunction.

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25

Mitochondrial DNA as a Potential Translational Biomarker of Mitochondrial Dysfunction in Drug-Induced Toxicity Studies

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CHAPTER MENU

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25.1 Introduction

Mitochondria are double-membrane organelles that produce the majority of cellular energy in eukaryotes in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). In addition to energy production, mitochondria have many other crucial cellular functions, including the regulation of intracellular calcium homeostasis and apoptosis (Wojtczak and Zablocki, 2008). Because of their role in energy production and other key cellular functions, damage to mitochondria can have a serious impact on the health of cells and tissues and can result in a variety of diseases (Wallace, 1999). In recent years it has become widely accepted that systemic damage to mitochondria, often termed acquired mitochondrial dysfunction, is involved in many common human diseases (Malik and Czajka, 2013; Michel et al., 2012; Wallace, 1999) as well as in drug-induced toxicity (Dykens and Will, 2008), leading to a growing interest in developing biomarkers of mitochondrial health.

Mitochondrial energy production is carried out within the double membrane of mitochondria via electron transport through a complex of proteins known as the electron transport chain. During mitochondrial ATP

synthesis, electron leakage from the electron transport chain can lead to the production of reactive oxygen species (ROS), which in normal conditions is involved in signaling. However excess ROS can lead to oxidative stress. In normal healthy cells mitochondria are present as an interconnected network or several networks rather than the old-fashioned view of solitary organelles (Bereiter-Hahn et al., 2008). Cellular mitochondrial content is regulated via mitochondrial biogenesis and degradation of mitochondria via mitophagy. The mitochondrial mass reflects the bioenergetics requirements of the host cell and can vary from tens to thousands of mitochondria per cell. The number of mitochondria in different cell types therefore varies widely, for example, a brain cell may have around 2000 mitochondria (Uranova et al., 2001), a white blood cell may have less than a hundred (Selak et al., 2011), and oocytes may contain several hundred thousand mitochondria (Duran et al, 2011; Piko and Matsumoto, 1976). The number of mitochondria in a particular cell type also can vary in response to environmental and physiological factors, for example, cellular redox balance or signaling pathways (Michel et al., 2012; Rodriguez-Enriquez et al., 2009). Damage to mitochondria, once it exceeds a threshold, can affect a range of important cellular functions and can contribute

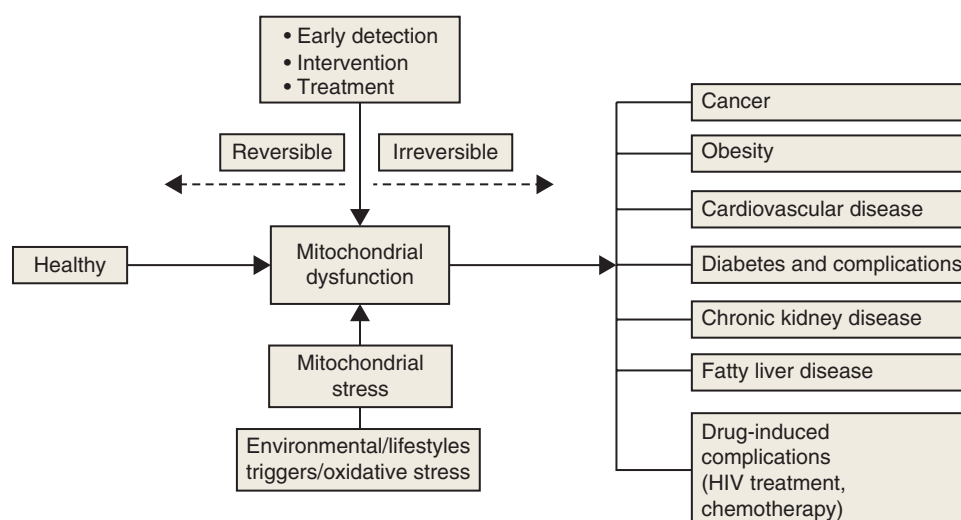


Figure 25.1 Mitochondrial dysfunction as an early event in disease. Environmental/lifestyle triggers such as high fat and/or glucose or drugs can result in oxidative stress and altered signaling, which in turn damages mitochondria in organs (e.g., kidney, heart, liver), cells (blood cells, adipocytes), and blood vessels; the damage may take decades to manifest itself and cause pathology. Identification of biomarkers for the early detection of metabolic and bioenergetic changes associated with these pathologies could allow intervention and prevention of irreversible bioenergetic dysfunction.

to the development of a large number of diseases (Michel et al., 2012; Wallace, 1999). Cells and tissues with high bioenergetic needs and consequently high mitochondrial mass are particularly sensitive to the impact of mitochondrial damage. Consequently, there is a strong need for translational biomarkers that can be used for early detection of potential mitochondrial dysfunction before irreversible damage to susceptible cells, tissues, and organs takes place (Figure 25.1).

Mitochondria are the only cytosolic organelles in eukaryotes that contain endogenous DNA outside of the nucleus. Mitochondrial DNA (mtDNA) is normally located within mitochondria as a small, circular extranuclear genome. Each mitochondrion can contain multiple copies of mtDNA (Bogenhagen, 2011; Falkenberg et al., 2007), and since cells contain many mitochondria, mtDNA is present as multiple copies within cells. The amount of cellular mtDNA has been shown to correlate with mitochondrial function and OXPHOS activity (Hock and Kralli, 2009; Williams, 1986), and this has led to studies using its quantity as a determinant of mitochondrial activity.

In the last decade, numerous studies have shown that mtDNA levels are altered in disease conditions in tissues and in circulation, and additionally mtDNA has emerged as a damage-associated molecular pattern (DAMP) with the potential to induce inflammation (Zhang et al., 2010). However, to date, few studies have attempted to use alterations in cellular or cell-free mtDNA in studies of drug toxicity. In this chapter, I propose the potential of using mtDNA levels in drug toxicity studies, both *in vitro*

and animal studies, as well as in clinical studies to monitor the effects of pharmacological compounds on mitochondrial health.

25.2 The Mitochondrial Genome

The human mitochondrial genome is 16,569 bp long and contains 37 genes, encoding 13 proteins and 24 transfer RNA and ribosomal RNAs crucial to mitochondrial function. The remaining mitochondrial proteins that are required to make functional mitochondria are coded for and transcribed from the nuclear genome, with resultant transcripts being translated into proteins at cytosolic ribosomes and transported into mitochondria for assembly (Scheffler, 2008). Mitochondria contain more than 1000 different proteins, some of which show tissue-specific profiles (Johnson et al., 2007; Smith et al., 2012). The correct functioning of all 37 genes encoded by the mitochondrial genome is crucial to make functional mitochondria and a functional electron transport chain. This is because mtDNA encodes 13 protein subunits crucial for the mitochondrial OXPHOS machinery as well as various RNAs required for mitochondrial protein synthesis. These components, together with nuclear-encoded proteins, result in the assembly of functional mitochondrial mass in cells and allow mitochondrial function and energy production in the form of ATP. Therefore, mtDNA has a crucial role in cells by acting as a template for both transcription and replication to generate functional mitochondria. Bioenergetically active tissues such as the brain,

Table 25.1 Key differences between the human mitochondrial and nuclear genomes.

	Mitochondrial DNA	Nuclear DNA
Cellular location	Mitochondria	Nucleus
Size (nt)	16,569	~30,000,000,000
Major function	DNA replication and transcription, signaling	DNA replication and transcription
Organization	Double-stranded circular molecule complexed with TFAM	Double-stranded duplex linear DNA molecules (chromosomes) complexed with histones to form chromatin
Genetic code	Different use of start and stop codons	Universal
Replication	Bidirectional from a single origin of replication	Numerous origins of replication
Transcription	Polycistronic mRNAs from two promoters	Highly regulated and mostly individual mRNA transcription from thousands of individual promoters
Introns/exons	No introns, very few noncoding regions, contiguous and overlapping	Contain introns and large stretches of noncoding regions
Inheritance	Maternal	Bi-parental
Replication	Independent of the cell cycle	Dependent on the cell cycle
Number of copies per cell	10s to many 1000s of copies of the mitochondrial genome—variable and can change in response to physiological stimuli	1–2 copies of the nuclear genome in the form of chromosomes (within which there are many repeated sequences)
Methylation	Resembles bacterial DNA (less methylated)	Methylated
Sequence identity	Contains very few regions that are unique (>90% is duplicated in the nuclear genome)	Contains pseudogenes known as NUMTs, which are identical to mtDNA and highly variable

heart, kidney, and muscle with a high mitochondrial content therefore can contain hundreds of thousands of copies of mtDNA per cell, whereas other tissues and cells such as blood cells with less mitochondrial mass contain considerably fewer mtDNA molecules per cell (Fernandez-Vizarra et al., 2011; Malik et al., 2016; Mercer et al., 2011).

Under normal conditions, the amount of mtDNA can change in response to cellular physiological signals, with cells maintaining a balance between mtDNA replication and transcription to allow mitochondrial biogenesis as needed. However, in certain disease conditions, this relationship breaks down, and cellular mtDNA content may increase in response to oxidative stress, but transcription and translation of mtDNA are blocked, leading to increased cellular mtDNA, which in time may become damaged. mtDNA damage could comprise mutations, deletions, and oxidation. The integrity and amount of mtDNA present in cells can have an impact on mitochondrial function (Czajka et al., 2015; Madsen-Bouterse et al., 2010). mtDNA damage can have downstream effects on cellular health, causing defects in the OXPHOS machinery and cellular signaling and subsequently leading to oxidative stress, an energy deficit, and eventually cell death. It may thus lead to release of cellular content including mtDNA into circulation, and if systemic mitochondrial dysfunction is present, the release of large amounts of cellular contents and mtDNA may compromise the body's capacity to clear circulating mtDNA.

Some of the key differences between the human mitochondrial and nuclear genomes are listed in Table 25.1. Eukaryotic DNA in the nuclear genome is organized as chromosomes, large double-stranded linear molecules stored as highly packaged and compact structures and stored as chromatin, a DNA–histone protein complex. In contrast, mtDNA exists as a small circular double-stranded DNA molecule organized into a nucleoprotein with the transcription factor A (TFAM) protein, termed a nucleoid. Nucleoids are found associated with the inner mitochondrial membrane (Bogenhagen, 2011; Falkenberg et al., 2007), and individual mitochondria can contain several copies of the mitochondrial genome (Navratil et al., 2007; Veltri et al., 1990). The differences between the mitochondrial and nuclear genome (Table 25.1) can significantly impact the methodology used for the measurement of mtDNA and are discussed in more detail later on (see Section 25.4).

25.3 Is Mitochondrial DNA a Useful Biomarker of Mitochondrial Dysfunction

The amount of mtDNA in a cell could provide a major regulatory point in mitochondrial activity, as the transcription of mitochondrial genes is proportional to

their copy number (Hock and Kralli, 2009; Williams, 1986). Indeed, mtDNA has been widely utilized as an indicator of cellular mitochondrial content. We previously proposed the hypothesis that mtDNA content measured as Mt/N (mitochondrial-to-nuclear genome ratio) is a biomarker of mitochondrial dysfunction (Figure 25.2, Malik and Czajka, 2013).

The premise of this theory is that the Mt/N value of a particular cell type changes in conditions of stress such as redox imbalance or other altered signaling. The initial response to increased cellular stress would be an adaptive response where Mt/N values would increase as a result of increased mitochondrial biogenesis. In conditions of persistent oxidative stress, alterations in Mt/N may represent a mixture of intact and functional mitochondrial genomes as well as damaged mtDNA fragments that have not been properly removed. Oxidative stress may eventually lead to the depletion of Mt/N alongside mitochondrial dysfunction resulting from damaged mtDNA and proteins. Accumulation of damaged mtDNA in the cell may lead to an inflammatory response as mtDNA is un-methylated and resembles bacterial DNA (Figure 25.2).

Oxidative stress is a common feature in many diseases including diabetes complications, cardiovascular disease, neurodegenerative disease, cancer, renal disease, and others (Halliwell and Gutteridge, 2007). Free radicals, also known as ROS, are produced as a side product of using oxygen for energy production and are highly reactive molecules with unpaired electrons. It has been estimated that approximately 5% of the oxygen being used in the body turns into ROS, as a consequence of electron leakage from the electron transport chain during OXPHOS (Adam-Vizi and Chinopoulos, 2006; Halliwell and Gutteridge, 2007; Turrens, 2003). With the exception of phagocytes, cells produce more than 95% of their intracellular ROS via the mitochondrial electron transport chain. Most cells are well equipped to deal with intracellular ROS as they have endogenous

antioxidant systems such as glutathione peroxidase, catalase, and superoxide dismutase (Nohl, 1991; Nordberg and Arner, 2001). These highly abundant cellular proteins, present in most cells, can sequester ROS by accepting electrons and becoming oxidized and are usually recycled by donating their electrons to chains of acceptors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Rydstrom, 2006). The cell's metabolic performance is closely related to its antioxidant response, and NADPH levels are central to the activity of many antioxidants (Kirsch and De Groot, 2001). Despite these endogenous antioxidant systems, when chronic ROS production occurs, the cell's ROS levels can exceed their detoxification and cause a shift in the redox balance. Free radicals that escape the cells' antioxidant response can oxidize proteins, lipids, and DNA molecules within the cell, leading to altered properties and cellular damage. Many common drugs cause mitochondrial oxidative stress (reviewed by Mehta et al., 2008), and many common diseases such as diabetes and its complications, cancer, and neurodegenerative disorders as well as aging have been shown to have redox impairment (Halliwell and Gutteridge, 2007; Wallace, 1999; Ying, 2008).

The mitochondrial life cycle controls cellular mitochondrial mass through both mitochondrial biogenesis, the synthesis of new mitochondria, and mitophagy, the degradation and removal of damaged mitochondria. Evidence indicates that both biogenesis and mitophagy may be impaired in conditions of oxidative stress. Abnormal signaling results in an adaptive response through enhanced production of mitochondria (Michel et al., 2012). Reduced removal results in the accumulation of damaged mitochondria (Kim et al., 2007) as is the case for diabetes where blockage of the electron transport chain at complex III results in accumulation of excess ROS (Giacco and Brownlee, 2010; Newsholme et al., 2007). As mtDNA is located close to the source of ROS production, the DNA itself can become damaged,

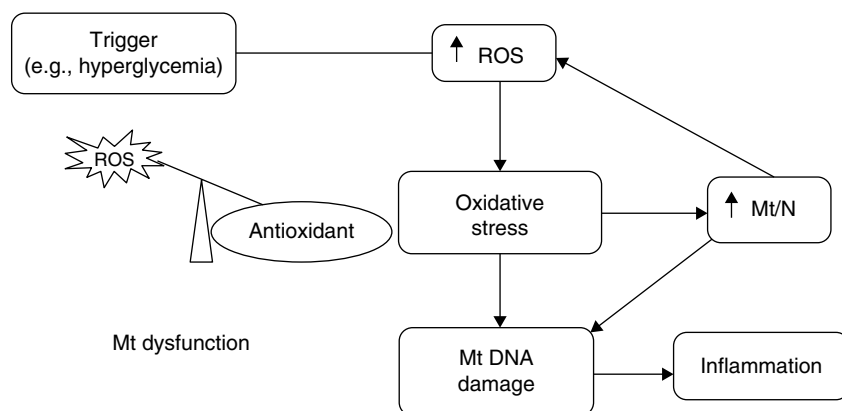


Figure 25.2 Schematic of the hypothesis that mitochondrial DNA can increase in response to oxidative stress as an adaptive response. Environmental/lifestyle triggers such as high fat and/or glucose or drugs result in oxidative stress and altered signaling, which leads to an early adaptive response of increased cellular mtDNA but over time causes systematic damage to mitochondria in organs (e.g., kidney, heart, liver) and cells (blood cells). Malik and Czajka (2013). Reproduced with permission of Elsevier.

resulting in accumulation of deletions and mutations (Bohr, 2002; Croteau and Bohr, 1997; Indo et al., 2007).

Accumulation of damaged mtDNA, alongside its ineffective clearance, may result in its release from damaged mitochondria and cells and cause a chronic innate inflammatory response. In such a scenario, mtDNA could contribute directly to pathology because unlike eukaryotic nuclear DNA that is often methylated at CpG motifs within DNA, mtDNA is largely un-methylated like bacterial DNA. Un-methylated DNA is known to cause immune responses via the intracellular Toll-like receptor (TLR)9 (Barbalat et al., 2011; Sparwasser et al., 1997). Injection of oxidized mtDNA directly causes inflammatory arthritis in mice (Collins et al., 2004). Zhang et al. (2010) showed that circulating mtDNA levels were markedly increased in trauma patients and provided a mechanistic explanation for this observation by showing that mtDNA could directly activate human neutrophils via TLR9 (Zhang et al., 2010). Accumulation of mtDNA in the cytosol of cardiomyocytes resulted in heart failure in a mouse model where the normal process of degradation of damaged mtDNA had been disrupted (Oka et al., 2012). Therefore, altered mtDNA levels may elicit an increased immune response, resulting in chronic inflammation and oxidative stress, thus contributing directly to pathogenesis. In parallel, loss of cellular mtDNA would cause reduced mitochondrial function and a bioenergetic deficit, which would further impair the cell's ability to repair cellular damage.

According to our hypothesis (Figure 25.2), in conditions of oxidative stress, the transcriptional and replication machinery of mitochondrial biogenesis will be upregulated as a maladaptive response, resulting in increased mitochondrial biogenesis via replication of the mitochondrial genome (Malik and Czajka, 2013). There are some studies in the literature supporting the view that ROS can lead to increased mitochondrial biogenesis. In human endothelial cells, homocysteine-induced ROS resulted in increased expression of TFAM and NRF-1 genes, and this effect was abolished by antioxidant treatment (Perez-de-Arce et al., 2005). In human lung fibroblasts, following treatment with hydrogen peroxide to induce oxidative stress, there was an increase in mitochondrial mass and mtDNA copy number (Lee et al., 2000). Upregulation of transcriptional machinery was shown to be protective against oxidative stress, for example, overexpression of recombinant TFAM *in vitro* and *in vivo* can stimulate mitochondrial biogenesis and reduce oxidative stress (Thomas et al., 2011). Lee and Wei proposed that mild oxidative stress leads to increased mitochondrial biogenesis and copy number and suggested that the stress response of cells in terms of mitochondrial copy numbers and biogenesis could be key in terms of the life or the death of the cell and should be

further investigated (Lee and Wei, 2005). Moreover, in a study of 156 healthy subjects, ranging from the ages of 25 to 80, it was found that mtDNA content in leucocytes was higher in volunteers with increased levels of oxidative stress (Liu et al., 2003).

We recently showed that growth of primary human renal glomerular mesangial cells in high glucose led to a rapid increase in cellular mtDNA in parallel with increased oxidative stress (Czajka et al., 2015) and that these changes preceded other measures of mitochondrial dysfunction. Interestingly, the increased mtDNA was not functional since mtDNA-encoded mRNAs were not upregulated in parallel. Instead, the mtDNA was damaged and there was upregulation of the TLR9 pathway in parallel (Czajka et al., 2015). These data support the hypothesis that oxidative stress can lead to early and detectable changes in mtDNA. Interestingly, we further showed that the mtDNA changes preceded mitochondrial dysfunction, since mtDNA changes were detectable within 24 h of growth in high glucose whereas cellular respiration remained functional until 8 days (Figure 25.3). This further supports the view that mtDNA changes take place early on and may be used as an indicator of mitochondrial dysfunction before damage to cellular respiration takes place. These data also suggest that early changes in mtDNA may cause a cascade of proinflammatory responses via the early activation of the TLR9 pathway.

25.4 Methodological Issues for Measuring Mitochondrial DNA Content

As discussed in more detail later on and previously described, disease-associated changes in mtDNA content from various body fluids have been reported in a broad range of human diseases, as well as in normal development, fertility, and exposure to environmental factors (Malik and Czajka, 2013). The use of body fluids for these studies is an attractive option as tissues and organs cannot easily be accessed, and most published studies have tended to use blood samples. A common method for measuring mtDNA content is to quantify a mitochondrial-encoded gene relative to a nuclear-encoded gene to determine the mitochondrial genome to nuclear genome ratio, which we have termed Mt/N (Malik et al., 2011). Earlier studies measuring Mt/N utilized hybridization (Rodriguez-Enriquez et al., 2009; Veltri et al., 1990), whereas more recent studies use real-time (Cavelier et al., 2000; D'Souza et al., 2007; Malik et al., 2011) or digital (Masser et al., 2016) quantitative PCR (qPCR), a highly sensitive technique that is fast,

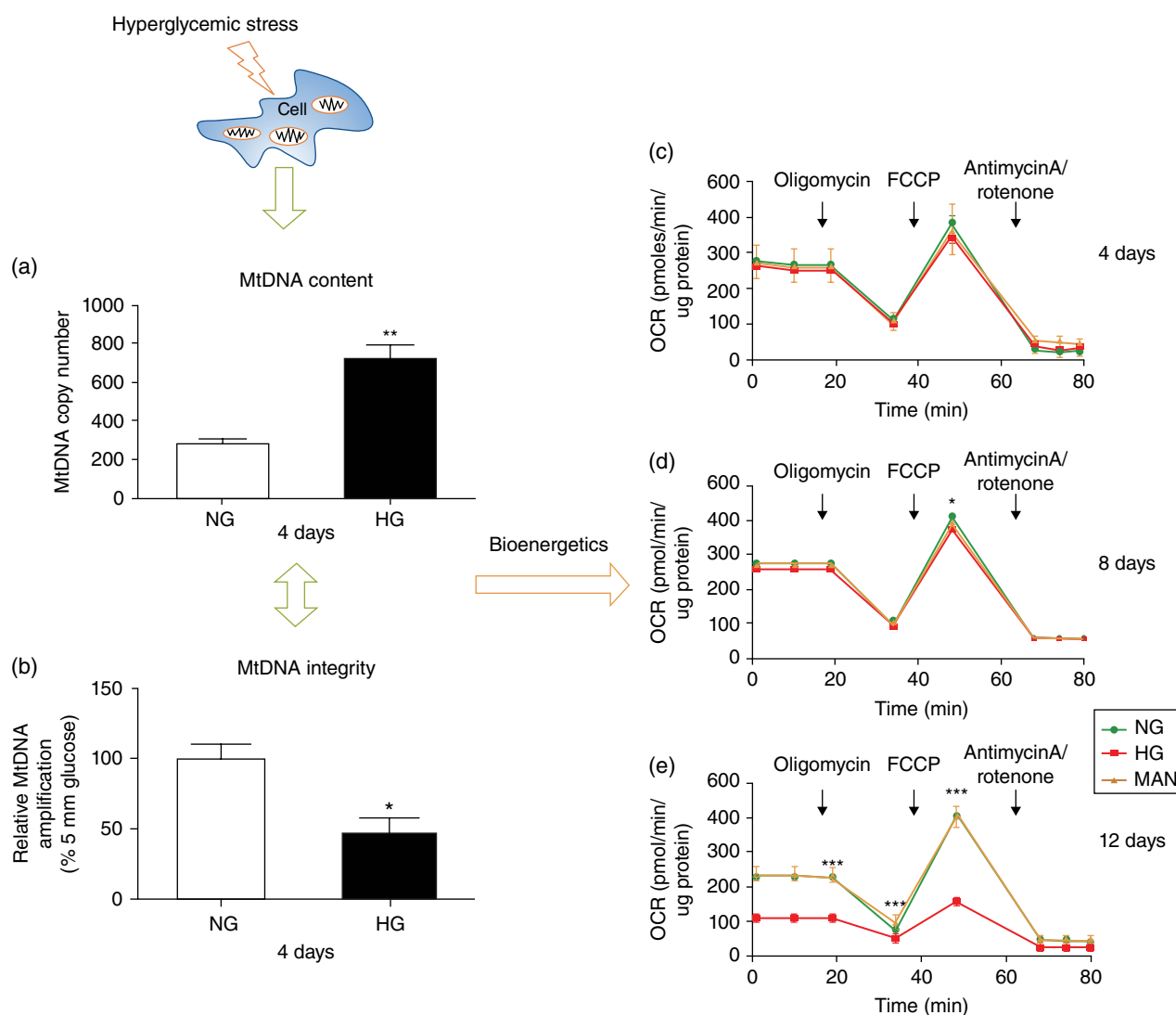


Figure 25.3 Changes in cellular mtDNA precede metabolic dysfunction in conditions of oxidative stress. Growth of HMCs in high glucose led to a significant increase in cellular mtDNA, which was detectable within 24 h and highly significant after 4 days. (a) However, the mtDNA was damaged as illustrated by reduced amplification of an mtDNA 8.6 kb fragment (b). Cells showed normal bioenergetic profile at day 4. (c) However, after 8 days, maximal respiration and reserve capacity were significantly reduced in hyperglycemic cells but unaffected in normoglycemic cells (d, e). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Czajka et al. (2015). Reproduced with permission of Elsevier.

adaptable for high throughput, and widely available. This has resulted in the utilization of this technique in determination of Mt/N in a large number of studies of clinical samples (reviewed in the next section).

mtDNA quantity in the periphery, in circulating peripheral blood cells as well as in cell-free fluid of blood such as plasma, is a highly feasible screening tool for translational studies. However, both increases and decreases in mtDNA have been reported in pathogenic conditions. Currently there is no standard for defining what constitutes an abnormal mtDNA quantity in different sample types, and data from different populations for specific diseases have been inconsistent. Many

methodologically based issues can significantly alter mtDNA values (Chiu et al., 2003; Hammond et al., 2003; Kam et al., 2013; Malik et al., 2011; Malik and Czajka 2012). These include (i) duplication of the mitochondrial genome in the nuclear genome, (ii) use of inappropriate nuclear primers, (iii) dilution bias, and (iv) template preparation problems. These problems can lead to serious errors and are likely to be in part responsible for the conflicting data in the literature. Many protocols widely used for mtDNA quantification do not meet the criteria of specificity and reproducibility as they fail to take into account either the co-amplification of nuclear regions with high identity to the mitochondrial genome or the

dilution effect (Malik et al., 2011). Furthermore, many published papers do not give the actual copy numbers and rely instead on relative values, which makes the data more difficult to interpret, especially if the samples being used comprise both cell-free and cellular mtDNA. (For a more detailed discussion of these methodological issues, see Malik and Czajka (2013), Ajaz et al. (2015), and Malik et al. (2016).)

25.5 Acquired Mitochondrial DNA Changes in Human Diseases

The aim of this section is to highlight the growing body of evidence that, when considered together, strongly supports the view that mtDNA is a potentially valuable and currently largely overlooked biomarker for drug toxicity studies. Our focus is on studies reporting changes in mtDNA quantity under disease conditions rather than mtDNA damage or deletions/mutations/haplotypes. The wider availability of qPCR as a methodology has led to a substantial increase in publications reporting changes in mtDNA content in human body fluids and tissues. Changes in mtDNA content have been described for a wide range of human diseases from cancer to diabetes as well as in development, aging, and exercise. We reviewed the literature and showed that dozens of studies had shown changes in mtDNA in a large number of diseases (Malik and Czajka, 2013). Since then the number of studies reporting changes in mtDNA in disease has risen even more sharply.

In the cancer field, altered mtDNA levels have been observed in peripheral blood cells, saliva, tumor tissues, and other body fluids in numerous studies (reviewed in Malik and Czajka, 2013), leading us to suggest that control of mtDNA copy number may be dysregulated in cancer. Altered mtDNA levels were proposed to contribute to the risk of cancer in the meta-analysis of numerous studies (Hu et al., 2016; Mi et al., 2015). The dysregulation of mtDNA levels may have direct consequences for drug therapy response in patients. For example, in one study, the level of mtDNA in breast cancer tissue correlated with patient response to anthracycline chemotherapy, with higher mtDNA levels showing lower drug sensitivity (Hsu et al., 2010), whereas in acute lymphoblastic lymphoma, reduced blood mtDNA after treatment was found to confer increased susceptibility to chemotherapy (Kwok et al., 2011). mtDNA copy number changes are widely described in cancers, and interestingly it has been found that mitochondrial dysfunction induced by chemical depletion of mtDNA or impairment of mitochondrial respiratory chain in cancer cells promotes cancer progression to a chemoresistant or

invasive phenotype. Qu et al. (2015) found that leukocyte mtDNA was an independent prognostic marker of colorectal cancer and could be used to stratify patients for chemotherapy. Chen et al. (2016) carried out a meta-analysis of 18 separate studies where mtDNA had been measured in 3961 cases from peripheral blood and/or tumor tissue. Their analysis suggested that increased mtDNA levels in peripheral blood predicted a poor cancer prognosis whereas a better outcome was presented among patients with elevated mtDNA levels in tumor tissues. Therefore, in the future, selective anticancer therapy development may benefit from using mtDNA alterations to inform drug design.

In the human immunodeficiency virus (HIV) field, the impact of therapy on measureable mtDNA changes is very clearly indicated by evaluation in patients undergoing HIV therapy and strongly linked to the risk of numerous drug-induced HIV complications. Antiretroviral therapy (ART), widely used for the treatment of HIV, can cause mitochondrial toxicity and many complications. Differences in mtDNA have been shown between the adipose tissue of HIV-infected and ART-treated subjects demonstrating that HIV therapy can impact mtDNA in organs as well as within the periphery, showing systemic effects of drug therapy (Buffet et al., 2005). The older ART drugs such as nucleoside reverse transcriptase inhibitors directly affect mtDNA replication and result in tissue-specific and organ-specific pathologies, and consequently, many studies have reported mtDNA changes in association with drug-induced complications in HIV patients. One direct mechanism of mtDNA damage is by the inhibition of DNA polymerase gamma, the enzyme that carries out mtDNA replication and that is particularly sensitive to certain antiviral drugs such as dideoxynucleoside inhibitors. As for various cancers, HIV treatment can cause significant changes in mtDNA, and therefore it is very likely that control of mtDNA copy number is compromised as a consequence of HIV infection and/or treatment. *In vitro* experiments showed that lymphoblast cells with increased mtDNA were more resistant to HIV therapy (Bjerke et al., 2008), suggesting that as in cancer, altered mtDNA could have consequences for HIV therapy.

Changes in mtDNA content have been described for metabolic disorders such as diabetes and obesity, as well as fertility, development, and aging (see Malik and Czajka, 2013). We have recently shown that circulating mtDNA levels were independently associated with risk of diabetic nephropathy (Czajka et al., 2015) and in a separate study circulating mtDNA levels and inflammation correlated with risk of diabetic retinopathy, the leading cause of adult blindness (Malik et al., 2015). The dysregulation of mtDNA content in metabolic diseases suggests that changes in mtDNA content correlate

with metabolic changes. Interestingly, the antidiabetic drug thiazolidinedione (TZD) was shown to result in increased mtDNA in adipose tissue of patients with diabetes, in parallel with increased fat storage and weight gain (Bogacka et al., 2005). Altered mtDNA levels have also been reported in liver disease, chronic renal failure, hemodialysis, and septic shock where mtDNA is believed to cause systemic inflammatory response syndrome (Malik and Czajka, 2013).

mtDNA changes have been reported to correlate with disease in human population-based studies of neurodegenerative disease including multiple sclerosis (Blokhin et al., 2008; Varhaug et al., 2016), Parkinson's disease (Pyle et al., 2016), Alzheimer's disease (Mathew et al., 2012), and Huntington's disease (Petersen et al., 2014) as well as for depression (Kim et al., 2011). mtDNA has become accepted as an activator of both inflammation and the innate immune response and has been shown to be the cause of organ injury (Oka et al., 2012). Additionally, cell-free mtDNA levels in circulation were shown to be a high risk factor for mortality in two different studies of patients in intensive care units. Circulating mtDNA levels have been shown to be predictive of mortality in patients admitted to intensive care units (Nakahira et al., 2013) and also correlated with traumatic injury and sepsis (Yamanouchi et al., 2013). mtDNA levels have also shown to be predictive of poor outcome/death in patients who have taken drug overdoses (McGill et al., 2014).

An interesting theme emerging from a large number of studies is the reports that suggest that mtDNA levels can correlate with and be an indicator of the effect of exposure to chemicals, drugs, or environmental toxins in humans. Occupational exposure to low-dose benzene can result in increases in circulating mtDNA, and this has been proposed to be a possible cause of increased incidence of leukemia in this population (Carugno et al., 2012). Exposure to the herbicide atrazine was shown to result in mitochondrial dysfunction and insulin resistance in an *in vivo* study (Lim et al., 2009). Using exfoliated cells from saliva, smokers were found to have increased mtDNA, and this increase was independent of age and alcohol intake (Masayeva et al., 2006).

Budnick et al. (2013) evaluated the impact of exposure to pesticides and found that circulating mtDNA showed both alterations in quantity and loss of integrity, leading the authors to propose that mtDNA has the potential to serve as a biomarker for recognizing vulnerable risk groups after exposure to toxic/carcinogenic chemicals. Even in a traditionally genetic disease with a clear nuclear mutation, mtDNA was proposed as a biomarker to follow the progression and treatment response of Huntington's disease by Disatnik et al. (2016). In their model system, they observed that both tissue and circulating levels of

mtDNA were changing at different stages of disease and in response to treatment.

Therefore a large body of evidence now exists, showing that mtDNA levels can be measured in human clinical samples and that disease-associated changes can be detected in populations. Indeed the evidence for reported alterations in mtDNA in body fluids of human patients in correlation with many diseases has grown rapidly, and in the previous section I have only been able to comment on a subset of these. What is clear is that there is widespread interest in using mtDNA as a biomarker in human populations, and with the mounting evidence for a link between patient drug response and circulating mtDNA levels, there is strong potential for the future use of this marker in the field of personalized medicine.

25.6 Conclusions and Future Directions

Mitochondrial dysfunction is a key issue in drug development, and off-target effects of many drugs may have an impact on mitochondria. Mitochondrial dysfunction contributes to drug toxicity and adverse side effects via many mechanisms in the cell (Mehta et al., 2008). Although structural similarities of drugs to electron acceptors and donors, assays based on redox dyes, and bioenergetics assays have been successfully employed for screens of mitochondrial effects, such assays do not easily lend themselves for noninvasive use in human samples. Furthermore, there is a need to develop biomarkers for early detection of mitochondrial dysfunction before tissue and organ damage. Because of its early adaptive response to oxidative stress by increased replication and blocked transcription, mtDNA may provide an indicator of mitochondrial stress prior to other indicators. In addition, mtDNA lends itself to rapid detection via methods such as qPCR and digital PCR, making it an attractive high-throughput biomarker. However, methodology issues have hindered the successful use of mtDNA as a biomarker and led to conflicting and unreproducible findings in some cases. Of particular note in this regard is the presence of nuclear mitochondrial DNA segments (NUMTs) in the nuclear genome that can skew data by co-amplifying nuclear genes when mtDNA levels are being assessed. In addition, assays currently in use seldom distinguish between cell-free and cellular mtDNA: the former is of importance as it may be an indicator of inflammation, and the latter is important as it may be an indicator of bioenergetic deficit in the cell. Nevertheless, mtDNA copy number measurements could be successfully utilized in drug toxicity studies. Carefully designed assays that measure absolute copy number and take account of the methodological issues

described previously could be used in numerous stages of drug development. For example, initial *in vitro* screens could utilize target cell lines to define if drugs in development have an impact on cellular mtDNA levels, and if they do, then titration studies could inform potentially safer levels of the drug. *In vivo* animal studies could be used to study the systemic effects of potential drugs on mtDNA levels in organs and cells over time and inform the potential leakage of mtDNA into the periphery, which would have implications for inflammation. Once clinical trials commence, mtDNA levels in peripheral blood—compartmentalized as PMBCs for cellular and plasma for cell-free, as well as in urine, compartmentalized as urinary pellet for cellular debris and cell-free urinary supernatant, or other body fluids, such as saliva, semen, or cerebrospinal fluid—could be used to monitor the impact of the drug under development on systemic mtDNA levels in patients.

In conclusion, the growing body of evidence showing dysregulated mtDNA levels in common diseases, both in cell-free and cellular samples, supports the view that mtDNA is a useful biomarker of mitochondrial dysfunction. Furthermore, emerging data from cancer, HIV, and other fields indicates that mtDNA levels may correlate with patient response to treatment and are strongly suggestive that the utilization of mtDNA as a biomarker in drug toxicity studies may be of great benefit in drug development.

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26

Predicting Off-Target Effects of Therapeutic Antiviral Ribonucleosides: Inhibition of Mitochondrial RNA Transcription

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26.1 Introduction

Therapeutic ribonucleoside inhibitors have been recognized as one of the most promising classes of antiviral compounds currently being developed to treat RNA virus infections. These compounds have been hailed as “game changers” because of their broad-spectrum antiviral activity and the high barrier for the virus to develop resistance mutations (Coats et al., 2014). Despite such promise, these compounds have been the source of some unfortunate drug failures in recent history (Coats et al., 2014). While several promising antiviral ribonucleosides have entered early-stage clinical trials, these ultimately were discontinued or put on “hold” as a result of severe adverse events (Coats et al., 2014). Remarkably, none of the compounds that failed because of patient toxicity were identified as high toxicity risks during preclinical testing. The cause(s) and source(s) of the toxicity were simply not understood. Only recently, however, does data suggest that toxicity has likely been, at least in part, the result of unintended inhibition of mitochondrial transcription mediated through the utilization of these antiviral ribonucleosides as substrates by the human mitochondrial RNA polymerase (POLRMT)

(Arnold et al., 2012a). The unintended inhibition of mitochondrial gene expression likely pushed mitochondrial function past a tolerable “threshold,” resulting in a precipitous decline in cellular function and severe organ toxicity. Moreover, preclinical toxicity was missed because of poor model systems and/or assays that would predict adverse effects, especially when there are changes to mitochondrial gene expression. Here, we review the *in vitro* biochemical and cell-based assays that can predict the potential of these compounds to cause changes to mitochondrial gene expression.

26.2 Therapeutic Ribonucleoside Inhibitors Target RNA Virus Infections

RNA virus infections represent one of the most significant public health threats in the United States and abroad today. Over the past several years, we have witnessed the emergence and reemergence of such pathogens as SARS coronavirus, West Nile virus, dengue virus, Zika virus, rhinovirus, Norwalk virus, and hepatitis C virus (HCV),

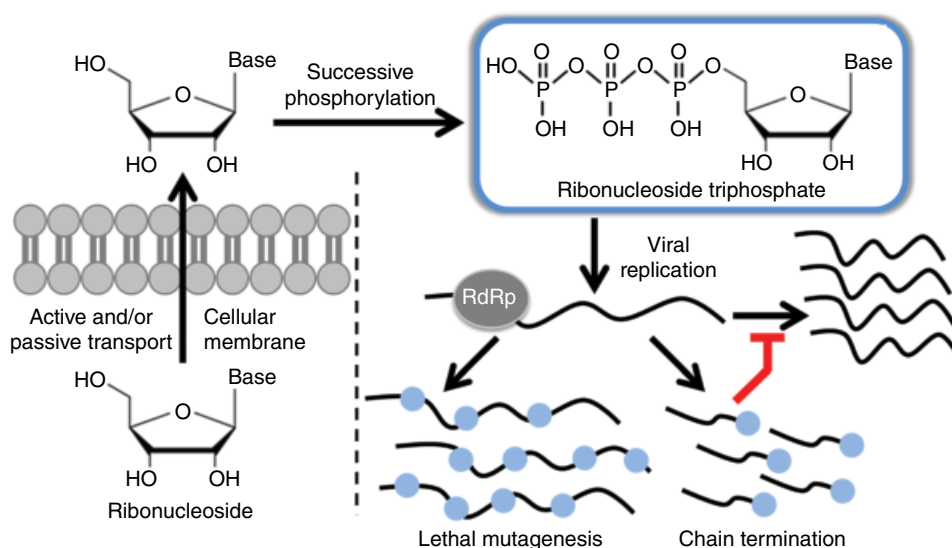


Figure 26.1 Nucleoside inhibitors target the viral RdRp and inhibit viral replication. See text for details.

to name a few. Given the dire circumstances surrounding RNA virus infections, there is an ongoing effort to develop direct-acting antivirals (DAAs) that can impede viral infection and eventually lead to a cure (Vermehren and Sarrazin, 2011; Williams, 2011). DAAs act by targeting and inhibiting the viral proteins or enzymes involved in the virus life cycle. One such target is the viral RNA-dependent RNA polymerase (RdRp). DAAs that target the viral RdRp include both non-nucleoside and nucleoside inhibitors (Brown, 2009; Coats et al., 2014). Nucleoside inhibitors are, in essence, analogues of the natural cellular ribonucleosides. Upon entering the cell, ribonucleosides require successive phosphorylation to the triphosphorylated form to elicit full activity (Figure 26.1). Oftentimes, ribonucleosides are administered as prodrugs that facilitate adsorption, distribution, and metabolism such that the conversion to the active triphosphorylated form is achieved orders of magnitude more readily than just administration of the ribonucleosides themselves (Brown, 2009; Coats et al., 2014). These compounds, once activated to the triphosphorylated form, target the active site of the viral RdRp and are substrates for these enzymes (Figure 26.1). Once incorporated, these compounds can either directly terminate RNA synthesis (chain terminators) or increase the number of tolerable mutations (mutators), eventually leading to lethal mutagenesis (Figure 26.1) (Brown, 2009; Graci and Cameron, 2008). Because these compounds target the conserved active site of the viral RdRp, they typically exhibit broad-spectrum antiviral activity, and there is a high barrier in the selection of virus resistance mutations (Brown, 2009; Coats et al., 2014).

In December 2013, sofosbuvir (prodrug of 2'-deoxy-2'-fluoro-2'-C-methyluridine) became the first antiviral ribonucleoside inhibitor to be clinically approved to treat

HCV infection. However, despite such promise, the majority of antiviral ribonucleoside inhibitors have been unable to achieve the same clinical success. This has mostly arisen because of severe adverse events that occurred during clinical trials. For example, the first two nucleoside analogues to enter clinical development, NM283 (prodrug of 2'-C-methylcytosine) and RG1626 (prodrug of 4'-azidocytosine), were discontinued because of their respective associations with dose-limiting gastrointestinal and hematologic toxicity (Coats et al., 2014). Following the observation of laboratory abnormalities associated with liver functional tests, PSI-938 (prodrug of 2'-deoxy-2'-fluoro-2'-C-methylguanosine) was also placed on clinical hold (Coats et al., 2014). Additionally, clinical development of BMS-986094 (prodrug of 2'-C-methylguanosine) was halted because of severe kidney and heart damage (Coats et al., 2014). As a result of these studies, IDX184 (prodrug of 2'-C-methylguanosine) was put on partial clinical hold and then ultimately terminated from further study (Coats et al., 2014). All of these failures led us to two questions: Why were there no indications of the potential of these compounds to cause such adverse events, and what were the origins of this toxicity?

26.3 Nucleoside Reverse Transcriptase Inhibitors (NRTIs) Mediate Mitochondrial Toxicity

It has long been recognized that the human mitochondrial DNA polymerase (Pol γ) has been an "off-target" of nucleoside reverse transcriptase inhibitors (NRTIs) used for the treatment of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections (Bailey and

Anderson, 2010; Lee et al., 2003; Lewis et al., 2003; McKenzie et al., 1995; White, 2001). The utilization of NRTIs by Pol γ led to the unintended inhibition of mitochondrial DNA (mtDNA) replication and an impairment of mitochondrial function (Bailey and Anderson, 2010; Lee et al., 2003; Lewis et al., 2003; White, 2001). NRTIs have caused a wide array of clinical phenotypes including cardiomyopathy, muscle and liver toxicity, peripheral neuropathy, lactic acidosis, lipodystrophy, and lipoatrophy (de Baar and de Ronde, 2008). Because mtDNA is present in vast excess of the level that is required to support mitochondrial function, reduction of mtDNA copy number will not manifest in preclinical assays traditionally employed to evaluate compound toxicity (Durham et al., 2005). The development of specific biochemical assays to reveal Pol γ inhibition was therefore required in order to assess the potential of preclinical candidates to elicit mitochondrial dysfunction (Anderson, 2010; Bailey and Anderson, 2010; Lee et al., 2003; Lewis et al., 2003). It was only after this realization that Pol γ was an unintended “off-target” that safer NRTIs were developed, resulting in reductions in toxicity as a result of mitochondrial dysfunction (Bailey and Anderson, 2010; Lee et al., 2003; Lewis et al., 2003).

26.4 Mitochondrial Dysfunction Is an Unintended Consequence of Clinical Drug Candidates

Mitochondria are cellular organelles typically known as the “powerhouse” of the cell. These tiny organelles produce the cell’s energy source in the form of ATP by a process known as oxidative phosphorylation (OXPHOS), and this process is absolutely essential for normal cellular function. Mitochondria contain their own genomic DNA (mtDNA), which needs to be replicated and transcribed to produce the rRNAs, tRNAs, and mRNAs required to produce key components of the OXPHOS machinery (Falkenberg et al., 2007). Disruption of the ability of mitochondria to replicate and express its genome as well as altering the integrity and activity of the OXPHOS machinery can severely affect the bioenergetic capacity of the cell and lead to effects associated with mitochondrial impairment (Wallace, 2005; Wallace et al., 2010). Unfortunately, many pharmaceuticals are being identified that alter mitochondrial function, leading to “off-target” side effects that are observed during and/or after clinical trials (Chan et al., 2005; Dykens and Will, 2007; Nadanaciva and Will, 2011; Wallace, 2008). This has led to a number of different classes of drugs to be either halted or recalled as a result of the unintended alteration of mitochondrial function (Chan et al., 2005; Dykens and Will, 2007; Nadanaciva and Will, 2011; Wallace, 2008).

While several clinical signs of drug-induced mitochondrial dysfunction can include modest-to-severe phenotypes such as lactic acidosis, exercise intolerance, nausea, and malaise, these may not often be observed (Will and Dykens, 2008). The problem is that impairment to mitochondrial function can lead to widely different phenotypic presentations among different tissues. While aerobically poised tissues with high-energy demands are likely the most affected, the clinical manifestations between these organs/tissues are not equal. In addition, cells and tissues will likely not be responsive as long as mitochondrial function is above a required “threshold” to support normal cellular and/or tissue function, but once passed, severe organ toxicity can result (Rossignol et al., 2003; Wallace, 2005). This is commonly called the “phenotypic threshold effect” (Rossignol et al., 2003). Other contributing factors are the general health and age of an individual, previous organ history, and the genetic variation of both the nuclear and mitochondrial genome. For example, many mutations are being discovered in nuclear-encoded factors directly involved in mitochondrial replication, transcription, and/or translation systems and can sensitize individuals to mitochondrial impairment (Tuppen et al., 2010; Wallace, 2010). For example, it has been shown that a single mutation in Pol γ can sensitize an individual to idiosyncratic drug-induced toxicity, whereby this mutation relaxes the specificity for utilization of a certain class of NRTI (Bailey et al., 2009; Yamanaka et al., 2007). The genetic variability of wild-type and mutant mtDNA, termed heteroplasmy, within a given cell or tissue can also dictate the outcome of a phenotypic presentation among individuals (Rossignol et al., 2003; Wallace et al., 2010). This is often the case in larger population studies where isolated adverse events are normally observed. Therefore, properly identifying compounds that cause mitochondrial dysfunction via “phenotypic threshold effects” is of great concern and is rapidly becoming more widely acknowledged within the drug development community (Chan et al., 2005; Dykens and Will, 2007; Dykens et al., 2007; Nadanaciva and Will, 2011; Wallace, 2008). Unfortunately, many problems exist in properly identifying mitochondrial toxicants because of a general lack of understanding of both mitochondrial function and dysfunction and a lack of suitable *in vitro* and animal models that enhance predictive capabilities that can be extrapolated to the clinic.

In the evaluation of cellular toxicity, cell-based assays routinely assess changes in cell viability or by measuring overall ATP output using luciferase-coupled assays (Crouch et al., 1993; McKim, 2010). While these traditional toxicity assays seemingly appear reliable in identifying compounds that cause cytotoxicity, there are major shortcomings with these approaches in predicting the toxicity of compounds that have the ability to cause

changes and/or alterations to mitochondrial function. First, the vast majority of cell lines employed are immortalized cancer cell lines grown in high concentrations of glucose. Under these conditions, the majority of ATP produced within the cell is almost exclusively produced by glycolysis and not OXPHOS. This is known as the Crabtree effect (Marroquin et al., 2007). Under these conditions, mitochondrial toxicants may have no effect on cell viability (Marroquin et al., 2007). Second, the durations of time that most such assays are performed are insufficiently long to obtain measurable changes in mitochondrial function that require mtDNA expression. Last, the chosen cell lines do not display a diverse genetic variation in what would normally be observed in a typical patient population. The limited diversity of both the nuclear genome and mtDNA can essentially desensitize cells to changes in proper mitochondrial function.

The lack of diversity is also a major shortcoming in evaluation of toxicity in relevant animal models. Animals are typically genetically identical, very young, and healthy and have no other risk factors or other underlying conditions that would lead to adverse events when treated with mitochondrial toxicants. Therefore, compounds that cause mitochondrial dysfunction are often overlooked during preclinical testing. These adverse events are then presented during later-stage clinical trials in human subjects, where the variability of the patient population is expanded. To circumvent this problem, a move toward using animals that are more sensitive to mitochondrial impairment is being explored (Dyken and Will, 2007; Nadanaciva and Will, 2011). This is routinely coupled with a more thorough analysis of tissues and organs likely to be sensitive to mitochondrial dysfunction and succumb to “phenotypic threshold” effects. In all, more sensitive and robust preclinical toxicity assays are needed that assess directly the impact potential drug candidates have on various aspects of mitochondrial function in order to circumvent the late-stage attrition often observed because of the clinical manifestations of drug-induced mitochondrial toxicity.

26.5 Mitochondrial Transcription as an “Off-Target” of Antiviral Ribonucleosides

Transcription of the mitochondrial genome is accomplished, with the help of accessory transcription factors, by the POLRMT, a nuclear-encoded single-subunit DNA-dependent RNA polymerase (DdRp or RNAP) that is related to the bacteriophage T7 class of single-subunit RNAPs (Arnold et al., 2012b). In addition to its role in transcription, POLRMT serves as the primase for

mitochondrial DNA replication. Therefore, this enzyme is of fundamental importance for both expression and replication of the human mitochondrial genome and absolutely essential for normal cellular function and indispensable in the production of the OXPHOS machinery. Until recently, no study addressed whether ribonucleoside analogs were substrates and/or inhibitors of POLRMT (Arnold et al., 2012a). However, it has now been shown that antiviral ribonucleosides are indeed substrates and inhibitors for POLRMT *in vitro* and in cells (Arnold et al., 2012a; Feng et al., 2016). The *in vitro* substrate utilization by POLRMT correlated with the inhibition of mitochondrial RNA transcription in cells and corresponding decreases in mitochondrial protein production and cellular respiration (Feng et al., 2016). Moreover, the efficiency of incorporation by POLRMT *in vitro* predicted outcomes in cells when normalized for intracellular metabolism of the antiviral ribonucleoside to the triphosphorylated form (Arnold et al., 2012a). Finally, evidence suggests that moderate levels of ribonucleoside analog incorporation by POLRMT increased the risk of *in vivo* mitochondrial dysfunction as dose-dependent toxicity studies in dogs resulted in significant mitochondrial swelling and lipid accumulation in hepatocytes along with gene signature changes linked to loss of hepatic function and increased mitochondrial dysfunction (Fenaux et al., 2016). As a result of these studies, it is postulated that the “off-target” inhibition of mitochondrial transcription has contributed to the unexpected attrition of antiviral ribonucleoside analogues in the clinic. Therefore, it is suggested that a comprehensive analysis and screening platform be initiated to test directly the potential of antiviral ribonucleosides to be substrates and inhibitors for POLRMT in both biochemical and cellular assays.

26.6 Evaluation of Substrate Utilization by POLRMT *In Vitro*

The biochemical tools used to study substrate utilization by POLRMT *in vitro* were recently developed (Smidansky et al., 2011). These advancements have allowed the determination of the utilization of antiviral ribonucleoside triphosphates by POLRMT and so predicting unwanted “off-target” inhibition of mitochondrial transcription (Arnold et al., 2012a). Utilization of antiviral ribonucleoside triphosphates by POLRMT can be determined by using RNA-primed DNA template nucleic acid scaffolds without the need for transcription factors and by assessing the fraction of extended primer in the presence of nucleotide substrate (Arnold et al., 2012a). These scaffolds consist of an annealed 5′-³²P-labeled 12-nt

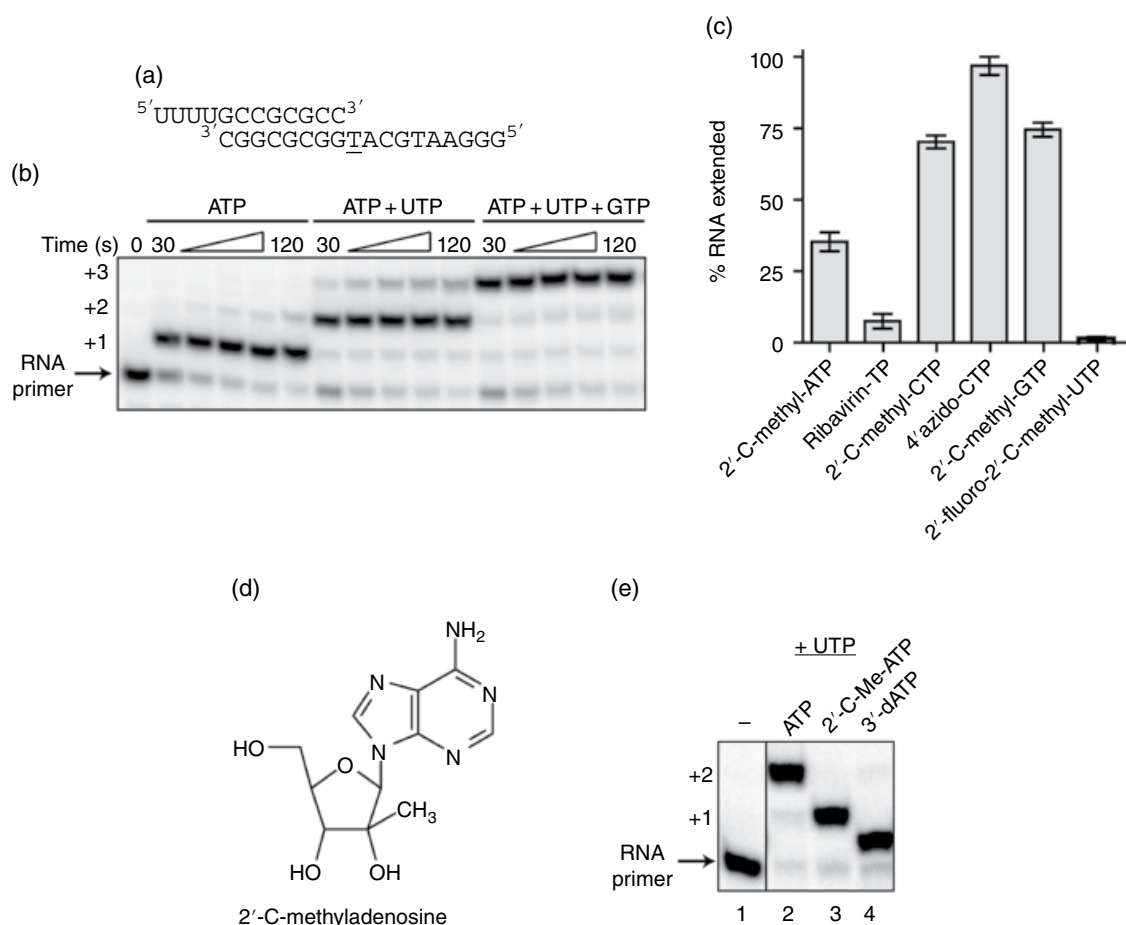


Figure 26.2 *In vitro* substrate utilization by POLRMT and inhibition of RNA synthesis. (a and b) Factor-independent assay for POLRMT-catalyzed nucleotide incorporation. (a) DNA/RNA scaffold. This scaffold consisted of a 12-nt RNA annealed to an 18-nt DNA, forming an 8 bp duplex region with a 4-nt 5'-RNA overhang and a 10-nt single-stranded DNA template. The first templating base is underlined. (b) Single- and multiple-nucleotide incorporation catalyzed by POLRMT. POLRMT is incubated with RNA/DNA-nucleic acid scaffold and either ATP, ATP, and UTP or ATP, UTP, and GTP for various amounts of time. It forms a stable elongation-competent complex and readily extends the RNA primer to $n+1$, $n+2$, and $n+3$ in the absence of transcription factors TFB2M and TFAM. (c) Antiviral ribonucleoside triphosphates are substrates for POLRMT. Percentage of RNA product relative to correct nucleotide (ATP, CTP, GTP, or UTP) is shown. Error bars represent s.e.m. (d and e) Inhibition of POLRMT-mediated transcription. (d) 2'-C-methyladenosine ribonucleoside analogue. (e) Non-obligate chain termination of RNA synthesis *in vitro*. Reaction products from POLRMT-catalyzed nucleotide incorporation in the presence of the next correct nucleotide substrate, UTP. Reactions containing 2'-C-methyl-ATP were unable to be extended to $n+2$, demonstrating the ability of this nucleoside analog to be non-obligate chain terminator for POLRMT once incorporated into nascent RNA.

RNA primer and 18-nt DNA template forming an 8-bp duplex with a 4-nt 5'-RNA overhang and a 10-nt single-stranded DNA template region (Figure 26.2) (Arnold et al., 2012a; Smidansky et al., 2011). To assess the incorporation of various ribonucleoside triphosphates with different base configurations, the appropriate complementary base residue is included as the first templating base in corresponding DNA template strands (Figure 26.2). The initial assays used to assess the incorporation tested a panel of purine and pyrimidine analogues that contain modifications to the base or ribose found in past and/or current clinical candidates for the treatment of HCV (Arnold et al., 2012a). The fraction of

the primer extended after a 30 s incubation of POLRMT in the presence of each nucleotide substrate at a concentration of 500 μ M normalized to correct nucleotide utilization (Figure 26.2) was determined (Arnold et al., 2012a). Under these conditions, all of the antiviral analogues tested except for 2'-deoxy-2'-fluoro-2'-C-methyluridine (triphosphate formed from sofosbuvir) were incorporated much more efficiently than ribavirin, suggesting that POLRMT has a relaxed specificity for incorporation and is a possible target for inhibition (Arnold et al., 2012a). Further studies by Feng et al. (2016) compared the incorporation with the corresponding natural NTP substrate at fixed saturating concentrations

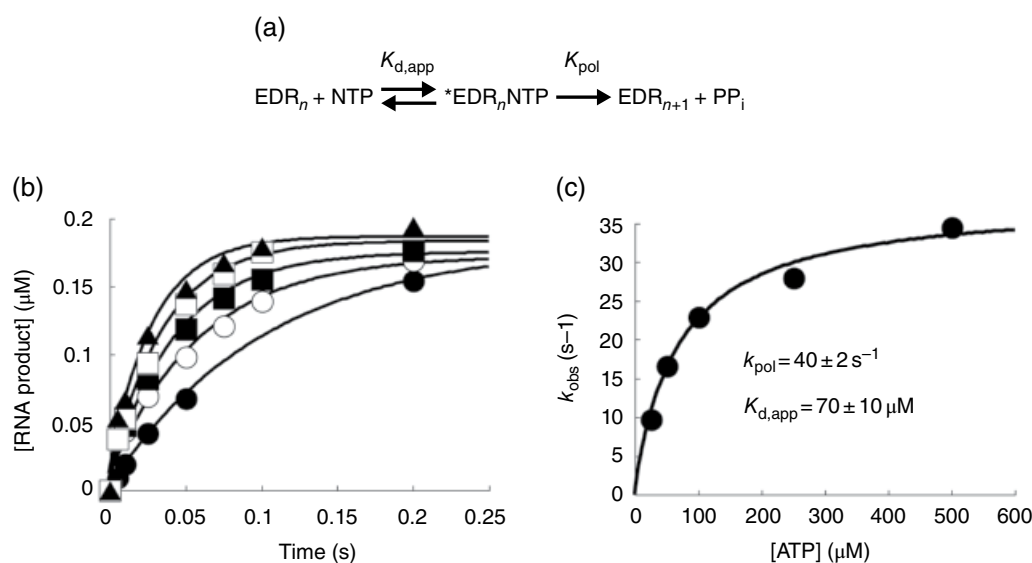


Figure 26.3 Determination of the efficiency of nucleotide incorporation by POLRMT. (a) Minimal mechanism for single-nucleotide incorporation. (b) Kinetics of POLRMT-catalyzed nucleotide incorporation. POLRMT was incubated with DNA/RNA scaffold and then rapidly mixed with various concentrations of ATP. At each ATP concentration quantitated, the RNA product was plotted as a function of time and fit to a single exponential yielding an observed rate constant, k_{obs} , for POLRMT-catalyzed nucleotide incorporation. (c) Estimation of k_{pol} and $K_{d,\text{app}}$. Values for k_{obs} were plotted as a function of ATP concentration and fit to a hyperbolic model that defines the mechanism in panel A, yielding estimates of the kinetic parameters k_{pol} , the maximal rate constant for nucleotide incorporation, and $K_{d,\text{app}}$, the apparent dissociation constant for the nucleotide substrate.

of nucleotide and were able to show that the triphosphates formed by BMS-986084, IDX184, 4'-azidocytidine, and 2'-C-methylcytidine served as excellent substrates and were incorporated by POLRMT similar to those of their corresponding natural rNTPs (Feng et al., 2016). In contrast, the active forms of sofosbuvir, PSI-938, mericitabine, and GS-6620 were all exceedingly poor substrates for POLRMT (Feng et al., 2016). As a first approximation, the efficient utilization by POLRMT illuminates the significant potential these compounds may have on altering mitochondrial gene expression. In addition, the lack of utilization by POLRMT suggests that “off-target” inhibition is not likely, and this is consistent with the advanced clinical development and eventual approval of sofosbuvir for treatment of HCV.

In addition to evaluating substrate utilization by POLRMT, several antiviral ribonucleoside compounds were tested for their ability to terminate RNA synthesis once incorporated by POLRMT into nascent RNA (Arnold et al., 2012a). In particular, modifications to the 2' or 4' position of the ribose ring have been shown to cause termination of RNA synthesis by viral RdRps (Brown, 2009). These compounds contain the required 3'-OH group for subsequent nucleotide incorporation, but because of their inability to support RNA extension after they are incorporated, the compounds are known as

non-obligate chain terminators. By using a template with thymine as the first templating base and adenine as the second templating base, the combination of ATP and UTP leads to the extension of the RNA primer by two nucleotides (Figure 26.3) (Arnold et al., 2012a). However, when using 2'-C-methyl-ATP and UTP, POLRMT produced only the +1 extension product, consistent with this analogue being a non-obligate chain terminator (Arnold et al., 2012a). The combination of 3'-dATP, an obligate chain terminator, and UTP was used as a control for chain termination. Further experiments also evaluated various cytidine analogues for their ability to inhibit elongation by POLRMT (Arnold et al., 2012a). In all cases, the non-obligate chain terminators were capable of terminating RNA synthesis by POLRMT.

26.6.1 Determination of the Efficiency of Incorporation by POLRMT

To more accurately identify ribonucleoside analogs that have the potential to be used as substrates and inhibit mitochondrial transcription, it is imperative to determine the efficiency of POLRMT-catalyzed nucleotide incorporation for a given ribonucleoside analogue. This is performed by determining the dependence of the pre-steady-state rate constant for nucleotide

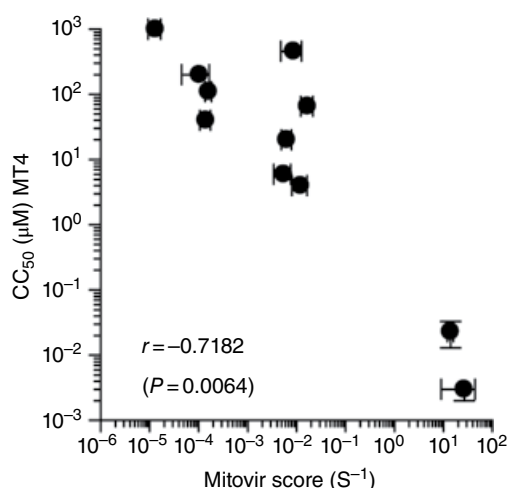


Figure 26.4 Predicting adverse effects of antiviral ribonucleosides during preclinical development: The mitovir score. Correlation between cytotoxicity (CC₅₀) and mitovir score for MT4 cells. Mitovir score: rate constant for incorporation calculated by using the experimentally determined kinetic parameters k_{pol} and $K_{\text{d,app}}$ and the intracellular concentration of nucleoside analog triphosphate [TP]; mitovir score = $k_{\text{eff}} (\text{s}^{-1}) = (k_{\text{pol}} * [\text{TP}]) / (K_{\text{d,app}} + [\text{TP}])$. Error bars represent s.d. Nonparametric (Spearman) correlations with r values shown. In parentheses are one-tailed P -values calculated from Spearman coefficients to provide a measure of statistical significance of correlation.

incorporation on nucleotide substrate concentration. This will reveal information concerning the binding of the incoming nucleotide, the maximal rate constant for nucleotide incorporation, and the specificity of nucleotide incorporation (Figure 26.4). These aspects of POLRMT behavior are summarized by the kinetic parameters $K_{\text{d,app}}$, k_{pol} , and $k_{\text{pol}}/K_{\text{d,app}}$, respectively, which define a minimal mechanism for nucleotide incorporation as shown in Figure 26.4. The observed rate constants for incorporation over a range of nucleotide concentrations are then fit to a hyperbolic model that defines the mechanism, yielding estimates of the kinetic parameters k_{pol} , the maximal rate constant for nucleotide incorporation, and $K_{\text{d,app}}$, the apparent dissociation constant for the nucleotide substrate.

This approach was taken to determine the kinetic parameters for a variety of different ribonucleoside analogues (Arnold et al., 2012a). It was found that the incorporation efficiency ($k_{\text{pol}}/K_{\text{d,app}}$) of each analogue was less than that of the correct nucleotide by at least one order of magnitude (Arnold et al., 2012a). In addition, only one analogue (2'-deoxy-2'-fluoro-2'-C-methyl-UTP) was incorporated with an efficiency less than ribavirin (Arnold et al., 2012a). The efficiency of incorporation of the second most inefficient analogue (2'-C-methyl-ATP) was still 10-fold higher than observed for

ribavirin (Arnold et al., 2012a). When comparing the efficiencies of nucleotide incorporation to the natural correct NTP substrate, the relative frequency of incorporation of each analogue can be calculated. Interestingly, it was found that these values ranged from 1 in 970,000 to 1 in 15 incorporation events (Arnold et al., 2012a). These data suggest a misincorporation frequency of approximately 1 in 238,000 for ribavirin, 1 in 970,000 for 2'-deoxy-2'-fluoro-2'-C-methyl-UTP, 1 in 26,000 for 2'-C-methyl-ATP, 1 in 3,900 for 2'-C-methyl-CTP, and 1 in 147 for 4'-azido-CTP (Arnold et al., 2012a). However, although the large differences in frequency connote potency, a single misincorporation event of either of the chain-terminating analogues would be sufficient to terminate RNA synthesis and inhibit mitochondrial transcription.

By determining the kinetic parameters, one can begin to identify substituents that have an influence on binding of both the incoming nucleotide and the required geometry for efficient catalysis. Coupling this information with structural studies could lead to an appreciation of the structure–activity relationships involved in POLRMT nucleotide selection. For example, modeling studies have suggested that the 4'-azido substitution was the most easily accommodated by POLRMT, with essentially no perturbation of the active site required for the inhibitor to bind (Feng et al., 2016). The kinetic parameters k_{pol} and $K_{\text{d,app}}$ for 4'-azido-CTP were only 5- and 30-fold different, respectively, compared with CTP consistent with this observation (Arnold et al., 2012a). Additionally, it was suggested that the dual substitutions of 2'-fluoro-2'-C-methyl had a more pronounced van der Waals clash with Tyr999, leading to a small shift in this residue toward 1', likely due to the loss of hydrogen bonding capacity with the fluorine (Feng et al., 2016). The combination of these substitutions had a pronounced impact on both kinetic parameters, $K_{\text{d,app}}$ and k_{pol} , culminating in a substantial decrease in the efficiency of POLRMT-catalyzed nucleotide incorporation (Arnold et al., 2012a). Overall, in an effort to balance potency with host toxicity in the design of future antiviral ribonucleoside compounds, it will be extraordinarily useful to compare and contrast the kinetic parameters with those obtained with viral RdRps as new nucleoside analogues can be analogued away from utilization by POLRMT and toward viral RdRp targets.

26.6.2 Determination of the Sensitivity to Inhibition: Mitovir Score

The ability of antiviral ribonucleosides to be incorporated *in vivo* will depend on the sizes of the intracellular antiviral ribonucleoside triphosphate pool and natural

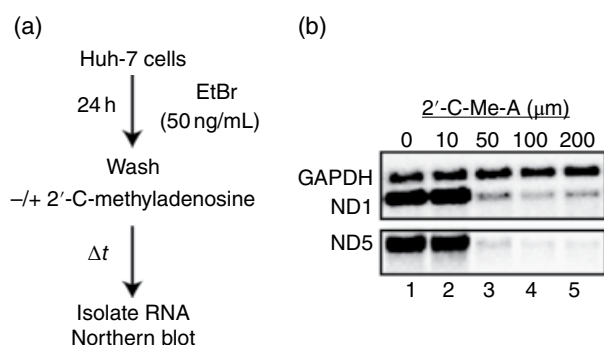


Figure 26.5 Inhibition of POLRMT-catalyzed RNA synthesis in cells. (a) Experimental design. Huh-7 cells were treated with ethidium bromide (EtBr) for 24 h to deplete mitochondrial transcripts from cells, washed and treated with 2'-C-methyladenosine, total RNA isolated, and Northern blots performed. (b) Northern blots of ND1, ND5, and GAPDH after EtBr treatment and recovery in the presence of 2'-C-methyladenosine (2'-C-Me-A). Cells treated with a minimum of 50 μM 2'-C-methyladenosine showed specific inhibition of mitochondrial transcription and the inability to produce both ND1 and ND5 transcripts.

ribonucleoside triphosphate pool with which the analogue competes. Phosphorylation of nucleoside analogues can occur either in the mitochondria or cytoplasm, with subsequent transport across the inner mitochondrial membrane (Dolce et al., 2001; Lai et al., 2004; Leung and Tse, 2007; Lewis et al., 2003; Scatena et al., 2007). The efficiency of phosphorylation is likely to be a critical factor that contributes to the size of the antiviral ribonucleoside triphosphate pool and the ability of antiviral ribonucleosides to cause unwanted toxicity, as has been demonstrated for NRTIs (Lewis et al., 2003). Interestingly, it was recently found that the total cellular pool of an antiviral ribonucleoside triphosphate was similar in magnitude to the intramitochondrial pool (Arnold et al., 2012a). The concentration of intracellular antiviral ribonucleoside triphosphate can then be used to adjust the efficiency of incorporation to yield an approximate frequency of incorporation of the antiviral ribonucleotide. This value was referred to as the mitovir score (mitochondrial dysfunction caused by antiviral ribonucleosides) since it provides a prediction of POLRMT-mediated mitochondrial dysfunction caused by the antiviral ribonucleoside and provides the rate constant for analogue incorporation by POLRMT adjusted for intracellular concentration of the triphosphorylated form of the analogue (Arnold et al., 2012a). This score is essentially an effective rate constant for the utilization of the antiviral rNTP calculated by using the values for k_{pol} and $K_{\text{d,app}}$ determined *in vitro* and the value for the concentration of antiviral rNTP present in cells (Arnold et al., 2012a). This score was strongly correlated with the

observed cytotoxicity in MT4 cells (Figure 26.5) (Arnold et al., 2012a). It was therefore suggested that the use of the MT4 cell line in combination with the mitovir score, which represents the combination of biochemical analysis and intracellular metabolism, can be appropriate to begin identifying antiviral ribonucleosides during preclinical development with the potential to cause adverse effects (Arnold et al., 2012a).

26.7 Direct Evaluation of Mitochondrial RNA Transcripts in Cells

The ability of antiviral ribonucleoside compounds to terminate RNA synthesis *in vitro* suggested that *in vivo* inhibition of transcription might occur. Also, given that the active triphosphorylated forms of the antiviral nucleosides did indeed accumulate in mitochondria to concentrations similar to that found in the cytoplasm suggests that direct assays to assess the impact on mitochondrial RNA transcription are required. To further examine the possibility that POLRMT is an off-target for these compounds and that this has biological consequences, cellular assays were developed that determined the ability of antiviral ribonucleoside compounds to specifically inhibit mitochondrial transcription in cells (Arnold et al., 2012a; Ehteshami et al., 2016; Feng et al., 2016). To address this directly, assays have been developed to visualize the impact of antiviral ribonucleosides on mitochondrial RNA transcription using Northern blots and RT-PCR (Arnold et al., 2012a; Ehteshami et al., 2016; Feng et al., 2016).

Northern blots allow for the detection and direct visualization of specific RNA transcripts by hybridization using a labeled DNA probe complementary to a portion of the RNA target sequence. This assay can be a useful read-out in determining whether cells treated with antiviral ribonucleoside inhibitors show any changes to mitochondrial transcription, such as termination of synthesis, abortive transcription, or general reduction of full-length RNA transcripts. Cellular assays were initially developed to determine the effect on newly synthesized mitochondrial RNA transcripts (Arnold et al., 2012a). This is performed whereby transcription is reversibly inhibited by low concentrations of ethidium bromide. Mitochondrial RNA transcripts are allowed to degrade during the initial ethidium bromide treatment; then, during the recovery phase when the ethidium bromide block is removed and mitochondrial transcription is resumed, cells are treated with or without the ribonucleoside analogue compounds. Mitochondrial transcription was inhibited for 24 h using

50 ng/mL ethidium bromide. This time is sufficient for the majority of mitochondrial RNA transcripts to be degraded because the time is much greater than the half-lives of mitochondrial RNAs (Piechota et al., 2006). The ethidium bromide was then removed and replaced with media lacking nucleoside or containing 2'-C-methyladenosine (Figure 26.5) or 4'-azidocytidine. Total RNA was isolated and processed for analysis by Northern blotting using probes for mitochondrial RNAs (ND1 and ND5) and a cellular RNA control (GAPDH). In both cases, the abundance of mitochondrial RNAs using both adenosine and cytidine analogues was reduced (Figure 26.5) (Arnold et al., 2012a). Additionally, cells treated with 2'-C-methyladenosine showed a dose-dependent reduction in the abundance of the mitochondrial RNA transcripts ND1 and ND5 (Figure 26.5), with no changes in the level of nuclear transcription, demonstrating the specificity of inhibition of mitochondrial transcription (Arnold et al., 2012a). Additionally, it was shown that while the main nuclear DdRp Pol-II had the capacity to incorporate chain-terminating nucleotides *in vitro*, the observation that Pol-II-dependent nuclear transcription was unchanged during treatment in cells was explained by the proofreading capability of transcription elongation factor S-II (TFIIS) (Arnold et al., 2012a), which promotes cleavage of the nascent RNA strand when Pol-II either pauses or misincorporates incorrect nucleotide substrates, thereby allowing for the efficient removal of incorrect nucleotide substrates and enhancing the fidelity of nuclear transcription (Sekine et al., 2012). The ability of non-obligate chain terminators to inhibit mitochondrial RNA transcription in cells suggests that mitochondrial factors do not exist to cleave the nascent RNA associated with a stalled elongation complex that would be analogous to TFIIS of multi-subunit RNA polymerases (Fish and Kane, 2002).

Subsequent studies by Ehteshami et al. (2016) used RT-PCR to show that BMS-986094, the prodrug of a guanosine nucleotide analogue (2'-C-methylguanosine), was sufficient to fully inhibit mitochondrial RNA replenishment, whereas both DAPN-PD1 and sofosbuvir showed no interference with the accumulation of mitochondrial RNA transcripts (Ehteshami et al., 2016). The selective inhibition of mitochondrial synthesis by BMS-986094 compared with DAPN-PD1 was attributed to the relative abundance of intracellular concentrations of 2'-C-methyl-GTP. While these two compounds are both metabolized to produce 2'-C-methyl-GTP upon treatment of cells, BMS-986094 produced greater than 87 times the intracellular concentration of 2'-C-methyl-GTP than DAPN-PD1 (Ehteshami et al., 2016). Together, these data support the hypothesis that inhibition of mitochondrial RNA transcription is dependent upon

both the relative efficiency of incorporation and intracellular concentrations of ribonucleoside triphosphate in cells as suggested by previous studies (Arnold et al., 2012a; Ehteshami et al., 2016).

26.8 Inhibition of Mitochondrial Function

Thus far, cell-based assays for general cytotoxicity have failed to reliably identify ribonucleoside inhibitors associated with clinical toxicity. While it is now known that ribonucleoside inhibitors are, indeed, substrates for POLRMT and can inhibit mitochondrial transcription *in vitro* and in cells and that the aforementioned assays provide the ability to evaluate the efficiency of nucleotide incorporation and inhibition of mitochondrial transcription, additional studies are warranted to assess the consequences of antiviral ribonucleoside treatment on various aspects of mitochondrial function. These studies are only now beginning to be performed. In addition to showing direct incorporation of ribonucleoside inhibitors *in vitro* and in cells, Feng et al. (2016) investigated the effects of these compounds on both mitochondrial protein synthesis and respiration (Feng et al., 2016). Using the MitoTox MitoBiogenesis kit (MitoSciences/Abcam), which uses quantitative immunocytochemistry to measure the protein levels of a nuclear DNA-encoded mitochondrial protein, succinate dehydrogenase (SDH-A), and mitochondrial DNA-encoded mitochondrial protein, cytochrome c oxidase (COX-I), it was found that while many ribonucleoside inhibitors showed no selective effect on reducing mitochondrial protein synthesis, 4'-azidocytidine showed selective inhibition with a CC50 of 50 μ M. In addition, co-incubation of BMS-986064 with ribavirin, which itself showed little effect, potentiated the mitochondrial and cellular toxicity of BMS-986094 (Feng et al., 2016). Further studies by Feng et al. showed that both 4'-azidocytidine and BMS-986094 reduced mitochondrial respiration as measured by the rate of oxygen consumption (OCR) of PC-3 cells after a 5-day treatment using a Seahorse extracellular flux analyzer (Feng et al., 2016). Last, Fenaux et al. (2016) showed that a monophosphate prodrug of 2'-ethynyluridine developed for the treatment of HCV was found to result in significant mitochondrial swelling and lipid accumulation in hepatocytes from 8-day toxicity studies in dogs with dosing at greater than 250 mg/kg/day (Fenaux et al., 2016). In addition, gene expression analysis showed selective depletion of mitochondrial transcripts and dose-proportional gene signature changes linked to loss of hepatic function, altered lipid metabolism, and increased mitochondrial dysfunction

(Fenaux et al., 2016). The triphosphate of this compound showed moderate levels of incorporation by POLRMT, which was attributed to the risk of *in vivo* mitochondrial dysfunction (Fenaux et al., 2016). In contrast, sofosbuvir at the same doses resulted in no evidence of liver toxicity, as is evidenced by clinical chemistry and histopathology results (Fenaux et al., 2016). In all, these studies have begun to expand the repertoire of assays to assess the direct effects ribonucleoside inhibitors can have on altering mitochondrial gene expression.

26.9 Conclusions

In this chapter we have described strategies to improve preclinical screening of therapeutic antiviral ribonucleosides for the potential to inhibit and/or to perturb

mitochondrial gene expression. The early standard for the preclinical safety assessment of therapeutic ribonucleosides has been less than adequate. Biochemical assays routinely evaluated the ability of ribonucleotides to inhibit DNA polymerases, cell-based assays had no direct readout on alterations to mitochondrial transcription, and animal models, by use of cell lines or whole organisms, were not sensitive enough to observe changes in mitochondrial function of sufficient magnitude to be detected. The current state of the art fills the gaps of the past by expanding the repertoire of preclinical toxicity assays to include direct measurement of the ability of antiviral ribonucleosides to be substrates and/or inhibitors of POLRMT *in vitro* and in cells. These advances offer the ability to identify ribonucleosides with the potential for disruption of mitochondrial gene expression and therefore toxicity.

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27

Imaging of Mitochondrial Toxicity in the Kidney

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CHAPTER MENU

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27.1 Mitochondria in the Kidney

The major function of the kidney is to maintain water, electrolyte, and acid–base homeostasis in the body. The kidneys receive a high percentage of the cardiac output (typically 20–25%) and filter about 180 L of fluid every day in the average adult, via the glomeruli. Only about 1% of this filtrate is finally excreted in the urine, so approximately 178 L have to be reabsorbed along the remainder of the nephron after the glomerulus. The bulk of this reabsorption (60–70%) takes place in the first part of the tubule (the proximal tubule (PT)), with the remainder taking place in the distal tubule (DT) and collecting duct (CD) (Figure 27.1). The first part of the PT is convoluted (PCT) to increase the surface area for reabsorption. The later part is straight (PST) and descends from the cortex down into the medulla, via the medullary rays.

PT cells are highly adapted to perform such a large amount of solute transport. They have an extensive apical membrane brush border and express a multitude of different solute transporters at the apical membrane

(Figure 27.1). Most transport is coupled directly or indirectly to the movement of sodium, which is driven by the highly expressed basolateral Na^+/K^+ -ATPase. Water follows the movement of sodium by solvent drag, either across the cells (through aquaporins) or via the paracellular route. Certain solutes (phosphate, glucose, bicarbonate, and amino acids) are mainly or exclusively reabsorbed in the PT, while other solutes (e.g., calcium, potassium, and chloride) can also be taken up further down the nephron by the DT and CD. The size cutoff for the glomerular filtration barrier is approximately 70 kDa, so some albumin and many hundreds of low molecular weight proteins (LMWPs) are normally filtered. These are then also reabsorbed in the PT, via receptor-mediated endocytosis (Christensen et al., 2009).

The transport of such large amounts of fluid places huge energy demands on the PT. Accordingly, PT cells have a very high density of mitochondria to generate ATP via oxidative phosphorylation (OXPHOS). Mitochondria in the PT have a very distinctive elongated appearance and basolateral striated distribution (Figure 27.1), sitting

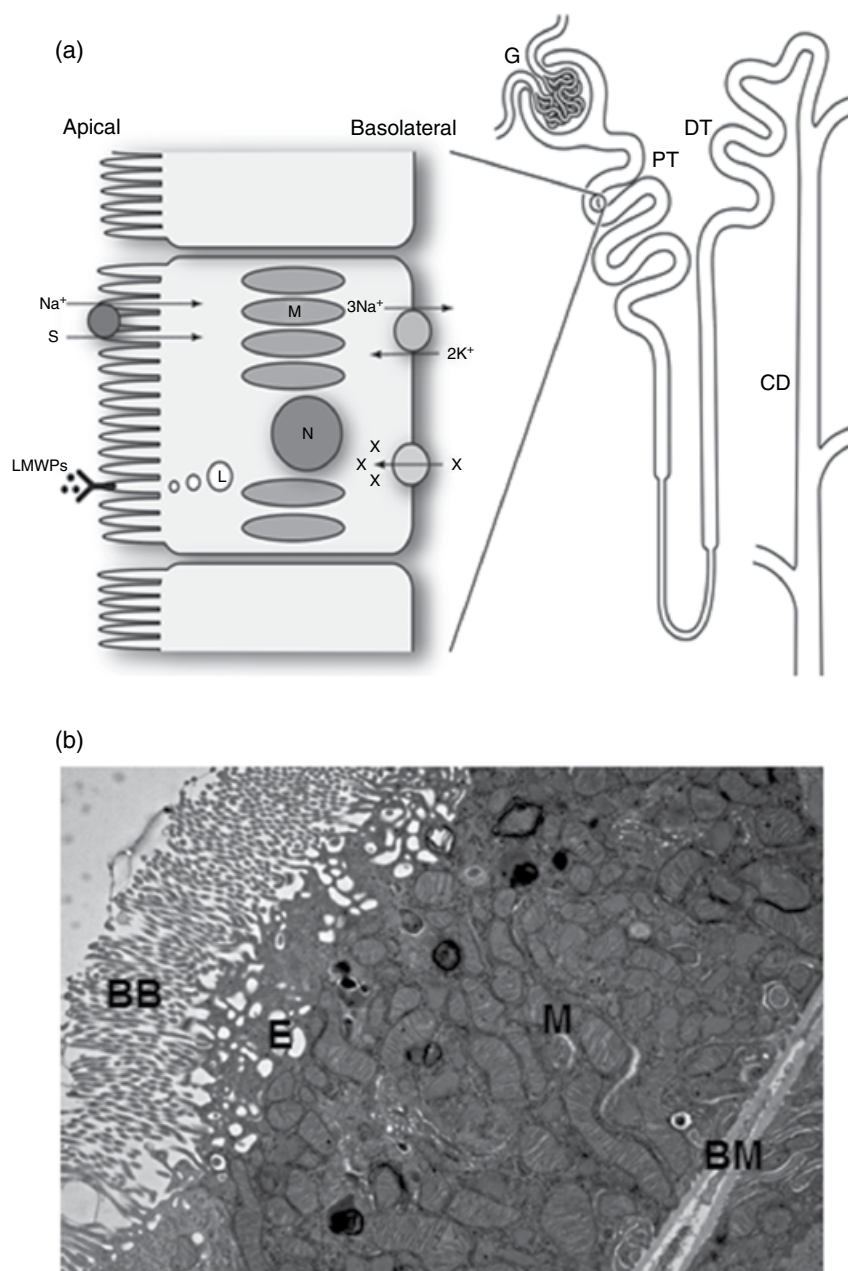


Figure 27.1 Solute handling and uptake of drugs in the nephron. (a) The majority of fluid filtered by the glomerulus (G) is reabsorbed along the proximal tubule (PT). Further reabsorption takes place in the distal tubule (DT) and collecting duct (CD). Various solutes (S) are cotransported across the PT apical membrane with Na^+ , while low molecular weight proteins (LMWPs) are taken up by receptor-mediated endocytosis into endosomes and eventually lysosomes (L). Sodium transport is driven by the basolateral Na^+/K^+ -ATPase, which requires ATP generated by mitochondria (M) located in close proximity (N, nucleus). A number of toxic drugs (X) can rapidly accumulate into PT cells via transporters expressed in the basolateral membrane and induce mitochondrial dysfunction and acute kidney injury. (b) Electron micrograph of a mouse PT showing the characteristic morphological features, including a highly developed apical membrane brush border (BB), numerous subapical endosomes (E), and a high density of elongated mitochondria (M) lying in a striated distribution close to the basolateral membrane (BM). Part A: Hall et al. (2014). Reproduced with permission of Oxford University Press. (See insert for color representation of the figure.)

in close proximity to Na^+/K^+ -ATPase pumps, which are the main consumers of ATP in these cells. For reasons that are not fully yet explained, PT cells have a very limited ability to generate ATP via anaerobic glycolysis

(Bagnasco et al., 1985), which renders them particularly vulnerable to mitochondrial insults.

Based on differences in ultrastructure, three distinct subtypes of PT cell can be distinguished (termed S1–S3)

(Christensen et al., 2012). S1 cells are located in the first part of the PCT, S2 in the latter part of the PCT and early PST, while S3 are found exclusively in the PST. The changing morphology of these cells reflects changes in function along the PT. In the early parts of the PT, the main function is uptake and transport of filtered solutes, thus S1 cells have a highly developed brush border and are densely packed with mitochondria. These features are less prominent in S2 cells. More distally the PT becomes a secretory epithelium, excreting endogenous organic ions and exogenous drugs into the urine, and S3 cells typically have fewer mitochondria.

27.2 Drug Toxicity in the Kidney

Along with the liver, the kidney represents a major excretory pathway for drugs and is therefore a common target for drug toxicity. PT cells express a range of xenobiotic transporters at the basolateral membrane—including organic cation transporters (OCTs) and organic anion transporters (OATs)—which are capable of taking up drugs from the blood stream (Launay-Vacher et al., 2006). As such, drugs can accumulate at high concentrations within PT cells. They are then excreted into the urine across the apical PT membrane by transporters such as p-glycoprotein and multidrug resistance-associated proteins (MRPs), which are also ATP dependent.

Many therapeutic drugs are toxic to mitochondria, mostly in ways that remain poorly understood. The unfortunate combination of multiple drug uptake pathways, high mitochondrial density, and limited glycolytic capacity renders the PT particularly vulnerable to drug-induced toxicity. The long list of drugs associated with kidney toxicity continues to grow with time and includes antibacterials (e.g., gentamycin, vancomycin), antivirals (e.g., tenofovir, cidofovir), chemotherapies for cancer (e.g., cisplatin, ifosfamide), antiepileptics (e.g., valproate), and iron chelators (e.g., deferasirox) (Izzedine et al., 2003; Perazella and Moeckel, 2010; Hall et al., 2014). This is a major clinical problem that limits the development and application of otherwise effective treatments. In addition, environmental toxins (e.g., cadmium) and herbal remedies (e.g., aristolochic acid) can also cause kidney dysfunction (Thevenod, 2003).

The clinical sequela of mitochondrial toxicity in the PT depends on the severity and ranges from asymptomatic increases in urine LMWP excretion (so-called tubular proteinuria) to significant wasting of solutes, resulting in systemic depletion and overt consequences like osteomalacia (the renal Fanconi syndrome), and in the most severe cases, to rapid and life-threatening declines in renal excretory function (acute kidney injury (AKI)) (Hall et al., 2014). As discussed in earlier chapters,

mitochondria have a range of other important intracellular roles beyond ATP production—including modulation of calcium signaling, biosynthesis of macromolecules, maintenance of redox state, and activation of cell death pathways—so drug-induced mitochondrial damage is likely to have multiple adverse downstream consequences for PT cells. There is currently a large amount of research centered on the role of mitochondria in the pathogenesis of all forms of AKI, and recent studies in rodents have suggested that mitochondrial-targeted therapies (e.g., antioxidants) could be of benefit (Tabara et al., 2014; Hall and Schuh, 2016).

27.3 Fluorescence Microscopy

Various methods are available to investigate mitochondrial structure or function in the kidney, including electron microscopy, oxygen consumption assays, and metabolomic screening. However, fluorescence microscopy is currently the only method available that allows simultaneous combined assessment of mitochondrial morphology, dynamics, and function *in situ* in living cells and therefore represents a powerful tool in mitochondrial research. Crucially, changes in mitochondrial parameters can be followed in real time in response to toxic insults, in parallel with other important readouts of cellular function, such as calcium signaling or solute trafficking. Mitochondria have a complex symbiotic relationship with their host cell, and it is becoming increasingly clear that mitochondrial function is adapted in each cell to the specific requirements of the host. Unfortunately, immortalized cell lines derived from kidney tubules often rapidly lose their primary phenotype. Moreover, they are typically much more glycolytic than cells *in vivo* (Hall et al., 2010), which limits their suitability as experimental models for drug toxicity. Therefore, wherever possible it is important to make measurements in native tissue.

27.3.1 Multiphoton Microscopy

Standard confocal microscopy with a single-photon excitation laser is an appropriate methodology to image mitochondria in isolated cells or tubules; however, it has significant limitations when working with intact organs, due to issues such as phototoxicity and poor tissue penetration. Multiphoton microscopy (MPM), also widely known as two-photon microscopy, offers solutions to these problems. It utilizes a longer wavelength excitation laser (typically in the range 700–1000 nm), pulsed at very high frequency, and relies on the principle that two low-energy photons arriving at the same point simultaneously can excite a fluorescent molecule (Helmchen and

Denk, 2005). There are several practical advantages to MPM when working with intact tissue. First, tissue penetration is much deeper, as longer wavelength light is less scattered. Second, phototoxicity is reduced, which allows repeated imaging of the same section over time. Third, multiple fluorescent probes with different emissions can be excited simultaneously at a single wavelength. Finally, nonfluorescent signals can be elicited, such as second harmonics from structures like collagen.

27.4 Assessment of Mitochondrial Function with Fluorescence Microscopy

In the last few years, there has been a rapid expansion in the number of fluorescent probes developed that can provide real-time readouts of mitochondrial function. However, many of these are essentially confined currently to usage with cultured cells *in vitro*, due to practical difficulties with effectively loading cells in living animals. Nevertheless, several important mitochondrial signals can be imaged in intact kidney tissue, both *ex vivo* and *in vivo*, using endogenous and exogenous fluorescent molecules; and these will be discussed in the following sections.

27.4.1 Mitochondrial Membrane Potential and pH

The proton pumping activity of OXPHOS complexes I, III, and IV generates a potential difference across the inner mitochondrial membrane ($\Delta\psi_m$), which is central to mitochondrial function and drives several key processes, such as ATP synthesis, calcium uptake, and protein import (Nicholls, 2004). Conversely, dissipation of this potential is typically associated with activation of cell death pathways. $\Delta\psi_m$ can be assessed in living cells using lipophilic cationic dyes (e.g., tetramethylrhodamine methyl ester (TMRM) or rhodamine 123), which accumulate into the mitochondrial matrix (Duchen et al., 2003). Fluorescence intensity, which is dependent on the degree of dye accumulation, is taken as a readout of the magnitude of the potential. While widely used and very effective, certain precautions have to be taken when working with these dyes. Firstly, they can be extruded by cells via efflux pumps (e.g., p-glycoprotein). Secondly, when illuminated with a laser, they can generate toxic reactive oxygen species (ROS). Finally, if they accumulate into mitochondria at a very high concentration, auto-quenching of the signal can occur, meaning that an increase in $\Delta\psi_m$ can actually be reflected as a decrease in fluorescence signal. For this reason, they are typically

used at low concentrations for experiments (e.g., 25–100 nM). Other $\Delta\psi_m$ -dependent dyes are also available, such as JC-1 and the MitoTracker probes, but these also have limitations (Brand and Nicholls, 2011).

Proton pumping across the mitochondrial inner membrane also generates a pH gradient (ΔpH_m) (Santo-Domingo and Demaurex, 2012). Although this makes some contribution to the overall electrochemical gradient driving protons back through the ATP synthase (the proton motive force), it is thought to be relatively small in comparison to $\Delta\psi_m$. Nevertheless, there may be some situations where ΔpH_m might play an important physiological role, and it can be assessed in living cells using pH-sensitive fluorescent probes. One commonly used dye is SNARF (Peti-Peterdi et al., 2012), which has the advantage of two separate emission wavelengths, thereby providing a ratiometric readout of mitochondrial pH.

27.4.2 Mitochondrial Redox State

NADH is the substrate for OXPHOS complex I and is naturally fluorescent in the reduced state, but not when oxidized to NAD^+ . It can be excited by UV light (single-photon excitation) or at wavelengths around 700–750 nm (two-photon excitation). It represents a useful endogenous mitochondrial marker in living tissues, but bleaches quickly when imaged repetitively. FADH_2 transfers electrons to complex II, and in contrast to NADH it is only fluorescent in the oxidized state (FAD^{2+}). It is typically excited at 450–500 nm (single photon) or around 900 nm (two photon). Combined imaging of both NADH and FAD^{2+} can provide insights into mitochondrial redox state, and the signals can be calibrated by using OXPHOS inhibitors or uncouplers to generate maximally reduced or oxidized conditions, respectively (Duchen et al., 2003; Hall et al., 2009). However, in practice FAD^{2+} signals are often quite weak, which can limit their usefulness in intact tissues.

27.4.3 Mitochondrial Reactive Oxygen Species Production

Mitochondria are thought to be a major source of ROS generation, probably as a by-product of normal OXPHOS activity (Murphy, 2009). Drug-induced mitochondrial damage could therefore lead to increased ROS production and oxidative stress. Several ROS-sensitive fluorescent probes are commercially available and widely used, including dihydroethidium (also known as hydroethidine), 2',7'-dichlorofluorescein (DCF), and CellROX. They are often marketed as being specific for certain ROS species (e.g., O_2^- or H_2O_2); however, in reality there is probably a lot of cross-reactivity, and dyes may behave differently within living cells from pure

in vitro assays. Most ROS probes work on the principle that they only become fluorescent upon oxidation; thus the rate of increase in fluorescence signal over time is taken as a readout of the rate of ROS generation. Since there are other non-mitochondrial sources of ROS within cells (e.g., NADPH oxidase), measurement of mitochondrial ROS requires the usage of probes that are selective for these organelles. One such example is MitoSOX, which is a cationic derivative of dihydroethidium that accumulates into mitochondria. However, the drawback of probes like MitoSOX is that changes in fluorescence signal can be confounded by changes in $\Delta\psi_m$ (Polster et al., 2014).

Glutathione (GSH) is an important intracellular antioxidant, which also plays an important role in the conjugation and excretion of drugs in the PT (Lash, 2005). Depletion of GSH is probably an important mechanism via which drugs can induce oxidative stress. The intracellular level of GSH can be imaged in live cells using monochlorobimane (MCB), which forms a fluorescent adduct with low molecular weight thiols (Keelan et al., 2001).

27.5 Ex Vivo Imaging of Mitochondria in the Kidney

Widely used *ex vivo* experimental models in renal research include tissue slices and the isolated perfused kidney (IPK). Primitive kidneys (organoids) grown from stem cells may also represent a useful model in the future for drug toxicity screening (Davies, 2015). These models all have the advantage that organ architecture is relatively well preserved, but experimental conditions can be more tightly controlled than *in vivo*. For example, in toxicology experiments drugs can be directly applied to the tissue at known concentrations. However, there are also several limitations to *ex vivo* models. Firstly, cells within the tissue may be damaged by the isolation process (e.g., due to brief periods of ischemia). Secondly, the normal concentration gradients that exist in the kidney may be obliterated. Thirdly, the nervous innervation of the kidney (which has an important role in regulating function) is lost. Finally, in the specific case of the IPK hemodynamic changes occur in the normal microperfusion of the organ.

We have shown previously that live imaging of mitochondria can be performed in both kidney slices and the IPK and that there are striking differences in intrinsic mitochondrial signals between different nephron segments (Hall et al., 2009, 2011). Since most of our experience to date has been with the slice model, we have focused the following discussion on this.

27.5.1 Live Imaging of Kidney Slices

Fresh slices of kidney tissue can be cut (typically about 200 μm thickness) from either the cortex or medulla of the kidneys isolated from anaesthetized mice or rats using a standard vibrotome. In our experience, when incubated on ice in an oxygenated buffer containing metabolic substrates, they remain usable for up to about 6–8 h. For imaging experiments they are placed in an open organ bath chamber on a microscope stage (underneath an upright objective) and perfused with oxygenated buffer at 37°C. Dyes are typically loaded via a recirculating perfusion system, until a steady-state signal has been achieved. Using this setup, we have shown that various aspects of mitochondrial function can be imaged in the tissue, including $\Delta\psi_m$, ΔpH_m , NADH, FAD^{2+} , ROS generation, and GSH levels (Figure 27.2) (Hall et al., 2009). Different tubular segments (i.e., PT, DT, and CD) can easily be identified by their characteristic morphology, and resolution is sufficient to perform detailed imaging of individual mitochondria within cells. Drugs can be applied via a perfusion system and responses in mitochondrial signals recorded in real time. For example, we have shown that the application of OXPHOS inhibitors and uncouplers lead to expected changes in mitochondrial redox state (as denoted by NADH/ FAD^{2+} signals) and energization (Hall et al., 2009).

The kidney slice model therefore represents a useful model to investigate nephron segment specific effects on mitochondria of suspected toxins, which can be directly applied at known concentrations and under tightly controlled physiological conditions. In the example depicted (Figure 27.3), the acute effects of sodium maleate on mitochondrial NADH and membrane potential can be observed in the PT. Maleate is an established mitochondrial toxin that accumulates in PT cells due to uptake via OATs and is thought to deplete intracellular coenzyme A, leading to inhibition of citric acid cycle activity and fatty acid metabolism (Zager et al., 2008). We have found that application of maleate (20 mg/mL) to kidney slices leads to an acute decrease in NADH in PTs, followed shortly afterward by spreading depolarization of mitochondria. Thus, live imaging can provide new information regarding the temporospatial effects of established toxins and could potentially also be used to screen new compounds for adverse mitochondrial effects in the kidney.

27.6 Intravital Imaging of Mitochondria in the Kidney

While there are several practical advantages to *ex vivo* methods like the kidney slice, measurements in living animals provide the most realistic model of the situation

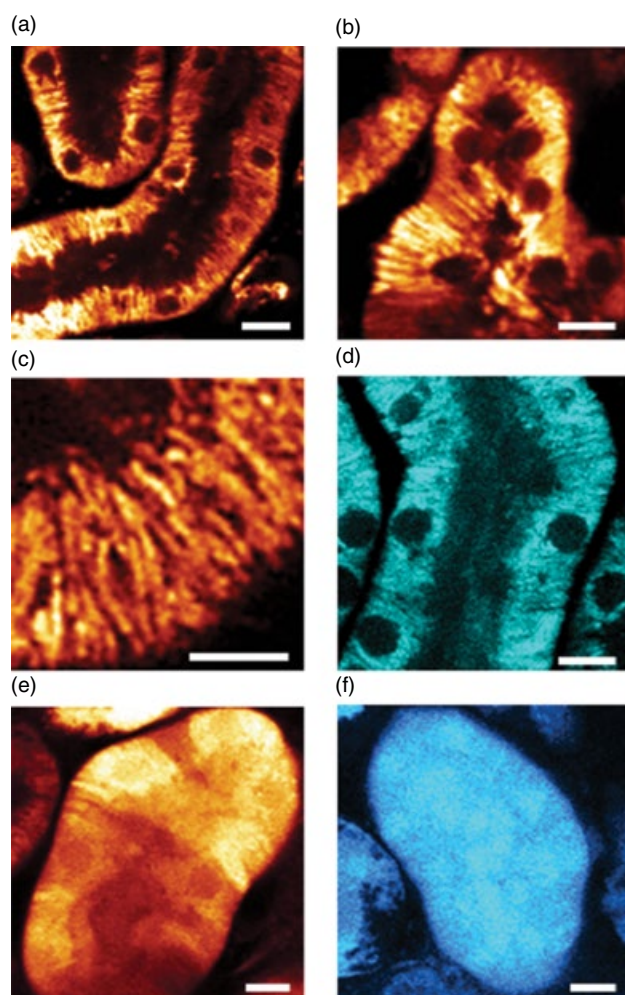


Figure 27.2 Multiphoton imaging of mitochondria in live mouse kidney cortex slices. A variety of aspects of mitochondrial function can be imaged in different nephron sections. (a–c) Mitochondrial membrane potential (measured using TMRM, excited at 850 nm) in proximal tubules (PTs) (a) and in a distal tubule (DT) (b). High resolution imaging of mitochondria in the PT, demonstrating the typical elongated morphology (c). (d–f) Mitochondrial NADH (excited at 720 nm) (d), mitochondrial pH (measured with SNARF, excited at 800 nm) (e), and the antioxidant glutathione (measured using monochlorobimane, excited at 720 nm) (f) in the PT. Scale bars = 10 μm in (a and b) and (d–f) and 5 μm in (c). (See insert for color representation of the figure.)

in humans. Methods to perform intravital imaging of the rodent kidney (using MPM) have now been established for over 10 years, thanks largely to the pioneering work of two groups (Molitoris and Sandoval, 2005; Peti-Peterdi et al., 2012). To prepare animals for experiments, the left kidney is externalized under anesthesia via a flank incision to enable access with the microscope objective. Experiments can be performed with either an upright or inverted microscope configuration, but the latter typically stabilizes the kidney better, leading to fewer movement artifacts. Dyes can be injected systemically via the

jugular or femoral vein; since not all dyes are efficiently taken up by the kidney *in vivo*, the range of signals that can be imaged is more limited than with *ex vivo* models, where dyes can be bulk loaded over time with a recirculating perfusion system. Nevertheless, we and others have shown that several important mitochondrial signals can be obtained *in vivo*, including NADH and $\Delta\psi_m$ (Figure 27.4) (Kalakeche et al., 2011; Hall et al., 2013).

Using intravital MPM, we have investigated how disease-causing insults affect mitochondrial function in the kidney. We have shown that in response to ischemia (a major cause of AKI in humans) mitochondria in the PT rapidly depolarize and fragment (Hall et al., 2013). Interestingly, mitochondria in neighboring DTs remain energized and elongated for considerably longer, suggesting that there are fundamental differences in how mitochondria in different nephron segments respond to the same insult. We have also investigated how mitochondria in the kidney respond to therapeutic drugs that cause nephrotoxicity. For example, we showed that the aminoglycoside antibiotic gentamicin induces mitochondrial damage after several days of repeated injections, but that these changes were preceded by alterations in other signals (e.g., from lysosomes) (Hall et al., 2013), suggesting that mitochondria are probably not the primary targets of this drug. More recently, we have investigated the effects of the anticancer drug cisplatin and have found clear evidence of mitochondrial toxicity 24 h post a single injection of the drug (Figure 27.4).

A major advantage of intravital imaging is that mitochondrial signals can be obtained simultaneously with other important readouts of kidney function, such as blood flow, tubular flow, and solute uptake and transport (Figure 27.4), thus enabling the study of the downstream consequences of mitochondrial toxicity on tubular function. For example, following intravenous injection of sodium maleate (a potent mitochondrial toxin that accumulates selectively into PT cells), we found that glomerular filtration of small molecules was preserved, but there was no longer any detectable apical uptake in PTs, suggesting a profound downstream effect on solute handling (Schuh et al., 2016). This observation fits nicely with the fact that humans with drug-induced kidney disease typically exhibit a solute wasting phenotype (renal Fanconi syndrome).

27.7 Recent Technical Developments in Intravital Kidney Imaging

While intravital microscopy is clearly a very powerful research tool, which has enabled numerous important new scientific insights to be gained, there are some

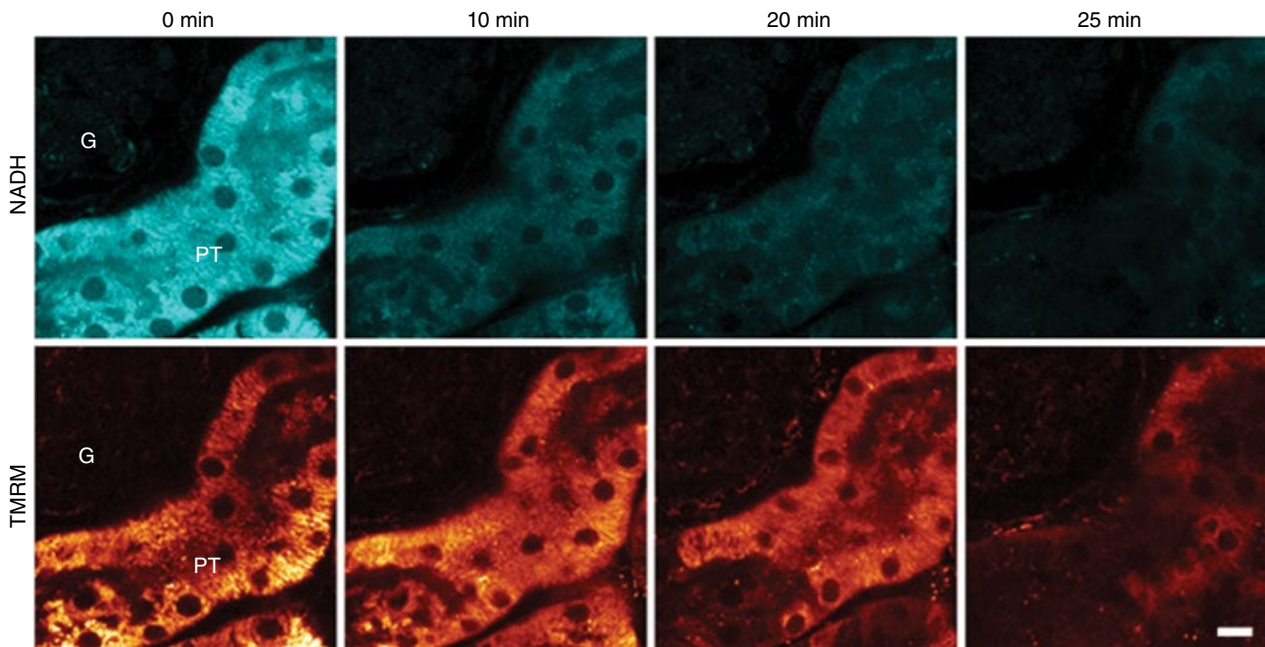
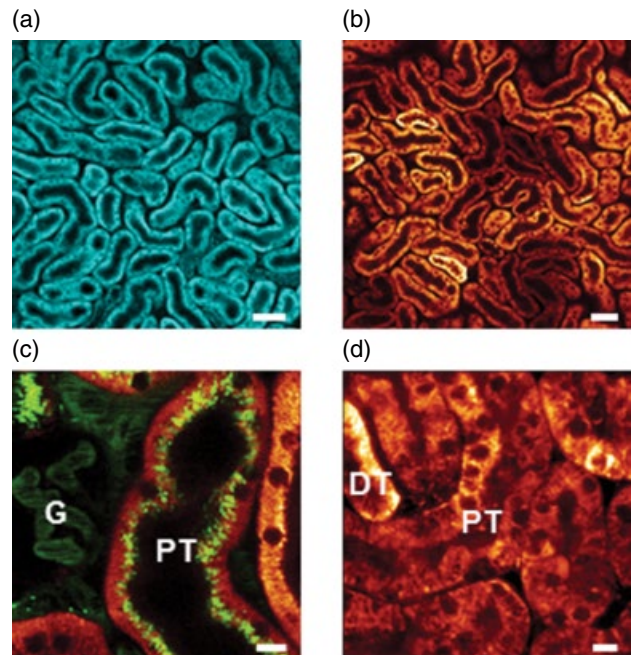


Figure 27.3 Multiphoton imaging of mitochondrial toxicity in live mouse kidney cortex. By adding the drug under investigation to the tissue perfusate, toxic effects on mitochondria in different nephron segments can be visualized in real time. In the example shown, the effects of the established nephrotoxin sodium maleate (20 mg/mL) are depicted on NADH (excited at 720 nm) and membrane potential (measured using TMRM, excited at 850 nm), two important readouts of mitochondrial function. Maleate causes an acute decrease in NADH signal in the proximal tubule (PT), which is followed by progressive loss of potential. G, glomerulus. Scale bar = 10 μ m. (See insert for color representation of the figure.)

Figure 27.4 Intravital multiphoton imaging of mitochondria in the mouse kidney. (a and b) Mitochondrial NADH (excited at 720 nm) (a) and membrane potential (measured using TMRM, injected intravenously, and excited at 850 nm) (b) in tubules of the outer kidney cortex. (c) Mitochondrial signals in the proximal tubule (PT) can be co-imaged with real-time uptake of filtered solutes across the apical membrane. In the example depicted, fluorescently labeled albumin injected intravenously can be visualized in the capillaries of the glomerulus (G) and in subapical endosomes of the PT. (d) Example image from a mouse injected with TMRM, acquired 48 h postinjection of cisplatin (30 mg/kg bodyweight), showing mitochondrial damage in PTs, but a normal appearance in the DT. Scale bars = 50 μ m in (a and b) and 10 μ m in (c and d). (See insert for color representation of the figure.)



practical constraints when working in the kidney that have limited progress in comparison with other organs, such as the brain. These include the fact that the kidney is a very optically dense organ, so tissue penetration is limited to the outer cortex, meaning that important

structures like glomeruli cannot be routinely be visualized. Moreover, the kidney emits a high amount of autofluorescence in the blue and green ranges, which can mask signals of interest. To address some of these technical challenges, we have recently constructed a new

microscope optimized for intravital kidney imaging. This machine is adapted for longer wavelength excitation and is coupled to a laser tunable up to 1300 nm. We have found that this setup offers several major advantages (Schuh et al., 2016). For example, due to the fact that longer wavelength light is less scattered in tissues, depth of imaging is significantly increased. Moreover, endogenous autofluorescence is much lower at longer wavelengths, meaning that the signal to background ratio is increased when working with far-red probes.

In parallel with improvements in lasers and microscopes, there is also a need to increase the number of fluorescent sensors that can be used for intravital kidney imaging, to increase the range of mitochondrial and metabolic readouts that can be obtained. Unfortunately, attempts to express genetically encoded fluorescent proteins using viral vectors (a technique routinely performed in the brain) have largely been unsuccessful in the kidney, although injection via the renal vein might work to an extent (Corridon et al., 2013). An alternative approach is to create transgenic animals stably expressing fluorescent reporters. For example, a mouse has been generated previously that expresses a photoactivatable protein in mitochondria in various organs (including the kidney), which allows the study of mitochondrial dynamics *in vivo* (Pham et al., 2012). Meanwhile, mice expressing calcium-sensitive reporters have been used recently to perform the first *in vivo* imaging of calcium signals in glomeruli (Burford et al., 2014) and PTs (Szebenyi et al., 2015). Of note, calcium is thought to be an extremely important regulator of mitochondrial function within cells.

It is probable that other recent technological developments will also further extend the capabilities of intravital imaging. For example, advances in adaptive optics could significantly increase the depth of imaging possible in light-scattering organs like the kidney (Wang et al., 2015). Meanwhile the increasing availability of automated microscopes and image analysis tools, coupled with exponential increases in computing power, will markedly increase productivity in live imaging work in the near future.

27.8 Conclusion

Drug toxicity in the kidney is a major clinical problem, but understanding of the underlying mechanisms remains limited, and there are very few effective preventative or treatment strategies available. Thus, there is an urgent

need for new progress in this field. The PT contains a high density of mitochondria and is the major target for most kidney toxic drugs. Live imaging with fluorescence microscopy (and in particular MPM) allows the detailed study of mitochondrial morphology, dynamics, and function in kidney tissue, either *ex vivo* (e.g., tissue slices) or *in vivo* in living rodents. Obtainable readouts of mitochondrial function include energization state ($\Delta\psi_m$), redox state (NADH/FAD²⁺), and rates of ROS generation. Importantly, changes in signal in response to toxins can be followed in real time and related to other parameters of kidney function. Recent technical advances, including longer wavelength imaging and generation of transgenic animals expressing fluorescent reporters, are significantly increasing the capabilities of intravital microscopy. In summary, in parallel with other developments in the mitochondrial research field, live imaging techniques have the potential to provide much needed breakthroughs in understanding how and why so many therapeutic drugs cause kidney disease in humans.

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Disclosures

None.

Conflicts of Interest

None.

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28

Imaging Mitochondrial Membrane Potential and Inner Membrane Permeability

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28.1 Introduction

A proton motive force (Δp) generated by respiratory chain components in the mitochondrial inner membrane drives mitochondrial ATP production (Nicholls and Ferguson 2013). Δp is made up mostly of a negative electrical potential difference ($\Delta\Psi_m$) across the mitochondrial inner membrane. Thus, $\Delta\Psi_m$ is the principal driving force for mitochondrial ATP synthesis to meet cellular energy demands. Inhibition of mitochondrial ATP production due to perturbations of $\Delta\Psi_m$ frequently occurs after injurious stresses and can have catastrophic consequences on cellular function. Consequently, $\Delta\Psi_m$ is widely measured in studies of the pathogenesis of cellular injury and death. Two approaches are frequently employed to assess $\Delta\Psi_m$ in response to drugs and toxicants. In the first, mitochondria are isolated from tissues and assessed for their ability to generate $\Delta\Psi_m$ either after direct exposure to toxicants or after tissue exposure to injurious stresses. In the second approach, $\Delta\Psi_m$ is measured *in situ* in cultured cells or the living animal.

28.2 Isolated Mitochondria

Before the development of cellular imaging technologies, $\Delta\Psi_m$ measurements were performed mostly in isolated mitochondria purified from tissues by homogenization and differential centrifugation in iso-osmotic sucrose or sucrose/mannitol solution (Table 28.1). After Teflon-on-glass homogenization, the crude homogenate is centrifuged at approximately 600 g to remove nuclei and incompletely disrupted cells. The supernatant is then centrifuged at approximately 10,000 g to sediment the mitochondria, which are resuspended and re-pelleted two or three more times to yield purified mitochondria.

Mitochondria isolated from liver originate mostly from hepatocytes, and contamination with mitochondria from non-parenchymal cells (endothelial, Kupffer, and stellate cells) is minor. Since liver mitochondria are quite homogeneous and can be obtained in large amounts, isolated liver mitochondria preparations are commonly used to study the effects of drugs and toxins on mitochondrial function, especially since the liver is a

Table 28.1 Isolation of rat liver mitochondria (Schneider 1948; Lemasters and Hackenbrock 1980).

Step comment
Preparation: Wash all glassware and utensils in deionized distilled water to remove detergent residues. Pre-chill.
Isolation medium: Ice-cold 0.25 M sucrose (2 mM K-Hepes buffer, pH 7.4, and 0.5 mM EGTA are optional).
Homogenization: Homogenize freshly excised rat liver cut in small pieces at 30% weight/volume with 3–4 strokes in Potter-Elvehjem tissue grinder with Teflon pestle. Use loose fitting pestle for first stroke to avoid excess pressure. Keep ice-cold at all times.
Centrifuge, 600 g × 10 min. Carefully remove supernatant for next step taking care not to disturb loose pellet of nuclei, erythrocytes, and partially broken cells.
Centrifuge, 10,000 g × 15 min. Aspirate, muddle pellet with precooled glass or Teflon rod in a few drops of medium and resuspend in 40 ml. Use EGTA-free medium for this and subsequent steps. Muddle around any erythrocytes at bottom of pellet so that they are not resuspended.
Centrifuge, 10,000 g × 10 min. Repeat twice. Aspirate supernatant and fluffy surface layer of pellet. Resuspend final pellet in ~1 ml of medium. Measure protein and adjust concentration to 50 or 100 mg protein/ml.

frequent target of drug toxicity. Similarly, left ventricular heart mitochondria can be isolated in large numbers and high purity from a variety of species, including beef and common laboratory animals. However, many heart mitochondria are enmeshed in the myofibrillar apparatus and are difficult to release without digestion of the myofibrils with proteolytic enzyme preparations, which may alter aspects of mitochondrial function.

Isolation of mitochondria from brain tissue is more problematic because of the intrinsic cellular heterogeneity of the nervous system. Isolation of mitochondria from whole brain results in a heterogeneous population of mitochondria that originates from different regions of the brain, such as the cortex, hippocampus, cerebellum, striatum, and substantia nigra. These mitochondria differ in size, enzyme content, and apparent susceptibility to toxicants. For example, systemic administration of complex II (succinate dehydrogenase) inhibitors, such as 3-nitropropionate, induces selective degeneration of striatal neurons that resembles pathologically and behaviorally Huntington's disease (Brouillet et al. 2005; Tunez et al. 2010). Similarly, complex I (NADH-ubiquinone oxidoreductase) inhibition with rotenone targets dopaminergic neurons in substantia nigra, producing a Parkinson-like syndrome in rats (Panov et al. 2005). Therefore, mitochondrial preparations pooling different brain regions are not ideal to study the effects of neurotoxins on mitochondria. Isolation of mitochondria from specific regions of the brain, such as cerebral cortex, hippocampus, and striatum, is possible, but yields will be very low from laboratory animals like rats and mice (Battino et al. 1995; Sims and Anderson 2008).

Mitochondrial fractions from the brain are typically contaminated with synaptosomes. Synaptosomes have a negative plasmalemmal membrane potential ($\Delta\Psi_p$) and therefore accumulate positively charged membrane potential indicators, which can lead to incorrect

inferences regarding $\Delta\Psi_m$. Different strategies are employed to remove synaptosomes from mitochondrial preparations. Percoll density gradient centrifugation removes synaptosomes based on density differences of mitochondria and synaptosomes to yield metabolically active and well-coupled mitochondria (Sims and Anderson 2008). Another well-established method to remove synaptosomes is to permeabilize them with a small amount of digitonin. Digitonin preferably binds to cholesterol-containing membranes lysing plasma membranes but sparing mitochondria (Fiskum et al. 1980; Sims and Anderson 2008). A disadvantage of the digitonin method is that residual amounts of digitonin may alter the permeability of the outer mitochondrial membrane, promoting mitochondrial swelling and dysfunction (Brustovetsky et al. 2002). In that respect, density gradient centrifugation may be the preferred method to isolate synaptosome-free mitochondria. For many types of studies, synaptosomal permeabilization rather than removal of synaptosomes may be sufficient to give mitochondria direct access to added reactants, inhibitors, and other substances of interest. Mutant recombinant perfringolysin O, a cholesterol-dependent cytolysin derived from *Clostridium perfringens*, forms oligomeric pores in cholesterol-containing membranes to provide more specific plasma membrane permeabilization than digitonin without adverse effects on mitochondrial function (Divakaruni et al. 2014).

Methods of mitochondrial isolation are optimized to select healthy mitochondria over damaged mitochondria, which becomes a problem when studying the effects of drugs and toxicants. Damaged mitochondria are often swollen, sediment differently from healthy mitochondria, and are not recovered in differential centrifugation and density gradient procedures. Thus, mitochondrial damage after *in vivo* drug/toxin exposure may be underestimated in isolated mitochondrial preparations.

Measurement of marker enzymes of the inner membrane, like succinate dehydrogenase, is useful to monitor mitochondrial recovery.

28.2.1 Nernstian Distribution of Fluorescent Probes

Membrane permeant cationic fluorescent dyes are commonly used to measure $\Delta\Psi_m$ in isolated mitochondria and intact cells. These dyes have a delocalized positive charge and accumulate electrophoretically in response to the electrical potential (Johnson et al. 1981; Emaus et al. 1986; Ehrenberg et al. 1988). At equilibrium, distribution of the permeant dye is dictated by the Nernst equation:

$$\Delta\Psi = -59 \log \left(\frac{F_{in}}{F_{out}} \right) \quad (28.1)$$

where $\Delta\Psi$ is the electrical potential difference in millivolts, F_{in} is cationic fluorophore concentration inside mitochondria (or other compartment of interest), and F_{out} is fluorophore concentration outside. Because $\Delta\Psi_m$ can be as great as -180 mV, as much as a thousand-fold concentration gradient can form across the mitochondrial inner membrane. These fluorophores appear to be specific for mitochondria, because $\Delta\Psi_m$ is so much more negative than other cellular compartments, but potential-indicating fluorophores will be taken up into any negatively charged compartment. Typically, $\Delta\Psi_p$ is negative. Thus, cationic fluorophores are first taken up by $\Delta\Psi_p$ and then by $\Delta\Psi_m$. In this way, $\Delta\Psi_p$ magnifies mitochondrial uptake, and if $\Delta\Psi_p$ decreases, mitochondria will lose fluorescence even if $\Delta\Psi_m$ is unchanged.

28.2.2 Monitoring Membrane Potential in Isolated Mitochondria

In isolated mitochondria, uptake of membrane potential-indicating fluorophores into the matrix space leads to self-quenching of the fluorophores and a red shift of their absorbance spectrum that varies linearly with $\Delta\Psi$. Thus for fluorophores like rhodamine 123, safranin, tetramethylrhodamine methyl ester (TMRM), and others, a decrease of total fluorescence or change of absorbance of mitochondria suspended in a cuvette signifies mitochondrial polarization (Akerman and Wikstrom 1976; Emaus et al. 1986; Scaduto and Grotyohann 1999). These cuvette-based techniques have also been adapted to multi-well plate readers to monitor $\Delta\Psi_m$ in all the wells of 24- and 96-well microtiter plates virtually simultaneously (Blattner et al. 2001).

28.2.3 Pitfalls with Potential-Indicating Fluorophores

A variety of potential-indicating fluorophores are available for membrane potential measurements. The recognition of the possible drawbacks related to the use of different fluorophores is critical in order to achieve meaningful results. The most common problems associated to the probes are interference with cell and mitochondrial metabolism, phototoxicity especially when imaging is performed by laser scanning confocal microscopy, and probe binding to cellular proteins. Inhibition of mitochondrial respiration increases in the order of tetramethylrhodamine methyl ester (TMRM) < rhodamine 123 < tetramethylrhodamine ethyl ester (TMRE) $\ll$ DiOC₆ (Rottenberg and Wu 1998; Scaduto and Grotyohann 1999). Rhodamine 123 at high matrix concentrations inhibits the mitochondrial ATP synthase of oxidative phosphorylation (Emaus et al. 1986). DiOC₆, which is frequently used in flow cytometric studies, strongly inhibits mitochondrial respiration. Therefore, DiOC₆ should be used at less than 1 nM loading concentration to avoid this effect (Rottenberg and Wu 1997). Of the commonly used probes, TMRM appears to be the least toxic to mitochondrial metabolism (Scaduto and Grotyohann 1999).

Rhodamine 123 is mostly used in the matrix quenching mode in isolated mitochondria and intact cells. Rhodamine 123 as well as TMRM can accumulate in mitochondria to levels that exceed those predicted by the Nernst equation (Emaus et al. 1986). Excess accumulation may be due to fluorophore stacking and formation of aggregates. Formation of aggregates causes fluorescence quenching and a red shift of absorbance. In cuvette and multi-well assays, decreased fluorescence due to quenching signifies an increase of $\Delta\Psi_m$ (Figure 28.1) (Emaus et al. 1986; Scaduto and Grotyohann 1999; Blattner et al. 2001). Similarly in cultured cells, increased fluorescence signifies depolarization, and a transient 50% increase in total rhodamine 123 fluorescence occurs in cultured hepatocytes after mitochondrial depolarization with HgCl₂ (Nieminen et al. 1990). The increase in total cellular rhodamine 123 fluorescence represents unquenching of fluorescence when the probe moves out from mitochondria into the cytosol. Subsequently fluorescence is lost as cytosolic Rh123 diffuses across the plasma membrane into the extracellular medium. Aggregate formation and quenching are dependent on fluorophore concentration and can be minimized by using lower loading concentrations.

Cationic fluorophores used to measure $\Delta\Psi_m$ are redistribution probes, since the probes must physically redistribute between intra- and extramitochondrial compartments as $\Delta\Psi_m$ changes. Such redistribution

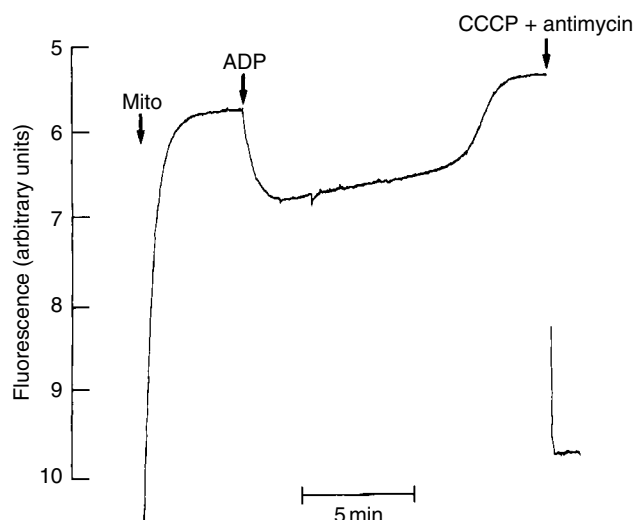


Figure 28.1 Mitochondrial membrane potential monitored by fluorescence quenching of rhodamine 123. Fluorescence was excited at 503 nm, and emission was measured at 527 nm. Additions are 0.167 mg protein/ml rat liver mitochondria (Mito), 300 μ M ADP, 1.3 μ M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, an uncoupler), and 1.6 μ g/mL antimycin (complex III respiratory inhibitor) to a basic reaction medium containing 150 mM sucrose, 5 mM MgCl_2 , 5 mM disodium succinate, 3.2 μ M rotenone, 200 nM Rh123, and 5 mM KPi and K-HEPES buffers, pH 7.4. Adapted from Emaus et al. (1986). Reproduced with permission of Elsevier.

takes time, and temporal resolution of changes of $\Delta\Psi_m$ from simple intensity measurements is typically several seconds or longer. Nicholls has developed kinetic models that utilize the rate as well as the magnitude of fluorescence changes to improve temporal resolution (Nicholls 2006, 2012). This approach requires characterization of the kinetics of probe redistribution across both the mitochondrial and plasma membranes that are in general different for different cell types.

Measurement of total cellular fluorescence of potential-indicating fluorophores has its drawbacks, because total fluorescence does not detect changes in individual mitochondria but instead averages all mitochondria. For intact cells, moreover, total fluorescence will be influenced by changes of both $\Delta\Psi_m$ and $\Delta\Psi_p$. Therefore, fluorescence microscopy has become a more attractive method, as originally shown for rhodamine 123 (Johnson et al. 1980). Fluorescence microscopy, especially confocal and multiphoton microscopy, permits observations of the heterogeneity of mitochondrial responses. For example, after exposure of rat pheochromocytoma 6 cells to staurosporine, mitochondria release TMRM in an asynchronous manner (Heiskanen et al. 1999). Confocal images reveal that some cellular mitochondria abruptly depolarize, whereas other mitochondria remain fully polarized for a longer period. In this example, the heterogeneity of the mitochondrial response within

individual cells would not have been detected without high-resolution confocal imaging.

Like rhodamine 123 and TMRM, cationic JC-1 accumulates into mitochondria electrophoretically. Inside mitochondria, JC-1 forms precipitates, called J-aggregates, in a concentration-dependent fashion (Reers et al. 1991, 1995). Other probes may also form aggregates, but in contrast to rhodamine 123 and TMRM, J-aggregation formation by JC-1 leads to a shift from green to red fluorescence emission instead of simple fluorescence quenching. Thus, mitochondrial hyperpolarization increases red fluorescence. Agents that abolish $\Delta\Psi_m$, for example, protonophores, then cause loss of red fluorescence, and the ratio of red to green fluorescence reflects the magnitude of mitochondrial polarization (Reers et al. 1991). Many studies have used a ratio of red and green fluorescence as a semiquantitative measure of $\Delta\Psi_m$ in intact cells (Scanlon and Reynolds 1998; Gillan et al. 2005). One of the disadvantages of using JC-1 is that the probe equilibrates very slowly across the plasma membrane. Mitochondria near the plasma membrane surface may accumulate more JC-1 and therefore appear to have higher membrane potential than those further away from the plasma membrane. Therefore, apparent heterogeneity of $\Delta\Psi_m$ between individual mitochondria within a cell may simply be due to uneven distribution of JC-1 (or other cationic probe) and not due to $\Delta\Psi_m$ variations. Longer incubations may be required to avoid this artifact by allowing added fluorophores to equilibrate between all mitochondria.

Another problem associated with JC-1 is that its red-fluorescing microprecipitates within a single mitochondrion give the impression that $\Delta\Psi_m$ is heterogeneous even within one mitochondrion. Confocal images of JC-1-loaded hepatocytes show these red-fluorescing JC-1 precipitates inside the JC-1 monomer-labeled matrix (green fluorescing) of individual mitochondria (Figure 28.2). The fact that the red-fluorescing J-aggregates do not fill the entire mitochondrial matrix indicates that the aggregates are indeed microprecipitates. In Figure 28.2, aggregates are seen to be localized to the lateral margins of mitochondria presumably in association with mitochondrial membranes. Heterogeneity of red and green fluorescence in single JC-1-loaded mitochondria thus represents the physical distribution of microprecipitates, and such heterogeneity does not indicate variation of $\Delta\Psi_m$ within the single mitochondria. Formation of physical aggregates of JC-1 within mitochondria limits the use of the probe for high-resolution imaging of cellular and mitochondrial $\Delta\Psi$.

During mitochondrial depolarization, most potential-indicating fluorophores are released from mitochondria. However, a group of cationic fluorophores, called MitoTracker probes, accumulate electrophoretically into mitochondria but are retained within mitochondria even

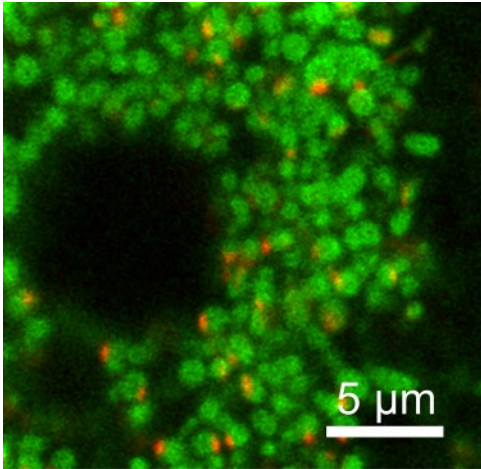


Figure 28.2 Red and green mitochondrial fluorescence after loading with JC-1. A cultured mouse hepatocyte was loaded with 100 nM JC-1 for 30 min in Krebs–Ringer–Hepes (KRH) buffer at 37°C, and green and red fluorescence was imaged by multitrack confocal microscopy using 488 and 543 nm excitation light, respectively. Red inclusions within green fluorescing mitochondria are JC-1 J-aggregates. (See insert for color representation of the figure.)

after depolarization. Inside mitochondria, chloromethyl groups of MitoTracker probes form covalent adducts with protein sulfhydryls (Macho et al. 1996). Once the probes have formed adducts, fluorescence of the probes becomes independent of $\Delta\Psi_m$. Since MitoTracker fluorescence is retained even after fixation, MitoTrackers are commonly used also to co-localize mitochondria in immunocytochemical preparations. However, the intensity of MitoTracker fluorescence will depend on many factors, including the time, typically many minutes, required to form covalent bonds with protein thiols. In general, MitoTracker fluorescence should not be used as a measure of changes of $\Delta\Psi_m$. However, MitoTracker Green used in combination with TMRM can be used to visualize selectively mitochondria undergoing depolarization (Elmore et al. 2001).

28.3 Imaging of Membrane Potentials in Single Intact Cells

Confocal and multiphoton microscopy creates submicron optical sections through single cells. With this resolution, fluorophore accumulation of individual mitochondria can be quantified in comparison with extramitochondrial fluorophore concentration. Using confocal microscopy to measure the intracellular distribution of membrane potential-indicating fluorophores, the distribution of intracellular electrical potential (Ψ) relative to the extracellular space can then be calculated using Equation 28.1. From differences of Ψ between subcellular compartments,

individual membrane potentials can be determined, such as $\Delta\Psi_p$ (cytosolic minus extracellular Ψ) and $\Delta\Psi_m$ (mitochondrial minus cytosolic Ψ).

In most cells, $\Delta\Psi_p$ ranges from -30 to -100 mV and $\Delta\Psi_m$ ranges from -120 to -160 mV. Because these $\Delta\Psi$'s are additive, mitochondria are 150 – 260 mV more negative than the extracellular space. Such large $\Delta\Psi$'s correspond to fluorophore concentration ratios between mitochondria and the extracellular space that can exceed $10,000$ to 1 (Equation (28.1)). Such large gradients cannot be imaged using a conventional linear scale of 256 gray levels per pixel (8-bit pixel memory). Rather 12- to 16-bit memory, sequential imaging at different laser powers or a nonlinear logarithmic (gamma) scale must be used instead (Chacon et al. 1994). Gamma scales, which are commonly used in scanning electron microscopy, logarithmically compress input signals into the available 256 gray levels of pixel memory. Earlier models of confocal microscopes, such as the Bio-Rad MRC-600, included gamma imaging circuits, but gamma circuitry unfortunately is no longer available in most current laser scanning confocal/multiphoton microscope systems. Fortunately, newer confocal microscope systems permit acquisition of images with 12- or 16-bit pixel depth to allow direct calculations of $\Delta\Psi$.

28.3.1 Image Acquisition and Processing

Typical fluorophore loading concentrations are 100 – 500 nM. To maintain equilibrium distribution, fluorophore concentration is then decreased to one-third to one-fourth the loading amount during all subsequent incubations. After cells have been loaded with a fluorophore, confocal/multiphoton images may then be collected. At least two images are required. The first image is an optical section through the specimen of interest. The second image is that obtained after refocusing inside the glass coverslip. The latter image serves as a background image. Both images must be collected using identical instrumental settings of laser power, gain, and brightness. Image oversaturation (pixels at highest gray level) and undersaturation (pixels with a zero gray level) must be avoided. Care must be taken to avoid laser-induced photodamage. A low laser power setting ($\leq 1\%$ for older instruments, $\leq 0.1\%$ for some newer instruments) should be used to minimize photodamage, especially if serial imaging over time of the same area of the specimen will be performed. If the microscope can collect images with pixel depth of 12 or 16 bits (4,096 and 65,536 gray levels, respectively), then additional images may not be needed. However, if pixel depth is only 8 bits (256 gray levels) or if the ratio of fluorescence intensity between mitochondria and the extracellular space exceeds the gray level range, then additional images at approximately 10 times greater laser power must be collected both in the cell and within the glass coverslip, since fluorescence varies linearly with

excitation intensity for 1-photon confocal imaging. For 2-photon multiphoton imaging, however, fluorescence is proportional to the square of excitation intensity.

With confocal microscopes equipped with multitracking systems, lower and higher laser power images can be collected simultaneously one line at a time. Each line (row of pixels) in the images is sequentially collected. A first line scan is performed at lower laser power followed immediately by a second line scan at higher laser power, a process that is repeated with each succeeding line in the image. Multitracking capability permits the two images to be acquired simultaneously and eliminates problems associated with specimen movement and focus drift. When images at higher laser power are collected, oversaturation of mitochondria will be evident, but oversaturation in higher power images does not pose a problem, since only the unsaturated pixels of higher power images will be used to quantify areas of weak fluorescence intensity in the extracellular space.

After processing images collected through the sample and within the glass coverslip, maps of the intracellular distribution of Ψ can be generated by a three-step procedure: (i) background subtraction, (ii) quantitation of extracellular fluorescence, and (iii) calculation of Ψ for each pixel gray level to create a pseudocolor map of Ψ .

28.3.2 Background Subtraction

Light detectors, such as photomultiplier tubes commonly used in confocal microscopes, generate signals even in the absence of light. This background signal must be subtracted from the signal collected in the presence of light in order to obtain an output truly proportional to fluorescence intensity. In confocal microscopy, images collected in the plane of the coverslip represent this background, since added fluorophore cannot penetrate into the glass. Pixel intensities in the background should be uniform and without gradients across the field of view. Such gradients or shading usually signify a correctable misalignment of the microscope optics. A mean pixel value is calculated for all the pixels of each background image using image analysis software (e.g., ImageJ, Adobe Photoshop, MetaMorph). This average background value is then subtracted from every pixel of the corresponding specimen image. Alternatively, background can be subtracted on a pixel-by-pixel basis. The background-corrected images represent the true relative distribution of fluorescence intensity within the images.

28.3.3 Fluorescence of the Extracellular Space

Since fluorescence should be the same everywhere in the extracellular space, all areas of the extracellular space in the background-subtracted specimen image are selected,

and average pixel intensity is determined. In the case extracellular fluorescence is too weak, then extracellular fluorescence can be measured in the same way but at higher laser power. Division (after background subtraction) of mean extracellular fluorescence from the higher laser power image by the power ratio between the higher and lower power images then yields an estimate of extracellular fluorescence for the lower power image.

28.3.4 Pixel-by-Pixel Calculation of Ψ

Using Equation (28.1), a value for Ψ ($\Delta\Psi$ relative to the extracellular space) can be calculated for every pixel of the background-corrected image on the assumption that fluorescence intensity is proportional to monovalent cationic fluorophore concentration. To display the intracellular distribution of Ψ , the images can be pseudo-colored by assigning different colors to specific mV ranges of Ψ . To determine the pixel value corresponding to a specific mV value of Ψ , Equation 28.1 is rearranged:

$$P_i = \text{antilog} \left(\frac{\log P_{\text{out}} - \Psi}{59} \right) \quad (28.2)$$

where P_{out} is average background-subtracted pixel intensity in the extracellular space and P_i is the pixel value representing a particular mV value of Ψ . Ranges of pixel intensity values corresponding to specific ranges of Ψ can be calculated from Equation (28.2). Individual colors are then assigned to these different ranges.

Figure 28.3 illustrates a pseudocolored image of a TMRM-loaded adult feline cardiac myocyte imaged by confocal microscopy. The difference of Ψ between the extracellular space (where Ψ is zero) and the cytosol/nucleus represents $\Delta\Psi_p$, whereas the difference between the cytosol/nucleus and mitochondria represents $\Delta\Psi_m$. In the cardiac myocyte, the cytoplasm was so densely packed with mitochondria that cytosolic Ψ needs to be estimated from the nucleus, which has the same potential as the cytosol. Thus, $\Delta\Psi_p$ was estimated to be about -100 mV. This $\Delta\Psi_p$ is consistent with the plasmalemmal polarization of excitable cells. Since mitochondrial Ψ is up to -220 mV, $\Delta\Psi_m$ is in the range of -120 mV. In hepatocytes, $\Delta\Psi_p$ is about -30 mV, but $\Delta\Psi_m$ remains -120 mV (not shown) (Chacon et al. 1994; Zahrebelski et al. 1995).

Mitochondrial diameter in hepatocytes and cardiac myocytes is about $1 \mu\text{m}$. Since the thickness of confocal optical sections is slightly less than $1 \mu\text{m}$, some mitochondria occupy the entire thickness of the optical slices, but other mitochondria occupy only part of the thickness of the section. Mitochondria that only partially occupy the confocal optical sections contribute to apparent heterogeneity and underestimation of $\Delta\Psi_m$. Thus,

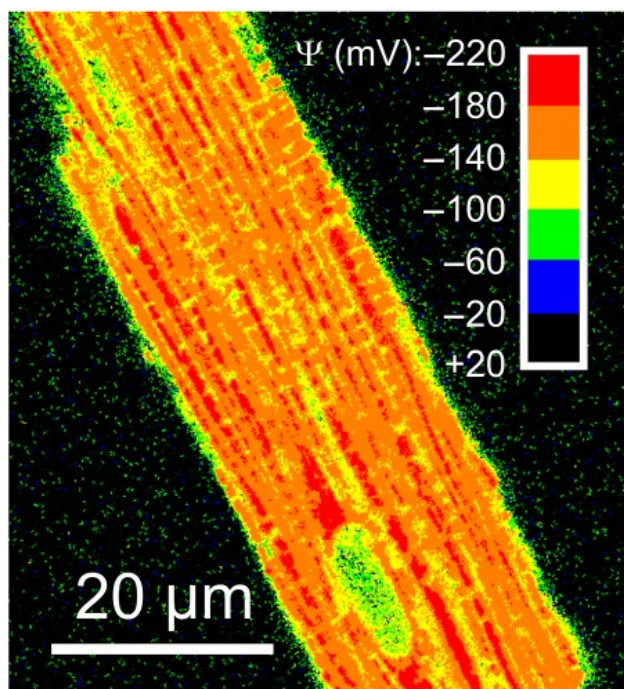


Figure 28.3 Distribution of electrical potential in a cardiac myocyte. An adult feline cardiac myocyte was loaded with 200 nM TMRM for 20 min at 37 °C, and TMRM fluorescence was imaged by confocal microscopy using 543 nm excitation and a 565–615 nm emission filter. The distribution of Ψ is displayed in pseudocolor, as described in the text. (See insert for color representation of the figure.)

the highest values of Ψ_m most likely reflect true mitochondrial $\Delta\Psi$. In other cell types, such as cancer cells, mitochondrial diameter is much less than 1 μm , which makes it more difficult to estimate $\Delta\Psi_m$ correctly.

Super-resolution techniques, such as stimulated emission depletion (STED) microscopy and Airyscan laser scanning confocal microscopy, improve axial resolution. By STED microscopy, axial resolutions approaching 100 nm are possible, but the range of usable non-phototoxic STED fluorophores remains somewhat limited (Klar et al. 2000; Neupane et al. 2014). Nonetheless, TMRM can be used to visualize $\Delta\Psi_m$ in living cells by STED microscopy (Glancy et al. 2015).

Airyscan super-resolution microscopy achieves an axial resolution of 400 nm and is compatible with essentially all fluorophores used for confocal microscopy of living cells, including cationic indicators of $\Delta\Psi$. By collecting adjacent Airyscan confocal images at different focal depths (z-stacks), three-dimensional visualization or rendering of datasets is possible using commercial software such as Imaris (Bitplane AG, Zurich). Figure 28.4 illustrates a volume rendering of TMRM-labeled mitochondria in a primary rat hepatocyte. Color coding reflects relative median TMRM intensity of each mitochondrion, whereas

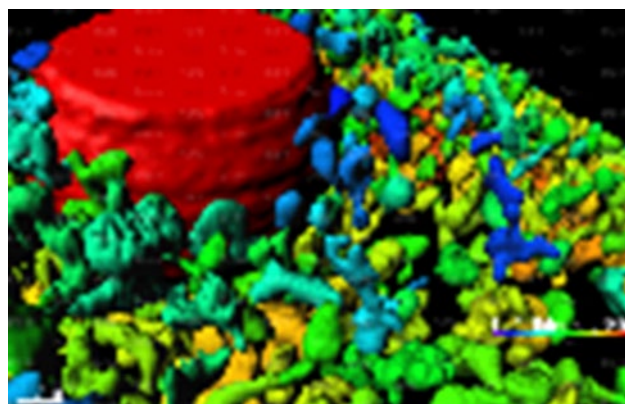


Figure 28.4 Airyscan super-resolution volume rendering of TMRM-labeled mitochondria in a primary rat hepatocyte. Mitochondrial color represents median TMRM fluorescence intensity from low (blue to green) to high (yellow to red). The nucleus is segmented based on PicoGreen labeling and is colored red. (See insert for color representation of the figure.)

the nucleus is shown in red. Contour-dependent shading enhances perception of three dimensions. Notably, $\Delta\Psi_m$ for individual mitochondria within the hepatocyte shows heterogeneity, an effect that is not due to sectioning artifact. In particular, a subpopulation of low potential (blue) mitochondria resides in the perinuclear region (Figure 28.4).

28.4 Mitochondrial Permeability Transition

In the mitochondrial permeability transition (MPT), opening of nonselective, highly conductive permeability transition (PT) pores causes the inner membrane of mitochondria to become permeable to molecules of up to 1500 Da, which leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude mitochondrial swelling (Hunter et al. 1976; Halestrap and Richardson 2015; Biasutto et al. 2016; Hurst et al. 2017). Cyclosporine A (CsA) and various of its analogues inhibit PT pore opening. The molecular composition of PT pores remains uncertain. In one model, PT pores form from the adenine nucleotide translocator (ANT) in the inner membrane, the voltage-dependent anion channel (VDAC) in the outer membrane, and cyclophilin D (CypD), a CsA binding protein from the matrix. Findings that the MPT still occurs in mitochondria that are deficient in ANT, VDAC, and even CypD challenge the validity of this model (Kokoszka et al. 2004; Basso et al. 2005; Baines et al. 2007). More recently, PT pores are proposed to form within F_1F_0 -ATP synthase dimers at the interface between

monomers or within the c-ring of F_0 (Bernardi et al. 2015; Jonas et al. 2015). Spastic paraplegia 7 (SPG7), an AAA-type membrane protease in mitochondria, and the inorganic phosphate carrier of the inner membrane may also participate in PT pore formation (Halestrap and Richardson 2015; Shanmughapriya et al. 2015). In another model, PT pores form from damaged misfolded membrane proteins that aggregate at hydrophilic surfaces facing the bilayer to create aqueous transmembrane channels (He and Lemasters 2002). CypD and other molecular chaperones close these nascent PT pores to protect mitochondria from depolarization and swelling. CypD confers sensitivity to Ca^{2+} by which increased matrix Ca^{2+} opens the PT pores, an action antagonized by CsA. When nascent pores formed by misfolded protein aggregates exceed the chaperones available to regulate their conductance, unregulated PT pore opening occurs. Such unregulated PT pores are insensitive to CsA inhibition and no longer require Ca^{2+} for opening. The protein misfolding model accounts for why the MPT occurs in ANT-deficient mitochondria (Kokoszka et al. 2004), since misfolding of other mitochondrial membrane proteins causes PT pore formation in the absence of ANT. Mitochondria from CypD knockout mice are desensitized to onset of the Ca^{2+} -induced MPT, and higher Ca^{2+} is needed to induce the MPT (Basso et al. 2005), which is consistent with a role of CypD as the Ca^{2+} sensor of the MPT. Much evidence supports a critical role of the MPT in cell necrosis and apoptosis from oxidant stress, Ca^{2+} toxicity, warm ischemia/reperfusion, cytokines, Fas ligation, bile acid toxicity, ethanol, acetaminophen, and a variety of other stresses to hepatocytes, cardiac myocytes, and other cell types (Waldmeier et al. 2003; Lemasters et al. 2009; Readnower et al. 2011; Lemasters 2013; Halestrap and Richardson 2015; Ong et al. 2015).

28.4.1 Swelling Assay of the MPT

When the MPT occurs, $\Delta\Psi_m$ collapses, and onset of the MPT can be followed with potential-indicating fluorophores. However, the MPT is not the only cause of mitochondrial depolarization. A more specific assay is to follow mitochondrial swelling as assayed by a decrease of absorbance. At onset of the MPT, large amplitude swelling occurs driven by the colloid osmotic force of protein in the matrix. For a dilute mitochondrial suspension (0.2–0.5 mg protein/mL), this swelling leads a decrease of absorbance of 0.2–0.4 units, which is discernable even to the naked eye (Figure 28.5). The requirements for this *in vitro* assay in isolated mitochondria are (i) an absence of Mg^{2+} since Mg^{2+} strongly inhibits the MPT; (ii) a source of energy, such as

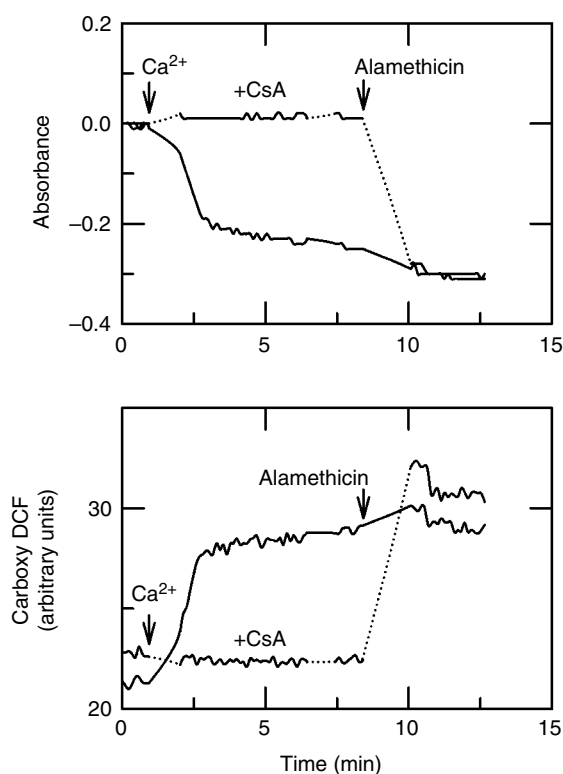


Figure 28.5 Mitochondrial swelling and release of carboxydichlorofluorescein after induction of the mitochondrial permeability transition. Rat liver mitochondria were loaded with 5 mM carboxyDCF diacetate during isolation. CarboxyDCF-loaded mitochondria (0.5 mg/mL) were added to incubation buffer containing 100 mM KCl, 20 mM Tris, 20 mM K-Hepes, 10 mM NaCl, 1 mM KH_2PO_4 , 5 mM succinate, 20 μ M EGTA, 2 μ M rotenone, and 1 μ g/mL oligomycin. Absorbance change at 620 nm (top) and carboxyDCF fluorescence excited at 485 nm (bottom) were determined using a multi-well fluorescence plate reader. $CaCl_2$ (100 μ M) was added after 1 min (first arrow). Alamethicin (5 μ M, second arrow) was added to permeabilize and induce swelling of all mitochondria. Dotted lines indicate gaps in measurements as additions were made.

respiratory substrate (e.g., succinate plus rotenone); (iii) 1–3 mM Pi that promotes Ca^{2+} uptake as well as independently promoting the MPT; and (iv) boluses of $CaCl_2$ to induce MPT onset. In such an assay, addition of Ca^{2+} causes large amplitude swelling (decrease of absorbance) beginning after a delay of several seconds to even a few minutes (Figure 28.5). A variety of reactive chemicals (e.g., phenylarsine oxide, diamide) act to lower the threshold of the Ca^{2+} -induced MPT, whereas MPT inhibitors (e.g., CsA) increase the threshold for Ca^{2+} . Importantly at onset of the MPT, all Ca^{2+} that has been taken up into the matrix is abruptly released. Thus, MPT onset can also be monitored by Ca^{2+} release using Ca^{2+} fluorophores added to the extracellular medium, such as Fluo-5 N (Blattner et al. 2001).

28.4.2 Release of Carboxydichlorofluorescein

At onset of the MPT, trapped solutes inside mitochondria are released into the medium. To assess this aspect of PT pore opening, mitochondria can be ester loaded with carboxydichlorofluorescein diacetate during isolation. Matrix esterases release carboxydichlorofluorescein free acid (carboxyDCF) that remains trapped in the matrix space. Like other fluorophores in the matrix, the fluorescence of carboxyDCF is partially quenched. At onset of the MPT, carboxyDCF is released into the medium with unquenching of fluorescence. Hence, an increase of carboxyDCF fluorescence indicates increased nonspecific permeability of the mitochondrial inner membrane to carboxyDCF (Figure 28.5).

28.4.3 Visualizing the MPT in Intact Cells

Many of the ion-indicating fluorophores used for optical microscopy of living cells are multivalent organic anions. To load these molecules into cells, the charged acids are neutralized by forming acetate or acetoxymethyl esters, which are membrane permeable. After incubation with the esters, cytosolic esterases regenerate the free acid form of the fluorophores, which become trapped in the cytosol. The temperature of loading strongly affects intracellular distribution. In cells like cultured hepatocytes and cardiac myocytes, warm loading at 37°C promotes nearly exclusive loading into the cytosol and nucleus, whereas cold loading leads to additional loading into mitochondria, lysosomes, and possibly other intracellular organelles (Nieminen et al. 1995; Trollinger et al. 1997; Kim et al. 2006; Zhang and Lemasters 2013).

Calcein is a pentacarboxylic acid fluorophore whose fluorescence is independent of physiological changes of intracellular ions except for quenching by chelatable Fe^{2+} and Cu^{2+} (Epsztejn et al. 1997). Calcein acetoxymethyl ester (AM) loaded at 37°C localizes almost exclusively to the cytosol and nucleus (Figure 28.6). After warm ester loading, mitochondria exclude calcein and appear instead as small round dark voids in the green calcein fluorescence. These voids correspond exactly to mitochondria, as shown by simultaneous loading with TMRM (Figure 28.6). Mitochondria exclude calcein because the PT pores are closed and the inner membrane is otherwise impermeable to this very polar 623 Da solute. However at onset of the MPT, PT pores open and permit calcein to enter into the matrix, as illustrated in Figure 28.6 for a rat hepatocyte exposed to the oxidant chemical *tert*-butyl hydroperoxide. As a consequence, the dark voids disappear. Simultaneously, TMRM fluorescence disappears, indicating mitochondrial depolarization. Inhibition of

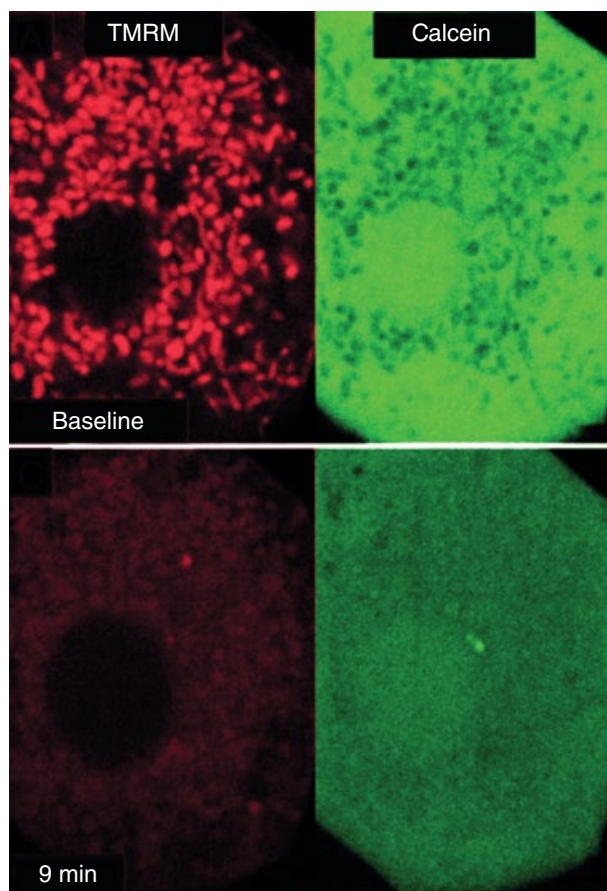


Figure 28.6 Increased mitochondrial inner membrane permeability in a rat hepatocyte induced by *tert*-butylhydroperoxide. A cultured rat hepatocyte was loaded with TMRM (left panel) and calcein (right panel). Note that dark round voids in the green calcein fluorescence coincide with red TMRM labeling of mitochondria. After 9 min exposure to 100 μM *tert*-butylhydroperoxide, dark mitochondrial voids filled with green calcein fluorescence. Simultaneously, mitochondrial release red TMRM fluorescence. These events signified onset of the MPT. Adapted from Nieminen et al. (1995). Reproduced with permission of Portland Press. (See insert for color representation of the figure.)

these changes by CsA then supports the conclusion that the inner membrane permeabilization and depolarization are specifically due to the MPT.

In contrast to loading at 37°C, calcein AM loading at 4°C causes entry of calcein into both cytosol and mitochondria (Nieminen et al. 1995). Presumably during warm loading, cytosolic esterases are so active that calcein AM is hydrolyzed before it can enter mitochondria. At 4°C, esterase activity is slowed, allowing movement of unhydrolyzed calcein AM into mitochondria where mitochondrial esterases cleave the ester to entrap calcein free acid. The temperature dependence of ester loading is both probe and cell type specific, but the loading technique works well in both cultured hepatocytes and adult

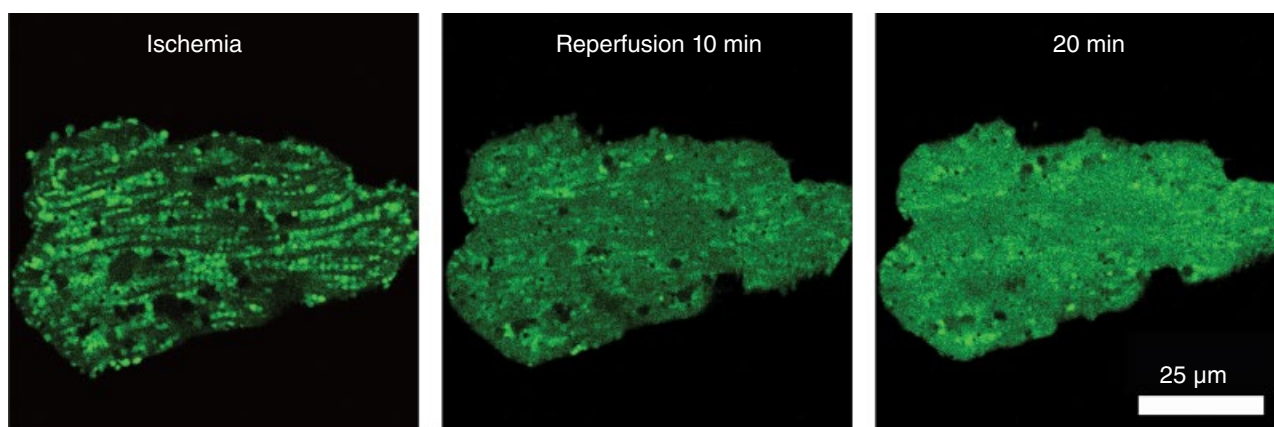


Figure 28.7 Inner membrane permeabilization after ischemia/reperfusion in rat myocytes visualized by mitochondrial calcein release after cold ester loading/warm incubation. An adult rat cardiac myocyte was cold-loaded with calcein AM and subjected to 3 h of simulated ischemia at 37°C at pH 6.2 followed by reperfusion at pH 7.4 for 10 and 20 min. Green calcein fluorescence was retained by mitochondria at the end of ischemia. After reperfusion, mitochondria began to release calcein, signifying inner membrane permeabilization. For experimental details, see Kim et al. (2006). Reproduced with permission of American Physical Society. (See insert for color representation of the figure.)

heart cardiac myocytes (Trollinger et al. 1997; Kim et al. 2006; Zhang and Lemasters 2013).

The plasma membrane gradually releases fluorophores from the cytosol through an organic anion carrier (Wieder et al. 1993). Fluorophores trapped in mitochondria, however, are retained. Thus, when cold calcein AM loading is by warm incubation of several hours, calcein localization becomes almost exclusively mitochondrial (Figure 28.7) (Trollinger et al. 1997). Now at onset of the MPT, instead of calcein moving into the mitochondria, calcein moves out, as illustrated in Figure 28.7 for a cardiac myocyte after ischemia/reperfusion (Kim et al. 2006). Due to unquenching, overall calcein fluorescence also becomes brighter after release.

Due to differences in esterase activity and intracellular distribution in different cells types, specific localization of calcein to cytosol or mitochondria may not be possible. In this instance, calcein AM may be loaded in the presence of 1 mM CoCl_2 (Petronilli et al. 1999). Co^{2+} strongly quenches calcein but enters only the cytosol. Hence, mitochondria but not cytosol fluoresces after calcein ester loading with CoCl_2 . Then after onset of the MPT, release of calcein into the cytosol and quenching by Co^{2+} lead to loss of calcein fluorescence. This technique for visualizing the MPT in living cells has proven useful in various cancer cell lines (Petronilli et al. 1999; Lam et al. 2001).

When physiologically occurring heavy metals increase, particularly Fe^{2+} (but not Fe^{3+}), calcein fluorescence quenches and consequently calcein fluorescence intensity decreases. The source of Fe^{2+} is typically lysosomes, which release Fe^{2+} after alkalinization or lysosomal membrane disruption. Quenching can be distinguished

from nonspecific leakage by a decrease of intracellular fluorescence even when cells are incubated with calcein free acid in the extracellular space. Reversal or prevention of quenching by iron chelators like desferal and dipyrindyl confirm that an increase of chelatable Fe^{2+} is causing the quenching (Uchiyama et al. 2008; Kon et al. 2010; Zhang and Lemasters 2013).

28.4.4 Plasma Membrane Permeability

Directed compartmental loading of calcein also allows direct observation in cells of changes of permeability of the plasma membrane. For example, at the onset of cell death after toxic and hypoxic injury, trapped cytosolic calcein is lost almost instantaneously (Zahrebelski et al. 1995). Similarly, when calcein is in the extracellular space, the fluorophore enters the cell interior at onset of cell death. Other extracellular fluorophores also enter cells at onset of cell death, such as propidium iodide that binds to DNA in the nucleus with an enhancement of fluorescence (Lemasters et al. 1987).

28.5 Conclusion

Quantitative confocal/multiphoton microscopy of the intracellular distribution of membrane permeant cationic fluorophores provides a minimally perturbing means to measure dynamically both mitochondrial and plasmalemmal $\Delta\Psi$ in living cells. The method eliminates artifacts associated with measurement of $\Delta\Psi$ by flow cytometry and related non-microscopic techniques.

A limiting factor is time resolution, since several seconds are required for cationic fluorophores to reestablish a new steady-state equilibrium after a change of $\Delta\Psi$. Unlike virtually any other technique, confocal/multiphoton microscopy permits nondestructive serial observation of the $\Delta\Psi$ s of populations of cells and their individual mitochondria. Similarly, confocal microscopy can be adapted to visualize directly onset of inner membrane permeabilization due to the MPT in single mitochondria of living cells.

Live cell, three-dimensionally resolved confocal and multiphoton microscopy is now an indispensable tool for studying mitochondrial function and pathophysiology.

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Quantifying Skeletal Muscle Mitochondrial Function *In Vivo* by ^{31}P Magnetic Resonance Spectroscopy

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CHAPTER MENU

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29.1 MRS Methods in Skeletal Muscle

Magnetic resonance spectroscopy (MRS) methods offer a valuable way to study some aspects of cellular metabolism and physiology *in vivo*, but correct interpretation of the data depends on understanding both the underlying mechanisms and the nature of the measurement. In skeletal muscle, in particular, phosphorus magnetic resonance spectroscopy (^{31}P MRS) can detect and quantify a small set of metabolites that usefully occupy a central place in cellular ATP turnover and cellular pH homeostasis. Correctly interpreted, these measurements allow useful inferences about processes that are otherwise difficult to access in noninvasive ways suited to multiple measurements and clinical studies. Correct interpretation requires attention both to the technical aspects of the measurements and to the metabolic and cellular physiological background. In this chapter we discuss the application of this methodology to the assessment and quantification of muscle mitochondrial function *in vivo*.

MRS methods *in vivo* depend on measuring signals from cellular metabolites. We will begin by describing briefly the three main ways in which they can be used to measure cellular metabolic fluxes. First, we mention for completeness a method that is outside the scope of this review: ^{13}C MRS with exogenous tracers. An application of this to study muscle mitochondrial metabolism is the use of $[2\text{-}^{13}\text{C}]\text{acetate}$ infusion to measure tricarboxylic acid cycle flux in resting muscle (Befroy et al., 2009). As discussed elsewhere (Kemp, 2008a; Kemp & Brindle, 2012), the main interpretative difficulty is that while resting muscle measurements of tricarboxylic acid cycle flux (or of ATP turnover or of O_2 consumption) undoubtedly report on an aspect of mitochondrial function, they have no direct relationship to mitochondrial capacity in the sense we elucidate in the succeeding text.

There are two other approaches, both using ^{31}P MRS. One is ^{31}P MRS magnetization transfer: the attempt to use this to probe mitochondrial metabolism in resting muscle presents several interpretative difficulties, which are discussed in detail elsewhere (Kemp, 2008a; Kemp & Brindle, 2012; Sleight et al., 2016) and to which we will return later.

A different approach underpins most of the work we are concerned with here, assessing metabolic fluxes by analysis of the kinetics of dynamic ^{31}P MRS measurements in exercise and recovery. The metabolic capability of skeletal muscle for large changes of ATP turnover in support of changing mechanical output presents a key opportunity for estimation of net fluxes. ^{31}P MRS measurements of the exercise and post-exercise recovery kinetics of cytosolic pH and the cytosolic concentrations of phosphocreatine (PCr), orthophosphate (Pi), and ADP contain much information about two main areas of metabolism. One is cellular pH homeostasis, which involves the processes of what one might call H^+ production, consumption, buffering, and efflux (Kemp et al., 1994, 2001b, 2009). In the present context we will not need to discuss this further. The other is energy metabolism, in the sense of ATP production and ATP use and their regulation. An important aspect of this is the use of ^{31}P MRS measurements to assess muscle mitochondrial function, in the sense of oxidative ATP synthesis, its regulation in relation to ATP demand, and its pathophysiology. This is the main subject of this chapter.

29.2 The Metabolic and Physiological Background to ^{31}P MRS Studies of Muscle

The essential metabolic background recently has been reviewed (Kemp et al., 2014). For present purposes we can simplify muscle energy metabolism to a system of ATP supply and demand, buffered by the CK reaction, which equilibrates PCr, creatine (Cr), ATP, ADP, and H^+ . ADP and inorganic phosphate (Pi) are 1:1 stoichiometric products of ATP hydrolysis and also substrates for ATP synthesis by oxidative metabolism and anaerobic glycolysis. Any temporary mismatch between ATP supply and demand is buffered by PCr breakdown, such that PCr falls if ATP uses outruns supply, with negligible change in [ATP]. In terms of metabolic regulation, ATP turnover is largely demand driven, and CK-related metabolites play an important role as feedback regulators of ATP supply. We will discuss in the next section how this model can be used in the analysis of dynamic ^{31}P MRS data.

As mentioned, ^{31}P MRS gives access to some key parts of this system. The muscle ^{31}P MRS spectrum contains phosphomonoester (PME), Pi, phosphodiester (PDE) and PCr, and the γ (doublet), α (doublet), and β (triplet) peaks of ATP. In resting muscle PME comprises largely the hexose monophosphates (HMPs) and glycolytic intermediates glucose 6-phosphate (~80%) and fructose 6-phosphate (~15%) (Kemp et al., 2001b); inosine

monophosphate can also contribute post-exercise when there has been loss of the adenine nucleotide pool. Muscle PDE comprises metabolites of phospholipid breakdown. We can use spectral data to calculate cytosolic pH (from the chemical shift difference between Pi and PCr), the free concentrations of ADP and AMP and the free energy of ATP hydrolysis (ΔG_{ATP}) (essentially from pH and [PCr], as explained later), and free $[\text{Mg}^{2+}]$ (from the chemical shift of β -ATP (Iotti et al., 2000)). Typical published values in normal resting human muscle (Kemp et al., 2007) are [PCr] 33 mmol (l cytosolic water) $^{-1}$, more concisely expressed as 33 mM, [Pi] 4 mM, pH 7.0, [ADP] 14 μM , ΔG_{ATP} -63 kJ mol^{-1} , [TCr] 43 mM, and [ATP] 8 mM.

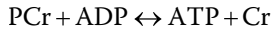
29.3 Physiological Principles in the Quantitative Analysis of Dynamic ^{31}P MRS Data

Next we consider the application of two key physiological principles to the quantitative interpretation of ^{31}P MRS data, particularly for the study of mitochondrial metabolism and function. This has been reviewed in detail elsewhere (Kemp et al., 2014).

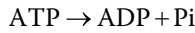
The first principle is that ATP supply must equal ATP demand. Considering first the supply side, in muscle ATP can be produced in two ways: anaerobically by glycolysis, mainly from glycogen to lactate (at a flux we will call L) and, more efficiently (at a flux we call Q), by oxidation in the tricarboxylic acid cycle of acetyl coenzyme A derived either from glycolysis or from β -oxidation of fatty acids and the subsequent oxidation of reducing equivalents via the mitochondrial electron transport chain. On the demand side, ATP is used (at a flux we will call U) by a variety of ion pumps including the sarcolemmal Na^+/K^+ -ATPase and the sarcoplasmic Ca^{2+} -ATPase and by the myosin ATPase that generates the force, which is the muscle's mechanical output. We can distinguish a basal component (U_B) independent of force generation from the component that increases with mechanical output, much of which is due to the myosin ATPase (Zhang et al., 2006). It is usually assumed that basal ATP demand (i.e., not being used to support force generation) is the same during exercise and recovery as at rest (some evidence on this point is discussed elsewhere (Kemp et al., 2014)). Thus as resting glycolysis is negligible in muscle, we can identify U_B with the resting oxidative ATP synthesis rate Q_B .

ATP demand can switch on very quickly during exercise, while glycolytic and oxidative ATP generation takes time to respond. Catastrophic ATP depletion is avoided by what is sometimes called the temporal buffering

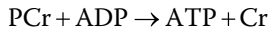
action of the CK system. Ignoring a number of complications relating to the multiple charged species involved, CK catalyzes this reaction:



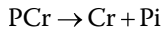
In skeletal muscle CK is always near equilibrium and works such that any temporary mismatch between ATP demand and supply is met almost entirely by a change in [PCr], not [ATP]. This means that ATP hydrolysis



is matched by PCr breakdown



and the result is the sum of these two, the apparent “splitting” of PCr



This is sometimes loosely called “PCr hydrolysis,” a reaction that does not, however, actually occur.

Thus ATP demand is equal to the sum of glycolytic ATP synthesis, oxidative ATP synthesis, and PCr breakdown: algebraically

$$U = L + Q + -\delta[\text{PCr}] / \delta t \quad (29.1)$$

(where δ is used to mean either small measurable spectrum-to-spectrum increments or differentials derived from fits).

The principles are the same during recovery from exercise. Now ATP synthesis is overwhelmingly oxidative (so $L \approx 0$) and directed almost exclusively toward PCr resynthesis (so $U \approx 0$) (some evidence on this point is discussed elsewhere (Kemp et al., 2014)). Equation (29.1) now becomes

$$Q \approx \frac{\delta[\text{PCr}]}{\delta t} \quad (29.2)$$

Oxidative ATP synthesis rate can therefore be estimated directly as the absolute rate of PCr resynthesis.

The relationships imposed by the CK equilibrium mean that acute changes in [PCr] are matched by opposite changes in Cr so that total creatine ($[\text{TCr}] = [\text{Cr}] + [\text{PCr}]$) remains constant and changes in [PCr] are also matched approximately by opposite changes in Pi so that $[\text{PCr}] + [\text{Pi}]$ remains approximately constant.

Free cytosolic ADP concentration, whose important role in metabolic regulation we discuss later, can be estimated from ³¹P MRS measurements (supplemented by some other data) as

$$[\text{ADP}] = \left\{ ([\text{TCr}] / [\text{PCr}]) - 1 \right\} [\text{ATP}] / (K_{\text{CK}} [\text{H}^+]) \quad (29.3)$$

where $K_{\text{CK}} = 1.66 \times 10^9 \text{ L mol}^{-1}$. This calculation uses either a measured or (more commonly) assumed value of [TCr] (which is not of course detectable by ³¹P MRS) and a value of [PCr] that can either be measured by specially calibrated ³¹P MRS methods or more commonly calculated from PCr/ATP and an assumed “normal” value of [ATP] (see succeeding text). It can be seen from Equation (29.3) that [ADP] tends to increase when [PCr] falls or when pH rises, but it is important not to confuse this algebraic truism with a statement about causal mechanisms (Kemp et al., 2014).

The free energy of ATP hydrolysis, whose importance to mitochondrial function we discuss later, can be estimated as

$$\Delta G_{\text{ATP}} = \Delta G_{\text{ATP}}^{\circ} + RT \ln([\text{ADP}][\text{Pi}] / [\text{ATP}]) \quad (29.4)$$

where $\Delta G_{\text{ATP}}^{\circ} = 32 \text{ kJ mol}^{-1}$ and RT (gas constant $\times$ absolute temperature) = 2.57 kJ mol^{-1} . More complex expressions are available for Equations (29.3) and (29.4), taking more detailed account of ionization states and metal binding (Kemp et al., 2001b).

A much more complicated picture of cellular energy metabolism could be constructed, with a more detailed account of the metabolic machinery of ATP production and use (Schmitz et al., 2012), and of compartmentation and interactions involving subcellular components such as mitochondrial CK (Saks et al., 2010). However, the implications of these refinements for the practical interpretation of ³¹P MRS data remain unclear.

The second principle underpinning the interpretation of ³¹P MRS data in skeletal muscle relates to metabolic regulation. For much of the dynamic range, at least until fatigue supervenes, the regulation of ATP supply is dominated by ATP demand (Jeneson et al., 2000). The important thing for the interpretation of ³¹P MRS data is how this is brought about. For oxidative ATP synthesis, attention has long been focused on closed-loop negative feedback by CK-related metabolites. The general concept (Kemp et al., 2014) is that oxidative ATP synthesis is some function (f) of a feedback signal (X):

$$Q = f(X) \quad (29.5)$$

In this X is related in some way to the fall in [PCr], which results from any tendency toward shortfall in ATP supply. The concentrations of ADP, Cr, and Pi, which all increase as PCr falls, have all been considered as contributors to this notional signal X , and there is currently no general agreement on this point. However, the precise model adopted does not much affect the practical interpretation of ³¹P MRS data (Kemp et al., 2014).

Perhaps surprisingly, this general model seems to be compatible with detailed computational simulation of

oxidative metabolism (Schmitz et al., 2012). The possibility of an additional direct activation of oxidative ATP synthesis, independent of closed-loop feedback, often called “open-loop,” “feed-forward,” or “parallel” activation (Korzeniewski, 1998), is discussed elsewhere (Kemp et al., 2014).

29.4 Approaches to Measuring Mitochondrial Function *In Vivo*

It is worth pausing to clarify what we mean by “mitochondrial function” in this context. It is a dynamic property, clearly, having to do with measurement of metabolic fluxes rather than of concentration per se. The relevant fluxes are clearly those of oxidative ATP synthesis, and these concepts might in principle be framed in terms of the carbon fluxes (substrate to CO₂), the O₂ flux (in a fixed stoichiometric relation to the carbon flux), or the ATP synthesis rate itself (mainly oxidative phosphorylation); the relationship of the latter two is expressed as the coupling ratio P:O (or P:O₂) (Brand, 2005).

What we will call muscle “mitochondrial capacity,” also known as muscle “oxidative” or “aerobic” capacity (Kemp et al., 1993b), is a concept distinct from metabolic flux (Befroy et al., 2009) but can be thought of as a maximum rate of mitochondrial ATP synthesis under conditions of actual or notional maximal activation. As this way of putting it makes clear, there are basically two ways to assess mitochondrial capacity *in vivo*: to measure the rate of mitochondrial ATP synthesis (or some surrogate) in maximal exercise or to make measurements during submaximal exercise from which the maximum rate can be inferred (Kemp et al., 2014). For practical reasons discussed in the succeeding text, it is almost impossible to achieve truly maximal exercise of any muscle group of interest in an MR scanner. ³¹P MRS studies therefore are almost exclusively of the second submaximal/inferential kind. There are several different approaches, which have been reviewed in detail (Kemp et al., 2014) and will be mentioned later on, but it is often convenient to treat these together as yielding “³¹P MRS-based measures of mitochondrial function” (MMMF). We can define an absolute MMMF as one that is intended to correspond quantitatively to a maximal rate of mitochondrial ATP synthesis and so has units of metabolic flux such as mM/min. A relative MMME, by contrast, is one for which this is not so (Kemp et al., 2014).

It is worth mentioning that in contrast to ³¹P MRS, measurements of oxygen consumption ($\dot{V}O_2$) *in vivo* during maximal exercise are feasible. Noninvasive measurements of whole-body $\dot{V}O_2$ using expired-gas spirometry test the integrated response of the whole

cardiorespiratory/vascular/muscular system, as well as measurement of whole-body $\dot{V}O_2$ at peak exercise; this also allows studies of whole-body $\dot{V}O_2$ kinetics, to which the contribution of the exercising muscle can to some extent be reconstructed by analysis and modeling (Rossiter, 2011). Measurement of absolute $\dot{V}O_2$ in specific muscles or muscle groups requires invasive arterio-venous difference (AVD) methods; these can be used in exercise, including maximal single- or two-limb exercise. Published AVD $\dot{V}O_2$ data from maximal exercise have been reviewed in detail elsewhere (Kemp et al., 2014).

29.5 Some Practical and Experimental Considerations in ³¹P MRS Studies of Muscle

Much ³¹P MRS muscle work has used pulse-and-acquire signal acquisition methods and physical location by surface coil, which works reasonably well for accessible muscles. Most human ³¹P MRS studies are of quadriceps (vastus lateralis and rectus femoris), calf muscle (soleus and gastrocnemius), or plantar flexors (tibialis anterior), which are accessible in whole-body scanners and can be easily exercised (Barker & Armstrong, 2010). Studies of upper limb muscles (finger flexors, first dorsal interosseus) were formerly popular using smaller magnets.

Single voxel localization has, until recent developments in high-field human scanners (Fiedler et al., 2016; Meyerspeer et al., 2011), required an often unacceptable trade-off with time resolution, even more so with spectroscopic imaging methods (Cannon et al., 2013), although this can be improved using gated multiple-repetition methods (Forbes et al., 2009) or specialized imaging sequences (Parasoglou et al., 2013b).

To quantify ³¹P MRS spectra, signal intensity estimates derived by an appropriate fitting method are either treated in relative terms or calibrated by special techniques to yield absolute concentrations. There is surprisingly poor agreement about these in healthy human muscle (Kemp et al., 2007). A common approach is to collect a fully relaxed resting spectrum (i.e., one using a long repetition time to minimize T_1 effects of longitudinal relaxation) to establish baseline Pi/ATP and PCr/ATP and then use a shorter repetition time for rest-exercise-recovery acquisition. For some kinetic analyses, only relative changes in PCr are important, and resting muscle PCr/ATP and Pi/ATP are often interpretable on the assumption that [ATP] has a “normal” value and does not change during exercise and recovery. Note that for calculation of [ADP] and ΔG_{ATP} (Equations (29.3) and (29.4)), any assumptions about [ATP] and [TCr] do not “cancel out.”

Much published work has used purpose-built exercise equipment and a variety of bespoke exercise protocols: in human studies this is most commonly isometric or concentric (shortening) exercise, sometimes with a poorly defined component of eccentric (lengthening) exercise. Incremental (ramp) protocols are convenient for assessing a range of intensities in a single session but are more complicated to analyze in detail.

Exercise physiologists define levels of exercise intensity based on responses of circulating lactate and measured $\dot{V}\text{O}_2$ (Rossiter, 2011): in *moderate* exercise below the lactate threshold (LT), $\dot{V}\text{O}_2$ rapidly reaches a steady state; in *heavy* exercise between LT and the “critical power” (CP), $\dot{V}\text{O}_2$ shows a “slow component,” a slow increase beyond the expected steady state; in *very heavy* exercise above CP, both the $\dot{V}\text{O}_2$ slow component and the circulating lactate increase until the limit of tolerance is reached; in *severe* exercise the metabolic rate exceeds the maximal aerobic capacity ($\dot{V}\text{O}_{2\text{MAX}}$) from the start. Most ^{31}P MRS studies of oxidative ATP metabolism use what amounts to *moderate* or *heavy* exercise, often not tightly defined, as higher intensities are difficult to achieve in the physical constraints of the MR scanner. The general lack of agreement on exercise equipment, setup, and protocols means that there are few agreed “reference ranges” for dynamic MR parameters.

29.6 ^{31}P MRS Studies in Resting Muscle

Muscle’s dynamic responses are conditioned by the resting state from which start, and some resting measurements are interpretable in terms of pathological or physiological change. We begin, therefore, by summarizing what is known about ^{31}P MRS-detectable metabolites that are regulated in resting muscle. This has been reviewed in detail elsewhere (Kemp et al., 2014).

In normally perfused muscle at rest (for which we use the suffix $_B$, for “basal”), ATP is supplied almost exclusively by oxidation of fatty acids in the fasting state and glucose postprandially. Any metabolite that functions as a mitochondrial feedback signal, as in Equation (29.5), should presumably be maintained by that feedback mechanism at a value appropriate to basal ATP demand, in accordance with

$$Q_B = f(X_B) \quad (29.6)$$

This description probably applies to [ADP]: it must settle at the value necessary to drive basal oxidative ATP synthesis at the rate of basal ATP demand. This then defines $[\text{Cr}]/[\text{PCr}]$ according to Equation (29.3) (with the causal logic running “left to right”). It is perhaps also true

of [Pi], which along with [ADP] defines ΔG_{ATP} according to Equation (29.4): if ΔG_{ATP} is the key mitochondrial feedback signal, it must settle at the value necessary to drive basal oxidative ATP synthesis at the required rate. However, resting muscle cytosolic [Pi] is also dependent (according to pump-and-leak principles) on sarcolemmal Na^+ -dependent Pi uptake, although the details of this are still relatively unclear. Thus [ADP] must presumably adapt to a cytosolic [Pi], which depends on sarcolemmal transport physiology, such that ΔG_{ATP} is at the correct value to drive basal oxidative ATP synthesis. Similarly, total cell creatine ([TCr]) depends on sarcolemmal Na^+ -dependent Cr uptake balanced by efflux of creatine and creatinine, and so [PCr] must settle at the value required to make [ADP] the right value to drive basal ATP synthesis. On similar principles resting cell pH is set by processes of H^+ efflux, dominated by the Na^+/H^+ antiporter, and is in effect the pH at which net H^+ efflux is near zero. Lastly [ATP], an important system parameter even though it does not change much during anything less than severe exercise demand is set by the balance of adenine nucleotide synthesis and breakdown.

In fact we do not know whether observed resting ^{31}P MRS abnormalities in mitochondrial diseases or other states of modified mitochondrial function (e.g., training) are quantitatively consistent with the relevant versions of Equation (29.6). The high [Pi] and low [PCr] (thus high [ADP]) typically seen in classical mitochondrial myopathies appear to make sense in these terms (Taylor et al., 1994). However, we usually do not know how particular disease or other states affect basal ATP turnover Q_B ; we usually lack measured values of [TCr], which makes calculated [ADP] and ΔG_{ATP} potentially misleading, and we have no agreed models of how ADP and Pi contribute to the general feedback signal we have called X .

Finally in this discussion of resting muscle measurements, we mention a manipulation of resting muscle that is sometimes used in the context of assessing mitochondrial function: cuff ischemia, which cuts off vascular O_2 delivery and H^+ efflux. Once residual oxidative ATP synthesis has depleted muscle O_2 content, basal ATP demand (U_B) is met by PCr breakdown, which can be measured directly:

$$U_B \approx \frac{-\delta[\text{PCr}]}{\delta t} \quad (29.7)$$

And then increasingly also by glycogenolysis, which can be estimated from pH and PCr changes in ways discussed elsewhere (Kemp et al., 2014).

As noted previously, measurements of resting muscle ATP turnover do reflect an aspect of mitochondrial function, but this has no obvious relationship to the concept of mitochondrial capacity (Kemp et al., 2014).

However, there is an interesting application of combined ^{31}P MRS and appropriately calibrated near-infrared spectroscopy (NIRS) measurements in resting ischemia: dividing the measured rate of ATP turnover (Equation (29.7)) by the initial rate of fall of muscle O_2 content yields an estimate of the P:O ratio, a measure of mitochondrial coupling (Marcinek et al., 2005).

29.7 ^{31}P MRS Magnetization Transfer Methods

As mentioned previously, in magnetization transfer methods, magnetic labeling is used to measure exchange flux. For practical reasons this is usually applied to resting muscle, and there have been two main applications with claimed potential relevance to mitochondrial metabolism.

First, CK activity can be measured as PCr/ATP exchange (Parasoglou et al., 2013a). But what does this mean? In general, exchange flux depends on the relevant enzyme activity and the concentrations of substrates and products: for CK the rate expression is available (McFarland et al., 1994), and the relevant metabolites can be measured, calculated, or assumed. However, the main physiological importance of CK in skeletal muscle appears to be simply that it is near equilibrium (see earlier). The measured exchange flux is therefore probably best considered a non-causal marker of pathology. Despite the intimate links between CK and mitochondria, this measurement appears to be unconnected with mitochondrial function in the senses that concern us here.

Second, Pi/ATP exchange measured by magnetization transfer in resting muscle has been argued to reflect mainly oxidative ATP synthesis, on the assumptions that this is unidirectional (so that exchange flux $\approx$ net flux) and that other contributions (notably that mediated by the near-equilibrium glycolytic enzymes GAPDH and PGK) are relatively small (Befroy et al., 2009). However, observed rates of Pi/ATP exchange are much larger than known rates of oxidative ATP synthesis in resting muscle, so one or both of these assumptions must be wrong (Kemp & Brindle, 2012). Recently this measurement has been extended in human muscle to steady-state exercise, showing that this discrepancy is approximately independent of ATP turnover measured from initial rates of post-exercise PCr resynthesis (Kemp, 2008a; Kemp & Brindle, 2012; Sleight et al., 2016). An earlier proposal that resting Pi/ATP exchange can be used as a measure of mitochondrial capacity also fails because of the clear distinction, in demand-driven cellular ATP turnover, between a rate and a capacity. However, some interesting correlations between Pi/ATP exchange and measures of

resting ATP turnover and mitochondrial capacity (Schmid et al., 2012) remain unexplained (Kemp & Brindle, 2012).

29.8 Muscle Exercise Responses Studied by ^{31}P MRS

Information from ^{31}P MRS data about mitochondrial capacity proper is in practice only obtainable for dynamic responses to exercise and recovery. For present purposes we shall ignore the basal component Q_B (Kemp et al., 2014). Regardless of exercise intensity, it is worth distinguishing “initial exercise,” the slightly ill-defined phase before significant glycolytic or oxidative ATP synthesis, when ATP is supplied almost entirely by PCr breakdown:

$$U \approx \frac{-\delta[\text{PCr}]}{\delta t} \quad (29.8)$$

The initial-exercise rate of PCr breakdown (for which we use the subscript $_0$) is often conveniently measured from an exponential fit:

$$-\left(\frac{\delta[\text{PCr}]}{\delta t}\right)_0 = k_{\text{exer}}(-\Delta[\text{PCr}]_{\text{SS}}) \quad (29.9)$$

where k_{exer} is the exponential rate constant of PCr and $-\Delta[\text{PCr}]_{\text{SS}}$ is the fitted steady-state fall.

The kind of exercise that most straightforwardly tests mitochondrial function is what might be loosely called “oxidative” exercise. In physiological terms (see aforementioned text), this means moderate intensity exercise, below the LT, where glycolysis can be neglected. Equation (29.1) now becomes

$$U \approx Q - \delta[\text{PCr}]/\delta t \quad (29.10)$$

The kinetics of PCr in these circumstances are mono-exponential, which implies a linear steady-state relationship between oxidative ATP synthesis and [PCr]:

$$-\frac{\delta Q}{\delta[\text{PCr}]} \approx \frac{-Q}{\Delta[\text{PCr}]} = k_{\text{exer}} \quad (29.11)$$

and thus also similar mono-exponential kinetics of oxidative ATP synthesis (Funk et al., 1990; Kemp et al., 1994, 1998; Mahler, 1985). In principle k_{exer} reflects mitochondrial function exactly as does the rate constant of PCr resynthesis in recovery (k_{reco}), which we discuss in the succeeding text; these are the on- and off-kinetics of a first-order system. Similar arguments apply to the relationship between oxidative ATP synthesis rate and

ΔG_{ATP} (Kemp et al., 1994; Meyer, 1988). Note that there is a close parallel between the analysis of PCr kinetics measured by ^{31}P MRS and of pulmonary $\dot{V}\text{O}_2$ kinetics measured by pulmonary spirometry (Rossiter, 2011).

A simple interpretation of ^{31}P MRS measurements in steady-state exercise below the LT takes mechanical output as a surrogate for oxidative ATP synthesis:

$$U_{\text{ss}} \approx Q_{\text{ss}} = f(X_{\text{ss}}) \quad (29.12)$$

Other things being equal, impaired mitochondrial function will lead to bigger steady-state changes in putative mitochondrial regulators or their correlates (e.g., [PCr], [Pi], and [ADP]), in other words bigger X for a given Q . In practice this argument is complicated by possible differences in relative work output, contractile costs, and glycolytic contribution to ATP turnover. Much of the ^{31}P MRS work in this area has therefore focused on recovery from exercise, which we consider next.

29.9 Mitochondrial Function Studied by ^{31}P MRS in Recovery from Exercise

This has recently been reviewed in detail (Kemp et al., 2014). PCr resynthesis is a measure of the rate (more strictly, the suprabasal rate) of oxidative ATP synthesis (Equation (29.2)) and given its mono-exponential kinetics when pH change during exercise is small (in the off-phase of “purely oxidative” exercise considered previously):

$$\frac{\delta[\text{PCr}]}{\delta t} = k_{\text{reco}}(-\Delta[\text{PCr}]) \quad (29.13)$$

where k_{reco} is the rate constant of PCr in recovery and $-\Delta[\text{PCr}]$ is the fall below basal. In fact, even though PCr recovery follows more complicated kinetics after acidifying exercise, the earlier phase is often conveniently close to mono-exponential.

We noted previously (as Equation (29.2)) the useful case of the initial rate of PCr recovery, which is generally assumed (Kemp et al., 2014) to be a measure of end-exercise (subscript E) oxidative ATP synthesis. In terms of exponential kinetics, this can be written as

$$Q_{\text{E}} \approx k_{\text{reco}}(-\Delta[\text{PCr}]_{\text{E}}) \quad (29.14)$$

As mentioned previously, muscle “mitochondrial capacity” can be thought of as a maximum rate of mitochondrial ATP synthesis under some actual or notional maximal activation, and in ^{31}P MRS studies we are dealing with the notional, inferential case. There are several

approaches to such “ ^{31}P MRS-based Measures of Mitochondrial Function (MMMF),” each implicitly or explicitly assuming a model of the regulation of oxidative ATP synthesis, and it is useful here to begin with a general approach (Kemp et al., 2014). The basic assumption about mitochondrial regulation is Equation (29.5), and from this Q_{MAX} (“mitochondrial capacity”) is the extrapolated maximal rate:

$$Q_{\text{MAX}} = f(X_{\text{MAX}}) \quad (29.15)$$

where X_{MAX} is the notional value of X at some maximal stimulation and/or mechanical output. We will consider briefly three specific approaches (Kemp et al., 2014).

The linear analysis mentioned previously for “oxidative” exercise predicts that post-exercise PCr recovery kinetics will be mono-exponential with a rate constant k_{reco} , which is roughly proportional to mitochondrial capacity. Thus k_{reco} is a relative MMMF, and the inversely related time constant $1/k_{\text{reco}}$ and the half-time $\ln(2)/k_{\text{reco}}$ are inverse relative MMMFs. To obtain an absolute MMMF from this analysis, one approach is to extrapolate the defining first-order linear relationship between $\delta[\text{PCr}]/\delta t$ and $-\Delta[\text{PCr}]$ to notional complete PCr depletion. This amounts to taking $-\Delta[\text{PCr}]$ as X in Equation (29.15), so that

$$Q_{\text{MAX}} = k_{\text{reco}}[\text{PCr}]_{\text{B}} \approx k_{\text{reco}}[\text{TCr}] \quad (29.16)$$

where $[\text{PCr}]_{\text{B}}$ is resting [PCr]. In literal terms such extrapolation is impossible, as intense exercise lowers cell pH, which slows PCr recovery (i.e., decreases k_{reco}) for reasons that can be satisfactorily explained in terms of the interactions of pH with the CK equilibrium (Kemp et al., 2014) if it is assumed that the dominant feedback signal X is [ADP], acting as in mitochondrial incubation *ex vivo*. Good evidence for this assumption is the well-known approximately hyperbolic relationship between [ADP] and oxidative ATP synthesis rate measured from recovery (Equation (29.14)) (Boska, 1994; Jeneson et al., 1995, 1996, 2009; Kemp et al., 1993a, b, 2002; Thompson et al., 1995) or its surrogates such as aerobic-exercise work rate (Equation (29.12)) (Chance et al., 1985).

For any candidate model of feedback control of mitochondrial ATP synthesis, the question arises whether a single relationship holds convincingly across the whole dynamic range. For the ADP model, it has long been realized that a simple hyperbolic relationship to [ADP] cannot, given observed values of resting [ADP], account for the whole dynamic range of ATP turnover rates without unrealistic assumptions about basal ATP turnover. A cooperative sigmoid relationship gives a better fit (Cieslar & Dobson, 2000; Jeneson et al., 1996, 2009; Layec et al., 2013a), and it is currently debated to what

extent this can be explained computationally (Kemp et al., 2014). Empirically, though, extrapolating PCr resynthesis rate to “infinite” [ADP] (“zero” [PCr]) gives an estimate of mitochondrial capacity that is largely independent of end-exercise pH:

$$Q_{\text{MAX}} = \left(\frac{\delta[\text{PCr}]}{\delta t} \right)_E \left\{ 1 + \left(\frac{K_m}{[\text{ADP}]_E} \right)^n \right\} \quad (29.17)$$

where $[\text{ADP}]_E$ is end-exercise [ADP], K_m is the apparent [ADP] for half-maximal flux, and n is the Hill coefficient defining sigmoidicity. Coupled with simple assumptions about the pH dependence of H^+ efflux, this ADP feedback model can account semiquantitatively for many of the observed relationships between PCr and ADP recovery and end-exercise pH in exercise at high enough intensity to lower cell pH (Kemp et al., 2014).

The same is true of the alternative “nonequilibrium thermodynamic” model (Westerhoff et al., 1995) that assigns causal primacy to the sigmoid relationship between oxidative ATP synthesis and ΔG_{ATP} . This can also be used to extrapolate an estimate of mitochondrial capacity (Kemp et al., 2014):

$$Q_{\text{MAX}} = \frac{-\left(\delta[\text{PCr}] / \delta t \right)_E \left\{ a \cdot \exp \left[(\Delta G_E - c) / (RT) \right] - 1 \right\}}{\left\{ a \cdot \exp \left[(\Delta G_E - c) / (RT) \right] + b \right\}} \quad (29.18)$$

In this expression a is an empirical constant, b is a constant relating to thermodynamic reversibility, c is “static head” (the value of ΔG_{ATP} at which the flux theoretically is zero), and ΔG_E is end-exercise ΔG_{ATP} . The near-linear slope ($\delta Q / \delta \Delta G_{\text{ATP}}$) of the middle part of this sigmoid relationship, defined on the basis of a useful electrical analogy as “mitochondrial conductance” (Meyer, 1988), can also be used as a relative MMMF, and a simple analysis based on the ADP model (Kemp et al., 1998) equates it approximately to $6Q_{\text{MAX}}/RT$. A purely empirical sigmoid function has also been used to fit $Q/\Delta G_{\text{ATP}}$ relationships and so to estimate a maximum value Q_{MAX} (Jeneson et al., 1997, 2009). Some formal relationships between [ADP] and ΔG dependence, as well as some general properties of closed-loop feedback, are summarized elsewhere (Kemp, 1994, 2006a, b, 2008b, 2009; Kemp et al., 1998).

Finally, an additional relative MMMF based on simple feedback models is the ADP recovery time constant (or the half-time, an inverse MMMF) (Argov et al., 1996; De Stefano et al., 1996; Kemp et al., 1995; Taylor et al., 1994).

The improved signal/noise ratio attainable in MRS at high field (7 T), and consequent improvement in spatial and temporal resolution, will throw light on how well the relationships on which these analyses of mitochondrial function depend truly conform to the theoretical

assumptions and to what extent they are preserved in the face of changes in exercise intensity and pattern (Fiedler et al., 2016).

29.10 Validating MRS-Based Measures of Mitochondrial Function

MMMFs reflect a system property with contributions from a number of different factors: these include the number of mitochondria, the amount and the activity per mitochondrion of all the respiratory chain components and the enzymes of fat and carbohydrate oxidation, and the vascular supply of O_2 and its diffusion across the capillary wall and through the myocyte to the mitochondria (Kemp, 2004).

The constraints of the CK equilibrium mean that all of these MMMFs correlate reasonably closely (when the effects of pH on PCr recovery are accounted for as alluded earlier) (Edwards et al., 2013). The validity of a particular MMMF might be tested in several ways. Most straightforwardly, in states where there are changes in intrinsic mitochondrial function or mitochondrial numbers (states such as training, aging, primary mitochondrial diseases, and secondary causes of mitochondrial dysfunction), we can ask: does an MMMF change detectably in the right direction? Further, across a range of such states: does an MMMF correlate with other relevant measures? Correlations of MMMF with *ex vivo* measures (such as respiration rate in skinned fibers or isolated mitochondria) would be expected where an appreciable amount of the primary physiological variation or pathophysiological abnormality is mitochondrial (e.g., training, aging, and primary mitochondrial disease), but not in states where the lesion is mainly extramitochondrial (e.g., peripheral vascular disease). MMMF would be expected to correlate with *in vivo* measures such as whole-body $\dot{V}\text{O}_{2\text{MAX}}$, although whole-body measurements can be limited by factors that do not necessarily affect skeletal muscle metabolism in single MR-accessible muscles (e.g., cardiorespiratory function). In clinical conditions affecting muscle mitochondrial function, whatever the pathophysiology, we would expect MMMF to correlate with $\dot{V}\text{O}_{2\text{MAX}}$ where the muscle sampled by ^{31}P MRS is sufficiently typical of the mass of muscle contributing to whole-body gas exchange.

In fact MMMF all respond broadly as expected to physiological manipulations and pathophysiological situations (Kemp et al., 2014). Some examples follow. MMMFs increase with suitable aerobic training in healthy subjects (Forbes et al., 2008; Larsen et al., 2013) and several diseases (Adamopoulos et al., 1993; Cordina et al., 2013b; Sala et al., 1999; Stratton et al., 1994; Taivassalo et al., 2001; van Tienen et al., 2012).

MMMFs in cross-sectional studies usually decline with age (Layec et al., 2013b; Taylor et al., 1997). Across populations of differing ages, they correlate with measures of whole-body aerobic performance (Edwards et al., 2013; Hunter et al., 2001, 2002; Larson-Meyer et al., 2000; McCully et al., 1993; Takahashi et al., 1995), and with measurements *ex vivo* of mitochondrial numbers, enzymes, and respiratory chain components (Bajpeyi et al., 2011; Conley et al., 2000; Hunter et al., 2002; Johannsen et al., 2012; Lanza et al., 2011; Larson-Meyer et al., 2001; McCully et al., 1993; Paganini et al., 1997; Praet et al., 2006). They are decreased as expected in mitochondrial myopathy (Argov et al., 1996; Arnold et al., 1985; Taivassalo et al., 2001; Taylor et al., 1994) and Friedreich ataxia (Nachbauer et al., 2013). MMMFs decreased as expected in diseases in which there is impaired vascular O_2 delivery: these include peripheral vascular disease (Kemp, 2004; Kemp et al., 1995, 2001a), where muscle perfusion is reduced, and congenital heart disease (Adataia et al., 1993; Cordina et al., 2013a), where blood O_2 content is reduced. There are also more complex cases. In chronic cardiac disease (Kemp et al., 1996; Mancini et al., 1989), renal disease (Kemp et al., 2004; Marrades et al., 1996), and pulmonary disease (Mannix et al., 1995; Sala et al., 1999), decreased MMMF are likely multifactorial, with contributions from impaired O_2 delivery and O_2 diffusion and the loss of mitochondria and mitochondrial components. The possible role of abnormalities of muscle mitochondrial function and lipid metabolism in the pathophysiology of insulin resistance in general, and type 2 diabetes specifically, is debated, as reviewed elsewhere (Kemp et al., 2014). Decreased MMMFs in McArdle's disease (Kemp et al., 2009), which may seem surprising, presumably reflect impaired glycogenolytic supply of pyruvate for oxidation rather than mitochondrial dysfunction as such, highlighting the need for caution in interpretation.

It would clearly be interesting to know whether an observed decrease in an MMMF in some disease or physiological state can be quantitatively explained by an observed decrease in, for example, mitochondrial content, or in the activity of a particular mitochondrial component, or in some extramitochondrial parameter such as capillarity. However, such assessment would require a quantitative analysis by no means yet worked out for any particular case. Moreover, empirical data relating such abnormalities are very sparse.

The most stringent test of a claimed absolute MMMF is whether it yields values similar to other measures, such as $\dot{\text{V}}\text{O}_{2\text{MAX}}$ expressed for the same muscle volume, and *ex vivo* measures of mitochondrial maximal ATP synthesis fluxes extrapolated back to *in vivo* conditions. As we have discussed in detail elsewhere (Kemp et al., 2014), this comparison is made difficult by formidable technical issues, a consequent lack of directly comparable

data, and gaps in our understanding of some pertinent physiology. Comparison of MMMF with other measures of oxidative function such as $\dot{\text{V}}\text{O}_2$ data during maximal exercise is also complicated by a number of factors: these include the choice of correct P:O for cross-calculation, the lack of agreement between the absolute maximal values that emerge from the ^{31}P MRS different models, and the influences of systemic cardiac–pulmonary–vascular limitations on maximal $\dot{\text{V}}\text{O}_2$ by muscle groups of different sizes and in different kinds of exercise (Kemp et al., 2014). Resolving these issues will take major advances in systems physiology.

It has been argued that some apparently anomalous data, for example, post-exercise transient undershoot of ADP below resting values (Argov et al., 1996), are evidence of parallel activation (i.e., open-loop control) of mitochondrial ATP synthesis (Kemp et al., 2014). The implications are important: if there were significant parallel activation in exercise, PCr recovery kinetics could be dominated by the intrinsic off-kinetics of that process rather than conventional mitochondrial properties; furthermore it could no longer be assumed that the end-exercise and the immediate initial-recovery rates of oxidative ATP synthesis are the same (Equation (29.2)). This is still unresolved.

29.11 Conclusions and Summary

Examination of the detailed evidence, particularly comparison with results of invasive methods, supports the generally accepted picture reviewed here. Several methodological limitations have been discussed previously. There are still disagreements about, for example, stoichiometric factors and the best exercise protocols. Muscles of considerable clinical interest (e.g., diaphragm) remain inaccessible. There is in general a lack of direct validation, largely because of the technical difficulty of combining MRS with invasive measures and of interleaving ^{31}P MRS with other MR measures. With a few exceptions (van den Broek et al., 2007), there is a lack of detailed datasets covering wide ranges of exercise conditions. No MRS methods can resolve cellular-scale heterogeneity, and technical limitations constrain spectroscopic imaging approaches to larger-scale heterogeneity (Cannon et al., 2013). Validated computational models of glycolytic and oxidative ATP synthesis, and of cardiopulmonary and vascular function, and of H^+ efflux would put physiological interpretation on a more solid foundation. Higher-field working and novel pulse sequences (Meyerspeer et al., 2011) have some potential to avoid the limitations discussed here.

Nevertheless, ^{31}P MRS has advantages in terms of safety, spatial and temporal resolution, suitability for repeated measurements, and time courses. In practice

the most reliable way to assess muscle mitochondrial function by ^{31}P MRS is probably to use moderate exercise that causes little change in pH and take the PCr recovery rate constant as a relative MMMF. Strictly speaking all MMMFs depend on the intactness of the whole cardiovascular/respiratory/muscle O_2 delivery and usage system, whose overall performance can also be assessed by whole-body $\dot{\text{V}}\text{O}_{2\text{MAX}}$ or $\dot{\text{V}}\text{O}_2$ kinetics. However, typical “MR” exercise is unlikely to be significantly limited by cardiopulmonary function, and so MMMFs reflect mainly “intramuscular” properties: the numbers and function of skeletal muscle mitochondria and the adequacy of microvascular O_2 delivery.

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List of Symbols and Values

$k_{\text{exer}}, k_{\text{reco}}$	exponential PCr rate constants in exercise, recovery
L	glycolytic ATP synthesis rate
$-\Delta[\text{PCr}]$, $-\Delta\text{pH}$	fall in [PCr], pH below basal
Q	oxidative ATP synthesis rate
Q_{B}	basal ATP turnover rate (oxidative)
Q_{MAX}	estimated maximum Q (“mitochondrial capacity”)
U	total ATP usage rate
$\dot{\text{V}}\text{O}_2$	O_2 consumption rate
$\dot{\text{V}}\text{O}_{2\text{MAX}}$	peak or maximum $\dot{\text{V}}\text{O}_2$
X	generalized ATP turnover feedback signal
B	resting muscle or non-contraction related
0	initial exercise
E	end of exercise
MAX	maximal exercise
SS	steady-state exercise
K_{CK}	$1.66 \times 10^9 \text{ l mol}^{-1}$
K_{AK}	1.12
$\Delta G_{\text{ATP}}^\circ$	32 kJ mol^{-1}
RT	2.57 kJ mol^{-1}

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Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants

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Volume II

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Foreword

The field of mitochondrial medicine is enjoying a renaissance driven largely by advances in molecular biology and genetics. The first draft sequence of the human mitochondrial genome in 1981 provided the critical blueprint that enabled the identification of the first point and single large-scale deletion mutations of mitochondrial DNA (mtDNA) in 1988. To date, more than 270 distinct mtDNA point mutations and hundreds of mtDNA deletions have been identified. Subsequent sequencing of the human nuclear genome in the early 2000s helped to catalyze the discovery of approximately 1000 nuclear genes that, together with the mtDNA, encode the mitochondrial proteome. With a complete parts list, it has been possible to delve deep into the molecular basis of Mendelian mitochondrial disorders, with more than 200 nuclear disease genes now identified. There is now widespread consensus that mitochondrial dysfunction contributes to a spectrum of human conditions, ranging from rare syndromes to common degenerative diseases to the aging process itself.

Today, there is great excitement that in the coming decade, new medicines will become available that alleviate disease by targeting mitochondria. At the same time, there is widespread appreciation that many drugs fail clinical trials because of their mitochondrial liabilities. *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* represents one of the most important textbooks for those hoping to target mitochondria, as well as for those wanting to avoid mitochondrial side effects. It is a deep and thoughtful resource that will appeal to basic scientists, clinicians, and professional drug developers.

Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants provides ample reminders of the intimate connections between mitochondria, pharmacology, and toxicology. Some of the most widely used tool compounds for investigating mitochondrial physiology, such as antimycin and oligomycin, are indeed natural products that serve as a chemical warfare in the microbial world. The fact that antimicrobial agents are often toxic to mitochondria is not surprising given the hypothesized proto-bacterial origin of mitochondria. These overlapping effects of such drugs are perhaps best

illustrated by aminoglycosides and linezolid antibiotics, which not only inhibit bacterial protein synthesis but are also well known to cause neurotoxicity such as hearing loss, peripheral neuropathy, and optic neuropathy through impairment of mitochondrial translation. Pharmacogenetics contributes to these toxicities with the well-established link between the m.1555A>G variant that predisposes to aminoglycoside-induced deafness.

Toxic side effects of clinically important and investigational new drugs for viruses have historically provided fundamentally new insights into the replication of mtDNA. One of the earliest anti-HIV agents, zidovudine (azidothymidine (AZT)), is a nucleoside analogue that effectively inhibits viral reverse transcriptase but, in some patients, inhibits the mitochondrial polymerase gamma, leading to depletion of mtDNA particularly in muscle and causing myopathic weakness. These mitochondrial toxicities exposed the reliance and vulnerability of the mitochondrial genome to disruptions of the deoxynucleotide pool substrates for mtDNA replication. These toxicities also serve as a reminder that the mtDNA replication machinery of mitochondria actually resembles that of viruses.

Mitochondrial toxicity is such a common side effect in humans; a thorough understanding and surveillance of these off-target effects are required for the successful development of new medicines. A vivid case in point is fialuridine or 1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil (FIAU), a nucleoside analogue that was tested for therapeutic efficacy for hepatitis B infection but tragically caused fatal liver failure and death in 5 of 15 patients and forced liver transplantations in two other patients.

Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants takes a rather systematic approach to mitochondrial pharmacology and toxicology and for this reason will be of use to even those outside of strict drug discovery. It begins with a scholarly introduction to the nuances of mitochondrial drug transport and detoxification systems, illustrated with specific case studies (Chapters 1–5). It then reviews cardinal features of mitochondrial toxicity at the organ level, highlighting some of the dose-limiting toxicities of very commonly used

and lifesaving drugs (Chapter 6–12). One of the greatest challenges in our field lies in measuring mitochondrial function. *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* dedicates many chapters (Chapters 13–29) to reviewing modern technologies for measuring mitochondrial function *in vitro*, *ex vivo*, and *in vivo*. Although these technologies represent the current state of the art, they have their limitations, and much research is required to pioneer new, facile biomarkers and technologies that are sensitive, specific, and minimally invasive. The text then progresses to reports from the clinic (Chapters 30–40) as well as from environmental biology (Chapters 41–45) that offer additional vignettes and examples of drug–mitochondria interactions.

The book *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants* is very timely. While genetics and genomics have driven much progress in mitochondrial medicine for the past few decades, we anticipate that chemical biology may represent one of the most exciting new frontiers. We applaud Yvonne Will, James Dykens, and all of their contributors for assembling this new two volume book entitled: *Mitochondrial Dysfunction Caused by Drugs and Environmental Toxicants*. This textbook will be an important canon in the future of mitochondrial medicine and more broadly in modern drug discovery.

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Part 4

Reports from the Clinic

30

Statin and Fibrate-Induced Dichotomy of Mitochondrial Function

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30.1 Introduction

The mitochondrial respiratory chain (MRC) (Figure 30.1) is located in the inner mitochondrial membrane and plays a central role in cellular energy generation (Wallace, 1999). The MRC consists of four enzyme complexes: complex I (NADH: ubiquinone reductase; EC 1.6.5.3), complex II (succinate: ubiquinone reductase; EC 1.3.5.1), complex III (ubiquinol: cytochrome *c* reductase; EC 1.10.2.2), and complex IV (cytochrome *c* oxidase; EC 1.9.3.1) (Hatefi, 1985). The MRC together with complex V (ATP synthase; EC 3.6.3.14) undertakes the process of oxidative phosphorylation and is consequently known as the oxidative phosphorylation system (Hatefi, 1985, Wallace, 1999). In this process, electron transport between complex I and IV is coupled to proton pumping at complexes I, III, and IV, which creates a proton motive gradient between the inner and outer mitochondrial membranes. Complex V then utilizes this proton gradient to synthesize ATP from ADP and inorganic phosphate (Pi) (Rahman and Hanna, 2009). As protons

are transferred through the F_o sector of complex V, this causes a conformational change in the F₁ sector of the enzyme, which provides energy for ATP synthesis by means of “rotary catalysis” (Jonckheere et al., 2012). In addition to the enzyme complexes, the electron carriers, coenzyme Q₁₀ (CoQ₁₀) and cytochrome *c*, are also essential for oxidative phosphorylation with a deficiency in CoQ₁₀ biosynthesis, representing a potentially treatable form of MRC disorder (Hargreaves, 2014). As far as the authors are aware, there have been no reported cases of cytochrome *c* deficiency that suggests that a deficit in the level of this cytochrome may not be compatible with life. Recent studies have indicated that although the enzymes in the oxidative phosphorylation system can exist as discrete entities, they may also be present in the inner mitochondrial membrane in the form of super enzyme complexes. These “supercomplexes” are composed of aggregates of complexes I, III, and IV, complexes I and III, and complexes III and IV (Lapuente-Brun et al., 2013). Mitochondrial DNA (mtDNA) encodes for 13 of the approximately 90 protein

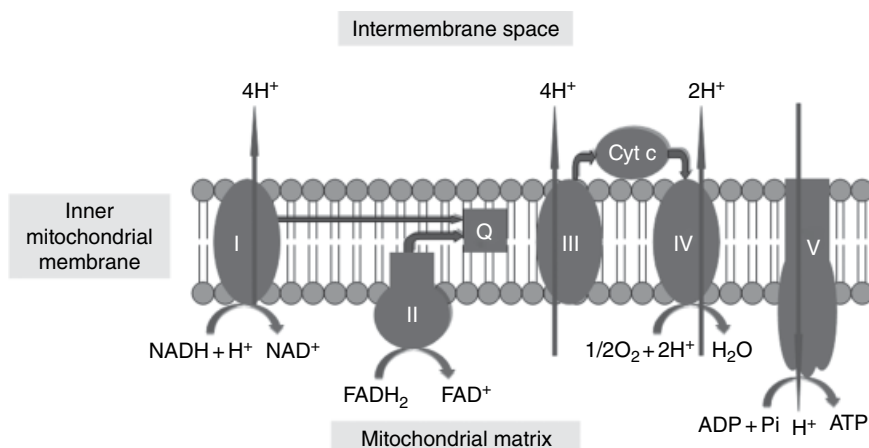


Figure 30.1 Diagram of the mitochondrial respiratory chain (MRC) and complex V illustrating proton (H⁺) movement during oxidative phosphorylation, where Cyt c, cytochrome c; Q, coenzyme Q₁₀.

subunits that constitute the MRC and complex V, the remainder being encoded for by nuclear DNA (nDNA) (Saraste, 1999). In contrast to the other enzyme complexes, complex II is entirely nDNA encoded (Saraste, 1999). mtDNA in contrast to nDNA is not protected by histones or DNA-binding proteins and therefore is vulnerable to free radical-induced oxidative damage (Wei, 1998). Furthermore, impairment of oxidative phosphorylation system enzyme activities may also result from oxidative damage to their protein subunits as well as the inner mitochondrial membrane phospholipids (Zang et al., 1990). This therefore indicates that in addition to genetic causes that have an incidence of 1 in 5000 live births (Rahman and Hanna, 2009), defects in oxidative phosphorylation may also be caused by secondary factors that result in oxidative stress generation such as disease pathophysiology or “off-target” drug-induced mitochondrial toxicity. In addition to oxidative stress generation, pharmacotherapy may also cause mitochondrial impairment by the direction inhibition of the enzyme complexes, uncoupling of oxidative phosphorylation, and/or impairment of mtDNA replication (Hargreaves et al., 2016).

Although a number of pharmacotherapies have been associated with mitochondrial toxicity, there are some classes of drugs that have been associated with both an impairment and an improvement in mitochondrial function. This chapter will illustrate this phenomenon by focusing on two pharmacotherapies, statins and fibrates, which are used in the treatment of dyslipidemia, highlighting the putative mechanisms responsible for their contrasting effects on mitochondrial function.

30.2 Statins

Statins competitively inhibit the enzyme, HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway and subsequently cholesterol synthesis

(Figure 30.2), and were introduced in 1987 to treat hypercholesterolemia (Hargreaves et al., 2005). A number of clinical trials with statins have suggested an acceptable safety profile; however, statin-associated muscle symptoms (SAMS) have been increasingly reported with this pharmacotherapy, resulting in skeletal muscle dysfunction in up to 25% of statin users who exercise (Sinzinger et al., 2002; Stroes et al., 2015). These myopathic side effects include myalgia, myositis, muscle weakness, and in rare cases rhabdomyolysis, but in many patients SAMS may be more subtle and may occur with or without serum creatine kinase (CK) elevation (Sathasivam, 2012). In view of their pleiotropic nature, the myopathic side effects of statin therapy may arise from a number of causes; however this chapter will outline the evidence for mitochondrial dysfunction as a contributing factor to these adverse side effects of the drug.

30.3 Effect of Statin Treatment on Endogenous CoQ₁₀ Status

Although the pathogenesis of statin-induced muscle dysfunction has yet to be fully elucidated, studies have associated statin treatment with a decrease in mitochondrial function together with an attenuation of energy production (Stroes et al., 2015). Lactic acidosis and elevated plasma lactate:pyruvate ratios have also been reported in patients following statin therapy, indicating a perturbation in oxidative phosphorylation (De Pinieux et al., 1996; Goli et al., 2002). In view of the commonality of the CoQ₁₀ and cholesterol biosynthetic pathways (Figure 30.2) together with the essential electron carrier role of CoQ₁₀ in the MRC, a number of studies have assessed whether a statin-induced deficit in endogenous CoQ₁₀ status may be a contributing factor to the mitochondrial dysfunction observed in patients. The majority of these studies have assessed the effect of statin therapy on circulatory CoQ₁₀ status by measuring the

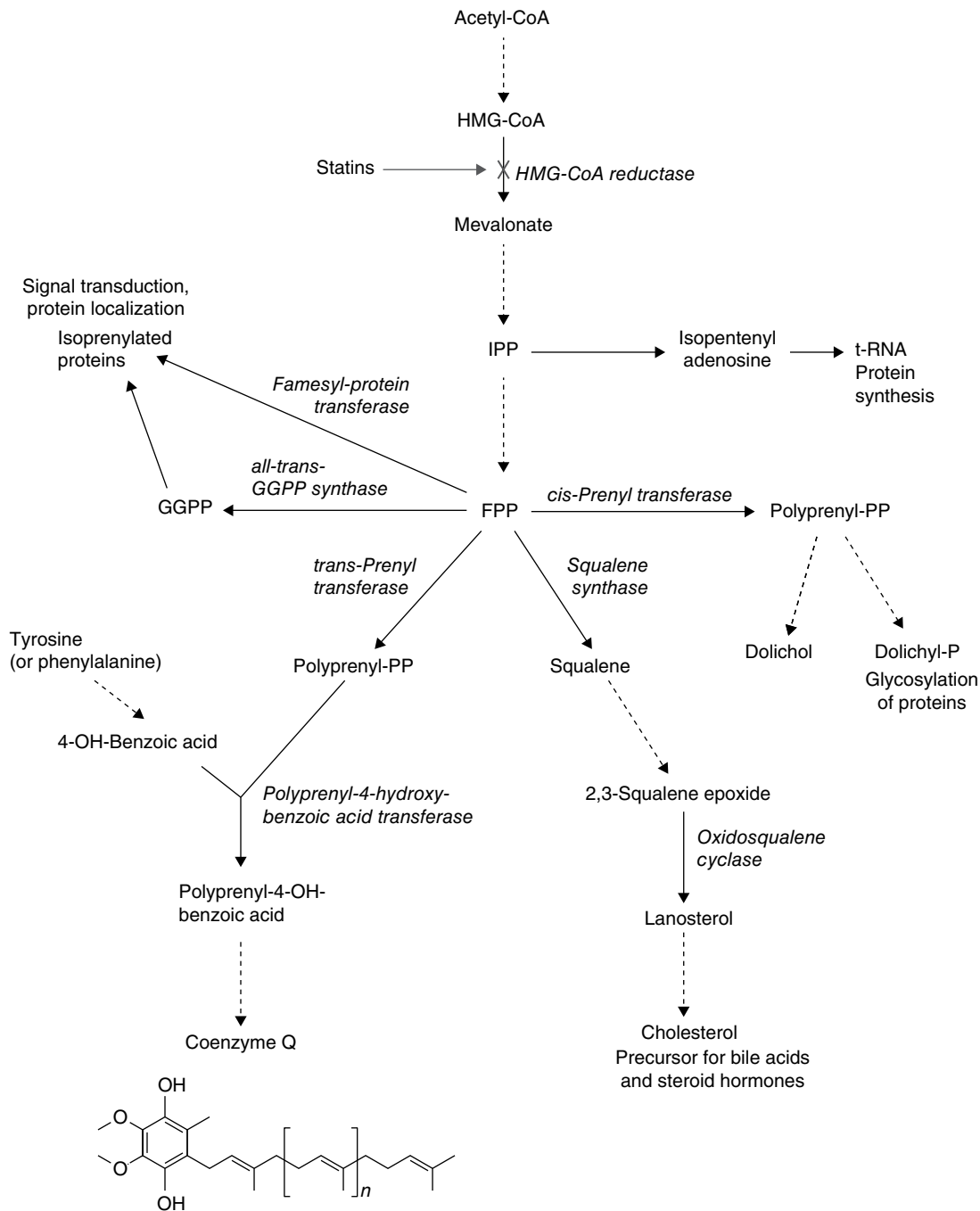


Figure 30.2 Mevalonate pathway, where PP, pyrophosphate.

levels of this isoprenoid in both serum and plasma (Banach et al., 2015). The majority of these studies have found evidence of a decrease in circulatory levels of CoQ₁₀ following statin treatment ranging from an approximate 27–50% reduction (Hargreaves et al., 2005). In the circulation, the majority of CoQ₁₀ is carried by the lipoproteins, predominantly the low-density lipoprotein (LDL) fraction (Hargreaves, 2003). In view of this, it has been suggested that plasma and serum CoQ₁₀ status

should be normalized to either total serum lipid or cholesterol levels (Hughes et al., 2002). Since statins target the liver causing a decrease in LDL synthesis, the diminution in plasma/serum CoQ₁₀ status following statin therapy may simply reflect the decrease in the circulatory level of LDL induced by this pharmacotherapy (Hargreaves et al., 2005). Indeed, in the majority of studies, the decrease in plasma/serum CoQ₁₀ status is nullified once the fall in circulatory total lipid or

cholesterol is taken into account (Hargreaves et al., 2005). In contrast, the decrease in circulatory CoQ₁₀ status in some studies appears to be more pronounced than the diminution in lipid/cholesterol levels, indicating a possible statin-induced inhibition of CoQ₁₀ biosynthesis (Ghirlanda et al., 1993; Watts et al., 1993; Human et al., 1997). In these studies, however, the possibility of a preexisting CoQ₁₀ deficiency or increased oxidative stress may have had an effect on circulatory levels of CoQ₁₀ (Hargreaves et al., 2005).

Since plasma/serum may not be the most appropriate surrogate to gauge tissue levels of CoQ₁₀ following statin treatment, a few studies have assessed the effect of this pharmacotherapy on muscle CoQ₁₀ status. This would seem appropriate as the major side effects associated with statin therapy are of muscle origin (Sinzinger et al., 2002; Stroes et al., 2015). Surprisingly, however, few studies have assessed the effect of statin therapy on muscle CoQ₁₀, and of these the results have been contradictory with few studies showing evidence of a decrease in CoQ₁₀ status following this pharmacotherapy. Since the incidence of statin-associated adverse effects appears to be dose dependent and exacerbated by drugs that affect their metabolism/detoxification (Duncan et al., 2009), it is not surprising that evidence of decrease in muscle CoQ₁₀ status has been reported under these conditions. In the study by Päivä et al. (2005), evidence of an approximately 34% decrease in skeletal muscle CoQ₁₀ status was reported following 8 weeks of treatment of 16 hypercholesterolemia patients taking a high dose (80 mg/day) of simvastatin. Interestingly, no associated myopathic side effects were reported although one patient was found to have a threefold increase in plasma hepatic transaminase levels. The authors suggested that the deficit in muscle CoQ₁₀ status may reflect the concomitant decrease in mitochondrial enrichment or volume as indicated by the decrease in the activity of the mitochondrial marker enzyme, citrate synthase (Päivä et al., 2005). Evidence of muscle CoQ₁₀ deficiency (77 pmol/mg; reference interval: 140–580 pmol/mg) has been reported in a patient who experienced rhabdomyolysis and renal failure following the increase in the dosage of simvastatin from 20 to 40 mg a day (Duncan et al., 2009). Importantly, the patient was also being treated with cyclosporine that has been reported to inhibit the hepatic cytochrome P450 enzymes involved in the metabolism and detoxification of simvastatin, which has been associated with an increased bioavailability of the statin (Arnadottir et al., 1993). Interestingly, in a recent study in rats, evidence was reported of myopathy and associated muscle mitochondrial dysfunction (indicated by decreased ATP level, elevated lactate:pyruvate ratio, and abnormal mitochondrial morphology) following a high-dose atorvastatin

treatment (100 mg/kg; El-Ganainy et al., 2016). Coadministration of CoQ₁₀ was found to ameliorate both the statin-induced myotoxicity and mitochondrial dysfunction in the rats (El-Ganainy et al., 2016). Unfortunately, muscle CoQ₁₀ status was not assessed in this study, and therefore it is uncertain whether the adverse myopathic side effects of atorvastatin treatment were attributable to a decreased muscle CoQ₁₀ status (El-Ganainy et al., 2016).

In view of the invasiveness of a muscle biopsy, investigators have used other cell types to evaluate the effect of statin therapy on endogenous CoQ₁₀ status. In the study by Bargossi et al. (1994), platelets were used to evaluate the effect of simvastatin (20 mg/day) treatment on endogenous CoQ₁₀ levels in hypercholesterolemia patients and reported a 12.5% decrease from basal levels following 90 days of treatment. More recently, blood mononuclear cells (MNCs) were employed to assess the effect of the statin and rosuvastatin therapy on the CoQ₁₀ status of children with familial hypercholesterolemia (Avis et al., 2011). In this study a 32% decrease in MNC CoQ₁₀ status was reported following 29 weeks of statin treatment. The decrease in MNC CoQ₁₀ status was found to be insufficient to perturb cellular ATP synthesis, although we cannot dismiss the possibility that an upregulation in glycolysis may have maintained the cellular energy status (Avis et al., 2011). The possibility arises that the threshold to which CoQ₁₀ must fall below before oxidative phosphorylation becomes compromised may vary between cell types that may influence their susceptibility to statin-induced mitochondrial dysfunction. Furthermore, an underlying defect in CoQ₁₀ biosynthesis or the inheritance of a particular polymorphism in gene encoding for a CoQ₁₀ biosynthetic pathway enzyme, which has been reported in the case of the *COQ2* gene (which encodes for the enzyme, 4-hydroxybenzoate polyprenyltransferase), may increase the susceptibility of an individual to statin-induced myotoxicity (Oh et al., 2007).

30.4 Effect of Statin Treatment on Cerebral CoQ₁₀ Status

Statin therapy has also been associated with cognitive impairment with reports of memory loss, insomnia, depression, and cerebellar ataxia being reported (Langsjoen and Langsjoen, 2015; Teive et al., 2016). Although it is uncertain whether all statins can cross the blood–brain barrier (BBB), animal studies have indicated that the lipophilic statins, lovastatin, and simvastatin are able to cross the BBB and enter the brain (Shepardson et al., 2011). At present it is uncertain whether the neurological side effects associated with statin therapy

are linked to a diminution in cerebral CoQ₁₀ status. However, the reports of statin-induced cerebral ataxia (Negvesky et al., 2000; Berner, 2010; Teive et al., 2016), which is the most common clinical presentation of primary CoQ₁₀ deficiency (Emmanuele et al., 2012), have indicated the possibility that statins may be inducing this neurological dysfunction as the result of diminution in cerebral CoQ₁₀ status. Unfortunately, no assessment of endogenous CoQ₁₀ status was made in any of the studies that reported an association between statin therapy and cerebral ataxia (Negvesky et al., 2000; Berner, 2010; Teive et al., 2016). In order to assess the effect of statin therapy on cerebral CoQ₁₀ status, the authors exposed three adult rats to simvastatin (20 mg/kg) for 3 days and then assessed the total cerebral ubiquinone status (CoQ₁₀ + coenzyme Q₉; CoQ₉; predominant ubiquinone species in rat; Hargreaves, 2003) in comparison with three “vehicle”-treated controls. Following 3 days of statin treatment, there was a 14% decrease in total cerebral ubiquinone status in comparison with control levels (Figure 30.3), which was found to be insufficient to perturb the activities of the cerebral MRC enzymes (results not shown). Because there was only a marginal decrease in cerebral total ubiquinone status following simvastatin therapy, a longer-term study may be required, which takes into account the half-life of ubiquinone in tissues (>50 h) before the potential inhibitory effect of statin therapy upon cerebral ubiquinone biosynthesis can be confirmed or refuted (Thelin et al., 1992). A recent *in vitro* study that assessed the effect of statin therapy on a human BBB cell model reported the ability of this pharmacotherapy to induce the increased expression of LDL receptors on the BBB cell surface (Pinzón-Daza et al., 2012). Since LDLs are the major carriers of CoQ₁₀

in the circulation (Hargreaves, 2003), this indicates that statins may also have the potential to increase cerebral CoQ₁₀ status.

30.5 Effect of Statin Treatment on Oxidative Phosphorylation

Impairment of oxidative phosphorylation has been associated with adverse side effects of statin treatment (Golomb and Evans, 2008), with the lipophilicity of the statin being an important determinant of this mitochondrial toxicity (Kaufman et al., 2006). Lipophilic statins (cerivastatin, simvastatin, and fluvastatin) may be able to penetrate biological membranes more easily than hydrophilic ones (rosuvastatin and pravastatin) as the result of passive diffusion (Hargreaves et al., 2005; Schirris et al., 2015). However, statins can also be transported into cells through the monocarboxylate transporter, MCT4, whose variability in expression may also influence tissue levels (Dobouchaud et al., 2000). MCT4 is predominantly expressed in the glycolytic fast-twitch fibers of skeletal muscle, which appear to be more sensitive to statin-induced necrosis than their oxidative counterparts (Wilson et al., 1998; Westwood et al., 2005). Although evidence of mitochondrial toxicity has been reported at therapeutic levels of the drug, 0.1–1 μM (Sirvent et al., 2005b), higher concentrations of statins as the result of tissue accumulation and drug–drug interactions may be required to elicit these adverse side effects (Kaufman et al., 2006; Luh and Karnath, 2003). Nonetheless, an underlying defect in oxidative phosphorylation may also render the patient more susceptible to the mitochondrial toxicity and the adverse side effects of statins at lower doses (Hargreaves et al., 2016). This is illustrated by patients with the subclinical presentation of the mitochondria DNA syndrome, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), who presented with evidence of severe myotoxicity following statin therapy (Chariot et al., 1993; Thomas et al., 2007; Tay et al., 2008). A number of inhibitory mechanisms have been suggested to account for the ability of statins to impair oxidative phosphorylation, which will be outlined in the following section. In addition, the putative role of statins in mitochondrial biogenesis will also be discussed.

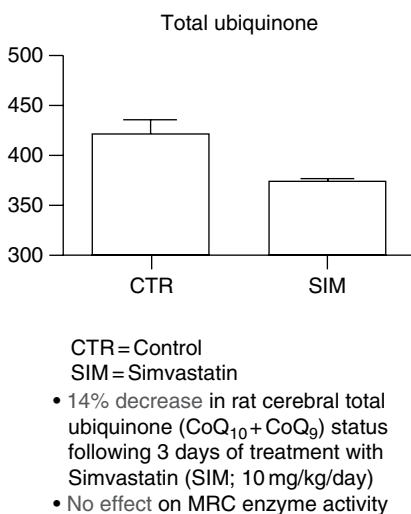


Figure 30.3 Effect of simvastatin on rat cerebral ubiquinone status.

30.5.1 Impairment of Isoprenylation

The possibility arises that the loss of MRC enzyme activity may simply result from an impairment in the isoprenylation of the enzyme. Isoprenylation is involved in the posttranslational modification of proteins and

requires the attachment of either a farnesyl or geranylgeranyl group, which is a product of the mevalonate pathway, to a protein to facilitate its attachment to cell membranes and/or to enhance protein–protein interactions (Zhang and Casey, 1996). In the study by Arenas et al. (2003), evidence of decreased muscle MRC complex IV activity (35% of control level) in association with muscle weakness, myalgia, and elevated plasma creatinine kinase (CK) activity (50,000 U/L; normal: <200 U/L) was reported in a patient following 3 weeks of treatment with the lipophilic statin cerivastatin and the fibrate gemfibrozil. Immunoblot studies indicated a reduction in the protein levels of the COX I and COX II subunits of the enzyme, leading the authors to speculate that the loss of enzyme activity was the result of impaired assembly or increased instability of the holoenzyme (Arenas et al., 2003). Within the COX I subunit of complex IV, a farnesyl chain acts as an anchor to attach the heme aa₃ prosthetic group to the subunit (Diaz et al., 2005). Furthermore, the COX I subunit is thought to act as a transmembrane scaffold for the enzyme subunits of complex IV (Tsukihara et al., 1996). Therefore, a decrease in the level of farnesyl as the result of the combined gemfibrozil and cerivastatin therapy may have resulted in a loss of structural integrity of complex IV as speculated by Arenas et al. (2003), which may have caused a concomitant decrease in enzyme activity (Arenas et al., 2003). In contrast, the inability of mevalonate treatment to prevent statin-induced mitochondrial dysfunction and cytotoxicity in rat skeletal muscle myoblasts puts into question the involvement of an impairment in isoprenylation as a causative mechanism for the mitochondrial toxicity of this class of drugs (Kaufman et al., 2006).

30.5.2 Uncoupling Oxidative Phosphorylation

In vitro studies using rat mitochondria have reported the ability of cerivastatin to significantly inhibit glutamate-/malate-driven state 3 respiration, although no evidence of MRC enzyme impairment was detected apart from a moderate loss of complex V activity (Nadanaciva et al., 2007). The impairment of oxidative phosphorylation was attributed to a possible inhibitory effect of the drug on mitochondrial membrane substrate/ion channels, although a study by Kaufman et al. (2006) has indicated the ability of cerivastatin to directly uncouple oxidative phosphorylation despite the concentrations of cerivastatin used in this *in vitro* study (100 μ M), vastly exceeding that reported in the plasma of patients (8 nmol/L; Muck, 2000). It was suggested that the tissue level of this drug may achieve such a high concentration either because of its high lipophilicity or as the result of drug–drug interactions (Kaufman et al., 2006). Gemfibrozil is

thought to magnify the toxicity of statins, especially cerivastatin by impeding their metabolism and therefore increasing their bioavailability (Prueksaritanont et al., 2002b). However, the ability of gemfibrozil to enhance the mitochondrial toxicity of cerivastatin under *in vitro* conditions indicates that these compounds may work synergistically to impair oxidative phosphorylation (Nadanaciva et al., 2007). Cerivastatin was withdrawn from the market in 2001 after its association with 52 deaths attributed to rhabdomyolysis-induced kidney failure (Furberg and Pitt, 2001). It is uncertain however whether mitochondrial dysfunction was a causative factor in the cerivastatin-induced fatal myotoxicity as no investigations were undertaken to confirm/refute this possibility. The lipophilic statins, simvastatin, lovastatin, and fluvastatin, have also been reported to uncouple oxidative phosphorylation in rat liver mitochondria although, as in the case of cerivastatin, at supra-pharmacological doses (100 nmol/mg of protein; Nadanaciva et al., 2007). At present it is uncertain whether statins could reach concentrations as high 100 nmol/mg of protein in skeletal muscle of patients even allowing for variability in MCT4 expression between individuals. An *in vitro* study using immortalized human muscle rhabdomyosarcoma cells has indicated that simvastatin and fluvastatin may accumulate up to intracellular concentrations of 25–30 nmol/mg of protein following a 30 min incubation with 100 μ M of the statin (Kobayashi et al., 2008). However, the intracellular concentrations of these statins were not found to increase further upon longer periods of incubation putting into question the validity of the supra-pharmacological doses used in *in vitro* studies to assess statin-associated mitochondrial toxicity.

30.5.3 Direct Interaction with the Enzyme Complexes of the Oxidative Phosphorylation System

Significant inhibition of MRC complex I activity has been reported in human skeletal muscle fibers following acute doses of simvastatin (10, 30, and 100 μ M) (Sirvent et al., 2005a). In this study, impairment of MRC complex I activity was indicated by a reduced respiration rate with pyruvate/malate and glutamate/malate substrates. Although in this study there was evidence of a marginal loss of MRC complex IV activity following statin treatment, this degree of inhibition has been reported not to affect the global respiratory rate (Mazat et al., 1997). The authors speculated that the interaction of simvastatin with MRC complex I may cause a blockage of electron transference within the enzyme itself or between complexes I and III, causing an impairment of activity (Sirvent et al., 2005a). A subsequent study using muscle

fibers from patients treated with statins at therapeutic doses also revealed evidence of a deficiency in MRC complex I activity together with a marginal loss of MRC complex IV activity, which was associated with a deregulation of muscle calcium homeostasis (Sirvent et al., 2012). *In vitro* studies have indicated that the lipophilic statins simvastatin, atorvastatin, and fluvastatin at concentrations, 100–200 μ M, are able to directly impair glutamate-driven respiration, inhibit fatty acid β -oxidation, dissipate the mitochondrial membrane potential, and induce mitochondrial permeability transition (Kaufman et al., 2006). In addition, a study employing an immunocapture technique has indicated that statins at 150 μ M are able to cause direct inhibition of the enzyme complexes of the oxidative phosphorylation system with simvastatin being the most pronounced and causing a loss of MRC complexes I, II–III (succinate cytochrome *c* reductase), and IV together with an impairment of complex V. Pravastatin and atorvastatin caused no inhibitory effect (Nadanaciva et al., 2007). Although the mitochondrial toxicity induced by statins in these *in vitro* studies occur at doses that vastly exceed therapeutic levels, they indicate that statins have the potential to immediately perturb oxidative phosphorylation. The mechanism(s) by which they elicit these effects have yet to be elucidated, but it may involve a direct interaction with the enzyme complexes and/or a competition with the natural substrates for the binding site of the enzyme.

Of note, statin therapy has been associated with a decrease in the level of muscle mtDNA by an as-yet unknown mechanism (Stringer et al., 2013). Unlike nucleoside reverse transcriptase inhibitors (Hargreaves et al., 2016), statins have not been associated with an impairment of mtDNA polymerase gamma, and although an increase in oxidative stress may account for a loss of mtDNA content, no evidence of this was determined in the study by Stringer et al. (2013). The normal level of nDNA-encoded, MRC complex II activity in conjunction with a loss of the mtDNA and nDNA-encoded complexes I, II–III, IV, and V activities following statin treatment in the study by Nadanaciva et al. (2007) would complement evidence of mtDNA depletion. However, the loss of mitochondrial enrichment/volume has been associated with statin therapy (Päivä et al., 2005), and this may account for the decrease in mtDNA levels associated with this drug therapy (Stringer et al., 2013), although this would not explain the global loss of enzyme activity reported by Nadanaciva et al. (2007), since isolated rat liver mitochondria were used in this study. A deficit in CoQ₁₀ status should also be taken into account in view of its involvement in DNA replication and repair through its role in pyrimidine synthesis (Lopez-Martin et al., 2007).

30.5.4 Oxidative Stress

An *in vitro* study by Warner et al. (2000) indicated that statin treatment may have an inhibitory effect on selenoprotein synthesis. Statins are thought to interfere with the isopentenylolation of selenocysteine-tRNA (Sec-tRNA), which governs the expression of all selenoproteins by decreasing the level of available isopentenyl pyrophosphate, a product of the mevalonate pathway and required for the maturation of Sec-tRNA (Endo et al., 1976). Accordingly, a decreased level of the selenoprotein antioxidant enzyme, glutathione (GSH) peroxidase, together with increased markers of oxidative stress (increased levels of malondialdehyde and oxidized LDL) and reduced levels of GSH has been reported in patients following statin therapy (Palazhy et al., 2015). *In vitro* studies using immortalized human liver Hep G2 cells also reported the ability of statins (at clinically relevant concentrations) to decrease the expression of GSH peroxidase, which was coupled with increased cellular reactive oxygen species (ROS) production (Kromer and Moosmann, 2009). Therefore, in view of the susceptibility of the MRC to oxidative stress-induced inactivation (Kowaltowski and Vercesi, 1999), the possibility arises that as a result of their ability to perturb the synthesis of both GSH peroxidase (Palazhy et al., 2015) and CoQ₁₀ (Hargreaves et al., 2005), statin treatment may result in a secondary loss of MRC enzyme activity. This may be a possible explanation for the impairment of MRC complex IV activity in conjunction with the deficit in muscle CoQ₁₀ status reported in two patients following statin therapy (Duncan et al., 2009).

There is increasing evidence to suggest that ROS may be a double-edged sword: although they can be toxic to cells, they may also play an important role in cell signaling involved in the antioxidant defense network.

30.5.5 Statin Lactones

Although statins are generally administered in their active acid forms, their myotoxic side effects together with their inhibitory effect on MRC complex III activity have been associated with their lactone forms (Skottheim et al., 2008; Schirris et al., 2015). The conversion of statins from their acid to lactone forms is thought to be catalyzed by both glucuronidation via uridine diphosphate (UDP)-glucuronosyltransferase activity and by fatty acid β -oxidation (Prueksaritanont et al., 2002a). Although under normal circumstances statin lactones do not exceed the level of their corresponding acid forms in plasma (Skottheim et al., 2008), the highly polymorphic nature of UDP-glucuronosyltransferases may result in increased conversion rates in individuals with corresponding elevations in circulatory lactone levels (Stormo et al., 2013). The study by Schirris et al. (2015)

suggested that statin lactones have the ability to compete with ubiquinol, the reduced form of CoQ₁₀ (Hargreaves, 2003) for the Qo binding site of MRC complex III in C212 myoblasts, causing a diminution in enzyme activity. The acid forms of the statin failed to inhibit MRC complex III activity. Furthermore, decreased complex III activity was also determined in the muscle biopsies from patients with statin-induced myopathies together with a decreased ATP synthetic capacity (Schirris et al., 2015). Although the level of cellular ubiquinol was not assessed in this study, the ability of substrate level stimulation of the fatty acid β -oxidation or glyceraldehyde 3-phosphate pathways to attenuate statin-induced inhibition of MRC activity has indicated a rationale for therapeutic strategies to increase endogenous ubiquinol status in patients experiencing the myotoxic side effects of statin therapy. The importance of fatty acid β -oxidation in maintaining the ubiquinol pool is indicated by the association between carnitine palmitoyltransferase 2 (CPT II) deficiency and statin-induced myopathy (Hur et al., 2014) as well as the ability of carnitine supplementation to ameliorate statin-associated myotoxicity (Arduini et al., 2004). In addition, the potential of statins to induce a deficit in endogenous CoQ₁₀ status (Hargreaves et al., 2005) or the presence of an underlying defect in CoQ₁₀ biosynthesis (Oh et al., 2007) may also have a bearing on patient ubiquinol status and their potential to induce inhibition of MRC complex III activity.

30.5.6 Mitochondrial Biogenesis

In addition to the detrimental effects of ROS on cellular metabolism, there is growing evidence in the literature that ROS may also play an important role in cell signaling (Sano and Fukuda, 2008). It has been proposed that mitochondrial biogenesis and induction of antioxidant enzyme synthesis may be triggered by low levels of ROS generation (Gems and Partridge, 2008). ROS is thought to interact with genetic signaling pathways that regulate the expression of genes involved in mitochondrial genesis and the antioxidant defense network. The adaptive response of mitochondria to low levels of ROS is referred to as “mitochondrial hormesis” (Ristow and Zarse, 2010). It has been speculated that low levels of ROS mediate “mitochondrial hormesis” through interaction with the peroxisome proliferator-activated receptor gamma coactivators, PGC-1 α and PGC-1 β , which have been identified as regulators of mitochondrial biogenesis and ROS detoxification (St-Pierre et al., 2006; Schultz et al., 2007; Ventura-Clapier et al., 2008). These regulatory factors appear to associate with the transcription factors already bound at the promoter region of target genes and induce an upregulation of gene expression (Scarpulla, 2006).

In the study by Bouitbir et al. (2012), atrial muscle biopsies from statin-treated patients were found to have a decreased level of oxidative stress compared with those of patients not receiving statin therapy. The decreased oxidative stress correlated with an increase in mRNA expression of the antioxidant enzymes copper–zinc and manganese superoxide dismutase (CuZnSOD and MnSOD, respectively) together with an increased mRNA expression of PGC-1 (PGC-1 α and PGC-1 β). In addition, the maximal oxidative capacity (maximal respiration rate measured with glutamate/malate substrates in the presence of ADP) was also found to be increased in the atrial muscle biopsies from statin-treated patients compared with those from the non-treated patients. In view of the important roles PGC-1 α and PGC-1 β play in both the regulation of CuZnSOD and MnSOD expression and mitochondrial biogenesis (Wang and Wong, 2010; Ibarra-Lara et al., 2012), it was speculated that statins may elicit their beneficial effects to cardiac tissue via the stimulation of the PGC-1 signaling pathway (Bouitbir et al., 2012). Interestingly, skeletal muscle biopsies from patients with statin-induced myopathy were found to have an increased level of oxidative stress, decreased oxidative capacity, and reduced PGC-1 mRNA expression (Bouitbir et al., 2012). The ability of statins to downregulate the expression of PGC-1 mRNA in skeletal muscle should also be considered as a possible mechanism for statin-induced mitochondrial toxicity. Further experiments in rats using atorvastatin indicated that statins acted through “mitohormesis” mechanism in cardiac muscle, stimulating mitochondrial biogenesis and cellular antioxidant defense by ROS-induced expression of PGC-1 α + PGC-1 β (Bouitbir et al., 2012). In contrast, atorvastatin treatment resulted in rat skeletal muscle generating a greater degree of ROS in comparison with cardiac muscle that appeared to overwhelm the PGC-1 signaling pathway (Bouitbir et al., 2012). This effect was attributed to the lower cellular antioxidant status of skeletal muscle in comparison with that of the heart. Administration of the antioxidant quercetin to the treatment regime ameliorated the detrimental effects of the statin although there was no evidence of “mitohormesis.” The apparent failure of atorvastatin in the presence of quercetin to stimulate the PGC-1 signaling pathway in skeletal muscle may indicate that either there was no “window of opportunity” for statin-induced ROS to induce PGC-1 expression or statins do not act through this pathway in skeletal muscle. Importantly, if the cellular antioxidant status is a critical factor in dictating an individual’s susceptibility to statin-induced myopathy, then clinical evaluation of this parameter prior to and during this pharmacotherapy together with antioxidant administration may prove beneficial

to patients. The cause of the statin-induced ROS generation was not investigated in the study by Bouitbir et al. (2012), and it is suggested that the reader refers to the previous section, "Oxidative Stress," for more detail on this subject.

30.6 Fibrates

Fibrates are synthetic amphipathic carboxylic acid compounds that are used in the treatment of hyperlipidemia, although they are mainly used specifically for the treatment of hypertriglyceridemia (Katsiki et al., 2013). In addition, fibrates have also been shown to ameliorate insulin resistance and glucose intolerance in diabetes (Kobayashi et al., 1988; Lee et al., 2002). The actions of fibrates are thought to be predominantly mediated by their activation of the nuclear receptors, peroxisome proliferator-activating receptors (PPARs), although the PPAR- α isoform is thought to be their major target (Lee et al., 1995; Staels and Fruchart, 2005). Once activated, these receptors regulate the transcription of more than 80 genes involved in lipid metabolism, energy balance, and inflammation (Plutzky, 2004). All fibrates are administered as esters and have to be converted by both blood and tissue esterases into their active PPAR agonist carboxylic acid form (Najib, 2002). In general fibrates are considered to be well tolerated, demonstrating an excellent profile; however this drug regime has been associated with myopathy both in monotherapy (Hodel, 2002) and in conjunction with statin therapy (Wierzbicki et al., 2003). Several studies have also linked fibrate treatment to hepatotoxicity in rodent models (Bhardwaj and Chalasani, 2007). The adverse side effects associated with fibrate treatment are thought to be independent of their PPAR- α activation and as a possible consequence of their direct effect on mitochondrial function (Zhou and Wallace, 1999). In view of the number of available fibrates, the following section will refine itself to four commonly used fibrates in clinical medicine: fenofibrate, clofibrate, gemfibrozil, and bezafibrate.

30.7 The Effect of Fibrate Treatment on Mitochondrial Respiratory Chain Function

In vitro studies have indicated that the mitochondrial toxicity associated with fibrate treatment may be caused by their prodrug ester forms rather than by the active fibric acid moiety (Brunmair et al., 2004; Wilk et al., 2015).

It has been suggested that fibrates induce mitochondrial dysfunction by different molecular mechanisms (Brunmair et al., 2004). The fibrate fenofibrate has been reported to inhibit MRC complex I activity at concentrations reported in patients on this pharmacotherapy (Brunmair et al., 2004; Zungu et al., 2006; Wilk et al., 2015). The cause of this inhibition is uncertain, but it may result from the binding of fenofibrate to a hydrophobic region of the enzyme, resulting in an impairment of complex I activity (Scatena et al., 2004). This is given further credence by the study of Yamada et al. (2013), which indicated that the site of inhibition was in the ubiquinone dehydrogenase portion of MRC complex I. Furthermore, this study also indicated that fenofibrate may also induce uncoupling of oxidative phosphorylation. The association between fenofibrate treatment and oxidative stress generation may also be a contributing factor to the inhibition of MRC complex I activity in view of the susceptibility of complex I to free radical-induced oxidative damage (Hargreaves, 2014). However, this would not explain the reported reversible nature of fenofibrate-induced MRC complex I deficiency (Wilk et al., 2015). It has been suggested that fenofibrate-induced complex I dysfunction may contribute to the ability of the drug to activate PPAR- α (Zhou and Wallace, 1999) in addition to its ability to improve glucose tolerance and insulin insensitivity in diabetes (Brunmair et al., 2004). The inhibitory effect of fenofibrate on complex I activity has also been suggested to contribute to the antitumor effects of the drug (Wilk et al., 2015). Treatment of mouse glioblastoma cells with fenofibrate was found to induce depletion of cellular ATP as a consequence of MRC complex I inhibition, which activated autophagy causing extensive tumor cell death (Wilk et al., 2015).

Clofibrate has been reported to cause a direct dose-dependent depolarization of mitochondrial membrane potential in rat liver mitochondria (Zhou and Wallace, 1999). The increase in ADP-stimulated respiration induced by clofibrate treatment coupled to the decreased ADP/O ratio, a measure of the efficiency of oxidative phosphorylation, suggested that this compound was acting as an uncoupler of this process (Zhou and Wallace, 1999). However, a subsequent study using rat muscle mitochondria has indicated that clofibrate may also inhibit MRC complex I activity at a concentration of 100 μ M, which, within the plasma range found in patients (Taylor and Chasseaud, 1977), induced an approximate 27% loss of enzyme activity. This degree of inhibition of MRC complex I activity has been reported to be sufficient to perturb oxidative phosphorylation (Davey et al., 1998). In contrast, a study by Nadanaciva et al. (2007) using a novel immunocapture technique instead

of a spectrophotometric enzyme activity assay indicated that in rat liver mitochondria, the impairment of oxidative phosphorylation induced by clofibrate was via its inhibitory effect on MRC complex V activity rather than that of complex I. Although the cause of its inhibitory effect on MRC activity is unknown, clofibrate treatment has been associated with an increase in intracellular and mitochondrial ROS production, which may result in this impairment (Qu et al., 2001). The mechanism by which clofibrate induces an increase in ROS production has yet to be fully elucidated and may just be a direct consequence of the collapse of the mitochondrial membrane potential and therefore a secondary consequence of the MRC impairment elicited by the drug (Qu et al., 2001). Alternatively, it may simply result from the peroxisomal proliferation induced by the fibrate (Schrader and Fahimi, 2006).

Gemfibrozil has been reported to be a potent inhibitor of oxidative phosphorylation, although the mechanism by which it elicits its inhibitory effect is as yet unknown and it does cause a marginal loss of MRC complex I activity (Zhou and Wallace, 1999; Nadanaciva et al., 2007). It has been suggested that gemfibrozil may act as an uncoupler of oxidative phosphorylation in addition to its ability to induce the mitochondrial permeability transition pore (Zhou and Wallace, 1999). The latter may either result from an impairment of oxidative phosphorylation or result in a loss of mitochondrial membrane potential. The effect of bezafibrate treatment on mitochondrial function appears to be contradictory. In rat liver mitochondria, bezafibrate up to a concentration of 200 μ M was not found to perturb oxidative phosphorylation (Zhou and Wallace, 1999). However, in human cell lines, bezafibrate was found to cause a significant inhibition of MRC complexes I–III (NADH cytochrome *c* reductase) activity at a concentration of 100 μ M (Scatena et al., 2003). In a study by Zhou and Wallace (1999), only succinate-dependent respiration was determined, which would not detect a defect in MRC complex I activity. In further support of the potential inhibitory effect of bezafibrate on MRC complex I activity, a study by Yamada et al. (2013) reported an approximate 40% inhibition of NAD-linked state 3 respiration following immediate exposure of rat liver mitochondria to 200 μ M of bezafibrate. This fibrate was also reported to cause some weak uncoupling of oxidative phosphorylation (Yamada et al., 2013). Of the four fibrates discussed in this section, bezafibrate appears to elicit the lowest level of impairment of oxidative phosphorylation as evaluated by *in vitro* assessment. However, since the majority of these studies assessed mitochondrial function following the immediate addition of the fibrate to the assay, the differences in mitochondrial toxicity of this group of compounds cannot be attributed to an effect of their

metabolism, but may be influenced by their lipophilicity (Yamada et al., 2013).

30.8 Fibrates in the Treatment of Oxidative Phosphorylation Defects

The induction of mitochondrial biogenesis has been suggested as a potential therapeutic strategy for the treatment of mitochondrial diseases (Wenz et al., 2008). One of the mechanisms by which this has been achieved is by pharmaceutical upregulation of the transcription cofactor, PGC-1 α , which is considered to be a major regulator of mitochondrial biogenesis and function (Andreux et al., 2013). PGC-1 α is thought to upregulate not only the expression of nuclear genes encoding components of the oxidative phosphorylation system but also mtDNA genes through the modulation of transcription factor A, mitochondrial (TFAM), (Finck and Kelly, 2006). PPAR activation has been reported to increase PGC-1 α expression (Liang and Ward, 2006), and therefore pharmaceuticals that can act as PPAR agonists have shown some promise in the treatment of mitochondrial disease (Schon et al., 2010). Among these compounds, the PPARs agonist, bezafibrate, has been reported to improve mitochondrial function in myoblasts and fibroblasts from patients with MRC disorders (Bastin et al., 2008) as well as ameliorating mitochondrial dysfunction in animal models of these disorders (Kanabus et al., 2014). In view of the ability of fibrates to cross the BBB (Deplanque et al., 2003), a study by Ioannou et al. (2010) investigated the ability of bezafibrate to stimulate MRC enzyme activity in human astrocytoma cells, and the authors reported an increase in both MRC complex IV activity and cellular ATP status following treatment. These results have indicated the potential of bezafibrate to treat mitochondrial encephalopathies.

To date, in humans, a clinical trial to assess the efficacy of bezafibrate treatment in the fatty acid β -oxidation disorder, CPT II, deficiency has shown some promising clinical results (Bonfont et al., 2010), and a clinical trial to assess the utility of bezafibrate in the treatment of mitochondrial myopathy is being considered at the Royal Victoria Hospital, Newcastle, United Kingdom (ClinicalTrials.gov Identifier: NCT02398201). It is of note that, among the fibrates, bezafibrate is the only compound to demonstrate some therapeutic potential in the treatment of mitochondrial dysfunction. This property of the drug may be associated with its low level of mitochondrial toxicity in comparison to other fibrates (see previous section). In addition, bezafibrate is a pan-agonist, activating all three of the PPAR isoforms (α , δ + γ) in comparison with other fibrates that

preferentially target the α isoform (Schoonjans et al., 1997). Furthermore, since activation of the δ rather than α isoform of PPAR has been associated with induction of PGC-1 α expression, this may also explain why bezafibrate rather than the other fibrates is an appropriate candidate for this therapeutic approach to treat mitochondrial disease (Hondares et al., 2007).

30.9 Conclusion

In view of their pleiotropic modes of action, it is perhaps not surprising that therapeutic drugs have the potential to both inhibit and improve mitochondrial function. In addition to the antidyslipidemic classes of drugs discussed in this chapter, antiepileptics (Finsterer and

Zarrouk Mahjoub, 2012; Carrasco-Pozo et al., 2015) and the antioxidant drug idebenone (Giorgio et al., 2012) may also exhibit this dichotomy toward mitochondrial function. As indicated by statins and fibrates, the positive or negative effect of drugs on mitochondrial function may be tissue specific (Sano and Fukuda, 2008) and depend on the degree of activation or metabolism of the particular pharmacotherapy (Laizure et al., 2013; Schirris et al., 2015). In addition, the degree of ROS generated by the drug may also have an important bearing on these parameters, indicating the importance of the antioxidant status in predisposing the tissue to either drug-induced mitochondrial genesis or toxicity (Bouitbir et al., 2012). Therefore, antioxidant monitoring and replenishment strategies should be considered for pharmacotherapies that influence mitochondrial function.

References

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31

Friend or Foe: Can Mitochondrial Toxins Lead to Similar Benefits as Exercise?

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CHAPTER MENU

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31.1 Beneficial Effects of ROS and Mitotoxin Exposure

Reactive oxygen species (ROS) have historically been touted as toxic by-products of metabolic pathways and have been causally linked to a myriad of chronic diseases (Fukui and Moraes, 2008; Ristow, 2006; Tatsuta and Langer, 2008; Wiederkehr and Wollheim, 2006). They include the superoxide anions, hydroxyl radicals, and hydrogen peroxide that are extremely reactive to macromolecules (nucleic acids, proteins, and lipids) (Cross et al., 1987). Mitochondria are the primary site of cellular ROS production as a result of electron leakage from mitochondrial electron transport chain complexes I and III (Murphy, 2009; Quinlan et al., 2013). One of the prominent theories of aging, called the “free

radical theory of aging,” proposes that mitochondrial ROS-induced oxidative damage to macromolecules contributes to aging and associated comorbidities (Harman, 1956, 1972). However, evidence from both *in vitro* and *in vivo* molecular studies of model organisms depicts ROS as signaling molecules that are indispensable in maintaining cellular redox homeostasis, organismal physiology, and adaptive responses (De Haes et al., 2014; Ray et al., 2012; Schieber and Chandel, 2014a; Sena and Chandel, 2012; Wood et al., 2003).

Acute increases in cellular ROS levels via minute amounts of mitotoxins can increase longevity from yeast to mice (Hekimi et al., 2011). Exposure of *Caenorhabditis elegans* to paraquat, a chemical generator of mitochondrial ROS, is sufficient to increase their lifespan (Yang and Hekimi, 2010). ROS-dependent increase in signaling pathways is deciphered as the mechanism that promotes lifespan in mitochondrial mutants in *C. elegans* (Lee et al., 2010). These studies have introduced the concept

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known as “mitohormesis” and have called into question the free radical theory of aging by implicating ROS as pro-longevity signals rather than pro-aging agents (Hekimi et al., 2011; Ristow and Schmeisser, 2011; Schulz et al., 2007; Tapia, 2006). Now, ROS signaling has also been implicated to play a positive role in wound healing, autophagy, transcriptional regulation, hypoxia survival, intracellular pH homeostasis, cell differentiation, ischemic preconditioning, heat shock, and innate immunity (Di Meo et al., 2016; Hamanaka et al., 2013; Johnson et al., 2012; Lebuffe et al., 2003; Madamanchi et al., 2001; Schieber and Chandel, 2014b; Sihvola and Levonen, 2016; Tormos et al., 2011; Vanden Hoek et al., 1998; West et al., 2011; Xu and Chisholm, 2014).

On a similar note, metformin, currently the most popular anti-diabetic and prospective anti-aging drug, also appears to be a mitochondrial toxin. It inhibits mitochondrial electron chain complex I, which ultimately activates AMP-activated protein kinase (AMPK) signaling and drives pro-metabolic adaptations (Batandier et al., 2006; Ota et al., 2009; Stephenne et al., 2011). Additionally, metformin is reported to have anti-proliferative and anti-tumorigenic effects (Hankinson et al., 2017; Kourelis and Siegel, 2012). The underlying molecular mechanisms of the beneficial effects of metformin remain largely unknown. The most accepted opinion is that chronic metformin treatment reduces overall ROS production (Cahova et al., 2015; Garg et al., 2017; Hou et al., 2010). However, recent studies have suggested that *in vitro* treatment of various cell lines with metformin increases intracellular ROS production (Chan and Miskimins, 2012; Queiroz et al., 2014). It is therefore tempting to hypothesize that metformin works, at least in part, through the generation of ROS pulses, which, similar to the acute effects of exercise (Section 31.4), activates AMPK and other pathways, leading to an induction of mitochondrial biogenesis and antioxidant pathways that ultimately lead to lower ROS, and other pro-metabolic outcomes of metformin treatment.

Knowing that ROS is linked to both cellular physiology and pathology, it is prudent to decipher the factor(s) that dictate the beneficial nature of the ROS at the molecular level. It is worth noting, however, that ROS pulses likely represent only one aspect of the beneficial effects of mitotoxins. For example, as respiratory chain inhibitors, mitotoxins are likely to lower the energy charge (ATP/AMP ratio), which mimics another aspect of exercise, leading to an induction of mitochondrial biogenesis and possibly other pro-metabolic outcomes. In the sections to follow, we will, however, concentrate on the ROS arm of the phenomenon, because this (perhaps more elusive) aspect was the focus of our recent research.

31.2 Window of Opportunity for ROS and Mitotoxins: Low Concentration and Short Time

If “too much of a good thing can be bad,” can a little bit of a bad thing be good? The various aforementioned examples demonstrating the potential positive effects of moderate exposure to mitotoxins echo the general philosophical assertion that various potentially detrimental effects on a living organism naturally induce protective mechanisms, resulting in increased resilience to the original insult, but, because protective mechanisms are generally not very specific and because an insult typically induces a spectrum of protective mechanisms, the overall result often is an overall improved healthspan, longevity, and so on. A very powerful demonstration of this principle is the positive effect of repeated low doses of radiation, one of the most implicated of genotoxic insults, in conditioning against subsequent high radiation doses (Phan et al., 2012). Of note, the mechanism of radiation genotoxicity also includes ROS (Bonner, 2003).

The hormetic nature of ROS eliciting a beneficial response is dictated by the levels of ROS, the temporality of ROS production, and the capacity of endogenous ROS scavenging mechanisms (Hamanaka and Chandel, 2010). The pathologies, where ROS is shown to play a negative role, are usually characterized by chronic production of high levels of ROS, causing irreversible damage to macromolecules and an “exhausted” antioxidant system, which together culminates in redox imbalance that promotes cell death or abnormal metastatic cell growth (Bialas et al., 2016; Chandrasekaran et al., 2016; Hempel and Trebak, 2017; Ly et al., 2017; Newsholme et al., 2016). Contrarily, both *in vivo* and *in vitro* studies have demonstrated that stimuli (including physical activity, calorie restriction, hypoxia, and heat shock) that induce temporal spikes in intracellular ROS production mitigate chronic ROS-induced pathologies by inducing an adaptive response (Madamanchi et al., 2001; Radak et al., 2008; Schieber and Chandel, 2014b; Schmeisser et al., 2013; Schulz et al., 2007; Zarse et al., 2012). Unlike chronic and excessive ROS production, physiological stimuli and/or minute quantities of mitotoxins induce temporal ROS production that work within a therapeutic window to induce signaling events downstream of insulin/insulin-like growth factor 1 (IGF-1) receptors, AMPK, mechanistic target of rapamycin (mTOR), sirtuins, and antioxidant production that ensure cellular redox and metabolic homeostasis (D’Autreaux and Toledano, 2007; Hardie et al., 2006; Vachharajani et al., 2016; Yuyun et al., 2013). Together, these results suggest that low concentrations of mitotoxins promote controlled transient ROS production that activates

homeostatic and adaptive pathways critical for cellular and organismal metabolism and survival.

31.3 Endurance Exercise, a Greatly Beneficial, Transient ROS-Generating Activity, Causes Translocation of p53 to Mitochondria

Can mitotoxins producing ROS exert any beneficial effects in mammals including humans? Intriguingly, endurance exercise, obviously a highly beneficial activity in mitigating various metabolic diseases, is intimately related to a transient production of ROS (Boccatonda et al., 2016; Radak et al., 2008). In particular, each bout of endurance exercise transiently produces spikes of mitochondrial ROS (Powers et al., 2011; Steinbacher and Eckl, 2015). Mitochondrial ROS generated by exercise are expected, as any ROS, to be damaging to the cell and in particular to mtDNA. Instead, exercise appears somehow to circumvent these anticipated negative consequences (Chen et al., 2016; Safdar et al., 2016b). There are at least two nonexclusive possibilities. First, it is well established that exercise-induced ROS may directly mediate the positive effects of exercise (e.g., via ROS-dependent signaling that induces metabolic pathways to modulate homeostatic recovery, as discussed in the “Introduction”) (Steinbacher and Eckl, 2015). Alternatively or in addition, with each bout of endurance exercise, ROS spikes may activate protective mechanisms, such as DNA repair, which, once activated, may assume an ameliorative role beyond merely neutralizing the immediate consequences of a ROS spike (Cartee et al., 2016; Chen et al., 2016; Hawley et al., 2014; Safdar et al., 2016b). They may also ensure resilience against other potential threats and help to revitalize cells by clearing chronically oxidatively damaged macromolecules and organelles, such as ones associated with aging, metabolic diseases, or an unhealthy lifestyle (Cartee et al., 2016; Hawley et al., 2014). This possibility has not been fully explored experimentally, and the evidence thus far is more correlative than causal. Intriguingly, there is evidence that such protective mechanism(s) may indeed exist: it has been previously demonstrated that p53, a tumor suppressor protein, is translocated to the mitochondria following acute bouts of endurance exercise (Saleem and Hood, 2013). p53 has been recently increasingly recognized as an mtDNA repair enzyme, which works both in excision repair and enhancing the fidelity of the mitochondrial DNA polymerase gamma (Achanta et al., 2005; Bakhanashvili et al., 2008, 2009; de Souza-Pinto et al., 2004). We have also demonstrated that DNA repair activity in mitochondrial extracts

ex vivo is inhibited by immunodepletion of p53 (Safdar et al., 2016b). Thus translocation of p53 to mitochondria after a bout of endurance exercise is consistent with the hypothesis that exercise activates some protective mechanisms. Many questions remain, however. First, it is not clear whether p53 translocation is caused specifically by ROS spike or by some other effect of exercise.

31.4 Mild Exposure to Mitochondrial Toxins *In Vitro* Recapitulates a Beneficial Endpoint of Endurance Exercise (Translocation of p53 to Mitochondria)

Endpoints of such a complex activity as exercise are necessarily multifaceted and involve both local and systemic intra- and intercellular and organ effects. We therefore used a simplified *in vitro* system (Safdar et al., 2016b). Fibroblasts in culture were treated with rotenone, an established mitochondrial ROS inducer, to emulate the exercise-related ROS spikes: abrupt increase in the ROS levels due to the addition of rotenone is a fair approximation of an ROS spike. Intriguingly despite the excessive simplicity of this system, we were able to reproduce the phenomenon of translocation of p53 into mitochondria. It is highly probable therefore that ROS spikes are the specific signal for translocation of p53 in muscle upon exercise. Intriguingly, fibroblasts that were pretreated with an antioxidant, *N-acetylcysteine*, show significant reduction in mitochondrial translocation of p53 when exposed to rotenone. Together these data imply, rather counterintuitively, that a signal as simple as a ROS spike generated by a mild exposure to a mitotoxin may be sufficient to recapitulate at least some of the beneficial processes thought to be specifically associated with exercise. This in fact is a very radical concept hinting potential pharmaceutical approaches.

Interestingly, translocation of p53 to mitochondria occurs only at moderate rotenone concentrations. At higher concentrations, p53 leaves the mitochondria and translocates to the nucleus. Apparently, mitochondria and the nucleus are competing for the common pool of p53. The migration of p53 to the nucleus at higher rotenone concentrations may reflect the possibility that at these concentrations ROS levels go above and beyond the therapeutic window and are sufficiently high to significantly damage nuclear DNA. Damaged nuclear DNA apparently is a much more powerful attractor for p53, since classically the function of p53 is to cause cell cycle arrest and promote DNA repair in response to nuclear DNA damage (Sahin and DePinho, 2012).

p53 translocation to mitochondria can be caused merely by a mitotoxin-derived ROS spike, but does it actually activate repair of the damaged mtDNA? More generally, will an ROS spike alone cause, at least in part, an amelioration similar to that associated with exercise? Or, alternatively, are additional outcomes of exercise needed to initiate this beneficial process? The experiments described later in this chapter indicate that ROS spikes alone can prove beneficial.

31.5 Progeroid mtDNA Mutator Mouse: A Test Ground for the Similarity between the Effects of Mitotoxin Exposure and Exercise

mtDNA mutator (PolG) mouse is a genetically engineered mouse in which mitochondrial DNA polymerase gamma (*polg1*), the sole polymerase replicating mtDNA, lacks proofreading ability, resulting in a greatly elevated mtDNA mutational burden (Kujoth et al., 2005; Trifunovic et al., 2004). These mice are particularly suitable for testing the interplay of oxidative stress, mtDNA repair, and the beneficial effects of mitotoxins and exercise. Indeed, they are under chronic oxidative stress, display measurable DNA damage, and present with a severe premature aging phenotype, which, most remarkably, is nearly fully reversible by endurance exercise (Dai et al., 2010; Safdar et al., 2011, 2016b; Trifunovic et al., 2004).

In our recent work we demonstrated that in the PolG mouse, exercise causes a boost of DNA repair in mitochondria and that this repair activity is not only abolished *ex vivo* with immunodepletion of mitochondrial p53, but also endurance exercise in a transgenic muscle-specific p53 knockout mouse model in PolG background failed to ameliorate mtDNA damage (Safdar et al., 2016b). This strongly implies that exercise stimulates mtDNA repair by translocating p53 into the mitochondria in a way similar to normal mice. The effects of exercise in PolG mice are much more dramatic than in the wild-type mice and include, in addition to other endpoints, a measurable reduction of non-mutational mtDNA damage.

In particular, we have used the difference in the number of mutations as detected by short-range (3kb) versus long-range (16kb) single-molecule PCR as a measure of non-mutational damage in mtDNA (Safdar et al., 2016a). We observed an excess of PCR-induced mutations in 3kb assay in mtDNA from sedentary but not exercised PolG mice. We interpret this as an excess of non-mutational damage in PolG mtDNA that is converted into mutations during PCR (Figure 31.1a). Exercise causes a notable reduction of so measured mtDNA damage compared to the sedentary PolG mouse. This finding is consistent with the observed decrease in 8-hydroxydeoxyguanosine

(marker of DNA oxidation) abundance in mtDNA as measured by immunoblotting (Safdar et al., 2016a). Here we measure non-mutational chemical DNA damage, and not true mtDNA mutations. Note that true mtDNA mutations are generally very difficult to induce even by most powerful exogenous mutagens, because of the overwhelmingly high rate of the endogenous mtDNA mutations (Coller et al., 1998; Marcelino et al., 1998). Thus the PolG mouse provides a convenient, measurable, and highly beneficial model to study the molecular aspects of exercise adaptation. Experiments in wild-type fibroblasts described previously hint that this endpoint of exercise might be equally evoked by an ROS spike caused by a mitotoxin exposure, because such spikes cause translocation p53, an mtDNA repair factor, into mitochondria.

The natural purpose of the ROS-initiated repair appears to be merely the cleanup of the damage (probably infinitesimally small) caused by the acute ROS pulse, whether it was caused by exercise or mitotoxin. In the case of PolG cells and mice, however, the same mechanism apparently also clears chronic damage that keeps the system in the deteriorated condition in the *sedentary* PolG mouse. We believe that an acute ROS spike in response to exercise may activate, in addition to mtDNA repair by p53, metabolic pathways that result in mtDNA complementation as a result of alteration in mitochondrial reticulum dynamics (fusion vs. fission) and the removal of “excessively bad” mitochondria via mitophagy (Ju et al., 2016; Romanello and Sandri, 2015). In other words, if we extend the analogy between PolG mice and the aged organism, these ROS pulses appear to be rejuvenating rather than merely maintaining.

31.6 Mutational Analysis Hints Existence of the “Good” and the “Bad” mtDNA and Evokes Alternative Hypotheses

The p53–mtDNA repair hypothesis is highly attractive as it ties together several diverse observations made by us and published in the literature. However elegant, the repair hypothesis is not the only one possible.

Of note, the results of our mutational analysis of the 3kb/16kb mtDNA fragments, that is, a decrease of mutational load of 3kb, but not in 16kb fragments as a result of exercise, can be explained only if we assume that there were two subpopulations of mtDNA in the cell: the “pristine” and the “damaged.” The damaged mtDNA is amplified by 3kb but not by 16kb PCR, so 3kb PCR fragments (but not the 16kb fragments) end up containing additional artificial mutations originating from the damaged nucleotides in DNA converted into mutations by PCR. The damaged mtDNA would be expected to

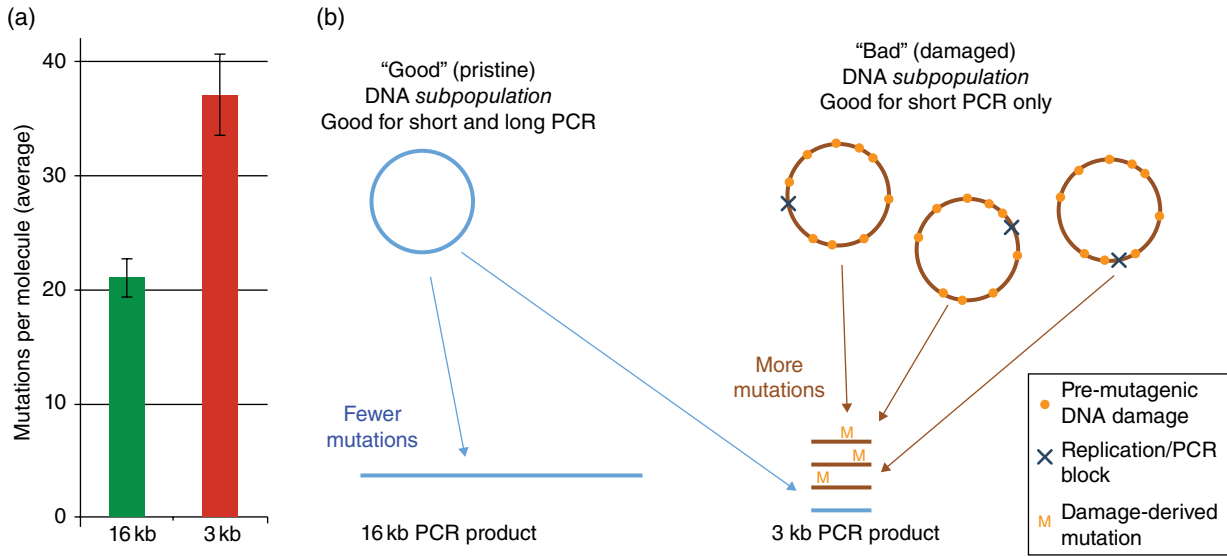


Figure 31.1 Evidence for the existence of subpopulations of mtDNA molecules with different levels of non-mutational DNA damage. (a) Apparent mutational load in muscle of PolG mtDNA “mutator” mouse is higher on shorter (3 kb) PCR fragments than longer ones (16 kb) (Safdar et al. 2016a). This observation is puzzling because true mutations are not expected to affect amplifiability of DNA by PCR. (b) To explain lower levels of mutations in the longer PCR fragments we recall that *apparent* mutations detected by PCR-based approaches are known to include, in addition to the true mutations, a significant proportion of artificial mutations, which result from error-prone bypass of chemically damaged DNA nucleotides (Khrapko and Vijg, 2009). Additionally, the number of mtDNA molecules amplifiable with 16 kb primers is typically approximately 10 times lower than those amplifiable with 3 kb primers which implies that a majority of molecules in the sample contain at least one molecule impassable lesion, such as strand break or bulky adduct (black crosses). If so, the lack of mutations on long PCR fragments implies that molecules with impassable lesions also contain an excess of bypassable error-prone damage sites, as illustrated in panel (b). In other words, a subpopulation of ‘good’ molecules (16 kb amplifiable) is devoid both of impassable lesions and damaged sites. This implies that damage is distributed non-randomly, and that the ‘good’ subpopulation is not merely a shoulder of a broad distribution of damage levels, but a group of molecules systematically protected from damage.

originate from the “bad,” that is, oxidatively damaged, “worn-out” mitochondria. This logic is illustrated and explained in Figure 31.1.

If there are two types of mitochondria, then the observed amelioration of mtDNA damage does not necessarily require DNA repair. Instead (or in addition), it can be achieved by the selective elimination of the “bad” mitochondria initiated by an ROS spike. Indeed, recent studies show that ROS exposure may result in an initiation of the removal of “bad” mitochondria or “bad” mitochondrial parts. We therefore consider two other mechanisms that may work instead or in addition to the p53–mtDNA repair mechanism.

31.6.1 Mitotoxins May Ameliorate Accumulation of mtDNA Damage by Alteration of Mitochondrial Dynamics and Activation of Mitophagy Resulting in Removal of the “Bad” Mitochondria

Studies assessing the accumulation of dysfunctional mitochondria in aged tissues have shown that molecular pathways that ensure the quality control of cellular mitochondria are impaired with aging (Masiero et al., 2009;

Romanello and Sandri, 2015; Zaglia et al., 2014). Ample experimental and clinical data demonstrate that mitochondrial quality is mediated by the alteration of mitochondrial fusion and fission that is paired with mitochondrial autophagy (also known as mitophagy) (Diot et al., 2016; Sun et al., 2016). Exercise-mediated ROS spikes induce mitochondrial fusion and fission that results in shuffling of good mitochondria (metabolically functional) from bad mitochondria (depolarized dysfunctional mitochondria with accumulated mtDNA and macromolecular oxidative damage) (Hood et al., 2016; Romanello and Sandri, 2015; Vainshtein and Hood, 2016; Vainshtein et al., 2015b). These bad mitochondria are then preferentially removed by mitophagy. This scenario has been widely proposed in the literature (Hood et al., 2016; Romanello and Sandri, 2015; Vainshtein and Hood, 2016; Vainshtein et al., 2015b). This explanation indeed looks very reasonable as both exercise and the increase of ROS level have been shown to activate autophagy, and mitophagy, in particular (Vainshtein et al., 2015a, b). The “repair” and the “mitophagy” hypotheses are not mutually exclusive and may both be responsible for the amelioration of mtDNA by exercise and/or temporal pulses of ROS.

31.6.2 Mitotoxins May Ameliorate Accumulation of mtDNA Damage by Activating Mitochondria-Derived Vesicle Trafficking and/or by Formation of Mitochondrial Spheroids

Recent studies have shown that under conditions of mitochondrial oxidative stress, nanovesicles, called MDVs, can be formed from mitochondria (McLelland et al., 2014; Roberts and Fon, 2016; Soubannier et al., 2012). It is shown that MDVs bud off of damaged mitochondria and are degraded in the lysosome-independent canonical autophagy pathway (Soubannier et al., 2012). MDVs are shown to contain oxidized proteins and are hypothesized to regulate mitochondrial quality (i.e., keep good ones and remove bad ones) faster than mitophagy (McLelland et al., 2014; Soubannier et al., 2012). In addition to MDVs, some studies have shown the formation of mitochondrial spheroids as a novel mitochondrial remodeling and quality control mechanism (Ding et al., 2012a, b; Yin and Ding, 2013). Mitochondrial spheroids are structurally unique mitochondria with a squeezed mitochondrial matrix and require ROS for their formation (Ding et al., 2012a, b). Mitochondrial spheroids are detected in CCCP-treated mouse embryonic fibroblasts and in mouse liver exposed to acetaminophen, acute alcohol, or high-fat diet, all of which can be classified as mitotoxins that produce ROS (Ni et al., 2013). In addition to oxidized mitochondrial macromolecules, mitochondrial spheroids also contain endoplasmic reticulum, lipid droplets, and so on (Ding et al., 2012a, b). There is a paucity of research investigating the formation of MDVs and mitochondrial spheroids in response to ROS spikes caused by acute bouts of endurance exercise. However, it seems highly plausible that in addition to the upregulation of mtDNA repair, mitochondrial fusion/fission paired with mitophagy, and cellular antioxidants, an alternative way that endurance exercise or hormetic quantity of mitotoxins would alleviate mtDNA damage is through the removal of oxidatively damaged mitochondrial components (mtDNA, lipids, and proteins) via MDVs and/or mitochondrial spheroids.

31.7 *Ab Absurdo*: Lack of Exercise May Result in Increased Damage

The opposite of exercise is a lack of it, for example, bed rest. One would expect that a person on a bed rest would not be exposed to exercise-related ROS pulses. One may expect that, as a result, in the absence of a force drawing p53 into mitochondria, the levels of mitochondrial p53 will decline with time, making mtDNA vulnerable to the

ever-present basal mitochondrial ROS. If so, one may expect an increase of mtDNA damage as a result of prolonged bed rest. Interestingly, some of us (CC-S and KK) have shown, in a preliminary study (Kraytsberg et al., 2016), that the levels of mtDNA deletions in muscle increased after 4-week-long bed rest. Of note, there were no serious pathological changes in muscle detected in this study where bed rest allowed some residual movement in bed (Brooks et al., 2010); however, another study showed a +25% coordinated reduction in nuclear and mitochondrial DNA-encoded mitochondrial transcripts, resulting in lower content of mitochondrial electron transport chain proteins and enzyme activity after 2 weeks of leg immobilization (Abadi et al., 2009). This increase in deletion burden may be cautiously interpreted as an indication of increased mtDNA damage. While this result is quite preliminary, it is worth mentioning that without the conceptual framework built in this chapter (i.e., ROS-induced repair), it would be really difficult to explain the increase in mtDNA deletion load in an inactive muscle.

31.8 Conclusions, Disclaimers, and Perspectives

It appears that ROS pulses produced either by bouts of exercise or exposure to low doses of mitotoxins share many similarities in their physiological effects. In particular, both appear to cause the translocation of p53 into mitochondria, where p53 boosts mtDNA repair. A surprising corollary is that mitotoxins that are commonly considered extremely and universally harmful may in fact be beneficial if administered in the right way.

This process makes a lot of sense as an adaptation that ablates acute mtDNA damage resulting from ROS bursts produced during natural exercise. However, in situations where significant *chronic* damage is already present (such as the progeroid PolG mouse, or naturally aged tissues), such bursts *additionally* have the potential to profoundly ameliorate the chronic deteriorative condition.

While much work needs to be done to confirm and extend the observations described herein, the most ambitious and exciting direction of research stemming from the data and logic of this chapter might be to try to administer carefully adjusted doses of mitotoxins in whole animals. While metformin has been in use for years now, other mitotoxins may be worth trying as well. PolG mice appear to be an ideal model for such an experiment. An informed search among mitotoxins may help to find those where toxicity is minimized while potential benefits are maximized. For example, paraquat

and rotenone are known to be associated with Parkinsonism (Nandipati and Litvan, 2016), but other mitotoxins do not necessarily have this drawback. Furthermore, mitotoxins with a short half-life may be more suitable to produce ROS pulses while minimizing damaging long-term effects.

As a note of caution, some of the data used in our reasoning in this chapter are still preliminary, and conclusions are intentionally controversial and provocative. The purpose of this chapter is not to review well-established facts, but rather to elicit interest toward

this poorly explored but exciting and potentially high payoff research field.

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32

Involvement of Mitochondrial Dysfunction on the Toxic Effects Caused by Drugs of Abuse and Addiction

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32.1 Introduction

Drug abuse represents a serious public health problem worldwide, carrying serious social and health burdens. The most feared and debated issues concerning drug abuse are related to their toxic potential and addiction. Addiction is, in fact, a major health alteration resulting in maladaptive behavioral changes associated with alterations in neural plasticity (Kutlu and Gould, 2016). It is widely accepted that the initial reinforcing effects of most drugs of abuse rely heavily upon the induction of large and rapid increases in the release of dopamine (DA). DA is a multifaceted neurotransmitter involved in the fine-tuning of motor and cognitive function, and regulation of reward and motivation, in the *nucleus accumbens* (Baler and Volkow, 2006). With continued use of drugs of abuse, learning deficits emerge along with cognitive inflexibility, which, combined with previously formed maladaptive drug-context/drug-cue associations,

contribute to the maintenance and reinforcement of addiction (Kutlu and Gould, 2016).

Current and emerging studies are focused on the involvement of mitochondria-dependent pathways in the neuronal toxicity and addiction caused by drug abuse, since mitochondria are key players of adenosine 5'-triphosphate (ATP) synthesis, calcium buffering, and apoptosis signaling in neurons. Once thought to be solitary and rigidly structured, mitochondria are presently acknowledged as highly dynamic organelles. The dynamic processes involved in regulating mitochondrial function enable organelle recruitment to critical subcellular compartments, content exchange between mitochondria, mitochondrial shape control, mitochondrial communication with the cytosol, and mitochondrial quality control. When mitochondrial function is disrupted, cellular dysfunction ensues, which ultimately may lead to neuronal damage and brain injury.

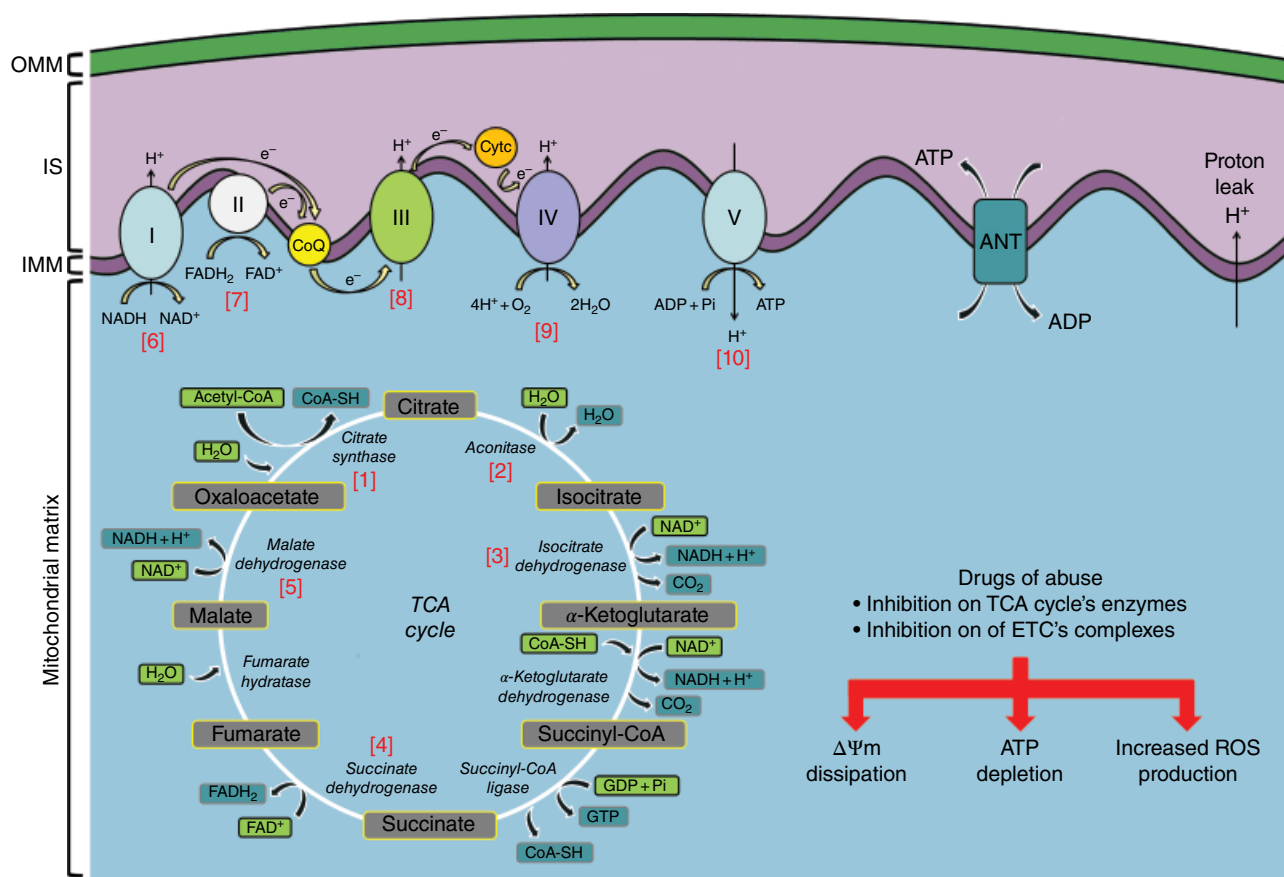


Figure 32.1 Modulation of mitochondrial tricarboxylic acid (TCA) cycle and electron transport chain (ETC) functioning by drugs of abuse and addiction. **TCA cycle:** Mediators of TCA cycle and enzymes described to be targeted by drugs of abuse. The TCA cycle consists of a series of chemical reactions catalyzed by enzymes occurring inside the mitochondria, particularly in the mitochondrial matrix. Drugs of abuse have been described to modulate the activity of key enzyme of the TCA cycle, including citrate synthase [1], aconitase [2], isocitrate dehydrogenase [3], succinate dehydrogenase [4], and malate dehydrogenase [5]. **ETC:** Mediators of the mitochondrial ETC and proteins described to be targeted by drugs of abuse. The ETC, also called mitochondrial respiratory chain, comprises five multi-subunit protein complexes located in the inner mitochondrial membrane (IMM). The adenosine nucleotide translocator (ANT), an IMM's protein, is responsible for the electrogenic 1 : 1 exchange of ADP for ATP. *In vivo* studies have reported the inhibition of the ETC complexes I [6], II [7], III [8], IV [9], and V [10] by drugs of abuse, which have been supported by additional *in vitro* settings. Inhibition of ETC functioning may trigger $\Delta\Psi_m$ dissipation, ATP depletion, and increased ROS formation. OMM, outer mitochondrial membrane; IS, intermembrane space; IMM, inner mitochondrial membrane. Adapted from Barbosa et al. (2015). Reproduced with permission of Springer. (See insert for color representation of the figure.)

This chapter highlights the evidences supporting the involvement of mitochondrial dysfunction on the toxic effects caused by drug abuse and addiction.

32.2 The Tricarboxylic Acid Cycle as a Target Pathway

The tricarboxylic acid (TCA) cycle is a central part of the energetic metabolism that contributes to the generation of ATP by the chemical breakdown of carbohydrates, fats, and proteins. It consists of a series of chemical reactions, catalyzed by enzymes occurring inside mitochondria, called

the mitochondrial matrix (Raimundo et al., 2011). A schematic representation of the mitochondrial TCA cycle is illustrated in Figure 32.1. Generically, the TCA cycle can be divided into two stages: oxidative, in which citrate (six carbon atoms) is converted to succinyl-CoA (four carbons), releasing two CO₂ molecules, and reductive, the successive oxidations of succinate to fumarate, fumarate to malate, and malate to oxaloacetate. The first reaction of the cycle is the condensation of acetyl-coenzyme A with oxaloacetate to form citrate, catalyzed by citrate synthase. Citrate is subsequently converted to isocitrate by aconitase, an enzyme that contains nonheme 4Fe–4S cluster. The conversion of isocitrate to α-ketoglutarate is the first oxidative decarboxylation of the cycle, catalyzed

by isocitrate dehydrogenase, and occurs in three forms: nicotinamide adenine dinucleotide oxidized form (NAD^+)-dependent and localized at mitochondria (i), as well as nicotinamide adenine dinucleotide phosphate oxidized form-dependent and localized at either mitochondria (ii) or the cytoplasm (iii). The α -ketoglutarate dehydrogenase complex catalyzes the conversion of α -ketoglutarate to succinyl-coenzyme A (succinyl-CoA) and CO_2 . Succinyl-CoA is the precursor for heme synthesis in animals. Subsequently, succinyl-CoA generates succinate and guanosine 5'-triphosphate (GTP) or ATP, by a reaction catalyzed by succinate-CoA ligase (SUCL), which is a dimer of an α subunit (SUCLG1) and one of the β subunits, either ATP-forming (SUCLA2) or GTP-forming (SUCLG2). Succinate is oxidized to fumarate by succinate dehydrogenase (SDH). The SDH reaction is part of both the citrate cycle and the electron transport chain (ETC), where it is referred as complex II. All other oxidative steps of the cycle generate nicotinamide adenine dinucleotide reduced form (NADH) to feed complex I of the ETC, whereas the electrons removed from succinate are channeled through flavin adenine dinucleotide reduced form to ubiquinone. Fumarate hydratase catalyzes the hydration of the double bond in fumarate, generating malate. The last reaction of the cycle recycles oxaloacetate from malate. This reaction is catalyzed by malate dehydrogenase (MDH) and couples the oxidation of malate with the reduction of NAD^+ (Raimundo et al., 2011).

Increased evidences suggest that drugs of abuse might target critical enzymes of the TCA cycle. Cocaine (20 mg/kg, i.p.) did not cause alterations on TCA cycle substrates in mice brain, as measured 25 min after drug administration (King et al., 1975). A further study, using rat heart tissue, also sustained that the TCA cycle potential was not sensitive to chronic cocaine treatment (25 mg/kg, i.p., 6 times/week, during 12 weeks), thereby supporting the observations revealed in brain tissue (Morris et al., 1994). Nevertheless, a study investigating cocaine's effects on gene expression of mitochondria-related proteins involved in the maintenance of energetic metabolism and mitochondrial function, in the prefrontal cortex of cocaine-abusing individuals that died from different causes, found an increased transcription of MDH gene (Lehrmann et al., 2003). This strongly suggests that cocaine interference on TCA cycle intermediates could be related with long-lasting abuse of the drug.

Previous studies have clearly suggested a pivotal role for mitochondria in mediating street heroin neurotoxic effects. In rats, 5 min after heroin administration (1 mg/kg, s.c.), a generalized depression of brain energy metabolism was noticed (Pontieri et al., 1997). A recent study also revealed an acceleration of the TCA cycle functioning, as traduced by increased levels of aconitase and citrate products in rat's urine and/or serum, following

heroin administration (twice daily for 10 days, at a dose of 3 mg/kg on the first day and then increasing by 1.5 mg/kg/day, to a final dose of 16.5 mg/kg/day. Heroin exposure was then stopped for 4 days, after which the rats were administered with heroin, again at 16.5 mg/kg/day, for 4 days) (Zheng et al., 2013). Thus, it is reasonable to consider these peripheral metabolites as surrogates characterizing the metabolic effect of heroin on central nervous system (CNS) function.

In vivo studies have also demonstrated a significant inhibition of the citrate synthase and SDH activities in several areas of rat brain, including prefrontal cortex, hippocampus, striatum, and amygdala, around 2 h after multiple administrations of methamphetamine (METH) (0.5, 1 or 2 mg/kg, i.p., single administrations or 0.25 mg/kg, i.p., once daily, for 15 days) (Feier et al., 2012, 2013). Furthermore, METH-induced decreased activity of MDH was also found in rat hippocampus, striatum, and amygdala around 2 h after the last dose of a repeated administration of the drug (0.25 mg/kg, i.p., once daily, for 15 days) (Feier et al., 2013). Studies addressing the influence of METH (4 $\times$ 10 mg/kg, i.p., once every hour) on mitochondrial metabolic networks found decreased urinary levels of TCA cycle's intermediates, including aconitase, α -ketoglutarate, malate, fumarate, succinate, oxaloacetate/pyruvate, and isocitrate/citrate, in rat's urine collected between 0 and 24 h after the last METH injection (Shima et al., 2011). In the urine samples collected between the 72 and 96 h period, no differences were found in these markers, as compared with control rats (Shima et al., 2011). These findings suggest that the inhibitory effect of METH on TCA cycle functioning could be reversible and, therefore, time limited. Other studies investigating protein expression profiles, using a 2-DE-based proteomics approach, have also reported a decreased expression of isocitrate dehydrogenase and aconitase in rat's amygdala 4 h after METH administration (1 mg/kg, i.p.) (Iwazaki et al., 2008).

In rats, around 2 h after single administration of d-AMPH (2 mg/kg, i.p.), an inhibition of the citrate synthase activity in amygdala and striatum was also revealed (Feier et al., 2012). In turn, reduced SDH activity was also reported in rat's striatum around 2 h after d-AMPH administration (2 mg/kg, i.p.) (Feier et al., 2012). Similarly, long-term exposure to d-AMPH (i.p., once daily, for 20 days—initial dose of 5 mg/kg/day and subsequently increased by 1 mg/kg every 5 days, up to a total of 8 mg/kg/day on days 16–20) also resulted in an inhibition of citrate synthase in rat's brain 24 h after the last d-AMPH injection (Valenzuela et al., 1987). Using another administration scheme (2 mg/kg, i.p., once daily, for 7 days, followed by another single injection of 2 mg/kg at the 15th day), similar reductions in citrate synthase, SDH, and MDH activities were documented in prefrontal

cortex, striatum, and hippocampus of rats, 2 h after the last d-AMPH injection (Valvassori et al., 2013).

Increased levels of TCA cycle's intermediates, including fumarate and malate, were also reported in brain tissue of rats 60 min after AMPH administration (5 mg/kg, i.p.). Likewise, increased levels of α -ketoglutarate were reported even after 5 min (2.5 mg/kg, i.p.) or 30 min (5 or 15 mg/kg, i.p.) of exposure to AMPH (Berntman et al., 1978). Thus, since METH was shown to reduce urinary levels of TCA cycle's intermediates between the 0 and 24 h period after administration (Shima et al., 2011), this suggests the existence of a biphasic effect of these drugs on the TCA cycle functioning: a stimulation at early stages, probably as a result of their acute effects, followed by a phase of reduced mitochondrial activity, which may result from a direct or indirect inhibition of the TCA cycle's enzymes.

The β -keto amphetamine (cathinones, β -KA) designer drugs, including mephedrone (4-methylmethcathinone (4-MMC)), show a large degree of structural similarity to amphetamines. However, little is currently known about whether these substances also share the potential neurotoxic of their non-keto amphetamine counterparts or what mechanisms could be involved.

An affection of TCA cycle caused by cannabinoids has been also described. *In vitro* studies with crude mitochondrial fraction isolated from pig brain reported a decreased citrate synthase activity following exposure to Δ 9-tetrahydrocannabinol (50 μ M; the main psychoactive compound of cannabis/marijuana) or to the agonist of the CB₁ cannabinoid receptor, AM251 (Singh et al., 2015).

There is considerable evidence that ethanol also adversely alters mitochondrial function. A recent study in pregnant rats revealed that maternal ethanol exposure (ethanol administration in the tap water, from the gestation day 5 to 21 days' postpartum, as follows: 2.5% (v/v) during 2 days, 5% (v/v) during 4 days, 10% (v/v) during 4 days, 15% (v/v) during 6 days, and finally 20% (v/v) during the additional period of the treatment) decreased glutamine synthetase activity and induced aspartate aminotransferase enzymatic activity in the hippocampus of 21-day-old pups (Cesconetto et al., 2016). The authors suggested that in the hippocampus of ethanol-exposed offspring, glutamate is preferentially used as a fuel in TCA cycle instead of being converted into glutamine, which indicates a TCA function imbalance, in order to supply specific neuronal alterations caused by ethanol.

Long-lasting use of tobacco/nicotine is known to exert pathological effects on almost all systems and tissues, including the CNS. A study based on histochemical methods dissected the nicotine's effects on the activity of different TCA enzymes in frontoparietal regions and subcortical nuclei of the rat brain (Turégano et al., 2001). It was shown an increased activity for MDH and SDH in

the superior layers (I, II, and III) of the frontoparietal cortex (cingulate, motor, and somatosensory regions) following both short (4 mg/kg/day, i.p., for 3 days) and long-lasting (2 mg/kg/day, i.p., for 15 days) administration of nicotine (Turégano et al., 2001). These observations support the hypothesis that nicotinic cholinergic drugs can have metabolic and long-lasting stimulant effects on cortical circuits, which could, in part, be attributed to an altered mitochondrial function.

Taking into account the previously referred findings, the TCA cycle's enzymes described to be targeted by drugs of abuse and addiction are indicated in Figure 32.1.

32.3 Effects on the Mitochondrial Electron Transport Chain

Mitochondria provide cellular energy by converting oxygen and nutrients into ATP via oxidative phosphorylation (Ho et al., 2012), which occurs at the ETC, localized in the inner mitochondrial membrane. A schematic representation of the mitochondrial ETC is illustrated in Figure 32.1. Although glycolytic metabolism of glucose is highly relevant in many organs, in the brain, ATP is mainly produced via oxidative phosphorylation. Oxidation of glucose in the TCA cycle supplies high-energy electrons in the form of NADH or flavin adenine dinucleotide reduced form to undergo oxidative phosphorylation, which involves the flow of these high-energy electrons along the ETC, from complex I (NADH dehydrogenase) and complex II (SDH) to complex IV (cytochrome *c* oxidase (COX)) and finally to molecular oxygen (Ho et al., 2012).

Along the flow of electrons through the ETC, there is a concomitant pumping of protons in complexes I, III (ubiquinol–cytochrome *c* oxidoreductase), and IV, from the mitochondrial matrix to the mitochondrial intermembrane space, creating an electrochemical gradient, also known as proton motive force, across the inner membrane (Mitcheel, 1961). Complex V, also called ATP synthase, utilizes this electrochemical gradient to drive adenosine 5'-diphosphate (ADP) phosphorylation to generate ATP, by channeling the protons back to the matrix (Mitcheel, 1961; Hatefi et al., 1975). The proton motive force has two components: the mitochondrial membrane potential ($\Delta\Psi_m$), which arises from the net movement of positive charge across the inner mitochondrial membrane, and the pH gradient. At any given time, the $\Delta\Psi_m$, typically between -150 and -180 mV, reflects the balance between processes that contribute to the generation of the proton gradient and those that consume it (Ho et al., 2012; Vafai and Mootha, 2012).

The nicotinamide nucleotide transhydrogenase, which plays an important role in ROS homeostasis, relies on

the proton motive force to regenerate mitochondrial nicotinamide adenine dinucleotide phosphate reduced form. Furthermore, it is coupled to solute and ion transport across the inner membrane, by which its collapse can halt essential biosynthetic reactions, such as Fe-S clusters' biogenesis and protein import. The importance of the proton motive force, namely the $\Delta\Psi_m$, is exemplified by the fact that glycolytic ATP can be consumed by complex V that is run in reverse to defend $\Delta\Psi_m$ during states of ETC inhibition (Vafai and Mootha, 2012).

In vitro studies with rat brain isolated mitochondria showed that cocaine (1 mM, for 1 min) impairs mitochondrial respiration by causing a direct inhibition of complex I, without affecting complex II activity (Cunha-Oliveira et al., 2013). In fact, a previous study by the same research group has already found an amelioration of cocaine cytotoxicity in mitochondrial DNA-depleted cells lacking mitochondrially encoded ETC subunits (Cunha-Oliveira et al., 2006). This suggests that cocaine might directly target the ETC function.

A human study investigating the gene expression of mitochondrial proteins related to mitochondrial function and energetic metabolism maintenance, in the prefrontal cortex of cocaine-abusing individuals that died from different causes, revealed increased transcription for COX subunit 6C and ATP synthase subunit C3 genes (Lehrmann et al., 2003). An *in vivo* study used quantitative real-time PCR assays to determine the numbers of copies of mitochondrial deoxyribonucleic acid (mtDNA) and of messenger ribonucleic acid (mRNA) corresponding to two mitochondrial proteins. A significant elevation of nicotinamide adenine dinucleotide dehydrogenase subunit I (NDI) and nicotinamide adenine dinucleotide dehydrogenase subunit VI of mitochondrial complex I was found in the prefrontal cortex and hippocampus of rats during early cocaine abstinence (3 days after 12 days with 2-h daily sessions of cocaine self-administration—the animals were given free access to the drug. Pressing on the active lever resulted in a 5-s infusion of 0.1 mL 0.5 mg/kg cocaine. Each infusion was followed by a 20-s time-out when rats could not receive the drug despite pressing the lever) (Sadakierska-Chudy et al., 2017). However, in a recent study, with immortalized CHME-5 microglial cells, cocaine (0.5 μ M, 24 h) was linked to a significant decrease of mitochondrial SDH iron-sulfur subunit of complex II, ubiquinol-cytochrome *c* reductase core protein II of complex III, and ATP synthase expression (Samikkannu et al., 2016). Thus, as previously suggested (Manoli et al., 2007), the cocaine-induced increased expression of ETC-related genes could be related to an acute response to stress, in which increased gene expression predominates.

A previous *in vitro* study investigated the requirement of a functional ETC for heroin-induced neurotoxicity (Cunha-Oliveira et al., 2007). By using NT-2 rho⁰

cells, which lack a functional ETC, no alterations indicative of heroin toxicity were found, as compared with NT-2 rho⁺ cells, thereby suggesting that street heroin neurotoxicity was independent of a fully functional ETC (Cunha-Oliveira et al., 2007). A human study including 36 individuals with heroin-related spongiform leukoencephalopathy (neurologic injury related to heroin inhalation) revealed a significant increase of ETC complex I activity in peripheral white blood mitochondria (5.6 ± 2.4 U/mL), as compared with control subjects (4.2 ± 2.1 U/mL) (Zhou et al., 2013). This indicates that a disturbed activity of ETC complex I could have a major role in the pathogenesis of heroin spongiform leukoencephalopathy.

Increased evidences suggest that a fully functional mitochondrial ETC is also crucial in preventing the neurotoxic effects of amphetamines. This theory received particular attention after the observation that inhibitors of the ETC could enhance MDMA- (Nixdorf et al., 2001) and METH-induced (Albers et al., 1996) neurotoxicity, both in mice (Albers et al., 1996) and rats (Nixdorf et al., 2001). Whereas the direct infusion of MDMA (100 μ M, at 1.8 (Darvesh and Gudelsky, 2005) or 2 μ L/min (Nixdorf et al., 2001), for 8 h) into the rat's striatum did not affect DA or 5-HT tissue content, as measured 5 days (Darvesh and Gudelsky, 2005) or 7 days (Nixdorf et al., 2001) later, coadministration of MDMA with malonate (inhibitor of mitochondrial complex II) produced long-term depletion of both DA and 5-HT content (Nixdorf et al., 2001; Darvesh and Gudelsky, 2005). Similarly, infusion of malonate (2–3 μ mol) in *caudate nucleus*, followed by two systemic administrations of METH (5, 2.5 mg/kg, i.p., 2 h interval), resulted in greater damage to dopaminergic neurons (DA depletion and TH inhibition), 5–7 days later, than the observed for METH or malonate alone (Albers et al., 1996).

Other studies revealed that substrates of energy metabolism attenuated MDMA- (Darvesh and Gudelsky, 2005), METH- (Stephans et al., 1998), and d-AMPH-induced (Wan et al., 1999) neurotoxicity. Perfusion of nicotinamide (precursor for the electron carrier molecule NADH, 1 mM) or ubiquinone (an electron-carrying coenzyme of the ETC, 100 μ M) in rat's striatum, beginning 2 h prior to the first MDMA injection (10 mg/kg, i.p., every 2 h, four times) and ending 6 h after the last injection of MDMA, significantly attenuated MDMA-induced 5-HT depletion 5 days later (Darvesh and Gudelsky, 2005). Striatal infusion of decylubiquinone (ubiquinone analogue) or nicotinamide for 6 h, beginning immediately after the last METH injection (3 $\times$ 10 mg/kg, i.p., every 2 h, followed by a last i.p. injection of 5 mg/kg, 2 h later), significantly attenuated the METH-induced rat striatal DA depletions, as measured 1 week later (Stephans et al., 1998). In the same way,

pretreatment with nicotinamide (500 mg/kg, i.p.) 3 h before d-AMPH administration (10 mg/kg, i.p.), significantly attenuated d-AMPH-induced acute striatal reduction of the ATP/ADP ratio (3 h after d-AMPH administration) and long-term striatal DA depletion (7 days later) in rat (Wan et al., 1999). Despite this, the first direct evidence that AMPH-like compounds may interfere with the ETC function was provided by Burrows and coworkers (Burrows et al., 2000). It was demonstrated that both METH (4 × 10 mg/kg, i.p., every 2 h) and MDMA (4 × 15 mg/kg, i.p., every 2 h) induced a significant reduction of the complex IV activity in the *substantia nigra*, *nucleus accumbens*, and striatum of rats, 2 h after the last injection of the drugs (Burrows et al., 2000). Following these studies, many reports have revealed the potential of amphetamines to interfere with the ETC function.

Many studies have reported an inhibition by amphetamines of mitochondrial ETC complexes' activity, namely, complex I (Feier et al., 2012, 2013), complexes II–III (Brown et al., 2005; Feier et al., 2012, 2013), and complex IV (Prince et al., 1997; Feier et al., 2012, 2013), in striatum and other DA-containing brain areas of rat. Consistently, METH (5 mg/kg, i.p., four times, 2 h interval) was also found to profoundly decrease the COX activity in the mitochondrial fraction of rat's frontal cortex 12 h after the last injection (Bachmann et al., 2009). Furthermore, a significant decrease in complex I activity in mitochondrial P2 homogenate from mice's brain was also associated with METH, both *in vivo* (10 or 20 mg/kg, i.p., twice, 2 h apart), 5 days after the last METH injection, and *in vitro* (1–10 μ M), in a concentration-dependent manner, after a 60 min exposure period (Thrash et al., 2010). In turn, decreased expression of complex I, as revealed 24 h after METH administration (30 mg/kg, i.p., one daily, for 7 days), was also associated with METH-induced neurotoxicity in mice (Klongpanichapak et al., 2006). Likewise, in SH-SY5Y cells, METH (1.68 mM, 24 or 48 h) was also shown to cause a time-dependent decrease in the expression of complex IV (subunits I, II, and IV) and complex V (β subunit), although no alterations were found on the expression profile of complexes I, II, and III (Wu et al., 2007). Furthermore, an upregulation of cytochrome *c* oxidase subunit I (COXI) gene and a downregulation of the genes codifying for nicotinamide adenine dinucleotide dehydrogenase subunit II (NDII) in the *substantia nigra* (Barrett et al., 2001) and ventral midbrain (Xie et al., 2002), 12 h after METH administration (single dose of 45 mg/kg, subcutaneous (s.c.)), were linked to drug-induced dopaminergic neuronal injury in mice. Of note, the COXI gene expression alterations revealed by microarray hybridization in the ventral midbrain, which were observed even after 24 h of METH exposure (single

dose of 45 mg/kg, s.c.), were consistently correlated with changes in mRNA levels (Xie et al., 2002). Notably, by preventing METH-induced hyperthermia (one cage containing METH-treated animals was placed on ice during the entire dosing regimen), the inhibition of mitochondrial complexes II–III observed in striatal mitochondria of rats administered with METH (10 mg/kg, s.c., four times, 2 h interval) was not rescued 1 h after the last injection, thus suggesting that METH's effects on mitochondrial function are independent of its hyperthermic effects (Brown et al., 2005). Furthermore, the coadministration of the *N*-methyl-D-aspartate receptor antagonist MK-801 or the peroxynitrite scavenger 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) attenuated the METH-induced inhibition of the mitochondrial complex II (Brown et al., 2005), thus providing strong evidence for a correlation among METH-induced effects on glutamatergic system (excitotoxicity), oxidative stress, and mitochondrial function inhibition (Brown et al., 2005).

d-AMPH (2 mg/kg, i.p., once daily, for 14 days) was shown to induce a marked inhibition of complexes I, II, III, and IV of the ETC in rat's hippocampus, striatum, prefrontal frontal cortex, and amygdala, as measured around 2 h after the last injection (Valvassori et al., 2010; Moretti et al., 2011). A reduction on the activity of complex I in the amygdala and complexes III and IV in hippocampus, striatum, prefrontal frontal cortex, and amygdala was also found in rats about 2 h after intraperitoneal administration of a single dose of 2 mg/kg of d-AMPH (Feier et al., 2012). Despite this, previous studies in NT2 rho⁰ cells (cells with trace-to-no complex I, II/III, and IV activity, but with normal $\Delta\Psi_m$, as compared with NT2 rho⁺ cells (Cardoso et al., 2001)) revealed more pronounced toxic effects, as compared with the effects observed in NT2 rho⁺ cells, following AMPH exposure (1 or 2 mM, for 24 h), indicating that the absence of fully functional ETC renders cells more sensitive to AMPH's toxic effects (Cunha-Oliveira et al., 2006).

MDMA (10 mg/kg, i.p., four times, 2 h interval) was also shown to cause an inhibition of complex I and II activity in rat's striatum, 12 h after the last injection (Quinton and Yamamoto, 2006). In mouse's striatum, high doses of MDMA (10, 20, 30 mg/kg, i.p. every 2 h) were also shown to decrease the activity of mitochondrial complex I, 1, 3, 6, 12, or 24 h after the last MDMA injection (Puerta et al., 2010). Furthermore, deletions in the genes coding for NDI and NDII subunits of the mitochondrial complex I and for COXI of the mitochondrial complex IV were found in isolated mitochondria from several brain areas of rat, including the prefrontal cortex, hippocampus, striatum, *raphe nuclei*, amygdala, *substantia nigra*, and ventral tegmental area, 2 weeks after MDMA administration (10 mg/kg, i.p., four times, 2 h

interval) (Alves et al., 2007, 2009a). Notably, the effects of MDMA on mtDNA were almost completely rescued by coadministration of the monoamine oxidase (MAO)-B inhibitor selegiline (Alves et al., 2007) or acetyl-L-carnitine (Alves et al., 2009a). In these studies, apart from the deletions on mtDNA, MDMA also decreased the expression patterns of the subunits NDII and COXI of the mitochondrial complexes I and IV, respectively (Alves et al., 2007, 2009a, b), an effect almost completely prevented by coadministration of the MAO-B inhibitor selegiline (Alves et al., 2007) or acetyl-L-carnitine (Alves et al., 2009a), but not by inhibiting MAO-A with clorgyline (Alves et al., 2009b) (to a better understanding about the involvement of MAO in the neurotoxic effects of MDMA and other amphetamines, see Section 32.5).

In vitro studies with the β -keto amphetamine designer drugs, such as 4-methylmethcathinone (1 or 2 mM) and 3,4-methylenedioxymethcathinone (2 mM), indicate that these drugs produce changes in mitochondrial respiration in SH-SY5Y cells (den Hollander et al., 2014, 2015), thereby providing an important first insight into the mechanisms of cathinone cytotoxicity.

Initial studies in rat brain have found the Δ 9-tetrahydrocannabinol (10 μ M) as an effective inhibitor of the NADH-oxidase activity *in vitro* (Bartova and Birmingham, 1976). *In vitro* studies with crude mitochondrial fraction isolated from pig brain reported a decreased activity for ETC complexes II/III and IV following exposure to Δ 9-tetrahydrocannabinol (50 μ M) (Singh et al., 2015). In agreement with this observation, a study in mitochondrial extracts isolated from rat brain associated Δ 9-tetrahydrocannabinol exposure (30 μ M, for 30 min) to an impairment on ETC complexes I, II, III, and IV (Wolff et al., 2015), thereby supporting a putative role for alterations in ETC for cannabis effects in the brain.

As recently reviewed (Jung, 2015), many studies corroborate that ethanol exposure decreases the activity of ETC complexes in laboratory animals and humans. In *in vitro* studies, brain mitochondria isolated from ethanol-treated (2×2 g/kg, 2 h apart) 7-day-old mouse pups displayed a transient decrease in oxygen consumption (Lamarche et al., 2013). In another study, an intermittent ethanol exposure for 5 weeks (25% ethanol in 5% sucrose by means of intragastric intubation, three times a day, for 4 days) was tested. The 4-day ethanol-exposure period was followed by a 3-day ethanol withdrawal period (five cycles of ethanol exposure and withdrawal were performed). This experimental protocol was shown to markedly decrease the complex IV activity in the prefrontal cortex and cerebellum of both young (3- to 4-month-old) and aged rats (29- to 30-month-old) (Jaatinen et al., 2003). In another study, to evaluate ethanol-induced brain mitochondrial dysfunction, rats were orally administered with

a 20% (v/v) aqueous solution of alcohol ethanol (5 g/kg of body weight/day) for 60 days. Ethanol-treated rats showed significantly lowered activities of complexes I, II, and IV, as compared with the control littermates (Reddy et al., 2010). Taken together, these studies provide strong evidence for an involvement of ETC dysfunction in the toxic effects caused by ethanol abuse.

Increasing evidences have also established a role for ETC dysfunction in the neuronal effects caused by tobacco/nicotine. Initial studies in purified rat brain mitochondria revealed the nicotine's ability to bind and inhibit the ETC complex I, with an EC_{50} of 0.331 μ M (Cormier et al., 2001). In rats exposed to nicotine (1 mg/kg body weight/day, for 7 days), isolated mitochondria from different regions of the brain (cerebral hemisphere, cerebellum, and diencephalons) displayed reduced activity for ETC complexes I, II, and III (Das et al., 2009). In another study, by using a microarray consisting of 4793 clones derived from a mouse DA cDNA library, the gene expression patterns for six brain regions (amygdala, hippocampus, nucleus accumbens, prefrontal cortex, striatum, and ventral tegmental area) of rats administered with nicotine for 7 days was profiled. A modulation of components of the mitochondrial ETC was revealed, including COXI, COXII, COXIII, cytochrome *b*, NADH dehydrogenase (complex I) subunit 4, and NADH dehydrogenase (complex I) subunit 6, by nicotine in multiple brain regions (Wang et al., 2009).

Taking into account the previously referred findings, the ETC complexes described to be targeted by drugs of abuse and addiction are presented in Figure 32.1.

32.4 Drugs of Abuse Might Target Mitochondrial Biogenesis

The mitochondrial biogenesis, the beginning of mitochondria "life cycle", encompasses the coordinated synthesis of nuclear DNA- and mtDNA-encoded proteins, together with membrane synthesis and the proper targeting and folding of ETC subunits. Nevertheless, the continuous mitochondrial renewal is sustained by a physiological equilibrium between organelles' biogenesis and selective degradation by autophagy (mitophagy) (Herrmann et al., 2012; Zhu et al., 2013). An increased mitochondrial biogenesis reflects a long-term adaptive response, although it is not always required to meet transiently increased energetic needs. Thus, transient changes in energy demands can be met by increases in the expression of a mitochondrial subset of genes or of crucial regulators and by the enhancement of mitochondrial function (Hock and Kralli, 2009). Some physiological states, such as caloric restriction, endurance exercise, long-lasting cold exposure, and exogenous agents,

including thiazolidinediones and resveratrol, were described to increase mitochondrial biogenesis (Hock and Kralli, 2009; Onyango et al., 2010).

A complex network of hormone- or growth factor-initiated intracellular signaling pathways and the resultant activation of nuclear transcription factors regulate mitochondrial biogenesis (Zhu et al., 2013). Among these, peroxisome proliferator-activated receptor gamma coactivator 1 α , nuclear respiratory factors 1 and 2 (which control expression of nuclear DNA-encoded mitochondrial structural proteins) (Wu et al., 1999), and transcription factor A mitochondrial (a nuclear DNA-encoded transcription factor crucial for replication, transcription, and maintenance of mtDNA) (Kang and Hamasaki, 2005) are the major players. Additionally, other factors, including hormones, second messengers (such as calcium, endothelial nitric oxide synthase, or cyclic adenosine 5'-monophosphate), and kinase pathways (including protein kinase A, mitogen-activated protein kinases, or adenosine 5'-monophosphate-activated protein kinase catalytic subunit α -2), control mitochondrial biogenesis at different levels (by regulating PGC-1 α expression, protein localization, or posttranslational modifications) (Devaux et al., 2010; Zhu et al., 2013). Additionally, the resulting pre-proteins need to be imported, processed, and efficiently assembled in mitochondria, through complex processes, tightly regulated by posttranscriptional mechanisms, and the target of the rapamycin (TOR) pathway (Devaux et al., 2010).

Accumulated evidences along the years suggest that drugs of abuse might target mitochondrial biogenesis. In animal models of addiction and in cultured cells, Yue-Mei Feng and coworkers (2013) examined the effect of morphine on mtDNA levels. A significant reduction in mtDNA copy number in the hippocampus of morphine-exposed mouse and rats was found (10 mg/kg, s. c., twice per day, at 12 h intervals, for 10 days). Furthermore, in cultured C6 cells, morphine exposure (100 μ M, for 24 h) also caused a reduction in mtDNA copy number. In addition, an elevation of mitochondrial mass was observed in morphine-treated PC12 cells (100 μ M, for 24 h) (Feng et al., 2013). The authors suggested that the increased mitochondrial mass could be a manifestation of mitochondrial dysfunction, as the remaining mitochondria may be larger due to structural disorganization in the presence of morphine. Notably, a reduction of the mtDNA copy number in the peripheral blood of female heroin addicts was also shown, 0, 3, and 6 months after heroin withdrawal (Feng et al., 2013), thereby supporting the findings observed in experimental models.

Using a microarray hybridization approach, Xie and coworkers (2002) and Barrett and coworkers (2001) correlated an upregulation of COXI gene and a downregulation of the genes codifying for NDII subunit of the ETC in the ventral midbrain and substantia nigra of mice,

respectively, to METH-induced dopaminergic neuronal injury, as measured 12 h after METH administration (45 mg/kg, s.c.). Notably, the expression patterns revealed by microarray hybridization were consistently correlated with changes in mRNA levels and attenuated by DAT inhibition with WIN35428 (12.5 mg/kg, i.p., immediately before METH) (Xie et al., 2002). In the same way, a study evaluating the molecular mechanisms underlying METH's neurotoxic effects linked increased mitochondrial mass and decreases in mtDNA copy number and mitochondrial protein content per mitochondrion to the neurotoxicity induced by METH (1.68 mM, 48 h) in human dopaminergic neuroblastoma SH-SY5Y cells (Wu et al., 2007). Other studies indicating an impairment of the protein kinase B (Akt)/mammalian target of TOR pathway in METH-induced neurotoxicity (Kongsuphol et al., 2009; Gonçalves et al., 2012; Li et al., 2012) strongly suggest a role for alterations in mitochondrial biogenesis in its neurotoxic effects.

A study with the hallucinogenic amphetaminic compound DOI, a potent serotonin 2A receptor agonist, revealed increased mitochondrial biogenesis in rabbit renal proximal tubular cells following drug exposure (10 or 20 μ M, 24 h), as ascertained by the increased mitochondrial respiratory rate under normal or uncoupled state (Beeson et al., 2010). Another study published in the same year, using the same experimental model, revealed increased activity of the PGC-1 α promoter and increased expression of ATP synthase β and nicotinamide adenine dinucleotide dehydrogenase 1 sub-complex 8 following DOI exposure (3 or 10 μ M) for 24 h, thereby revealing, unequivocally, the DOI's ability to modify mitochondrial biogenesis (Rasbach et al., 2010). Furthermore, by using the 5-HT receptor antagonist AMI-193 or by genetic silencing of PGC-1 α , DOI-induced PGC-1 α expression and biogenesis of mitochondrial proteins were blocked, respectively, suggesting that 5-HT receptors may mediate the transcription of this mitochondrial biogenesis regulator (Rasbach et al., 2010). Despite this, although it remains unclear whether similar effects may mediate DOI's neuronal effects, these evidences strongly suggest that similar mechanisms may be involved at brain level.

Animal studies analyzing the effects of a single intragastric ethanol administration (5 g/kg) found exacerbated mtDNA deletion in mouse brain, with a maximal effect about 10 h after ethanol administration. Additionally, the mtDNA depletion was followed by a gradual increase in mtDNA synthesis (Mansouri et al., 2001). Further *in vivo* studies associated ethanol (22.7 g/kg/day and gradually increased, accounting for ~32.9% of the total caloric intake after 1 week of intragastric alcohol feeding and ~38.4% of the total caloric intake after 4 weeks of intragastric alcohol feeding) to alterations in mitochondrial morphology, with increased number of elongated mitochondria, and

enhanced mitochondrial biogenesis in the mouse liver by upregulating PGC-1 α expression (Han et al., 2012). In the same line, an increased expression of mitochondrial biogenesis genes, including peroxisome proliferator-activated receptor gamma coactivator-1 α and mitochondrial transcription factor A, and mitochondrial DNA, but decreased mitochondrial mass, was observed in ethanol-administered rats for 150 days (diet composition: 16% protein, 5% carbohydrate, 45% fat, and 34% EtOH calories) (Caro et al., 2014). This apparent contradiction could come from the fact that the steady state of mitochondrial mass is determined by the balance between mitochondrial biogenesis and mitochondrial degradation via autophagy (mitophagy). Thus, this suggests that a hypothetical exacerbated mitochondrial degradation could not be adequately balanced by a concomitant increase in mitochondrial biogenesis, resulting in lower mitochondrial mass.

By exposing rats to daily environmental tobacco smoke (300, 100, and 0 $\mu\text{g}/\text{m}^3$ total suspended particulate), from postnatal day 8 (PD8) to PD23, Fuller and coworkers (Fuller et al., 2012) reported an increased mitochondrial biogenesis in the cerebellar cortex, as traduced by increased mitofilin-stained mitochondria. This study strongly suggests that mitochondrial pathways could be involved in the functional deficits seen in environmental tobacco smoke-exposed children.

32.5 Mitochondrial Quality Control and Drugs of Abuse

Mitochondria have developed several homeostatic mechanisms (Tatsuta and Langer, 2008; Baker et al., 2011). The first line of defense is provided by a highly conserved intra-organellar proteolytic system that conducts the surveillance of protein quality control within mitochondria. Molecular chaperones and energy-dependent proteases monitor the folding and assembly of mitochondrial proteins and selectively remove excess and damaged proteins from the organelle (Koppen and Langer, 2007; Tatsuta and Langer, 2008). The second level of mitochondrial quality control arises from the dynamic nature of the organelle itself. Mitochondria are constantly fusing and dividing, mediated by dynamin-like guanosine 5'-triphosphatases (GTPases) in the inner and outer mitochondrial membranes, assigning an important role for components regulating mitochondrial dynamics (Otera and Mihara, 2011). However, severe damage of mitochondria impairs fusion and results in fragmentation of mitochondria, which are then selectively removed by an autophagic process, termed mitophagy (Fischer et al., 2012). The selective degradation of defective mitochondria is critical for proper neuronal function, health, and survival, since it protects them from releasing oxidants and apoptosis triggers. At this level, phosphatase and tensin homolog-induced

putative kinase 1 (PINK1)/Parkin signaling pathway acts as a key regulator of mitophagy, thus allowing degradation of small and surrounding fusion-deficient mitochondria with sustained depolarization (Twig and Shirihai, 2011). Parkin is localized in the cytosol, but translocates to depolarized mitochondria, where it ubiquitinates protein targets (Narendra et al., 2008). The adaptor protein p62 then binds ubiquitinated mitochondrial proteins and LC3 on autophagosomes, thus recruiting autophagic membranes for mitochondrial clearance (Youle and Narendra, 2011). PINK1 is a serine/threonine kinase that recruits Parkin to depolarized mitochondria. In mitochondria with intact membrane potential, PINK1 is imported and degraded by presenilin-associated rhomboid-like protein (Jin et al., 2010). In mitochondria with reduced membrane potential, PINK1 is no longer degraded and accumulates in the outer mitochondrial membrane, where it can recruit Parkin through direct interaction (Kim et al., 2008; Matsuda et al., 2010). Through its E3 ubiquitin ligase activity, Parkin also ubiquitinates mitochondrial proteins, including VDAC1 (Geisler et al., 2010), Miro (Wang et al., 2011; Liu et al., 2012), and fusion mediators [mitofusins (Mfns)] (Gegg et al., 2010). Additionally, PINK1/Parkin axis also regulates turnover of specific ETC components (Vincow et al., 2013), suggesting additional roles for the PINK1/Parkin pathway in regulating mitophagy.

A recent study tightly associated the neuronal death caused by cocaine (1 μM , 48 h) to downregulation of both PINK1 and Parkin. In addition, immunocytochemistry analysis revealed Parkin-ring-like structures surrounding fragmented mitochondria of hippocampal neurons, thus indicating a role for autophagy in this effect (De Simone et al., 2016).

Studies in cultured C6 cells reporting a restoration of the morphine-induced (100 μM , for 24 h) mtDNA copy number loss by knocking down the atg7 gene strongly suggest the involvement of autophagy in that effect (Feng et al., 2013). Consistent with this notion, aggregation of LC3B in the soma of cultured mouse cortical neurons upon morphine exposure was observed, thereby indicating morphine-induced degradation of dysfunctional mitochondria via autophagy (Feng et al., 2013).

A recent study demonstrated that the METH (2 mM, 12 h)-induced cell death in a dopaminergic neuronal model, the N27 cell line, was closely associated with induction of mitophagy (Lin et al., 2012). Furthermore, overexpression of LC3 partially protected against METH (2 mM, 24 h)-induced apoptotic cell death, as measured the extent of DNA fragmentation, suggesting a neuroprotective role for autophagy in METH-induced neurotoxicity (Lin et al., 2012). Additionally, striatal tissue isolated from METH-administrated (4 $\times$ 20 mg/kg, i.p., 2 h interval) rats also exhibited elevated LC3 1 and 7 days' post-METH administration, suggesting that METH-induced

autophagy may serve as an adaptive strategy for inhibiting mitochondria-mediated apoptotic cell death (Lin et al., 2012).

Another study, in PC12 cells, also provided robust cellular evidence that upon METH exposure, PINK1 increased the total number of mitochondria, concurrently recruited beclin 1 (a protein essential for initiation of the autophagic process), Parkin and ubiquitin and enhanced the clearance of damaged mitochondria. Furthermore, in the absence of functional PINK1, and upon METH-induced autophagy stress, a failure of the autophagy system at large was observed, with marked accumulation of dysfunctional mitochondria and dramatic increase of apoptotic cell death (Lenzi et al., 2012). In accordance with these findings, Castino and coworkers (2008) showed typical apoptotic cell death in PC12 cells exposed to METH (1 μ M, 12 h) when the autophagy/mitophagy pathway was impaired or blocked.

Using Parkin knockout mice, Takamatsu and coworkers (2011) attempted to clarify the role of Parkin in MDMA-induced hyperthermia, one of the causal factors of neuronal damage. Notably, an enhanced hyperthermic response was observed in either heterozygous or homozygous knockout mice for Parkin administered with MDMA (30 mg/kg, i.p.), as compared with respective wild-type littermates (Takamatsu et al., 2011). Taken together, these data suggest the PINK1/Parkin signaling pathway as a promising target to counteract the toxic effects mediated by METH and MDMA.

In vivo studies revealed that knockout of Parkin exacerbates ethanol-induced behavioral impairment, as well as DA depletion in the striatum of mice administered with ethanol (liquid diet, with ethanol comprising 35.8% of total calories, for 10 days, and a 6.6% concentration of ethanol for an additional period of 8 days). Furthermore, since the ratio of LC3-II/TOMM20 was significantly lower in ethanol-fed Parkin knockout mice, as compared with ethanol-fed Parkin wild-type littermates, it suggests that the absence of Parkin leads to the downregulation of autophagy (Hwang et al., 2017). This strongly indicates a major role for autophagy in preventing the ethanol effects in the brain.

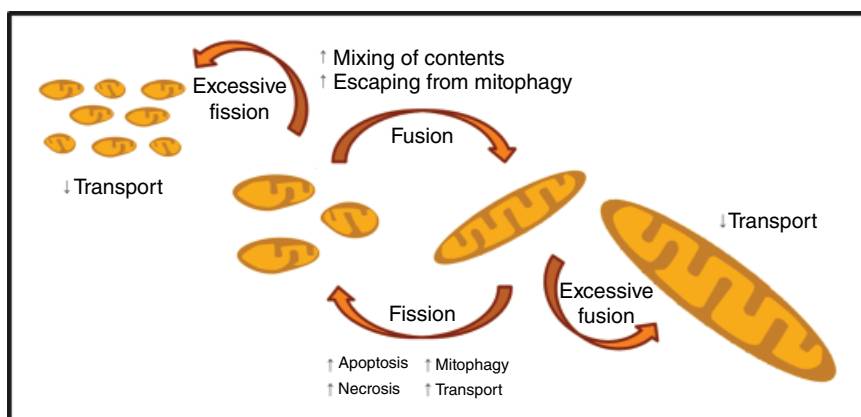
32.6 Mitochondrial Fusion/Fission Equilibrium Is Affected by Drugs of Abuse

Mitochondria are highly dynamic and complex organelles that, as well as undergoing regulated movement throughout the cell, continuously alter their shape, ranging between two opposite processes, fusion and fission, in response to several stimuli and cellular metabolic

demands. These opposite processes are crucial in sustaining the robust structure and function of mitochondria (Saxton and Hollenbeck, 2012). Mitochondrial shapes can range from punctuate structures to tubular networks, which are probably physically contiguous structures constructed via fusion of single bean-like-shaped mitochondria (Popov et al., 2005). Continuous movements of mitochondria allow a physical contact that facilitates their fusion, thus creating single large mitochondria. Mitochondrial fusion allows the exchange of mtDNA and other matrix components between neighboring mitochondria, including damaged or senescent mitochondria, thus conferring protection against mtDNA mutations and maintaining a healthy oxidative phosphorylation (Chan, 2006; Ranieri et al., 2013) (Figure 32.2). Fission events permit mitochondrial separation to daughter cells during mitosis (Taguchi et al., 2007), control organelle size and shape for an efficient mitochondrial distribution in neuronal processes (Ishihara et al., 2004; Kageyama et al., 2012), allow the recovery of damaged mitochondria for degradation by mitophagy (Twig et al., 2008), and regulate apoptosis through segregation of the most critically injured mitochondria (Youle and van der Bliek, 2012) (Figure 32.2).

Mitochondrial fusion is a multistep process that requires the coordinated fusion of both outer and inner mitochondrial membranes, ultimately resulting in mixing of matrix contents. In mammalian cells, three large GTPases mediate mitochondrial fusion. Mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), located in the outer membranes of adjacent mitochondria, induce outer membrane fusion, whereas optic atrophy 1 protein (OPA1), a protein residing in the intermembrane space, mediates fusion of the inner membranes (Song et al., 2009). Mfns are highly homologous GTPases anchored to the outer membrane, with their amino- and carboxyl-terminals directed to the cytosol (Rojo et al., 2002), which, by forming homo- or hetero-protein complexes, allow mitochondrial tethering and fusion through GTP hydrolysis (Koshiba et al., 2004). Whereas the Mfn's amino-terminal GTPase domain is required for fusion activity, carboxyl-terminal domain coordinates the docking of mitochondria to one another through antiparallel binding to the carboxyl-terminal domains of Mfn molecules on adjacent mitochondria. Additionally, Mfn1 appears to mediate tethering of mitochondria more efficiently than Mfn2 (Ishihara et al., 2004). OPA1 acts as a hetero-oligomeric complex, formed by a larger-size OPA1 (L-OPA1) and the smaller-size OPA1 (S-OPA1), in the inner mitochondrial membrane fusion (Cipolat et al., 2004). However, in mediating mitochondrial fusion, OPA1 functionality appears to require Mfn1, but not Mfn2 (Cipolat et al., 2004). Additionally, OPA1 was also described to play a key role in cristae organization of the inner mitochondrial

Figure 32.2 Cellular and physiological roles of mitochondrial fusion/fission. A correct balance between fusion and fission generates discrete mitochondria that may be transported over long distances. Additionally, fission events facilitate cell death through apoptotic or necrotic features and mitophagy, whereas fusion allows content mixing and can block mitophagy. However, an excessive mitochondrial fusion or fragmentation generate mitochondria that present defective transport.



membrane (Cipolat et al., 2006; Frezza et al., 2006), by a mechanism independent of the mitochondrial fusion (Frezza et al., 2006).

Since knockout of Mfn1 or Mfn2 results in the formation of small fragmented mitochondria (Chen et al., 2007a), and cells lacking Mfns exhibit several cellular defects, including poor cell growth, heterogeneity of inner mitochondrial membrane potential and decreased respiration (Chen et al., 2003), this indicates that mitochondrial fusion plays a crucial role in maintaining mitochondrial functionality.

Mitochondrial fission is mediated by dynamin-related protein 1 (Drp1), a member of the dynamin family of GTPases. When mitochondrial fission is stimulated, Drp1 translocates from the cytosol to specific sites on the mitochondrial outer membrane and homo-oligomerizes, forming spiral chains around mitochondria, which, using GTP hydrolysis, constrict and twist tubule to initiate mitochondrial fission (Smirnova et al., 2001; Ingberman et al., 2005). Nevertheless, it has been suggested that tubules of the endoplasmic reticulum wrap around and squeeze mitochondria at the early stage of division, prior to the assembly of Drp1 filaments on mitochondria (Friedman et al., 2011). After mitochondrial fission, Drp1 spirals likely disassemble from mitochondria for future rounds of mitochondrial fission (Smirnova et al., 2001; Ingberman et al., 2005).

In yeast, Drp1 attaches to mitochondria by binding to mitochondrial fission protein 1 (Fis1), a protein anchored to the mitochondrial outer membrane, through the adaptor protein Mdv1 (or its paralogue Caf4) (Mozdy et al., 2000). However, in mammals, though a yeast Fis1 orthologous has been identified (hFis1) (James et al., 2003), orthologous for Mdv1 and Caf4 are not described, and the evidences supporting a role for hFis1 in mitochondrial fission are mixed. Supporting a role in fission events, overexpression of Fis1 in mammalian cells promoted mitochondrial fragmentation, and Fis1 inhibition resulted in mitochondrial elongation (Yoon et al., 2003).

Similarly, in Fis1-null cells, mitochondria presented an elongated and interconnected morphology, when compared with their respective wild-type counterparts, thus indicating that Fis1 plays a role in fission events (Losón et al., 2013). Despite this, in another study it was shown that the conditional knockdown of Fis1 did not disrupt mitochondrial morphology or Drp1 recruitment to mitochondria (Otera et al., 2010). Similarly, a recent study also reported an independence on Drp1 in Fis1-regulated mitochondrial fission (Onoue et al., 2013). Thus, in mammals other outer mitochondrial membrane proteins have been described as putative mitochondrial receptors for Drp1. One of the molecules is mitochondrial fission factor (mff), whose its knockdown resulted in mitochondrial elongation (Gandre-Babbe and van der Bliek, 2008), by a mechanism independent of Fis1 (Losón et al., 2013). Subsequent observations indicating that Mff and Drp1 co-localize in puncta on mitochondria and that the exogenous expression of Mff increased the amount of Drp1 recruited to mitochondria and induced extensive mitochondrial fission (Otera et al., 2010) led to consider Mff as a strong candidate for Drp1 receptor in the outer mitochondrial membrane.

Alternatively, mitochondrial elongation factor 1 (Zhao et al., 2011), also identified as MiD49/51 (Palmer et al., 2011), was reported to promote mitochondrial fusion instead of fission, which, by blocking GTP binding to the GTPase domain of Drp1, does not allow the conformational change of Drp1 multimers required for membrane fission. However, it remains unclear why MiD49/51 causes mitochondrial elongation instead of fission. According to one model, Fis1 can partially reverse the elongated mitochondrial phenotype by sequestering mitochondrial elongation factor 1 and, consequently, unblocking Drp1 GTP hydrolysis, thus leading to organelle fission (Zhao et al., 2011; Oettinghaus et al., 2012). On the other hand, a recent study demonstrated that MiD49/51 overexpression enhanced mitochondrial recruitment of Drp1, though an increased inhibitory

phosphorylation of Drp1 on Serine637 was observed (decreased phosphorylation of Drp1 at Serine637 facilitates its recruitment to mitochondria (Cereghetti et al., 2008)). However, after carbonyl cyanide *m*-chlorophenyl hydrazone treatment, this phosphorylation was reduced, and mitochondrial fission ensued, suggesting that MiD49/51 may recruit Drp1 to mitochondria, but maintains it in an inactive state until a cellular signal triggers fission (Losón et al., 2013). In addition, the observation that B-cell lymphoma extra long (Bcl-xL) caused a significant increase in Drp1 GTPase activity in cultured hippocampal neurons also suggests that Bcl-xL may act as a GTPase-activating protein for Drp1 in mediating mitochondrial fission (Li et al., 2008).

Increased evidences suggest that drugs of abuse and addiction might target fusion/fission mechanisms, resulting in alterations of mitochondrial fusion/fission equilibrium.

The cell death caused by METH (300 μ M, 24h) in rat hippocampal progenitor neural cells was associated with Drp1 oligomerization and consequent translocation to mitochondrial surface, by a calcium-independent mechanism, resulting in a phenotype of increased mitochondrial fragmentation (Tian et al., 2009). Furthermore, METH (2mM, 12h)-induced neurotoxicity in mesencephalic dopaminergic cells was closely associated with more rounded and swollen mitochondria (Lin et al., 2012).

Recently, studies conducted by our research group showed a perturbation of mitochondrial fusion/fission equilibrium associated with MDMA. Using cultured neurons from mice hippocampus, MDMA (1.6mM), following a short incubation period of 90 min, led to an increased fragmentation of axonal mitochondria, a phenotype closely correlated with an impairment of axonal mitochondrial trafficking (Barbosa et al., 2014a). Similar results were further described for the mixture of MDMA and 6 of its major metabolites [α -MeDA, N-Me- α -MeDA, 5-(GSH)- α -MeDA, 5-(GSH)-N-Me- α -MeDA, 5-(NAC)- α -MeDA, and 5-(NAC)-N-Me- α -MeDA], at *in vivo* relevant concentrations (each compound at a equimolar concentration of 10 μ M), following an exposure period of 24 h (Barbosa et al., 2014b).

Additionally, another study associated a disruption of the inner mitochondrial membrane cristae to METH-induced neurotoxicity (Lin et al., 2012). Thus, this suggests that AMPH-like drugs, namely, METH, may target key regulators of mitochondrial cristae remodeling, such as Opa1 (Cipolat et al., 2006; Frezza et al., 2006), mitofilin (Yang et al., 2012), or PINK1/Parkin pathway (Poole et al., 2008).

An enlarged mitochondrial profile was also perceived in the cerebellar cortex of adult rats after 3 or 6 months of ethanol intake (Tavares and Paula-Barbosa, 1983), suggesting an abnormal organelle fission.

Despite this, more studies are needed to clarify whether these proteins and pathways are perturbed by

drugs of abuse and addiction and what pathways drive to alterations in these proteins and/or mechanisms.

32.7 Mitochondrial Distribution under the Influence of Drugs of Abuse

In neurons, essential constituents and mitochondria, which are generally synthesized or generated in the cell body, are delivered through the neuronal processes to their final destination, the synaptic terminals. Owing to their unique metabolic requirements, these areas do not display a uniform mitochondrial distribution. The large size of many neurons (up to 1 m in humans), which precludes rapid diffusion of ATP from one end of the cell to the other, implies, therefore, that the energy production must be spatially matched to local energy usage. In neurons, areas with high energy requirements such as presynaptic and postsynaptic terminals, active growth cones and axonal branches, myelination boundaries, sites of axonal protein synthesis, and nodes of Ranvier are enriched in mitochondria, compared with other cellular domains (Sheng and Cai, 2012). Furthermore, though mitochondrial biogenesis may occur locally in the axon, generation of new organelles mainly occurs within the cell body. Additionally, as the degradation of organelles, such as mitochondria, ensues in the cell body, dysfunctional mitochondria need to return to the soma for an efficient degradation (mitophagy) through the autophagy-lysosomal system (Ashrafi and Schwarz, 2013). As such, neurons require highly efficient mechanisms for mitochondrial transport regulation from and to the cell body to enable the rapid redistribution of mitochondria to different areas in order to supply metabolic requirements.

The cytoskeleton, constituted by microtubules, actin filaments, and neurofilaments (also called intermediate filaments), is responsible for the maintenance of the highly specialized structure of neurons, also allowing axonal growth and coordinated transport and stable docking events (Sau et al., 2011). Microtubules, formed by polymers of α - and β -tubulin, are polarized structures with the “plus” end toward to the terminal and the “minus” end toward to the cell body. However, dendrites present a mixed polarity (Sau et al., 2011; Saxton and Hollenbeck, 2012). Cytoskeletal filaments are linked to microtubule-associated proteins, such as tau protein, which permit their stabilization (Saxton and Hollenbeck, 2012). Kinesin motors, linked to the outer mitochondrial membrane protein Miro1/2, through the adaptor proteins Trak1/2 (also known as OIP106 and OIP98/Grif-1, respectively) mainly drive “plus” end-directed mitochondrial transport, whereas cytoplasmic dynein mediates mitochondrial transport toward the “minus” ends of microtubules (MacAskill et al., 2010; Sheng and Cai, 2012; Schwarz, 2013).

Since neuronal mitochondrial trafficking is tightly regulated in response to changes in the local energetic state and metabolic demand, it requires the existence of highly coordinated mechanisms to regulate the movement of these organelles along neuronal processes and their docking to supply specific biological needs. At this level, cytosolic calcium (Rintoul et al., 2003; Chang et al., 2006, 2011; Macaskill et al., 2009; Wang and Schwarz, 2009), microtubule-associated proteins (Ebner et al., 1998; Stamer et al., 2002; Jiménez-Mateos et al., 2006; Stoothoff et al., 2009; Llorens-Martín et al., 2011), and PINK1/Parkin (Weihofen et al., 2009) are perhaps the best characterized regulators of mitochondrial transport, though other regulators like mitochondrial calcium (Chang et al., 2011), Mfn2 (Misko et al., 2010, 2012), or histone deacetylases (HADCs) (Chen et al., 2010; Kim et al., 2010) were also described.

The apparent relevance of impaired neuronal trafficking of intracellular organelles and materials in the context of the neurotoxicity caused by amphetamine compounds was firstly provided by Callahan and coworkers (Callahan et al., 2001). In that study, to further appraise the 5-hydroxytryptaminergic neurotoxic potential of MDMA and fenfluramine, the authors used tritiated proline to examine anterograde transport along ascending axonal projections originating in the rostral *raphe nuclei* of rats. They found a marked reduction in anterograde transport of labeled material to various forebrain regions known to receive 5-hydroxytryptaminergic innervation, 3 weeks after MDMA exposure (20 mg/kg, s.c., twice daily, for 4 days) or fenfluramine (10 mg/kg, i.p., four times, 2 h interval) administration. Furthermore, these transport perturbations were associated with lasting decrements in 5-HT axonal markers (5-HT and 5-hydroxyindoleacetic acid content), thus suggesting a role for impaired axonal transport in developing neurotoxic effects (Callahan et al., 2001). However, this study could not differentiate between neuronal mitochondrial trafficking and transport of other intracellular organelles or materials, not allowing, thus, a clear appraisal of the real impact of AMPH-like drugs on mitochondrial trafficking.

One year later, another study, using a microarray hybridization approach, reported a decreased expression of the microtubule-associated protein tau in the mouse ventral midbrain 24 h after METH administration (45 mg/kg, s.c.) (Xie et al., 2002), suggesting a feasible alteration of mitochondrial trafficking by METH. In support of this observation, MDMA was further reported to increase tau phosphorylation in mice hippocampus (Busceti et al., 2008) and to disrupt microtubular system in 5-hydroxytryptaminergic axons of rat frontal cortex (Ádori et al., 2011).

In the last years, two studies reported an alteration of mitochondrial movement in hippocampal neurons by

monoamine neurotransmitters 5-HT and DA. Acting through the serotonin 1A receptor subtype, 5-HT increased Akt activity and, consequently, decreased glycogen synthase 3 β (GSK3 β) activity, thus promoting axonal transport of mitochondria (Chen et al., 2007b). Although the molecular target of the Akt–GSK3 β signaling pathway remained unclear, this observation highlights the possibility that 5-HT might act as an extracellular modulator in regulating neuronal ATP distribution by controlling axonal mitochondrial distribution. By contrast, DA and dopamine D₂ receptor agonists inhibited mitochondrial movement via the same Akt–GSK3 β signaling cascade (Chen et al., 2008), suggesting that the distribution of neuronal mitochondria may occur through a conserved regulatory mechanism. Additionally, the Akt–GSK3 β signaling pathway likely achieves this through the regulation of kinesin–cargo interactions (Morfini et al., 2002; Pigino et al., 2003). Considering that the acute and sustained release of monoamine neurotransmitters, including 5-HT and DA, from nerve endings is by far the most studied acute effect of amphetamines in the brain (Capela et al., 2009; Carvalho et al., 2012), these studies provided strong evidences that amphetamines' brain effects may rely on neuronal mitochondrial trafficking alterations.

Recent studies conducted by our research group constituted the first direct evidence that AMPH-like compounds, namely, MDMA, target the neuronal mitochondrial trafficking (Barbosa et al., 2014a, b). In one of those studies, MDMA (1.6 mM), after a short incubation period of 90 min, was shown to reduce the overall mitochondrial movement along axonal processes of cultured neurons from mice hippocampus in a time-dependent manner. This effect was closely associated with increases in intracellular free calcium levels, both in cell body and neuronal extensions, increased phosphorylation of tau protein, at Thr181 residue, and excessive fragmentation of axonal mitochondria (Barbosa et al., 2014a). Overexpressing experiments with wild-type Miro1 or its mutant version (Miro1 Δ EF) lacking the EF hand calcium-binding domains (it does not respond to calcium levels alterations) revealed an independence on Miro1 regulatory functions through cytosolic calcium in MDMA-induced mitochondrial trafficking impairment (Barbosa et al., 2014a). Nevertheless, experiments in tau-null neurons revealed a partial reversion of mitochondrial transport deficits in a similar fashion to those observed in wild-type neurons overexpressing a GSK3 β kinase death construct (GSK3 β Δ 9, which lacks kinase activity—in neurons, tau phosphorylation is largely dependent on GSK3 β) (Barbosa et al., 2014a), supporting a main role for tau protein and GSK3 β in MDMA's mitochondrial trafficking phenotype. Lastly, mitochondrial fusion/fission-regulated events were also shown to play a crucial

role in MDMA-induced mitochondrial trafficking arrest. Overexpression of wild-type Mfn2 or Drp1 K38A (mutant Drp1 construct that lacks fission properties) partially recovered the MDMA's mitochondrial transport phenotype, although overexpression of Mfn2 R94Q (mutant Mfn2 construct that lacks fusion and transport properties) did not (Barbosa et al., 2014a). This indicates that fully functional Mfn2 was required to reverse MDMA's effects and suggest that MDMA might alter fusion mechanisms.

Since MDMA and its metabolites have been shown to coexist in the brain following peripheral administration of MDMA (Chu et al., 1996; Jones et al., 2005; Erives et al., 2008), our research group introduced a new approach, in which parent compound and 6 of its major

in vivo metabolites [α -MeDA, N-Me- α -MeDA, 5-(GSH)- α -MeDA, 5-(GSH)-N-Me- α -MeDA, 5-(NAC)- α -MeDA, and 5-(NAC)-N-Me- α -MeDA] were combined as a mixture, at *in vivo* relevant concentrations (each compound at a equimolar concentration of 10 μ M), and its effects on neuronal mitochondrial trafficking were further appraised. Using this experimental design, a reduction in overall mitochondrial motility along axonal processes of cultured hippocampal neurons following exposure to the mixture for 24 h was shown. Additionally, this effect was shown to rely on mitochondrial fusion/fission-dependent mechanisms, since the overexpression of wild-type Mfn2 or Drp1 K38A almost completely rescued the mitochondrial trafficking arrest caused by this mixture (Barbosa et al., 2014b).

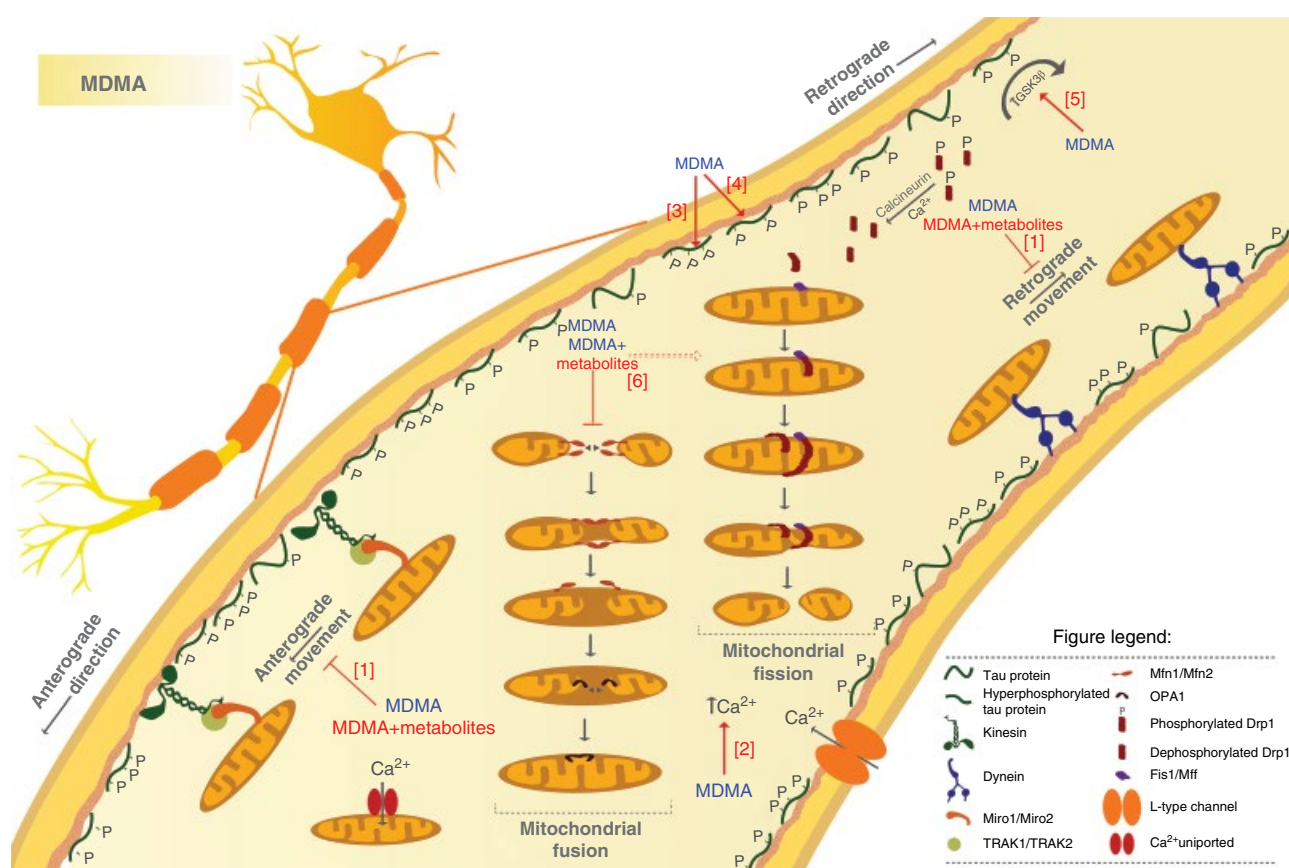


Figure 32.3 Postulated mechanisms for the mitochondrial trafficking impairment induced by MDMA and its metabolites in cultured hippocampal neurons. MDMA, as well as the mixture of MDMA and its metabolites, impair axonal transport of mitochondria, in both anterograde and retrograde directions [1]. Intracellular calcium raises, both in the cell body and neuronal extensions [2], are associated with the transport deficits induced by MDMA, though these effects most likely rely on Miro1-independent mechanisms [2]. Nevertheless, MDMA increases tau phosphorylation at threonine 181 residue [3], which contributes partially to its effects on mitochondrial neuronal trafficking, most likely due to microtubules destabilization [4]. This increase in tau phosphorylation promoted by MDMA appears to be mediated by glycogen synthase 3 β (GSK3 β) [5]. Furthermore, MDMA, as well as the mixture of MDMA and its metabolites, increase the fragmentation of axonal mitochondria. Overexpression of functional mitofusin 2 (Mfn2) or dynamin-related protein 1 (Drp1) K38A, a Drp1 construct that downregulates mitochondrial fission, partially rescue the trafficking deficits triggered either by MDMA or by the mixture of MDMA and its metabolites. Nevertheless, overexpression of Mfn2 R94Q, a Charcot-Marie-Tooth neuropathy 2A (CMT2A) mutant protein with impaired fusion and transport properties, does not recover the MDMA's and the mixture's mitochondrial phenotype, indicating that MDMA, as well as the mixture of MDMA and its metabolites, might alter mitochondrial fusion/fission mechanisms [6] and, thus, compromise mitochondrial transport. Adapted from Barbosa et al. (2015). Reproduced with permission of Springer.

Taking into account the previously referred findings, a mechanistic pathway for the deregulation of neuronal mitochondrial trafficking caused by MDMA and by the mixture of MDMA and its metabolites was postulated, which is illustrated in Figure 32.3.

In vitro studies have been also investigating the mitochondrial patterning in iPS cells and neural progenitor cells following ethanol exposure, as well as in neurons derived from neural progenitor cells. Following ethanol exposure (70 mM, for 7 days), mitochondria clustered more prominently in the perinuclear area of the cells, as revealed 7 days later, thereby indicating a defective mitochondrial distribution into the cell (De Filippis et al., 2016).

Other studies, analyzing the impact of recognized neurotoxins, such as MPP⁺, on mitochondrial transport, have reported disrupted mitochondrial motility in dopaminergic neurons, by oxidative stress-related pathways (Kim-Han et al., 2011), and in PC12 cells, by modification of microtubule dynamics (Cartelli et al., 2010). Nevertheless, to date it remains unclear whether similar mechanisms may mediate the mitochondrial distribution impairment caused by drugs of abuse and addiction.

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32.8 Concluding Remarks

Accumulating evidence has clearly implicated mitochondria as a direct target of drugs of abuse and addiction. Mitochondrial damage promoted by these drugs relies on the inhibition of mitochondrial ETC, perturbations of mitochondrial clearance mechanisms, and interference with mitochondrial dynamics, including mitochondrial biogenesis, fusion/fission, distribution, and mitophagy. Additionally, other studies indicate that the toxicity caused by drugs of abuse and addiction depends on the activation of several mitochondrial pathways. Although it is difficult to attribute a causal association of mitochondrial toxicity to cellular damage, it seems reasonable to consider that they might be closely interconnected. On the other hand, it is also clear that disrupted mitochondrial function may readily be secondary to other events, since it is difficult to attribute any cell injury caused by toxic substances to mitochondrial dysfunction as a primary cause. As such, the importance of these effects to pharmacological actions and toxicity caused by drugs of abuse and addiction requires further research.

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33

Drug-Induced Mitochondrial Toxicity during Pregnancy

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Mitochondria are the powerhouse of the cell. Their main function is energy production coupled to cell respiration in the mitochondrial respiratory chain (MRC). They also have other important functions including lipid, carbohydrate, or protein catabolism, steroid and heme group synthesis, calcium homeostasis, and thermogenesis. However, under pathogenic conditions, mitochondria are a major source of reactive oxygen species (ROS) production and a key factor in the induction of apoptosis (Scheffler 2008; Herst et al. 2017).

Mitochondria are present in most cells of our body, so that impairment of mitochondrial bioenergetics, biogenesis, dynamics, or turnover may endanger cell survival and tissue homeostasis, leading to organ failure and ultimately threatening the life of the patient (Garrabou et al. 2011).

Importantly, they are critical in maintaining the energetic and metabolic supply essential for fertilization, implantation, and embryo development, all of which are highly energy-demanding processes, as are embryonic

cell division, migration, and differentiation. Moreover, they also regulate apoptosis development when selective pruning is crucial for successful embryo development (Nunnari et al. 2012). Consequently, any toxic agent for mitochondria is potentially able to impair oocyte or fetal viability, hampering fertility and prompting further obstetric complications.

Mitochondria contain their own genetic material encoding for 13 MRC proteins (Anderson et al., 1981), while the remaining mitochondrial proteins (thousands in mammals) are encoded by the nucleus. Therefore, intergenomic communication between these two sites is essential for normal mitochondrial function and physiologic processes as pregnancy develops. Some characteristics of primary mitochondrial diseases may arise in the case of xenobiotic mitochondrial toxicities. For instance, in the presence of mitochondrial embryonic alterations at the genetic level, wild-type and mutated mitochondrial DNA (mtDNA) molecules may coexist within a given mitochondria and cell (heteroplasmy). In this case, the type, severity, and onset of clinical manifestations derived from mitochondrial impairment will depend on

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the number of mutated molecules (threshold effect) and differential distribution of these mutated mitochondrial genomes within the various tissues (mitotic segregation), all of which underlie the clinical complexity of mitochondrial toxic disorders (Garrahou et al. 2011). We should not forget that genetic lesions in nuclear DNA can also have mitochondrial consequences independent of mitochondrial genetics and that direct interference of alternative pathways independent of genetics may also arise (e.g., by interfering MRC function, altering oxidative stress or protein synthesis) (Pon and Schon 2007).

In the context of pregnancy, mitochondrial toxicity may be manifested and exclusively restricted to the carrying mother (maternal toxicity) or may be translated into the embryo (fetal toxicity). It can be manifested as infertility, as early pregnancy loss (in case of interference with implantation processes), or as obstetric complications during pregnancy (in case of further interference into fetal development). Fortunately, only few cases of lethal consequences for the newborn have been reported as due to mitochondrial toxicity (Nau et al. 1981; Poirier et al. 2015). However, milder forms of mitochondrial toxicity have been described (Barret et al. 2003; Hernández et al. 2012; Morén et al. 2015; Noguera-Julian et al. 2015; Hernandez et al. 2016), which may further contribute to the development of diseases during adulthood, such as injurious fetal cardiac remodeling (Barker 1999; Crispi et al. 2010; García-Otero et al. 2016; Timpka et al. 2016).

Many compounds can yield mitochondrial damage, and some of them are widely and frequently used in clinical settings. As discussed in this book, off-target effects of drugs can yield mitochondrial toxicity with clinical consequences, and it is a justified inference that such toxicity may entail severe consequences during fertile stages and pregnancy, taking into account that many drugs are able to cross the placental barrier and reach the fetus.

Herein we describe the mechanisms of action and potential secondary mitochondrial effects of drugs currently used in clinical practice during pregnancy in an attempt to increase awareness and caution about their management.

33.1 Mitochondria in Human Fertility

In vitro fertilization studies in humans have demonstrated that adequate mitochondrial function is essential for successful egg fertilization and uterine implantation, the first steps of fetal development (Reynier et al. 2001; Jansen and Burton 2004; Santos et al. 2006). Indeed, mitochondria are maternally inherited, and the oocyte is the sole gamete responsible for providing the mitochondrial population for the future embryo. They also affect male fertility since, although spermatozoa do not provide mitochondria to the future embryo, mitochondrial function is

essential for flagellum motility and, thus, fertilization (Guo et al. 2017). Consequently, reproductive gametes are highly susceptible to mitochondrial toxicity.

The mitochondrial bottleneck, defined as the reduction of the number of mtDNA molecules per mitochondria during meiotic oogenesis, is also crucial in the development of the future embryo (Stewart and Chinnery 2015). Briefly, mitochondrial number increases during oogenesis, accounting for approximately 23% of the ooplasm at the end of the process (Santos et al. 2006), which represents up to 100,000 mitochondria per mature oocyte. However, although the number of mitochondria increases, mtDNA levels remain constant, resulting in reduced mtDNA content per mitochondria, estimated into 100,000-600,000 molecules per mature oocyte. The aim of this evolutionary strategy is to ensure the presence of only a few molecules of mtDNA per mitochondrion to avoid heteroplasmic segregation through the maternal lineage (Santos et al. 2006). This renders the oocyte mitochondria highly susceptible to mtDNA depletion or mutagenic factors, such as infections, environmental toxins, drugs of abuse, and medications, which may exert secondary effects including infertility or obstetric complications (López et al. 2008).

33.2 Mitochondrial Toxicity in Human Pregnancy

33.2.1 Risk Categories of Mitochondrial Toxic Drugs According to their Capacity to Cause Birth Defects during Pregnancy

Once the oocyte is fertilized and implanted, mitochondria remain essential to fuel adequate fetal development. Thus, exposure to mitochondrial hazards should be minimized. Notwithstanding, some therapeutic schedules are unavoidable.

During pregnancy, acute disorders, such as migraine or infectious diseases, may require short-term treatment with potential mitochondrial toxic medication. Similarly, chronic conditions such as epilepsy, depression, or asthma may coexist and require treatment. Mothers are giving birth at increasingly older ages, which in turn increases the likelihood of chronic conditions and coexistence of pathologies associated with pregnancy such as hypertension, diabetes, and mood disorders, among others. Additionally, in early pregnancies, women who are not yet aware of their condition may use medication, increasing the risk of suffering its secondary effects.

The Food and Drug Administration (FDA) (USA) and Therapeutic Goods Administration (TGA) (AU) have established risk categories to indicate the potential of a therapeutic drug, when administered during pregnancy, to cause birth defects. Table 33.1 summarizes risk

Table 33.1 The Food and Drug Administration (FDA) (USA) and Therapeutic Goods Administration (TGA) (AU) classification for mitochondrial toxic drugs potentially used during pregnancy depending on their association to birth defects. Source: Food and Drug Administration (www.fda.gov) and Therapeutic Drugs Administration (<https://www.tga.gov.au/node/4013>)

Food and Drug Administration (USA)	Therapeutic Goods Administration (AU)	Drugs that exert mitochondrial toxicity and may be used in pregnancy
A Adequate and well-controlled studies have failed to demonstrate a risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters)	A Drugs that have been taken by a large number of pregnant women and women of childbearing age without any proven increase in the frequency of malformations or other direct or indirect harmful effects on the fetus having been observed	Erythromycin (TGA), paracetamol (TGA), lidocaine (TGA), miconazole (TGA)
B Animal reproduction studies have failed to demonstrate a risk to the fetus, and there are no adequate and well-controlled studies in pregnant women	B1 Drugs that have been taken by only a limited number of pregnant women and women of childbearing age, without an increase in the frequency of malformation or other direct or indirect harmful effects on the human fetus having been observed. Studies in animals have not shown evidence of an increased occurrence of fetal damage	Erythromycin (FDA), azithromycin (FDA), lidocaine (FDA), didanosine (FDA), saquinavir, nelfinavir (FDA), ritonavir (FDA), nevirapine (FDA), amphotericin B (FDA)
	B2 Drugs that have been taken by only a limited number of pregnant women and women of childbearing age, without an increase in the frequency of malformation or other direct or indirect harmful effects on the human fetus having been observed. Studies in animals are inadequate or may be lacking, but available data show no evidence of an increased occurrence of fetal damage	Didanosine (TGA), nelfinavir (TGA)
	B3 Drugs that have been taken by only a limited number of pregnant women and women of childbearing age, without an increase in the frequency of malformation or other direct or indirect harmful effects on the human fetus having been observed. Studies in animals have shown evidence of an increased occurrence of fetal damage, the significance of which is considered uncertain in humans	Linezolid (TGA), clarithromycin (TGA), clozapine (FDA), stavudine (TGA), zidovudine (TGA), lamivudine (TGA), abacavir (TGA), indinavir (TGA), ritonavir (TGA), nevirapine (TGA), ketoconazole (TGA), amphotericin B (TGA)
C Animal reproduction studies have shown an adverse effect on the fetus, and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks	C Drugs that, owing to their pharmacological effects, have caused or may be suspected of causing harmful effects on the human fetus or neonate without causing malformations. These effects may be reversible. Accompanying texts should be consulted for further details	Clarithromycin (FDA), rifampicin (FDA), linezolid (FDA), amitriptyline, clomipramine, fluoxetine, sertraline, haloperidol, risperidone, clozapine (TGA), paracetamol (FDA), aspirin (TGA), diazepam (TGA), bupivacaine, zalcitabine (FDA), stavudine (FDA), zidovudine (FDA), lamivudine (FDA), abacavir (FDA), indinavir (FDA), ketoconazole (FDA), miconazole (FDA), amiodarone (TGA)
D There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks	D Drugs that have caused, or are suspected to have caused or may be expected to cause, an increased incidence of human fetal malformations or irreversible damage. These drugs may also have adverse pharmacological effects. Accompanying texts should be consulted for further details	Aminoglycosides (gentamicin, amikacin), aspirin (FDA), phenytoin, phenobarbital, carbamazepine, valproic acid (TGA), diazepam (FDA), zalcitabine (TGA), amiodarone (FDA)
X Studies in animals or humans have demonstrated fetal abnormalities, and/or there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience, and the risks involved in the use of the drug in pregnant women clearly outweigh potential benefits	X Drugs that have such a high risk of causing permanent damage to the fetus that they should not be used in pregnancy or when there is a possibility of pregnancy	Thiamphenicol, valproic acid (FDA)

Many other therapeutic drugs are responsible for mitochondrial toxicity, but their administration is restricted during pregnancy. FDA and TGA may differ in the classification of the drugs depending on local policies, as stated in Table 33.1. Source: Food and Drug Administration (www.fda.gov) and Therapeutic Drugs Administration (<https://www.tga.gov.au/node/4013>)

categories for drugs administered during pregnancy that have been associated with mitochondrial toxicity. These agents have varying mitochondrial mechanisms of toxicity and potencies, as discussed in this chapter.

In June 2015 the FDA classification was replaced with meaningful information to patients and healthcare providers in order to allow better patient-specific counseling and informed decision making. While the new labeling improves the old format, it still does not provide definitive treatment guidance to avoid adverse events so that clinical interpretation is still required on a case-by-case basis.

33.2.2 Clinical Spectrum of Mitochondrial Toxicity during Pregnancy

Given their ubiquitous distribution and varying energetic demands of different tissues, mitochondrial toxicity can be translated into a wide spectrum of clinical symptoms that, in case of pregnancy, can affect both the maternal and the fetal compartment (Andreu and Gonzalo-Sanz 2004, Blohm 2017). Thus, it is difficult to predict the clinical picture or achieve a definite diagnosis for mitochondrial toxicity, not to mention that there are few avenues for therapeutic management.

Mitochondrial toxicity usually affects those tissues that are most energy demanding, such as the nervous system, cardiac and skeletal muscle, renal, liver, and lymphoid tissues. In the case of nonpregnant adults, the symptoms range from myopathy, neuropathy, encephalopathy, and lactic acidosis to lipodystrophy and sensory pathology such as deafness and blindness (Taylor and Turnbull 2005). However, in the context of pregnancy, mitochondrial insufficiencies have been linked to higher prevalence of obstetric complications including gestational diabetes mellitus, preterm delivery, miscarriage, stillbirth, intrauterine growth restriction, preeclampsia, premature rupture of membranes, or postnatal sudden infant death (Mando et al. 2014).

As seen in Table 33.1, a variety of drugs that are used in the clinical setting may yield mitochondrial toxicity and most of them cross the placental barrier reaching the fetus (Latini et al. 1984; Hendrick et al. 2003; Pacifici 2005, 2006; De Santis et al. 2011; Antonucci et al. 2012; Singh et al. 2016). Of all the drugs herein exposed, the less likely to reach the fetus are local anesthetics and antifungals, mainly due to the limited dosage and local effect, that hamper reaching significant blood concentrations, if any.

Although mitochondrial toxicity is widely documented in nonpregnant adults, little information is known within the context of human pregnancy, particularly in terms of *in utero* exposure and potential impact on fetal development and newborn health. Most of the data are restricted

to *in vitro* studies, cell lines, and animal models, and only few observational studies have been done in patients. Of course, studies are also needed to characterize placental permeability and hence fetal exposures.

Therapeutic drugs exert their mechanism of action through different means and, as a secondary effect of medication, mitochondrial physiology can be disturbed at different levels, such as causing genetic alterations (through depletion, deletions, and/or point mutations), MRC dysfunction (Zhang et al. 1990; Davey et al. 1998), deregulation of oxygen consumption, increase of ROS levels (Wallace and Melov 1998), depolarization of membrane potential, inhibition of mitochondrial protein synthesis (Scatena et al. 2007), imbalance of mitochondrial dynamics, or mitochondrial biogenesis, among others (see Figure 33.1). The different kinds of toxic mechanisms, together with the threshold effect and the mitotic segregation phenomenon, in case of mitochondrial genetic alterations, explain the heterogeneous clinical manifestations observed in exposed pregnant women and their newborn, regardless of similar type, time, and dose exposure to the toxic compound (Gröber 2012).

33.2.3 Classes of Mitochondrial Toxic Drugs Administered during Pregnancy

Numerous drugs have been described to potentially cause mitochondrial toxicity, such as antineoplastics (including flutamide, tamoxifen, and doxorubicin), antifungals (such as sodium azide), antidiabetics (metformin), fibrates, and antimalarials. Since their use is not indicated in pregnancy, the interested reader is referred to other sources of bibliography for a complete description of their off-target effects (Morén et al. 2014, 2016). Rather, the focus of this chapter is the description of drugs potentially administered during pregnancy having potential mitochondrial toxicity and their clinical consequences.

33.2.3.1 Antibiotics

The endosymbiotic origin of mitochondria (Margulis 1975) explains the homology shared between mitochondria and prokaryotes and why some antibiotics can exert off-target mitochondrial damage leading to severe clinical manifestations including fetal death (Lang et al. 1999). This is the case of some antibiotics, including aminoglycosides, rifampicin, linezolid, and macrolides, which act as inhibitors of bacterial and mitochondrial protein synthesis, eventually leading to well-known side effects, including deafness (Torres-Ruiz et al. 2011), peripheral neuropathy (Bressler et al. 2004), hyperlactatemia, or lactic acidosis (Del Pozo et al. 2014) in nonpregnant adults (Hong et al. 2015). Of this class of antibiotics, thiamphenicol has been used for abortive

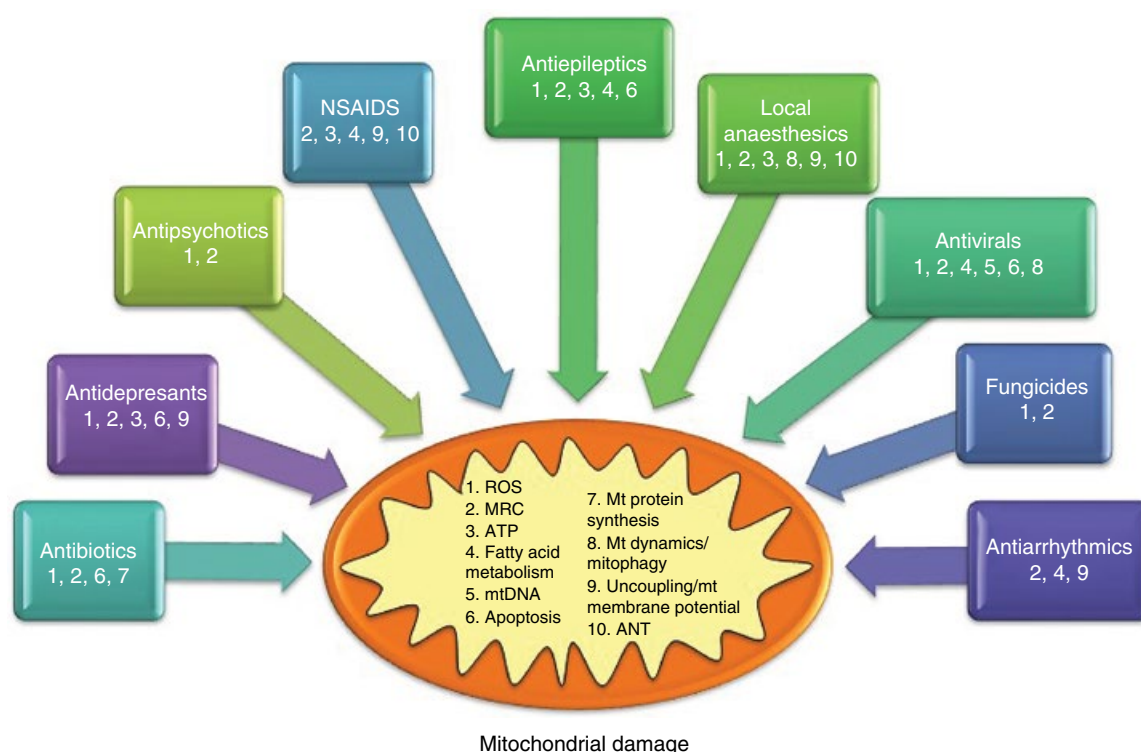


Figure 33.1 Drug-induced mitochondrial toxicity. Different degrees of evidence (in experimental models, nonpregnant humans, or human pregnancies) sustain the mitochondrial toxicity of drugs. The central mitochondria depict 10 mitochondrial alterations caused by exposition to the described drugs. The external squares represent the specific mitochondrial toxicity (in numbers) studied in experimental models (green), in nonpregnant humans (not necessarily excluding studies in experimental models; in purple), or in human pregnancies (not necessarily excluding studies in experimental models or nonpregnant humans; in blue). NOTE: The administration of the aforementioned drugs must always be prescribed by a clinician and only recommended when the therapeutic benefits exceed the potential adverse effects derived from the mitochondrial toxicity of these drugs, which should be closely monitored. ANT, adenine nucleotide translocator; ATP, adenosine triphosphate; MRC, mitochondrial respiratory chain; Mt, mitochondrial; mtDNA, mitochondrial DNA; NSAIDS, nonsteroidal anti-inflammatory drugs; ROS, reactive oxygen species.

purposes due to the severe mitochondrial toxicity for the exposed fetus (Nau et al. 1981). Macrolides, such as erythromycin, have been additionally associated with ROS-induced alteration of potassium channel kinetics, which disrupts cardiomyocyte conductance and enhances apoptosis even at concentrations lower than those to be hepatotoxic in animal models (Guo et al. 2010; Salimi et al. 2016). In population-based studies of nonpregnant adults, macrolides are associated with increased rates of cardiac defects (Bar-Oz et al. 2012; Lin et al. 2013; Bérard et al. 2015), but little is known in the context of human pregnancies.

33.2.3.2 Antidepressants

Depression is the most prevalent of the mental disorders requiring treatment during pregnancy and reproductive age. Tricyclic antidepressants (TCA) such as amitriptyline, clomipramine, and desipramine were the first drugs administered for mood disorders. Their therapeutic effect is achieved by the inhibition of serotonin, norepinephrine, and dopamine reuptake into presynaptic

vesicles in the brain in a nonselective manner, leading to significant side effects, such as memory loss. This type of drugs secondarily inhibits the enzymatic activities of MRC complexes I, II/III, and IV (Abdel-Razaq et al. 2011), thereby increasing oxidative stress and apoptosis in experimental models (Lee et al. 2015).

Selective serotonin reuptake inhibitors (SRIs) are better tolerated than TCA, making them the most widely prescribed antidepressants worldwide. Fluoxetine inhibits mitochondrial respiration at complex I (Hroudová and Fišar 2012), has effects on ATPase activity, and interferes with the physical characteristics of the inner mitochondrial membrane in animal models, which could be responsible for the gastrointestinal discomfort caused by this drug in nonpregnant adults (Curti et al. 1999). It has also been reported that fluoxetine prevents apoptosis by inhibiting mitochondrial permeability transition pore opening and cytochrome *c* release in a cell model (Nahon et al. 2005). However, no adverse effects directly related to fluoxetine have been reported in newborns of exposed women (Riggin et al. 2013). Sertraline uncouples mitochondrial oxidative

phosphorylation and inhibits the activities of CI and V, as demonstrated in an experimental model (Li et al. 2012). Although additional data are needed (Tuccori et al. 2009), sertraline has been shown to induce apoptosis in experimental models (Chen et al. 2014) and has been suggested to be responsible for an increased risk of neonatal pulmonary hypertension (Kieler et al. 2011; Andrade 2012).

Further studies are needed to confirm the safety of TCA and SRIs in the context of human pregnancy.

33.2.3.3 Antipsychotics

Although less prevalent than depression, other mental disorders such as schizophrenia, bipolar disorder, and psychotic depression may develop or become exacerbated during pregnancy. Antipsychotic drugs should be carefully managed in this setting. Mitochondrial toxicity has been reported to be the potential base of extrapyramidal disorders (Casademont et al. 2007), as well as polymorphisms in cytochrome p450 and aging, among others. Antipsychotics have been classically described to inhibit MRC complex I function (Przedborski et al. 1992; Maurer and Möller 1997), leading to an increase of oxidative stress in nonpregnant adults (Martins et al. 2008; Raudenska et al. 2013). Differential severity of molecular and clinical toxicity has been established from high to low potency as follows: haloperidol, risperidone, and clozapine (Casademont et al. 2007). Despite these toxicities, we are unaware of any studies of mitochondrial toxicity of neuroleptic drugs during human pregnancies. It is reported, though, that antipsychotics are associated with increased gestational weight and diabetes as well as with increased risk of preterm birth. The effects of antipsychotics on low birth weight or malformations are inconclusive, but these effects have been reported to be a primary cause for treatment withdrawal (Contreras-Shannon et al. 2013; Gonçalves et al. 2014).

33.2.3.4 Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been prescribed worldwide to reduce inflammation and pain and are contraindicated during pregnancy, except for paracetamol and, in case of recurrent pregnancy loss or high risk of preeclampsia, aspirin. They have been reported to induce uncoupling of oxidative phosphorylation, to increase resting state respiration, to decrease ATP synthesis and mitochondrial membrane potential, to inhibit adenine nucleotide translocase, and to alter mitochondrial lipid metabolic pathways in animal-derived experimental models, which may account for NSAID-induced gastrointestinal injury in nonpregnant adults (Matsui et al. 2011). However, little information is available on the potential effects in fetal development and obstetric complications of human pregnancies.

33.2.3.5 Antiepileptics

The administration of an adequate treatment plays a pivotal role in the well-being of the mother and the developing fetus in those pregnant women with preexistent epilepsy and those who develop seizures secondary to eclampsia. Unfortunately, this therapeutic approach is not free of adverse effects. For example, barbiturates have been shown to impair mitochondrial lipid metabolic pathways (Santos et al. 2008), and benzodiazepines may increase apoptosis in experimental models (Contreras-Shannon et al. 2013). Differential mitochondrial toxicity has been described *in vitro* with potencies from high to low with phenytoin, phenobarbital, and carbamazepine (Santos et al. 2008), inhibiting mitochondrial respiration and ATP synthesis. Valproic acid has also been strongly associated with mitochondrial toxicity through the interference of β -oxidation pathways, mitochondrial respiration, and increasing ROS metabolism (Rumbach et al. 1986; Silva et al. 2008; Komulainen et al. 2015) in experimental models. All these molecular alterations have been associated with hepatotoxicity in nonpregnant adults. Valproic acid has been additionally related to severe teratogenic effects, such as neural tube defects (Ornoy 2009). Unfortunately, scarce data are available regarding mitochondrial toxicity of these drugs in human pregnancies (Tomson et al. 2011; Battino et al. 2013).

33.2.3.6 Local Anesthetics

Local anesthetics, such as bupivacaine or lidocaine, have been reported to induce mitochondrial damage through uncoupling of oxidative phosphorylation, inhibiting mitochondrial ATPase or other MRC complexes, as well as increasing ROS and mitophagy in experimental models and patients (Szewczyk and Wojtczak 2002; Nouette-Gaulain et al. 2011). However, their use during pregnancy might be unavoidable if an intervention is needed, and, consequently, the study of derived mitochondrial toxicity in human pregnancy becomes pressing. Indeed, it has been reported that mitochondrial toxicity is associated with postoperative pain and in one case of postanesthetic myopathy (Hogan et al. 1994).

33.2.3.7 Antivirals

The use of antivirals is essential in the treatment of infection and prevention of transmission of infections caused by human immunodeficiency virus (HIV) or hepatitis C virus (HCV). Although their administration is considered safe for the mother and child, they have been associated with mitochondrial toxicity, which may trigger related adverse effects as preeclampsia, stillbirth, preterm birth, and low birth weight (Suy et al. 2006; Townsend et al. 2007; Haeri et al. 2009; Rudin et al. 2011), as well as fetal remodeling potentially leading to disease in adulthood (García-Otero et al. 2016). Importantly,

there are still lingering concerns about their short-term (Barret et al. 2003) or long-term health consequences (García-Otero et al. 2016) and the ramifications of their effects on lipid, glucose, intermediary, and mitochondrial metabolism (Kirmse et al. 2013).

The potential clinical risks associated with antiretroviral exposure in HIV pregnant women and their fetuses and infants have been described in observational studies with varying degrees of evidence and conflicting results (Brocklehurst and French 1998; Lambert et al. 2000; Thorne et al. 2004; Tuomala et al. 2005; Hernandez et al. 2017). Such risks have been mainly attributed to nucleoside reverse transcriptase inhibitors (NRTI) and, to a lesser extent, protease inhibitors (PI) and non-NRTI. First-generation NRTIs (didanosine, zidovudine, and stavudine) have long been associated with mitochondrial toxicity due to inhibition of the endogenous mitochondrial γ -DNA polymerase, the enzyme responsible for mtDNA replication and repair (Brinkman et al. 1998). γ -DNA polymerase inhibition affects the copy number and quality of mtDNA through point mutations, deletions, and depletions, ultimately triggering mitochondrial dysfunction, as has been documented in both experimental models and patients. Over time, damaged mitochondria become unable to perform metabolic functions, leading to apoptosis (Viora et al. 1997; Brinkman et al. 1999; Mallal et al. 2000; Petit et al. 2005; Opii et al. 2007; Langs-Barlow et al. 2015). Clinically, these molecular drawbacks have been linked to myopathy, hyperlactatemia, lactic acidosis, polyneuropathy, or pancreatitis in nonpregnant adults (Lewis and Dalakas 1995; Cherry and Wesselingh 2003; Garrabou et al. 2009). Fortunately, second-generation NRTIs are being commercialized with reduced mitochondrial and clinical toxicity (Morén et al. 2012; Mouton et al. 2016). Protease inhibitors are associated with mitochondrial and apoptotic effects in nonpregnant adults and clinically to metabolic syndrome and lipodystrophy, although there remains some controversy (Phenix et al. 2001; Estaquier et al. 2002). Some non-NRTIs have been weakly linked to apoptosis in nonpregnant adults, and few adverse clinical manifestations have been associated with their use.

Most of the mitochondrial studies performed in HIV pregnancies have described an increased frequency of adverse obstetric events in the HIV cohort (Poirier et al. 2003; Shiramizu et al. 2003; Aldrovandi et al. 2009; Hernández et al. 2012). Additionally, most of the studies have described different degrees of evidence of mitochondrial toxicity in pregnant women and their newborn, occasionally accompanied by the development of apoptosis in maternal, fetal, or placental tissue (Hernández et al. 2012; Ross et al. 2012; Morén et al. 2015; Noguera-Julian et al. 2015; Hernandez et al. 2016). Recent evidence points to alterations in mitochondrial dynamics and

mitophagy in cohorts of HIV pregnant women undergoing treatment (Guitart-Mampel et al. 2017). However, no direct causal effects between mitochondrial toxicity and adverse pregnancy outcomes have been demonstrated in most of these studies (Jao et al. 2017), perhaps because of their reduced statistical power. A few studies directly associate mitochondrial toxicity with rare but lethal antiretroviral exposure in children born from HIV mothers (Poirier et al. 2015). Further studies in larger cohorts are needed, see Chapter 34 for more information on HAART therapy and mitochondrial toxicity.

33.2.3.8 Antifungals

Vaginal fungal infections may also appear in the course of pregnancy. Antifungals have been reported to inhibit complex I and II enzymes of the MRC necessary for mitochondrial oxidative phosphorylation, potentially leading to increased ROS production in experimental models (Rodriguez and Acosta 1996). Examples of these drugs, mainly used topically, are ketoconazole, miconazole, and amphotericin B. As is the case for many drugs, few data are available on the potential obstetric complications in human pregnancies.

33.2.3.9 Antiarrhythmics

Exceptionally, antiarrhythmics may need to be administered to a pregnant woman. Amiodarone is used in the treatment of many ventricular and supraventricular arrhythmias and is particularly useful for converting atrial fibrillation. Its more serious adverse effects include thyroid dysfunction, hepatocellular liver damage, or pulmonary fibrosis. The hepatotoxicity mechanism could be immune or mitochondrial mediated, as an impairment of mitochondrial function, with uncoupling of oxidative phosphorylation, inhibition of MRC complexes I–III, and inhibition of fatty acid β -oxidation as has been demonstrated in experimental conditions (Spaniol et al. 2001). Again, scarce data is available in the context of pregnancy and human beings.

33.3 Therapeutic Approach of Drug-Induced Mitochondriopathies

Although acute or chronic exposure to the above mentioned mitotoxic drugs may be considered potentially harmful during conception or pregnancy, few studies have evaluated the clinical consequences and potential toxicities of such exposure in human beings. Thus, exposure to these drugs should be minimized or avoided, if possible, during pregnancy, except under specific clinical circumstances; it is always preferable to prevent, rather than to deal with, the secondary effects of therapies.

When prescription is mandatory, clinicians should be aware of early signs of mitochondrial toxicity to avoid further manifestation of adverse clinical effects. If symptoms appear, clinicians should plan strategies to revert the manifestation of symptoms. Whenever there is clinical evidence of suspect, early management should help to achieve faster mitochondrial and clinical recovery. The discontinuation of such exposure should be considered as the most efficient therapeutic approach. Unfortunately, this may not always be possible. If the withdrawal of the given mitotoxic drug is not feasible, then secondary effects should be treated. To date, mitochondrial treatments are exclusively supportive and symptomatic with limited proven efficacy and, again, not yet evaluated in pregnant women. Such mitochondrial treatments may consist of the administration of certain mitochondrial-targeted drugs (vitamins, enzymatic cofactors, or antioxidants), performing blood transfusions, hemodialysis, dietary measures (Finsterer 2010; Monteiro et al. 2014), and physiotherapy (Andreu and Gonzalo-Sanz 2004; Pedrol et al. 2005; Artuch et al. 2006; Finsterer 2009).

Despite of the restricted possibilities, these strategies have been demonstrated to modify the onset, evolution, and outcomes of patients suffering from mitochondrial toxicities. This has been widely shown in HIV-infected nonpregnant adults. Current guidelines of HIV infection and antiviral treatment in case of mitochondrial toxicity promote strategies based on (i) substituting antivirals to less toxic regimens for mitochondria, (ii) reducing antiretroviral doses, (iii) changing antiviral schedules to nucleoside-sparing therapies, or (iv) implementing structured treatment interruptions (Mussini et al. 2005; Negrodo et al. 2009; Negrodo et al. 2010). Similarly, in case of symptomatic hyperlactatemia and lactic acidosis as consequences of mitochondrial toxicity during HIV management in nonpregnant adults, concomitant administration of mitochondrial treatments (L-carnitine, B6 and C vitamins, thiamine, and hydroxocobalamin) has been associated with faster and most efficient recovery of patients (Pedrol et al. 2005). The utility of these supportive measures remains controversial as long as they are not tested in pregnant women.

In case of human pregnancies, novel approaches based on *in silico* modeling and system pharmacology, aimed to design and predict on-target and off-target effects of a drug before administration, should be considered in order to maximize its therapeutic action and minimize its toxic consequences (Bai et al. 2014). This way, the assessment of the therapeutic/safety index would be taken into account during drug development to prompt an adequate benefit/risk ratio.

Additionally, the implementation of biomarkers to monitor mitochondrial toxicity derived from the intake

of these drugs should also be considered in the clinical settings and in the cohort of pregnant women and those wishing to conceive.

33.4 Conclusions

Few therapies, if any, are free of adverse effects. The aforementioned therapeutic drugs currently used in medical practice may trigger mitochondrial toxicity, which may ultimately underlie adverse clinical events. Although their manifestations are usually subclinical, they may occasionally lead to serious off-target effects in both mother and fetus. Most of these adverse mitochondrial effects are manifested through infertility or obstetric complications. However, mitochondrial toxicity may also contribute to fetal diseases and, by triggering irreversible physiologic alterations and fetal remodeling, to pathology emerging during adulthood.

As the current mitochondrial therapies are symptomatic rather than therapeutic, the prevention of mitochondrial toxicities is the best prophylactic choice.

As a matter of course, medication should be avoided in case of pregnant women or those wishing to conceive, unless strongly needed. In case of compulsory treatment, medical advice has to take precedence over secondary mitochondrial events. Clinicians should be aware of these toxicities and circumventing strategies (especially treatment interruption or substitution of toxic drugs by alternative compounds).

Further studies are needed to better understand these mitochondrial toxicities in human pregnant women based on observational clinical case studies or large epidemiologic cohorts follow-up, especially in the case of chronic or unavoidable treatments.

In case of mandatory or accidental therapeutic exposures to these mitotoxic drugs, there is the crucial need to (i) find less toxic substances, (ii) establish novel or already described biomarkers into clinical settings for monitoring toxicity during pregnancy, and (iii) elaborate guidelines to assess risk–benefit ratios in cases of therapy administration.

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Mitochondrial Toxicity in Children and Adolescents Exposed to Antiretroviral Therapy

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34.1 Introduction

In the late 1990s, the implementation of highly active antiretroviral (ARV) therapies (HAART) dramatically changed the prognosis in vertically acquired human immunodeficiency virus (HIV) infection. The benefit obtained from HAART in terms of morbidity and mortality in HIV-infected children is beyond question, but ARV-related toxicities in pediatric patients have been increasingly reported over the past 20 years (Brewinski et al., 2011; Alam et al., 2012; Piloya et al., 2012; Espiau et al., 2016). Worldwide, current recommendations agree that all children and adolescents should be put on HAART as soon as HIV infection is diagnosed, leading to potential lifelong exposure to these drugs and to the adverse effects associated with them (WHO, 2010; Panel CDC Child, 2016; Foster et al., 2017). Together with long-term metabolic complications, complex HAART regimens, insufficient pharmacokinetic and pharmacodynamic data, and lack of appropriate formulations represent a challenge for compliance to medications in HIV-infected children and ultimately may lead to viral failure and emergence of drug-resistant viral mutations.

ARV-associated metabolic complications were initially documented in adult patients, but all of them have now been reported in pediatric and adolescent patients.

These include mitochondrial toxicity, lipodystrophy syndrome (abnormal body fat distribution, including lipoatrophy, lipohypertrophy, and the mixed pattern), dyslipidemias, insulin resistance, cardiovascular and cerebrovascular risk, low bone mineral density, and renal toxicity (reviewed in Fortuny et al., 2015). In the late 1990s, HIV-infected teenagers from America and Europe were shown to be affected initially in case series and then in cohort studies. Later, and because of the widespread availability of ARV therapy, metabolic adverse events have also been described in resource-limited settings (Brewinski et al., 2011; Piloya et al., 2012) and in younger children (Hazra et al., 2013; Langat et al., 2013). Although exposure to certain ARVs plays a central role in the development of these clinical syndromes, most of them have also been reported in the naïve patient underscoring the deleterious effects of chronic inflammation due to uncontrolled HIV infection, also in the development of metabolic comorbidities.

On the other hand, routine use of HAART in HIV-infected pregnant women has led to a dramatic decrease in HIV mother-to-child transmission (MTCT) rates to below 1–2% in the last 15 years. Actually, the latter represents one of the major successes since the HIV pandemic began in the mid-1980s. Recommended HAART regimens during pregnancy include a dual nucleoside

reverse transcriptase inhibitor (NRTI) plus a third drug, usually a protease inhibitor or a non-NRTI. HIV-exposed infants routinely receive zidovudine (ZDV) prophylaxis for the first 4–6 weeks of life (WHO, 2010; Panel CDC Pregnancy, 2016). ARV-related short- and long-term toxicity in the HIV-exposed uninfected (HEU) infant remains of concern, with hematological and mitochondrial toxicity being the most commonly observed consequences (reviewed in Sollai et al., 2015).

The available studies in the pediatric population are limited by the number of patients, the observational nature of the studies, and the lack of long-term follow-up and appropriate control groups of HIV-uninfected peers. Ethnic background and different stages of growth and development, such as puberty, are also known to have an influence on the emergence or worsening of some of these adverse events, as well as some other HIV-unrelated factors such as malnutrition in resource-limited settings, obesity, coinfections, amount of solar exposure, and genetic background. Moreover, differences in definitions and methods used to determine some of these conditions undermine results and confound comparisons between studies.

34.2 Mitochondrial Toxicity in Children and Adolescents Infected with HIV

Mitochondrial alterations have long been associated with HIV infection and with ARV drugs in adulthood and children (Foster and Lyall, 2008; Koczor and Lewis, 2010). NRTIs are known to inhibit both HIV reverse transcriptase and DNA polymerase gamma, the endogenous enzyme responsible for replication and repair of the mitochondrial DNA (mtDNA) genome (Brinkman et al., 1998; Carr and Cooper, 2000; Lim and Copeland, 2001; Côté et al., 2002). This leads to a reduction in mtDNA production and to an increase in the number of point mutations and deletions. Protease inhibitors have also been described as causing mitochondrially induced apoptosis (Dowell et al., 2000). Many of the metabolic adverse events in HIV-infected persons are ascribed to, to varying extents, mitochondrial toxicity due to inhibition of mitochondrial DNA polymerase gamma and secondary depletion of mitochondrial DNA in many tissues including fat, muscle, peripheral blood mononuclear cells, and others (Saitoh et al., 2008; Morén et al., 2011a, Sharma et al., 2013; Takemoto et al., 2017; 2012).

In some cohorts of HIV-infected children, chronic, symptom-free hyperlactatemia (below 5 mmol/L; normal values up to 2.1 mmol/L) has been reported in 17–68% of patients (Desai et al., 2003; Noguera et al., 2003; van Ramshorst et al., 2014). In our experience, all

children with elevated lactate concentrations (peak values up to 4.9 mmol/L) were receiving at least one NRTI, with optimal immunological and virological responses, and, in half of the cases, lactate values spontaneously returned to normal despite no changes in treatment. Regression analysis revealed that a younger age at the beginning of HAART was the only risk factor for hyperlactatemia (Noguera et al., 2003). In a recent study in rural South Africa in which 80% of the patients were receiving stavudine, hyperlactatemia was associated with the development of lipodystrophy, higher baseline CD4 counts, and longer HAART treatment duration (van Ramshorst et al., 2014). Such hyperlactatemia in HIV-infected children shows a chronic, compensated, and asymptomatic evolution and has a poor predictive value for symptomatic lactic acidosis.

Symptomatic hyperlactatemia and lactic acidosis are rare in all age groups and usually occur within the first months after starting HAART. Why only a very small percentage of these children develop clinical symptoms is likely a reflection of the mitochondrial threshold effect (Brinkman et al., 1998). In the last 10 years, the increased availability of more tolerable and safer NRTIs, plus new ARV drugs, has yielded a decline in the incidence of symptomatic hyperlactatemia and lactic acidosis.

The clinical presentation of lactic acidosis is usually insidious and unspecific and can include gastrointestinal symptoms (nausea and vomiting, abdominal pain), dyspnea, peripheral neuropathy, and systemic symptoms (fatigue, weakness, weight loss, and myalgias), developing over several days or presenting as fulminant multi-organ failure often including hepatic and pancreatic involvement. The diagnosis of lactic acidosis is by exclusion, and other conditions potentially leading to secondary acidosis such as sepsis and diarrhea, among others, need to be ruled out. Elevated blood lactate levels (usually >5 mmol/L) together with elevated anion gap metabolic acidosis or elevated alanine levels will confirm the diagnosis. In such cases, the concomitant determination of amylase, lipase, serum albumin, and hepatic transaminases is recommended. However, in the absence of symptoms, the routine measurement of serum lactate is not recommended, because of its low specificity (Brinkman, 2001; Panel CDC Child, 2016). In any case, because of ongoing glycolytic erythrocyte metabolism, the proper collection and processing of venous blood for lactate determination is critical to avoid false elevated levels. Prolonged tourniquet application is discouraged, and, ideally, venous blood needs to be collected in a prechilled fluoride–oxalate-containing tube, put on ice, and processed within 15–30 min of collection (Andersen et al., 2013).

Symptomatic hyperlactatemia with lactic acidosis has been reported with all available NRTIs (Church et al., 2001; Rey et al., 2003; Rosso et al., 2003; Shah, 2005), but

stavudine and didanosine carry the highest risk, especially when the two drugs are used together; in fact, the use of stavudine is discouraged in the most recent treatment guidelines, while the use of didanosine is either no longer considered a first-line option or discouraged (WHO, 2010; Bamford et al., 2015; Panel CDC Child, 2016). Other risk factors for lactic acidosis that have been described in the adult patient are female gender, advanced HIV infection, CD4 cell counts below 350/mm³, African–American race, hepatitis C virus coinfection, pregnancy, elevated body mass index, and coadministration of other drugs with either stavudine or didanosine, such as metformin, tetracycline, tenofovir, and ribavirin (Panel CDC Child, 2016).

Lactic acidosis is probably the only metabolic adverse event in which immediate discontinuation of all ARV agents is mandatory. Early diagnosis and implementation of supportive therapy (intravenous fluids including bicarbonate infusions, oxygen, respiratory support, etc.) is critical, because lactic acidosis is associated with high mortality rates in the adult patient (33–58%). Together with supportive therapy, supplementation with high-dose vitamins (thiamine and riboflavin), carnitine, vitamin C, or coenzyme Q10 has been recommended. A nucleoside-sparing regimen or a HAART combination including *mitochondrion-friendly* nucleosides, including abacavir, tenofovir, and lamivudine/emtricitabine, is recommended for resuming treatment in patients previously affected with an episode of symptomatic hyperlactatemia; close clinical follow-up and monthly monitoring of lactate during the first months of a new HAART regimen is highly recommended in these cases.

In the research setting, the pathogenesis of HIV- and HAART-associated mitochondrial dysfunction in HIV-infected patients has been much less studied in children and adolescents compared with adults. In pediatric patients, most of the available studies have focused on the analysis of mitochondrial metabolism in peripheral blood mononuclear cells (PBMCs), which retains the tissue of choice because of the ease of sampling, despite other limitations (Maagaard et al., 2006). Our group has demonstrated a mild depletion of mtDNA in a cohort of asymptomatic HIV-infected children on HAART when compared with healthy controls (Morén et al., 2011b). Similar levels of mitochondrial RNA, complex IV protein subunit content, and enzymatic activity (measured in PBMCs) were observed between these groups, and these findings were not altered by mitochondrial abundance. Previous studies in smaller case series of HIV-infected pediatric patients had reported no differences in mtDNA levels (Cossarizza et al., 2002; Rosso et al., 2008), while others had identified only didanosine as a mtDNA suppressor (Saitoh et al., 2007). A recent study has reported rates of mtDNA depletion from a large cohort of

HIV-infected HAART-treated Chinese children and adolescents (Liu et al., 2013) that were consistent with our results. In a subsequent analysis, we demonstrated lower levels of mtDNA and a reduction of mitochondrial function among HIV-infected children and adolescents affected with body fat distribution abnormalities as compared with patients who were asymptomatic. These findings were independent of the use of HAART or control of viral replication (Morén et al., 2012). A mitochondrial basis for the development of body fat abnormalities has been reported in adults (Garrahou et al., 2011). In this context, it should be noted that the potential protective role of antioxidant supplementation in the development of lipodystrophy is under investigation (Milazzo et al., 2010).

In the first study (Morén et al., 2011b), the mitochondrial genome depletion was not reflected in differences in transcription, structural, or functional activities. However, in a detailed analysis of the mitochondrial respiratory chain (MRC) that included a control group of healthy controls, we observed a significant reduction of the general CI-III-IV activity (but not in isolated MRC complexes) in HAART-treated children and a reduction by 50% in global oxygen consumption in naïve patients (measured in PMBCs) (Morén et al., 2013). In the latter study, a trend toward an increase in apoptosis was also observed. These findings not only confirmed the deleterious role of HIV itself on mitochondrial function that has been extensively described in adults (Miró et al., 2004; Cummins and Badley, 2010) but also suggested a generalized mitochondrial impairment in the HIV-infected child due to both HIV and HAART, rather than a specific profound and localized damage to any of the complexes of the MRC. More recently, a three-fold increase in mtDNA mutations, as measured by sequencing, was reported in a small series of HIV-infected HAART-treated children in China (Ouyang et al., 2016). Although these patients did not show signs or symptoms consistent with mitochondrial dysfunction, some of the mutations that were identified (i.e., G15043A and A15662G) have been associated with mitochondrial diseases.

In the early 2000s, different strategies regarding HAART were considered when an adult patient developed mitochondrial-associated clinically relevant toxicity, including (i) NRTI dose reduction, (ii) interruption of therapy, (iii) use of an NRTI-sparing regimen, and (iv) switching to a *mitochondrion-friendly* NRTI. Given the current availability of new ARV drugs that are more potent and less toxic, and after the results of the SMART trial (Strategies for Management of Antiretroviral Therapy; El-Sadr et al., 2006), the two first strategies have become obsolete in both pediatric and adult patients. Nonetheless, we reported a partial restoration of mtDNA levels and a decrease in plasma lactatemia after planned interruption of HAART in a small case

study of HIV-infected children (Noguera et al., 2010). We also demonstrated a significant reduction in cytochrome *c* oxidase activity among untreated HIV-infected children and in those receiving HAART including “first”-generation NRTIs (ZDV, didanosine, and stavudine), but not in patients receiving lamivudine/emtricitabine, abacavir, or tenofovir (Morén et al., 2012).

Overall, these findings are consistent with the large body of evidence available on the pathogenesis of HIV-related mitochondrial toxicity in the HIV-infected adult patient (Côté et al., 2002; Miró et al., 2003, 2004), but their clinical implications in children remain unclear, especially in the long term. Further longitudinal studies in large cohorts of MTCT HIV-infected children and adolescents are needed to better illuminate mitochondrial toxicity pathways and their clinical significance in the pediatric population.

34.3 Mitochondrial Toxicity in HIV-Uninfected Infants That Were Perinatally Exposed to Antiretrovirals

As is the case with HIV-infected children and adolescents, the benefit obtained from the use of ARV to prevent HIV MTCT far outweighs the ARV-associated fetal and infant toxicity that has been thus far described. In the last 10 years, the use of the more toxic NRTIs has been progressively abandoned in the treatment of HIV infection, and this includes pregnant HIV-infected women. The use of didanosine and/or stavudine is discouraged in pregnancy, while ZDV remains the leading alternative option (Panel CDC Pregnancy, 2016). Newer NRTIs show a much lower propensity to cause mitochondrial toxicity (Curran and Ribera, 2011). However, the long-term risk of this exposure in these otherwise healthy children remains a concern, and long-term investigations are required, especially as antenatal ARV drugs and regimens continue to evolve. Hematological and mitochondrial adverse events have been the most commonly reported among HEU infants to date (Panel CDC Pregnancy, 2016).

Hematological toxicity was early described in HEU infants exposed to ZDV during the third trimester of pregnancy, delivery, and neonatal period (Connor et al., 1994). This mainly consists of macrocytic anemia, usually non-symptomatic and self-limited by the age of 3 months. Exposure to maternal or neonatal combined ARV therapy increases the severity of anemia, seldom requiring discontinuation of prophylaxis or therapeutic interventions (Dryden-Peterson et al., 2011). Small decreases in other cell lines (platelet, lymphocyte, CD4 and CD8 cell, and neutrophil counts) have also been

described; unlike hemoglobin levels, these decreases may persist through age 18–24 months, or even longer in the case of neutrophil and lymphocyte counts (Bunders et al., 2005). These findings have shown very little clinical relevance to date, and the timing and need of hematologic monitoring in non-symptomatic HEU infants are uncertain; most experts recommend retesting hemoglobin levels and neutrophil counts when diagnostic HIV tests samples are obtained (Panel CDC Pregnancy, 2016).

Genetic and biochemical findings consistent with mitochondrial injury have been described in HEU children, ultimately leading to infrequent clinical syndromes that are similar to those of inherited mitochondrial diseases (Blanche et al., 1999; Barret et al., 2003; Brogly et al., 2007). The French cohort was the first to raise the alert about the risk of clinically significant mitochondrial toxicity in a group of eight ARV-exposed HIV-uninfected children with severe biological and neurological abnormalities consistent with mitochondrial dysfunction, such as delays in cognitive development, motor abnormalities, seizures, abnormal cerebral magnetic resonance imaging, and hyperlactatemia, either with a typical histologic pattern or with a deficit in the enzymologic study of the respiratory chain complexes. Two of these patients died (Blanche et al., 1999). The 18-month incidence of mitochondrial disease in this cohort was 0.26%, as compared with 0.01% in the French general population (Barret et al., 2003), and they also reported a higher risk of febrile seizures below the age of 18 months (Landreau-Mascaro et al., 2002). Using the same diagnostic criteria, the Pediatric AIDS Clinical Trials Group protocols 219/219C retrospectively identified 20 cases (out of 1037, 1.6%) of possible mitochondrial disease among HIV/ARV-exposed healthy children in the United States (Brogly et al., 2007). Of note, the median age at clinical onset in the two largest case studies of mitochondrial disease in HEU children was 7 months (Blanche et al., 1999) and 16 months (Brogly et al., 2007). It is thus possible that early mitochondrial toxicity may persist until later in life and should always be kept in mind by physicians caring for HEU children, with special attention paid to the long-term follow-up of this population into adulthood. In fact, the development of significant organ system abnormalities of unknown etiology in HEU children should prompt the evaluation for potential mitochondrial dysfunction (Panel CDC Pregnancy, 2016), particularly if the nervous system or the heart is affected (Williams et al., 2010; Nozyce et al., 2014; Sibude et al., 2015).

Hyperlactatemia rates ranging from 13 to 68% have been reported in HEU cohorts, with infants generally symptom-free and showing a trend toward normalization during the first year of life (Alimenti et al., 2003; Noguera et al., 2004; Ekouevi et al., 2006). A recent study

in the United States reported a significantly lower rate (3.4%) of hyperlactatemia, but included both infants and children up to 12 years of age (Crain et al., 2011). In our experience, elevated lactate levels were associated with gestational exposure to didanosine, but not to other ARV drugs or regimens, neither during pregnancy nor later. Three female infants (out of 127, 2.4%) developed neurologic symptoms consistent with mitochondrial dysfunction together with hyperlactatemia (Noguera et al., 2004). In any case, routine measurement of serum lactate is not recommended in HEU infants because of its low predictive value for relevant toxicity and should only be considered if a patient develops severe clinical symptoms of unknown etiology, particularly neurologic symptoms (Panel CDC Pregnancy, 2016). As mentioned previously, in neonates and infants, venous blood samples for lactate determination need to be appropriately obtained and processed to avoid false-positive results. If lactate levels are elevated in a symptomatic infant, ARV should be discontinued if the patient remains on prophylaxis, and supportive therapy should be initiated.

The mitochondrial toxic effect derived from HIV itself has been well demonstrated in ARV-naïve HIV-infected patients, but cannot be directly invoked in the HEU child. Therefore, in this population, other explanations for mitochondrial dysfunction are needed, including not only NRTI-related toxicity but also possible NRTI-unrelated mechanisms, especially mitochondrial toxicity affecting maternal tissues associated with fetal development, such as placenta and cord blood (Shiramizu et al., 2003; Divi et al., 2007; Gingelmaier et al., 2009; Hernández et al., 2012). It is likely that this phenomenon is the final result of different converging causes and pathogenic pathways.

With regard to mtDNA content in PBMCs of HEU infants, some studies have shown mtDNA depletion (Poirier et al., 2003; Aldrovandi et al., 2009), while others have reported increased mtDNA levels when compared with ARV-unexposed controls (Côté et al., 2008; McComsey et al., 2008; Ross et al., 2012). Methodological differences regarding how mtDNA results were reported, methods for PBMC isolation, platelet contamination, timing of blood sampling, and type and duration of ARV exposure may explain these contradictory findings. We

recently longitudinally assessed mtDNA content in a large cohort of HEU infants and found stable amounts during the first year of life (Noguera-Julian et al., 2015). These results are consistent with those reported by Aldrovandi and colleagues in a cohort of 411 healthy HIV-unexposed pediatric patients aged 0–18 years, in whom PBMC mtDNA levels did not show age-related differences (Aldrovandi et al., 2009). We also observed lower complex IV enzymatic activity in HEU infants at all time points when compared with healthy controls, and that this activity showed a linear trend toward normalization with age. These data nicely reflect a reversible mitochondrial dysfunction that tends to gradually normalize over the first year of life. Moreover, these data are consistent with the previously reported normalization of plasma lactate levels in these patients (Noguera et al., 2004), although we found no correlation between lactate levels and complex IV activities (Noguera-Julian et al., 2015). Previous studies had failed to demonstrate significant differences in MRC function, assessed by means of the complex II/IV ratio of cytochrome *c* oxidase in frozen neonatal PBMCs obtained within the first 48 h of life between HEU and unexposed neonates (McComsey et al., 2008; Ross et al., 2012). Interestingly, we observed an inverse correlation between complex IV enzymatic activity and mtDNA up to the age of 6 months (Noguera-Julian et al., 2015), suggesting that an increase in mtDNA content initially counteracts mitochondrial toxicity in the HEU infant (Brogly et al., 2011).

No differences have been reported between HEU children and healthy controls in mtDNA deletions or mitochondrial haplotypes (Brogly et al., 2011), mitochondrial RNA content (Côté et al., 2008), or telomere length (Poirier et al., 2003; Imam et al., 2012). Conversely, AC/TG mtDNA mutations were more common both in HIV-infected mothers and their uninfected infants, raising concerns about long-term consequences as these mutations have been associated with aging and age-associated diseases (Jitratkosol et al., 2012). In an American study including over one million newborns, the rate of abnormal newborn metabolic screens in HEU infants was almost double that for the general population (2.2% vs. 1.2%), and most of these disorders were related to mitochondrial metabolism (Kirmse et al., 2013).

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35

Drug-Induced Mitochondrial Cardiomyopathy and Cardiovascular Risks in Children

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35.1 Introduction

Mitochondria are the power plants of the cells and supply most of the energy needed by the cell. The principal function of mitochondria is to make energy in the form of ATP. Often, this function is thought to be the only one of this organelle. However, mitochondria are much more than simple cellular batteries. They are important in regulating cell survival, proliferation (McBride et al., 2006), and death (Kroemer et al., 2007), as well as calcium homeostasis (Hajnoczky et al., 2006). Organs, such as cardiac and skeletal muscles and the brain, are metabolically more active and depend more on mitochondrial-produced energy than do other cells (Szewczyk and Wojtczak, 2002). As a result, drug-induced mitochondrial failure increases damage to these critical organs.

Mitochondrial impairment is an inadvertent side effect of many medications that causes various organ toxicities, especially to the heart, given its high-energy demand. Thus, preventing and treating this impairment and understanding its implications for drug development are important. This chapter describes the mechanisms and

the short- and long-term consequences of HIV medications and cancer chemotherapies that are associated with mitochondrial dysfunction and its cardiac consequences in children.

35.2 HIV Therapy

Adults and children with human immunodeficiency virus (HIV) are now living longer because of advances in antiretroviral therapy (ART). However, HIV is still a chronic illness. The long-term complications of the infection and its treatment are increasingly apparent in both adults and children. Children differ from adults in that most have been exposed to HIV their entire lives (even *in utero*) and are likely to be exposed to ART for most of the rest of their lives. Furthermore, these exposures occur during vulnerable stages of development: *in utero*, infancy, and hormonal changes of puberty.

Not only has ART helped control disease progression and has extended life for HIV-infected patients, but it also has emerging and progressive longer-term complications,

Table 35.1 Nucleoside reverse transcriptase inhibitor toxicities in HIV-infected patients and their relation to mitochondrial pathways.

Clinical features	NRTI(s)	Mitochondrial pathway			
		Mitochondrial structure	mtDNA depletion	Decreased mitochondrial proteins	Altered mitochondrial function
Skeletal myopathy	Zidovudine	Abnormal, ragged red fibers	Yes	Yes	Yes
Cardiomyopathy	Zidovudine, stavudine	Abnormal	ND	ND	ND
Neuropathy	Zalcitabine, stavudine, didanosine	Abnormal	Yes	ND	ND
Macrocytosis	Zidovudine, stavudine	ND	ND	ND	ND
Pancreatitis	Didanosine	ND	ND	ND	ND
Hepatic steatosis	Zidovudine, stavudine, didanosine	ND	Yes	ND	ND
Lactic acidosis	Zidovudine, stavudine, didanosine	NA	NA	NA	ND
Lipodystrophy	Stavudine, zidovudine	Abnormal	Yes	ND	Yes
Acute kidney injury (AKI) ^a	Tenofovir ^a	Yes ^a	Yes ^a	ND ^a	ND ^a

Gerschenson and Brinkman (2004). Reproduced with permission of Elsevier.

NA, not applicable; ND, not determined; NRTI, nucleoside reverse transcriptase inhibitor.

^aNot in the original table.

such as mitochondrial abnormalities (Dagan et al., 2002; Gerschenson and Brinkman, 2004; Shikuma et al., 2005; Barlow-Mosha et al., 2013). Metabolic consequences have been reported in up to 60% of HIV-infected children (Jaquet et al., 2000; Arpad et al., 2001; Miller et al., 2009) and include dyslipidemia, insulin resistance (IR), and body composition changes (lipoatrophy and lipohypertrophy) (Carr et al., 1998; McComsey and Leonard, 2004; Bitnun et al., 2005; Sanchez Torres et al., 2005; Dzwonek et al., 2006; Kim et al., 2007; Miller et al., 2008).

Mitochondrial dysfunction secondary to ART is drug and organ specific (Table 35.1). This specificity was first described in 1988, in HIV patients treated with high doses of the nucleoside reverse transcriptase inhibitor (NRTI), zidovudine (Gertner et al., 1989). These patients experienced mitochondrial-related skeletal muscle myopathies that were identified by histological and electron microscopy examination of biopsy tissue. Histologically, ragged red fibers were observed, which is the accumulation of mitochondria with paracrystalline inclusions in the subsarcolemmal space (Dalakas et al., 1990). Concentrations of mtDNA, RNA, and proteins in the muscles of these patients were decreased but were reversed in 50% of patients after the drug was withdrawn (Dagan et al., 2002). These findings led to the hypothesis that NRTIs inhibit mtDNA replication, causing these myopathies. HIV-infected adults receiving ART are more likely to have altered mtRNA and mtDNA than

HIV-infected but ART-naïve individuals (McComsey et al., 2008), indicating that mitochondrial abnormalities cannot be explained by HIV infection alone.

Several studies have now reported that mitochondrial function is affected by HIV and ART and perhaps by cytokines through a diverse array of mechanisms, including direct increases in reactive oxygen species (ROS) (Hulgan et al., 2003; McComsey and Morrow, 2003), uncoupling of mitochondria (Pace et al., 2003; McComsey et al., 2006), decreases in ATP concentrations in adipocytes and pre-adipocytes (Gerschenson et al., 2006), and apoptosis (Gerschenson and Brinkman, 2004). The combination of ART and HIV in targeting mitochondria is a multi-hit process (Figure 35.1). The peripheral blood mononuclear cells (PBMCs) of untreated HIV-infected patients have lower mtDNA content than that of healthy controls (Miro et al., 2004). Zidovudine inhibits the mitochondrial adenylate, kinase, and adenosine nucleotide translocator in isolated rat liver mitochondria (Gerschenson and Brinkman, 2004), as well as thymidine phosphorylation in rat heart and liver mitochondria (McKee et al., 2004; Lynx et al., 2006; Susan-Resiga et al., 2007). HIV polypeptides, such as gp120, can increase oxidative stress (Louboutin et al., 2010), and Tat induces apoptosis (Kruman et al., 1998). Thus, decreased mtDNA concentrations do not appear to be a prerequisite for mitochondrial dysfunction. As a result, evaluating mitochondrial function is critical when considering ART-associated mitochondrial toxicity.

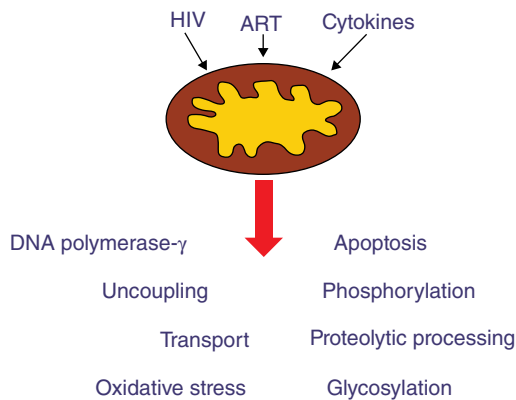


Figure 35.1 Multi-hit hypothesis of mitochondrial dysfunction from HIV, ART, and cytokines. Mitochondria are targeted at multiple functional levels by ART drugs, HIV, and cytokines, all of which alter mitochondrial DNA replication, energy metabolism, and apoptosis. Gerschenson and Brinkman (2004). Reproduced with permission of Elsevier.

NRTIs inhibit mtDNA polymerase- γ and deplete mtRNA, which in turn impair oxidative phosphorylation (OXPHOS), alter ATP concentrations, and cause cell apoptosis and peripheral subcutaneous lipoatrophy, with or without visceral fat accumulation (Gerschenson and Brinkman, 2004). Protease inhibitors inhibit Glut4 (Hruz et al., 2008), transcription factors (e.g., PPAR- γ , SREBP-1 [ritonavir]) (Mallon et al., 2005), and fatty acid synthase (saquinavir and ritonavir) (Bastard et al., 2002; Mallon et al., 2005); decrease adipocyte differentiation (Caron et al., 2003); and cause cellular apoptosis (Domingo et al., 1999) and IR (Dube et al., 2005). These inhibitors, such as efavirenz, can suppress lipogenic pathways of adipocytes *in vitro* (Rodriguez de la Concepcion et al., 2005).

The most common oxidation products of mtDNA include 8-oxo-deoxyguanosine, and high concentrations of the base modifications have been detected in monkeys treated with NRTIs (Bialkowska et al., 2000) and in HIV patients on a stavudine-based regimen (Gerschenson et al., 2009). In some studies, up to a third of HIV-infected children reportedly have chronic symptom-free hyperlactatemia (normal values up to 2.1 mmol/L) (Desai et al., 2003; Noguera et al., 2003). Mitochondrial dysfunction, hyperlactatemia, and several symptomatic cases have also been described in HIV-uninfected ART-exposed healthy infants (Blanche et al., 1999; Noguera et al., 2004; Brogly et al., 2007).

Clinical features of mitochondrial dysfunction in HIV include lipodystrophy, hepatic steatosis, lactic acidosis, skeletal myopathy, cardiomyopathy, and peripheral neuropathy (Gerschenson and Brinkman, 2004). Zidovudine was the first NRTI used in 1985 to treat HIV infection, and sustained treatment with high doses caused serious myopathies in many patients (Yarchoan et al., 1986;

Scruggs and Dirks Naylor, 2008). Currently, in combination with other ART drugs, the daily dose of zidovudine has been decreased, which substantially decreased the incidence of myopathy. Although *in vitro*, other NRTIs are more toxic than zidovudine, and skeletal myopathy has been described predominantly with zidovudine (Loutfy et al., 2003). In contrast, only a few HIV patients have experienced dilated cardiomyopathy during NRTI treatment, and only two had mitochondrial abnormalities (Tanuma et al., 2003).

High-dose zidovudine has been associated with mitochondrial cardiomyopathy in rats (Lewis et al., 1991) and worsened cardiac dysfunction in adult transgenic mice with AIDS (Lewis et al., 2000). This cardiomyopathy altered the steady-state abundance of mtDNA, mitochondrial RNA, and mitochondrially encoded polypeptides in affected tissues and was associated with molecular evidence of cardiac remodeling (Lewis et al., 1992). However, in contrast to the animal studies, in a prospective study, zidovudine was not associated with acute or chronic abnormalities in left ventricular structure or function in HIV-infected infants perinatally exposed to the drug (Lipshultz et al., 1992, 2000).

In 24 children with symptomatic HIV infection, echocardiograms were acquired immediately before zidovudine therapy was started and at a mean of 1.32 years after therapy began. Progressive left ventricular dilatation caused progressive elevation of ventricular afterload, which depressed ventricular performance, but intrinsic ventricular contractility remained normal (Lipshultz et al., 1992). The study also followed 382 infants without HIV infection (36 with postpartum zidovudine exposure) and 58 HIV-infected infants (12 with postpartum zidovudine exposure) with serial echocardiograms (Lipshultz et al., 2000). Zidovudine exposure was not associated with marked echocardiographic abnormalities in mean left ventricular fractional shortening, end-diastolic dimension, contractility, or mass in either non-HIV-infected or HIV-infected infants. Thus, current ART regimens appear to have reduced the incidence of myopathies.

Initial concerns about pediatric HIV-associated cardiovascular disease (CVD) came from studies in fetal monkeys, in which transplacental exposure to NRTIs resulted in mitochondrial abnormalities, including decreased mtDNA copies per cell and OXPHOS (Gerschenson et al., 2000, 2004). Additional studies in 1-year-old monkeys after NRTI treatment found mitochondrial damage and increased concentrations of mtDNA in the heart (Divi et al., 2005). HIV-infected adults have a higher risk of early CVD than that of the general population because of the HIV infection itself and ARV treatment (Bavinger et al., 2013; Post et al., 2014).

The global risk of premature CVD in children and adolescents with vertically acquired HIV infection appears to be increasing. Concentrations of cardiac markers, such as carotid intima-media thickness, were higher in ART-treated, HIV-infected children than in age-, gender-, race-, and body mass index (BMI)-matched uninfected controls, suggesting an elevated cardiovascular risk (McComsey et al., 2007). The US obesity epidemic among youth is also affecting the HIV-infected children, with prevalence rates up to 12% (Jacobson et al., 2011). Together with obesity and other HIV-unrelated factors, abnormal body fat distribution, lipid abnormalities, and altered glucose metabolism increase the risk of future CVD in these children (Morrison et al., 2007). Finally, uncontrolled HIV-related inflammatory and immunologic factors also contribute to cardiovascular risk (Kuller et al., 2008).

Recently, in a large cohort of perinatally HIV-infected adolescents in the United States, Pathobiological Determinants of Atherosclerosis in Youth (PDAY) risk scores for coronary arteries and abdominal aorta were 1 unit or higher (McMahan et al., 2005) in 48 and 24%, respectively, indicating an increased CVD risk. The highest scores were predicted by HIV disease severity and boosted protease inhibitor use (Patel et al., 2014). In a prospective cohort study, concentrations of biomarkers of vascular dysfunction were higher in HIV-infected children than in HIV-exposed but uninfected children, as were CVD risk factors, including unfavorable lipid concentrations and active HIV replication.

Among perinatally HIV-infected youth with and without IR matched on demographics, pubertal develop-

ment, current ARV exposures, and HIV disease severity, measures of multiple components of mitochondrial respiration were lower in those with IR than in those without (Takemoto et al., 2017), which suggests that IR increases the risk of CVD. In PBMCs, mitochondrial respiration was lower in those youth with IR compared with those without. The positive correlation between non-mitochondrial respiration and fasting glucose concentrations suggests damage to the electron transport chain. These findings point to a disruption in the use of nutrients to produce energy in the insulin-resistant group. This disruption suggests dysfunctional mitochondria or a breakdown in the hepatic- β -cell feedback loop, both of which can account for the differences in values of glucose, insulin, and mitochondrial respiration. This correlation also suggests that IR may be associated with an alternative and less-efficient means of energy production (Figure 35.2). Concentrations of mitochondrial respiration markers were lower in HIV-infected youth with IR than in those without; thus, the disordered mitochondrial respiration may be a potential mechanism for IR in this population.

There is no proven, effective therapy for NRTI-associated mitochondrial toxicity, other than withdrawing the implicated agent. Even then, symptoms may not resolve completely. Similarly, there are no established methods for preventing therapy-related mitochondrial toxicity. Toxicities depend on the combination of individual drugs and ART and should be monitored at each clinic visit (Welch et al., 2009). Some small studies have found benefits from antioxidants, such as antioxidant supplementation (with vitamin E, beta-carotene, *N*-acetylcysteine,

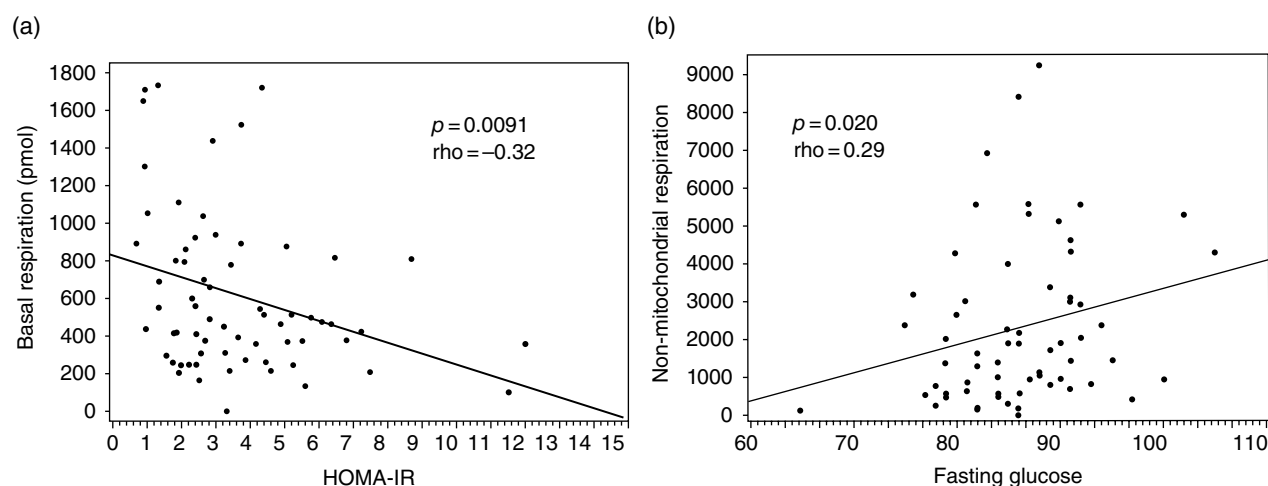


Figure 35.2 Metabolic correlations associated with mitochondrial respiratory characteristics. Data are from 66 children perinatally infected with HIV. (a) Relationship between basal respiration and homeostatic model assessment with insulin resistance (HOMA-IR) with and without insulin resistance. Basal respiration was inversely related to insulin-resistant status as determined by HOMA-IR ($p = 0.009$, Spearman's $\text{rho} = -0.32$). (b) Non-mitochondrial respiration was positively correlated with fasting glucose ($p = 0.02$, Spearman's $\text{rho} = 0.29$). Takemoto et al. (2017). Reproduced with permission of Wolters Kluwer Health.

Table 35.2 Recommended lifestyle habits and monitoring of metabolic and renal adverse effects in the routine follow-up of HIV-infected children and adolescents.

Recommended lifestyle habits

Calorically appropriate low-fat diet, especially in patients with a high BMI or dyslipidemias

Sufficient calcium and vitamin D intake

Regular aerobic exercise; weight-bearing exercise after puberty

Smoking avoidance or cessation

Avoidance of other toxic substances, illicit or not

Monitoring proposal for routine follow-up of HIV-infected children and adolescents

Calculate BMI from body weight and height measurements at each visit; measure blood pressure every 3–6 months

Assess metabolic toxicities at each visit, including abnormal patterns of body fat distribution

In the absence of symptoms, measuring lactate concentrations is not recommended

Laboratory monitoring for dyslipidemias and glucose homeostasis before and 4–12 weeks after HAART initiation or switch and at least every 6–12 months thereafter in the absence of ongoing toxicity concerns

Periodically measure vitamin D concentrations, especially in the presence of several risk factors for low bone mineral density

If available, acquire dual-energy X-ray absorptiometry scans every 1–2 years, especially among children with abnormal findings or in the presence of several risk factors for low bone mineral density

Calculate estimated glomerular filtration rate from proteinuria and glycosuria measurements at least every 6–12 months and every 3–6 months if tenofovir is used

Fortuny et al. (2015). Reproduced with permission of Wolters Kluwer Health.

selenium, *Ginkgo biloba* extracts, and nutritional supplements), but optimal prevention and monitoring strategies have not been determined (Lopez et al., 2003; Cherry et al., 2005). In the absence of proven pharmacological and other medical treatments, lifestyle modifications (e.g., healthy diet and exercise; avoiding smoking and other toxic substances) are probably the most important therapeutic approach to preventing and treating complications in HIV-infected children (Welch et al., 2009) (Table 35.2).

35.3 Cancer Therapy

One of the major successes of pediatric oncology is that children with childhood cancers are now living substantially longer and healthier lives because of advances in chemotherapeutic agents. Improved 5-year survival rates, from less than 58% in the 1970s to 83% today

(Ward et al., 2014), have increased the number of long-term survivors of childhood cancer in the United States to an estimated 420,000 (Armstrong and Ross, 2014). Most of this success is attributed to the widely used and highly effective anthracycline, doxorubicin. Anthracycline chemotherapeutics, such as doxorubicin and daunorubicin, are cytotoxic agents used to treat a spectrum of childhood malignancies, including acute lymphoblastic leukemia (ALL), acute myelogenous leukemia, Hodgkin disease, and non-Hodgkin lymphoma. However, over the years, multiple long-term follow-up studies have discovered that doxorubicin treatment is associated with late cardiac effects years later (Lipshultz et al., 1991; Lipshultz and Adams, 2010; Lipshultz et al., 2013a). In fact, cardiotoxicity is the most serious shortcoming of anthracycline-based treatment of cancer (Barry et al., 2007).

Chemotherapeutic medications are designed to interfere with rapidly dividing neoplastic cells. However, in the process, they can adversely affect normal cell division, especially in tissues with rapid turnover. The cardiovascular system's regenerative capacity is limited, so potential adverse effects from these chemotherapeutic agents may be unpredictable. Cardiotoxic effects have been documented for several classes of cancer drugs, including anthracyclines (e.g., doxorubicin and epirubicin), alkylating agents (e.g., busulfan and cyclophosphamide), antimetabolites (including 5-fluorouracil and cytarabine), antimicrotubule agents (e.g., vinca alkaloids), targeted agents (e.g., lapatinib and trastuzumab), and radiation (Simbre et al., 2005; Fulbright, 2011). Of these, anthracycline-related cardiotoxicity is particularly well studied.

The mechanisms of anthracycline-induced cardiotoxicity remain uncertain. Once anthracycline enters the cell, it forms complexes with iron and reduces the quinone moiety to subquinone. This reduction leads to a cascade production of ROS, which causes some of the deleterious effects on the cell and its components and that ultimately kills cells. Oxidative stress also induces nitric oxide synthase and produces nitric oxide and peroxynitrite, which inactivate myofibrillar creatine kinase and other important cardiac enzymes (Mihm and Bauer, 2002; Fogli et al., 2004). This damage, and the fact that cardiomyocytes divide too slowly to replace damaged cells, leads to organ damage.

Several other mechanisms for anthracycline-related cardiotoxicity have been postulated, including the induction of apoptosis, the production of vasoactive amines, the formation of toxic metabolites, the upregulation of nitric oxide synthetase, and the inhibition of transcription and translation (Ito et al., 1990; Peng et al., 2005; Chen et al., 2007). Anthracyclines uncouple the electron transport chain, which creates highly ROS and impairs phosphorylation and ATP synthesis (Simunek et al.,

2009). Anthracyclines also disturb mitochondrial calcium homeostasis, destabilize the mitochondrial membrane, decrease ATP production, and ultimately kill the cell.

Cardiomyocytes exposed to anthracyclines show other changes, which may or may not depend on the oxidative pathway. These changes include depleted cardiac stem cells (De Angelis et al., 2010), impaired DNA synthesis (Kalyanaraman et al., 2002), impaired cell signaling that triggers cell death (Jurcut et al., 2008), altered gene expression (Rusconi et al., 2004), inhibited calcium release from the sarcoplasmic reticulum (Lowis et al., 2006), impaired formation of the protein titin in sarcomeres (Lebrecht et al., 2005), and impaired mitochondrial creatine kinase activity and function (Ryberg et al., 2008). These subcellular processes often continue for weeks after anthracycline exposure, which may provide insight into the mechanism of chronic cardiomyopathy (Tokarska-Schlattner et al., 2006).

Given the high-energy demands of the heart, 45% of myocardial volume is comprised of mitochondria, making them more abundant in cardiac tissue than in other tissues (Marin-Garcia et al., 2001; Gottlieb and Gustafsson, 2011). Cardiac cells are thus more susceptible to free radical damage because of their highly oxidative metabolism, relatively poor antioxidant defenses, and high concentration of cardiolipin, a phospholipid concentrated in the inner mitochondrial membrane and critical to cell respiration. Cardiolipin accumulates anthracycline in the cardiomyocytes at concentrations 100-fold higher than those in plasma. Anthracyclines are thought to inhibit the respiratory chain by binding to cardiolipin or by interacting with mtDNA (Lebrecht and Walker, 2007).

Doxorubicin damages mtDNA by several mechanisms. Anthracyclines can cause acute mitochondrial toxicity. Early mitochondrial lesions have been found in cardiomyocytes, and the mitochondrial ultrastructure is altered rapidly after doxorubicin infusion (Ferrans, 1978). The rapid kinetics of mitochondrial impairment after acute doxorubicin exposure are likely to result from either the direct ROS-mediated respiratory complex inactivation or the ability of the Fe^{3+} -doxorubicin complex to bind to cardiolipin (Goormaghtigh et al., 1990; Keizer et al., 1990).

Mutations in mtDNA have been identified at low concentrations of doxorubicin after short-term treatment (Adachi et al., 1993; Serrano et al., 1999). Acute doxorubicin exposure also reduces mtDNA copy numbers in rat hearts (Hixon et al., 1981). Doxorubicin may be metabolically transformed into a quinone methide and, through this intermediate, binds covalently to mtDNA (Abdella and Fisher, 1985).

The mitochondria are injured slightly during acute doxorubicin treatment. The lesions then accumulate with time and ultimately impinge on the bioenergetic

capacity of the organelles, even when the initial doxorubicin insult is no longer present. This mechanism hinges on the fact that the respiratory chain dysfunction associated with the mtDNA damage can subsequently liberate ROS at the respiratory chain. In turn, ROS may either attack the respiratory chain itself or damage the mitochondrial genome (Corral-Debrinski et al., 1991; Richter, 1992).

MtDNA depletion, mtDNA mutations, and respiratory chain dysfunction that begun during anthracycline chemotherapy may lead to formation of ROS and a cycle of secondary mtDNA and respiratory chain insults that accumulate long after treatment. Such events would explain, in part, the molecular mechanism of “dose memory” and delayed-onset cardiomyopathy (Lebrecht et al., 2003; Lebrecht et al., 2005). In fact, mitochondrial dysfunction resulting in bioenergetics crisis is an apparent hallmark of doxorubicin-related cardiotoxicity (Tokarska-Schlattner et al., 2006).

Lastly, doxorubicin inhibits topoisomerase II, an ATP-dependent nuclear enzyme that catalyzes the knotting–unknotting and catenation–decatenation reactions during normal replication of double-stranded DNA (Tsai-Pflugfelder et al., 1988). Doxorubicin binds to the topoisomerase II–DNA complex and inhibits the broken DNA strands from religating. This process can also induce single- or double-stranded DNA breaks (Tewey et al., 1984) and a quantitative defect of mtDNA copy number (mtDNA depletion) (Lawrence et al., 1993). Both qualitative (mutations) and quantitative (depletion) mtDNA lesions may interact synergistically and severely compromise the synthesis of mtDNA-encoded respiratory chain subunits.

Recently, information about doxorubicin-induced topoisomerase-II β (Top2 β) alterations has partially clarified the mechanism of doxorubicin-induced toxicity (Zhang et al., 2012; Khiati et al., 2014). The Top2 β -doxorubicin-DNA ternary cleavage complex induces DNA double-strand breaks, killing cells (Lyu et al., 2007). Furthermore, deletion of the Top2 β gene in cardiomyocytes of mice protects the cells from doxorubicin-induced DNA double-strand breaks and transcriptome changes that are responsible for defective mitochondrial biogenesis and ROS formation (Zhang et al., 2012). In patient’s peripheral blood leukocyte, Top2 β concentrations were higher in anthracycline-sensitive patients. This was associated with decreased LV ejection fraction [LVEF] $\geq 10\%$ from baseline and $\text{LVEF} < 50\%$, (despite receiving a cumulative doxorubicin dose $\leq 250 \text{ mg/m}^2$) than in anthracycline-resistant patients (who received a cumulative doxorubicin dose $\geq 450 \text{ mg/m}^2$ with $\text{LVEF} \geq 50\%$), which suggests the potential use of Top2 β as a surrogate marker for susceptibility to anthracycline-induced cardiotoxicity (Vepongsa and Yeh, 2014). Additionally,

nuclear-encoded mitochondrial topoisomerase I single-nucleotide polymorphisms is associated with doxorubicin cardiotoxicity and could also be examined as a biomarker (Khiati et al., 2014; Nitiss and Nitiss 2014).

Doxorubicin-associated cardiotoxicity is likely at least partially caused by semiquinone free radicals generated by doxorubicin–iron complexes in the myocardium. Furthermore, one animal study found a dose-dependent increase of doxorubicin in mitochondria, indicating that doxorubicin accumulates in the mitochondria and increases mitochondrial iron concentrations (Ichikawa et al., 2014). Reaction of these free radicals with oxygen (ROS) leads to lipid peroxidation and ultimately DNA damage (Vile and Winterbourn, 1990; Wallace, 2003). In addition, dexrazoxane, a cardioprotectant, chelates iron, thereby reducing iron from forming doxorubicin–iron complexes and, as a result, reducing free radical DNA damage (Lipshultz et al., 2004).

Preclinical studies have associated doxorubicin exposure with irreversible cardiac mitochondrial dysfunction (Zhou et al., 2001; Lipshultz et al., 2016). Clinical histological studies have observed doxorubicin-related structural abnormalities, and one recent study of childhood ALL survivors found that gene polymorphisms of mitochondrial expression that influences translational control may affect mtDNA replication and possibly indicate susceptibility to doxorubicin (Kwok et al., 2011). In late doxorubicin cardiotoxicity in mice, increased peripheral blood lymphocytes contained mitochondrial mutations that were related to respiratory chain defects and late-onset cardiomyopathy (Lebrecht et al., 2003). These studies indicate that doxorubicin has deleterious effects on mitochondrial structure and that DNA could affect mtDNA copy numbers and OXPHOS.

Further, preclinical studies on anthracycline exposure in mitochondria have reported disrupted OXPHOS, particularly in the OXPHOS enzyme activities of NADH dehydrogenase (complex I) and cytochrome *c* oxidase (complex IV) activity (Chandran et al., 2009; Lebrecht et al., 2010), as well as changes in the mtDNA in the form of mutations, deletions, and reduced copy numbers per cell in heart tissue (Lebrecht and Walker, 2007; Lebrecht et al., 2007). If the mitochondria of doxorubicin-exposed cancer survivors are indeed impaired, through either DNA intercalation or oxidative stress, their mtDNA may undergo clonal expansion to compensate for mutations or deletions that could lead to normal OXPHOS and ATP production in the myocardium (Lee and Wei, 2005). Studies in rats treated with dexrazoxane and doxorubicin show that concomitant dexrazoxane treatment prevented doxorubicin-induced cardiac mitochondrial dysfunction by maintaining OXPHOS activities and mtDNA integrity (Lee and Wei, 2005; Lebrecht et al., 2007).

Anthracycline-related cardiotoxicity can present within a week or decades after the initial treatment (Goorin et al., 1990). Early toxicity is not a prerequisite for toxicity presenting decades later (Trachtenberg et al., 2011). In fact, whether any time limit exists is uncertain. Cardiotoxicity is often classified by when the signs and symptoms appear. Acute-onset cardiotoxicity occurs within a week of treatment; early-onset chronic progressive cardiotoxicity occurs between 1 week and 1 year; and late-onset cardiotoxicity occurs 1 year after treatment (Table 35.3).

Several risk factors contribute to cardiac toxicity in survivors of childhood cancers (Table 35.4). A higher cumulative anthracycline dose is associated with an

Table 35.3 Different types of anthracycline cardiotoxicity.

Characteristic	Acute cardiotoxicity	Early-onset, progressive cardiotoxicity	Late-onset, progressive cardiotoxicity
Onset	Within the first week of anthracycline treatment	<1 year after completing anthracycline treatment	≥1 year after completing anthracycline treatment
Risk factor dependence	Unknown	Yes ^a	Yes ^a
Clinical features in adults	Transient depression of myocardial contractility	Dilated cardiomyopathy	Dilated cardiomyopathy
Clinical features in children	Transient depression of myocardial contractility	Restrictive cardiomyopathy, dilated cardiomyopathy, or both	Restrictive cardiomyopathy, dilated cardiomyopathy, or both
Course	Usually reversible on discontinuation of anthracycline	Can be progressive	Can be progressive

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^a See Table 35.4 for risk factors.

Table 35.4 Risk factors for anthracycline-induced cardiotoxicity.

Risk factors	Features
Total cumulative dose	Most important predictor of abnormal cardiac function
Age	For similar cumulative doses, younger age predisposes to greater cardiotoxicity (especially <5 years of age)
Length of follow-up	Longer follow-up reveals higher prevalence of myocardial impairment
Sex	Females more vulnerable than males for similar doses
Concomitant mantle irradiation	Evidence of enhanced cardiotoxicity; not clear whether additive or synergistic
Others	Concomitant exposure to cyclophosphamide, bleomycin, vincristine, amsacrine, or mitoxantrone may predispose to cardiotoxicity; trisomy 21 and black race have been associated with a higher risk of early clinical cardiotoxicity
Rate of anthracycline administration	Higher rate was thought to predispose to greater toxicity, but current trials in children do not support this finding

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increased risk of late cardiac dysfunction (Lipshultz et al., 1991; Nysom et al., 1998). Cardiac damage has been found even in patients who received doses less than 240 mg/m², suggesting that there is no true “safe” dose of anthracycline (Darzy and Shalet, 2009). Cancer survivors also increase their risk for CVD if they develop metabolic syndrome and/or type 2 diabetes mellitus. These patients may have adjunct radiotherapy that put them at a higher risk for CVD than the general population. Even cancer survivors who are not overweight or obese may have a higher prevalence of fatty liver and metabolic abnormalities (Tomita et al., 2011). This paradox was recently reported in a cross-sectional comparison of 319 childhood cancer survivors at least 5 years after diagnosis to 208 siblings (Steinberger et al., 2012).

The 2006 and 2010 American Heart Association scientific statements describe the most effective strategies for improving physical activity among people of all ages (Marcus et al., 2006; Artinian et al., 2010). As in the general population, long-term childhood cancer survivors can also have one or more of the traditional risk factors for atherosclerosis, which could further increase the risk of future cardiovascular complications beyond that directly related to cancer therapies. Physical inactivity, obesity, tobacco use, and diabetes mellitus are some of the most commonly explored traditional modifiable atherosclerotic risk factors (Franco et al., 2011). An

improved understanding of the lifetime cardiovascular risk associated with these factors in long-term childhood cancer survivors may help guide treatment and predict any potential additional cardiovascular risk of specific cancer therapies, such as anthracyclines (Towbin et al., 2006).

Serum cardiac biomarkers, such as cardiac troponin T (cTnT), cardiac troponin I (cTnI), and N-terminal pro-brain natriuretic peptide (NT-proBNP), have been studied to determine whether they can detect this cardiotoxicity. In fact, cTnT and NT-proBNP have been validated as surrogate endpoints for monitoring late cardiotoxicity in long-term cancer survivors (Lipshultz et al., 2012). Cardiac troponins are specific for myocardial damage, and increased concentrations indicate irreversible cardiomyocyte cell death in patients treated with anthracycline (Lipshultz et al., 1997; Cardinale et al., 2004). In 134 children with high-risk ALL receiving moderate doses of anthracyclines, elevated serum cTnT and NT-proBNP concentrations during the first 90 days of treatment were significantly associated with structural cardiac abnormalities (Lipshultz et al., 2012). Studies may determine whether these markers can be used to tailor anticancer therapy to improve future outcomes.

In 2007, the American Society of Clinical Oncology Survivorship Expert Panel identified the need for optimal screening and monitoring strategies and evidence-based treatment options for long-term cardiac and pulmonary toxicity secondary to chemotherapy or radiotherapy in symptomatic and asymptomatic cancer survivors (Carver et al., 2007). Unfortunately, this guideline and several others are not consistent and are based on limited evidence. As a result, the optimal timing and frequency of cardiovascular monitoring in cancer survivors remains controversial. The American Heart Association recommends close monitoring of cardiac function, but it is not specific about the modality or frequency (Hunt et al., 2005). The Children’s Oncology Group released risk-based guidelines for long-term cardiac morbidity (Landier et al., 2004). Antineoplastic drugs not only cause early morbidity and mortality but can also lead to long-term, subclinical damage that can manifest later in life. Thus, minimizing or preventing this initial damage through various strategies as early as possible is important (Figure 35.3) (Goorin et al., 1990; Lipshultz and Sallan 1993; Lipshultz et al., 1997; Simbre et al., 2001; Lipshultz et al., 2002; Lipshultz and Colan 2004; Lipshultz et al., 2005; Simbre et al., 2005; van Dalen et al., 2005; Wouters et al., 2005; Lipshultz, 2006; Alvarez et al., 2007; Barry et al., 2007; Lipshultz, 2007; Scully and Lipshultz, 2007; Lipshultz et al., 2008; Lipshultz and Adams, 2010; Franco et al., 2011; Trachtenberg et al., 2011; Zerra et al., 2013).

Dexrazoxane, an ethylene diamine tetraacetic acid, such as bisdioxopiperazine, becomes hydrolyzed within

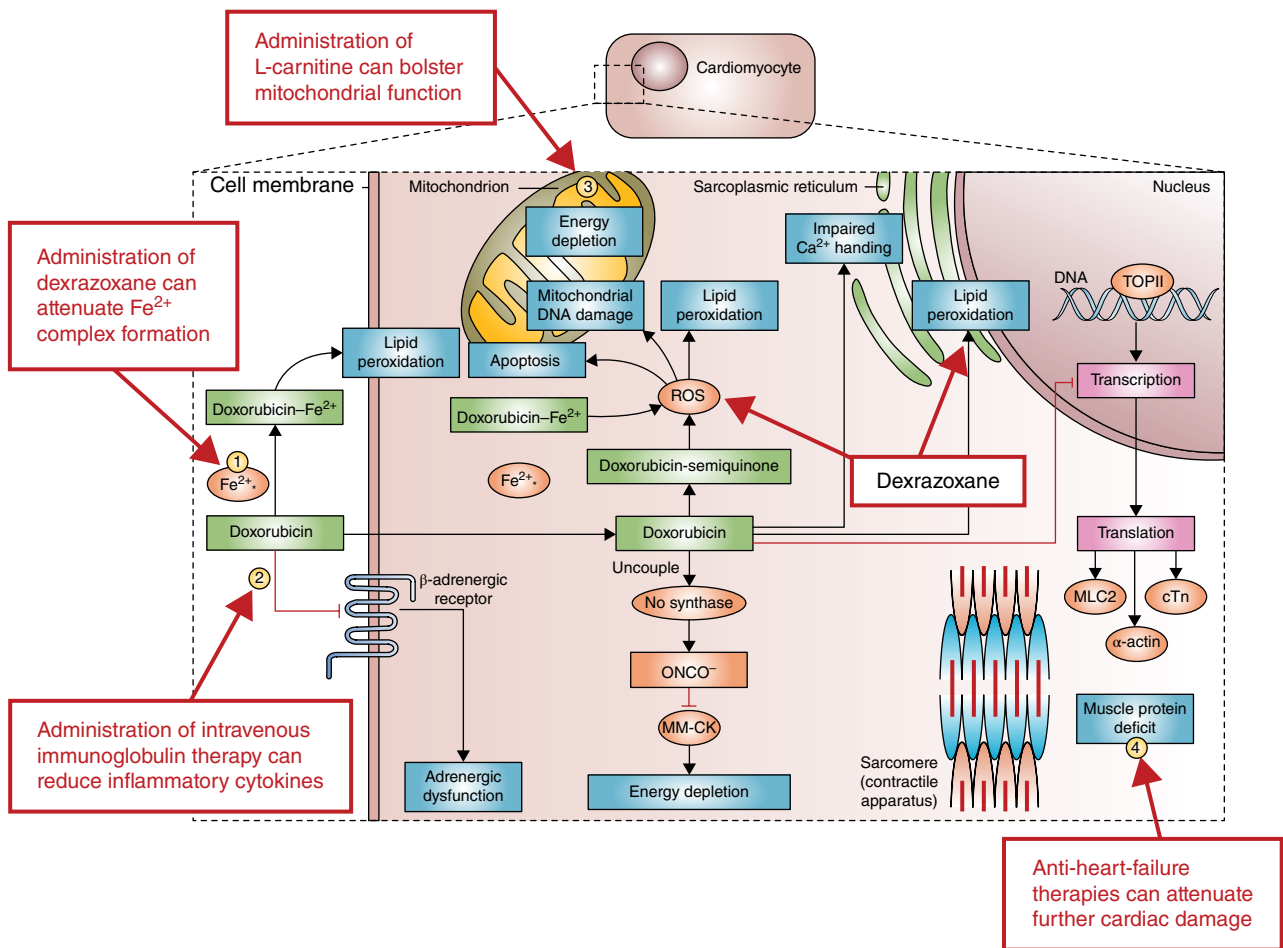


Figure 35.3 Potential opportunities for cardioprotection in cardiomyocytes. Lipshultz et al. (2013b). Reproduced with permission of Nature Publishing Group.

the cardiomyocyte to form a potent chelator of heavy metals, especially iron. Hence, it acts by chelating intracellular iron and decreasing free radical formation (Minotti et al., 2004). It provides long-term cardioprotection without decreasing the efficacy of doxorubicin in patients with high-risk ALL (Lipshultz et al., 2010). Despite the variety of potential cardio protective agents tested, specifically by controlling ROS production and oxidative stress, dextrazoxane reduces the incidence and severity of anthracycline-related cardiomyopathy (Lipshultz et al., 2004, 2010; Lebrecht et al., 2007). The cardioprotective activity of dextrazoxane has been attributed to its transformation into a metabolite, ADR-925, which displaces iron from anthracycline–iron complexes or chelates free or loosely bound cellular iron, thereby preventing site-specific oxidative damage in cardiac tissue (Lipshultz et al., 2004, 2010; van Dalen et al., 2011; Lipshultz et al., 2013b).

Gene polymorphisms of mitochondrial expression may affect mtDNA replication and possibly indicate sus-

ceptibility to doxorubicin in children with ALL (Kwok et al., 2011). Doxorubicin intercalates into mtDNA, disrupting genes encoding for OXPHOS polypeptides. In another study, mtDNA and OXPHOS enzyme activities were assessed in PBMCs of survivors of ALL treated with doxorubicin, with or without dextrazoxane (Lipshultz et al., 2016). As a compensatory response to impaired mitochondrial function, mtDNA copy numbers within each cell were higher in patients who received doxorubicin alone. Patients receiving both doxorubicin and dextrazoxane had a lower number of mtDNA copy numbers per cell, indicating that dextrazoxane preserves mitochondrial structure and function during doxorubicin treatment. At a median of 7.8 years after treatment, the median number of mtDNA copies per cell for patients treated with doxorubicin alone was significantly higher than it was for those who also received dextrazoxane (medians, 1106.3 and 310.5; $p = 0.001$) (Figure 35.4). The number of PBMC mtDNA copies per cell was higher in doxorubicin-treated survivors, and the concomitant use

of dexrazoxane was associated with a lower number of mtDNA copies per cell. Overall OXPHOS activity did not differ between groups, possibly because of a compensatory increase in mtDNA copies per cell to maintain mitochondrial function in the setting of mitochondrial dysfunction. This finding in survivors was concerning because the long-term sustainability of this compensatory response is questionable.

Doxorubicin-treated, long-term survivors of childhood ALL have significantly more mtDNA mutations or polymorphisms than do healthy children (Lipshultz et al., 2007). With a median of 8.5 years after diagnosis, the proportion of 167 ALL children with sequence variants (63 variants in 51 children, or 30.5%) was 2.4-fold higher than the proportion of 55 control children with sequence variants (8 sequence variants in 7 children, or 12.7%).

Dexrazoxane decreases the incidence and severity of both acute and late anthracycline-induced cardiotoxicity in children with solid tumors (Choi et al., 2010). The Dana–Farber Cancer Institute’s Childhood Acute Lymphoblastic Leukemia Consortium has incorporated dexrazoxane into all its clinical protocols involving anthracyclines since 2000 with no adverse effects. The use of dexrazoxane for cardioprotection in patients receiving doxorubicin has been endorsed by national organizations, such as the American Heart Association (Lipshultz et al., 2013a).

Anthracycline-related cardiotoxicity should be managed according to contemporary guidelines for treating systolic and diastolic hypertension, lipid disorders, diabetes mellitus, and behaviors that may increase the risk of heart failure (e.g., smoking, excessive alcohol consumption, and illicit drug use). Healthcare providers should periodically evaluate children for signs and symptoms of heart failure in those at high risk for heart failure and, in patients with known atherosclerotic vascular

disease, should follow current guidelines for secondary prevention. In patients with a strong family history of cardiomyopathy, and in those receiving cardiotoxic interventions, noninvasive evaluation of LV function (i.e., LVEF) is indicated. No studies have evaluated strategies to prevent heart failure in survivors of childhood cancer.

35.4 Conclusion

Advances in treatment have increased survival in children with HIV infection and cancer, but their cardiovascular-related health burden will increase as they age. We need to understand how to better identify the adverse cardiac effects of the various medications used to treat these diseases. Mitochondrial alterations and/or dysfunction are part of the pathogenesis of a wide range of diseases including unrelated disorders, such as diabetes and autism in children. Further, iatrogenic mitochondrial dysfunction explains many adverse reactions from medications. Even then, data supporting evidence-based guidelines for cardiovascular monitoring during various therapies are not definitive. Cardiovascular effects may appear decades after treatment and are often progressive and irreversible. The aim of vigilant monitoring is to identify signs of cardiac disease early enough to potentially prevent, slow, or reverse the deterioration of the structure and function of the heart. More research is needed to identify tailored therapies that will decrease the risk of cardiotoxicity without reducing efficacy. Mitochondrial toxicity testing may also advance drug development. By understanding the mechanisms underlying drug-induced mitochondrial damage, it may be possible to develop strategies and guidelines to decrease the potentially toxic effects of medications.

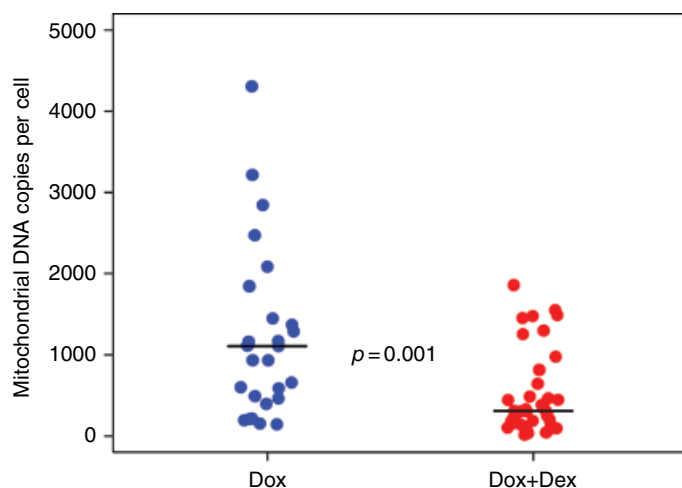


Figure 35.4 Mitochondrial DNA concentrations for doxorubicin alone, without dexrazoxane versus dexrazoxane administered before each dose of doxorubicin. DEX, dexrazoxane; DOX, doxorubicin; DOX+DEX, administration of dexrazoxane before every doxorubicin treatment. Horizontal black bars indicate median. Lipshultz et al. (2016). Reproduced with permission of John Wiley & Sons.

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36

Role of Mitochondrial Dysfunction in Linezolid-Induced Lactic Acidosis

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CHAPTER MENU

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Linezolid is the only commercially available oxazolidinone, a class of antibiotics discovered in the late 1970s and particularly active against Gram-positive bacteria. In the United States, it is approved for the treatment of community-acquired pneumonia, nosocomial pneumonia, and skin infections caused by *Streptococci* or *Staphylococcus aureus* (including methicillin-resistant strains) and for the treatment of infections caused by vancomycin-resistant *Enterococcus faecium* (Diekema and Jones, 2001). Maximal duration of therapy approved by the Food and Drug Administration (FDA) is 28 days.

Globally, the prevalence of methicillin-resistant *S. aureus* (MRSA) ranges from 20 to 80% (World Health Organization, 2014). In the United States, the Centers for Disease Control and Prevention estimate that MRSA was responsible for >80,000 invasive infections and >11,000 deaths in 2011 (US Department of Health and Human Services, 2013). In accord with these findings, in many European countries, >25% of *S. aureus* are resistant to methicillin (European Centre for Disease Prevention and Control, 2015).

Enterococcus is a commensal of humans that can cause difficult-to-eradicate nosocomial infections. Multidrug-resistant *Enterococcus faecalis* and *E. faecium* are becoming more common in both the United States and Europe. Up to 30% of healthcare-associated infections due to *Enterococcus* in the United States involve vancomycin-resistant strains, with an estimated annual mortality of 1300 patients (US Department of Health and Human Services, 2013). The prevalence of vancomycin resistance among *E. faecium* in Europe is similarly increasing (European Centre for Disease Prevention and Control, 2015).

The reported trends and prevalence in potentially life-threatening infections caused by multidrug-resistant Gram-positive bacteria pose a serious challenge to healthcare providers and explain the increasing use of linezolid (Monnet and Giesecke, 2014).

Linezolid is a safe drug and is used orally or intravenously. It is oxidized by the liver and is eliminated via non-renal (70%) and renal (30%) routes (Diekema and Jones, 2001). The most common side effects are diarrhea

(4%), nausea (3%), and headache (2%). Other less common side effects are thrombocytopenia and myelosuppression, peripheral and optic neuropathy, and serotonin syndrome (especially in case of concomitant use of serotonergic agents) (Rubinstein et al., 2003). Although some of these side effects might be attributed to mitochondrial dysfunction, current knowledge on this issue remains scarce.

Lactic acidosis can develop during linezolid use. When no other risk factors can be identified, it possibly reflects mitochondrial dysfunction induced by linezolid.

The primary aim of this chapter is to examine the role of mitochondrial impairment in the etiology of linezolid-induced lactic acidosis, with a special emphasis on critically ill subjects who are at higher risk of death.

36.1 Mechanisms Responsible for Lactic Acidosis in Critically Ill Subjects

Lactic acid is a product of glycolysis and is normally produced (mainly by the skeletal muscle) and consumed (mainly by the liver) at a rate of ≈ 1 mmol/h/kg of body weight in humans. As a result of the balance between production and consumption, lactate concentration in blood is normally stable and <2 mmol/L (Mizock, 1989). Lactic acidosis, the accumulation of lactate in blood, usually reflects a combination of overproduction and underutilization.

In the cytoplasm, glucose is oxidized to pyruvate (glycolysis) accompanied by the reduction of nicotinamide adenine dinucleotide (NAD^+ to NADH) and the production of 2 mol of ATP per mole of glucose. Glycolysis does not require oxygen and it proceeds in the presence (aerobic glycolysis) or absence (anaerobic glycolysis) of this gas. In the presence of oxygen, pyruvate is transported into mitochondria where it is oxidized to carbon dioxide and water via the Krebs cycle and oxidative phosphorylation. During oxidative phosphorylation, 30–32 mol of ATP are released per mole of glucose that is fully oxidized, that is, some 16-fold more than glycolysis. Oxidative phosphorylation is strictly aerobic and is responsible for 80–90% of whole-body oxygen consumption (Nelson and Cox, 2012).

When oxygen availability is impaired, glycolysis accelerates to compensate for the loss of oxidative ATP generation. Under these conditions, cytosolic lactate dehydrogenase regenerates NAD^+ by reducing pyruvate to lactate that exits the cells and increases circulating levels (Kreisberg, 1984; Mizock, 1989). However, glycolysis can only retard the decline in ATP. Most cells are imperiled by the unavailability of oxygen, and the ensuing ATP depletion, and will die via apoptosis or necrosis

depending on severity and duration of anoxia (Lieberthal et al., 1998).

Lactic acidosis in critically ill subjects commonly develops due to tissue hypoxia (type-A lactic acidosis) and thus mainly reflects lactate overproduction via accelerated glycolysis (Arieff and Graf, 1987). Less frequently, lactic acidosis develops without clinical evidence of tissue hypoxia (type-B lactic acidosis). For example, it can occur when oxygen use is impeded by an intrinsic mitochondrial defect (after exposure to mitochondrial inhibitors such as cyanide or metformin at toxic dose), when aerobic glycolysis is overly stimulated (increased sympathetic tone) or when lactate clearance is largely limited (acute and massive liver disease) (Kreisberg, 1984; Mizock, 1989). However, most cases presenting as type-B lactic acidosis during critical illness are actually due to clinically occult tissue hypoxia, that is, they represent type-A lactic acidosis.

36.2 Mechanisms Responsible for Tissue Hypoxia in Critically Ill Subjects

Tissue hypoxia reflects an imbalance between oxygen supply and demand (Snyder and Pinsky, 1987). Its most common cause is low oxygen delivery due to low hemoglobin concentration, low arterial oxygen saturation, and/or low blood flow (cardiac output and its effective distribution in tissues) (Barcroft, 1920).

Tissue hypoxia can also develop when oxygen demand increases and oxygen delivery does not. This can be the case of a subject with shivering, agitation, increased work of breathing, fever or hyperthermia, and a preexisting, sufficiently advanced cardiopulmonary disease (Snyder and Pinsky, 1987).

Finally, oxygen metabolism can derange even if oxygen supply is not limited. For example, when mitochondrial function is impaired by an intrinsic factor such as structural or xenobiotic inhibition of oxidative phosphorylation, oxygen utilization ceases independently from oxygen supply. This condition is known as “dysoxia” (Robin, 1977), “histotoxic hypoxia,” or “cytopathic hypoxia” (Fink, 1997) and yields type-B lactic acidosis.

36.3 Relationship between Lactic Acidosis and Oxygen-Derived Variables

The relationship between oxygen delivery and consumption is usually biphasic. Under normal conditions, oxygen supply (≈ 1000 mL/min, in humans) largely exceeds oxygen demand (≈ 250 mL/min). When oxygen delivery

suddenly starts to decline, oxygen consumption initially remains normal. This can be seen as an increase in oxygen extraction—the ratio between oxygen that is taken up by tissues and oxygen that is delivered to those same tissues—and a decrease in venous oxygen content. In other words, if oxygen delivery decreases, the hemoglobin unloads more oxygen in the capillaries and returns to the heart with lower (venous) oxygen saturation. As oxygen supply declines further, a critical threshold is reached below which tissue oxygen consumption starts to diminish. The blood lactate concentration then abruptly increases, reflecting accelerated anaerobic metabolism (Schumacker and Cain, 1987; Snyder and Pinsky, 1987; Ronco et al., 1993).

The most common cause of tissue hypoxia in critically ill subjects is low oxygen supply. Therefore, (type-A) lactic acidosis is usually associated with low arterial hemoglobin concentration, low arterial oxygen saturation, or low cardiac output as well as with low venous oxygen saturation (Kasnitz et al., 1976). Interventions that effectively ameliorate oxygen delivery, including blood transfusion, inhalation of oxygen, fluid resuscitation, and infusion of vasoactive drugs, usually increase oxygen consumption (Bihari et al., 1987) and resolve lactic acidosis (Rivers et al., 2001).

However, for venous oxygen saturation to decline, the mitochondria must be able to use oxygen, as they normally do. As a paradox, if mitochondria cease to function, oxygen consumption virtually stops and venous oxygen saturation progressively increases up to the arterial value. Accordingly, (type-B) lactic acidosis due to mitochondrial poisoning is typically associated with abnormally high, and not low, venous oxygen saturation (Peddy et al., 2006; Protti et al., 2010, 2012). Moreover, it does not benefit from interventions that increase oxygen delivery because the underlying problem is inefficient or lacking of oxygen utilization.

36.4 Incidence and Risk Factors of Linezolid-Induced Lactic Acidosis

Linezolid-induced lactic acidosis is defined as lactic acidosis that develops during linezolid use in the absence of any other major, and more common, risk factor, such as tissue hypoxia.

From August 2011 to August 2016, 90 cases of lactic acidosis, out of 2801 adverse events associated with linezolid use, were reported to the FDA Adverse Event Reporting System (personal communication). Lactic acidosis thus represented 3% of total adverse events. In 63 of these 90 cases, linezolid was considered the sole cause of lactic acidosis that proved fatal in 43% of the patients (27 fatalities). Similarly, over the same period, 6175

adverse events were reported to the European drug reaction report system of which 243 were for lactic acidosis (4%) with mortality of 15% (37 fatalities) (<http://www.adrreports.eu>). Even if these data are likely to underestimate its true incidence, linezolid-induced lactic acidosis is probably rare.

We searched in PubMed human studies published in English using “linezolid AND lactic acidosis” and “linezolid AND mitochondrial toxicity” as keywords. We retrieved 30 publications describing 57 cases in total, 20 in children and 37 in adults. As for the adults (17 females and 20 males), mean age was 62 ± 17 years, duration of therapy was 40 ± 31 days, lactate and arterial pH at the time of diagnosis were 14 ± 8 mmol/L and 7.12 ± 0.16 , respectively. Signs and symptoms at presentation ranged from abdominal discomfort, nausea, and vomiting to life-threatening respiratory failure and shock. Mortality was 15% (3 fatalities) among children and 19% (7 fatalities) among adults (Table 36.1).

In five studies (six subjects), lactic acidosis was attributed to linezolid toxicity despite the concomitant presence of cardiovascular insufficiency which, as discussed earlier, is a more common and reasonable cause of the syndrome. In three of these studies, however, lactic acidosis persisted after the resolution of shock and was only resolved after linezolid was withdrawn (Carson et al., 2007; Lee et al., 2013; Abou Hassan et al., 2016). In some studies, it was not possible to attribute lactic acidosis to linezolid toxicity because of coexisting tissue hypoxia (Im et al., 2015) or concomitant use of other potentially toxic drugs (such as nucleoside reverse transcriptase inhibitors) (Carson et al., 2007). In most of the remaining studies, no obvious cause of lactic acidosis, other than linezolid use, could be identified.

One probable risk factor of linezolid-induced lactic acidosis is long-term drug use. Almost 60% of the cases reported in Table 36.1 occurred during prolonged (>28 days) treatment. Even so, peak lactate level was not associated with duration of linezolid use ($p=0.88$ for Pearson's correlation). Linezolid dose, when reported, was always the same (600 mg twice a day).

One other risk factor could be renal failure, since linezolid is partly eliminated by the kidneys. Only 8, out of 57, subjects with linezolid-induced lactic acidosis had acute or chronic renal failure when they started assuming linezolid. Their lactate level (16 ± 9 vs. 13 ± 8 mmol/L, $p=0.30$ at Student's *t*-test) and mortality (13% vs. 21%, $p=0.60$ at chi-squared test) were not higher than those of subjects with initially normal renal function. Therefore, kidney injury seems to have a minor (if any) role in the development of the syndrome.

The importance of polymorphisms in mitochondrial DNA (mtDNA) was investigated in 6 studies and is discussed in the following text.

Table 36.1 Cases of linezolid-induced lactic acidosis in adults, retrieved in PubMed in September 2016.

Reference	Age	Sex	Reason for using linezolid	Days of therapy	Lactate (mmol/L)	Arterial pH	Clinical presentation	Outcome
Apodaca and Rakita (2003)	52	F	Invasive nocardiosis	77	10	NA	Nausea, vomiting, myelosuppression	Alive
Bernard et al. (2003)	81	M	MRSA osteomyelitis	21	18	6.90	Confusion, hypertension, tachycardia	Dead
Palenzuela et al. (2005)	49	F	<i>Mycobacterium abscessus</i>	58	13	NA	Nausea, diarrhea	Alive
	74	F	VRE infection	47	18	NA	Nausea, vomiting, confusion	Dead
Soriano et al. (2005)	25	M	Knee prosthesis infection	90	7	NA	Mild asthenia	Alive
	75	M	Nocardia cerebral abscess	80	3	NA	Severe asthenia	Alive
	55	M	MRSA knee prosthesis infection	45	5	NA	Mild asthenia	Alive
De Vriese et al. (2006)	63	F	MRSA prosthetic joint infection	120	25	NA	Optical neuropathy, myopathy, acute kidney injury	Alive
Garrabou et al. (2007)	65	M	Methicillin-resistant <i>S. epidermidis</i> knee prosthesis infection	35	5	NA	None	Alive
	74	F	MRSA hip prosthesis infection	44	3	NA	Mild asthenia	Alive
	83	M	Methicillin-resistant <i>S. epidermidis</i> knee prosthesis infection	30	5	NA	Gastrointestinal discomfort	Alive
Carson et al. (2007)	35	F	<i>Mycobacterium avium</i> complex infection in AIDS	35	23	7.16	Diarrhea, abdominal pain, pancreatitis, shock	Alive
Wiener et al. (2007)	80	F	VRE bacteremia	19	19	7.03	Dyspnea, chest pain, thrombocytopenia, anemia	Alive
Scotton et al. (2013)	81	F	Mycobacterium tuberculosis complex paravertebral abscess	12	19	7.24	Nausea, vomiting, anemia	Alive
Kopterides et al. (2005)	70	M	MRSA bacteremia	7	13		None	Alive
Boutoille et al. (2009)	48	M	MDR Mycobacterium tuberculosis	105	12	7.32	Dyspnea, abdominal pain	Dead
Velez and Janech (2010)	36	M	VRE bacteremia	42	13	7.31	Abdominal pain, nausea, altered mental status	Alive
De Bus et al. (2010)	55	F	Prosthetic hip infection	50	13	7.27	Weakness, hitching, abdominal pain, nausea, vomiting, hepatic failure	Alive
Carbajo et al. (2011)	79	M	Prosthetic knee infection	49	3	7.25	Asthenia, anorexia, nausea, vomiting, pancytopenia	Alive
Contou et al. (2011)	81	M	<i>Enterococcus faecium</i> bacteremia	0	16	7.03	Confusion, tachypnea, tachycardia	Alive

Cope et al. (2011)	20	M	MRSA pneumonia in a subject with mitochondrial disease	2	7	NA	Tachypnea, tachycardia, vomiting	Alive
Miyawaki et al. (2013)	75	M	MRSA pyogenic spondylitis	72	25	7.09	Dyspnea, hypothermia, coma	Alive
Lee et al. (2013)	56	F	Urosepsis	1	4	7.37	Septic shock, respiratory failure, refractory lactic acidosis	Alive
Del Pozo et al. (2014)	72	F	Nocardiosis	63	3	7.25	Hypoglycemic coma, diarrhea, vomiting	Alive
	43	M	Pulmonary tuberculosis	56	7	7.29	Nausea, asthenia, diarrhea, pancytopenia, acute kidney injury	Alive
Sawyer et al. (2014)	63	M	Chest-wall abscess, pulmonary nocardiosis	85	23	6.96	Dyspnea, abdominal pain, altered mental status	Alive
Djibré et al. (2014)	70	M	Sternal manubrium infection	15	23	6.89	Vomiting, drowsiness, dyspnea, hypothermia, sepsis	Dead
Hsu et al. (2015)	38	F	Urinary VRE infection	12	24	6.92	Dyspnea, altered mental status, thrombocytopenia, anemia	Alive
Im et al. (2005)	64	M	Soft tissue infection in a subject with diabetes	42	20	6.91	Nausea, shock, acute kidney injury	Dead
	77	M	MRSA knee prosthesis infection	28	16	7.10	Tachypnea, tachycardia	Dead
	52	F	Urinary VRE infection	7	NA	7.19	Tachypnea, tachycardia, shock	Alive
	76	M	Diabetic foot osteomyelitis	5	5	NA	None (routine lactate testing)	Alive
	69	F	MRSA spine implant infection	35	5	NA	None (routine lactate testing)	Alive
Holmaas et al. (2014)	70	M	Toe infection	23	29	7.01	Somnolence, dyspnea, anemia	Alive
Johnson et al. (2015)	34	M	VRE endocarditis	12	24	7.07	Somnolence, hypoglycemia, pancreatitis	Alive
Abou Hassan et al. (2016)	74	M	Prosthetic knee infection	35	21	6.90	Shock, myelosuppression	Alive
Protti et al. (2016)	64	M	Respiratory failure in a lung transplant recipient	5	10	7.30	Shock, acute renal failure	Dead

MDR, multidrug resistant; MRSA, methicillin-resistant *Staphylococcus aureus*; NA, non-available; VRE, vancomycin-resistant *Enterococcus*.

36.5 Relationship between Linezolid-Induced Lactic Acidosis and Oxygen-Derived Variables

We have recently reported the case of a 64-year-old man who was admitted to intensive care with acute respiratory failure, 5 months after single lung transplant for pulmonary fibrosis. Computed tomography showed ground glass opacities at the right lung (graft). Initial treatment included linezolid (1200 mg/day IV, continuous infusion), meropenem, amphotericin B, and noninvasive ventilation. By day 3, all microbiological examinations were negative. Suspecting acute graft rejection, methylprednisolone (1 g/day) was started, while antimicrobials were continued. On day 4, respiratory failure worsened. Mechanical ventilation and low-flow venovenous extracorporeal carbon dioxide removal were initiated. Vasodilatory shock and acute renal failure were treated with increasing dose of catecholamines and continuous venovenous hemodialysis. On day 5, linezolid was discontinued because of refractory severe lactic acidosis possibly related to its use. On day 7, the man died of multiple organ failure. Postmortem examination revealed bilateral pulmonary edema and no bowel ischemia (Protti et al., 2016).

Oxygen-derived variables and blood lactate levels recorded day by day are shown in Figure 36.1. Severe lac-

tic acidosis progressively developed despite normal, or even above normal, venous oxygen saturation. Oxygen delivery slightly decreased over time, from 644 to 504 mL/min/m², while oxygen consumption substantially declined, from 172 to 52 mL/min/m². As a result, the oxygen extraction ratio dropped from 0.27 to 0.10, a direct reflection of impaired mitochondrial oxidative phosphorylation.

These data strongly resemble those reported in cases of inadvertent metformin accumulation or cyanide intoxication, where some components of the mitochondrial respiratory chain (complexes I and IV, respectively) are largely inhibited (Peddy et al., 2006; Protti et al., 2010, 2012). Linezolid-induced (type-B) lactic acidosis thus reflects an inability of mitochondria to use otherwise available oxygen.

Of note, linezolid should be considered the most probable cause of lactic acidosis only after excluding other, more common, explanations. This can be very difficult as subjects receiving linezolid usually suffer from some degrees of hypoxemia, anemia, or low cardiac output (possible complications of the infection being treated). Progressive worsening of lactic acidosis in the face of the correction of these abnormalities supports an alternative diagnosis. Even so, no specific symptom, sign, or diagnostic test available at the bedside can definitely prove that lactic acidosis is factually due to linezolid toxicity.

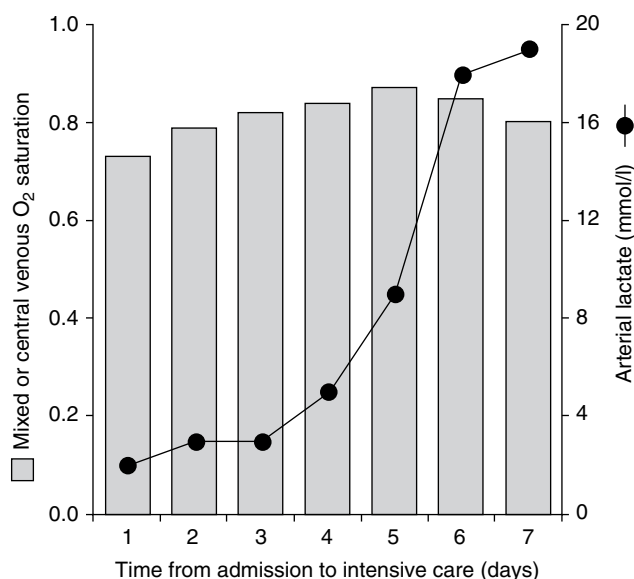


Figure 36.1 Oxygen-derived variables during linezolid-induced lactic acidosis. Changes over time of central (from day 1 to 3; normal value is around 0.70) or mixed (from day 4; normal value is around 0.65) venous oxygen (O₂) saturation in a man who developed linezolid-induced lactic acidosis during his stay in intensive care. Linezolid (1200 mg/day) was continuously infused from days 1 to 5.

36.6 Mitochondrial Ribosomes and Translation

Most of the mitochondrial proteins, including subunits of respiratory chain complex II and citrate synthase, are encoded by nuclear DNA (nDNA). They are synthesized in cytoplasm and then imported in mitochondria. However, some subunits of respiratory chain complexes I, III, and IV are encoded by mtDNA and synthesized by mitochondrial ribosomes (Beattie et al., 1966; Sandell et al., 1967). Intra-mitochondrial translation of messenger RNAs (mRNAs) into proteins relies on transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) that are encoded by mtDNA. All the other components required, including initiation factors, elongation factors, termination factors, and proteins forming the mitochondrial ribosomes, are encoded in the nucleus.

The critical steps of intra-mitochondrial translation are grossly the same as those of translation in the cytoplasm (Mai et al., 2017). However, organelles devoted to translation (ribosomes) inside mitochondria clearly differ from their cytosolic, and bacterial, counterparts (De Silva et al., 2015). Mammalian mitochondrial ribosomes are made up of a small (28S) subunit and a large (39S)

subunit that together form the 55S complex. Each subunit contains a single rRNA: 12S for the small subunit and 16S for the large subunit. Compared with cytosolic (80S) and bacterial (70S) ribosomes, mammalian mitochondrial ribosomes contain much more mitochondrial ribosomal proteins and less rRNA (Chrzanowska-Lightowlers et al., 2011). As a result, their structure is quite porous and their average density is relatively low.

36.7 How Linezolid Exerts its Therapeutic—and Toxic—effects

Linezolid inhibits bacterial growth by interfering with bacterial protein synthesis, as do many other antibiotics. However, its molecular target, that is only partially understood, is probably unique and suggests why linezolid is often active against multidrug-resistant bacteria.

Bacterial ribosomes are made of up two subunits that together form the 70S complex. The small (30S) subunit contains 16S rRNA associated with 21 mitochondrial ribosomal proteins. It is part of the translation process, along with the mRNA, the tRNA carrying *N*-formylmethionine (fMet-tRNA) that initially enters the P-site, the guanosine triphosphate (GTP) that fuels the reaction, and the initiation factors IF1, IF2, and IF3. The large (50S) subunit contains 5S rRNA, 23S rRNA, and 36 proteins. It assembles with the initiation complex and, together with the elongation factor Ef-Tu and GFP, facilitates the entry of the second aminoacyl-tRNA to the A-site of the ribosome. The energy released by the hydrolysis of GTP activates the peptidyl transferase center (PTC): the amino acid at the P-site is transferred to the A-site where it is bound to the nascent polypeptide. The uncharged tRNA exits the P-site, the newly formed peptide in the A-site moves to the P-site and a new aminoacyl-tRNA enters the A-site. Elongation continues until the ribosome reaches a stop codon on the mRNA (termination) (Laursen et al., 2005).

In vitro experiments suggest that oxazolidinones inhibit bacterial protein synthesis by interfering with the early stages of translation. They possibly impede the assembly of the initiation complex or the binding of the first fMet-tRNA to the P-site (Swaney et al., 1998). More probably, they prevent the entry or the proper placement of the aminoacyl-tRNA in the active site of the PTC by binding to the A-site on the 23S rRNA of the large subunit of bacterial ribosomes (Leach et al., 2007). Several observations support this conclusion: (i) puromycin, which binds specifically to the A-site, prevents oxazolidinones from inhibiting peptidyl transfer when it is present at high concentration (Bobkova et al., 2003); (ii) similarly to chloramphenicol, linezolid is thought to affect the accuracy of translation that depends on the

ribosomal A-site (Thompson et al., 2002); and (iii) most of the mutations associated with bacterial resistance to oxazolidinones (such as the G2576U) are located next to the PTC on the 23S rRNA (Meka and Gold, 2004).

Insights into the mechanisms responsible for the therapeutic effects of oxazolidinones are important to understand the potential mitochondrial toxicity of these antibiotics. Mitochondria are quite similar to bacteria, because of their endosymbiotic origin. Therefore, mitochondrial ribosomes can be unintended off-targets of drugs acting on the bacterial ribosomes. Experiments performed with photoreactive substrates in living cells have shown that oxazolidinones can interact with mammalian mitochondrial (but not cytoplasmic) ribosomes, at positions A2451 and U2506 of the 16S rRNA of the large subunit, the same positions to which they cross-link in *S. aureus* ribosomes (Leach et al., 2007). This nonselective binding can interfere with mammalian mitochondrial protein synthesis and function. It can thus trigger (type-B) lactic acidosis, a clear sign of mitochondrial intoxication (Figure 36.2).

36.8 Mitochondrial DNA Polymorphisms and Susceptibility to Linezolid

mtDNA sequence polymorphisms can influence the individual response to specific drugs. For example, some mutations of the mitochondrial 12S rRNA cause hypersensitivity to aminoglycosides that can manifest as hearing loss (Jing et al., 2015).

As discussed earlier, the therapeutic activity of linezolid depends on the interaction with the bacterial 23S rRNA, while some toxic effects depend on the interaction with the human mitochondrial 16S rRNA. Some genetic polymorphisms of the bacterial 23S rRNA confer resistance to linezolid (Kloss et al., 1999; Prystowsky et al., 2001; Meka and Gold, 2004), whereas some genetic polymorphisms of human mitochondrial 16S rRNA (such as A2706G and G3010A) increase the risk of linezolid toxicity (Carson et al., 2007; Velez and Janech, 2010; Protti et al., 2016). However, there is a huge discrepancy between the very high frequency of these genetic polymorphisms of human mitochondrial ribosomes and the very rare occurrence of linezolid-induced lactic acidosis. In our database of subjects (mostly Italian) with fully sequenced mtDNA, A2706G and G3010A substitutions were as frequent as 61 and 15%, respectively. These percentages are even higher in pan-ethnic cohorts, up to 80% in the general population according to the human mtDNA mutation database (<http://www.mtddb.igp.uu.se>). Therefore, these mitochondrial rRNA polymorphisms act only as weak risk factors. Otherwise, linezolid-induced lactic acidosis would be a much more common event.

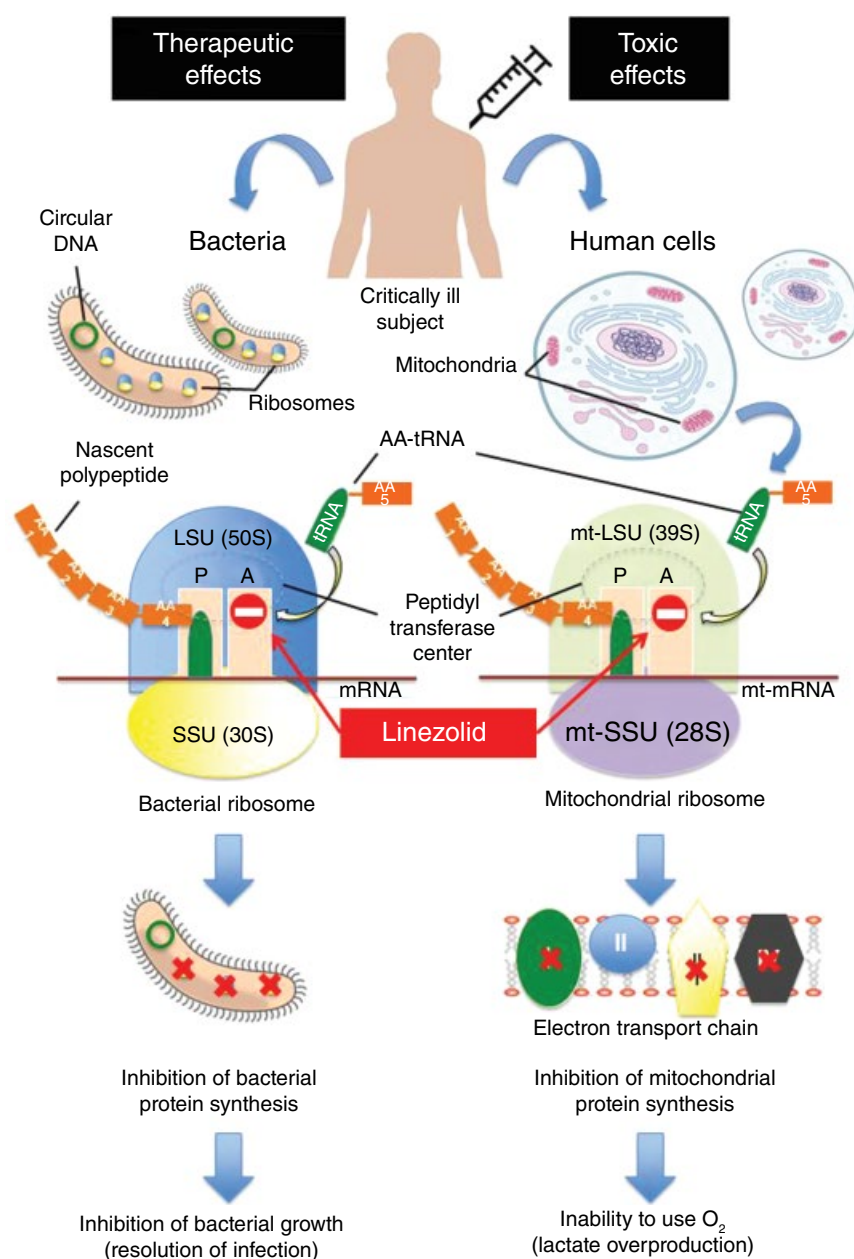


Figure 36.2 Therapeutic and toxic effects of linezolid. Bacterial ribosomes are made up of two subunits: a large 50S subunit (LSU) and a small 30S subunit (SSU). The large subunit contains the P-site where the peptidyl tRNA is formed, the A-site where the aminoacyl-tRNA (AA-tRNA) enters, and the peptidyl transferase center (PTC) where the amino acid at the P-site is transferred to the A-site and bond to the nascent polypeptide. Linezolid exerts its therapeutic effect by binding to the A-site and thus preventing the entry or the proper placement of the AA-tRNA in the active site of the PTC. As a result, bacterial protein synthesis and growth are inhibited. Mitochondrial ribosomes are structurally similar to their bacterial counterparts. They control the synthesis of some components of the respiratory chain complexes I, III, and IV. In rare cases, linezolid interacts with mitochondrial ribosomes, interferes with mitochondrial protein synthesis, impairs oxygen consumption, and thus accelerates anaerobic glycolysis. Lactate overproduction leads to (type-B) lactic acidosis.

Another source of variability of linezolid toxicity is possibly represented by ancient and highly frequent single-nucleotide polymorphisms (SNPs) that define groups of phylogenetically related mtDNA genotypes (mtDNA haplogroups) (Wallace et al., 1999). These mitochondrial sequences are responsible for subtle functional changes

that may affect disease occurrence or drug susceptibility. Subjects belonging to mtDNA haplogroup J1, for instance, may be particularly susceptible to linezolid toxicity (Pacheu-Grau et al., 2013). Notably, the SNPs that define this haplogroup are very close to the ribosomal PTC sequences. The haplogroup J1 is present in 9% of

non-Hispanic white people in the United States (Mitchell et al., 2014) and in 8–9% of the European population. Therefore 8–9% of the population of these two countries may be at higher risk for developing lactic acidosis during treatment with linezolid (Abou Hassan et al., 2016).

In conclusion, it is difficult to define a causal relationship between certain mtDNA polymorphisms and linezolid-induced lactic acidosis because of the moderate prevalence of these polymorphisms in the general population and the infrequent availability of mtDNA sequence of subjects who have possibly developed the syndrome. Even so, testing the mitochondrial sensitivity to the most frequently used ribosomal antibiotics (constructing a sort of “mitochondrial antibiogram”) might be of benefit (Pacheu-Grau et al., 2013).

36.9 Mitochondrial Toxicity of Linezolid

Since 2003, lactic acidosis during prolonged use of linezolid has been thought to reflect mitochondrial toxicity (Apodaca and Rakita, 2003). Few years later, it was demonstrated that oxazolidinones are able (i) to decrease the rate of mitochondrial incorporation of radiolabelled methionine (i.e., mitochondrial protein synthesis) in several animal tissues and (ii) to inhibit proliferation of human cells in a time- and dose-dependent manner. Treated human cells exhibited low levels of COX1 (a subunit of complex IV encoded by mtDNA) at Western blot analysis. Washing and reseeded these cells in the absence of oxazolidinones resulted in resumption of growth. Remarkably, these toxic effects were not noted in cells without mtDNA (rho-0) (Nagiec et al., 2005; McKee et al., 2006).

As for human studies, De Vriese and colleagues described mitochondrial structure, mitochondrial respiratory chain enzymes activity and mtDNA content of muscle, liver and kidney tissue samples taken from a subject who have developed optic neuropathy, encephalopathy, myopathy, lactic acidosis, and renal failure after using linezolid for 4 months. At histology, the liver and kidney looked normal and the skeletal muscle only had atrophy and a low number of subsarcolemmal mitochondria. At electron microscopy, few mitochondria had minor alterations of cristae architecture. Even so, biochemical assays revealed severe inhibition of respiratory chain complex I (kidney, muscle) and complex IV (liver, kidney, and muscle) that are mainly encoded by mtDNA. No alteration was noted in the activity of complex II, the only respiratory chain complex entirely encoded by nDNA. The activity of citrate synthase, which grossly reflects mitochondrial content, did not differ between case and controls. SDS-PAGE analysis of respiratory

chain subunits in the liver and muscle confirmed the selective depletion of respiratory chain complexes encoded by the mtDNA. Sequence analysis did not reveal any quantitative or qualitative defect in mtDNA. These findings were replicated in rats treated with linezolid for 2 (5 mg/kg/day) or 4 (250 mg/kg/day) weeks. Animals treated for short time and with low dose had minor variations in the activity of complex IV in the muscle and liver. Those treated for long time and with high dose suffered from severe reduction in the quantity and activity of mtDNA-encoded respiratory chain complexes. Citrate synthase activity and mtDNA content were well preserved (De Vriese et al., 2006).

Garrabou and colleagues noted a reduction in complex IV activity and COX-II protein level (a mtDNA-encoded subunit of complex IV) in peripheral blood mononuclear cells taken from five subjects with linezolid-induced lactic acidosis. All these abnormalities, including lactic acidosis, resolved after linezolid was discontinued (Garrabou et al., 2007).

In the case we have reported, the large decrease in systemic oxygen consumption and extraction (see earlier) was coupled with clear signs of defective mitochondrial protein translation in skeletal muscle biopsies taken at autopsy. In fact, skeletal muscle respiratory chain complexes I, III, and IV were largely inhibited (by 40–50% compared with controls), but complex II was not, with selective decrease in the level of mtDNA-encoded subunits. Lack of expected compensatory responses, such as induction of stress-response mitochondrial chaperones and acceleration of mitochondrial turnover, possibly contributed to these changes (Protti et al., 2016).

Similar biochemical defects were also found in the muscle of an elderly Lebanese subject with linezolid toxicity. Bilateral globus pallidus lesions, which are common during other human primary mitochondrial encephalomyopathies, were also noted (Abou Hassan et al., 2016).

The bulk of these data clearly demonstrates that the use of linezolid, especially if prolonged, can impair the activity of several components of the respiratory chain. This toxic effect is the consequence of abnormal mitochondrial translation.

36.10 Conclusion

Linezolid-induced lactic acidosis is rare—but potentially lethal—and reflects the untoward interaction between the drug and mitochondrial ribosomes that are structurally and functionally similar to bacterial ribosomes. The inhibition of mitochondrial protein synthesis depletes the electron transfer system of key components triggering acceleration of glycolysis with corresponding increased lactate production that yields (type-B) lactic acidosis.

Lactic acidosis should be attributed to linezolid only after excluding other, more common, causes (such as tissue hypoxia). Normal-to-high oxygen delivery, high venous oxygen saturation, and lack of response to interventions to increase systemic oxygen delivery all suggest a primary defect in oxygen utilization at the level of the mitochondria.

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37

Metformin and Lactic Acidosis*Jean-Daniel Lalau**Department of Endocrinology and Nutrition, Amiens University Hospital, Amiens, France***CHAPTER MENU**

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37.1 Introduction

While metformin is acknowledged as having a primary role in the treatment of type 2 diabetes mellitus, many cases of lactic acidosis have been reported in metformin-treated diabetic patients. In fact, these case reports featured all the possible permutations of metformin treatment with diseases capable of triggering lactic acidosis—especially in the context of shock syndromes (Lalau et al., 2017). Metformin's variable contribution to lactic acidosis is reflected by use of the ambiguous term “metformin-associated lactic acidosis” (MALA).

It is therefore essential to distinguish between “generic” MALA on one hand and a condition due exclusively to metformin (i.e., metformin-induced lactic acidosis (MILA)) on the other. In clinical practice, typical settings for MILA include metformin intoxication (due to overdosing) and acute metformin accumulation (due solely to acute kidney failure).

37.2 Metformin-Induced Lactic Acidosis

It is well known that metformin affects lactate metabolism (Bailey et al., 1992). In the intestine, metformin increases lactate production by accelerating glycolysis and by shifting glucose metabolism from aerobic pathways to

anaerobic pathways (Arieff et al., 1980; Radziuk et al., 1997). Postprandial lactate production by the small intestine (Bailey et al., 1992) and poor lactate uptake by hepatocytes (Radziuk et al., 1997) increase the plasma lactate level. If the recommended dose is adhered to, metformin's effects on glycemia control and glucose oxidation do not significantly modify the blood lactate concentration because lactate is converted to glucose in the liver (via the Cori cycle) (Stumvoll et al., 1995). In contrast, abnormally high levels of metformin accentuate the inhibition of hepatic gluconeogenesis and increase the level of anaerobic metabolism by the hepatocytes; this impairs lactate elimination and prompts a further decline in lactate uptake (Chu et al., 2003; von Mach et al., 2004). It was recently reported that metformin suppresses gluconeogenesis in rodents by inhibiting the hepatic redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase. Thus, metformin indirectly decreases the substrate fluxes of lactate and glycerol to gluconeogenesis and the flux of electrons to the mitochondrial respiratory chain (Madiraju et al., 2014).

In fact, metformin interferes with cell respiration only when mM concentrations (normally found solely in the intestine) are reached (He and Wondisford, 2015). The intestine is physiologically primed to promote glycolysis and the formation of lactate from glucose (Adeva-Andany et al., 2014). As long as the liver is working normally, hyperlactatemia is highly unlikely to result from intestinal lactate production. Conversely,

blood lactate levels may rise rapidly if the liver's uptake or metabolism of lactate is impaired because this organ is by far the main site for lactate recycling.

37.3 Further Complications of the Debate

The causal role of metformin as the triggering agent in lactic acidosis is difficult to assess because (i) metformin accumulation (even when major) does not necessarily lead to hyperlactatemia (Lalau and Race, 2000), and (ii) hyperlactatemia (even when major) does not necessarily lead to acidosis. Indeed, although the term "lactic acidosis" is used very frequently, a cause–effect relationship between lactate production and acidotic status has never been clearly established—at least in muscle tissue (Marcinek et al., 2010).

Hence MILA is not necessarily a "pure" phenomenon; it may merely reveal underlying defects in lactate metabolism and/or the acid–base balance.

37.4 In Clinical Practice

In situations with a risk of metformin accumulation, the following information is essential for sensibly assessing the relationship between metformin and lactic acidosis and, ultimately, for establishing a prognosis: (i) the metformin dose level, (ii) the time since administration of the last dose, (iii) the time interval between the last dose of metformin and collection

of the blood sample used to assay metformin, and (iv) the presence of any concomitant medications (in a context of metformin intoxication).

Once these factors are taken into account, it becomes clear that true MILA is very rare. In a review on metformin intoxication, Dell'Aglio et al. (2009) cited our reports (Lalau et al., 1998) but omitted the available information on multidrug intoxication in 11 of the 13 cases. In fact, this type of information is critical when determining the prognosis of an individual patient, since the course of lactic acidosis may be influenced by concurrent intoxication with drugs that alter ventilation.

Lastly, it should be remembered that lactate is not toxic *per se*. In fact, lactate production is the hallmark of impaired cellular energy production, and lactate can even substitute for glucose as a metabolic substrate under critical conditions (anaerobic conditions, in particular) via the tricarboxylic acid cycle entry (when it can be used as a gluconeogenic substrate, oxidized, or transaminated into alanine). Similarly, it is difficult to still consider metformin as a toxic drug. Although massive metformin accumulation may alter mitochondrial respiration, hyperlactatemia is highly unlikely to result from intestinal lactate production as long as the liver is able to eliminate the excess lactate. Furthermore, a study of a cohort of septic patients with lactic acidosis showed that the mortality rate was much lower in those who were treated with metformin—even though a high lactate concentration is classically associated with a poor prognosis in this setting (Doeniyas-Barak et al., 2016). This finding shows that metformin's beneficial effects are impressive—even in the most critical situations.

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Lessons Learned from a Phase I Clinical Trial of Mitochondrial Complex I Inhibition

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In 2014, a first-in-human clinical trial of a drug intended to treat symptomatic peripheral artery disease (PAD) via activation of 5' AMP-activated protein kinase (AMPK) was performed. By the time this phase I trial was prematurely halted, two healthy volunteers required endotracheal intubation and mechanical ventilation for severe metabolic acidosis. This section will describe experience from this clinical trial that may inform future development of this class of drugs. Further details may be found in Low Wang et al. (2015).

PAD affects approximately 8.5 million people in the United States including 12–20% of individuals older than age 60 (CDC 2016 fact sheet). It is usually caused by atherosclerotic narrowing of arteries supplying the lower extremities. However, revascularization of large arteries supplying the lower limbs alone is not always sufficient to normalize function and eliminate claudication, the calf muscle cramping, and pain with walking that occurs in PAD that may severely limit function (Muller et al. 2013; SVS 2015). Skeletal muscle and mitochondrial dysfunction, as well as endothelial cell dysfunction with impairment of endothelial nitric oxide synthase (eNOS), are additional factors that contribute to the functional limitations observed in PAD (Kiani et al. 2013; Pipinos et al. 2008). Activation of AMPK with R118, an indirect AMPK activator, was demonstrated to increase eNOS production and improve exercise capacity in a mouse model of PAD (Baltgalvis et al. 2014). R118 is a small molecule that was being investigated by a pharmaceutical company called Rigel Pharmaceuticals, Inc. for

potential use in PAD. R118 does not activate AMPK directly, but rather inhibits mitochondrial respiration by inhibiting mitochondrial complex I with blunting of NADH oxidation (Baltgalvis et al. 2014). Mitohormesis, the intermittent production of reactive oxygen species in mitochondria leading to adaptation to subsequent stress (Ristow and Zarse 2010; Tapia 2006), has been proposed as a potential mechanism for the beneficial effect of partial inhibition of mitochondrial respiration (Baltgalvis et al. 2014). Investigators have demonstrated increased mitochondrial number and efficiency in response to weak RNA interference of a component of mitochondrial complex I (Owusu-Ansah et al. 2013) and improved mitochondrial function in response to exercise (Ascensao et al. 2005) and caloric restriction (Anson et al. 2003). Both rodent and primate data supported the safety of R118 (Baltgalvis et al. 2014, Rigel investigator's brochure 2013): at the 20 mg/kg/day dose in Sprague-Dawley rats, minimal reversible gastrointestinal adverse effects were seen; the 30 mg/kg/day dose used to achieve similar serum exposure in cynomolgus monkeys resulted in emesis and glucosuria, but no remarkable elevations in lactic acid concentrations.

The phase I trial of R118 was designed to investigate the safety and tolerability of R118 and to characterize the pharmacokinetic and pharmacodynamic profile of R118 in healthy human volunteers. The design was single center, randomized, placebo controlled, and double blinded with the exception of the initial dosing group, in which the first 2 subjects were randomized 1 : 1 to receive

Table 38.1 Number and severity of adverse events.

	Treatment group					
	1		2	3	4	Total
	Placebo (n=4)	30 mg aq susp, fasted (n=5)	75 mg aq susp, fasted (n=5)	75 mg aq susp, fed (n=5)	74 mg cap (2–37 mg/cap), fasted (n=5)	R118 (n=20)
Number (%) of subjects with at least 1 AE	1 (25%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)	20 (100%)
Number of AEs	1	9	16	21	31	77
Number of AEs by severity						
Grade 1 (mild)	1	5	10	9	6	30
Grade 2 (moderate)	0	4	6	12	21	43
Grade 3 (severe)	0	0	0	0	4	4
Number of SAEs	0	0	0	0	6	6
Number of AEs leading to hospitalization	0	0	0	0	7	7
Number of AEs leading to intubation	0	0	0	0	3	3

R118 versus placebo and the remaining four subjects all received R118. Subsequent dosing employed a randomization ratio of 5:1 for R118 versus placebo.

Frequent monitoring of lactic acid and beta-hydroxybutyrate concentrations was performed within 24 h of dosing, and individual stopping rules for elevated lactic acid were incorporated into the trial protocol. The dose of R118 was carefully chosen based upon pharmacology studies performed in rodents and a safety factor of 20-fold below the no observed adverse effect levels (NOAEL) of 30 mg/kg/day in a cynomolgus monkey study that showed no evidence of lactic acidosis during 28 days of dosing. The original dosing protocol was amended after the trial began because of gastrointestinal adverse effects experienced by the human subjects.

Dosing group 1 involved a 30 mg dose of R118 in an aqueous suspension in a fasted subject as compared with one subject receiving placebo and was followed by 30 mg dosed in four additional subjects. This dose was tolerated except for mild to moderate nausea (all subjects), vomiting (two of the five subjects), and cold sweats (one of the five subjects) in the subjects randomized to receive R118 (Table 38.1). There were two subjects in this first dosing group who had mild lactic acid elevations lasting longer than 16 h post-dosing. The three subsequent dosing groups were randomized 5:1 as noted earlier. Group 2 received a single dose of 75 mg administered in an aqueous suspension, in the fasted state. This was not tolerated as well by the subjects, with all subjects experiencing at least one adverse event (AE) that was graded moderate in 37.5% of subjects and mild in 62.5%: four

subjects experienced nausea, four subjects experienced vomiting, two subjects experienced dizziness, and one subject experienced diarrhea (Low Wang et al. 2015). Three subjects exhibited mild elevations in lactic acid concentrations, which lasted for longer than 16 h in a single subject. Group 3 involved a single dose of 75 mg in fed subjects, but unfortunately the number and severity of AEs increased further. These were predominantly gastrointestinal in nature (nausea and vomiting, with diarrhea in one subject) along with headache in a single subject and presyncope in a single subject. Two of the five subjects receiving R118 exhibited more marked elevations in lactic acid. Group 4 turned out to be the final dosing group and involved a single dose of 74 mg (2–37 mg capsules) dosed to subjects in the fasted state using an encapsulated spray-dried dispersion of R118 in an effort to improve tolerability. This form of dosing resulted in approximately threefold higher R118 exposure than the aqueous suspension, but there was large interindividual variation, as was observed with the aqueous suspension (Low Wang et al., 2015). Group 4 exhibited a higher degree of lactic acid elevation, with two of five subjects developing lactic acidosis severe enough to require intubation. The R118 serum levels peaked earlier than lactic acid levels (Figure 38.1). Lactic acid levels in each subject in group 4 are shown in Figure 38.2.

The first of the two subjects requiring intubation did not have any significant medical history, did not smoke or use illicit drugs, and used alcohol only occasionally. He was a 51-year-old African–American man who had not taken any concomitant medications within 15 days of

Figure 38.1 Mean R118 and lactic acid concentrations in healthy volunteers in group 4 after a single dose of R118. R118 levels are denoted with squares, while lactic acid levels are denoted with circles. Low Wang et al. (2015). Reproduced with permission of John Wiley & Sons.

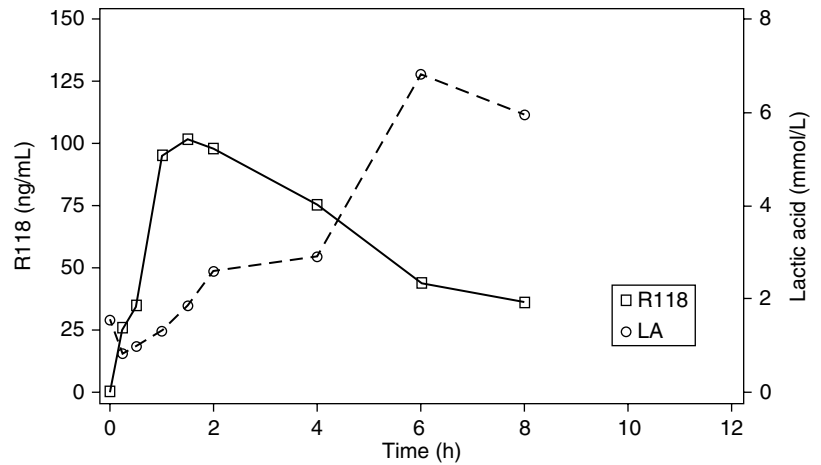
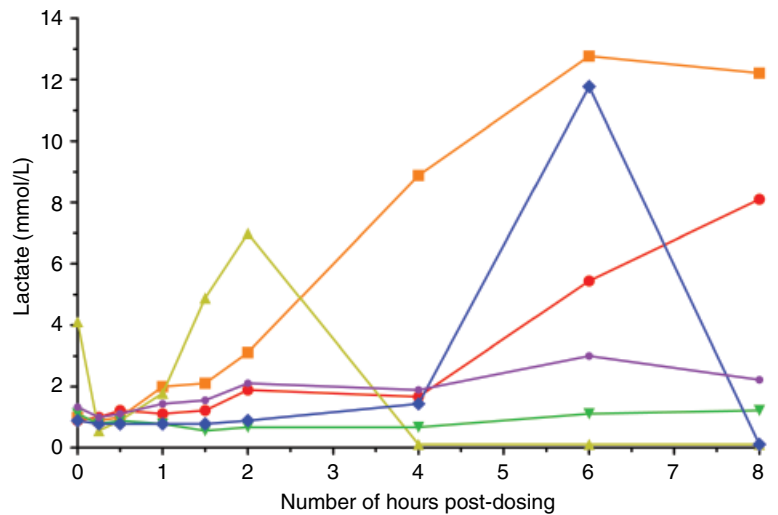


Figure 38.2 Serum lactate concentrations within 8 h of dosing in group 4 including the subject receiving placebo (inverted triangles). Each line represents lactate concentrations for a single subject over time. Lactate concentrations for subject 1 are denoted by the larger circles, while those for subject 2 are denoted by squares. Lactate concentrations were markedly elevated in 2 of the subjects (denoted by diamonds and triangles, respectively), but this did not correlate with the clinical presentation, and these subjects did not require intubation.



dosing. He began vomiting 1 h after receiving the 74 mg dose of R118. He was given 1 L of intravenous (IV) normal saline and promethazine 25 mg IV, but he became progressively agitated, with acute mental status changes and reported hypotension, so he was transported to the local emergency room (ER). Upon presentation, he was found to be hypothermic to 90.9°F. Within minutes of arrival and receiving diphenhydramine 50 mg IV, he developed severe hypotension with a blood pressure of 60/0 mmHg and unresponsiveness. He was intubated to preserve his airway and given benztropine 1 mg IV. At that point, he was also found to have acute kidney injury with a serum creatinine of 1.64 mg/dL and severe metabolic acidosis with arterial blood gas showing pH 6.63 at 5 h post-dosing. He was given three doses of sodium bicarbonate 50 mEq IV, and serum lactate 6.5 h post-dosing was greater than 20 mmol/L (reference range 0.9–1.70). He was transferred to the intensive care unit (ICU) and started on vasopressors including norepinephrine and vasopressin. After undergoing hemodialysis for

metabolic acidosis and receiving ICU supportive care, subject 1's lactate normalized 28 h post-dose, and he was discharged in good condition on day 7 post-dosing.

Subject 2 was a 54-year-old Hispanic man who had a medical history significant for hypercholesterolemia, long-standing tinnitus, lower back pain, and treated depression. He used tramadol as needed for his back pain but had not taken any concomitant medications within 15 days of taking the blinded 74 mg dose of R118. He did not smoke or use illicit drugs and had only 1 alcohol-containing drink per week. Approximately 5 h after the R118 dose, he experienced multiple episodes of vomiting. He was given 1 L of IV fluid but began to report abdominal pain and was noted to be tachypneic, so he was transported to a local ER. His lactic acid level was noted to be 16.6 (units and reference range not available). He was intubated and transferred to the ICU. He underwent hemodialysis for his metabolic acidosis (pH 7.138, serum bicarbonate 7.5 mEq/L). He was extubated 2 days after dosing and was off vasopressors and

following commands, but then became more agitated with increasing lactate (to 6 mEq/L), so he was reintubated and hemodialysis was restarted. By 3 days post-dosing, his serum lactate had normalized, and he was extubated 5 days after dosing but remained unresponsive until 7 days after initial dosing of R118. By day 8, he was awake and responding to commands, and he was finally discharged on day 11.

The other three subjects who had received 74 mg of R118 capsules in phase A4 developed nausea and vomiting and were hospitalized for observation but did not require intubation and were discharged in good health the day following dosing of R118.

In retrospect, the lactate levels in group 3 suggested a possible narrow therapeutic window for R118. What was unexpected from preclinical studies was the marked difference in pharmacokinetics of the uncoated encapsulated form of R118 administered in group 4. The final dosing phase of R118 resulted in severe toxicity that was likely multifactorial, with a longer half-life, markedly increased maximum concentration ($C_{\max}$), and area under the curve (AUC) than what had been observed in preclinical studies with presumed increased tissue accumulation of R118 (Figure 38.3), and possible stronger inhibition of complex I (Table 38.2). In mitohormesis, the “right” amount of stress from reactive oxygen species appears to induce an adaptive response that increases resilience to stress and protects against subsequent harm from a similar stress (Ristow and Zarse, 2010, Tapia 2006). However, with this narrow therapeutic window, creating just the right degree of stress by inhibiting mitochondrial complex I may be difficult, as demonstrated in this first-in-human clinical trial of R118. An additional

consideration is that the severe adverse metabolic consequences with R118 were seen in the resting condition. The intended use of the drug was to improve exercise performance in PAD, yet exercise per se can increase muscle and systemic lactate concentrations, which would be expected to further exacerbate the adverse effects of mitochondrial complex I inhibition.

In addition, there was the difficulty of translating animal studies to humans. Although a primate study showed similar serum R118 concentrations after dosing with R118 in aqueous suspension versus capsules, these two

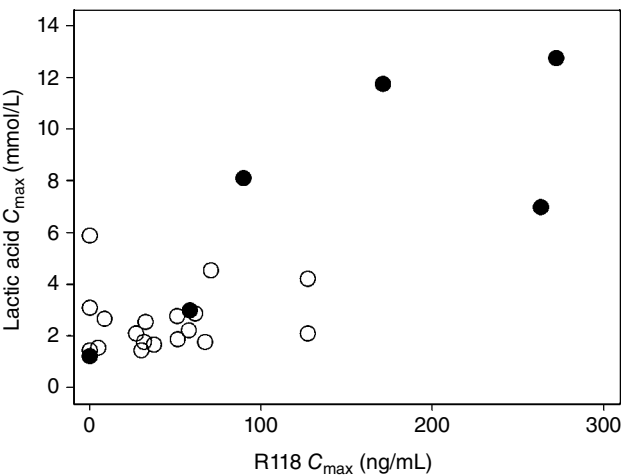


Figure 38.3 Correlation between peak $C_{\max}$ of R118 and peak lactate concentrations in each dosing group. Groups 1, 2, and 3 are represented by open circles. Group 4 is represented by filled circles. Low Wang et al. (2015). Reproduced with permission of John Wiley & Sons.

Table 38.2 Pharmacokinetic (PK) parameters for R118.

Group	1		2		3		4	
	30 mg suspension, fasted (N=5)		75 mg suspension, fasted (N=5)		75 mg suspension, fed (N=5)		74 mg capsules, fasted (N=5)	
PK parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$T_{\max}$ (h)	1.10	0.548	1.60	1.39	0.80	0.274	2.7	2.22
$C_{\max}$ (ng/mL)	26.4	19.5	55.9	42.0	74.8	30.2	171	97.4
AUC last ^a (ng·h/mL)	166	145	519	295	341	205	1570	1220
AUC _{0-∞} ^a (ng·h/mL)	232	206	657	352	440	197	1810	1370
$T_{1/2}$ (h)	30.1	14.7	47.2	18	84.4	37	39.3	11.5

Low Wang et al. (2015). Reproduced with permission of John Wiley & Sons; Group names changed from A1 to A4 in original manuscript to 1–4 in current chapter for consistency.

Note that the mechanism of clearance and volume of distribution is not known for R118.

SD, standard deviation.

^a AUC estimates for group 4 may be overestimated due to the fact that some subjects had missing time points while being treated at the hospital.

forms of dosing resulted in markedly different exposures in humans. Complex I contains 14 subunits, of which seven are derived from mitochondrial DNA. Although the complex I subunits are highly conserved among mammals, mitochondrial DNA from old world monkeys (including the cynomolgus monkeys studied for this compound) are distinct enough from human mitochondrial DNA to lack the ability to restore oxidative phosphorylation when combined with human rho0 cells (Kenyon and Moraes 1997). The differences in mitochondrial genome-derived complex I subunits may

account for the inability to predict drug toxicity for this compound using these preclinical and primate models.

It is quite possible that inhibition of complex I may still be an important target for drug development. Unfortunately, as was observed in this clinical trial, the interindividual pharmacokinetics of R118 varied dramatically (Figure 38.2), and serum R118 levels did not necessarily predict degree of lactic acidosis (Low Wang et al. 2015). The experience from this phase I clinical trial was a dramatic demonstration of how essential it is to utilize the right preclinical model to translate safety data to humans.

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Pharmacological Activation of Mitochondrial Biogenesis for the Treatment of Various Pathologies

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39.1 Introduction

Mitochondria orchestrate a wide range of metabolic pathways and are central to many critical cellular functions, including adenosine triphosphate (ATP) production, apoptosis, ion homeostasis, antioxidant defenses, and reactive oxygen species (ROS) production. As such, mitochondria are integral to the preservation of cell and tissue function necessary for human health. In the presence of toxicants or cellular stresses, mitochondria can be damaged, leading to increased ROS production, suppressed oxidative phosphorylation (OXPHOS) proteins, and diminished ATP production (Funk and Schnellmann 2012; McGill et al. 2012; Gibbs et al. 2016; Pisano et al. 2016). Thus, it is not surprising that mitochondrial dysfunction is important in a variety of acute and chronic disease processes across multiple organ systems.

Cells are capable of survival in a remarkable range of environments and disease states due, in part, to the plastic nature of mitochondria. Mitochondria are highly dynamic organelles able to adapt their size, shape, and organization in response to both internal and external stimuli. Dynamic quality control of mitochondrial mechanisms, including fission and fusion, mitophagy, and biogenesis, grants mitochondria the necessary plasticity and protection under cellular stress to meet metabolic needs for cell repair and regeneration (Friedman and Nunnari 2014). Because disruption of mitochondrial quality control mechanisms has been implicated in various diseases, strategies aimed at maintaining and restoring these cellular processes have received increasing attention. Specifically, pharmacological induction of mitochondrial biogenesis (MB), the generation of new, functional mitochondria, has proven to be a promising therapeutic target for a wide range of

acute and chronic diseases characterized by mitochondrial dysfunction (Whitaker et al. 2016).

39.2 Regulation of MB

MB is a multifactorial process involving the integration of strictly regulated transcriptional events, lipid membrane and protein synthesis/assembly, and replication of mitochondrial DNA (mtDNA) (Ventura-Clapier et al. 2008). Mitochondria are unique among extranuclear organelles in that they contain their own circular genome consisting of a small number of genes necessary for the architecture and function of mitochondria. Due to this limited coding capacity, MB requires coordination of both the mitochondrial and nuclear genomes (Scarpulla 2008). There are a number of nuclear transcription factors that increase expression of genes involved in OXPHOS, mitochondrial import and export systems, antioxidant defense, and mitochondrial gene transcription; however, these nuclear transcription factors are also involved in other cellular events independent of MB. In turn, the nuclear-encoded peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family of transcription coactivators, comprised of PGC-1 α , PGC-1 β , and PGC-related coactivator (PRC), have emerged as selective regulators of MB and respiratory function (Scarpulla 2008).

The PGC-1 coactivators serve as docking platforms that recruit additional protein complexes responsible for the transcription of nuclear genes involved in MB. PGC-1 α is the most studied and founding member of this family and is a positive regulator of MB and respiration, adaptive thermogenesis, and gluconeogenesis (Scarpulla 2008). Importantly, the expression of PGC-1 α is highly inducible by physiological cues signaling for increased energy needs, such as exercise, cold exposure, caloric restriction, oxidative stress, and cell division (Handschin and Spiegelman 2006). Due to the highly inducible nature of PGC-1 α , its activation has become an attractive therapeutic strategy for inducing MB.

PGC-1 α is also regulated through a host of posttranslational modifications, including methylation, acetylation, small ubiquitin-like modifier (SUMO)ylation, and phosphorylation. These modifications allow fine-tuning of PGC-1 α activity in a context-dependent manner. Deacetylation by silent mating type information regulation 2 homologue (sirtuin, SIRT) 1 and methylation by protein arginine methyltransferase 1 (PRMT1) increases PGC-1 α activity, whereas acetylation by general control nonderepressible 5 (GCN5) and SUMOylation by SUMO1 negatively impacts its function (Rodgers et al.

2005; Teyssier et al. 2005; Lerin et al. 2006; Rytinki and Palvimo 2009). Phosphorylation of PGC-1 α can increase or decrease its activity depending on the phosphorylating kinase. For example, p38 mitogen-activated protein kinase (MAPK) phosphorylation increases PGC-1 α activity, while AKT phosphorylation inhibits its activity (Puigserver et al. 2001; Li et al. 2007). Finally, PGC-1 α and other transcription factors associated with MB can be activated by nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) and Ca²⁺-dependent signaling (Wu et al. 2002; Handschin et al. 2003; Nisoli et al. 2004). In summary, multiple cellular mechanisms allow for the tight control of MB and mitochondrial homeostasis necessary to meet energy demands under physiological and pathological conditions.

The activating effects of PGC-1 α on MB are exerted through its coactivation of nuclear respiratory factors (NRFs), transcription factors that increase the transcriptional activity of numerous mitochondrial genes (Wu et al. 1999). The induction of NRFs by PGC-1 α increases the expression of not only mitochondrial transcriptional factor a (TFAM), but also other mitochondrial subunits of electron transport chain (ETC) complexes, such as ATP synthase, cytochrome *c*, and cytochrome oxidase IV. Following transcription and translation of the TFAM gene, the protein translocates to the mitochondrial matrix where it stimulates mtDNA replication and mitochondrial gene expression (Ventura-Clapier et al. 2008). In addition to NRFs, PGC-1 α also interacts with and coactivates other transcription factors, including peroxisome proliferator-activated receptors (PPARs), thyroid hormone, glucocorticoids, estrogen, and estrogen-related α and γ receptors for the increased expression of OXPHOS genes, mitochondrial transporters, antioxidant proteins, and other mitochondrial transcription factors (Ventura-Clapier et al. 2008).

39.3 Mitochondrial Dysfunction in Disease

Given the importance of mitochondria in a plethora of integral cellular processes, deregulation of mitochondrial homeostasis can have debilitating consequences, including loss of ATP-dependent cellular functions, increased ROS production, and oxidative damage. Therefore, mitochondrial dysfunction has been implicated in the pathophysiology of many disease states. In the following passages, and summarized in Table 39.1, we will discuss the contribution of mitochondrial dysfunction in several acute and chronic pathologies, as well as the enhancement of MB as a potential therapeutic strategy for each.

Table 39.1 Partial list of diseases characterized by mitochondrial dysfunction and evidence of MB as therapeutic interventions for each.

Disease	Mitochondrial dysfunction	Tested MB treatments	References
<i>Acute diseases</i>			
Ischemia/reperfusion			
● Myocardial infarction	Ischemia: ↓OXPHOS proteins, ↓ATP synthesis, ↓ATP-dependent cellular processes, ↓ion homeostasis	↑Exercise: ↓oxidative stress	Jiang et al. (2014)
● Acute kidney injury	Reperfusion: ↑ROS, ↑oxidative stress, ↑Ca ²⁺ , mPTP opening, ↑cell death	↑TFAM: ↑survival PDE inhibitors, formoterol, LY344864: ↑mitochondrial and renal function	Ikeuchi et al. (2005) Whitaker et al. (2013), Jesinkey et al. (2014a), and Garrett et al. (2014)
● Spinal cord injury		↑PGC-1α: ↓cell death, ↑function	Hu et al. (2016)
● Stroke		↑PGC-1α: ↓oxidative stress, ↓cell death Resveratrol: ↑neuron survival	St-Pierre et al. (2006); Dasgupta and Milbrandt (2007), and West et al. (2007)
<i>Chronic diseases</i>			
Neurodegenerative			
● Alzheimer's disease	↓MB, ↑mitochondrial size and structural damage, ↓content, ↑fragmentation, ↑mtDNA mutations	Bezafibrate: ↑PGC-1α, ↑behavior Resveratrol, TZDs, AICAR: ↑function, ↓cell death	Dumont et al. (2012), Kim et al. (2007), Cheng et al. (2016), and Vingtdoux et al. (2010)
● Parkinson's disease	↑mtDNA mutations (complex I), ↑oxidative damage, ↓PGC-1α, ↓OXPHOS, ↓homeostasis	Resveratrol: ↑function, ↓cell death	Blanchet et al. (2008) and Long et al. (2009)
● Huntington's disease	↓MB, ↓PGC-1α, ↓complex I-III activity, ↑mtDNA deletions, ↓mitochondrial genes	Resveratrol: ↑function, ↓cell death	Ho et al. (2010)
● Amyotrophic lateral sclerosis	↑Deformed mitochondria, ↓ETC activity, ↓ATP, ↑ROS, ↑oxidative stress, ↑Ca ²⁺ dysregulation	Resveratrol: ↑function, ↓cell death	Kim et al. (2007)

Arrows indicated increase (↑) and decrease (↓). AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; ATP, adenosine triphosphate; Ca²⁺, calcium ion; ETC, electron transport chain; MB, mitochondrial biogenesis; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; PDE, phosphodiesterase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1-α; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor A; TZDs, thiazolidinediones.

39.4 Acute Diseases

39.4.1 Ischemia/Reperfusion

Loss of mitochondrial function, and the subsequent energy deficit, is a major contributor to the tissue damage that occurs following ischemia (Stallons et al. 2013; Whitaker et al. 2016). The mechanisms underlying the resulting tissue damage are consistent, regardless of the site of injury or the method of ischemia induction. Deficient oxygen delivery halts OXPHOS, resulting in loss of mitochondrial membrane potential and eventually decreased ATP synthesis. This dysfunction is critical, as the organ systems that are most susceptible to ischemic injury have high energy requirements. Neurons,

for example, are greatly dependent upon efficient ion transport across the plasma membrane to maintain electrical homeostasis, conduct action potentials, and accommodate the release/uptake of neurotransmitters (Adibhatla and Hatcher 2010). Additionally, the kidney and heart are reliant upon ATP for the regulation of blood filtration and muscle contraction/relaxation, respectively (Hausenloy and Yellon 2013; Stallons et al. 2013; Whitaker et al. 2016).

While reperfusion following ischemia allows for reoxygenation and the reintroduction of nutrients to damaged tissue, the reinstatement of blood flow is also characterized by oxidative damage and inflammation (Moskowitz et al. 2010). Cells bordering the central site,

while hypoxic, are not solely dependent upon the vessel blocked during the ischemic event and as such are able to survive due to partial perfusion from surrounding vasculature. Upon reperfusion, however, the inrush of oxygen into these hypoxic peripheral cells leads to the generation of ROS, a large portion of which are formed from the ETC of dysfunctional mitochondria (Hausenloy and Yellon 2013; Sanderson et al. 2013). This increased ROS production not only overwhelms endogenous antioxidant defenses, but also leads to Ca^{2+} overload and opening of the mitochondrial permeability transition pore (mPTP). mPTP opening releases Ca^{2+} into the intermembrane space and allows the influx of ions and water into the mitochondrial matrix, simultaneously resulting in loss of the electrochemical gradient required to produce ATP and causing the matrix to swell, expanding the inner membrane until the outer membrane ruptures (Hirsch et al. 1998; McEwen et al. 2011). Rupturing releases accumulated Ca^{2+} , as well as ROS and proapoptotic proteins into the cytosol, initiating lipid/protein oxidation and apoptotic cell death (Sesso et al. 2004; Kalogeris et al. 2014). Therefore, therapeutics aimed at restoring mitochondrial homeostasis could aid in rescuing organ function following ischemic/reperfusion (I/R) injury.

39.4.2 Myocardial Infarction (MI)

Unfortunately, strategies targeting specific aspects of mitochondrial dysfunction following I/R have proven largely inconsistent or unsuccessful (Andreaddou et al. 2009; Kalogeris et al. 2014). For example, while beneficial as pretreatments, antioxidant-based treatments have shown limited, if any, therapeutic advantage when administered during or after reperfusion (Kalogeris et al. 2014). When administered at the time of reperfusion, the mPTP inhibitor cyclosporine A (CsA) enhanced mitochondrial function and decreased infarct size in the post-I/R heart *in vivo* (Jurado et al. 1998; Lim et al. 2007; Skyschally et al. 2010); however, data from human studies investigating CsA for the treatment of myocardial infarction (MI), which occurs following decreased blood flow to cardiac muscle, have been mixed (Piot et al. 2008; Ottani et al. 2016). Regardless of any positive results, CsA is toxic, making it less than ideal as a therapeutic (Caramelo et al. 2004; Schenk et al. 2010; Rabchevsky et al. 2011; Szalowska et al. 2015). Evidence suggests, however, that enhanced MB may be an advantageous method for the treatment of MI. Increased MB following exercise was found to lessen oxidative stress in rats following MI (Jiang et al. 2014). Additionally, overexpression of TFAM has been correlated with increased survival following permanent ligation of the coronary artery in mice (Ikeuchi et al. 2005). While the

aforementioned studies are preliminary and few in number, they indicate the potential therapeutic efficacy of augmenting MB for the treatment of MI.

39.4.3 Acute Kidney Injury (AKI)

There is constant movement of ions and small molecules within the kidney epithelium and, as such, renal epithelial cells are heavily dependent on mitochondrial function and the production of ATP to facilitate active transport. Rapid and persistent mitochondrial damage, and the subsequently increased ROS and loss of ATP and antioxidant defenses, largely contributes to the cell death that occurs with acute kidney injury (AKI) (Jassem et al. 2002; Jassem and Heaton 2004; Feldkamp et al. 2005; Bellomo et al. 2012; Funk and Schnellmann 2012; Stallons et al. 2013). The mortality rate of AKI can range from 10 to 80% depending on the specific patient population assessed, strongly demonstrating the need for new, more efficacious therapeutic interventions (Thadhani et al. 1996; Stallons et al. 2013). Recently, PGC-1 α and MB have gained popularity as potential treatment strategies following AKI. PGC-1 α expression has been found to correlate with renal function, with levels decreasing after AKI and partially restoring during recovery (Tran et al. 2011; Funk and Schnellmann 2012). In addition to these findings, our laboratory previously demonstrated that pharmacological activation of MB beginning 24h following I/R-AKI, when injury is maximum, accelerates mitochondrial and renal function recovery in mice. Interestingly, comparable results were observed when MB was induced using phosphodiesterase (PDE) inhibitors (Whitaker et al. 2013), the 5-HT_{1F} receptor agonist LY344864 (Garrett et al. 2014), or the Food and Drug Administration (FDA)-approved β_2 -adrenergic receptor agonist formoterol (Jesinkey et al. 2014a). These data not only strongly suggest the potential of MB as a treatment for AKI, but also indicate that the precise mechanism of MB induction is not critical to its therapeutic effect.

39.4.4 Spinal Cord Injury

Spinal cord injury (SCI) is a debilitating condition with no meaningful therapy. The initial injury causes disruption of vasculature within the spinal cord, resulting in vasoconstriction (Tator and Fehlings 1991; Baptiste and Fehlings 2006; Graumann et al. 2011), and within minutes to hours following this primary injury, the subsequent local decrease in oxygen delivery contributes to a self-propagating secondary cascade. Ischemia is a key mechanism of secondary injury post-SCI, with the degree of functional loss being proportional to the degree of ischemia (Tator and Fehlings 1991). Additional consequences of secondary injury include neuronal cell

death (Beattie et al. 2002; Anwar et al. 2016), progressive axon demyelination (Totoiu and Keirstead 2005), inflammation (Qiao et al. 2010; Qiao et al. 2015), and mitochondrial dysfunction. Loss of mitochondrial homeostasis results in decreased ATP production and inactivation of ATP-dependent ion pumps required for regulation of ion concentrations and reuptake of the excitatory neurotransmitter glutamate. This dysfunction ultimately leads to excitotoxicity, Ca^{2+} overload, and the eventual initiation of cell death cascades, all of which are hallmarks of SCI and further exacerbate injury (Choi and Rothman 1990; Rowland et al. 2008; Oyinbo 2011). Studies suggest that restoration of mitochondrial function shortly after SCI may be an efficacious therapeutic strategy. Unfortunately, despite *in vivo* data using treatments aimed at specific components of mitochondrial function, such as enhancing antioxidant defenses (Pandya et al. 2014; Patel et al. 2014) or inhibiting opening of the mPTP (Ibarra et al. 1996a, b; Rabchevsky et al. 2001; Ibarra et al. 2003; McMahon et al. 2009), there remains no approved pharmacological intervention. Enhanced MB, however, is a largely unexplored strategy for targeting multiple aspects of mitochondrial function for the treatment of SCI. Hu et al. (2015, 2016) recently reported that not only is PGC-1 α expression decreased in the spinal cord after contusive SCI in rats, but also spinal lentiviral overexpression of PGC-1 α immediately after injury attenuates neuronal cell death and promotes functional recovery, suggestive of the potential benefit of pharmacologically increasing PGC-1 α and MB following injury.

39.4.5 Stroke

Excessive ROS generation has long been implicated in neuronal cell death in various neurological and neurodegenerative pathologies, including cerebral ischemia (Chen et al. 2011; Sanderson et al. 2013). Similar to the consequences observed with SCI secondary injury, the lack of oxygen and nutrients being supplied to the brain disrupts neuronal homeostasis, ultimately leading to cell death. Multiple studies have indicated protective effects of PGC-1 α and endogenous MB following cerebral ischemia. Neuronal PGC-1 α is necessary for the generation of several proteins with antioxidant activity, including glutathione peroxidase, uncoupling protein 2 (UCP2), and superoxide dismutase 2 (SOD2) (St-Pierre et al. 2006). Furthermore, overexpression of PGC-1 α has been suggested to protect neurons from oxidative stress and subsequent cell death, while knockdown of the protein has the opposite effect (St-Pierre et al. 2006; Chen et al. 2010). Interestingly, ischemic brain injury has been shown to induce PGC-1 α and MB, presumably as an endogenous repair mechanism (Gutsaeva et al. 2008; Yin et al. 2008). These findings largely implicate PGC-1 α ,

and MB, as a potential neural protectant against ischemia-induced neuronal loss. Therefore, exogenously augmenting MB may facilitate these protective properties. For example, the polyphenol resveratrol, a main component of wine, has been observed to induce MB in neurons and, in a separate study, to protect against neonatal cerebral ischemia (Dasgupta and Milbrandt 2007; West et al. 2007).

39.5 Chronic Diseases

39.5.1 Neurodegenerative Diseases

Mitochondrial dysfunction and oxidative stress are hallmarks of many neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), the two most common neurodegenerative disorders (Yan et al. 2013). Postmortem analysis of the brain tissue of AD patients revealed mitochondrial structural damage, increased mitochondrial size, and decreased mitochondrial number (Hirai et al. 2001), indicating the disrupted nature of mitochondrial homeostasis in the diseased brain. Further supporting this phenomenon is alterations in mitochondrial fusion and fission proteins in the AD brain, suggestive of increased mitochondrial fragmentation (Yan et al. 2013). Detrimental mutations in mtDNA are present in various brain regions of both AD and PD patients (Khan et al. 2000; Coskun et al. 2012). Importantly, variants in mtDNA- or nuclear-encoded mitochondrial complex I genes, leading to altered OXPHOS, have been connected to increased predisposition to PD (Coskun et al. 2012). The dopaminergic neurons of the substantia nigra are particularly susceptible to mtDNA mutations and oxidative damage due to high amounts of prooxidant iron and low amounts of the antioxidant glutathione (Bender et al. 2006). Additionally, several genetic defects converging on mitochondrial pathways have been discovered in both sporadic and familial PD, strongly implicating loss of mitochondrial homeostasis in the disease. For example, in autosomal recessive juvenile parkinsonism, mutations in parkin and PTEN-induced putative kinase 1 (PINK1), components of mitophagy, result in failed clearance of damaged mitochondria, leading to persistent elevation of ROS in the substantia nigra and eventual neuron loss (Yan et al. 2013).

Huntington's disease (HD) is a lethal autosomal dominant disorder caused by a mutation of the huntingtin (Htt) gene and characterized by striatal degeneration and behavioral and cognitive defects. Patients with HD have metabolic deficits in the brain and muscle, as well as reduced complex I, II, and III activity in the striatum (Antonini et al. 1996; Tabrizi et al. 1999; Feigin et al. 2001). mtDNA mutations are also involved in the

pathology of HD, with increased mtDNA deletions being observed in the brains of HD patients compared with controls (Banoei et al. 2007). Importantly, the mutated form of Htt directly impairs mitochondrial function through interaction with PGC-1 α , leading to decreased expression of mitochondrial genes, including TFAM, altering mitochondrial function, and homeostasis (Cui et al. 2006; McGill and Beal 2006; Weydt et al. 2006; Chaturvedi and Beal 2013). Furthermore, postmortem analysis revealed decreased PGC-1 α and downstream target gene expression, as well as reduced mitochondrial content and MB in HD brain and muscle tissue (Chaturvedi et al. 2009; Kim et al. 2010).

Mitochondrial dysfunction is related to many of the proposed mechanisms of neurodegeneration that occurs with amyotrophic lateral sclerosis (ALS), a motor neuron disorder that generally results in death due to respiratory failure within 5 years of onset (Cozzolino and Carri 2012). Deformed, often swollen and vacuolated, mitochondria have been observed in the muscles and motor neurons of ALS patients and are thought to contribute to the formation of ALS-specific Bunina bodies (Hart et al. 1977; Sasaki et al. 2007; Cozzolino and Carri 2012). Analysis of central nervous system (CNS) tissue from ALS patients has consistently uncovered alterations in ETC activity, although the precise dysfunction has varied between cohorts (Bowling et al. 1993; Fujita et al. 1996; Wiedemann et al. 2002). In skeletal muscle, however, consistent deficiencies in complexes I and IV have been observed (Vielhaber et al. 2000; Crugnola et al. 2010). This loss of mitochondrial function is accompanied by increased ROS production and oxidative stress, as well as Ca²⁺ dysregulation and excitotoxicity. Importantly, the motor neurons affected by ALS have a low Ca²⁺ buffering capacity and are therefore highly vulnerable to excitotoxicity (Van Den Bosch et al. 2000), strongly indicating the need for improved mitochondrial function in these neurons. Interestingly, mitochondrial dysfunction has also been found in tissues unassociated with ALS, including in the liver and lymph nodes, of ALS patients (Nakano et al. 1987).

Current therapeutic treatments for these neuropathologies are palliative, as there exists no method of stopping or slowing progression of the diseases. There is substantial evidence suggesting that augmenting MB is a potential therapeutic strategy for neurodegenerative disorders. For example, not only is PGC-1 α decreased in animal models of PD, but loss of PGC-1 α also increases susceptibility to the disease (St-Pierre et al. 2006). Similarly, dysregulation of PGC-1 α has been shown to exacerbate lead-induced neurotoxicity, while modestly increasing PGC-1 α expression proved neuroprotective against the environmental toxicant associated with increased risk of developing PD.

Additionally, substantia nigral cells exposed to lead increased PGC-1 α transcription, likely as an endogenous protection mechanism (Dabrowska et al. 2015).

Impaired MB has also been implicated in the mitochondrial dysfunction that accompanies AD, and overexpression of PGC-1 α was found to ameliorate this dysfunction (Sheng et al. 2012). Furthermore, bezafibrate, a PPAR agonist that activates PGC-1 α , was found to enhance behavior in a transgenic mouse model of AD (Dumont et al. 2012). Treatment with various thiazolidinediones (TZDs), selective PPAR γ agonists shown to enhance MB, has resulted in improved cognitive function in patients with early mild to moderate AD (Watson et al. 2005; Risner et al. 2006; Heneka et al. 2015; Cheng et al. 2016). Interestingly, treatment with resveratrol, which activates PGC-1 α via SIRT1 expression and increased AMP-activated protein kinase (AMPK), has resulted in functional improvement and decreased neuronal degeneration in animal models of PD, AD, HD, and ALS (Kim et al. 2007; Blanchet et al. 2008; Long et al. 2009; Ho et al. 2010; Whitaker et al. 2016). Rotenone is a widely used pesticide and is considered the principal environmental risk factor for the development of nonfamilial PD; chronic exposure to low doses of rotenone results in complex I dysfunction and oxidative stress, two major contributors to the pathology of PD. Recently, resveratrol was observed to enhance mitochondrial homeostasis following rotenone exposure both *in vivo* and *in vitro*, correlating with increased TFAM and PGC-1 α expression, indicating improved MB (Peng et al. 2016). Furthermore, a phase III clinical trial (NCT00678431) is underway investigating combinatorial treatment with resveratrol, glucose, and malate as a potential treatment option for mild to moderate AD (Wang and Chen 2016). Direct activation of AMPK using the adenosine analogue AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) was also found to be neuroprotective using a mouse model of AD (Vingtdeux et al. 2010). These studies, among others, indicate that pharmacological amplification of PGC-1 α and subsequent enhancement of MB may be an effective strategy for the treatment of various acute and chronic degenerative disorders.

39.6 Pharmacological Activation of MB

Several compounds that modulate MB have been identified via target-based and phenotypic screens, including kinases, as well as compounds that bind to and modulate specific G protein-coupled receptors (GPCRs), nuclear

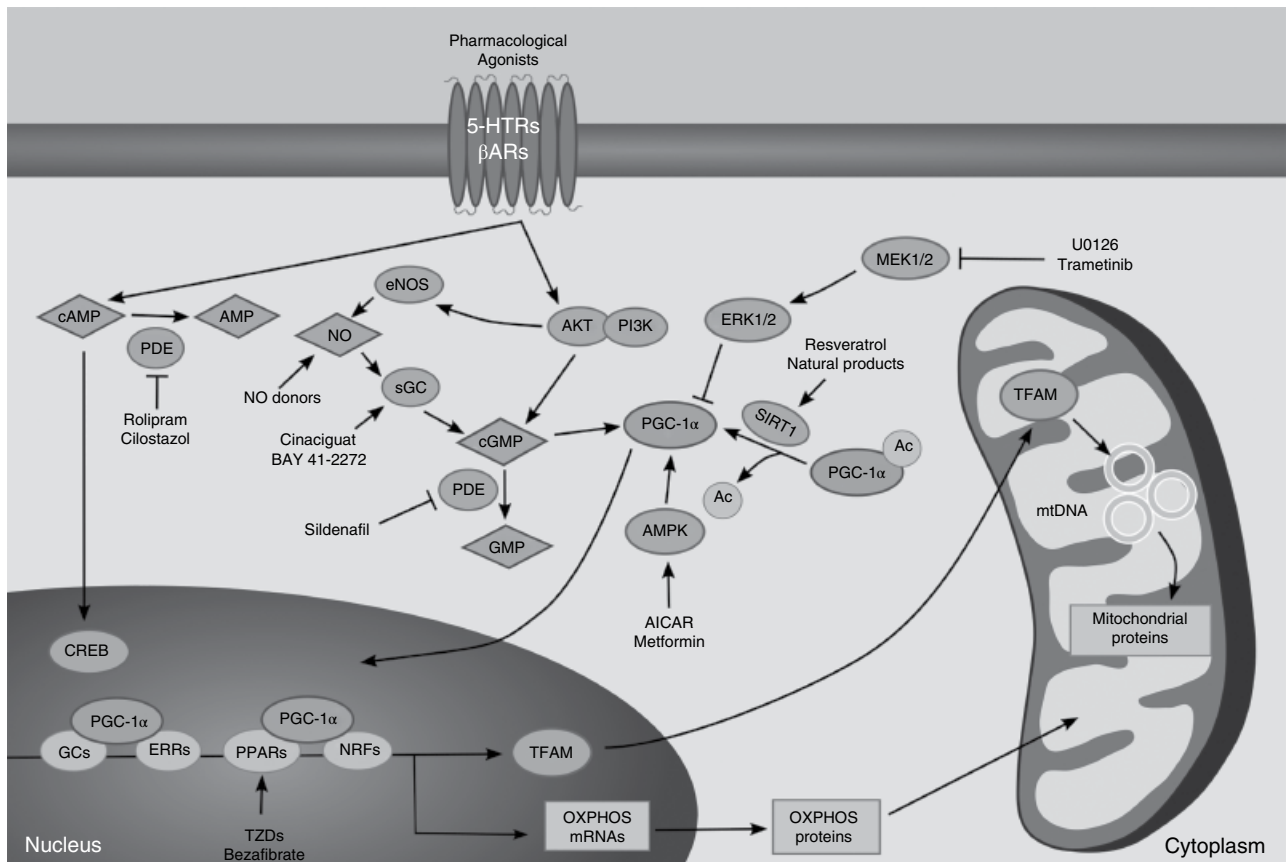


Figure 39.1 Pharmacological activation of MB. MB is a highly regulated cellular process that, while controlled by the central transcriptional coactivator PGC-1 α , involves an array of diverse pathways. Various pharmacological agents can augment MB by targeting different aspects of these pathways, including agonism of G protein-coupled receptors, increased AMPK and cGMP, enhanced SIRT1-mediated PGC-1 α deacetylation, and activation of coactivators that interact with PGC-1 α , culminating in increased expression of mitochondrial genes and MB. 5-HTRs, 5-hydroxytryptamine receptors; β ARs, β adrenergic receptors; Ac, acetyl group; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; Akt, protein kinase B; AMP, adenosine monophosphate; AMPK, adenosine monophosphate-activated kinase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CREB, cAMP response element binding; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; ERRs, estrogen-related receptors; GCs, glucocorticoids; GMP, guanosine monophosphate; MEK1/2, mitogen-activated protein kinase kinases 1/2; mtDNA, mitochondrial DNA; NO, nitric oxide; NRFs, nuclear respiratory factors; OXPHOS, oxidative phosphorylation; PDE, phosphodiesterase; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator 1- α ; PI3K, phosphoinositide 3-kinase; PPARs, peroxisome proliferator-activated receptors; sGC, soluble guanylate cyclase; SIRT1, sirtuin 1; TFAM, mitochondrial transcription factor A.

receptors, and other transcription factors. Hallmark compounds identified using this approach include modulators for PPARs, estrogen receptors (ERs), sirtuins (SIRTs), AMPK, and the cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cGMP. Animal models, and some human studies, have provided evidence supporting the efficacy of compounds that target MB for ameliorating mitochondrial dysfunction. In the following sections, we will discuss several mitochondrial biogenic drugs with therapeutic potential for the treatment of acute and chronic diseases, as well as their molecular targets. The affected pathways are outlined in Figure 39.1.

39.7 Kinase Modulators

39.7.1 AMPK

AMPK is an ubiquitously expressed heterotrimeric kinase that acts as a highly conserved energy sensor and participates in the regulation of energy-generating and consumption pathways. It turns on catabolic pathways to generate ATP while simultaneously switching off anabolic pathways that consume ATP. It also triggers increases in glucose uptake, OXPHOS, autophagy, and MB. It has been reported to directly phosphorylate PGC-1 α at two sites, activating its transcription

(Jager et al. 2007). An alternative mechanism by which AMPK may activate PGC-1 α function is via deacetylation catalyzed by the nicotinamide adenine dinucleotide (NAD)-dependent deacetylase SIRT1 (Rodgers et al. 2005).

Evidence of AMPK-induced PGC-1 α upregulation originated from a study that fed rats β -guanidinopropionic acid (β -GPA), a creatine analogue that results in ATP depletion. Muscle phosphocreatine, ATP, and ATP/AMP ratios were all markedly decreased in the β -GPA rats, leading to the activation of AMPK. Under these conditions, NRF-1 binding activity, cytochrome *c* content, and muscle mitochondrial density were all increased (Bergeron et al. 2001). These data suggest that AMPK plays an important role in promoting mitochondrial adaptation to energy stress and MB. Pharmacological activation of AMPK has been observed with multiple compounds, including AICAR and metformin (Rambert et al. 1961; Zhang et al. 2006). AICAR is an adenosine analogue AMPK activator, which has shown positive effects following cardiac and renal I/R injury through enhanced glucose uptake and SIRT1 expression, respectively (Russell et al. 1999; Lempiainen et al. 2012). AICAR has also been successful in chronic disease models, such as type 2 diabetes, by preventing insulin resistance in multiple tissues, as well as in AD through decreases in amyloid- β protein levels (Boon et al. 2008; Bikman et al. 2010; Vingtdoux et al. 2010). Table 39.2 summarizes AICAR-mediated MB in multiple tissues.

39.7.2 ERK1/2

MAPKs are serine–threonine kinases that mediate intracellular signaling associated with a variety of cellular activities, including cell proliferation, differentiation, survival, death, and transformation. Extracellular signal-regulated kinase (ERK1/2), a member of the MAPK family, has been implicated as a negative regulator of MB (Collier et al. 2016). Table 39.2 summarizes trametinib-mediated MB following renal I/R injury in mice. ERK1/2 reduces PGC-1 α and its downstream targets in a number of disease models. In animal models of PD, TFAM phosphorylation as a result of ERK1/2 activation decreased the transcription of mtDNA (Wang et al. 2014b). In AD, ERK1/2 activation impaired mitochondrial function, as evidenced by altered mitochondrial fission and fusion processes and reduced mitochondrial membrane potential (Gan et al. 2014). ERK1/2 activation is also a downstream event of ROS generation. Using renal proximal tubules cells (RPTC), our laboratory showed that following administration of the oxidant *tert*-butyl hydroperoxide (TBHP), ERK1/2 phosphorylation correlated with reduced complex I activity and ATP production (Nowak et al. 2006). Furthermore, I/R–AKI-induced ERK1/2 activation

preceded the initial decrease in renal PGC-1 α mRNA and loss of renal function (Collier et al. 2016).

ERK1/2 is thought to be the only substrate for mitogen-activated protein kinase kinase (MEK1/2) phosphorylation; therefore, pharmacological inhibition of ERK1/2 can be accomplished using MEK1/2 inhibitors. Treatment with the inhibitor U0126 was found to rescue hippocampal PGC-1 α , TFAM, and NRF-1 protein levels following amyloid- β injections in rats (Ashabi et al. 2012). Additionally, our laboratory has reported that inhibition of ERK1/2 using trametinib not only attenuates the early decrease in PGC-1 α but also prevents decreased renal function following I/R–AKI (Collier et al. 2016). These studies provide evidence that targeting negative regulators of MB is an effective strategy to induce MB and restore organ function following injury.

39.8 G Protein-Coupled Receptor Modulators

GPCRs are the largest and most diverse group of membrane receptors. These cell surface receptors are affected by both endogenous ligands and pharmacological agents. GPCRs are involved in an array of functions within the human body, and it is estimated that between one-third and one-half of all marketed drugs are GPCR ligands (Flower 1999; Robas et al. 2003). They consist of a single polypeptide that is folded into a globular shape and embedded within the cellular plasma membrane. As the name implies, GPCRs interact with G proteins, in that external signaling molecules bind to a GPCR, inducing a conformational change and triggering the interaction between the GPCR and a nearby G protein. G protein activation can affect the production of hundreds to thousands of downstream effectors, including those that modulate MB. Downstream effectors of GPCRs that can regulate mitochondrial function include cGMP, Ca²⁺, phosphoinositide 3-kinase (PI3K), and endothelial nitric oxide synthase (eNOS) (Dudzinski and Michel 2007; Tuteja 2009). While GPCRs are well-characterized regulators of a variety of cell processes, studies exploring GPCR ligands as inducers of MB are limited.

39.8.1 5-HT Receptors

5-Hydroxytryptamine (5-HT, serotonin) receptors have been classified into seven groups based on structure and signal transduction mechanisms. 5-HT receptors are predominantly GPCRs and are known to be involved in various CNS and peripheral functions, such as anxiety, sleep, blood vessel constriction, and gastrointestinal

Table 39.2 A selective list of pharmacological inducers of mitochondrial biogenesis.

Class	Drugs	MB effects	References
AMPK activators	AICAR	↑MB, ↑ATP production, ↑oxygen consumption in multiple tissues	Bikman et al. (2010), Boon et al. (2008), Lempiainen et al. (2012), Russell et al. (1999), and Vingtdoux et al. (2010)
MEK inhibitors	Trametinib	↑PGC-1α, ↑TFAM in RPTC, ↑recovery of renal function and MB following mouse I/R	Collier et al. (2016) and Nowak et al. (2006)
5HT agonists	LY344864	↑PGC-1α, ↑OXPHOS proteins, ↑mtDNA, ↑oxygen consumption, ↑recovery of renal function and MB following mouse I/R	Garrett et al. (2014)
	DOI		Rasbach et al. (2010)
	SB242084	↑PGC-1α, ↑OXPHOS genes, ↑oxygen consumption in RPTC	Harmon et al. (2016)
β-adrenergic agonists	Formoterol	↑PGC-1α, ↑OXPHOS proteins, ↑mtDNA, ↑oxygen consumption in RPTC, ↑recovery of renal function and MB following mouse I/R	Jesinkey et al. (2014a) and Wills et al. (2012)
CB1 antagonists	Rimonabant	↑OXPHOS genes, ↑oxygen consumption, ↑eNOS activation in mouse white adipocytes	Tedesco et al. (2008)
PDE inhibitors	Sildenafil	↑PGC-1α, ↑OXPHOS genes, ↑mtDNA, ↑oxygen consumption in RPTC, ↑recovery of renal function and MB following mouse folic acid-induced AKI	Whitaker et al. (2013)
	Vardenafil	↑PGC-1α, ↑mtDNA in human adipose tissue	De Toni et al. (2011)
	Rolipram	↑PGC-1α, ↑TFAM, ↑PGC-1α activity, ↑mtDNA, ↑AMPK in skeletal muscle	Park et al. (2012)
	Cilostazol	↑PGC-1α, ↑TFAM, ↑mtDNA, ↑ATP, ↑CREB in cultured endothelial	Zuo et al. (2013)
NO mimetics	DETA-NO	↑PGC-1α, ↑TFAM, ↑OXPHOS genes, ↑oxygen consumption in mammalian cultured models	Nisoli et al. (2004)
GC stimulators	Bay 41-2272	↑PGC-1α, ↑OXPHOS genes, ↑oxygen consumption in cultured models, protective in I/R and LPS models	Nisoli et al. (2004) and Salloum et al. (2012)
	Cinaciguat		
	Riociguat		
PPARγ agonists	TZDs	↑PGC-1α, ↑TFAM, ↑OXPHOS genes in adipose tissue and skeletal muscle in diabetes	Bogacka et al. (2005), Mensink et al. (2007), and Rong et al. (2007)
PPARα agonists	Fibrates	↑PGC-1α, ↑AMPK activation in multiple tissues	Ansquer et al. (2009), Grabacka et al. (2013), and Sarma (2012)
PPARδ agonists	GW501516	↑PGC-1α, ↑FAO in muscle and liver	Barroso et al. (2011) and Kleiner et al. (2009)
ER agonists	Estradiol	↑PGC-1α, ↑TFAM, ↑OXPHOS proteins, ↑oxygen consumption in multiple tissues	Capllonch-Amer et al. (2014), Irwin et al. (2008), Mattingly et al. (2008), and Sbert-Roig et al. (2016)
Sirtuin activators	Resveratrol	↑PGC-1α, ↑SIRT1, ↑mitochondrial function in multiple tissues	Csiszar et al. (2009), Feige et al. (2008), Funk and Schnellmann (2013), Libri et al. (2012), Menzies et al. (2013), Milne et al. (2007), Tong et al. (2013), and Wang et al. (2014a)
	SRT1720		
	SRT1460		
	SRT2104		
	SRT2379		
Natural products	Isoflavones	↑PGC-1α, ↑OXPHOS proteins, ↑oxygen consumption, ↑ATP, ↑SIRT1 activation in RPTC	Rasbach and Schnellmann (2008)
	Flavonoids	↑PGC-1α, ↑TFAM, ↑OXPHOS proteins, ↑SIRT1 in multiple tissues	Li et al. (2016), Ramirez-Sanchez et al. (2016), and Rayamajhi et al. (2013)

Arrows indicated increase (↑) and decrease (↓). 5-HT, hydroxytryptamine; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AKI, acute kidney injury; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; CB1, cannabinoid receptor 1; CREB, cAMP response element-binding protein; DETO-NO, diethylenetriamine/NO adduct; DOI, 2,5-dimethoxy-4-iodamphetamine; eNOS, endothelial nitric oxide synthase; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FAO, fatty acid oxidaton; GC, guanylate cyclase; I/R, ischemia/reperfusion; LPS, lipopolysaccharide; MB, mitochondrial biogenesis; mtDNA, mitochondrial DNA; NO, nitric oxide; OXPHOS, oxidative phosphorylation; PDE, phosphodiesterase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPAR, peroxisome proliferator-activated receptor; RPTC, renal proximal tubule cells; SIRT1, sirtuin 1; TFAM, mitochondrial transcription factor A; TZDs, thiazolidinediones.

motility (Vanhoutte and Luscher 1986; Leonard 1996; Nichols and Nichols 2008). 5-HT receptors have also been identified as targets for alterations in mitochondrial function, including apoptosis and mitochondrial membrane integrity (Nebigil and Maroteaux 2003). Our laboratory recently made the novel observation that several 5-HT receptor ligands can induce MB. The nonselective 5-HT receptor agonist α -methyl-5-HT increased mitochondrial respiration, a marker of MB, in RPTC (Garrett et al. 2014). We have also identified specific ligands of 5-HT₂ and 5-HT₁ receptors as potent inducers of MB. 2,5-Dimethoxy-4-iodoamphetamine (DOI), a 5-HT₂ receptor agonist, promoted renal MB *in vitro*, as evidenced by increased mitochondrial respiration genes and proteins, as well as rescued mitochondrial respiration following oxidant injury (Rasbach et al. 2010). Interestingly, the selective 5-HT_{2C} agonist CP809101 and the 5-HT_{2C} antagonist 6-Chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxamide dihydrochloride (SB242084) induced MB *in vitro* and in naïve mice. However, studies utilizing siRNA and knockout mice revealed that these ligands employ their mitochondrial biogenic effects through the 5-HT_{2A} receptor. Similarly, the 5-HT_{2A} agonist NBOH-2C-CN also elevated renal mitochondrial respiration, confirming that the 5-HT_{2A} receptor is a target for the induction of MB (Harmon et al. 2016). Table 39.2 summarizes 5-HT-induced MB pathways.

The 5-HT_{1F} receptor agonists LY344864 and lasmiditan were also found to be potent inducers of mitochondrial respiration and ETC genes *in vitro* and in renal, cardiac, and hepatic tissue in mice (Garrett et al. 2014). LY344864 treatment accelerated the recovery of renal function following I/R-AKI, correlating with restoration of mtDNA copy number (Garrett et al. 2014). Additionally, daily treatment with LY344864 reduced behavior impairments and increased mitochondrial gene expression and mtDNA content, indicative of enhanced MB, in a mouse model of PD (Scholpa et al. 2017). Recent data revealed that stimulation of the 5-HT_{1F} receptor induces MB through the activation of the stimulatory AKT/eNOS/cGMP pathway (Gibbs et al. 2017). Collectively, these data provide compelling evidence that exploitation of 5-HT receptors has a significant therapeutic role in the recovery of mitochondrial and organ function.

39.8.2 β -Adrenergic Receptors

Catecholamine-binding adrenergic receptors can be divided into two main groups, α and β , with dynamic roles in an array of functions β , with each group being further divided into classes. The β_1 -adrenergic recep-

tor is predominately expressed in cardiac tissue and pharmacologically targeted for heart rate stimulation and contractility. The β_3 receptor is localized mainly in adipose tissue and the bladder and, as such, is a target to treat overactive bladder. The ubiquitously expressed β_2 receptor is targeted by drugs used to treat asthma and chronic obstructive pulmonary disease (COPD). Adrenergic receptors have become an attractive target for MB due to their role in cold-induced MB. Studies revealed that in a cold environment, release of the catecholamine norepinephrine leads to the activation of β -adrenergic receptors, stimulation of adenylate cyclase, and an increase in intracellular cAMP, resulting in the phosphorylation of cAMP response element-binding protein (CREB), a transcriptional activator of PGC-1 α (Ye et al. 2013a). Thus, adrenergic receptor ligands can act as pharmacological modulators of MB.

The nonselective β -adrenergic receptor agonist isoproterenol increases PGC-1 α expression in brown adipose tissue in naïve mice (Muller et al. 2013). Interestingly, in models of cardiac dysfunction, the β_1 -selective antagonist metoprolol, with a reported pK_i of 7–7.6, enhances PGC-1 α activation and improves cardiac metabolism and function (Sharma et al. 2009; Alexander et al. 2015). The β_2 -adrenergic receptor has also been demonstrated as a target for MB. Formoterol, a long-acting β_2 -adrenergic receptor agonist, stimulates mitochondrial respiration, PGC-1 α mRNA, and PGC-1 α -dependent gene expression in RPTC and in naïve mouse kidney (Wills et al. 2012). Formoterol administration following I/R-AKI accelerated renal recovery, correlating with improved mitochondrial respiration (Jesinkey et al. 2014a). In addition to these renal effects, formoterol also induced MB in cardiac tissue and skeletal muscle (Wills et al. 2012; Jesinkey et al. 2014b). Table 39.2 summarizes formoterol-mediated MB following renal I/R injury in mice.

The identification of formoterol as an inducer of MB led to the screening of other β_2 -adrenergic receptor ligands. Interestingly, other such ligands, including clenbuterol and isoetharine, did not induce MB in RPTC (Peterson et al. 2013). Clenbuterol, however, was shown to activate receptor-interacting protein 140 (RIP140), a negative regulator of MB, leading to the suppression of PGC-1 α in muscle (Hoshino et al. 2012). Recent data from our laboratory revealed that both formoterol and clenbuterol increase cAMP; however, formoterol-induced MB occurs through G $\beta\gamma$ -dependent activation of the AKT/eNOS/cGMP pathway, which does not occur with clenbuterol, likely contributing to the differences in MB induction observed between the two agonists (Cameron et al. 2017). These data suggest

that functional selectivity based on spatial and chemical characteristics can be explored for the future development of pharmacological agents with structural similarities to β_2 agonists that induce MB.

39.8.3 Cannabinoid-1 Receptor

Cannabinoid type 1 (CB1) receptors are the most abundant GPCR expressed in the brain. CB1 receptors are also highly expressed in peripheral organs, especially white adipose tissue (WAT), where it functions to modulate energy metabolism. The endocannabinoid system is overactive in peripheral tissues as evidenced by markedly increased levels of the endocannabinoid arachidonoyl glycerol (2-AG), which has been correlated with body fat, visceral mass, and plasma insulin concentrations, in visceral fat of obese and diabetic subjects (Bluher et al. 2006). Previous studies suggest that CB1 receptor overstimulation causes a downregulation in MB in high metabolic tissues, partially explaining increased fat deposits and metabolic dysfunction in an obese setting (Tedesco et al. 2010). CB1 receptor-deficient mice are lean, resistant to high-fat diet, and have increased MB in WAT (Tedesco et al. 2008). Studies using the selective CB1 receptor agonist rimonabant persistently reduced body weight and liver steatosis and improved WAT metabolism and glucose uptake in skeletal muscle in obese mice. Additionally, rimonabant increased MB in cultured white adipocytes through an eNOS-dependent increase in AMPK activity, as evidenced by elevated mRNA expression of PGC-1 α , TFAM, and NRF-1 (Tedesco et al. 2008). Table 39.2 summarized rimonabant-mediated MB in mouse white adipocytes. These studies indicate that antagonism of the CB1 receptor stimulates fat metabolism through the activation of MB. Thus, pharmacological and genetic blockade of the CB1 receptor is a potential pharmacological strategy for the treatment of impaired energy metabolism.

39.9 Cyclic Nucleotide Modulators

The cyclic nucleotides cGMP and cAMP are ubiquitous second messengers that play a crucial role in MB. Following extracellular signaling, NO activates soluble guanylate cyclase (sGC), which produces cGMP, while stimulation of G_{α_s} activates adenylate cyclase activity, producing cAMP. These cyclic nucleotides either directly activate downstream kinases or are hydrolyzed by PDEs. cGMP and cAMP formations are disrupted in numerous pathological states with

mitochondrial dysfunction; therefore, modulators of cyclic nucleotides represent a class of potential therapeutics for the induction of MB in various diseases (Whitaker et al. 2016).

39.9.1 NO/cGMP Pathway

cGMP is a starting point for multiple signal transduction cascades. The biological importance of NO/cGMP signaling was first recognized for promoting vascular smooth muscle relaxation and platelet disaggregation (Karaki et al. 1988; Radomski et al. 1990). The effects of NO/cGMP signaling on differentiation of vascular smooth muscle in response to growth factors, vasoactive peptides, physical damage, and other stimuli have also been clearly demonstrated (Garg and Hassid 1989; Protter et al. 1996; Garcha and Hughes 2006). Results from these and other studies drove the search for therapies targeting the NO/cGMP pathway. Activation of the NO/cGMP signaling pathway through the use of NO donors such as diethylamine-*NONO*ate (DEA-*NONO*ate), cGMP mimetics, sGC stimulators such as cinaciguat and BAY 41-2272, and cGMP-selective PDE inhibitors such as sildenafil and vardenafil has been shown to increase PGC-1 α -dependent MB and mitochondrial function *in vivo* (Nisoli et al. 2004; De Toni et al. 2011; Salloum et al. 2012; Whitaker et al. 2013). Cinaciguat has also been shown to protect against cardiac I/R injury through protein kinase G (PKG)-dependent induction of hydrogen sulfide (H_2S), a known inducer of MB (Salloum et al. 2012). Bay 41-2272 induces PGC-1 α and other mitochondrial genes, increasing brown fat differentiation in mice (Nisoli et al. 2004). Sildenafil, a PDE5 inhibitor, causes accumulation of cGMP, leading to elevated mitochondrial respiration, ATP production, and ETC gene expression in RPTC and in the renal cortex of naïve mice (Whitaker et al. 2013). Sildenafil promotes recovery via MB and reduced renal tubular injury following AKI induced by folic acid, supporting the use of PDE inhibitors in models of toxicant-induced mitochondrial toxicity (Whitaker et al. 2013). See Table 39.2 for a summary of MB pathways mediated by NO donors, GC stimulators, and PDE inhibitors.

It is important to note that stimulation of MB through the 5-HT_{1F} receptor using LY344864 and through the β_2 -adrenergic receptor using formoterol increases eNOS phosphorylation and cGMP production *in vitro* (unpublished data). Activation of eNOS leads to the generation of NO from the endothelium, causing the activation of sGC and the subsequent production of cGMP. Interestingly, β -oxidation in eNOS knockout mice was reduced compared with wild-type mice. This was accompanied by reductions in energy expenditure and mRNA levels of PGC-1 α , TFAM, and NRF-1, indicating that

mitochondrial content is effected by the loss of eNOS-generated NO and implicating MB as an important downstream effect of NO (Le Gouill et al. 2007). In summary, NO/cGMP serves as a central regulator of MB in multiple signaling pathways, supporting the importance of this pathway as a target for MB.

39.9.2 cAMP/PKA/CREB Pathway

cAMP and its cellular effector protein kinase A (PKA) are responsible for the phosphorylation and activation of CREB. As discussed, CREB is a transcription factor that binds to CRE and recruits other coactivators to regulate transcriptional activity. The PGC-1 α promoter contains a CRE-binding site, and activation of CREB leads to increased transcriptional expression of PGC-1 α and downstream gene expression. CREB-induced activation of PGC-1 α in response to thermogenesis, gluconeogenesis, and exercise has been well-studied in several different tissues. In contrast, CREB activity has been shown to be reduced in cognitive and neurodegenerative disorders, such as AD and HD (DeMarch et al. 2007; Sheng et al. 2012).

Due to the tight regulation of cAMP via PDE inhibitors, these compounds are often used to activate the cAMP/PKA/CREB pathway and have proven to be effective in preclinical models of neurodegenerative diseases. Rolipram and cilostazol are cAMP-selective PDE inhibitors that have been found to induce PGC-1 α and mitochondrial activity in multiple *in vitro* models, indicating their potential for preclinical disease models (Park et al. 2012; Zuo et al. 2013). Rolipram has also been shown to improve synaptic conduction in an AD model, associated with CREB phosphorylation (Gong et al. 2004). Cilostazol was found to reduce neuroinflammation, infarct size, and apoptosis following ischemic brain injury (Choi et al. 2002). In addition to the effects in the CNS, cilostazol also reduced oxidant-induced mitochondrial dysfunction and MI size, also associated with CREB phosphorylation (Ye et al. 2013b; Chattipakorn et al. 2014).

Finally, recent studies propose the transportation of nuclear CREB to the mitochondria via chaperone proteins (Lee et al. 2005). Once inside the mitochondria, it is thought that CREB interacts with CRE sites in mtDNA, thereby regulating the expression of ETC proteins (De Rasmio et al. 2009). Mitochondrial CREB phosphorylation was shown to regulate the expression of mtDNA-encoded proteins, including NADH dehydrogenase subunit 1 (ND1) and ND6, mediated by PKA-induced CREB phosphorylation (Ryu et al. 2005; De Rasmio et al. 2009). Furthermore, disruption in mitochondrial CREB activity alters the expression of various mitochondrial genes, ultimately leading to impairment

of the ETC (Lee et al. 2005). In summary, numerous studies reveal the protective effects of CREB-induced MB through cGMP and PKA activation. See Table 39.2 for a summary of MB pathways mediated by rolipram and cilostazol.

39.10 Transcription Factor Modulators

As mentioned earlier, the MB program involves the integration of multiple transcriptional regulatory pathways controlling the expression of both nuclear and mitochondrial genes. While the transcriptional control of MB requires complex cooperation, nuclear-encoded transcription factor coactivators serve as major targets for the selective induction of MB. Members of the nuclear receptor superfamily, including PPARs and ERs, have been shown to regulate mitochondrial metabolism and function. Modulation of these pathways has demonstrated positive effects on MB, mitochondrial function, and disease outcomes in several animal and cellular models.

39.10.1 PPAR Agonists

PPAR isoforms (α , β/δ , and γ) have distinct tissue distributions and functions involved in lipid and fatty acid metabolism. PPAR γ , a well-characterized pharmacological target for the induction of MB, is highly expressed in white and brown adipose tissue and in endothelial cells. The primary function of PPAR γ is adipocyte proliferation and differentiation, with agonism increasing peripheral insulin sensitivity, potentially through MB and oxidative metabolism. TZDs such as pioglitazone and rosiglitazone are PPAR γ agonists used for the treatment of type 2 diabetes. While the effects of TZDs are pleiotropic, the capacity of TZDs to sensitize multiple tissues to the effects of insulin has been correlated with increased expression of mitochondrial proteins, suggesting that the induction of MB may be central to the clinical efficacy of these drugs (Bolten et al. 2007). Specifically, pioglitazone induced MB and reduces ROS production in subcutaneous adipose tissue (Bogacka et al. 2005), while rosiglitazone induced MB in human skeletal muscle of obese type 2 diabetic patients (Mensink et al. 2007; Rong et al. 2007). PPAR γ -induced MB also improved outcomes in renal and cardiac ischemic injury, neurodegenerative diseases, and multiple sclerosis models (Ito et al. 2003; Quintanilla et al. 2008; Bernardo et al. 2009; Wu et al. 2011; Zolezzi et al. 2013).

While the positive pharmacological effects of PPAR γ are the best characterized of the PPARs, data has also emerged describing PPAR α - and PPAR δ -induced augmentation of MB. PPAR α stimulation using fibrates increased PGC-1 α expression in models of cardiac disease, cancer, and metabolic disorders (Ansquer et al. 2009; Sarma 2012; Grabacka et al. 2013). Similarly, PPAR δ activation using GW501516 increased fatty oxidation and PGC-1 α expression in hepatic and muscle tissue (Kleiner et al. 2009; Barroso et al. 2011). Despite the promising data on PPAR α and PPAR δ agonists as inducers of MB, additional investigation is needed to determine the full therapeutic application and efficacy of these compounds. Table 39.2 summarizes MB pathways mediated by PPAR agonists.

39.10.2 ER Agonists

ERs, including ER α and ER β , are nuclear receptors important for hormone binding and transcription. A recent topic of interest is understanding how estrogen affects mitochondrial function and how this relates to sex-dependent differences in lifespan (Klinge 2008). Estradiol, a biologically active estrogen, was found to increase mtDNA copy number *in vitro* following 24, 48, and 72 h of exposure (Mattingly et al. 2008). In animal models, estradiol has also been shown to increase respiratory capacity and antioxidant defenses while reducing ROS production in the heart, brain, and skeletal muscle (Irwin et al. 2008; Capllonch-Amer et al. 2014; Sbert-Roig et al. 2016). More recently, estradiol was observed to induce TFAM expression, leading to increased mtDNA transcription, via a NRF-1-dependent mechanism (Scarpulla 2006). Table 39.2 summarizes estradiol-mediated MB pathways in multiple tissues. It is important to note that ERs are also located within the mitochondria, where they are predicted to bind to the D-loop of mouse and human mtDNA (Chen et al. 2008). Whether or not ERs directly or indirectly mediate mtDNA transcription is unknown; nonetheless, the presence of ERs in both the nucleus and mitochondria makes ERs a viable target for the modulation of mitochondrial respiratory function and MB.

39.11 Sirtuins

The SIRT family of proteins that act predominately as NAD-dependent deacetylases. In mammals, seven SIRT family members exist, including mitochondrial, cytosolic, and nuclear-localized SIRTs. Of this family, SIRT1 has received the most interest because of its

positive regulation of PGC-1 α (Canto and Auwerx 2009; Canto et al. 2009). As mentioned earlier, PGC-1 α is activated through promoter deacetylation, which primarily occurs via its interaction with SIRT1. SIRT1 also controls the acetylation of Forkhead box O (FOXO) transcription factors, which are important regulators of lipid and glucose metabolism (Brunet et al. 2004; Canto and Auwerx 2009). The acetylation status of FOXO directs it to certain targets, such as the PGC-1 α promoter (Canto and Auwerx 2009; Fernandez-Marcos and Auwerx 2011). Studies revealing SIRT1-induced activation of PGC-1 α led to interest in nutraceutical and pharmacological activators of SIRT1.

Resveratrol has been shown to increase SIRT1 activity in AMPK-dependent and independent mechanisms (Lagouge et al. 2006). It has also been shown to activate PPAR α and PPAR γ , which are both inducers of MB (Calleri et al. 2014; Takizawa et al. 2015). It also induces MB in a SIRT1-dependent manner in multiple cell types (Csiszar et al. 2009; Menzies et al. 2013; Wang et al. 2014a). Activation of SIRTs using resveratrol has been shown to be beneficial in animal models of metabolic diseases, improving lipid profiles and antioxidant defenses in diabetic and obese patients (Csiszar et al. 2009; Brasnyo et al. 2011; Timmers et al. 2011; Bhatt et al. 2012). As previously mentioned, resveratrol has also been found to be neuroprotective against the aforementioned neurodegenerative disorders. In addition to resveratrol, other natural products can increase SIRT1-mediated MB and metabolic functions, such as the isoflavones daidzein and genistein found in soybeans; the flavonoids quercetin, epicatechin, and green tea polyphenols found in fruits and cocoa, respectively; and a variety of other foods (Rasbach and Schnellmann 2008; Rayamajhi et al. 2013; Li et al. 2016; Ramirez-Sanchez et al. 2016). Interestingly, quercetin has been shown to increase mtDNA copy number and mitochondrial content in various brain regions of rats exposed to aluminum, a widely distributed element linked to several neurological diseases including AD, PD, and ALS, and known to induce oxidative stress and reduced MB (Sharma et al. 2013; Sharma et al. 2015). Furthermore, quercetin treatment also resulted in increased PGC-1 α expression in these rats, indicating enhanced MB (Sharma et al. 2015). PGC-1 α activation using green tea polyphenols rescued reductions in OXPHOS proteins and mtDNA content in a rat model of CsA-induced nephrotoxicity, consequently attenuating kidney injury and improving renal function. These data implicate natural products as a promising therapeutic strategy for toxicant-induced mitochondrial suppression to promote tissue repair and regeneration (Rehman et al. 2014).

Identification of natural SIRT1 activators encouraged the search for pharmacological inducers of SIRT1. As a result, a number of SIRT1 activators were developed, including SRT1720, SRT1460, and SRT2104. In models of type 2 diabetes, these compounds improve insulin, glucose, and fatty acid metabolism while also improving mitochondrial oxidative capacity (Milne et al. 2007; Feige et al. 2008; Libri et al. 2012). SRT1720 demonstrated protective effects across a variety of disease states in mice, including renal ischemic injury and MI, through activation of SIRT1/PGC-1 α (Funk and Schnellmann 2013; Tong et al. 2013). Furthermore, SRT1720 prevented the development of age-related metabolic diseases in mice by increasing insulin sensitivity and fatty acid oxidation and reducing inflammation and oxidative stress, therefore increasing lifespan (Mitchell et al. 2014). These data suggest that the use of SIRT1 activators may enhance disease outcomes by improving mitochondrial function. See Table 39.2 for a summary of MB pathways mediated by sirtuin activators and natural products.

39.12 Conclusions

Given the critical role of mitochondrial dysfunction in the development of multiple acute and chronic diseases, new therapeutic strategies to target MB to augment mitochondrial content and oxidative metabolism are actively under investigation. Preclinical and human studies indicate that induction of MB via various pathways promotes functional recovery in a variety of disease states, including metabolic disorders, I/R injuries, neurodegenerative, and cardiovascular pathologies. Unfortunately, however, relatively few drugs have been identified for the induction of MB, and such drugs are often functionally promiscuous. Therefore, a significant amount of work is still required to improve the safety and efficacy of MB-based therapeutics for the treatment of acute and chronic diseases.

Research on repurposing existing FDA-approved drugs is becoming a popular strategy in drug development, in part because of the known safety profiles of these agents. Interestingly, several classes of FDA-approved drugs are inducers of MB, such as rosiglitazone, formoterol, and sildenafil, suggestive of the safety of MB. It is also important to consider the MB-independent mechanisms of many of these drugs. For example, TZDs, estrogens, and SIRT1 activators can also stimulate off-target transcriptional programs that have been demonstrated to cause detrimental effects, such as hyperproliferation. Therefore, selective induction of MB has the potential to avert these toxic side effects.

In addition to the acute and chronic diseases mentioned herein, augmented MB also has the potential to aid in drug-induced mitochondrial toxicity. Several studies have investigated the effects of pharmacologically enhanced MB on mitochondrial toxicity in various organ systems, including the aforementioned rotenone- and lead-induced neurotoxicity and aluminum-induced oxidative stress. Interestingly, melatonin administration was found to reduce carbon tetrachloride-induced hepatocellular damage (Doherty 2000) and oxidative stress in rats while increasing mtDNA content, the expression of various MB proteins, including PGC-1 α and TFAM and mitophagy-related proteins. Additionally, melatonin treatment increased AMPK phosphorylation in these animals, an upstream activator of PGC-1 α (Kang et al. 2016). The environmental pollutant cadmium also induces hepatotoxicity via oxidative stress and mitochondrial dysfunction, including decreased SIRT1 activity, decreased deacetylation, and subsequent activation of PGC-1 α . Pretreatment with melatonin, however, was found to protect from these cadmium-induced effects *in vitro*, presumably via SIRT1-dependent PGC-1 α activation and increased MB (Guo et al. 2014). Although a great deal of work remains to be done, these recent studies indicate the potential for augmented MB and enhanced PGC-1 α as therapeutic interventions for a variety of pathologies converging on mitochondrial dysfunction and loss of mitochondrial homeostasis, including drug-induced mitochondrial toxicity.

Pharmacological induction of MB also has the capacity to activate MB in off-target organs, which may have harmful effects in the form of increased systemic oxidative stress and inflammation. Timing and duration of pharmacological intervention is also of importance when discussing the potential effectiveness of MB inducers. For example, MB prior to insult may be protective against mitochondrial dysfunction, but also presents the opportunity to propagate organ dysfunction due to increased mitochondrial content and ROS production. Furthermore, oxidative stress-induced pathways, such as mtDNA damage, that occur immediately after insult (i.e., I/R injury) may impair the function of the newly generated mitochondria. Additionally, prolonged treatment with agents targeting receptors may result in receptor desensitization and/or activation of antagonistic pathways, both of which could hinder MB signaling. By optimizing dosing strategies, we will gain insight on maximizing the efficacy of MB induction. Well-executed preclinical animal models investigating natural products and pharmacological inducers of MB will undoubtedly provide researchers and clinicians with valuable tools for developing MB-based strategies for therapeutic intervention of both acute and chronic diseases.

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Mitochondrial Toxicity Induced by Chemotherapeutic Drugs

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CHAPTER MENU

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40.1 Introduction

Cancer chemotherapy involves the use of drugs to treat cancer, usually as part of a multimodality therapy in combination with surgery and/or radiotherapy, with the final goal of achieving and maintaining cancer remission. This is often a long-term process, requiring the use of one or more chemical agents given in repeated doses, and the characteristics of the treatment are highly dependent on the tumor type (Airley 2009). Remission implicates the complete eradication of the disease for at least 1 month, and the treatment is aimed at preventing recurrence both at the primary tumor site and at distant sites (metastasis) (Airley 2009).

Ideally, chemotherapeutic agents should specifically kill cancer cells, with minimal collateral damage to normal cells. Thus, many forms of chemotherapy are focused on preventing cell division and inducing cell death by apoptosis, since cancer cells are more likely to be replicating during treatment than normal cells. However, cytotoxicity of chemotherapeutic drugs also affects normal cells, and anticancer treatments are

associated with significant dose-limiting side effects (Payne and Miles 2008). In addition, cancer cells may develop resistance to chemotherapy, which is associated with the upregulation of antiapoptotic mechanisms, which leads to decreased susceptibility to cytotoxic drugs (Hassan et al. 2014).

Although current chemotherapeutic agents are fairly successful in the treatment of cancer, patients often develop adverse effects that could force therapeutic alterations (Chu and Sartorelli 2015). Adverse effects can persist after cessation of chemotherapy for weeks, months, or even years, reducing long-term quality of life of these patients (Vichaya et al. 2015).

40.2 Mitochondria and Cancer Chemotherapy

Mitochondria play a central role in tumorigenesis (Tan et al. 2014; Tokarz and Blasiak 2014) and the use of drugs that target mitochondria provides an attractive anticancer approach (Fulda et al. 2010; Weinberg and Chandel 2015; Wen et al. 2013). Cancer cells often present higher levels of mitochondrial DNA (mtDNA) mutations and/or decreased mtDNA copy number, overproduction of

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mitochondrial reactive oxygen species (ROS), increased mitochondrial transmembrane electric potential ($\Delta\Psi_m$), and aberrant apoptotic machinery (Chatterjee et al. 2011; van Gisbergen et al. 2015). When defective mitochondrial respiration in cancer cells is restored by mtDNA acquisition, tumor-forming ability is regained (Berridge et al. 2015; Tan et al. 2015). These mitochondrial characteristics of cancer cells may provide selective therapeutic targets to promote tumor cell death and decrease drug resistance. Drugs that act directly on mitochondria, causing bioenergetic changes have been called mitocans (Guzman-Villanueva and Weissig 2016). These drugs can be naturally occurring, or chemically directed to cancer cell mitochondria through the conjugation of lipophilic cations or peptides with therapeutic compounds that target the antioxidant defenses and pro-apoptotic proteins (Chen et al. 2010; Guzman-Villanueva and Weissig 2016; Ralph et al. 2006), and can be combined with conventional chemotherapeutic agents (Ralph et al. 2006). Targeting the cancer cell powerhouses can take advantage of the higher $\Delta\Psi_m$ in tumor cells and aims to limit the supply of cellular building blocks and mitochondrial substrates, inhibit oxidative phosphorylation (OXPHOS), increase mitochondrial oxidative stress, or target the mitochondrial transition pore (mPTP) and the aberrant apoptotic machinery (Deus et al. 2014, 2015; Sandoval-Acuna et al. 2016). However, these drugs may not be so helpful against cancer stem cells, which were shown to be less reliant on mitochondria and are more difficult to kill with mitochondria-directed therapies (Song et al. 2017; Vega-Naredo et al. 2014). Nevertheless, mitochondria of tumor-initiating cells possess special characteristics, which may be used to create drugs that specifically target them (Yan et al. 2015, 2016).

Mitochondria are also involved in some of the adverse effects of classic chemotherapeutic agents, which often disturb mitochondrial function in nontarget cells (Figure 40.1) (Wang et al. 2010). Drug-induced mitochondrial dysfunction can be caused by the parent drug itself or by reactive metabolites produced by cytochrome P450 in the liver (Chen et al. 2016; Pereira et al. 2012a; Shirakawa et al. 2015), and every year a number of drug candidates do not reach the market due to toxicity issues, which are later shown to be associated with mitochondria (Nadanaciva et al. 2007). Mitochondrial disruption induced by chemotherapeutic agents affects predominantly high metabolic demand tissues/organs, such as the skeletal (Gilliam et al. 2016; Sorensen et al. 2016) and cardiac muscles (Carvalho et al. 2014; Monsuez et al. 2010; Sampaio et al. 2016), the liver (Ramadori and Cameron 2010), and the brain (Waseem et al. 2016; Xiao and Bennett 2012; Zheng et al. 2012). The circular structure of mtDNA allows an easier intercalation of chemotherapeutic agents that can lead to mitochondrial

dysfunction, since mtDNA has a higher rate of mutation and a more limited repair system compared with nuclear DNA (Figure 40.1a) (Scheede-Bergdahl and Jagoe 2013; Sorensen et al. 2016; Tuppen et al. 2010). On the other hand, some agents show a strong affinity to cardiolipin, one of the most abundant phospholipids in the inner mitochondrial membrane (IMM) and essential for electron transport chain (ETC) enzymatic activities, increasing the propensity to mitochondria-induced toxicity (Carvalho et al. 2014). Mitochondrial injury by chemotherapeutic agents can be caused by direct or indirect effects in this organelle, resulting in alterations of metabolic pathways and damage to mitochondrial components (Vichaya et al. 2015). The mechanisms of mitochondrial disruption include uncoupling of ETC followed by decreased ATP production (Figure 40.1e), inhibition of OXPHOS complexes (Figure 40.1b), oxidative stress, opening of mPTP, depletion of mtDNA, inhibition of tricarboxylic acid cycle (TCA) or β -oxidation (Figure 40.1g), and inhibition of membrane transporters (Begrache et al. 2011; Gogvadze et al. 2009). Increase in mitochondrial ROS was described as a main cause of chemotherapy-induced mitochondrial dysfunction (Rovini et al. 2011). Some chemotherapeutic drugs can directly induce oxidative stress via interaction of their chemical structure with oxygen, while others can decrease antioxidant defenses (Figure 40.1c, d) (Gilliam and St Clair 2011). Inside mitochondria, mtDNA and the iron-sulfur centers of aconitase and of complexes I, II, and III of the ETC are the most susceptible structures to oxidative damage (Guo et al. 2013). Chemotherapy-associated mitochondrial dysfunction may also involve mitochondrial p53 accumulation and formation of drug-mtDNA adducts, as a result of inflammatory and non-inflammatory-based mechanisms, which leads to apoptosis (Figure 40.1h) (Vichaya et al. 2015). In the next sections, we highlight the mitochondrial effects of some of the most well-known classes of anticancer drugs.

40.3 Conventional Chemotherapeutic Agents and Mitochondria

40.3.1 Alkylating Agents

Alkylating agents (AAs) are among the most widely used anticancer drugs and were the first effective nonhormonal drugs used in chemotherapy. Although they were used as chemical weapons during the World War I, their chemotherapeutic effects were only discovered in the 1940s (Tahmasbpour et al. 2015). Classes of AA comprise nitrogen mustards (e.g., mechlorethamine and cyclophosphamide), nitrosoureas, triazines, ethylenimides,

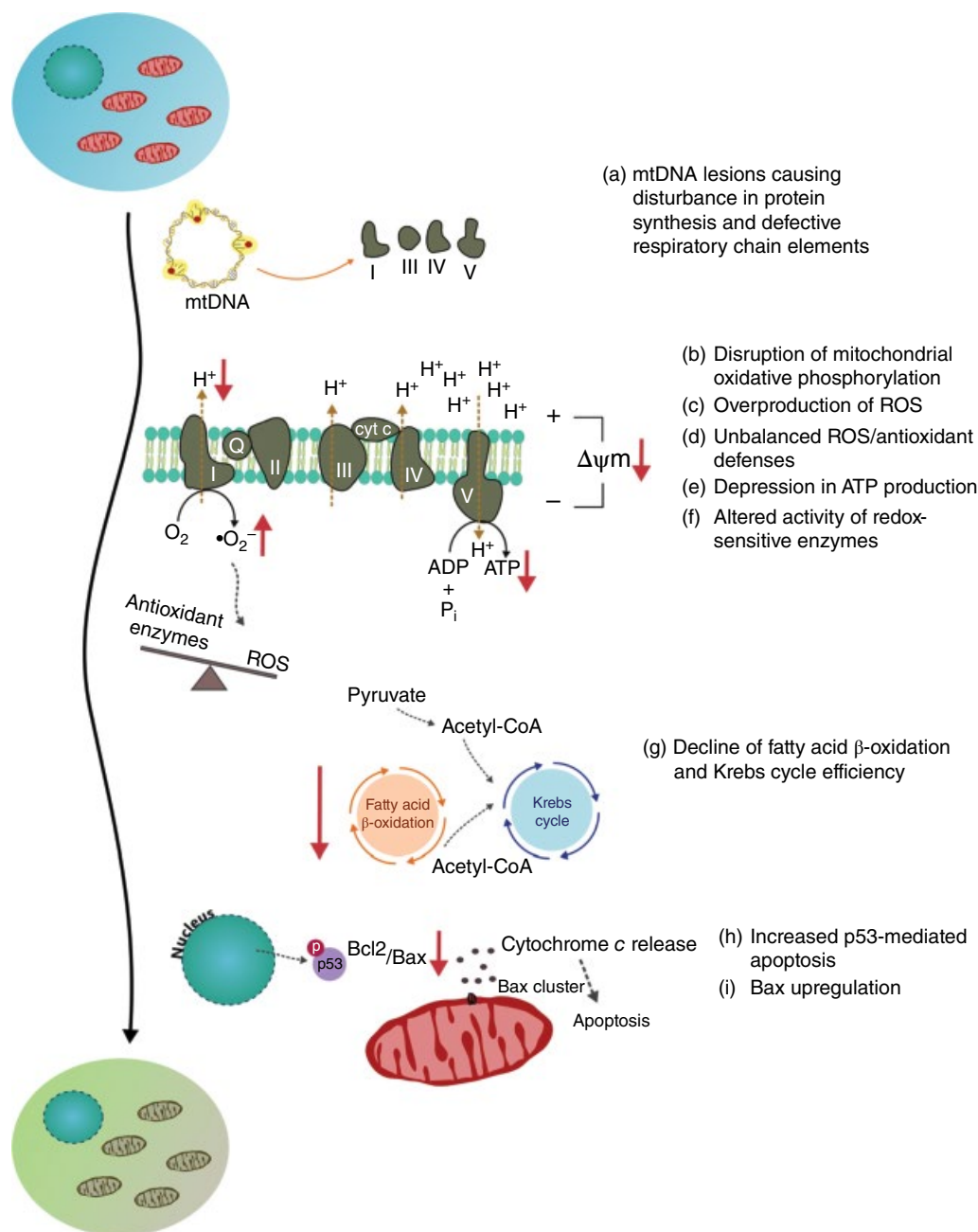


Figure 40.1 Main mechanisms by which anticancer drugs affect mitochondrial related functions. Some of the consequences of chemotherapeutic treatments include a decrease in mtDNA content and quality, increased oxidative stress, which leads to the oxidation of mitochondrial membranes and other structures such as proteins from electron transport chain, resulting in electron leak and even more mtROS production, decreased ATP content, collapse of $\Delta\Psi$, decreased mitochondrial biogenesis, inhibition of essential metabolic cycles, cytochrome c release from intermembrane space, caspase activation, and apoptosis. $\Delta\Psi$, mitochondrial transmembrane electric potential; cyt c, cytochrome c; mtDNA, mitochondrial DNA; Q, coenzyme Q.

and platinum-based compounds (e.g., oxaliplatin and cisplatin, Figure 40.2). These classes can further be grouped into two types: drugs that react directly with biological molecules and drugs that require a metabolic activation to form an intermediate, which in turn reacts with the biological molecules (Damia and D'Incalci 1998;

Sorensen et al. 2016). Cytotoxic AAs are widely used in chemotherapy of solid tumors, such as breast, ovarian, and lung cancer and leukemia and lymphoma malignancies (Taha et al. 2015). AAs mainly target DNA by attaching an alkyl group to the nucleic acid, reacting mainly with the N7 of guanine, although they can also alkylate

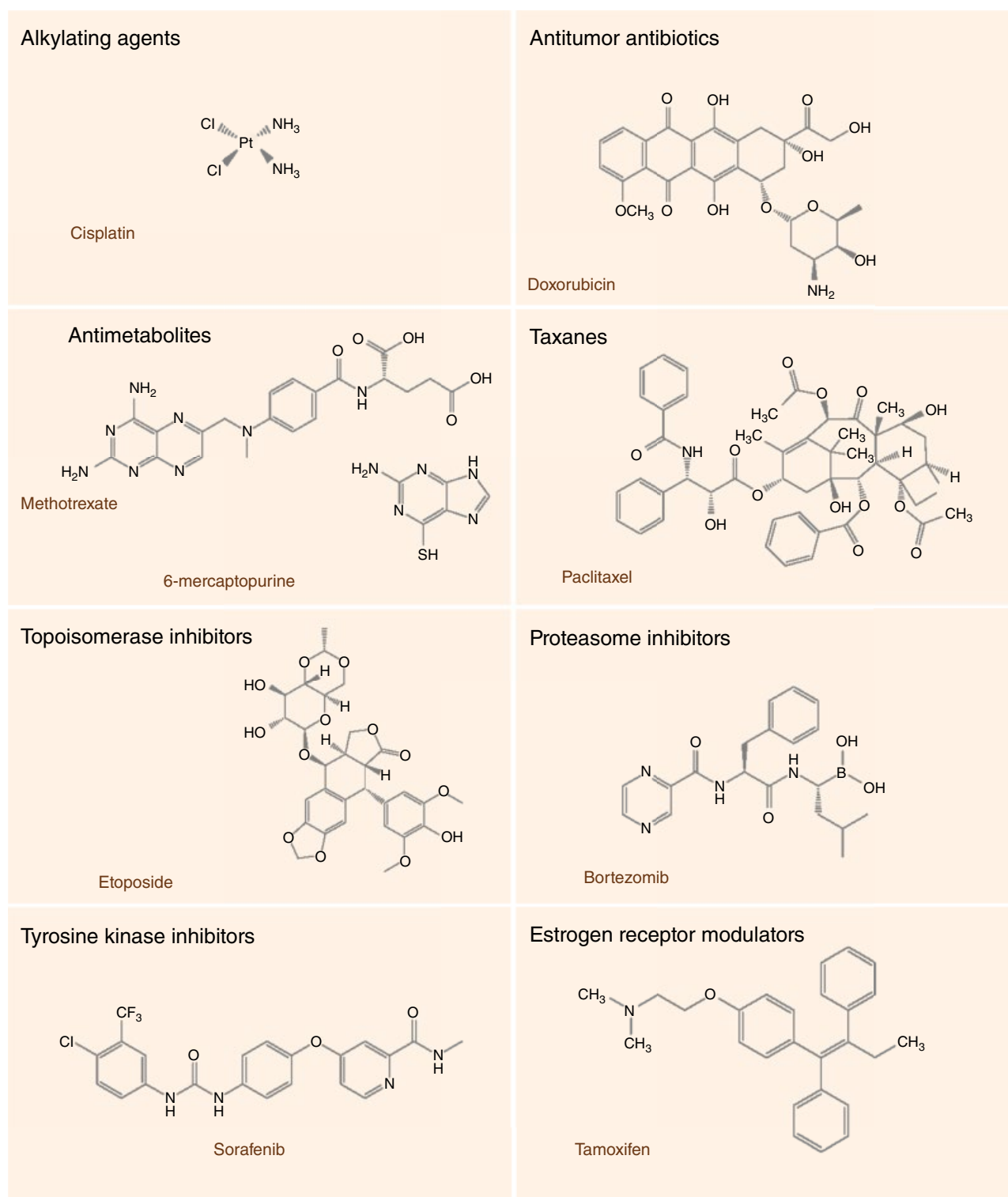


Figure 40.2 Molecular structures of elements from the different classes of anticancer drugs focused on this chapter.

other DNA positions, including O6 and N1 of guanine; N7, N3, and N1 of adenine; N3 of cytosine; and O4 of thymine (Damia and D'Incalci 1998). Thus, AAs can induce double-strand breaks that block DNA replication

and transcription and trigger DNA repair mechanisms. If repair is not efficient, apoptosis or other cell death pathways can be activated (Mourtada et al. 2013). AAs also form adducts with RNA, lipids, and proteins, as well

as increase ROS, and all these interactions lead to inhibition of nucleic acid and protein syntheses and disruption of energy metabolism, ultimately triggering cell death (Tahmasbpour et al. 2015; Zanotto-Filho et al. 2016). However, the cytotoxic effects of AAs are mostly due to the reactions with DNA, and since most of the major AAs are bifunctional, having two reactive groups, they can react with both strands of the DNA molecule by simultaneously alkylating two groups, leading to DNA crosslinkings (Kufe et al. 2003).

The antitumor properties of AAs affect mainly the proliferative cancer cells, as these cells are more susceptible to DNA damage, although they may also present cytotoxic effects in normal cells that proliferate quickly, including in bone marrow, gastrointestinal tract, and testicles. Thus, despite the promising efficiency of these compounds, the side effects limit their clinical usage (Kufe et al. 2003; Waissbluth et al. 2017). Mitochondria seem to be a key player in the deleterious consequences of these compounds in somatic cells (Druzhyna et al. 2008). Dysregulation of mitochondrial energy homeostasis through mutations in mtDNA or in nuclear-encoded mitochondrial proteins lead to negative side effects (Gilliam et al. 2016). It is also extensively described that AAs increase oxidative stress and reduce antioxidant defenses in different types of cells (Gilliam et al. 2016; Kufe et al. 2003; Taha et al. 2015; Tahmasbpour et al. 2015; Zanotto-Filho et al. 2016). The most prevalent forms of toxicity induced by AAs are nephrotoxicity, neurotoxicity, and ototoxicity, but the most severe toxic consequence during treatment with AAs occurs in the renal system and is usually the dose-limiting side effect (Rybak et al. 2009; Taha et al. 2015). AAs and their 4-hydroxy metabolites are excreted by the urinary tract where they may interact with local cells, react with DNA, and increase ROS (Taha et al. 2015). Oxidative stress heavily affects bladder cell mitochondria, leading to degeneration of this organelle due to swelling, membrane rupture, and destruction of cristae, resulting in permeabilization of the bladder epithelium membrane and consequent inflammation, which ultimately ends in necrosis (Taha et al. 2015). In skeletal muscle, the adverse effects of AA chemotherapy include muscle atrophy and dysfunction, insulin resistance, weakness, and fatigue, which strongly reduce the quality of life and increase the mortality of the patients (Sorensen et al. 2016). It has been shown in the C2C12 myotube cell line that oxaliplatin-induced reversible inhibition of key respiratory enzymes and interacted with complex IV, dysregulating electron flow through ETC (Sorensen et al. 2016). This dysfunction of electron transference increased the production of superoxide anion and peroxynitrite, leading to augmented toxicity (Tahmasbpour et al. 2015). The chronic side effects of the AA treatment are also related

to mitochondrial dysfunction and increased mitochondrial ROS production, due to nDNA and mtDNA mutations (Sorensen et al. 2016). Once increased ROS levels are the main cause of toxicity, a conjugation of antioxidants and chemotherapy seems to be a good strategy to overcome the side effects (Bhattacharya et al. 2001; Taha et al. 2015).

40.3.2 Antitumor Antibiotics

Antitumor antibiotics are another class of agents used in chemotherapy. These chemotherapeutic drugs affect the enzymes involved in DNA replication and transcription and can interfere with all phases of cell cycle (Sorensen et al. 2016). Among this class of drugs, anthracyclines are the most used in chemotherapy. Anthracyclines were first identified in the 1950s in the soil bacterium *Streptomyces peucetius*. This antibiotic family is composed of four main anthracyclines: doxorubicin (DOX, also known as adriamycin), daunorubicin, epirubicin, and idarubicin, with DOX being the most effective in cancer therapy (Simůnek et al. 2009; Volkova and Russell 2011). Concerning the chemical structure, these agents have a rigid planar tetracyclic ring with quinone moieties and an amino sugar attached to the tetracyclic ring (Figure 40.2). These molecules are highly effective antineoplastic agents and are extensively used to treat numerous types of adult and pediatric cancers (Menna et al. 2007; Smith et al. 2010; Volkova and Russell 2011), although their antineoplastic mechanism still raises controversy. Initially, the mechanism responsible for anticancer effects was attributed to intercalation of the planar ring into the DNA helix, which unwinds nucleic acids and causes a stereochemical disorder, blocking transcription and replication, and preventing the rapid growth of cancer cells (Branco et al. 2012). Anthracyclines also bind to mtDNA and to proteins involved in DNA replication and transcription, and these bonds lead to inhibition of DNA, RNA, and protein syntheses, leading to cell death (Sardão et al. 2009a; Yang et al. 2014). Nowadays, topoisomerase II is recognized as a main cellular target of these class of drugs, which acts by stabilizing the enzymatic reaction when the cut DNA strands are covalently linked to the enzyme, blocking subsequent DNA resealing, and inducing cell death (Pommier et al. 2010). Increased production of ROS in the presence of anthracyclines was also described as another antineoplastic mechanism (Kratz et al. 2006; Nitiss 2009).

The success of the treatment with anthracyclines is hampered by the dose-related side effects, such as hematopoietic suppression, vomiting, extravasation, impaired muscle function, and gastrointestinal disorders (Gouspillou et al. 2015; Kratz et al. 2006; Pereira et al. 2011). Although several organs are affected, the heart

seems to be the main target, with several forms of cardiotoxicity described, including acute (pericarditis and arrhythmias), early (left ventricular systolic dysfunction), or late onset (chronic cardiomyopathy and congestive heart failure) (Pereira et al. 2012a; Sardão et al. 2009a; Zhang et al. 2009). In a subchronic DOX toxicity model, Pereira et al. (2012b) demonstrated that cardiac mitochondria were more affected than their liver or kidney counterparts, suffering more alterations including decreased mitochondrial oxygen consumption and $\Delta\Psi_m$, which was also demonstrated by Serrano et al. (1999), using low doses of DOX injected weekly. DOX-induced cardiotoxicity has a strong mitochondrial component and oxidative stress on cardiac cells has been implicated as a major player (Sardão et al. 2009b; Zhang et al. 2011) (Figure 40.3). DOX structure contains a quinone moiety that interacts with cellular dehydrogenases, mainly NADH dehydrogenase (complex I) of the ETC, being reduced to an unstable semiquinone that can then react with oxygen and generate ROS (Vejpangsa and Yeh 2014; Volkova and Russell 2011). Since mtDNA is more susceptible to oxidative stress than nDNA, it has been reported to be damaged by DOX-induced ROS, or directly by DOX, leading to ETC failure and further increasing ROS production (Palmeira et al. 1997; Sardão et al. 2009a). In addition, DOX administration increases endothelial nitric oxide synthase (NOS) and inducible NOS activities, resulting in unbalanced production of NO and consequent harmful effects, while DOX can also be metabolized by NOS, leading to further production of superoxide anion (Garner et al. 1999; Vasquez-Vivar et al. 1997). This increase in NO levels, in cardiomyocytes, could result due to a dysregulation of iron homeostasis by DOX (Aldieri et al. 2002). The formation of DOX-iron complexes also favors hydroxyl radical generation through the Fenton reaction, acting by recycling ferric anion. However, it is thought that there is not enough free iron to complex with DOX and cause cardiomyopathy by itself (Octavia et al. 2012). The resulting oxidative stress damages different mitochondrial structures, through protein and lipid peroxidation, oxidation of mtDNA, and induction of mPTP (Sardão et al. 2009a). Moreover, DOX can undergo a two-electron reduction, being converted into doxorubicinol, which is more potent in dysregulating calcium and iron homeostasis (Menna et al. 2008; Minotti et al. 1998). Calcium plays an essential role in muscle contraction and in the regulation of important mitochondrial enzymes, such as pyruvate dehydrogenase and ATP synthase (Zhao et al. 2014). Interplay between oxidative stress and alterations in calcium homeostasis after DOX treatment may also play a role, since increased ROS levels stimulate calcium release from the sarcoplasmic reticulum (SR), which is located closely to mitochondria, leading to an accumula-

tion of calcium in mitochondria, with a further increase in mitochondrial ROS (Lebrecht et al. 2010; Octavia et al. 2012). The combination of calcium dysregulation and oxidative stress may trigger the opening of the mPTP, increasing the permeability of the IMM and disrupting the energy-generating processes (Carvalho et al. 2014; Octavia et al. 2012; Solem et al. 1996; Zhang et al. 2009; Zhou et al. 2001). In this context, the cardiac bioenergetics may be disturbed in different ways, through the oxidation of mtDNA and proteins, formation of complexes between DOX and cardiolipin, or direct interaction of DOX with ETC complexes (Lagoa et al. 2014; Zhao et al. 2014). In fact, a significant increase in extracellular acidification and lactate production, a by-product of glycolysis, and the decrease in OXPHOS suggest a shift to glycolysis after DOX treatment (Carvalho et al. 2010). Altogether, these processes caused by DOX treatment create a bioenergetic crisis in cardiomyocytes that could trigger cell death signaling, leading to autophagy, apoptosis, or necrosis (Octavia et al. 2012). Pro-apoptotic factors such as Bax (Figure 40.1h) and Bak can be directed to mitochondria after DOX treatment, leading to disruption of mitochondrial membrane permeability, allowing the release of several apoptotic initiators, including AIF and cytochrome *c* (Pereira et al. 2011; Volkova and Russell 2011), which lead to the activation of caspases-9 (Sardão et al. 2009a) and -3 (Octavia et al. 2012). DOX-induced cardiomyocyte apoptosis has been shown to be associated with increased expression and activation of the p53 tumor suppressor protein, leading to transcriptional activation of pro-apoptotic Bcl-2 proteins (Sardão et al. 2009a). DOX treatment also induces p53 translocation to mitochondrial membranes, leading to mitochondrial dysfunction and cell apoptosis (Sardão et al. 2009a). Using a chemical inhibitor of p53, pifithrin- α , a blockage of apoptosis via the inhibition of p53 downstream activity was observed (Sardão et al. 2009a), affecting Bax and MDM2 expression, which were increased by DOX treatment (Liu et al. 2004). However, p53-independent cell death may also occur through DOX-induced oxidative stress, also involving cytochrome *c* release and caspase-3 activation (Tsang et al. 2003).

40.3.3 Antimetabolites

Antimetabolites are a class of chemotherapeutic drugs that, because of their similarity to endogenous molecules, block nucleic acid synthesis by limiting deoxyribonucleoside triphosphate availability, target enzymes involved in *de novo* biosynthesis of purines and pyrimidines, and/or get misincorporated into nucleic acids (Grem and Keith 2005). These drugs interfere with DNA and RNA syntheses and are cell cycle specific, being active during G1 and S phases (Payne and Miles 2008).

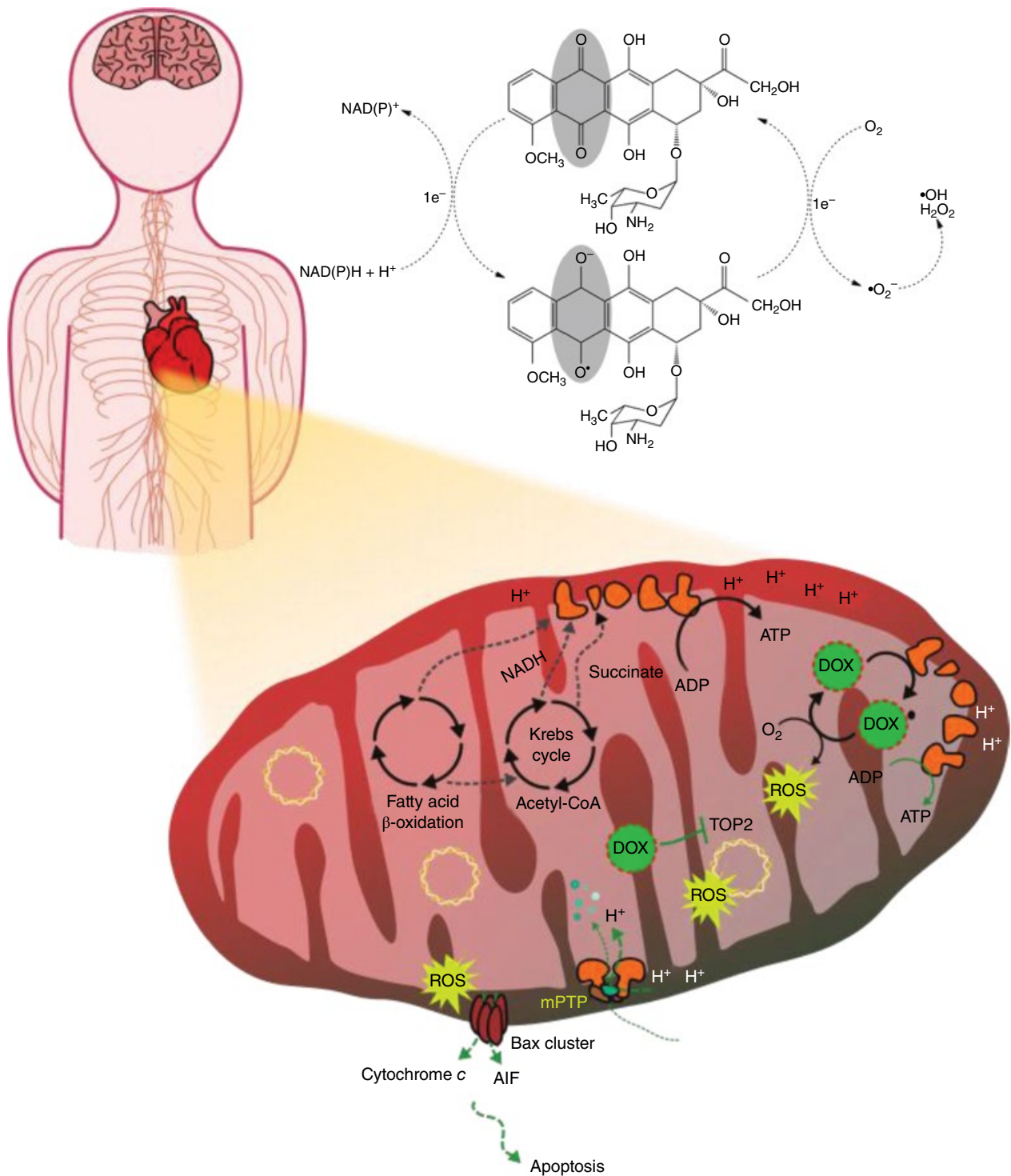


Figure 40.3 Overview of molecular reactions and mitochondrial alterations associated with development of doxorubicin cardiotoxicity. DOX accepts electrons from NAD(P)H-dependent flavoproteins generating a semiquinone form. Molecular oxygen can accept the electron from this semiquinone, forming superoxide anions. Subsequent mechanisms based on free iron and antioxidant enzymes may induce the generation of other ROS (H_2O_2 and $\cdot\text{OH}$). AIF, apoptosis inducing factor; DOX^{•-}, doxorubicin semiquinone radical; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; TOP2, topoisomerase II.

Antimetabolites are classified based on the molecules with which they interfere, such as pyrimidine antagonists (5-fluorouracil- 5-FU), purine antagonists (6-mercaptopurine, Figure 40.2), and folic acid antagonists (methotrexate, Figure 40.2) (Payne and Miles 2008). Once these drugs are converted into analogues of cellular nucleotides, they cause DNA damage and consequent apoptosis (Hwang et al. 2015; Olaussen and Postel-Vinay 2016; Parker 2009; Sorensen et al. 2016). Antimetabolite molecules are commonly used in the treatment of leukemia and breast, ovary, and gastrointestinal tract cancers (Sorensen et al. 2016).

5-FU, one of the most commonly used antimetabolites, and its prodrug capecitabine cause severe toxic effects in about 15–30% of the patients, including diarrhea, mucositis, and neurotoxicity, which could result from decreased dihydropyrimidine dehydrogenase activity (Lunenborg et al. 2016). However, the decreased activity or content of this enzyme just partly explains the side effects of 5-FU, and cardiotoxicity seems to be independent of it (Lischke et al. 2015). One of the metabolites produced by 5-FU catabolism is fluoroacetate, which inhibits the enzyme aconitase from the TCA cycle, impairing mitochondrial energy metabolism, inducing ATP depletion, and leading to cardiotoxicity (Lischke et al. 2015). This drug is also associated with myocardial ischemia, once it reduces endothelial NO synthase leading to coronary artery vasospasm, and also induces endothelium-independent vasoconstriction via protein kinase C (Cameron et al. 2016). The use of methotrexate is also limited by off-target toxicity, including intestinal toxicity, cardiotoxicity, nephrotoxicity, and hepatotoxicity, which are probably caused by increased ROS accumulation and consequently with lipid and protein peroxidation (Gautam et al. 2016). Treatment with azathioprine, which belongs to the purine analogues, being a prodrug for mercaptopurine, leads to hepatotoxicity due to ATP depletion and to a decrease in reduced glutathione, mainly in mitochondria, leading to cell death (Tapner et al. 2004).

40.3.4 Taxanes

Taxanes are a class of diterpenes used as chemotherapeutic drugs. One of these drugs is paclitaxel (Figure 40.2), which is used in the treatment of several types of solid tumors such as breast, ovarian, and lung cancer, exerting its antineoplastic effects on microtubules during the cell cycle (Mukhtar et al. 2014). Paclitaxel binds to β -tubulin in microtubules, stabilizing the microtubule lattice and suppressing depolymerization, leading to mitotic arrest during the G2/M phase of the cell cycle (Gornstein and Schwarz 2014). Thus, paclitaxel is highly effective against proliferating cancer cells. Despite the

promising antineoplastic action, paclitaxel properties turn it toxic to healthy tissues as well, most prominently to the peripheral nervous system. Even though neurons are not proliferative cells, they are also affected. The sensory symptoms in the peripheral nervous system initiate in the distal extremities and include sensory loss, temperature hypersensitivity, paresthesia, and neuropathic pain syndrome (Gornstein and Schwarz 2014). Persistent painful chemoneuropathy was reported to last months to years after cessation of paclitaxel treatment (Boyette-Davis et al. 2013). The effects on the microtubules are generally considered responsible for paclitaxel-induced neurotoxicity, but other off-targets should not be discarded because microtubule dynamic instability, despite being mostly accepted as responsible for the toxicity in microtubule-rich axons, has not been definitively demonstrated (Gornstein and Schwarz 2014). In a rat model of paclitaxel-induced painful peripheral neuropathy, atypical (swollen and vacuolated) mitochondria were observed in C-fibers and myelinated axons and these morphological alterations were suggested to result from the opening of the mPTP and consequent mitochondrial swelling (Flatters and Bennett 2006). On the other hand, the mPTP is associated with calcium movement (Jaggi and Singh 2012), and the mitochondria play a key role in intracellular calcium homeostasis. Impaired mitochondrial calcium uptake or increased leakage of mitochondrial calcium may be responsible for the increase in calcium-mediated neuronal excitability (Gleichmann and Mattson 2011). Rapid calcium release from mitochondria was observed after paclitaxel treatment in pancreatic acinar cells (Kidd et al. 2002), and calcium chelating agents significantly improve neuropathic pain in a rat model of paclitaxel-induced pain (Siau and Bennett 2006). In another study, paclitaxel was shown to induce cytochrome *c* release from mitochondria isolated from human neuroblastoma cells, which was prevented by cyclosporine A, a drug that binds to cyclophilin D and leads to desensitization of the mPTP (Andre et al. 2000). As mitochondria are a major source of ROS, pharmacological approaches aimed to decrease ROS levels were investigated as preventive measures for neuropathy (Fidanboyly et al. 2011). The global inhibition of ROS using the nonspecific ROS scavenger phenyl *N-tert*-butylnitrone (PBN) has been shown to improve paclitaxel-induced hypersensitivity and pain behaviors (Fidanboyly et al. 2011; Kim et al. 2010). Furthermore, since hepatic metabolism plays a pivotal role in the elimination of paclitaxel, its administration in patients with liver impairment should be handled with care (King and Perry 2001). In isolated liver mitochondria, paclitaxel caused the dissipation of $\Delta\Psi_m$ and induced ROS formation, in a concentration-dependent manner, and the release of cytochrome *c* from the intermembrane space (Varbiro et al. 2001).

40.3.5 Topoisomerase Inhibitors

Topoisomerase enzymes play an essential role in the high-order structuring of DNA, locally unwinding DNA duplex whose fixed ends cause stress by DNA supercoiling (Chen et al. 2013). Topoisomerases are divided into classes I and II, depending on whether one strand is cleaved and the opposing strand passes through it, or the DNA passes through a double-strand gap generated by the enzyme in the duplex DNA, respectively (Champoux 2001). Etoposide (Figure 40.2) is a class II topoisomerase (TOP2) inhibitor. It increases the levels of TOP2:DNA covalent complexes, introducing high levels of transient protein-associated breaks in the DNA (Nitiss 2009). Etoposide has been used to treat a wide variety of cancers, including lung, germ-cell malignancies, leukemias, lymphomas, and neuroblastomas (Belani et al. 1994). However, toxic side effects are associated with this treatment and include bone marrow suppression, nausea, vomiting, alopecia, and, at higher concentrations, mucositis and liver toxicity (Johnson et al. 1983). In rat liver mitochondria, etoposide was shown to increase mitochondrial sensitivity to calcium-induced swelling, depolarization of $\Delta\Psi_m$, and calcium release from mitochondria (Custodio et al. 2001). According to the authors, etoposide increased mitochondrial sensitivity to calcium-dependent induction of mPTP. In agreement, cyclosporine A was shown to inhibit or revert these effects (Custodio et al. 2001) and avoided the release of cytochrome *c* from mitochondrial intermembrane space into the cytosol, reducing the loss of cell viability (Karpnich et al. 2002).

40.3.6 Targeted Therapy

40.3.6.1 Proteasome Inhibitors

Bortezomib (BTZ, Figure 40.2) is an antineoplastic drug, firstly described as an inflammation inhibitor, but whose cytotoxic action rapidly changed its main application to cancer therapy (Goldberg 2011). BTZ belongs to the protease inhibitor class of chemotherapeutic agents since its reversible binding to 26S proteasome subunit leads to the inhibition of protein degradation and, consequently, to increased apoptosis (Chen et al. 2011). Approved in 2003 to be used primarily in myeloma treatment, BTZ was also investigated in the treatment of solid tumors, but the low efficiency and lack of improvement in patient responses encouraged the development of novel proteasome inhibitors (Chen et al. 2011). Despite being relatively well tolerated both when used as single agent and in combination with other drugs, its use is limited by peripheral neurotoxicity. Particularly in the case of chemotherapy-related neurotoxicity, BTZ is associated to severe dose-limiting peripheral neuropathy, characterized by sensory loss,

paresthesias, dysesthesias, and numbness, as well as reduced tendon reflexes and autonomic innervation in the skin (Argyriou et al. 2008; Carozzi et al. 2015; Cavaletti and Nobile-Orazio 2007). Mitochondrial damage has been suggested as an important key factor on BTZ-induced side effects. In rats, BTZ-induced painful peripheral neuropathy was associated with long-lasting dysfunction in axonal mitochondria, which presented deficient complex I- and complex II-mediated respiration, as well as decreased ATP production (Zheng et al. 2012). Mitochondrial dysfunction in cancer cell lines also seems to be determinant for BTZ cytotoxic action, involving not only ROS generation (Du et al. 2009) but also dysregulation of calcium influx (Landowski et al. 2005).

40.3.6.2 Monoclonal Antibodies

Trastuzumab is a humanized monoclonal antibody directed against the extracellular domain of human epidermal growth factor receptor 2 (HER2) and is mainly used in the treatment of women with HER2-positive breast cancers (Slamon et al. 2001). The HER2 gene is overexpressed in 25–30% of breast cancers and the abnormal high levels of the encoded protein increase the aggressiveness of the tumor (Slamon et al. 1987). Trastuzumab has been used as single agent or in combination with standard chemotherapy regimens, such as anthracyclines, and while this last class is suggested to induce irreversible cardiotoxicity, at least if lately diagnosed, trastuzumab-induced cardiac changes are usually reversible (Florescu et al. 2013). A number of mechanisms are proposed to be behind trastuzumab-induced cancer cell death, such as interference with the HER2 dimerization required in signal transduction, induction of antibody-dependent immune cell-mediated cytotoxicity (Arnould et al. 2006), or enhanced activity of the cell cycle inhibitor p27, inducing G1 cell cycle arrest and tumor growth inhibition (Le et al. 2003). Despite the improvement provided by trastuzumab treatment in the survival rate and cancer recurrence, multiple cardiotoxic events were reported. In a follow-up study (median 32.6 months) conducted by Guarneri et al., 28% of patients experienced cardiac adverse events (Guarneri et al. 2006). The high incidence of cardiac dysfunction was not surprising because of the significant percentage of patients with a previous history of cardiac disease or prior exposure to anthracyclines (Guarneri et al. 2006). Similar results were previously obtained when trastuzumab was used in combination with both anthracyclines and cyclophosphamide (cardiotoxicity in up to 27% of the patients), but the percentage decreased in patients receiving trastuzumab alone (about 7%) (Riccio et al. 2016; Slamon et al. 2001). Trastuzumab-induced cardiotoxicity is not totally understood in terms of molecular mechanisms, and the clinical management

currently relies on echocardiography to identify risk situations based on the reduction left ventricular ejection fraction (LVEF) (Florescu et al. 2013). However, some studies started to mechanistically highlight the toxic action of trastuzumab. HER2 belongs to epidermal growth factor (EGF) receptor family and is known to heterodimerize with other HER receptors, including HER4, upon the binding of ligands called neuregulins, which results in a series of auto- and trans-phosphorylations in the receptors, determining the elicited downstream signaling cascade (Wieduwilt and Moasser 2008). HER2-related signaling is required for a normal embryonic development and plays an important role in cardiac myocyte functioning. The HER2/HER4 heterodimerization has been postulated to be essential for cardiomyocyte function due to its role on the stimulation of survival signals upon stress and/or pressure overload (Albini et al. 2011). In mice, HER2 (also known as ErbB2) mutants exhibited multiple parameters of cardiomyopathy, including chamber dilation, wall thinning, and decreased contractility (Negro et al. 2004). HER2/ErbB2 overexpression has also been correlated with increased Bcl-xL/Bcl-xS ratio in the heart, which are antiapoptotic and pro-apoptotic proteins, respectively (Sysa-Shah et al. 2012). Trastuzumab-mediated inhibition of HER2/ErbB2 was associated with alterations of Bcl-xL/Bcl-xS balance, increased mitochondrial Bax oligomerization, reduction of the $\Delta\Psi_m$ and ATP depletion (Grazette et al. 2004), which may impair cardiomyocyte contractile function. ElZarrad et al. found that in mice heart, trastuzumab exerted an inhibitory effect on the expression of several genes related with cardiac contractibility, adaptation to stress, proliferation, wound healing, and mitochondrial function, with significantly less mitochondria found in the cardiomyocytes of the treated animals, which lacked the close contact organization predominantly observed in cardiomyocytes of the control group (ElZarrad et al. 2013). 3-Nitrotyrosine and 4-hydroxynonenal-protein adducts were also elevated in hearts of trastuzumab-treated animals, accompanied by increased caspase 3/7 activity, indicating the presence of oxidative stress and activation of apoptotic pathways, which were hypothesized to be the causes of the ultrastructural damages observed (ElZarrad et al. 2013).

40.3.6.3 Tyrosine Kinase Inhibitors

Sorafenib (Figure 40.2), a tyrosine kinase inhibitor (TKI), is used in the treatment of patients with unresectable hepatocellular carcinoma and advanced renal cell carcinoma and is usually better tolerated than most conventional chemotherapeutic drugs, with promising results observed in radioactive iodine refractory-differentiated thyroid cancer (Brose et al. 2014b). Sorafenib mainly inhibits vascular endothelial growth factor (VEGF) receptors-1,-2,-3, Raf kinases, RET (including RET/PTC), and

platelet-derived growth factor receptor β , thus acting toward the inhibition of angiogenesis and tumor cell proliferation (Wilhelm et al. 2006). Both VEGF and Raf pathways regulate diverse signaling cascades involved in different physiological processes and are critical to the homeostasis of many organs (Chen and Cleck 2009; Leicht et al. 2007). Thus, the blockage of these signaling pathways, apart from being responsible for the efficiency of the treatment, also leads to the toxic phenotype. Treatment interruptions and dose reductions are often recommended to manage the adverse effects associated with the treatment (Brose et al. 2014a). Some of the most common side effects are hand-foot skin reaction, rash, gastrointestinal distress, hypertension, and, although not so common, cardiovascular events, such as myocardial ischemia and congestive heart failure (Brose et al. 2014a; Li et al. 2015). VEGF/VEGF receptor signaling is essential in processes such as angiogenesis, vasodilation and maintenance of an antithrombotic state, and antiangiogenic therapies affect VEGF-related events including hypertension, wound healing, proteinuria, and arterial thromboembolic events (Chen and Cleck 2009). The cardiomyopathy associated with sorafenib treatment was hypothesized to rely on myocardial mitochondrial degeneration since at concentrations above 10 μM , sorafenib depleted ATP in a cardiomyoblast cell line (Will et al. 2008). In the same work but using isolated rat heart mitochondria, sorafenib was the most potent inhibitor of OXPHOS complexes activities at clinically relevant concentrations, among several TKI drugs (Will et al. 2008). Structural and functional changes induced by sorafenib were also analyzed using *in vivo* and *in vitro* cardiac models (French et al. 2010). Functional changes were milder than expected, with sorafenib inducing a slight but significant decrease in ATP levels and showing no effect on $\Delta\Psi_m$, although disrupted mitochondrial cristae were also found in sorafenib-treated rats (French et al. 2010). However, sunitinib, another TKI, promoted mitochondrial swelling, accumulation of dense deposits, matrix cavitation, and decrease in $\Delta\Psi_m$ in the same model in a dose-dependent manner (French et al. 2010). The exact mechanism of TKI-induced cardiotoxicity is unknown but could be related to inhibition of other kinases implicated in the regulation of mitochondrial function (French et al. 2010).

40.3.7 Estrogen Receptor Modulators

Tamoxifen (TAM, Figure 40.2) is an estrogen suppressor used in hormonal therapy in patients with estrogen receptor (ER) positive cancers, including breast cancer (Yang et al. 2013). ER-positive cancers are classified as such because of their responsiveness to estrogen that induces the proliferation of tumor cells and the progression of the disease (Yang et al. 2013). Estrogen is essential in female physiology, binding to ER- α and ER- β in their ligand-binding domains and inducing ER

association with some DNA sequences called estrogen response elements. This interaction leads to the recruitment of coactivators that catalyze the transcription of estrogen responsive genes important not only in physiological responses but also in the proliferation of breast cancer cells (Nilsson et al. 2001). TAM has the capacity to bind to ERs, disrupting the function of one of their domains, which compromises the normal estrogen-mediated gene expression and triggers genomic effects such as cytostasis (Johnston et al. 1995). In addition, there is evidence that TAM exerts its apoptotic properties through nongenomic mechanisms as well, even in ER negative cells (Jordan 2015). It is important to refer that, despite the success as a chemotherapeutic agent, TAM treatment is associated with an increased incidence of vaginal bleeding, ovarian cysts, endometrial thickening, decreased bone mineral density in premenopausal women, and, as a major concern, liver injury (Baum 2002; Ribeiro et al. 2014). Furthermore, long-term use of TAM may also induce secondary endometrial cancer in women, being considered a class I human carcinogen (Fisher et al. 1994). TAM works, as many other chemotherapeutic drugs, by inducing mitochondria-mediated apoptosis (Nazarewicz et al. 2007). The amine group of TAM can be protonated at physiological pH, followed by its translocation across the inner membrane into the mitochondrial matrix. The matrix alkaline environment favors the dissociation of the protons, leading to loss of $\Delta\Psi_m$ and impaired respiration (Cardoso et al. 2001). Furthermore, TAM promotes oxidative stress and apoptosis through mitochondria-dependent and nitric oxide (NO)-dependent pathways, by increasing NOS activity (Nazarewicz et al. 2007). It was previously described that mitochondria possess an NOS isoform that generates NO in response to increased intramitochondrial calcium concentration (Navarro and Boveris 2008; Valdez and Boveris 2007; Valdez et al. 2006). The formed NO not only controls mitochondrial bioenergetics via reversible regulation of cytochrome oxidase (complex IV) activity but also contributes to generate reactive nitrogen species (RNS), including peroxynitrite (Brown and Borutaite 2007). TAM increases calcium levels in mitochondria, stimulates mtNOS activity, and, at therapeutic concentrations, decreases oxygen consumption (Nazarewicz et al. 2007). Through mtNOS stimulation, lipid peroxidation is also increased, which inevitably destabilizes the supramolecular structure and fluidity of membranes, resulting in cytochrome *c* release from liver mitochondria (Nazarewicz et al. 2007; Tabassum et al. 2007). Other studies also showed TAM-related toxic effects in mitochondria, including increased levels of superoxide radicals and decreased Mn-SOD activity (Tabassum et al. 2007), collapse of $\Delta\Psi_m$ and ETC inhibition (Tuquet et al. 2000), as reviewed elsewhere (Ribeiro et al. 2014; Yang et al. 2013). Furthermore, TAM

decreased mtDNA synthesis in hepatic cells decreasing mtDNA copy number, which sensitized mitochondria to drug-induced effects due to a decrease in the synthesis of mtDNA-encoded respiratory chain subunits (Larosche et al. 2007). Moreover, β -oxidation was inhibited by TAM, presumably through the inhibition of carnitine palmitoyltransferase I (CPT1), which partly controls β -oxidation of long-chain fatty acids by regulating their entry into mitochondria (Larosche et al. 2007).

40.4 Mitoprotectants as Adjuvants in Chemotherapy

It is clear by now that chemotherapy is associated with toxic side effects of varying severity. This not only compromises the efficiency of the treatment, forcing the use of lower doses of drug or decreasing the number of cycles of chemotherapeutic regimen, but also entails a considerable cost in later treatments (Menna et al. 2008). Mitochondria are an important key target in toxic mechanisms, and thus many pharmacological strategies have been studied aiming to prevent those deleterious effects by protecting mitochondria. Excess ROS/RNS production is a result of the action of several chemotherapeutic agents, and oxidative stress has been established as one of the primary causes of mitochondrial dysfunction (Sinha et al. 2013). Consequently, cellular structures such as membrane lipids, proteins, and nuclear and mtDNA suffer oxidation and are damaged. The excessive oxidative damage that exceeds the cellular repair capacity results in disease and abnormal functioning of the biological systems (Turrens 2003). Membrane lipid peroxidation, for instance, affects membrane fluidity, permeability, and electron transport function in mitochondria (Radi et al. 1994). Highly associated with cancer therapy, fatigue was reported in more than 75% of cancer patients during the course of their disease and treatment (Vogelzang et al. 1997). Although underestimated, fatigue was also considered as one of the most distressing complaints among cancer patients and is often a reason why many patients discontinue the treatment (Liu et al. 2005). Among several factors including psychological conditions, sociocultural, and physiological factors, fatigue is also related with deficient energy supply and reduced efficiency of mitochondria (Ciregia et al. 2016; Gorman et al. 2015). Lipid replacement therapy (LRT) combined with antioxidants is a strategy that has been used in the treatment of certain clinical disorders, including chronic fatigue (Nicolson and Ellithorpe 2006). The treatment consists in providing high concentrations of undamaged lipids that will later replace the damaged membrane lipids and restore, or at least improve, the function of cellular structures (Nicolson 2005; Nicolson and Conklin 2008). The therapy is usually supplemented

with antioxidants to protect lipids from oxidation and ensure a safer delivery. A successful dietary supplement based on this principle is NTFactor®, which contains encapsulated components, including phospholipids, glycerophospholipids, and other types of lipids, that easily escape from the oxidation in the gut and reach the tissues without undue damage (Nicolson and Conklin 2008). Episodes of chemotherapy-induced fatigue, nausea, diarrhea, and other effects were reduced in patients with colon, rectal, and pancreatic cancers and an improvement in quality of life was reported, after the oral LRT (Colodny et al. 2000). Considering the link between toxicity and oxidative stress, many antioxidants have been tested *in vitro* and *in vivo*. In sensory neurons in culture, alpha-lipoic acid that is essential in cell energy metabolism, antioxidant regulation, and calcium homeostasis showed to be neuroprotective against cisplatin and paclitaxel-induced peripheral neuropathy (Melli et al. 2008). Other antioxidants such as vitamin A, vitamin E, *N*-acetylcysteine, or resveratrol have also been investigated, with some of them showing promising results, but others yielding some negative or contradictory findings (Arafa et al. 2014; Dresdale et al. 1982; Sterba et al. 2013; Wang et al. 1980). Unclear data may be due to a difficult translation of experimental results into chronic cardiotoxicity treatments (Gianni et al. 2008). BTZ (Section 40.3.6.1) is a promising drug in the treatment of childhood cancers, such as leukemia, but one worrisome side effect in this population is the possible suppression of bone growth (Eriksson et al. 2012). Humanin, a mtDNA-encoded peptide with proved protective action in brain disorders (Hashimoto et al. 2001), prevented BTZ-induced bone growth impairment in mice, while not interfering with its anticancer effects (Eriksson et al. 2014). In an anthracycline-treated group of children with acute lymphoblastic leukemia or non-Hodgkin lymphoma, coenzyme Q10 therapy was tested, and the antioxidant had a protective effect on cardiac function (Iarussi et al. 1994). Carvedilol, a neurohormonal antagonist that blocks adrenoceptors and has vasodilating properties, also showed promising results by preventing the inhibitory effects of DOX on mitochondrial respiration and the decrease in mitochondrial calcium loading capacity (Oliveira et al. 2004; Santos et al. 2002). In patients undergoing anthracycline therapy, carvedilol seemed to be an interesting protector of left ventricle function (Kalay et al. 2006). However, dexrazoxane (DEX) is, so far, the only approved method for anthracycline cardiotoxicity prevention with many clinical trials supporting its beneficial action (Lipshultz et al. 2010; Marty et al. 2006; Swain and Vici 2004). DEX was initially developed to be a chemotherapeutic agent due to its effects on TOP2 inhibition, but soon the attention moved to its cardioprotective effects against anthracycline-induced cardiotoxicity (Classen et al. 2003). In a study by Lipshultz et al., children

with acute lymphoblastic leukemia were followed during the treatment with DOX alone or DOX combined with DEX. The control group treated only with DOX showed a higher increase in serum levels of cardiac troponin T after the treatment compared with the group receiving the combined therapy (Lipshultz et al. 2012). Moreover, DEX also reduced the incidence and severity of early and late cardiotoxicity in children with solid tumors receiving DOX (Choi et al. 2010). Cardiac function was measured based on fractional shortening and systolic and diastolic left ventricular diameter, and the DOX plus DEX group presented significant fewer cardiac events (27.7% vs. 52.4%) and less severe congestive heart failure (6.4% vs. 14.3%), compared with the group treated with DOX alone (Choi et al. 2010). In addition, DEX seemed not to affect the response rates to chemotherapy (Elbl et al. 2006; Marty et al. 2006). Although the mechanism of cardioprotection is still not completely understood, the most accepted hypothesis proposes its ability to chelate intracellular iron and to decrease free radical formation (Sterba et al. 2013). At physiological conditions of pH and temperature, DEX undergoes hydrolysis, ultimately leading to the formation of its ring-opened metal ion-binding form, ADR925, which has a structure similar to EDTA, and can then either remove iron from the iron-ANT complexes or bind free iron, preventing oxygen radical formation (Sterba et al. 2013). However, more recently DEX-induced TOP2 β depletion was also suggested as essential for an effective cardioprotection against anthracycline cardiotoxicity (Jirkovska-Vavrova et al. 2015).

40.5 Conclusion

As described in the previous sections, chemotherapeutic agents affect different mitochondrial pathways. Some of these interactions are part of their therapeutic effects in cancer cells, but mitochondrial dysfunction resulting from treatment with chemotherapeutic agents also induces resistance to drug-induced apoptosis in cancer cells and affects the viability of normal cells, limiting their clinical utilization.

This chapter demonstrates that mitochondria are a critical mediator of chemotherapy-induced off-target toxicity. The multitude of structures and pathways in, and generating from, mitochondria increases the likelihood that chemotherapy may disturb bioenergetics and/or redox balances in that organelle. Despite what is described in this chapter, and facing the ever-growing list of mitochondrial findings, it is likely that the future will yield new evidence on how chemotherapy damages mitochondria in normal tissues, and how those organelles can be protected, opening the way for more effective and safer cancer treatments.

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Part 5

Environmental Toxicants and Mitochondria

41

The Mitochondrial Exposome

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41.1 Introduction

41.1.1 The Human Exposome

Humans are subjected to a diverse chemical experience that includes chronic as well as episodic exposures that vary spatially and temporally and in magnitude. While some environmental factors are known to contribute to disease, the relationship between many chemical exposures and human health is largely unknown. Identification of environmental stressors and their roles in human health and disease will improve the ability to implement preventative measures and reduce disease risk.

Despite completion of genome-wide association studies (GWAS) for a large number of chronic diseases, only a small number of identified variants describe risk (Thomas 2010). Interactions between genetic and environment/lifestyle risk factors are now suspected as important contributors to chronic disease burden; however, technologies that provide sequencing of environmental exposures are not available (Rappaport et al. 2014; Patel 2016; Rappaport 2016). In addition, exposure of different organs and cellular components may cause a range of toxicological responses. Thus, characterizing the contribution of environmental factors

to human health is inherently more complex than profiling the genome.

The need to include environmental exposures in understanding human disease led Christopher Wild to introduce the term exposome (Wild 2005), which he defined as “encompassing life-course environmental exposures (including lifestyle factors), from the prenatal period onwards.” The exposome was proposed as a complement to the genome, where life course of exposures and their interactions with the genome defines disease risk. Unlike the genome, exposures are transient at both short- and long-term time scales. Thus, quantitative assessment of chemical exposure over a lifetime poses substantial challenges. A more practical definition of the exposome was proposed by Miller and Jones (2014): “The cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes.” The exposome, as described in this framework, is not only limited to external chemical exposures but also includes processes internal to the body and socioeconomic influences (Rappaport and Smith 2010; Wild 2012). Importantly, by defining the exposome in terms of

cumulative measure of environmental influences and biological response, Miller and Jones (2014) acknowledge that the entirety of an individual's exposure does not need to be quantified, with exposure effect and maladaptation linking environment to health and disease.

The human metabolome is the expression of an individual's metabolic phenotype. The metabolome includes all endogenous metabolites, chemicals from human–environment interaction, and reactants arising from interaction of these compounds with enzymatic and bacterial processes. By extension, the metabolome provides a means to assess an individual's exposome. Application of metabolic profiling in human populations indicates that core metabolic processes only contribute to a fraction of the detected chemical species, with a large number of chemicals arising from environment and dietary sources. Current estimates suggest that upwards of one million chemicals are likely to contribute to the human metabolome (Uppal et al. 2016). Plants are likely to contain 10^5 to 10^6 small molecules (<2000 Da). Chemicals registered with the EPA for commercial use includes 10^5 unique species, with recent estimates from Toxic Substance Control Act indicating 70,000 are commonly used. A recent survey of 43,000 registered chemicals identified approximately 20,000 used directly in consumer products (Dionisio et al. 2015). Each of these is subject to conversion to other chemicals by the microbiome or following dispersion into the ecosystem. Thus, the contribution of the environment to defining the human metabolic phenotype likely exceeds the endogenous contribution due to central gene-directed metabolism.

41.1.2 The Mitochondrial Exposome

The human exposome is typically defined in terms of systemic exposures, with the goal of developing tools and techniques for measuring exposures for an individual. However, toxicity occurs at the microscale, where specific environmental agents or their metabolites interact with central biochemical pathways, resulting in changes that disrupt proper functioning and have the potential to lead to adverse outcomes. Mitochondria are particularly sensitive to chemical exposures. Accumulation of mutations in mitochondria has also been linked to premature aging, while interaction between mitochondria and environment is suspected in age-related diseases, including Parkinson's, Alzheimer's, and cardiovascular disease (Wallace 1999). In-depth characterization of these interactions, which we define as the *mitochondrial exposome*, is required to understand the specific environmental factors contributing to human mitochondrial disease. Thus, while the human exposome provides a construct for relating population-wide exposures to disease and human

health, the human mitochondrial exposome provides a framework for identifying environmental risks to mitochondria and possible associations with mitochondria-related diseases. This is highly relevant to drug-induced mitochondrial toxicities because the susceptible mitochondrial phenotype can be defined by the mitochondrial exposome.

41.1.3 Mitochondrial DNA Adductome

The causative role of chemical genotoxicity in mutagenesis is well documented (Delaney and Essigmann 2008). To date, the primary focus of genotoxicity has been on modifications occurring to nuclear DNA (nDNA). Although the exact mechanisms initiating carcinogenesis are not well understood, nDNA damage arising from adduct formation with electrophilic metabolites has been identified as a contributing factor. In addition to the nuclear genome, mitochondria contain a separately replicating genome. Oxidative damage to the mitochondrial genome is well recognized (Yakes and Van Houten 1997), but the effect of chemical genotoxicants on mitochondrial DNA (mtDNA) is not as well understood. The increase in mitochondrial dysfunction with age and age-related degenerative diseases, however, is consistent with an accumulation of mtDNA mutations acquired over a lifetime. The ability to characterize and identify specific mtDNA adducts arising from chemical exposures, referred to as the mtDNA adductome, provides a critical capability for assessing the role of mitochondrial dysfunction and environment in the pathogenesis of chronic diseases.

^{32}P -Postlabeling has been used to screen for the presence of nDNA adducts in multiple tissues and cell types; this method is, however, labor intensive, semi-quantitative, and nonspecific when multiple exposures are present (Balbo et al. 2014b). Recent development of liquid chromatography–mass spectrometry (LC-MS) approaches to characterize nDNA adducts demonstrates significant improvement for detecting and quantifying expected and unexpected adducts in both model systems and human samples. Low-resolution instrumentation may provide insufficient mass accuracy for elucidating possible chemical structures and associating DNA lesions with specific exposures (Singh and Farmer 2006). Thus, there is a benefit from high-resolution mass spectrometry (HRMS) platforms, which can support unambiguous identification and structural elucidation through multiple ion dissociation schemes and MS^n capabilities for detection, identification, and quantification of the mtDNA adductome (Balbo et al. 2014a).

The distribution and abundance of mtDNA lesions in humans that arise from chronic exposure to environmental pollutants is unknown. mtDNA lack the full

range of nDNA repair mechanisms, suggesting that the mtDNA adduct burden may be higher than that observed in nDNA and occur to a degree where heteroplasmy does not protect against mitochondrial dysfunction (Cline 2012). Development of methodologies for quantitative characterization of the mtDNA adductome provides a direct link between exposure to specific chemical species and underlying biological changes leading to disease. The use of analytical strategies providing untargeted measurements will allow detection of a wide array of changes without selecting specific analytes, which is more consistent with the varied and multicomponent exposure history during the course of a lifetime. Thus, understanding mtDNA effects from environmental exposure is expected to improve the ability to anticipate and manage drug-induced tissue injury.

41.1.4 Mitochondrial Genome and Proteome

Omic characterization of mitochondria has largely focused on the mitochondrial genome and proteome from various tissues, cells, and yeast strains (Anderson et al. 1981; Bibb et al. 1981; Rabilloud et al. 1998; Boore 1999; Millar et al. 2001; Da Cruz et al. 2003; Heazlewood et al. 2004; Pagliarini et al. 2008; Stefely et al. 2016). These efforts provide a better understanding of the evolutionary origin of mitochondria, protein synthesis, location of the proteins found within mitochondria, and how enzymes are formed and translated within the organelle structure. Alterations to the genome, discussed in Section 41.1.3, and specific characteristics of the proteome, discussed below, further support environmental exposures, that is, the mitochondrial exposome, as a potential determinant of mitochondrial susceptibility to drug-induced injury.

Within metazoan cells, mitochondria are the only organelle other than the nucleus containing a secondary genetic system required for biosynthesis. The mtDNA genome is separately replicating relative to the nucleus (Borst 1977) and encodes 13 proteins, with the remaining 99% encoded by nuclear genes and transported into the mitochondria (Bolender et al. 2008). The initial characterization of the mitochondrial genome was reported by Anderson et al. (1981) and represented the first component of the human genome to be successfully sequenced. The mtDNA genome in eukaryotic cells contain 16,569 base pairs, which encodes two ribosomal RNAs, 22 tRNAs, and 13 peptides that are constituents of complex I, complex III, cytochrome oxidase, and adenosine triphosphate (ATP) synthase (Scheffler 1999). Due to the limited mitochondrial proteins encoded within mtDNA compared to those required for

mitochondrial specific metabolic functions, nuclear-encoded proteins along with nuclear-mitochondrial genome interactions are essential for biogenesis and function.

The primary analysis of mitochondria using advanced chemical profiling platforms has focused on characterizing the mitochondrial proteome. To identify mitochondrial proteins, Taylor et al. (2003) isolated intact mitochondria from human heart tissue and used LC-MS to identify mitochondrial proteins. This approach provided detection of 615 proteins, with functionality assigned for 498 proteins related to mitochondrial function, including the citric acid cycle, glycolysis, apoptosis, redox processes, and oxidative phosphorylation (Figure 41.1a). Hydrophobicity of the identified proteins was also calculated using the Kyte-Doolittle algorithm (Kyte and Doolittle 1982) (Figure 41.1b); the results show possible sites exist within the mitochondrial protein structure for environmental chemical binding and accumulation. Similar techniques have been applied to a range of tissue to further characterize the mitochondrial proteome in mammals (Mootha et al. 2003; Forner et al. 2006; Kislinger et al. 2006; Calvo and Mootha 2010). To identify and create a database of mitochondrial proteins within different mice organs, Pagliarini et al. (2008) used genomics, mass spectrometry (MS)-based proteomics, green fluorescent protein tagging, and machine learning to develop a database of 1098 genes and associated protein expression for mitochondria obtained from 14 different tissues. This available database provides an important foundation for studies of environmental exposures and associated effects on mtDNA adductome, mitochondrial function, biological response, and impact on drug-induced toxicities.

41.2 Environmental Pollutants and Mitochondrial Toxicity

Mitochondria are recognized as secondary toxicological targets for many common environmental pollutants, including naturally occurring exogenous chemicals, environmental chemicals, and pharmaceuticals. Suspected environmental chemicals that act as mitochondrial toxicants include paraquat, cyanide, rotenone, particulate matter, polycyclic aromatic hydrocarbon quinines, dioxin, acrylamide, perfluorinated compounds, arsenic, and pentachlorophenol (Meyer et al. 2013). Toxicity occurs through multiple pathways, including disruption of mitochondrial pathways, mtDNA, and/or nDNA damage. In most cases, the action of these compounds was studied using *in vitro* exposures, with mitochondrial toxicity measured using functional assays or mtDNA mutations.

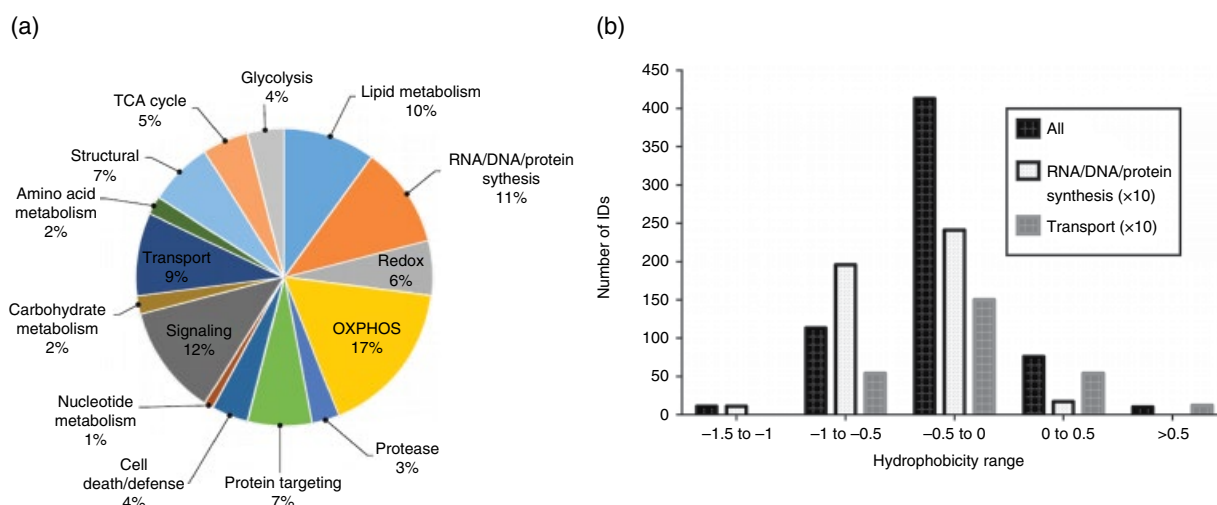


Figure 41.1 (a) Functional classification of mitochondrial proteins isolated from human heart tissue and identified using mass spectrometry-based proteomics. (b) Hydrophobicity range of identified mitochondrial proteins, based upon mean Kyte–Doolittle amino acid values. As reference, RNA/DNA/protein synthesis proteins, which tend to be more hydrophilic, and transport proteins, which tend to be more hydrophilic, were included for reference. Adapted from Taylor et al. (2003). Reproduced with permission of Nature Publishing Group.

41.2.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed and persistent environmental pollutants that are recognized as mitochondrial toxicants, either as the pure compound (van Grevenynghe et al. 2004; Babu et al. 2005) or bound to ultrafine particulate matter (Li et al. 2003b). Sources of PAHs include food, water, cooking, indoor air, vehicle exhaust, and other combustion products, with inhalation as the predominant exposure pathway (Menzie et al. 1992; Walker et al. 2016b). Toxicity of whole and hydrophobic/hydrophilic fractions of captured diesel exhaust particles was evaluated by Xia et al. (2004). When exposed to the particles and extracted organic material, the largest influence on mitochondrial viability was observed for the polar fraction, which increased reactive oxygen production and membrane potential in a dose-dependent manner. Similar behavior was observed for the nonpolar fraction, which included aromatics and parent PAH compounds. Membrane and Ca^{2+} -induced swelling were associated with exposure to both PAH fractions. Xia et al. (2004) hypothesized that this effect was occurring due to interaction of the PAH metabolites within the matrix itself, with the more polar quinone metabolites competing with ubiquinone and altering electron transfer in the inner membrane complexes. The results of this study have important implications for the mitochondrial exposure; specifically, mechanisms exist for the introduction of PAHs into the mitochondrial matrix, and sequestered environmental pollutants can directly interact with metabolic pathways.

41.2.2 Organohalogens

Environmental exposure to organohalogens, including organochlorine pesticides, polychlorinated biphenyls, brominated flame retardants, dioxins, and perfluorinated compounds, has been associated with negative health outcomes (Hatcher-Martin et al. 2012; Cohn et al. 2015; Walker et al. 2016a). Due to their recalcitrant properties, lipophilic nature, and tendency to bioaccumulate, organohalogens are widespread and persistent throughout the environment and have been detected at measureable levels in most human populations (Patel et al. 2016). Acute toxicity includes disruption to a range of metabolic pathways, many of which include secondary effects that alter mitochondrial function. Thus, there is the potential for mitochondrial disruption from low-dose chronic exposure to organohalogens. Primary toxicity is exerted through the generation of oxidative stress by increased formation of reactive oxygen species (ROS), which is suspected in the pathogenesis of many age-related diseases (Brieger et al. 2012; Hwang 2013).

To determine whether the pesticide methoxychlor caused mitochondrial dysfunction and generated oxidative stress, Gupta et al. (2006) used mitochondria isolated from mouse ovaries to evaluate how exposure altered function and overall cellular homeostasis. Methoxychlor caused oxidative damage both *in vitro* and *in vivo* due to altered mitochondrial respiration and H_2O_2 production. Decreased expression levels of Sod1, GPX, and CAT were also observed, which can contribute to accumulation of ROS, but these are cytoplasmic rather than mitochondrial enzymes. In support of the

mitochondrial effects, Schuh et al. (2005) found that methoxychlor acts as a complex I inhibitor in studies of brain mitochondria.

In vivo exposure to 3-methylsulfonyl-dichlorodiphenyldichloroethylene (msDDE) has also been associated with altered adrenal cortical mitochondria. Jonsson et al. (1991) evaluated mitochondrial structure using electron microscopy and irreversible protein binding following perinatal exposure to msDDE. Dose-dependent effects included reduction in the number of mitochondria, increased vacuolization, and reduction of cristae. Irreversible binding of msDDE was 50-fold higher within the mitochondrial fraction, which was attributed metabolic activation by 11 β -steroid hydroxylase.

The polybrominated diphenyl ether (PBDE) class of flame retardants results in mitochondrial dysfunction through a number of mechanisms, including changing mitochondrial depolarization, altered morphology, calcium homeostasis, triggering apoptosis, and ROS generation (Thorat et al. 1990; Voorspoels et al. 2007; Dingemans et al. 2008). For example, the toxicity of 2,2',4,5'-tetrabromodiphenyl ether (PBDE-49), a common component of commercial brominated flame retardant mixtures, causes mitochondrial dysfunction in neuronal tissue (Napoli et al. 2013). PBDE-49 disrupted mitochondrial morphology and caused loss of membrane potential and increased oxidative stress; uncoupling of electron transport occurred at lower exposure levels than inhibition of complexes IV and V. These results are consistent with the toxicity data reported for PBDE mixtures (Yu et al. 2008) and other brominated flame retardants (Birnbaum and Staskal 2004).

Perfluoroalkyl acids (PFAAs) have also been identified as mitochondrial toxicants. This class of chemicals includes perfluorooctanoic acid (PFOA) and perfluorosulfonic acid (PFOS), which have gained attention due to widespread persistence in groundwater and developmental risks associated with exposure (Giesy and Kannan 2002; Holzer et al. 2008; Napoli et al. 2013). Both *in vitro* and *in vivo* studies show that PFAAs and associated metabolites induce mitochondrial dysfunction. Adverse mitochondrial effects include induction of the mitochondrial permeability transition (MPT), uncoupling of oxidative phosphorylation (Starkov and Wallace 2002), stimulation of hepatic peroxisome proliferation (Berthiaume and Wallace 2002), and stimulation of mtRNA transcription (Walters et al. 2009). PFAAs share structural similarities with endogenous fatty acids and exhibit similar biological activities (Walters et al. 2009).

41.2.3 Contemporary Pesticides

Newer pesticides, including organophosphates, carbamates, triazines, and pyrethroids tend to be less persistent in the

environment (Barr and Needham 2002) than many earlier pesticides, for example, dichlorodiphenyltrichloroethane (DDT). Nevertheless, levels of these compounds have measured in human blood and urine (Patel et al. 2016), with pharmacokinetic modeling and exposure estimates indicating exposure occurs often enough to approach steady-state blood levels (Wetmore et al. 2012; Wambaugh et al. 2013). Examples of these chemicals exhibiting mitochondrial toxicity include rotenone, an important insecticide allowed for production of "organic" foods. Rotenone is a complex I inhibitor that results in ROS production (Li et al. 2003a). Other insecticides, permethrin and cyhalothrin, also inhibit complex I (Gassner et al. 1997). The herbicide, paraquat, acts as a mitochondrial redox cyler (Martinez and Greenamyre 2012), while atrazine inhibits F₁F₀-ATP synthase in sperm (Hase et al. 2008).

To increase the efficacy of the newer pesticides, synergists are also included in commercial formulations, including piperonyl butoxide (PBO) and *N*-octyl bicycloheptene dicarboximide (MGK 264). These compounds act as inhibitors of detoxification pathways, enabling longer biological half-lives of the active ingredients in pesticide formulations (Bernard and Philogene 1993). PBO, which is a cytochrome P450 inhibitor, has important implications for mitochondrial pathways utilizing this family of enzymes, including steroid biosynthesis and chemical detoxification.

41.2.4 High-Throughput Screening for Mitochondrial Toxicants

High-throughput screening (HTS) assays for testing mitochondrial toxicity enable screening and prioritization of a large number of environmental pollutants. Programs focusing on HTS assays, which include the Tox21 and ToxCast programs, use mammalian cell-derived enzymatic, receptor signaling, and protein-based assays representing a range of responses and pathways (Dix et al. 2007). Within the ToxCast program, screening assays are primarily pathway based, with biochemical activity represented by assay response (Sipes et al. 2013). These assays include mitochondrial specific pathways, such as CYP450 and fatty acid signaling pathways; however, none specifically test for exposure-related mitochondrial dysfunction or for the potential interaction of important therapeutic drugs with common environmental agents.

Unlike ToxCast, Tox21 uses a HepG2 hepatocyte cell line to rapidly screen for *in vitro* perturbations to mitochondria. In the study by Attene-Ramos et al. (2013), the use of a cell-based assay allowed the evaluation of 1408 chemical compounds at 14 dosing levels to test for mitochondrial toxicity by exposure-induced changes to

membrane potential. Following initial screening, 91 and 88 compounds at two different time points were classified as active and found to decrease mitochondrial membrane potential at 1 and 5 h, respectively. These results were confirmed by testing 38 active compounds using different metrics of mitochondrial function, including imaging-based measurement of membrane potential, lactate dehydrogenase (LDH) release, and changes in oxygen consumption rate (OCR). Additional testing identified 37 of the 38 compounds exhibiting activity after 5 h (Table 41.1). LDH results indicated that out of the 38 compounds, four had minimal cytotoxicity at 1 h and six compounds in total (including the initial four) were cytotoxic at 5 h. In addition, 31 compounds resulted in OCR changes at the IC_{50} , which suggests alteration in oxidative phosphorylation and subsequent inhibition of ATP synthesis.

Although many advances have improved the ability of HTS to assess toxic effects (Tice et al. 2013), the method does not provide direct evidence of short- and long-term toxicity to human populations. Furthermore, translating *in vitro* findings to quantitative risk assessment for human populations has proven challenging. Phenotyping of the mitochondrial exposome can be used for hazard identification by surveying populations for the occurrence of exposures, and prevalence can be used to prioritize toxicity. Thus, HTS provides important insights into relevant chemical species in the mitochondrial exposome, but does not directly address risks in human populations.

While the previously discussed studies are based on *in vitro* and acute dosage animal studies, they do provide insight into the potential for interaction between human mitochondria, environmental toxicants, and commonly used therapeutic agents. Thus, there is a critical need to identify exogenous chemicals that bioaccumulate within human mitochondria. Quantitative information on the levels of these chemicals will allow better assessment of whether realistic exposures result in appreciable accumulation of these agents and improve understanding of the mitochondrial exposome.

41.3 Bioaccumulation of Environmental Pollutants

The ability of environmental pollutants to cause alterations in mitochondrial function suggests interaction within mitochondria; however, it does not account for accumulation of chemicals arising from low-dose, chronic exposures. Many environmental pollutants are lipophilic and tend to bioaccumulate in human tissues (Malarvannan et al. 2013). Hydrophobic regions of cellular components, such as the phospholipid-based

inner membrane provide ideal microenvironments for sequestration of lipophilic compounds. Accumulated chemicals have the potential to exert toxicological activity through multiple mechanisms, including the parent compound, activated metabolites present in the mitochondria, and/or endoplasmic reticulum (Meyer et al. 2013). Studies evaluating chemical compartmentalization within mitochondria have been based on *in vitro* and animal models, with results indicating environmental chemicals can accumulate within mitochondria.

As discussed previously, PBDEs have been associated with increased ROS production and apoptotic cell death. In a study by Huang et al. (2010), the toxicity and accumulation of PBDE congeners were evaluated using an *in vitro* cerebellar granule neuron model cultured from 7-day-old mice. Figure 41.2 shows the distribution of PBDE congeners within different cellular components, including cytosolic, mitochondrial, nuclear, and microsomal fractions. Accumulation of the individual congeners within the mitochondria was exceeded only by the microsomal fraction, with congener-specific accumulation observed. Comparison of the lower ($1\ \mu\text{M}$) and higher ($10\ \mu\text{M}$) dose showed similar percent accumulation at both doses, which has important implications for exposure at environmentally relevant concentrations. Due to the higher pH in the mitochondrial matrix, it is well recognized that cationic, lyophilic compounds partition into mitochondria. However, the potential exists for accumulation of hydrophobic compounds within the mitochondrial matrix, even with the cytoplasm as the preferred environment for neutral compounds. For example, partitioning of hydrophobic chemicals to lipid membranes or cotransport with proteins is possible, and the direct contact of mitochondria with a cytoplasmic region enriched with environmental chemicals can facilitate transfer through diffusion and cotransport. Therapeutic studies have also identified compounds that selectively bind to mitochondria. These include sulfonylurea-related chemicals and anthracyclines (Hoye et al. 2008).

Although the potential exists for chemical accumulation within mitochondria and represents a central component of the mitochondrial exposome, limited to no information is available on the chemical profile in human mitochondria. While quantification of specific analytes is possible using traditional analytic approaches, methodologies providing greater chemical coverage are needed due to the wide variation in exposures across human populations. The use of HRM, which provides measure of 10,000–20,000 unique chemical species without *a priori* selection of analytes, has the potential to greatly improve the chemical characterization of human mitochondria. In the next section, we address application of HRM to the study of the mitochondrial metabolome and exposome.

Table 41.1 Thirty-eight compounds selected for confirmation analysis and further mechanistic studies, grouped based on oxygen consumption rate following IC₅₀ exposure.

Compound name	Classification
1,8-Dihydroxy-4,5-dinitroanthraquinone	Decreased OCR-irreversible uncoupling
2,2'-Methylenebis-(4-chlorophenol)	Decreased OCR-irreversible uncoupling
2,2'-Thiobis(4-chlorophenol)	Decreased OCR-irreversible uncoupling
3,4,5-Trichlorophenol	Decreased OCR-irreversible uncoupling
3,4-Dichlorophenyl isocyanate	Decreased OCR-irreversible uncoupling
4,4-Thiobis(6- <i>t</i> -butyl- <i>m</i> -cresol)	Decreased OCR-irreversible uncoupling
4-Cumylphenol	Decreased OCR-irreversible uncoupling
Captan	Decreased OCR-irreversible uncoupling
Diphenylurea	Decreased OCR-irreversible uncoupling
Emodin	Decreased OCR-irreversible uncoupling
Hexachlorophene	Decreased OCR-irreversible uncoupling
Kaempferol	Decreased OCR-irreversible uncoupling
Kepone	Decreased OCR-irreversible uncoupling
Nitazoxanide	Decreased OCR-irreversible uncoupling
<i>n</i> -Phenyl-2-naphthylamine	Decreased OCR-irreversible uncoupling
<i>o</i> -Benzyl- <i>p</i> -chlorophenol	Decreased OCR-irreversible uncoupling
<i>p</i> - <i>n</i> -Nonylphenol	Decreased OCR-irreversible uncoupling
<i>p</i> -Aminophenol	Decreased OCR-irreversible uncoupling
Phenmedipham	Decreased OCR-irreversible uncoupling
Tetra- <i>N</i> -octylammonium bromide	Decreased OCR-irreversible uncoupling
Hexamethyl- <i>p</i> -rosaniline chloride	Increased OCR-decreased FCCP OCR
Malachite green oxalate	Increased OCR-decreased FCCP OCR
4-Hydroxyphenyl retinamide	Suppressed FCCP OCR
Curcumin	Suppressed FCCP OCR
Genistein (4',5,7-trihydroxyisoflavone)	Suppressed FCCP OCR
Silibinin	Suppressed FCCP OCR
Basic red 9 (<i>p</i> -rosaniline HCl)	Decreased OCR and FCCP uncoupled OCR
Diethylstilbestrol (DES)	Decreased OCR and FCCP uncoupled OCR
Digitonin	Decreased OCR and FCCP uncoupled OCR
Phenyl mercuric acetate	Decreased OCR and FCCP uncoupled OCR
Zearalenone	Decreased OCR and FCCP uncoupled OCR
1,5-Naphthalenediamine	Decreased MMP, no change in OCR/FCCP OCR
2-Aminoanthraquinone	Decreased MMP, no change in OCR/FCCP OCR
Apigenin	Decreased MMP, no change in OCR/FCCP OCR
Formulated fenaminosulf (dexon)	Decreased MMP, no change in OCR/FCCP OCR
<i>o,p'</i> -DDD (mitotane)	Decreased MMP, no change in OCR/FCCP OCR
Resveratrol	Decreased MMP, no change in OCR/FCCP OCR
Riboflavin	Decreased MMP, no change in OCR/FCCP OCR

Adapted from Attene-Ramos et al. (2013). Reproduced with permission of American Chemical Society. Carbonilcyanide *p*-triflouromethoxyphenylhydrazon (FCCP) was added at the completion of the 1 h monitoring period to evaluate maximal mitochondria uptake and non-basal oxygen consumption rate (OCR).

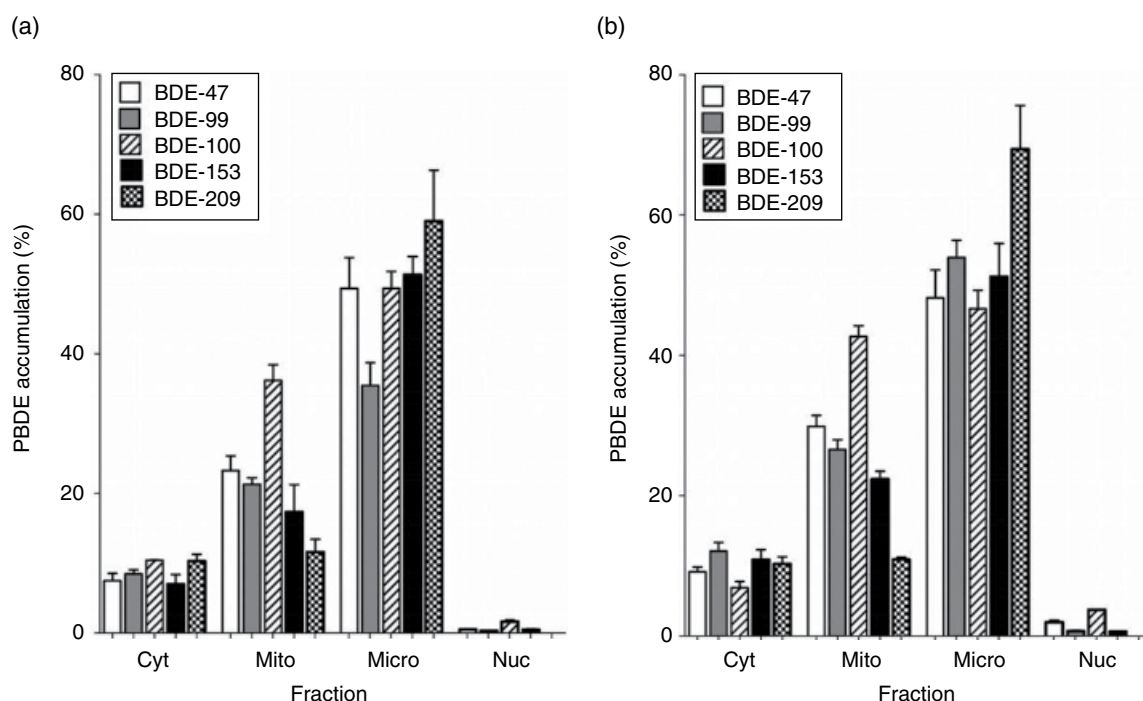


Figure 41.2 Percent PBDE accumulation within the cytosol (Cyt), mitochondria (Mito), microsomes (Micro), and nuclei (Nuc) fraction of cerebellar granule cells following exposure to (a) 1 μ M or (b) 10 μ M PBDE solutions for 24 h. Adapted from Huang et al. (2010). Reproduced with permission of Oxford University Press.

41.4 Mitochondria High-Resolution Metabolomics

Limited data exist on the metabolic profile of intact mitochondria, with primary focus on the measure of endogenous metabolites. In-depth characterization of chemical compartmentalization within mitochondria is required as a critical component of the mitochondrial exposome. HRM, developed as an advanced analytical framework for precision medicine and exposome research (Walker et al. 2016a), provides the capabilities necessary for phenotyping the mitochondrial exposome. By supporting quantification of large numbers of chemicals detected in human samples, HRM yields systematic biological and environmental chemical measurements required for profiling the exposome (Jones 2016).

Recent applications of HRM in human populations have shown that current instrumentation provides the sensitivity and selectivity to measure pollutants in human samples at environmentally relevant concentrations (Soltow et al. 2013; Jamin et al. 2014; Roca et al. 2014; Go et al. 2015; Rager et al. 2016). The ability to measure environmental toxicant burden in a systematic and quantitative way greatly enhances capabilities for internal exposure assessment, which traditionally relied on

external exposure monitoring, lifestyle factors, modeling, observational data, and targeted biomonitoring data. For example, in the study by Go et al. (2015), plasma from a healthy population of 153 individuals was analyzed using HRM profiling. Only 40% of the quantified environmental chemicals have previously reported reference levels, and the others were previously undetected in human populations. Similar results were obtained by Roca et al. (2014) and Jamin et al. (2014), where untargeted chemical measurements detected both common exposure (i.e., organophosphates and carbamate fungicides) and previously uncharacterized environmental chemical metabolites.

41.4.1 High-Resolution Metabolomics

No single platform will provide comprehensive coverage of all chemical species present within the mitochondria due to the diverse physiochemical properties of metabolites (Rappaport et al. 2014); however, current instrumentation and workflows provide sufficient chemical coverage for detailed screening. Nuclear magnetic resonance (NMR) spectroscopy and MS have been used for metabolic profiling of biological samples obtained from a thousand or more individuals to identify metabolic changes associated with aging, diet, and environment

(Holmes et al. 2008a, b; Menni et al. 2013; Dunn et al. 2015), in addition to being identified as one of the key technologies in exposome research (Wild 2012; Wild et al. 2013; Athersuch and Keun 2015; Go et al. 2015). Due to variations in methodology, chemical coverage by metabolomic platforms varies widely. Increased use of MS for metabolomics has largely been driven by advances in data analytic tools (Smith et al. 2006; Uppal et al. 2013; Yu et al. 2013; Libiseller et al. 2015), dedicated instrumentation (Park et al. 2012), and high-resolution, high scanning speed MS (Makarov et al. 2006; Kanu et al. 2008; Cribbs et al. 2014; Frediani et al. 2014), making measure of 15,000–20,000 unique features possible (Go et al. 2015a, b; Uppal et al. 2015). Current feasibility studies show increased coverage will be available with further development of data extraction tools and complementary ion characterization (Uppal et al. 2016).

HRM provides a number of advantages over traditional analytical approaches. High-resolution mass detection offers improved coverage of low abundance environmental, dietary, microbiome, and drug-related metabolites. Recent studies have shown that triplicate analysis enhances reliability of detection and improves quantification, especially for low abundance chemicals (Uppal et al. 2013). In addition, a simple extraction procedure (addition of solvent with internal standards followed by removal of protein precipitate) reduces error due to variation in efficiency of recovery or variability in detection of internal standard. Use of profile mode instead of centroid mode preserves ability to discriminate chemicals with very similar mass-to-charge ratio (m/z), and use of a data re-extraction routine with options to optimize parameter settings improves detection and quantitative accuracy (Uppal et al. 2013; Libiseller et al. 2015).

It is now possible to implement HRM for chemical surveillance in human populations, with studies showing external exposure can be linked to internal dose, biological response, and pathophysiological changes (Walker et al. 2016c; de Sousa Rodrigues et al. 2017). To characterize the overall influence of environment and phenotypic character on the mitochondrial exposome, evidence-based associations must be established. Untargeted profiling allows maximum chemical coverage and reduces bias by not limiting analytical targets to known pollutants, with detected chemical signals that are characterized after categorizing their importance. While initial validation requires confirmation with coelution and ion dissociation studies, development of cumulative detected metabolic feature databases and certified, well-characterized reference material (i.e., NIST SRM 1950 (Phinney et al. 2013)) provides direct identification and quantification based on instrumentally acquired information.

41.4.2 Metabolic Phenotyping of Intact Mitochondria

To date, characterization of the mitochondrial metabolome is limited. In a study by Roede et al. (2012), mitochondria isolated from wild type (WT) and thioredoxin-2 transgenic (TRX-TG) mice were profiled using HRM, which detected 2127 mitochondria-specific chemical features. Subsequent matching to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al. 2012) identified metabolites/features from a number of relevant pathways, supporting this approach for characterizing mitochondrial specific chemicals (Figure 41.3). Of the detected features, a large proportion was unidentified, suggesting uncharacterized environmental chemicals or metabolic intermediates. The results from this study provide an important framework for the use of HRM to characterize mitochondria isolates. First, these data demonstrate that exposure to environmental agents is universal. The mice selected for this study were housed in well-controlled facilities; however, exposure to environmental toxicants still occurred and was detected within mouse organ mitochondria. Second, these data illustrate the need to account for background exposures even when using laboratory-bred and housed animals. Based on the 2127 detected chemical features, Roede et al. (2012) utilized a metabolome-wide association study (MWAS) framework to differentiate metabolic differences related to gender and genetic phenotype. Results obtained from these two studies support the use of HRM for chemical

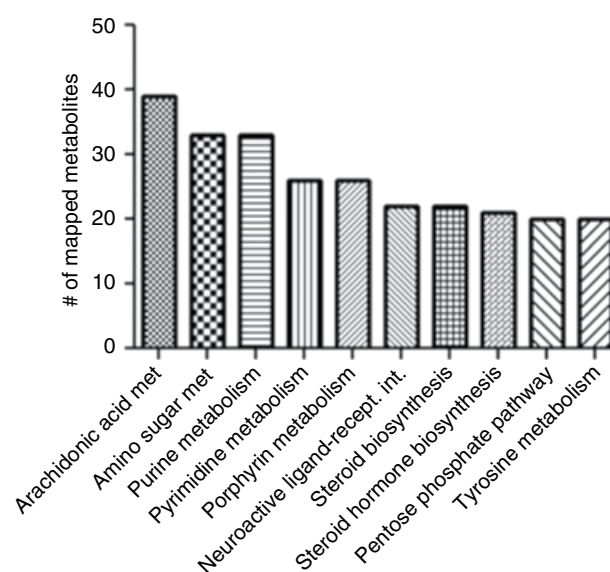


Figure 41.3 High-resolution metabolomic profiling of isolated mouse liver mitochondria detected metabolites from a range of metabolic pathways, including those specific to mitochondrial processes. Reproduced with permission of Roede et al. (2012).

characterization of mitochondria and use of HRM in a mitochondrial exposome framework.

Further characterization of the mitochondria metabolome described by Go et al. (2014b) provides additional evidence of HRM suitability to measure the mitochondrial exposome and as a tool for monitoring mitochondrial function *in vivo*. Annotation for the presence of

chemicals arising from environmental sources identified 140 mass-to-charge (m/z) features matching relevant chemicals, which included fungicides, herbicides, insecticides, pesticide synergist, plant growth regulators, plasticizers, food preservatives, flame retardant, and antiscald agent (Table 41.2). While not tested, Go et al. (2014b) speculate that the sources of these chemicals

Table 41.2 Environmental chemicals detected in mitochondria isolated from mouse liver.

Environmental chemical	Chemical formula	Classification	Detected m/z [M+H] ⁺	Delta	Mass error (ppm)
Propamocarb hydrochloride	C ₉ H ₂₁ ClN ₂ O ₂	Fungicide	225.1370	-0.0005	-2.174
Cyproconazole	C ₁₅ H ₁₈ ClN ₃ O	Fungicide	292.1225	-0.0014	-4.752
Triadimefon	C ₁₄ H ₁₆ ClN ₃ O ₂	Fungicide	294.0998	0.0006	1.947
Tridemorph	C ₁₉ H ₃₉ NO	Fungicide	298.3084	0.0020	6.758
Benzimidazole	C ₁₆ H ₁₄ F ₃ N ₃ O ₂ S	Fungicide	119.0606	-0.0002	-1.595
Piperonyl butoxide	C ₁₉ H ₃₀ O ₅	Pesticide synergist	339.2183	-0.0017	-5.121
Clopyralid	C ₆ H ₃ Cl ₂ NO ₂	Herbicide	191.9608	0.0006	3.049
Acetochlor	C ₁₄ H ₂₀ ClNO ₂	Herbicide	270.1258	-0.0002	-0.841
Butachlor	C ₁₇ H ₂₆ ClNO ₂	Herbicide	312.1725	0.0000	0.066
Propazine	C ₉ H ₁₆ ClN ₅	Herbicide	230.1146	0.0021	9.078
Atrazine GSH ^a adduct I ^b	C ₁₈ H ₃₀ N ₈ O ₆ S	Herbicide	487.2083	-0.0001	-0.291
Atrazine GSH adduct II ^b	C ₁₈ H ₂₉ N ₈ O ₆ SCl	Herbicide	521.1655	0.0037	7.041
Atrazine GSH adduct III ^b	C ₁₈ H ₂₉ N ₈ O ₇ SCl	Herbicide	537.1657	-0.0016	-2.930
Cyanazine GSH adduct ^b	C ₁₉ H ₂₉ N ₉ O ₆ S	Herbicide	512.2088	-0.0054	-10.477
Simazine	C ₇ H ₁₂ ClN ₅	Herbicide	202.0836	0.0018	8.835
Cyanazine	C ₉ H ₁₃ ClN ₆	Herbicide	241.0931	0.0032	13.066
Diethyltoluamide (DEET)	C ₁₂ H ₁₇ NO	Insect repellent	192.1371	0.0012	6.375
Acetamiprid	C ₁₀ H ₁₁ ClN ₄	Insecticide	223.0737	0.0008	3.464
Bifenazate	C ₁₇ H ₂₀ N ₂ O ₃	Insecticide	301.1533	0.0014	4.697
Cypermethrin	C ₂₂ H ₁₉ Cl ₂ NO ₃	Insecticide	416.0792	0.0023	5.535
Indoxacarb	C ₂₂ H ₁₇ ClF ₃ N ₃ O ₇	Insecticide	528.0787	-0.0007	-1.380
Pirimicarb	C ₁₁ H ₁₈ N ₄ O ₂	Insecticide	239.1527	-0.0024	-9.946
Ethephon	C ₂ H ₆ ClO ₃ P	Plant growth regulator	144.9811	0.0004	3.046
Forchlorfenuron	C ₁₂ H ₁₀ ClN ₃ O	Plant growth regulator	248.0574	0.0011	4.469
Daminozide	C ₆ H ₁₂ N ₂ O ₃	Plant growth regulator	161.0909	0.0012	7.487
Dimethyl phthalate	C ₁₀ H ₁₀ O ₄	Plasticizer	195.0639	0.0013	6.690
Monobutyl phthalate	C ₁₂ H ₁₄ O ₄	Plasticizer	223.0950	0.0015	6.706
Benzylbutyl phthalate	C ₁₉ H ₂₀ O ₄	Plasticizer	313.1411	0.0023	7.216
Di- <i>n</i> -heptyl phthalate	C ₂₂ H ₃₄ O ₄	Plasticizer	363.2507	0.0023	6.467
Diisodecyl phthalate	C ₂₈ H ₄₆ O ₄	Plasticizer	447.3435	0.0034	7.641
Phenoxyethanol	C ₈ H ₁₀ O ₂	Preservative	139.0744	0.0010	6.930
3-Iodo-2-propynylbutylcarbamate	C ₈ H ₁₂ INO ₂	Preservative	282.0013	-0.0027	-9.702
Triphenyl phosphate	C ₁₈ H ₁₅ O ₄ P	Flame retardant	327.0757	0.0024	7.249

Go et al. (2014b). Reproduced with permission of Oxford University Press.

Delta = theoretical m/z - detected m/z ; mass error = delta/(theoretical m/z) × 10⁶.

^a Glutathione.

^b LeBlanc and Sleno (2011).

included water, food, and bedding, which consist of cultivated vegetative products. In addition to detecting the parent compound, detoxification products consistent with mitochondrial defense mechanisms were detected. For example, the active compound for three different triazine herbicides (propazine, simazine, and cyanazine) was detected. Detoxification products of these compounds and other triazines were also detected, including three different atrazine–glutathione adducts and a cyanazine–glutathione adduct. Formation of glutathione adducts is a key mechanism in protecting mitochondria from oxidative stress, and detection of these metabolites using HRM approaches demonstrates that these techniques provide sufficient sensitivity and selectivity for broad chemical characterization. In addition to environmental chemical profiling of mouse liver mitochondria, Go et al. (2014b) investigated the feasibility of using circulating mitochondrial specific m/z features in plasma to evaluate mitochondrial dysfunction *in vivo*. Comparison of the mitochondrial metabolome to the plasma metabolome identified 30 metabolites specific to mitochondrial function that were also detected in human plasma from a cohort of 99 subjects. Overall, 1425 m/z features were detected in both the mitochondrial and human plasma metabolome, providing potential biomarkers for *in vivo* measure of mitochondrial function in humans.

A limited number of studies have used HRMS approaches to characterize specific aspects of mitochondrial biology or study toxic effects of exposure in animal models. Bird et al. (2011) isolated and extracted rat liver mitochondria for the hybrid targeted/untargeted analysis of cardiolipins and monolysocardiolipids. These lipids are essential for maintaining mitochondrial structure and are central to proper functioning of complexes I, III, IV, and V (Paradies et al. 2014). The mitochondrial lipids were characterized using different MS procedures, including reverse phase chromatography with data-dependent collision-induced dissociation. This methodology was then applied to evaluate the effect of diet on mitochondrial biochemistry. These data showed a link between the major fat component of diet and a specific monolysocardiolipid, suggesting that diet directly affects the mitochondrial metabolic footprint.

To investigate the toxicity of cadmium to liver mitochondria, Go et al. (2014a) used an integrated redox proteome and mitochondrial metabolome approach to measure antioxidant defense and associated metabolic changes. Comparison of cadmium-exposed mice to controls revealed almost half of the isotope-coded affinity tag (ICAT) identified cysteine-containing peptides were oxidized more than 1.5-fold following exposure. A statistically significant decrease in glutathione and glutathione disulfide and an increase in the cysteine redox potential were also observed, showing an increase in oxidative stress with increasing cadmium exposure.

HRM profiling identified changes in key mitochondrial pathways involved with fatty acid metabolism, including several carnitines and palmitoyl-coenzyme A. Thus, this study directly related acute exposure to cadmium to biological response evidenced by alterations within the mitochondrial metabolome. These results support the robustness and suitability of mitochondrial extraction and analysis to evaluate effects of environmental exposure on mitochondrial function.

The accumulating experience with HRM shows this to be an effective analytical platform for profiling the mitochondrial metabolome under experimental conditions in rodent models. Results show sample preparation and profiling methods enable detection of a large range of endogenous and exogenous chemicals. To date, there are no data available on the application of HRM to human mitochondria to determine the presence and distribution of environmental chemicals. In principle, the same approaches used for model studies could be used to study the relationship of endogenous chemical burden to the mitochondrial metabolic phenotype. Thus, a critical next step in developing the mitochondrial exposome will be to apply the approaches described previously to characterize isolates obtained from human tissues. An example of this approach is provided hereafter.

41.5 Case Study: Profiling the Human Mitochondrial Exposome

Due in part to the relative difficulty of obtaining tissue samples, the chemical burden of human tissues and subcellular fractions is largely unknown. In most epidemiology studies, body burden of environmental chemicals is estimated by measuring levels in easily obtainable biological fluids, such as urine or blood; however, this does not adequately reflect accumulation of lipophilic compounds in diverse body compartments. Development of HRM, which provides untargeted screening capabilities for a large number of low molecular weight chemicals in biological samples, has greatly improved the ability to characterize the exposome. Applying HRM to study isolated human mitochondria has the potential to provide new insight into the chemical burden arising from exposure to environmental chemicals and to identify potential toxicological targets. In this case study, we use HRM to profile and identify environmental chemicals in mitochondria isolated from human adrenals. These results represent the first characterization of the human mitochondrial exposome.

41.5.1 Adrenal Glands

The human body contains two adrenal glands, one located on the top of each kidney. Each gland is 4–6 cm long, 1 cm thick, and 4–6 g in weight (Lacroix and Clavier 2001).

Within each adrenal gland exist two separate compartments providing different biological functions. The inner compartment is the adrenal medulla and comprises 10–20% of the gland. The outer adrenal cortex fully surrounds the adrenal medulla and comprises the remaining 80–90% of the gland.

The adrenal cortex is the location for synthesis of hormones derived from cholesterol, which includes aldosterone, cortisol, corticosterone, dehydroepiandrosterone, and androstenedione (Parker and Rainey 2004). While cortisol and corticosterone are important in metabolism and stress response, dehydroepiandrosterone and androstenedione are common precursors for both male and female sex hormones, including testosterone, estradiol, and estrogen. Similar to the testes, steroid biosynthesis is rate limited by the transfer of cholesterol from the outer mitochondrial membrane to the inner membrane (Griffin and Ojeda 2004), which occurs via the steroidogenic acute regulatory (StAR) protein. Once within the inner membrane, steroidogenesis occurs by cholesterol side-chain cleavage (Lacroix and Clavier 2001).

The inner adrenal medulla is primarily responsible for secreting catecholamines, which provide critical functions related to the sympathetic nervous system (Griffin and Ojeda 2004). Hormones produced by the adrenal medulla include epinephrine and norepinephrine, which are synthesized from the hydroxylation of tyrosine through a pathway that includes levodopa and dopamine as precursors. Catecholamine synthesis pathways are almost identical to those within sympathetic nerves, with the medulla a modified sympathetic ganglion with postganglionic cells but no axons (Parker and Rainey 2004). The adrenal glands receive the highest rate of blood flow in the body on a per gram basis (Parker and Rainey 2004). Arterial blood enters through the outer cortex, flows through fenestrated capillaries between the cords of adrenocortical cells, and drains inwardly into venules in the medulla. This suggests that the adrenal gland could be highly exposed to internal levels of environmental chemicals based on a high blood flow/mass ratio. Furthermore, the adrenal gland plays a very important functional role in ensuring endocrine system health and has been reported to be the most highly targeted organ within the endocrine system by toxins (Rosol et al. 2001; Harvey et al. 2007). Thus, detection and identification of environmental chemicals within adrenal tissue mitochondria would improve estimates of exposure and allow more thorough evaluation of alterations to hormone production. Biomarkers of oxidative stress correlating with the presence of environmental chemicals in adrenal tissue will also provide means to improve exposure assessments. Adrenal tissues, especially within the

adrenal cortex, are high in unsaturated fatty acids, which is susceptible to lipid peroxidation (Gutteridge 1995).

While there are a number of diseases arising from adrenal dysfunction, chronic exposure to ambient environmental chemicals could have a more subtle influence on adrenal gland function (Takayanagi et al. 2000). Currently, limited data are available on how environmental exposures affect adrenal glands, even though a number of the enzymatic steps required for adrenal hormone synthesis are inhibited or induced by environmentally relevant chemicals. These include polychlorinated biphenyls, organochlorine pesticides, and plasticizers (Hornsby 1989; Xu et al. 2006; Harvey et al. 2007). In addition, exposure-induced alterations to steroidogenesis show that cytochrome enzyme overexpression results in increased production of androstenedione (Xu et al. 2006). Long-term disruption of adrenal mitochondria could impact overall adrenal gland health, and metabolic profiling of the tissue and mitochondria could provide insight into which pathways are most affected. Accordingly, we selected adrenal gland tissue for initial characterization of the mitochondrial exposome: application of the methods described hereafter can be easily extended to different tissues.

41.5.2 Methods

41.5.2.1 Adrenal Gland Selection Criteria and Procurement

Due to the challenges inherent in obtaining fresh organs from recently deceased patients and the lack of relevant information on environmental chemicals in human adrenals, we placed few limitations on donor characteristics. The adrenals were procured from the International Institute for the Advancement of Medicine (IIAM), an organization providing human organs and tissues for medical research, education, and development. Exclusion criteria included high body mass index (>45); evidence of current kidney bacterial infection; extended downtime (>30 min); serology positive for Anti-H I–II, HBsAg, HBcAb, Anti-HCV, Syphilis, Anti-CMV, and EBV; and the presence of health defects that would result in significant alteration in daily life. For our studies, single adrenal glands were obtained from deceased patients, with time of death no more than 36 h prior to tissue collection. The adrenal glands were removed by trained recovery teams and shipped using standard transport methods for transplantable organs, which included storage in preservation solution on ice.

41.5.2.2 Adrenal Gland Tissue Preparation

In this initial study, no attempt was made to separate the medulla from the cortex so the preparation is

expected to large represent the adrenal cortex mitochondria (80–90% of the adrenal is cortex). All samples were kept on ice within the original packaging until processing, and the initial dissection and homogenization were completed on ice in a 4°C room. The adrenal was rinsed three times with ice-cold calcium and magnesium-free HBSS, and the total weight of the gland was recorded. The adrenal was then sliced along the pressure ridge and sectioned into individual pieces of approximately 1 g. Each piece was washed with mitochondrial isolation buffer containing 2 mg/mL bovine serum albumin (BSA) and placed in a homogenization vessel containing 5 mL of isolation buffer. The tissue was homogenized using 15–20 strokes of a dounce homogenizer, and two isolates were prepared by pooling two homogenized tissue samples.

41.5.2.3 Mitochondria Isolation and Sample Preparation

Mitochondria were prepared using differential centrifugation (Savage et al. 1991; Roede et al. 2012), which provides an extract with approximately 90% purity and includes limited contamination from lysosomes and peroxisomes (Graham 2001). Briefly, the pooled homogenate was first centrifuged in an ice-cold isolation buffer containing BSA at 4°C for 5 min at 600×g to remove nucleic material and residual cell debris. The resulting supernatant was pipetted to a separate, chilled 15 mL centrifuge tube, and 3 mL of isolation buffer containing BSA were added to resuspend the pellet for rehomogenization. The suspension was then centrifuged at 4°C for 11 min at 11,000×g, the supernatant was discarded, and the mitochondrial pellet was resuspended in 2 mL of isolation buffer containing BSA.

The mitochondrial preparation was then used to create two equivalent fractions, one for HRM analysis and the other for characterization of mitochondrial integrity. The supernatant was transferred to two chilled microcentrifuge tubes, and centrifuged at 8000×g for 10 min. Pellet 1, which was collected for HRM analysis, was flash frozen in liquid N₂ and stored at –80°. Pellet 2 was used to evaluate mitochondrial viability via a MPT assay.

Mitochondria were extracted for HRM profiling using established methods (Roede et al. 2012; Go et al. 2014b). Briefly, an aliquot of isolate providing 400 µg protein was first diluted to 100 µL in deionized water. The suspension was then treated with two volumes acetonitrile containing ¹³C- and ¹⁵N-labeled internal standards, mixed on a vortex mixer, and allowed to stand on ice for 30 min. The extract was then centrifuged at 16,100×g at 4°C for 10 min. A 200 µL aliquot of the supernatant was transferred to a low volume autosampler vial, maintained in a refrigerated autosampler, and analyzed within 24 h.

41.5.2.4 High-Resolution Metabolomics

HRM of the mitochondria isolates was performed using liquid chromatography with HRMS (Park et al. 2012; Soltow et al. 2013). Analyte separation was accomplished by dual C₁₈ chromatography with acetonitrile gradient–formic acid gradient (total run time of 10 min) using an Ultimate 3000 high-performance LC system equipped with refrigerated autosampler, dual channel pumps, and heated column compartment. A Thermo Scientific Q-Exactive mass spectrometer was used for mass detection and operated with positive and negative electrospray ionization. Twelve replicates were analyzed for each extract, with 6 corresponding to each polarity mode. Feature detection and peak alignment were completed using apLCMS (Yu et al. 2009) and xMSanalyzer (Uppal et al. 2013), which provided the final metabolic feature table consisting of accurate mass *m/z* features based upon *m/z*, retention time and ion intensity.

41.5.2.5 Data Processing and Feature Annotation

A threshold filtering criteria of 1.5 (ratio of average abundance of feature in mitochondria isolate to average feature abundance in resuspension blank) was applied to identify features enriched in mitochondria. The *m/z* features were then characterized using ion intensities, retention time, and coefficient of variation (CV). Metabolite annotation was completed using the KEGG database and match accuracy of 10 parts per million (ppm). Environmental chemical classes were evaluated based on use and KEGG BRITE classification.

41.5.3 Results

41.5.3.1 Tissue Procurement

During the collection period, five adrenal glands were collected from five deceased donors. A summary of individual characteristics is given in Table 41.3. The individuals included a range of ages (27–71), geographic locations, and pathologies. All tissues were received within the designated delivery time following removal and were successfully dissected and processed for mitochondria.

Table 41.3 Adrenal tissues available for mitochondrial isolation.

ID	Source	Sex	Age	Cause of death
AG001	R adrenal	M	29	CVA 2nd ICH possible drug overdose suspected
AG002	R adrenal	F	56	Anoxia 2nd cardiovascular
AG003	R adrenal	M	27	HT 2nd GSW
AG004	R adrenal	F	46	CVA 2nd ICH
AG005	R adrenal	M	47	Anoxia 2nd asphyxiation

41.5.3.2 Mitochondria Isolation

The isolation procedure provided sufficient yield for metabolic profiling, with total mitochondrial protein concentrations ranging from 4,700 to 29,000 $\mu\text{g/mL}$. Isolation of viable mitochondria was achieved for all preparations, which was verified by the time-dependent drop in absorbance measured at 540nm when 15 μL of 2mM CaCl_2 due to MPT and swelling of the intact mitochondria. The associated cytosolic fraction and reference blank exhibited negligible change under the same conditions.

41.5.3.3 High-Resolution Metabolomics Results

Using apLCMS and xMSanalyzer for data extraction, 8746 and 9953 unique features were detected in positive and negative modes, respectively. To remove artifacts introduced during sample preparation, m/z features were referenced against blank buffer and filtered based upon fold change, resulting in 5387 (positive) and 6805 (negative) m/z features enriched in mitochondria. The abundance for the different m/z features varied over a nine-order-of-magnitude range, suggesting detection of both high and low abundance chemical species (Figure 41.4a). Median replicate CV for the six replicates analyzed for each sample was also shown to be quantitatively reproducible, with a median CV for all features of 23% (equivalent to standard error of the mean of 8.5% for $n = 6$) (Figure 41.4b).

Because this study was to evaluate whether low-level environmental chemicals could be detected within human mitochondria, the m/z features with high CV were included during annotation. Feature m/z and abundance was relatively consistent across the series of analyses. There was a significant decrease in number of features detected with increasing retention time and a similar decrease in abundance (Figure 41.4c).

The observed elution pattern suggested lower molecular weight chemicals eluted early, with greater retention of hydrophobic and larger molecular weight compounds, including lipids and environmental chemicals.

41.5.3.4 Characterizing the Mitochondrial Exposome

Matching feature m/z to the KEGG database provided accurate mass matches of 5823 m/z features, with 1554 and 1818 unique to positive and negative ionization, respectively. Due to the role of adrenal mitochondria in steroid biosynthesis, detected metabolites were mapped to the KEGG steroid biosynthesis pathways, which resulted in 73 matches using both positive and negative mode data (Figure 41.5). The ability to detect metabolites from this pathway in adrenal mitochondria shows that measures of metabolic function can be measured within the mitochondrial isolates, providing markers that can be used to assess exposure-related alterations to key metabolic pathways.

KEGG BRITE categorization, which provides a hierarchical classification scheme for different classes of endogenous and exogenous metabolites, was used to characterize the remaining matches (Figure 41.6). Both anthropogenic and biological metabolites were observed, supporting the use of HRM chemical characterization of the mitochondrial exposome. Limited data exists on body burden for many of these chemicals (Wambaugh et al. 2014; Rager et al. 2016), so use of untargeted HRM greatly improved the ability to detect unexpected and previously unidentified environmental exposures.

Annotated features within the pesticides and endocrine-disrupting BRITE categories were selected for further characterization. Using m/z features detected in positive and/or negative mode, 822 provided tentative

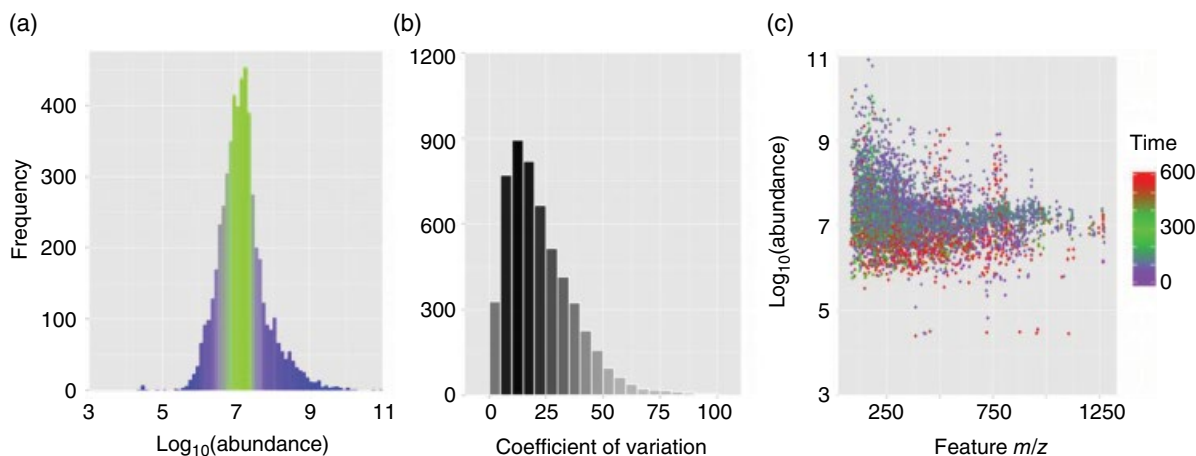


Figure 41.4 Characterization of high-resolution metabolomic profiling results for mitochondrial specific features. $\text{Log}_{10}(\text{abundance})$ (a), coefficient of variation (b), and m/z feature retention time (c) show untargeted profiling of mitochondria isolates provides coverage of a wide range of chemical signals. Distributions were similar for negative mode (data not shown).

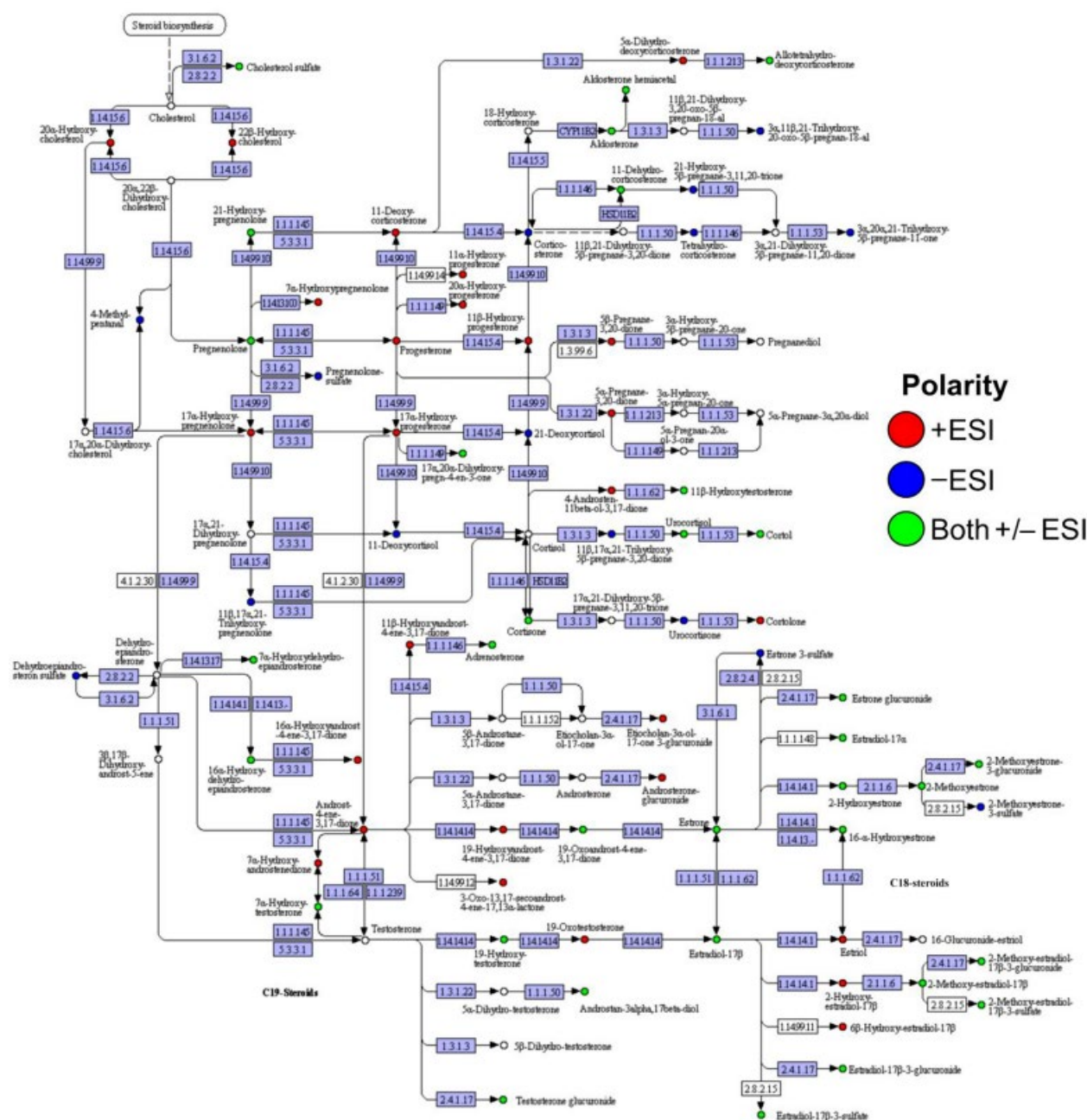


Figure 41.5 Annotation of both positive ESI and negative ESI m/z features for steroid biosynthesis metabolism. Coverage of this pathway provides important biological response measures for assessing exposure-related changes to adrenal pathways.

matches to these categories, with 224 and 346 unique to positive and negative, respectively. While these matches were based upon accurate mass alone and will require confirmation with reference standards, previous studies using coelution with authentic standards and in ion dissociation, we have found matches to known metabolic intermediates to be correct 60–80% of the time (Osborn et al. 2013). Figure 41.7 contains representative matches to different environmental chemicals from negative/positive

ionization polarities, and Table 41.4 provides a more detailed list, which includes chemical classification. Different types of environmental chemicals were detected, including pesticide synergists, chemical products, insecticides, fungicides, and halogenated chemicals.

The presence of these chemicals within the mitochondria has important implications for human health. For example, PBO was detected in all the individuals and confirmed by ion dissociation and coelution with authentic

standard. It is a potent CYP450 inhibitor (Willoughby et al. 2007), primarily used in insecticide formulations to increase the efficacy of treatment, widely applied in aerial spraying for mosquito control, and included in pesticide formulations for household use. Limited information is available on the effect of PBO to CYP450sc, the enzyme responsible during steroid biosynthesis for side-chain cleavage of cholesterol, which, following transport of the cholesterol into the mitochondrial inner membrane, is the next step in biogenesis for most steroidal hormones. Disruption of this metabolic pathway has the potential for adverse effects on health and growth.

Bisphenol A (BPA) is used for manufacture of polycarbonate plastics and resins and is recognized as a possible endocrine disruptor. BPA was detected within all adrenal mitochondrial samples, suggesting accumulation within human mitochondria. Further investigation of the

metabolic alterations from BPA exposure could provide plausible hypotheses on the mechanisms by which BPA effects hormonal production.

In positive ionization mode, matches to insecticides and fungicides were also detected. These include the commonly used carbamate insecticides (used in agricultural areas) and fungicides (added to produce to avoid molding during harvest and shipping). Additional environmental chemicals included chemicals used in industrial processes, commercial products, herbicides, and PAHs.

41.5.3.5 Case Study Conclusions

The results presented here show that the cytochrome P450 inhibitor—PBO—is present in human mitochondria. The results also show accurate mass matches (Level 5 identification by Schymanski et al. (2014)) to large numbers of other environmental chemicals, which will require confirmation by ion dissociation, coelution with standards, or other methods. Although some will be incorrect, available environmental chemical databases are biased toward commonly used chemicals, and our prior experience shows that a large fraction of matches are correct identifications. Thus, the results show that HRM profiling of mitochondria from human adrenal tissue is suitable for characterization of low-level environmental pollutants that have not previously been studied in human mitochondria.

The use of an untargeted methodology in tandem with ultrahigh accuracy MS enables detection of a broad range of chemicals that would be difficult and expensive if limited by a requirement for *a priori* selection of analytical targets and validation of targeted methods. The results support the suitability of HRM for environmental chemical surveillance and detection of exposure biomarkers in human tissues available for research. Such an approach could be very useful as a basis to understand environmental effects on adverse drug toxicities involving mitochondria.

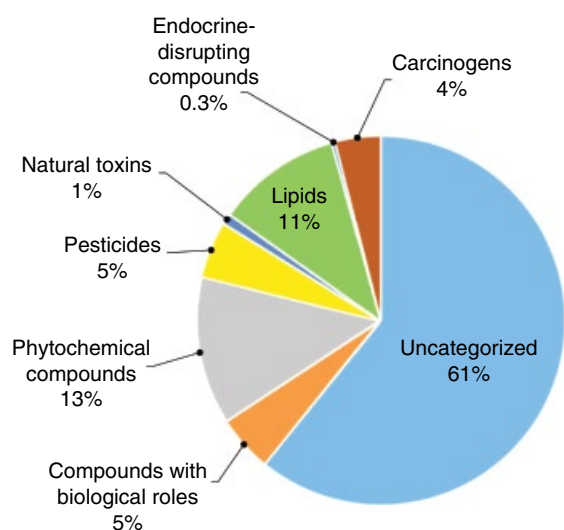


Figure 41.6 BRITE classification of annotated m/z features for positive ionization mode. Similar distributions were observed for negative mode (data not shown).

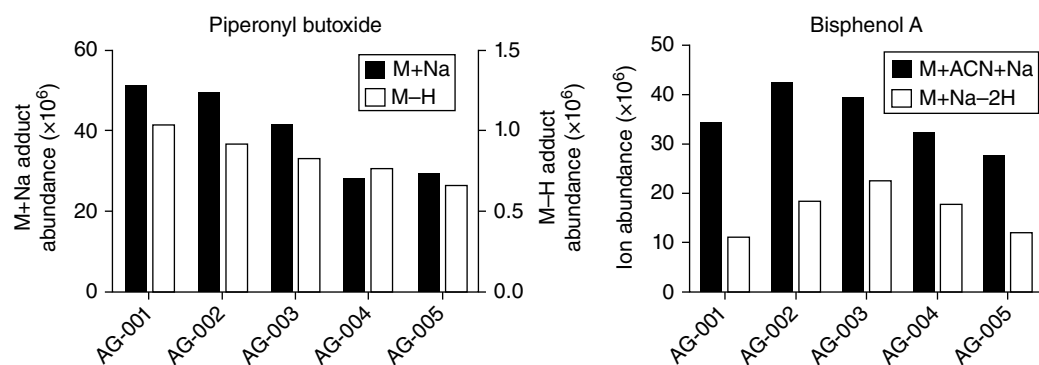


Figure 41.7 Detected environmental chemicals in adrenal mitochondria isolates with the potential to exert endocrine-disrupting effects. Piperonyl butoxide was confirmed using authentic reference standards.

Table 41.4 Matches to environmental chemicals detected in mitochondria isolated from human adrenal tissue.

Environmental chemical	Chemical formula	Classification	Detected m/z	Delta	Mass error (ppm)
Chlorfenethol	C ₁₄ H ₁₂ Cl ₂ O	Acaricide	289.0160	0.0003	0.98
Chloropropylate	C ₁₇ H ₁₆ Cl ₂ O ₃	Acaricide	339.0556	0.0008	2.23
Spiroxamine	C ₁₈ H ₃₅ NO ₂	Fungicide	298.2740	3.12E-05	0.10
Fuberidazole	C ₁₁ H ₈ N ₂ O	Fungicide	185.0702	-0.0008	4.27
Cycloate	C ₁₁ H ₂₁ NOS	Herbicide	216.1425	-0.0008	-3.87
Chlorsulfuron	C ₁₂ H ₁₂ ClN ₅ O ₄ S	Herbicide	358.0347	-0.0025	7.08
Paraquat	C ₁₂ H ₁₄ N ₂	Herbicide	250.1301	-0.0014	7.55
EPTC	C ₉ H ₁₉ NOS	Herbicide	190.1268	0.0009	4.57
Nicosulfuron	C ₁₅ H ₁₈ N ₆ O ₆ S	Herbicide	433.0930	0.0029	7.05
Styrene	C ₈ H ₈	Industrial precursor/intermediate	105.0704	-0.0005	-4.79
4-Aminobiphenyl	C ₁₂ H ₁₁ N	Industrial precursor/intermediate	170.0964	4.03E-05	0.24
4-Octylphenol	C ₁₄ H ₂₂ O	Industrial precursor/intermediate	251.1372	-0.0010	5.04
Metolcarb	C ₉ H ₁₁ NO ₂	Insecticide	166.0862	2.23E-05	0.13
2-Hydroxyphenyl methylcarbamate	C ₈ H ₉ NO ₃	Insecticide	168.0655	3.69E-05	0.22
Xylylcarb	C ₁₀ H ₁₃ NO ₂	Insecticide	180.1020	-0.0001	-0.46
Aldicarb sulfoxide	C ₇ H ₁₄ N ₂ O ₃ S	Insecticide	207.0799	-0.0001	-0.51
Propoxur	C ₁₁ H ₁₅ NO ₃	Insecticide	210.1125	-4.78E-05	-0.23
Fenclorophos	C ₈ H ₈ Cl ₃ O ₃ PS	Insecticide	364.8731	0.0023	7.03
Aminocarb	C ₁₁ H ₁₆ N ₂ O ₂	Insecticide	209.1266	-0.0019	9.25
Propamocarb	C ₉ H ₂₀ N ₂ O ₂	Insecticide	189.1598	0.0001	0.29
Chlorpyrifos	C ₉ H ₁₁ Cl ₃ NO ₃ PS	Insecticide	331.9257	0.0033	9.39
Chlorpyrifos-methyl	C ₇ H ₇ Cl ₃ NO ₃ PS	Insecticide metabolite	365.8650	-0.0012	3.68
Metaldehyde	C ₈ H ₁₆ O ₄	Molluscicide	177.1123	-0.0001	-0.66
Maleic hydrazide	C ₄ H ₄ N ₂ O ₂	Plant growth regulator	113.0355	-0.0010	-8.43
Daminozide	C ₆ H ₁₂ N ₂ O ₃	Plant growth regulator	161.0922	0.0001	0.46
Di- <i>n</i> -propyl phthalate	C ₁₄ H ₁₈ O ₄	Plasticizer	251.1272	0.0006	2.37
Di- <i>n</i> -butyl phthalate	C ₁₆ H ₂₂ O ₄	Plasticizer	279.1589	0.0001	0.53
Dimethyl phthalate	C ₁₀ H ₁₀ O ₄	Plasticizer	195.0644	-0.0008	3.90
Bis(2-ethylhexyl)phthalate	C ₂₄ H ₃₈ O ₄	Plasticizer	429.2406	0.0005	1.21
Anthanthrene	C ₂₂ H ₁₂	Polycyclic aromatic hydrocarbon	277.1035	-0.0024	-8.54

Delta = theoretical m/z - detected m/z ; mass error = delta/(theoretical m/z) $\times 10^6$.

Accumulation of the mitochondria burden of environmental chemicals is most likely a result from transfer of circulating levels of the chemicals to the organ and accumulation within the mitochondrial compartment. Understanding the mitochondrial exposome and how it is influenced by systemic exposures will be a critical part of linking mitochondrial dysfunction to environmental factors. For this, studies of environmental chemical distribution in animal models, especially determining half-life of chemicals within mitochondria, will be important to understand both short-term and long-term impacts of this accumulation.

41.6 Conclusions

Extensive data exist on the susceptibility of mitochondria to environmental toxins; however, there is a poor understanding of mitochondrial burden of chemicals in humans and the associated contribution to chronic disease. To address this knowledge gap, we introduce the concept of the mitochondrial exposome, defined as a measure of environmental stressors and associated biological response on mitochondrial function in humans. HRM profiling, which is based upon HRMS, allows for comprehensive characterization of the mitochondrial exposome through untargeted profiling of environmental chemicals and associated metabolic response. Measurement of the mitochondrial metabolic phenotype

provides biomarkers of biologically relevant dose, reducing the need for apical endpoints, and enables *in vivo* measurement of biochemical perturbations. This is comparable to high-throughput *in vitro* assays for chemical hazard identification because dose–response relationships can be evaluated for individual chemicals and mixtures; and chemical disposition, metabolism, and products can be evaluated for differential effects. Application of HRM to isolated mitochondria from human adrenal glands showing sufficient chemical coverage is available to assess the accumulated body burden of many anthropogenic compounds. While the results shown in this study are exploratory in nature, the techniques demonstrated here have broad applicability to characterize mitochondria from other tissues and species. Thus, continued development and measure in human populations are possible, providing avenues to develop new understanding of the environmental contribution to mitochondrial health.

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Central Mitochondrial Signaling Mechanisms in Response to Environmental Agents: Integrated Omics for Visualization

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CHAPTER MENU

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42.1 Introduction

Mitochondria function in aerobic metabolism plays a central role at the interface between an individual, defined as a functional genome, and associated environment, defined by environmental resources and threats (Go and Jones, 2014; Jones and Sies, 2015). With maturation of omics technologies, the central dogma of information storage in DNA, transcribed to RNA and translated into protein, emerges as integrated functional genome with epigenetic control of gene expression (Rydzanicz et al., 2013), posttranscriptional editing of RNA transcripts (Pineda et al., 2015), posttranslational modifications of the epiproteome (Dai and Rasmussen, 2007; Waldrup et al., 2014), and integrated control of biochemical reactions. This functional genome contains exposure memory systems (Jones, 2015), which enable the functional genome to remember exposures and improve utilization of resources and defend against challenges. Lifelong exposures of an individual, cumulatively termed the “exposome” (Miller and Jones, 2014; Wild, 2005), impact mitochondrial function but have not yet been well described.

Importantly, the exposome includes a broad range of chemicals derived from food, microbiome, pharmaceuticals, commercial products, and environmental exposures, but relatively few of these chemicals have been studied in the context of mitochondrial function and dysfunction (Go et al., 2014b).

Although containing only about 10% of the proteins encoded by the nuclear genome, the mitochondrial metabolome appears to include representative metabolites of up to 90% of the gene-directed metabolome. Mitochondrial metabolomics research has often focused on the citric acid cycle and energy metabolism, but mitochondria contain metabolic precursors and intermediates for mitochondrial replication (Walberg and Clayton, 1983), transcription (Edwards et al., 1982), translation (Ostrander et al., 2001; Pfisterer and Buetow, 1981), and posttranslational modifications (Bhattacharjee et al., 2009; Chavez et al., 2011). Many ancillary systems are also present, such as the ATP-dependent protease La (LON) (Ngo and Davies, 2007) that generates peptides, mitochondrial sirtuins (He et al., 2012) that generate acetylated and succinylated protein derivatives, and other mechanisms to modify bases in RNA.

The spectrum of metabolism in mitochondria is very broad, with enzymes catalyzing steps of purine and pyrimidine biosynthesis (Eriksson and Wang, 2008); carbohydrate (Kaminsky et al., 1982), amino acid (Guda et al., 2007), and fatty acid oxidation (Kunau et al., 1995); anabolic systems for biosynthesis; and elimination of nitrogen through ureagenesis (Christian and Spremulli, 2012; Sumegi and Srere, 1984). Metabolic pathways for energy metabolism include central processes for β -oxidation of fatty acids, including import of fatty acids as acylcarnitines (Kunau et al., 1995), which are deranged in many disease processes. Branched-chain amino acids (BCAA) are oxidized through branched-chain fatty acid intermediates (Crown et al., 2015), and these are also commonly altered in disease. Other pathways include porphyrin and heme biosynthesis (Richardson et al., 2010; Sano et al., 1959), terpene and terpenoid products, such as squalene precursors for cholesterol and vitamin D synthesis (Miller and Auchus, 2011; Schroepfer, 1981) and activation of vitamin D and biosynthesis of steroids, including sex hormones, mineralocorticoids and glucocorticoids (Miller, 2013). The terpene/squalene pathway also functions in biosynthesis of coenzyme Q, an essential electron carrier for mitochondrial bioenergetics (Tran and Clarke, 2007). Mitochondria also contain a spectrum of cytochromes P450 (Cyp) (Nebert and

Russell, 2002), glutathione *S*-transferases (Sato et al., 1977; Wiseman and Woods, 1977), and other detoxification enzymes, such as the rhodanese system for H_2S and thiocyanate metabolism (Aussignargues et al., 2012).

Overall, this extensive array of metabolism illustrates that mitochondria are intrinsic elements in the adaptive interface of the genome and exposome. As such, this implies that mitochondrial metabolomics will reflect adaptive and toxic responses to environment and also reveal perturbations as elements of toxicity and disease. The present article describes (i) new high-resolution metabolomics (HRM) as a practical approach for metabolic surveillance, (ii) application of HRM to measure mitochondrial metabolism, (iii) use of HRM with redox proteomics in a redox proteome $\times$ metabolome-wide association study (RMWAS) to understand mechanisms of mitochondrial cadmium (Cd) toxicity, (iv) combination of HRM in transcriptome $\times$ metabolome-wide association study (TMWAS) of environmental fungicide and herbicide toxicity, and (v) combination of the redox proteome, metabolome, and transcriptome in redox proteome $\times$ metabolome $\times$ transcriptome-wide association study (**RMTWAS**) of manganese (Mn) toxicity (Figure 42.1). We conclude with a conceptual framework to use these approaches for comorbidities and complex multimorbidities, such as those commonly observed

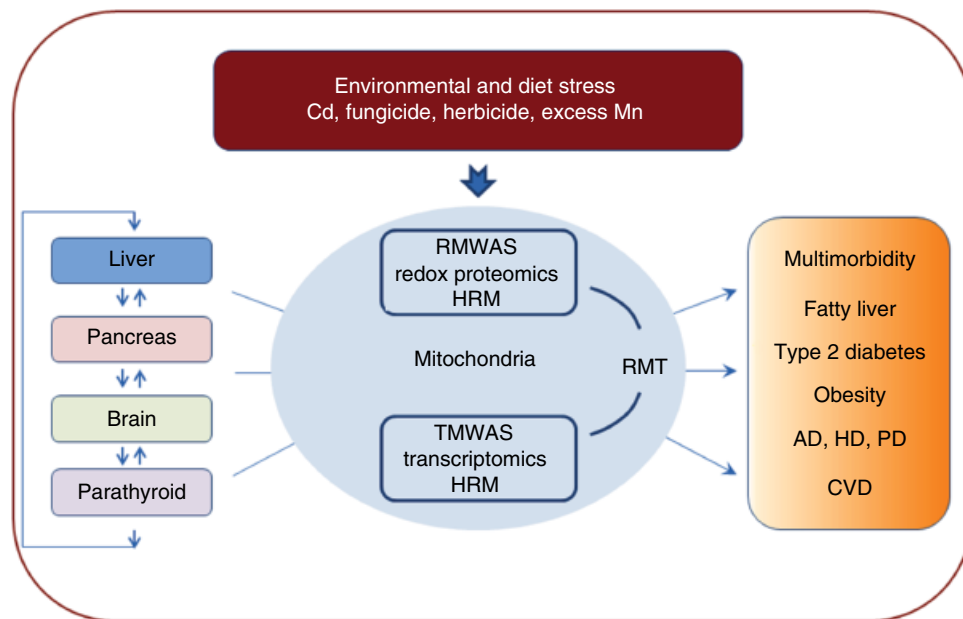


Figure 42.1 The use of integrated omics approaches for complex multimorbidities to understand central mitochondrial signaling mechanisms in response to environmental agents or drugs. Chronic exposures to environmental toxicants (cadmium, fungicide, herbicide, excess manganese) affect mitochondrial signaling and metabolism resulting in systematic dysfunctions of organs and contribute to development of multimorbidities. Application of high-resolution metabolomics (HRM) to measure mitochondrial metabolism and integrated omics approaches of HRM with redox proteomics (RMWAS), transcriptomics (TMWAS), and RMT (redox proteomics $\times$ HRM $\times$ transcriptomics) will advance our understanding of drug- or environmental agent-induced multimorbidities such as fatty liver disease associated with obesity and type 2 diabetes and further cardiovascular diseases (CVD) and neurodegenerative diseases (Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD)).

with drug-induced liver injury including fatty liver disease associated with obesity and type 2 diabetes, and further extended to neurodegeneration, cardiovascular disease, renal disease, and lung disease (Figure 42.1).

42.2 High-Resolution Metabolomics

Genome-wide association studies (GWAS) suggest that only 15–20% of diseases have a strong genetic component (Wild, 2005), with the remaining 80–85% likely having important exposome components (Rappaport and Smith, 2010). Consideration of chemicals of the exposome along with endogenous metabolism indicates that an extensive number of chemicals are present in biologic systems including pharmaceuticals, environmental chemicals, microbiome products, food additives, and non-nutritional phytochemicals, as well as endogenous chemical species (e.g., carbohydrates, amino acids, peptides, lipids, nucleic acids) and dietary compounds (e.g., vitamins, minerals) (Jones et al., 2012). Quantitative data show that endogenous metabolites are often three

to four orders of magnitude higher abundance in human samples than environmental chemicals (Rappaport and Smith, 2010). Thus, challenges for metabolomics include ability to measure a broad spectrum of metabolites covering an extensive range of concentrations.

HRM addresses these key needs by providing high-throughput, reproducible, and quantitative methods with extensive metabolic coverage (Go et al., 2015b; Walker et al., 2016) (Figure 42.2). HRM takes advantage of ultrahigh mass resolution and mass accuracy of Fourier transform mass spectrometry (FTMS) with three technical replicates to improve reproducibility and sensitivity for metabolic studies. With three technical replicates, deep data extraction with adaptive processing (apLCMS) (Yu et al., 2009) and xMSanalyzer (Uppal et al., 2013) and a quantification structure using reference standardization (Go et al., 2015b), large numbers of metabolites can be quantified (Figure 42.2). Detailed comparisons of different mass spectrometry approaches are available (Marshall and Hendrickson, 2008); special ion traps with a central electrode (Orbitrap (Makarov, 2000)) are of great utility, allowing measurement of greater than 20,000 metabolites in biological

HRM workflow

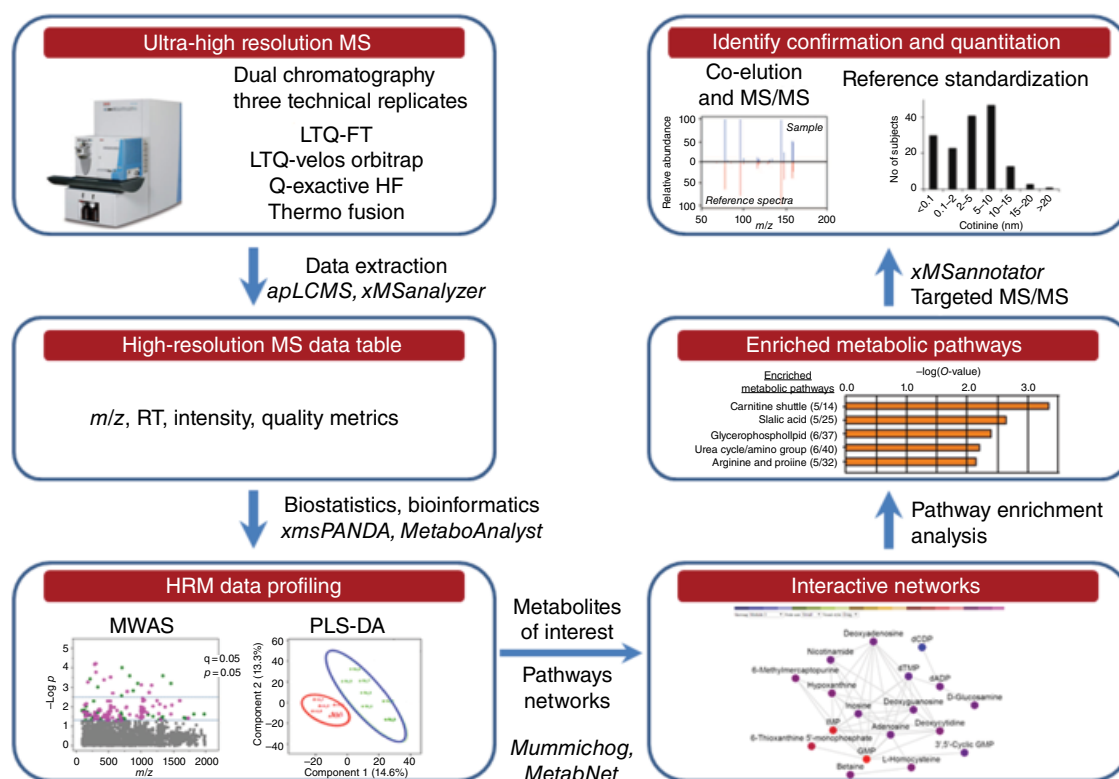


Figure 42.2 HRM workflow. HRM of high-throughput, reproducible, and quantitative measurement by ultrahigh mass spectrometry with computational tool for data extraction and analysis allows to measure greater than 20,000 metabolites and associated metabolic pathways and networks. See references (Go et al., 2014b; Jones, 2016; Walker et al., 2016) for details.

samples (Jones, 2016). In principle, one million or more chemicals can be measured (Uppal et al., 2016) including large numbers of low abundance metabolites.

42.3 High-Resolution Metabolomics of Liver Mitochondria

The mitochondrial metabolome within a specific cell type is a subset of an individual's metabolic phenotype, including endogenous metabolites, chemicals from individual–environment interaction, and products derived from mitochondrial activities. Because of the tissue and cellular heterogeneities, the extent of variation of the mitochondrial metabolome is unknown; one can expect, however, that this is extensive. Extensive research will be needed to gain a substantial understanding of the spectrum of endogenous metabolites as well as content of microbiome metabolites and dietary and environmental chemicals.

HRM was used in a pilot study of mouse liver mitochondria to gain an understanding of the spectrum of metabolites present in mitochondria (Roede et al., 2012). Mitochondria were isolated from male and female wild-type C57BL/6J mice and from mice overexpressing the mitochondrial protein thioredoxin-2 (Trx2). Half of the mice were less than 6 months old and half were greater than 12 months old. For the Trx2 transgenic mice, human Trx2 (Chen et al., 2002) was fused with a V5 epitope for detection of the transgene product (He et al., 2008). For mitochondrial isolation, livers were rapidly excised, homogenized in 22 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4), and 1 mM EGTA, and mitochondria were isolated by differential centrifugation (Savage et al., 1991). Mitochondria were extracted with acetonitrile and analyzed by HRM with a dual chromatography method using anion-exchange and reverse-phase chromatography (Soltow et al., 2013).

HRM results showing 6000–8000 metabolic features were detected for each column; many of the features were detected on both columns, and mitochondria were prepared with solutions containing biologic products that could contribute signals. Consequently, data were filtered to remove all features that were less than fourfold higher in mitochondria than in isolation medium and combined to yield a single set of 2127 metabolic features (Roede et al., 2012). High-resolution matches to Madison Metabolomics Consortium Database and METLIN, followed by mapping to Kyoto Encyclopedia of Genes and Genomes (KEGG), showed matches to 745 out of 1485 metabolites in the human pathways, or approximately 50% of all metabolites. With the recognition that

more than 70% of the metabolic signals were filtered out because the levels were not fourfold greater than found in the sucrose–mannitol isolation solution, the results show that the mitochondrial metabolome broadly mirrors all aspects of metabolism. Matches to metabolites in 136 out of 154 KEGG pathways were present, with the top 10 metabolic pathways including arachidonic acid metabolism, amino sugar and pentose phosphate metabolism, purine and pyrimidine metabolism, porphyrin metabolism, steroid and steroid hormone biosynthesis, tyrosine metabolism, and neuroactive ligand receptor intermediate metabolism (Roede et al., 2012). Comparisons of mitochondria from males and females showed that hundreds of metabolic features differ, with specific examples of amino acids (methionine, glutamate, leucine + isoleucine) higher in males and adenosine, amino-octadecanoic acid, and sphinganine higher in females. No significant differences were detected for mitochondria from Trx2 transgenic mice, but partial least squares discriminant analysis (PLS-DA) showed metabolic differences associated with 1-carbon metabolism, including methylated nucleotides, folate, and choline. No significant metabolic differences were observed between mitochondria from mice less than 6 months compared with mice greater than 12 months (Roede et al., 2012). Comparison of mitochondrial features and plasma features showed that 1425 were common, and these were used to select a list of 30 plasma markers of mitochondrial metabolism (Go et al., 2014b). This list includes substrates and products of mitochondrial enzymes in MitoCarta (Pagliarini et al., 2008), such as products of BCAA metabolism (3-methyl-2-oxovaleric acid, α -ketoisocaproate, α -ketoisovalerate), indicators of mitochondrial NAD-dependent metabolism (acetoacetate, β -hydroxybutyrate), heme biosynthesis (δ -aminolevulinic acid, protoporphyrinogen), coenzyme A biosynthesis (pantothenate), fatty acid metabolism (carnitine, multiple acylcarnitines), amino acid metabolism and the urea cycle (acetylglutamate, pyrroline-5-carboxylate, glutaric semialdehyde, saccharopine, kynurenine, fumarylacetoacetate), and 1-carbon metabolism (choline, dimethylcholine, sarcosine) (Go et al., 2014b). Thus, the data clearly show that mitochondrial metabolism is extensively interconnected with all aspects of cellular metabolism and that mitochondrial metabolites are present in plasma for use as indicators of mitochondrial function *in vivo*.

An important point, however, is that more than half of the metabolic features in mouse mitochondria had no database matches, indicating that mitochondria contain many uncharacterized metabolites. The chemical nature of these features can be inferred from multiple database matches to dietary chemicals (leucocyanidin),

drugs and drug metabolites (carbachol, lomefloxacin, *N*-desmethyltrifluoperazine, quinaprilat), and environmental chemicals (dihydrorotenone, 7-(acetyloxy)-3-(3-pyridinyl)-2H-1-benzopyran-2-one) (Roede et al., 2012). There is also a possibility that other uncharacterized metabolic pathways are present in mitochondria and that metabolites derived from the intestinal microbiome are present. The results point to an important need to extend mitochondrial biology to a more complete definition of the mitochondrial metabolome.

42.4 Integration of Mitochondrial Redox Proteomics and Metabolomics: RMWAS

We built upon this HRM analysis of mitochondria in research to investigate cadmium (Cd) effects on nonalcoholic fatty liver disease (NAFLD) (Go et al., 2015a). Cd contributes to diseases impacting many organ systems, for example, liver, kidney, lung, and others, but the underlying mechanisms are not well understood. Cd is an important environmental metal because it is present in food and accumulates in humans due to a 20-year biologic half-life. Previous studies showed that Cd affects activity and transcription of Trx reductase (Chrestensen et al., 2000; Sakurai et al., 2005) and that low environmental doses caused protein oxidation and stimulated inflammatory signaling (Go et al., 2013a, b).

To determine whether Cd-dependent oxidation of mitochondrial proteins was associated with metabolic effects, we exposed mice to Cd, isolated liver mitochondria, and measured the redox proteome and metabolome (Go et al., 2015a). Redox states of liver mitochondrial proteins were measured by redox proteomics–mass spectrometry using isotope-coded affinity tag (ICAT). Analyses of 2687 cysteine-containing peptides showed that 1667 peptides from 657 proteins were detected in both control and Cd-exposed samples. Forty-six percent of the peptides were oxidized more than 1.5-fold in mitochondria from Cd-treated mice compared with controls. Pathway analyses showed 86 pathways affected by Cd, including 24 Cys residues in proteins of BCAA metabolism and 14 Cys in proteins for acylcarnitine, carnitine, and fatty acid β -oxidation metabolism. Although no enzyme or transport data were available to suggest altered metabolic activities, protein oxidation is frequently associated with loss of activity. Thus, from this analysis, one can predict impact on BCAA and fatty acid metabolism.

Consistent with this prediction, HRM analysis showed that Cd treatment altered metabolites in BCAA and fatty

acid metabolism pathways. HRM data included 4745 features, with 250 increased by Cd and 292 decreased by Cd after FDR correction. Pathway enrichment analysis showed significant associations with 29 pathways associated with increased or decreased features in the mitochondria (Go et al., 2014a). The results showed increases in isobutyryl-CoA, succinyl-CoA, acetyl-CoA, and palmitoyl-CoA, consistent with impaired branched-chain fatty acid and β -oxidation pathways. HRM analyses also identified several metabolic features associated with carnitine/acylcarnitine system supporting β -oxidation, including carnitine, acetylcarnitine, propionylcarnitine, malonylcarnitine, palmitoylcarnitine, and stearyl carnitine. Cd-dependent inhibition of the acylcarnitine system was confirmed in studies of $^2\text{H}_3$ -palmitoylcarnitine uptake in mitochondria from Cd-treated mice compared with control mice. Together with the redox proteomics data, the results show that Cd induces oxidation of specific Cys in proteins with associated changes in intermediates of mitochondrial fatty acid metabolism.

This study provides straightforward methods to investigate drug-induced mitochondrial oxidative stress and associated effects on metabolism. Additionally, the results suggest that low-level environmental Cd impairs mitochondrial fatty acid metabolism. Such an effect could potentiate diet-induced or drug-induced fatty liver. HRM provides a functional measure of the consequences of oxidation in protein redox state so that a combined approach of redox proteomics and metabolomics (RMWAS) as described in the following text provides an efficient way to investigate central mechanisms of toxicity (Figure 42.3a). Continuous efforts have been made to advance our understanding of drug- and environmental agent-induced toxicity and disease; a similar integrated omics strategy with transcriptomics and metabolomics (TMWAS) (Figure 42.3b) was applied and described after the section of RMWAS.

Figure 42.4 illustrates an approach to combine redox proteomics and metabolomics data in developing a RMWAS. Data from Go et al. (2014a) for redox proteomics and metabolomics effects of Cd in mouse liver mitochondria were used for redox proteomics $\times$ metabolome integration using partial least squares regression (PLSR) and network() function implemented in R package mixOmics (Gonzalez et al., 2012; Le Cao et al., 2009). The network() function in the mixOmics package was used to generate the association matrix and visualize the associations (Gonzalez et al., 2012). The resulting visualization at a correlation threshold of 0.3 shows multiple central hubs of redox-sensitive Cys residues in proteins with a very complex network of metabolic interactions (Figure 42.4a). At a correlation threshold of 0.5, the overall structure is largely preserved

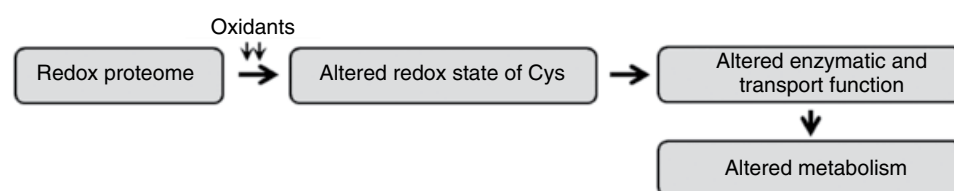
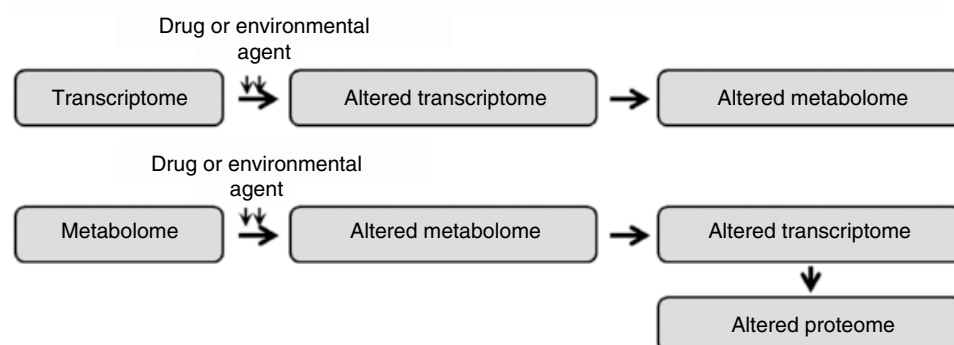
(a) RMWAS: redox proteome × metabolome-wide association study(b) TMWAS: transcriptome × metabolome-wide association study

Figure 42.3 Strategy of integrated omics approaches, RMWAS and TMWAS. Oxidants generated from drug and environmental agents affect redox states of proteome in a relatively short time frame. Oxidized protein redox state affects enzymatic and transport function leading to metabolic alterations (a, RMWAS). Similarly, such exposures could affect transcriptome and alter metabolome or vice versa depending on exposure time and dose (b, TMWAS).

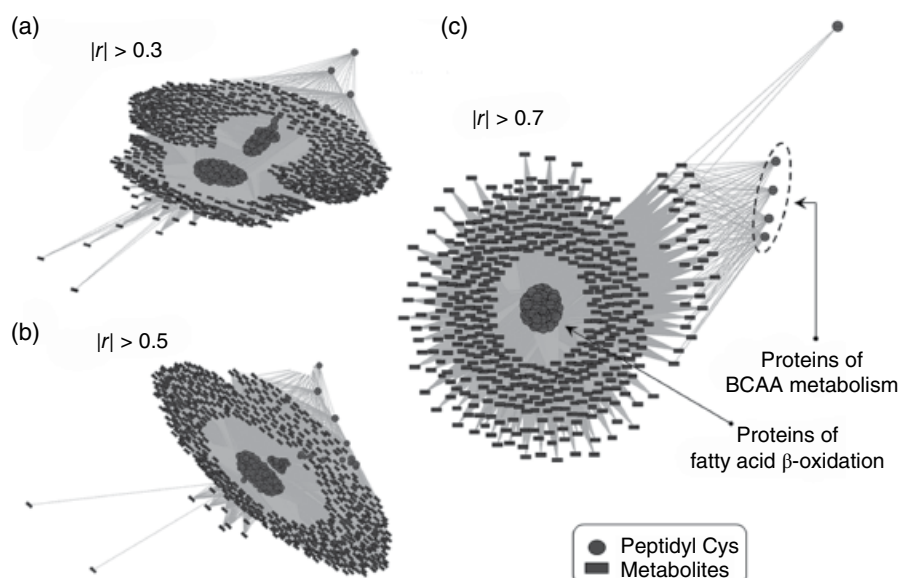


Figure 42.4 Visualization of integrated redox proteomics and metabolomics (RMWAS). The data of Cd-affected mitochondrial redox proteome and metabolome from the previous study by Go et al. (2014a) was used for RMWAS. The results of RMWAS were visualized by correlation threshold ($|r| > 0.3$ (a), $|r| > 0.5$ (b), $|r| > 0.7$ (c)). circle and rectangle represent oxidized protein peptide (peptidyl Cys) and altered metabolite in abundance by Cd, respectively. Adapted from Go et al. (2014a). Reproduced with Permission of Oxford University Press.

and shows two hubs of redox-sensitive Cys with multiple peripheral associations (Figure 42.4b). At a correlation threshold of 0.7, only one central hub remained, with redox-sensitive Cys in proteins associated with fatty acid

β -oxidation, including carnitine acyltransferases and proteins directly involved in β -oxidation (Figure 42.4c). Associated metabolites included acyl-CoAs and acylcarnitines (Go et al., 2014a). The more loosely connected

cluster of Cys residues on the right were associated with proteins involved in metabolism of BCAA and corresponding branched-chain fatty acids (Figure 42.4c). The metabolites connected to both the central hub and this cluster included branched-chain fatty acyl-CoAs. Notably, the correlations were negative, suggesting that the branched-chain fatty acyl-CoAs may be negatively impacting the normal function of β -oxidation.

42.5 Integration of HRM with Transcriptomics: TMWAS

A similar integrated omics approach was used with transcriptomics and metabolomics (TMWAS) in a Parkinson's disease model involving a combination of the herbicide paraquat (PQ) and fungicide maneb (MB) (Roede et al., 2014). Epidemiologic studies showed an association of PQ and MB exposures with Parkinson's disease, and experimental studies in neuroblastoma cell lines confirmed toxicity of PQ plus MB. Detailed understanding of mechanisms has been elusive, however, because the two chemicals did not appear to share common mechanisms.

A study to investigate mechanisms of combined toxicity of PQ and MB was conducted in a neuroblastoma cell line with PQ and MB concentrations that individually caused 20% cell death and together caused 50% cell death. Cells were treated and samples were collected under conditions where there was minimal cell death. HRM data were filtered to 1,358 metabolites and transcriptome array data was analyzed for 30,869 transcripts. Bioinformatics analyses showed that MB alone had far greater effect on metabolites and transcripts than PQ. The effect of the combination of PQ and MB was far greater than either MB or PQ alone. Pairwise comparisons of arrays were created for transcripts and metabolites so that the correlations could be filtered for statistical criteria and ranked according to strength of correlation. The resulting TMWAS showed that MB alone had a very strong central hub of transcripts correlated with a large cluster of metabolites, while PQ alone had only a weak association structure with no clearly defined hub or significant organization. Importantly, the combination of PQ and MB created a second network substructure in addition to the large central structure associated with MB alone.

The additional PQ+MB hub from the TMWAS contained four clearly discerned clusters of genes and associated metabolites. One of these clusters contained genes for two cation transporters and a cation transporter regulatory protein that is recognized as a proapoptotic protein. The cation transporters have previously been associated with enhanced mitochondrial accumulation

of PQ and manganese, thereby providing clear evidence that this transcript-metabolite cluster represents a hub contributing to the mechanism of toxicity. Specifically, MB altered expression of transport systems that facilitated PQ transport and toxicity (Roede et al., 2014).

The other three clusters included stress response genes and transporters linked to cytoprotective mechanisms. One of these was an early response cluster including heme oxygenase, a second was an antioxidant response cluster with increased expression of a transporter that supports GSH biosynthesis and cystine clearance, and a third cluster including PPAR α and increased nonessential amino acid transport. Together, the results show that most of the responses to the toxic combination of PQ and MB were adaptive responses rather than toxicologic responses. Only a small subset of the overall response pattern reflected the underlying toxicologic mechanism of the combined neurotoxicity of PQ and MB. The results clearly establish the principle that combined toxicity can occur through network-level interactions and show that TMWAS provides an effective way to investigate such complex mechanisms (Roede et al., 2014).

Although the experiments were selected to measure metabolic and transcriptional changes before substantial effects on protein abundance occurred, one must be aware that in this type of study, the causal relationship is not necessarily clear. In the RMWAS described above a relatively short time of exposure was used so the oxidation of proteins could be inferred to be causal in the changes in the metabolome (Figure 42.3a). Similarly, a relatively short time frame was used for the TMWAS (Roede et al., 2014). For longer exposures in a TMWAS, however, metabolic effects could cause changes in transcription, or changes in transcription could cause changes in protein abundance with secondary effects on metabolism (Figure 42.3b). Hence, caution is required in assigning causal relationships.

42.6 Three-Way Integration of Redox Proteomics, Metabolomics, and Transcriptomics to Create RMT Association Study for Mitochondrial Signaling in Manganese (Mn) Toxicity

The 2-way integration of metabolomics with redox proteomics or transcriptomics suggests that higher-scale integrations may provide an even more powerful and sensitive way to map functional networks associated with mitochondrial redox signaling. Mn exposure in a neuroblastoma SH-SY5Y cell model provides a model to test this development (Fernandes et al., 2016).

Mn is an abundant mineral that is nutritionally essential in mammals and causes toxicity with excess exposure (Smith et al., 2016). The dose–response curve for effects of Mn exposure on neurodevelopment endpoints at 12 and 36 months of age showed that both high and low Mn exposure can negatively influence child neurodevelopment (Claus Henn et al., 2010). Mn is an essential cofactor for the mitochondrial antioxidant protein superoxide dismutase-2 (SOD2), in addition to a diverse set of other enzymes required for biosynthesis and metabolism of lipids, carbohydrates, and amino acids (Burton and Guilarte, 2009; Takeda, 2003). The Food and Nutrition Board (FNB) of the Institute of Medicine established adequate intake (AI) levels of Mn at 2.3 and 1.8 mg Mn/day, respectively, for men and women (Institute of Medicine (US) Panel on Micronutrients, 2001).

Occupational toxicity from Mn is well documented (ATSDR, 2012a), and emerging evidence indicates that environmental Mn exposure from recycling waste, agricultural agents, fuel combustion, natural or anthropogenic contaminations of soil and water, and contaminated foods, infant formulas, food and nutritional products can also pose a threat in the general population (ATSDR, 2012a; Golub et al., 2005; Loranger et al., 1994; O’Neal and Zheng, 2015; Roede et al., 2011). Excess Mn exposures cause cognitive and behavioral deficits in a Parkinson’s disease-like neurological syndrome (Bouchard et al., 2011; Kwakye et al., 2015; Menezes-Filho et al., 2011; Myers et al., 2009). Neurological effects of occupational Mn are well documented by neurobehavioral tests of cognition, mood, and neuromotor activities (Beuter et al., 1999; Bouchard et al., 2005; Lucchini et al., 1995; Standridge et al., 2008; Wasserman et al., 2006). Mn-induced neurotoxicity involves oxidative damage, mitochondrial dysfunction, and apoptotic cell death (Anantharam et al., 2002; Galvani et al., 1995; Gavin et al., 1992; Maddirala et al., 2015; Milatovic et al., 2009; Oubrahim et al., 2001; Yoon et al., 2011; Zhang et al., 2004). Extrapolation of risks to the general population is difficult, however, and an interim guidance value for Mn, 0.16 mg/kg/day (ATSDR, 2012a), was set to be consistent with the upper limit of exposure set by the FNB. Importantly, the interim guidance maximum intake level of 11.2 mg/day for a 70 kg man is less than fivefold higher than the AI level. This narrow fivefold range between adequate and toxic intake levels emphasizes needs to understand the most critical functional deficits from Mn insufficiency and most sensitive functional disruptions from excess.

Mn concentrations in cells and human brain samples have been well characterized (Bowman and Aschner, 2014), enabling relevant dose–response studies to be performed with cell culture. Importantly, these studies demonstrate enhanced oxidant production and

apoptosis in HeLa cells treated with 0.5–2 mM MnCl_2 for 24 h (Oubrahim et al., 2001), increased oxidant production and decreased ATP in SH-SY5Y cells treated with 0.8 mM MnCl_2 for 24 h (Maddirala et al., 2015), inhibition of mitochondrial complex I in PC12 cell cultures (Galvani et al., 1995), and inhibition of ATP synthesis in isolated mitochondria *in vitro* (Gavin et al., 1992).

A Mn dose–response study showed that additions of Mn in the range of 1–10 μM to the culture medium for SH-SY5Y cells resulted in physiological cellular Mn levels ($\leq 16 \pm 1$ ng/mg protein) (Fernandes et al., 2016). These concentrations stimulated mitochondrial respiration, while concentrations in the range of 50–100 μM cellular Mn (toxicological; $> 37 \pm 2$ ng/mg protein) inhibited mitochondrial respiration and caused cellular thiol oxidation. Over the entire range of Mn concentration, Mn did not increase mitochondrial superoxide amount as measured by aconitase activity or MitoSOX fluorescence intensity. In contrast, Mn-treated cells had increased H_2O_2 production over the entire range as measured by MitoPY1. The results show that controlled Mn exposure provides a useful cell manipulation for toxicological studies of mitochondrial H_2O_2 signaling. This model establishes appropriate conditions to test tools for study of mitochondrial oxidant signaling that are mediated through redox proteomics, metabolomics, or transcriptomics mechanisms.

The principles for development of an integrated redox proteomics, metabolomics, and transcriptomics tool are derived from the concept of a targeted metabolome-wide association study (MWAS), originally used in a study of phenylalanine metabolism, with visualization by Manhattan plots (Go et al., 2015c), and further developed into a software tool, MetabNet, with visualization using Cytoscape (Uppal et al., 2015). An important capability of MetabNet is illustrated in Figure 42.5, which shows that global network structures are visualized at low stringency for correlation and subnetwork structures can be progressively visualized at greater stringency.

This approach allows arrays of omics data to be regressed against any quantitative parameter, such as Mn concentration or mitochondrial H_2O_2 production rate. In principle, one can observe multiparameter interaction structures by combining multiple omics arrays. A prototype tool for visualization of multi-omics integration is shown in Figure 42.6. By creating an array with pairwise tests for correlation among arrays, one can select top correlations, ranked by r and/or filtered by statistical criteria. With this approach, central hubs are readily visualized to connect metabolome and transcriptome response structures to redox proteome variations or proximal signaling events such as Mn-induced H_2O_2 production by mitochondria.

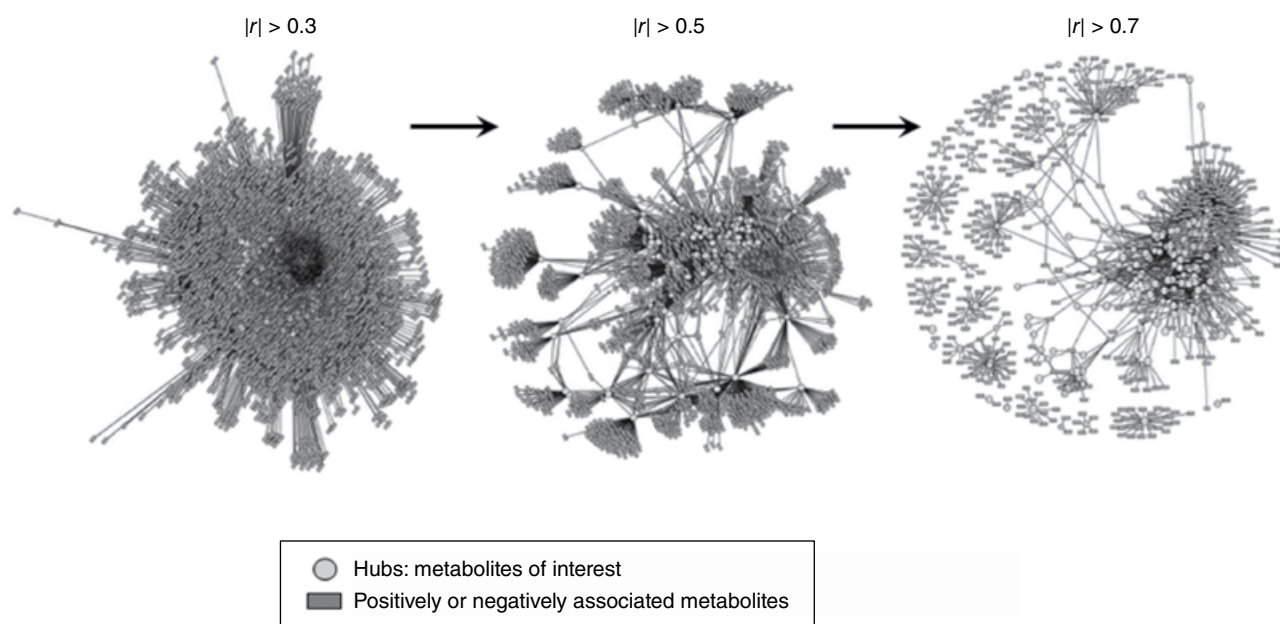


Figure 42.5 Network visualization capability of targeted MWAS by MetabNet. A capability of MetabNet (Uppal et al., 2015) is illustrated showing progressive visualization of subnetwork structures from the global network structures by increasing stringency of correlation threshold ($|r| > 0.3 \rightarrow |r| > 0.5 \rightarrow |r| > 0.7$).

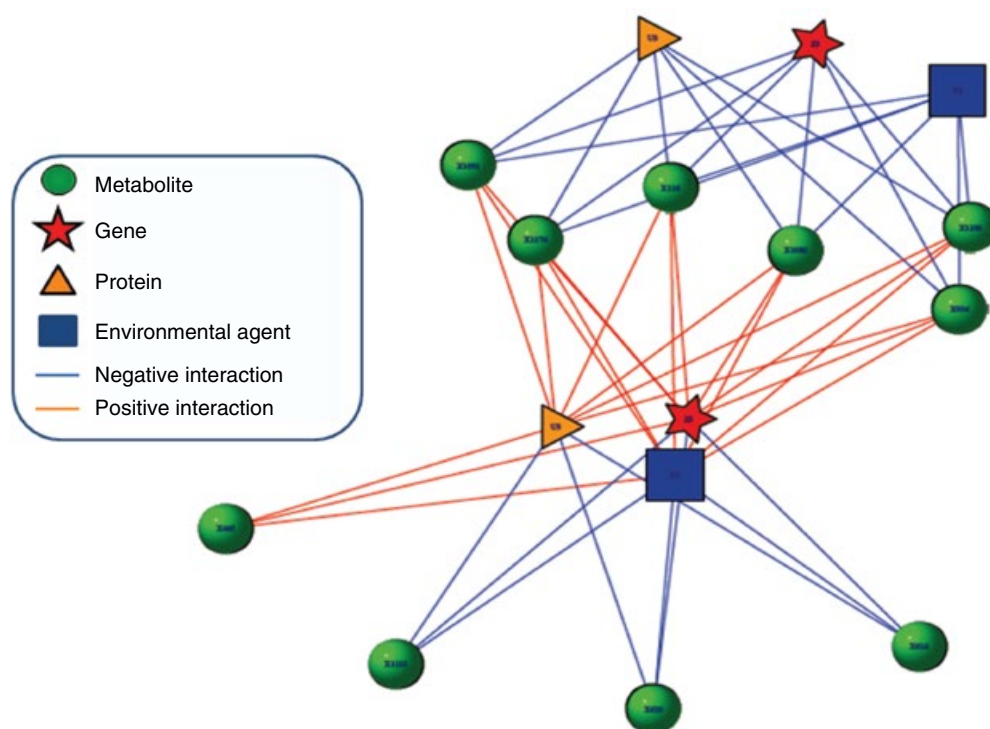


Figure 42.6 Visualization of multi-omics integrations. A conceptual representation of network structures resulted from multi-omics approaches (redox proteomics $\times$ metabolomics $\times$ transcriptomics).

42.7 Integrated Omics
Applications in Mitochondrial
Metabolic Disorder: Fatty Liver,
Diabetes, Obesity, and
Neurodegenerative Diseases

The availability of integrative omics tools allows new ways to study complex, interorgan disease processes, such as drugs, environmental agents, or dietary factors impacting mitochondria in different tissues. Useful applications for humans, however, are likely to require extensive data due to the heterogeneity of populations. For instance, in Figure 42.7 the redox metabolome is combined with clinical phenotype measures and HRM analyses for 500 individuals. The resulting network

structures at high stringency show separate hubs linked to the more reduced counterpart, GSH, and the more oxidized counterpart, cystine. Notably, by using MetabNet to obtain the broader associated metabolic network structure and Mummichog to test for pathway enrichment, one finds that the plasma redox metabolome is tightly linked to mitochondrial pathways of the citric acid cycle, BCAA metabolism, the urea cycle, and other central metabolic pathways. These data show that the principles developed from cell culture and animal models can be directly applied to human research to extend knowledge of complex biologic functions. The results demonstrate an opportunity to advance understanding of human disease by development of principles and tools through carefully controlled studies in experimental models. For instance, the challenge to

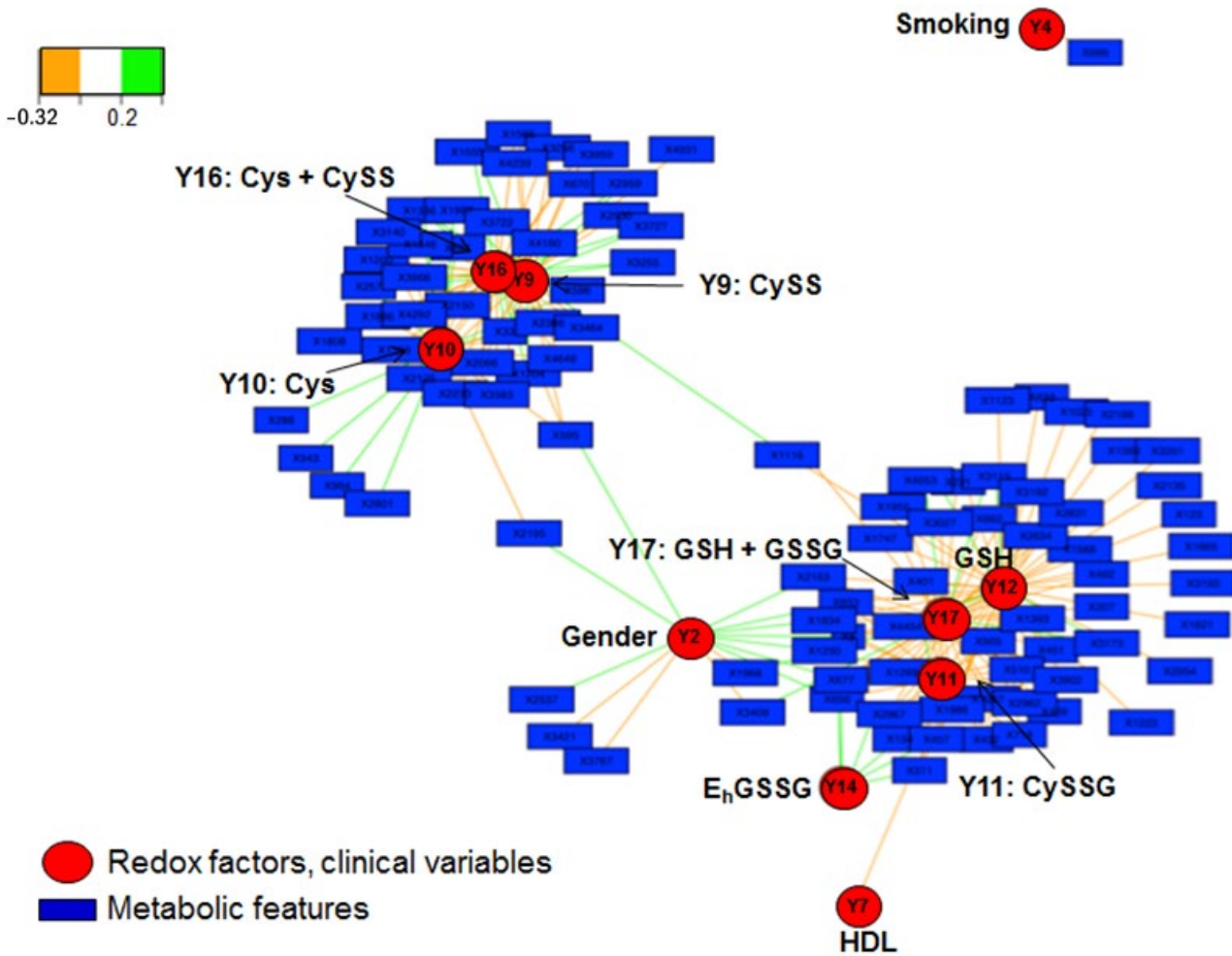


Figure 42.7 Application of integrated omics approaches in monitoring human health and disease. Application of multi-omics approaches in clinical measurement is illustrated. Metabolic features positively or negatively associated with redox factors (cysteine (Cys), cystine (CySS), glutathione (GSH), glutathione disulfide (GSSG), redox potential of glutathione (E_nGSSG), mixed disulfide (CySSG), total cysteine (Cys + CySS), total glutathione (GSH + GSSG)) and clinical variables (smoking status, gender, high-density lipoprotein (HDL)) are analyzed from 500 healthy individuals.

understand variable contributions of multiple low-level environmental exposures to human health is considerable because of the variability of thousands of low-dose exposures in the presence of many behavioral, dietary, and drug exposures with similar or greater impacts. Low-dose Cd potentiation of airway hyper-reactivity provides an effective example. Airway hyper-reactivity contributes to asthma and other diseases impacting the lungs. Addition of Cd to drinking water at levels that result in lung Cd levels similar to values in nonsmoking, non-occupationally exposed individuals caused an increase in reactivity measured by methacholine challenge (Chandler et al., 2016). Gene expression arrays with validation by real-time PCR showed that neuronal receptors represented by enriched olfactory, glutamatergic, cholinergic, and serotonergic gene sets were major inducible targets of low-dose Cd. Olfactory receptor gene sets were the most enriched, and these regulate chemosensory function and airway hypersensitivity. HRM showed that metabolites in pathways of glutamatergic (glutamate), serotonergic (tryptophan), cholinergic (choline), and catecholaminergic (tyrosine) receptors were also increased in the lung tissue. The glutamate receptor GRIN2A was found to be increased by Western blotting. Thus, the results clearly show that integrated omics can be very powerful to identify contributions of low-dose exposures to disease mechanisms.

The availability of integrated omics methods as described above raises the possibility that animal models can be used to address drug and environmental contributions to multimorbidities. Barnett et al. (2012) showed that most human diseases do not occur in isolation, but instead, multimorbidity is the most common disease process. By performing controlled low-level exposures in animal models, detailed analyses can be used to create network structures, which can be used to interpret complex human data.

As an example, one can consider potential benefits from integrated omics studies of interorgan toxicities from low-level Cd exposure. NAFLD, defined by hepatic steatosis without significant alcohol consumption, steatogenic medication, or hereditary disorders, includes a spectrum of disease ranging from a relatively benign deposition of fat (NAFL) to fat deposition with inflammation and fibrosis, termed nonalcoholic steatohepatitis (NASH) (Chalasani et al., 2012). NAFLD is common among Americans (28.8 million in 1988–1994 NHANES III (Lazo et al., 2013)) and is generally associated with obesity, diabetes mellitus, and dyslipidemia. Because obesity and diabetes are increasing, there is an expectation for a continuing increase in NAFLD in the coming decades (Michelotti et al., 2013). NAFLD is associated with higher overall and liver-related mortality in the general US population, and liver disease is a significant cause

of death among persons with NAFLD (Ong et al., 2008). Of particular concern, the prevalence of suspected NAFLD has more than doubled over the past 20 years in adolescents (Welsh et al., 2013), potentially foreboding a health crisis in coming decades.

Low-level Cd causes fatty liver in mice, and human epidemiological data also show that after multivariate correction for other factors such as smoking, the top quartile of exposures in NHANES III (cutoffs 0.65 and 0.83 $\mu\text{g/g}$ urinary creatinine for men and women, respectively) was associated with increased NAFLD, NASH, and risk of liver disease mortality (Hyder et al., 2013). Environmental contamination of food is the major source of Cd exposure for nonsmokers; daily intake of Cd for adult males and females is about 0.35 and 0.30 $\mu\text{g/kg}$, respectively (ATSDR, 2012b). The European Food Safety Authority recommended a tolerable intake of 2.5 $\mu\text{g/kg/week}$ (Authority, 2011), or 0.35 $\mu\text{g/kg/day}$, which approximates average Cd intake by US adults. Cd is not effectively excreted and accumulates in humans (10–35 years half-life) (Goyer, 1997; Peters et al., 2010).

The integrated redox proteomics and metabolomics data described above show that Cd impacts liver mitochondrial function and dyslipidemia. Evidence from several studies implicates mitochondrial dysfunction in NAFLD. Cd also contributes to pancreatic islet dysfunction, also likely due to mitochondrial dysfunction (Chang et al., 2013). The frequent occurrence of NAFLD with obesity and type 2 diabetes suggests common underlying mechanisms.

Application of integrated omics methods to plasma and multiple tissues (e.g., liver, pancreas, and adipose tissue) could provide the information whether low-dose Cd has similar impact. Studies of mitochondria from these tissues in Cd-treated mice could then be used to define common plasma markers reflecting sensitive redox proteomics, metabolomics, and transcriptomics responses. The results would considerably enhance understanding of potential multimorbidities linked to a common environmental agent. Such an approach could also be useful for drug-induced mitochondrial toxicities and extended to studies of aging and other at-risk conditions.

42.8 Summary and Perspective

Mitochondria are the powerhouse of most mammalian cells, and this has often led to a simplistic view that mitochondrial metabolomics can be assessed by measurement of a relatively small number of respiration-dependent metabolites. Energy production is only a fraction of the range of metabolism and function supported by mitochondria, and a broader view is needed to effectively address the contribution of mitochondrial

dysfunctions to disease. Plasma is a readily accessible body fluid, but its use to measure disease processes in specific organs is often limited by its interaction with all organ systems. On the other hand, mitochondria are present in most tissues, and toxic effects on mitochondria may simultaneously occur in many tissues. HRM of liver mitochondria show that thousands of metabolites are present, and conservative comparison of plasma and liver enzymes and metabolite contents provides a short list of candidate plasma markers of mitochondrial function. Studies of low environmental levels of Cd, Mn, and other environmental chemicals show disruption of mitochondrial function. Innovative approaches to combine HRM data with redox proteomics and transcriptomics provide a comprehensive approach to study mitochondrial signaling in response to dietary and environmental stresses. The approach has been found to be useful to measure both toxic and adaptive hub responses, and this has important implications for all aspects of drug and environmental mitochondrial toxicities. Prototype software has been developed to enable combined **RMTWAS** to detect mitochondrial oxidative

signaling pathways. Such software could be made available as an online tool to facilitate use for sensitive detection of mitochondrial signaling in response to environmental exposures (Uppal et al., <https://kuppall.shinyapps.io/xmwas/>). The progress in application of integrated omics approaches to understand toxicologic mechanisms suggests that the approach can be extended to complex interorgan disease processes, such as NAFLD linked to obesity, type 2 diabetes, and neurodegeneration. Such application to controlled mouse models with simultaneous studies of different organ systems could considerably enhance understanding of the contribution of environmental exposures to multimorbidities.

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43

Detection of Mitochondrial Toxicity of Environmental Pollutants Using *Caenorhabditis elegans*

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CHAPTER MENU

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43.1 What We Know about Pollutant Influences on Mitochondria

43.1.1 Introduction

A growing area of concern, and one that has been informed by pioneering pharmaceutical research, is the possibility that environmental pollutants or other environmental stressors may also cause mitochondrial toxicity (Brunst et al. 2015; Caito and Aschner 2015; Meyer et al. 2013). Although environmental pollutants rarely reach concentrations in people that are comparable with those achieved as part of a drug dosing regimen, there is cause for concern. First, there are clear cases of pollutants that are mitotoxics, as discussed in Section 43.1.2. Second, there are very large numbers of pollutants—close to 100,000—that are in the jurisdiction of the US Environmental Protection Agency (EPA) (Bell and Edwards 2015; Judson et al. 2009), and many can be detected in people (Porta 2012, Park et al. 2014, and chapter 47). Third, chemicals intended for industrial use, or produced accidentally, are not tested for toxicity nearly as rigorously as are drugs. If we were slow to realize the mitochondrial impacts of drugs—which are tested relatively carefully in preclinical and clinical trials, and

whose use and effects are carefully tracked and therefore easier to study epidemiologically—what pollutant-induced mitochondrial impacts might we be missing?

There are a number of theoretical reasons why we might expect mitochondria to be vulnerable to toxicant exposure, as recently reviewed (Caito and Aschner 2015; Meyer et al. 2013; Shaughnessy et al. 2014). These include:

- 1) The lipid-rich double membrane, which attracts lipophilic compounds
- 2) The presence of uptake pathways (channels and transporters) for essential metals such as calcium that may also take up toxic metals (Bridges and Zalups 2005)
- 3) The ability of compounds that are lipophilic but ionizable in the relatively high pH of the matrix to therefore be bioaccumulated in the matrix (Ross et al. 2005)
- 4) The normal production of ROS by the electron transport chain, which can be increased greatly under conditions such as inhibition of electron transport that hyper-reduce the ETC (Drose and Brandt 2012; Murphy 2009) or by the presence of redox cycling agents that can be reduced in the mitochondria (e.g., (Lopert and Patel 2016; Monks et al. 1992; Robb et al. 2015), but note that many of these chemicals can also redox cycle in the cytosol)

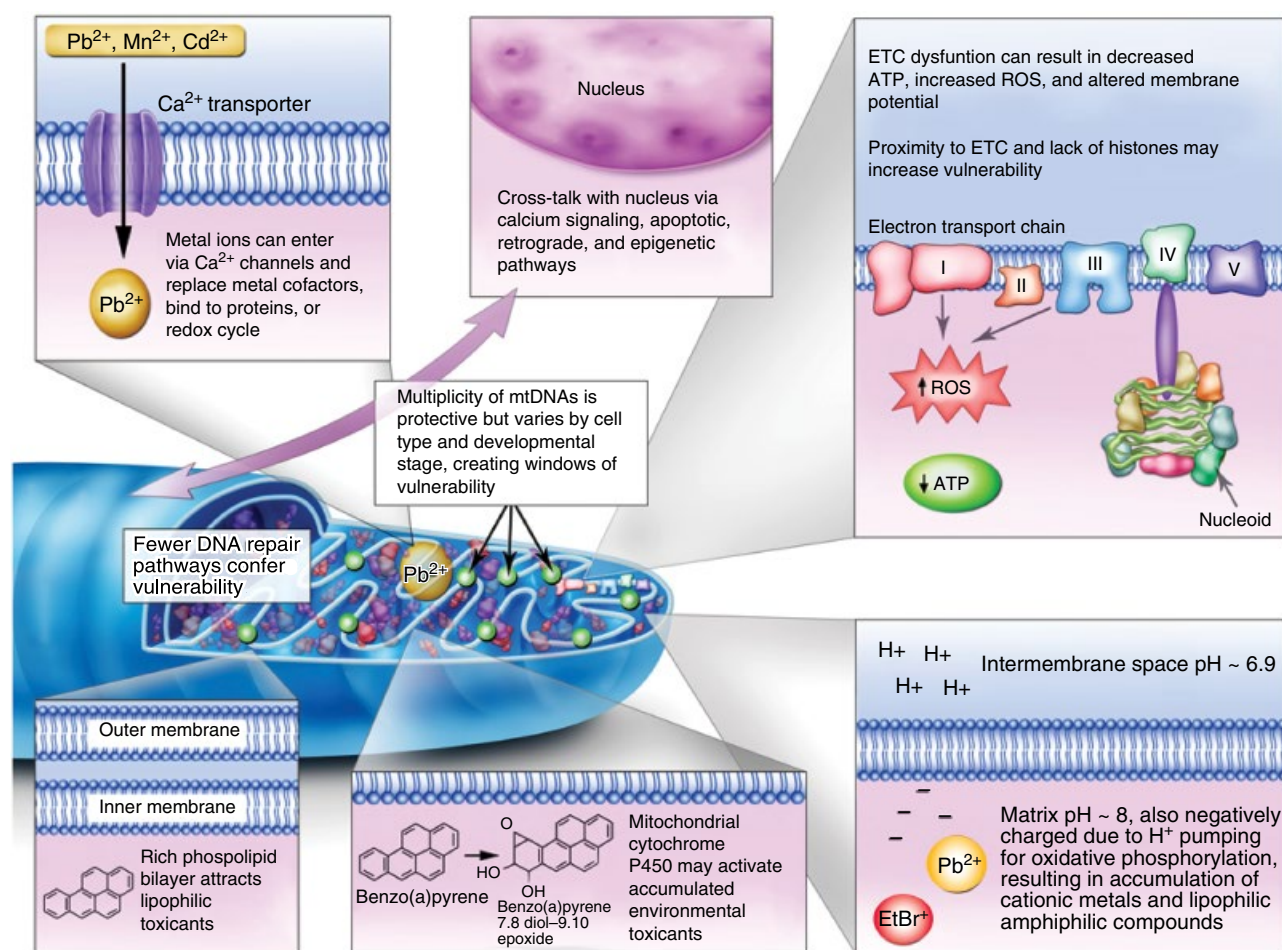


Figure 43.1 Theoretical reasons for mitochondrial sensitivity to exposures to environmental chemicals. Meyer et al. (2013). Reproduced with permission of Oxford University Press. (See insert for color representation of the figure.)

- 5) The presence of cytochrome P450 (CYP450) enzymes that can activate chemicals that are nonreactive in their “parent” form (see Chapter 7)
- 6) mtDNA vulnerabilities resulting from close association to sites of ROS generation and absence of some key DNA repair pathways (Alexeyev et al. 2013; Copeland and Longley 2014; Meyer et al. 2013; Scheibye-Knudsen et al. 2015; Van Houten et al. 2016)
- 7) The presence of large amounts of endogenous molecules that may exacerbate toxic reactions, such as iron (which can participate in Fenton chemistry) and cytochrome *c* (which can trigger apoptosis), and easily oxidized lipids, such as cardiolipin, which can mediate cytochrome *c* release and thus apoptosis (Czerniczyniec et al. 2013; Fariss et al. 2005)

However, it should be noted that in some cases, these processes may be protective, for example, resulting in appropriate apoptosis, mitophagy, or activation of antioxidant or other ROS-induced gene regulation

(Kagan et al. 2015) (Figure 43.1; reprinted with permission from (Meyer et al. 2013)).

In addition to these theoretical considerations, recent empirical evidence from screening efforts from the US EPA and National Toxicology Program (NTP), as well as a growing body of academic research, suggests that there are indeed many pollutants that affect mitochondria. Mitochondrial toxicity is rapidly emerging as a mode of action of many toxicants (Brunst et al. 2015; Meyer et al. 2013; Moreira et al. 2011; Pereira et al. 2009; Sabri 1998; Shaughnessy et al. 2010) and is one of the most common outcomes of *in vitro* toxicity screening efforts, including those of the NTP (Attene-Ramos et al. 2013, 2015; Houck et al. 2009; Shah et al. 2015; Wills et al. 2015). In this chapter, we summarize some of what is known about environmental mitotoxins and mechanisms of pollutant-induced mitochondrial dysfunction, outline some of the challenges and factors that complicate the research community’s ability to address this concern, and discuss the use of the nematode model organism *Caenorhabditis*

Table 43.1 Some important mechanisms of mitochondrial toxicity and representative environmental pollutants.

Mechanism of mitochondrial toxicity	Representative chemical or mixture
Mitochondrial DNA damage	Benzo[<i>a</i>]pyrene and some additional polycyclic aromatic hydrocarbons, mixtures such as creosote and air pollution, ultraviolet radiation, some aflatoxins, prooxidants such as paraquat
Protein inhibition (electron transport chain complex and other proteins)	Arsenite, lead, mercury, methylmercury, silver, cadmium, rotenone, pyraclostrobin, 3-nitropropionic acid
Redox cycling	2,4,6-Trinitrotoluene, pentachlorophenol, copper, iron, manganese, various quinones
Mitochondrial uncoupling	2,4-Dinitrophenol, pentachlorophenol, chlorfenapyr, arsenate

Note that each “mechanism” listed could in fact be further subdivided: for example, there are multiple kinds of DNA damage, and inhibition of different proteins has very different effects. Similarly, uncoupling can occur via proton transport by compounds like pentachlorophenol across the inner mitochondrial membrane or by ATP synthase-mediated addition of arsenate to ADP, generating an unstable molecule in which the ADP-arsenate bond quickly breaks; in both cases, but by different mechanisms, oxygen consumption is uncoupled from ATP production.

elegans to study mitochondrial toxicity, including a review of the mitochondrial biology of this organism, mitochondrial assays available, strengths and limitations of *C. elegans*, and select mitochondrial toxicity discoveries.

43.1.2 Environmental Mitotoxicants

There are a number of very well-known and specific mitochondrial poisons that exist in the environment, such as cyanide, carbon monoxide, and rotenone, which are discussed in general toxicology textbooks and do not need to be listed again. Perhaps the most interesting thing about them is the fact that many are plant, bacterial, or fungal toxins that target mitochondria via a variety of classes of chemical structure (e.g., rotenoids, antimycin A, cyanogens, oligomycin, monofluoroacetic acid, etc.) and mechanisms of toxicity (in these cases, inhibitors of ETC complexes I, III, IV, and V and the tricarboxylic acid (TCA) cycle, respectively) (Acamovic and Brooker 2005; Harborne 1988). The fact that multiple species throughout evolution have independently developed different toxicants that target mitochondria provides strong evidence for the sensitivity of organisms to mitochondrial toxicity.

Most of the toxins listed in the preceding paragraph are highly specific—so much so, in fact, that many are used in assays of mitochondrial function (e.g., Chapter 15)! However, relatively few cause toxicity in humans, except in unusual cases. Of greater human health concern is the very large number of chemicals to which we are exposed on a regular basis (Lioy and Rappaport 2011; Rappaport 2012; Rappaport et al. 2014) (see Chapter 41 of this volume for a discussion of the human “exposome”). Many of these may have some mitochondrial effects, but we anticipate that relatively

few will be truly specific in the sense that they target *only* mitochondrial function. For example, in Table 43.1, we have listed a number of important mechanisms of mitochondrial toxicity, along with representative environmental contaminants (note this is meant to be an illustrative, not comprehensive, list of chemicals and of mechanisms). Of these, 2,4-dinitrophenol is a specific mitochondrial uncoupler (Grundlingh et al. 2011), but many clearly have extramitochondrial targets and/or multiple different mitochondrial targets. For instance, metabolites of benzo[*a*]pyrene and aflatoxin B₁ cause a high level of DNA damage in the mitochondrial genome, and such damage cannot be repaired (in contrast to nuclear DNA damage), yet they and compounds that cause similar kinds of DNA damage clearly have very important effects via nuclear DNA damage, including mutations and carcinogenesis (Luch 2005; Smela et al. 2001). This is also true of shortwave ultraviolet (UV) radiation (Kasiviswanathan et al. 2012; Kozma and Eide 2014; Tulah and Birch-Machin 2013). Similarly, arsenic targets mitochondria (Prakash et al. 2016) and inhibits not only multiple mitochondrial enzymes (Bergquist et al. 2009; Hosseini et al. 2013; Naranmandura et al. 2011) but also many non-mitochondrial enzymes (Kitchin and Wallace 2008). The same is true of other metals that act by binding sulfhydryl and selenyl residues and affecting protein function, such as methylmercury (Antunes dos Santos et al. 2016)—and may in fact be true of all such metals. Arsenic is also an interesting example, as trivalent arsenicals act by protein inhibition, while pentavalent arsenate uncouples ATP production from oxygen consumption by mimicking phosphate during ATP production, resulting in an unstable ADP-arsenate bond that quickly breaks (Nemeti et al. 2010). Manganese is another example of an element that is mitotoxic via a wide range of mechanisms, including dopamine oxidation resulting in quinone formation, ETC inhibition, metal substitution, and altered

calcium homeostasis (Farina et al. 2013). Dioxin exerts the bulk of its toxic effects via binding to the aryl hydrocarbon receptor (AhR), and it is now clear that one of the many outcomes of AhR activation is mitochondrial toxicity (Shen et al. 2005). Thus, mitochondria are likely one (but not the only) target of many other AhR agonists. This list could go on; *the point we wish to emphasize is that the mitochondrial impacts of many chemicals can be important, even when the chemicals do not cause effects exclusively in mitochondria.* This does, of course, complicate dissection of mechanisms of action.

It would make little sense to try to summarize all the pollutants for which there is some evidence of mitochondrial effects. This would be a long list, yet would be out of date very quickly given how quickly research in this area is growing. We also note that in many cases, the evidence for mitochondrial toxicity as a primary effect, as opposed to a secondary effect resulting from toxicity in another cellular compartment, is weak (e.g., a decrease in membrane potential was observed, but it was not clear whether this was the primary effect of the exposure or whether it resulted from toxicity initiated at another cellular site). Finally, thousands of new chemicals are introduced to the environment annually, and in some cases entirely new classes of materials, such as nanomaterials, are developed. Therefore, any strategy we choose should address these as well. We now move to a discussion of strategies for testing potential mitotoxicity of environmental exposures and stressors.

43.1.3 How Should We Prioritize Environmental Chemicals and Stressors for Mitotoxicity Testing?

How should we prioritize which of the approximately 100,000 pollutant chemicals to test for mitochondrial effects? One approach is theoretical; for example, we have proposed that silver nanoparticles, designed to target microbes that are phylogenetically related to mitochondria, may therefore also target mitochondria (Maurer and Meyer 2016). Another approach is high-throughput screening, which we address in more detail later on. A final approach is to focus on chemicals or chemical mixtures that are used or occur at high levels, or to which very large numbers of people are exposed. For instance, we have investigated mitochondrial impacts of arsenic in part because arsenic exposure is so widespread (described as constituting the “greatest mass poisoning in human history” (Sen and Biswas 2013)) and methylmercury because of high levels of exposures resulting ultimately from burning of fossil fuels and small-scale gold mining (Futsaeter and Wilson 2013). Among the greatest environmental health impacts are those associated with air pollution (Brauer et al. 2012;

Gall et al. 2013; Smith et al. 2014). Both cigarette smoke (Ballinger et al. 1996; Fuller et al. 2012) and air pollution (Clemente et al. 2016; Grevendonk et al. 2016) cause mitochondrial toxicity, and in fact, in both cases, there is evidence that multiple different components of these very complex mixtures independently affect mitochondria, including particulate matter (PM) (especially ultrafine or “nano”-PM) (Li et al. 2015; Soberanes et al. 2009; Xia et al. 2004, 2007), polycyclic aromatic hydrocarbons (Backer and Weinstein 1980; Jung et al. 2009), acrolein (Mohammad et al. 2012), quinones (Knecht et al. 2013; Palmeira and Wallace 1997; Xia et al. 2004; Zhu et al. 1995), 2,4-dinitrophenol (Belloli et al. 2006; Harrison et al. 2005; Tremp et al. 1993), 2-ethylpyridine (Mansoor et al. 2014), nitrosamines (Bodhicharla et al. 2014; Braunbeck et al. 1992; Stepanov and Hecht 2009), and multiple metals (Cannino et al. 2009). Pesticides are widely used environmental chemicals, and there is evidence that many pesticides target mitochondria, including some organophosphates and organochlorines (Du et al. 2015; Gupta et al. 2006; Karami-Mohajeri and Abdollahi 2013; Lakroun et al. 2015) as well as well-studied examples such as rotenone and paraquat. Chemicals of emerging concern such as flame retardants (Behl et al. 2015; Jarema et al. 2015; Oliveri et al. 2015), triclosan (Shim et al. 2016; Weatherly et al. 2016), and newer-generation pesticides (Regueiro et al. 2015; Romero et al. 2010) have also been associated with mitochondrial toxicity, although the evidence is less comprehensive at this time. One difficulty is that the extent of exposure is not always well known; for example, there is exposure data (even defined very broadly, i.e., production volume) for less than 20% of the approximately 100,000 chemicals that fall under EPA’s mandate (Egeghy et al. 2012)! Another important challenge is understanding potential synergistic effects of mitochondrial toxicants (reviewed by Cedergreen (2014)), especially given the fact that mechanisms of mitochondrial toxicity can be quite disparate (Table 43.2).

43.1.4 Mechanistic Organization of Mitotoxic Effects

As we carry out mitotoxicity testing, we propose that just as gene ontologies have been used successfully to organize genes and gene products by biological roles, it will likely be helpful to organize chemicals by *how* they affect mitochondria. There are multiple ways in which mitochondrial “mechanisms of toxicity” could be organized. In Table 43.1, we used molecular-level events, but another very logical approach would be to focus on cellular or biological processes (e.g., ATP production, heme synthesis, calcium buffering, apoptosis, etc.). The recent advent of the “adverse outcome pathway” approach in toxicology

Table 43.2 Cellular and tissue-specific exposure route differences in physiological systems targeted by environmental mitotoxics in mammals and *Caenorhabditis elegans*.

	Mammalian		<i>C. elegans</i>	
	Major tissues	Tissue-specific cellular components	Major tissues	Tissue-specific cellular components
Nervous system (inhalation, ingestion, dermal exposure routes)	Brain Spinal cord	Neurons Astrocytes Oligodendrocytes Microglia Schwann cells	Nerve ring	Neurons Sensory and synaptic glia (glial system reviewed by Oikonomou and Shaham (2011))
Circulatory system (inhalation, ingestion, dermal exposure routes)	Heart Blood vessels	Cardiomyocytes Epicardial cells	Pharynx (developmentally similar to vertebrate heart (Haun et al. 1998))	
Respiratory system (inhalation exposure route)	Lungs Bronchi Trachea Larynx Pharynx	Type I and II pneumocytes Nonciliated bronchiolar ciliary cells	N/A	Pharynx

Mitochondrial toxicity can manifest as a result of multiple exposure routes and target tissues. However, there are important physiological differences between humans and *C. elegans* in the three “systems” discussed in the text as mitotoxics targets. This table describes some of the constitutive tissues and specialized cell types present within these exposure routes and target tissues in mammals that are absent in *C. elegans*.

offers another approach. AOPs offer a way to group chemicals based on common action and organismal outcome (Ankley et al. 2010; Villeneuve et al. 2014), which may be helpful for testing and regulating a very large number of chemicals (Edwards et al. 2016; Villeneuve 2015). However, work on mitochondrial AOPs is nascent, with only one relatively narrowly defined (“a consequence of inhibition of the respiratory chain leading to oxidative stress”) “mitochondrial dysfunction AOP” listed on the Wiki (<https://aopwiki.org/wiki/index.php/Event:177>). Understanding these mechanisms is critical to logically organize data, to inform us regarding which assays might be most predictive of *in vivo* toxicity by facilitating mechanism-based extrapolation, and, possibly, to develop biomarkers of mitochondrial toxicity in people. These would be of great use in epidemiological studies, which provide a critical complement to laboratory-based toxicity studies (Adami et al. 2011). Biomarkers are particularly useful in epidemiological studies of exposure to environmental chemicals as opposed to drugs, because exposure is typically much harder to quantify than it is for drugs that are prescribed and used relatively carefully. Proposed biomarkers include oxidized lipids (Roede and Jones 2010), mtDNA damage (Gonzalez-Hunt et al. 2016; Haugen et al. 2010; Sanders et al. 2014), mtDNA copy number (Brunst et al. 2015; Gebhard et al. 2014; Gonzalez-Hunt

et al. 2016; Kim et al. 2014), mtDNA methylation (Brunst et al. 2015), metabolomics (Chapter 27), and altered energetics (Chacko et al. 2014). Incomplete understanding of the biological regulation and significance of some of these outcomes remains challenging; for example, exposures have been reported to both increase and decrease mtDNA copy number, and the biological impact of methylation in the mtDNA remains unclear.

43.1.5 Testing Environmental Chemicals and Stressors for Mitotoxicity: Approaches and Considerations

Finally, what tests should we actually conduct? This text and others (Pon and Schon 2007) offer a wide range of mitochondrial assays. However, there are far too many environmental chemicals to permit careful mechanistic testing of them all. Current high-throughput screening efforts utilizing cell culture are focused on mitochondrial membrane potential and ATP content (Attene-Ramos et al. 2015), with follow-up in some cases by examination of oxygen consumption (Attene-Ramos et al. 2013) and/or medium-throughput, whole-organism work using organisms such as *C. elegans* and *Danio rerio* (Behl et al. 2015). In some cases primary cell lines have been used (Wills et al. 2015), which have much

more normal mitochondrial function than most immortalized lines. Not all have incorporated advances in understanding from the pharmaceutical field, such as the use of galactose in cell culture (Marroquin et al. 2007 and chapter 20). Ideally, assays should permit testing in the context of normal intercellular interactions, which can modulate or even mediate toxic effects. In addition, assays should incorporate important variables that can modify sensitivity (reviewed by (Meyer et al. (2013)). These include developmental stage, with early and late life postulated as sensitive windows of exposure; cell type and tissue differences, with post-mitotic and high-energy use cells potentially at most risk; genetic background with individuals with differences in genes involved in mitochondrial function or mitochondrial homeostasis likely at most risk (mitochondrial toxicity is likely to be significantly exacerbated in ~1 in 4000 persons who suffer from a mitochondrial disease (Chinnery et al. 2004; Dimauro and Davidzon 2005; Howell et al. 2005; Wallace 2005)); and physical condition including exercise, diet, and disease.

A further critical distinction is the difference between short-term high-level poisoning-type mitotoxicity and longer-term low-level exposures that could be chronic or intermittent or may occur at particularly critical developmental stages. We argue that the latter are more likely to be of importance to chronic and degenerative diseases, which are the major health threat of the future. For example, mitochondrial dysfunction may be important in neurodegenerative diseases (Farina et al. 2013; Kaminsky et al. 2015; Kubik and Philbert 2015), cancer (Robey et al. 2015; Salem et al. 2013), diabetes and metabolic syndrome (Kim and Lee 2014 and chapter 49), and cardiovascular disease (Finsterer and Ohnsorge 2013). While this is a relatively new area of inquiry, we and others have found that exposures leading to altered mitochondrial function early in development may lead to lifelong effects on mitochondrial function and related outcomes (Ditzel et al. 2016; Leung et al. 2013b; Wood et al. 2015), which may thus form an important subset of the health impacts of developmental exposures that can cause adult diseases (Birnbaum and Miller 2015). There is also evidence for multigenerational effects of mitochondrial exposures, and potentially even transgenerational effects of mitochondria dysfunction that are presumably mediated by epigenetic changes (Saben et al. 2016), which may themselves in some cases be causally related to mitochondrial function (Audano et al. 2014; D'Aquila et al. 2015; Shaughnessy et al. 2014). These changes may be both adaptive ("mitohormetic" is one term used to describe this process: (Naviaux 2014)) and maladaptive, depending on the context, and the potential for adaptation is itself likely affected by genetic background and timing of exposure.

There are multiple model systems available for mitotoxicity testing (many described in this text). In the succeeding text, we describe the strengths offered by *C. elegans*, a model system that combines relatively fast and affordable screening-type toxicity tests in a full-life history, *in vivo* model with powerful genetic and molecular tools for mechanistic work. We also address limitations of this model organism for mitochondrial toxicity, assays that are available, and some discoveries that have been made using *C. elegans*. We feel that no model system is sufficient by itself and hope that this chapter will facilitate the research community's incorporation of *C. elegans* into a spectrum of mutually complementary model systems.

43.2 Advantages of the *Caenorhabditis elegans* Model

43.2.1 Introduction

First developed by Sydney Brenner over 50 years ago as a model to study developmental and neurobiology, the nematode *C. elegans* has since emerged as an *in vivo* model for toxicologists (reviewed in Leung et al. (2008), Hunt (2016), Tejeda-Benitez and Olivero-Verbel (2016)). This species' small size (adults are ~1 mm in length), short developmental period (first larval stage to gravid adult in ~72 h) and lifespan (2–3 weeks), high reproductive rate (~300 offspring per hermaphrodite), ease of genetic manipulation, and low maintenance costs make *C. elegans* amenable to high-throughput toxicity testing. Many of these same advantages also make this species an attractive model for investigating mitochondrial toxicity *in vivo*. An *in vivo* model is advantageous because it preserves developmental stage- and tissue-specific differences in mitochondria, as well as intracellular signals typically lost *in vitro*, which is important as these signals can influence mitochondrial function. Although nematodes lack many of the well-defined organs found in mammalian models, they do have many well-defined tissues, including the cuticle, excretory system, gonad, hypodermis, intestine, muscles, neurons, and pharynx. These tissues can signal one another to control diverse biological processes such as mitochondrial metabolism, stress response, and aging (Bishop and Guarente 2007; Durieux et al. 2011; Libina et al. 2003; Taylor et al. 2014), thus demonstrating the importance of investigating mitochondrial function *in vivo*. In the succeeding text, we further highlight some of the biological and genetic advantages that make *C. elegans* an excellent *in vivo* model for investigating mitochondrial toxicity.

43.2.2 *C. elegans* Biology

Over the past 50 years, a great deal has been learned about *C. elegans* development, cell signaling, reproduction, and many other biological processes (reviewed in Corsi et al. (2015)). Furthermore, many significant scientific processes, including apoptosis (Hedgcock et al. 1983), miRNA (Lee et al. 1993), and RNA interference (RNAi) (Fire et al. 1998), have been discovered or mechanistically dissected in *C. elegans*. Notably, the lineage of all 959 somatic cells in adult hermaphrodites has been completely mapped (Kimble and Hirsh 1979; Sulston and Horvitz 1977; Sulston et al. 1983), while *C. elegans* also boasts the most comprehensively mapped neural network of any organism (Jarrell et al. 2012; White et al. 1986) (cell lineage and neural network visualized at www.wormatlas.org and www.wormweb.org). This broad understanding of nematode development and anatomy enables toxicologists to identify toxicant-induced developmental abnormalities. Of particular relevance to mitochondrial effects, a detailed understanding of neural networks and visualization of neuronal subtypes via GFP expression has allowed toxicologists to associate neurodegeneration with well-defined behavioral changes (Firnhaber and Hammarlund 2013).

C. elegans was also the first multicellular organism to have its genome sequenced and annotated (Consortium 1998). Although relatively small in size (100Mb, ~1/30 of the human genome), the *C. elegans* genome contains 20,444 protein-coding genes, 60–80% of which have human homologues (Corsi et al. 2015; Henricson et al. 2004; Kaletta and Hengartner 2006), while 40% of human disease genes have *C. elegans* orthologues (Culetto and Sattelle 2000). This high genetic homology has allowed researchers to investigate disease etiology and provides human relevance for gene–environment interaction studies. Furthermore, transparent nematodes were the first organism engineered to use GFP as a biological marker of gene expression (Chalfie 1994), a tool allowing researchers to characterize tissue-specific gene expression patterns throughout larval development and aging (expression patterns available at <http://gfpweb.aecom.yu.edu/index>, <https://transgeneome.mpi-cbg.de/transgeneomics/index.html>, while www.wormbase.org contains information on genome annotation, gene expression, and phenotypes associated with thousands of mutations). This robust understanding of *C. elegans* biology, combined with numerous online resources, provides toxicologists with the fundamental knowledge to investigate chemical-induced toxicity.

43.2.3 Mitochondrial Biology in *C. elegans*

As in mammals, mitochondria play many critical roles throughout the nematode lifespan, and mitochondrial function is highly conserved between *C. elegans* and

humans. The *C. elegans* mitochondrial genome is 13,769 bp in size (16,595 bp in humans) (Okimoto et al. 1992) and encodes 22 tRNAs, 2 rRNAs, and 12 ETC subunits (ATP8 is unconfirmed but may also be present (Breton et al. 2010)). Overall, it is estimated that each somatic cell in adults contains an average of 45–70 copies of mtDNA (Tsang and Lemire 2003). This value is considerably less than in human cells, which can contain hundreds to thousands of copies depending upon cell type and energetic demands. However, how mtDNA copy number varies by cell type in *C. elegans* remains unknown, except that copy number is higher in germ cells and oocytes (Leung et al. 2013b; Tsang and Lemire 2003). Nuclear-encoded subunits of the ETC are also highly conserved in *C. elegans* (Tsang and Lemire 2003), as is overall ETC structure and function (Murfitt et al. 1976). In addition to a fully functioning ETC, a complete Krebs cycle is also present, and fatty acid oxidation, glycolysis, and gluconeogenesis are also highly conserved (Kühnl et al. 2005; Murfitt et al. 1976; O’Riordan and Burnell 1989; Wadsworth and Riddle 1989; Zhang et al. 2013). Mitochondrial homeostasis pathways such as the unfolded protein response (Durieux et al. 2011), mitodynamics (fusion/fission) (Gandre and Van Der Blik 2007; Luz et al. 2015a), mitophagy (Palikaras et al. 2015a), mitochondrial antioxidant defenses (Hunter et al. 1997; Paek et al. 2012), and other pathways (Liu et al. 2014) are also conserved. The high conservation of mitochondrial metabolism between humans and *C. elegans* and the fact that 80% of human genes associated with inborn errors in metabolism have *C. elegans* orthologues (Kuwabara and O’Neil 2001) have permitted the use of *C. elegans* as a model for numerous metabolic diseases, including mitochondrial diseases (Dancy et al. 2016; Maglioni and Ventura 2016), lysosomal storage disorders (De Voer et al. 2008), lipid metabolism disorders (Zhang et al. 2013), and neurodegenerative diseases (Alexander et al. 2014; Lu et al. 2014; Peterson et al. 2008).

Although many major metabolic pathways are conserved between humans and *C. elegans* (reviewed in Braeckman et al. (2009)), some key differences in intermediary metabolism exist. For example, *C. elegans* appear to lack the urea cycle, as no functional urea cycle enzymes have been identified (Falk et al. 2008). Furthermore, the *C. elegans* genome contains only a single uncoupling protein homologue, *ucp-4*, which does not function as a classical uncoupler, but instead plays a role in succinate transport (Pfeiffer et al. 2011). Finally, the glyoxylate pathway (a variation of the Krebs cycle not found in humans), which allows developing nematodes to convert energy stored as lipids into carbohydrates (Wadsworth and Riddle 1989), is present and has been implicated in playing an adaptive role in ETC complex I dysfunction by shifting electron flow to

complex II, thus reducing ROS generation through complex I (Morgan et al. 2015).

Much is also known about how mitochondrial function changes throughout the lifespan of *C. elegans* (reviewed in Tsang and Lemire (2003)). Embryos maternally inherit 25,000 copies of mtDNA, and mtDNA copy number remains constant until the fourth larval stage (L4) when a fivefold increase occurs, followed by a further sixfold increase in adulthood. This large increase in mtDNA copy number is associated with germline and oocyte development (Tsang and Lemire 2002). Glycolysis and the glyoxylate pathway have also been shown to be highly active in developing embryos as stored lipids are converted into carbohydrates for energy utilization; however, upon hatching and during the first larval stage (L1), glycolysis and glyoxylate pathway activity begin to decrease and continue to decrease into adulthood, while a concomitant increase in Krebs cycle activity and mitochondrial oxidative phosphorylation occur (Tsang and Lemire 2003; Wadsworth and Riddle 1989). Finally, mitochondrial function and antioxidant capacity have been shown to decline steadily throughout the aging process (Brys et al. 2007; Tsang and Lemire 2002; Vanfleteren and De Vreese 1996), while the frequency of mtDNA mutations and deletions increases throughout aging (Melov et al. 1994, 1995). These trends (maternal inheritance of mtDNA, high glycolytic activity throughout development, reduced mitochondrial function and antioxidant capacity with aging, increased mtDNA mutations with aging) in mitochondrial function are similar to trends observed in humans and other mammalian models, further demonstrating the conservation of mitochondrial function throughout the nematode lifespan.

43.2.4 Mutagenesis and Mutant Availability

Availability of *C. elegans* strains carrying mutations or deletions in genes of interest allows researchers to easily investigate gene function, disease etiology, or gene–environment interactions. The *C. elegans* community, the *C. elegans* Knockout Consortium (celeganskoconsortium.omrf.org), and the *C. elegans* National BioResource Project of Japan (shigen.nig.ac.jp/c.elegans/mutants/index.jsp) have collectively isolated over 6,700 deletion alleles (Kwon et al. 2012), while the Million Mutations Project has established 2,000 mutagenized strains carrying 800,000 unique single nucleotide variants, resulting in approximately eight unique mutations for each of the 20,000 genes in the nematode genome (Thompson et al. 2013) (strains available through the *Caenorhabditis* Genetics Center (cbs.umn.edu/cgc)). Wide mutagenic coverage of the nematode genome has allowed researchers to isolate and study myriad strains with mutations in genes that play crucial roles in diverse mitochondrial

processes, including oxidative phosphorylation, fission, fusion, mitophagy, iron homeostasis, antioxidant defense, and mtDNA replication and repair (Bratic et al. 2009; Feng et al. 2001; Luz et al. 2015a; Ventura et al. 2005; Yang and Hekimi 2010). Although knockout strains are not yet available for all genes in the *C. elegans* genome, numerous techniques for genome-wide or site-specific mutagenesis are available (reviewed in Kutscher and Shaham (2014)), which allows new mutant strains to be easily generated.

43.2.5 RNA Interference

Gene silencing via RNAi was first discovered in *C. elegans* nearly two decades ago (Fire et al. 1998). Since then, its application has expanded to numerous species, providing researchers with a tool to rapidly investigate the function of any gene. In *C. elegans*, RNAi gene knockdown can be accomplished via several techniques (reviewed in Ahringer (2005)), including feeding, injection, and soaking (Tabara et al. 1998; Timmons and Fire 1998). However, the delivery method and concentration of double-stranded RNA must be considered, as dilution of RNAi against several mitochondrial ETC genes has been shown to have a nonmonotonic effect on nematode lifespan (Rea et al. 2007). Currently, two *C. elegans* RNAi libraries are available for purchase (Kamath et al. 2003; Rual et al. 2004) and collectively cover 94% of the nematode genome. The overall ease of RNAi administration, and breadth of RNAi libraries, has enabled researchers to perform large genetic screens for genes that control mitochondrial morphology (Ichishita et al. 2008) and identified numerous mitochondrial genes as playing critical roles in nematode lifespan (Hamilton et al. 2005; Lee et al. 2003).

Interestingly, *C. elegans* neurons are RNAi insensitive, as they lack the transmembrane protein SID-1, which is required for passive cellular uptake of RNAi (Tavernarakis et al. 2000; Winston et al. 2002). This observation has led researchers to generate RNAi-insensitive SID-1 mutants, which have then been rescued using DNA arrays (discussed further in the succeeding text) encoding SID-1 under the control of tissue-specific promoters, allowing researchers to investigate the effect of tissue-specific gene knockdown. Tissue-specific RNAi-sensitive strains include pan-neuronal (Calixto et al. 2010); GABAergic, dopaminergic, glutamatergic, and cholinergic neurons (Firnhaber and Hammarlund 2013); and hypodermis, intestine, and muscle sensitive strains (Qadota et al. 2007). These strains have been utilized to demonstrate the cell-non-autonomous nature of ETC dysfunction-mediated effects on nematode lifespan and to investigate tissue-specific mitochondrial gene–environment interactions (Durieux et al. 2011).

43.2.6 DNA Transformation

Ease of genetic manipulation is one of *C. elegans*' greatest strengths as a model organism, and thousands of transgenic strains overexpressing or ectopically expressing a gene or recombinant DNA have been generated (reviewed in Evans (2006)). Historically, DNA transformation has been accomplished via microinjection of extrachromosomal DNA arrays into the nematode germline (Mello and Fire 1995; Mello et al. 1991); however, mitotic instability and germline silencing can result in loss of, or mosaic expression of, the array (Kelly et al. 1997). Chromosomal integration can help solve stability issues, and arrays can be randomly integrated using gamma or UV irradiation (Broverman et al. 1993), or MosSCI technology can be used to insert the construct into a known location within the genome (Frøkjær-Jensen et al. 2008, 2012). Alternatively, DNA transformation can be accomplished via gene bombardment using a gene gun, which offers the major advantage of allowing researchers to isolate integrated strains directly (Praitis et al. 2001); however, gene bombardment is expensive compared with microinjection. The two methods are reviewed in more detail in Evans (2006). More recently the CRISPR–Cas9 system has been used to edit the nematode genome and generate transgenic lines (Chen et al. 2013; Friedland et al. 2013).

The aforementioned methods have allowed researchers to generate a multitude of stable transgenic strains that can be used to investigate various mitochondrial parameters, including steady-state ATP levels (Lagido et al. 2008), mitochondrial morphology (Benedetti et al. 2006), and redox status (Back et al. 2012b), while GFP expression driven by a promoter of interest allows researchers to monitor the induction of mitochondrial antioxidant and proteostasis genes (Anbalagan et al. 2012, 2013). These transgenic tools are discussed further in Section 43.4.

43.2.7 *C. elegans*: A Model of Expanding Utility in Toxicology

Due to the numerous advantages discussed in this chapter, use of the *C. elegans* model has greatly expanded in toxicology (reviewed in Hunt (2016)). Numerous studies have found that LC₅₀ (the concentration of a chemical required to kill 50% of a given sample) ranking in *C. elegans* correlates well with both mouse and rat toxicity ranking for many metals (Hunt et al. 2012; Williams and Dusenbery 1988), organophosphate pesticides (Cole et al. 2004), 100s of diverse compounds from the ToxCast™ phase I and II libraries (Boyd et al. 2016), and numerous other water-soluble chemicals (Ferguson et al. 2010; Li et al. 2013). However, higher concentrations are

typically required in nematodes, which is likely due to the nematode cuticle, a collagenous barrier that can limit the uptake of bulky or ionized compounds (Au et al. 2009; Partridge et al. 2008; Watanabe et al. 2005), as well as robust protective mechanisms that allow nematodes to survive in a variety of harsh environments.

Given nematodes' small size, low maintenance costs, and ability to be predictive of mouse and rat LC₅₀ ranking, it is surprising that more high-throughput toxicity screening has not been performed using *C. elegans*. Feasibility of high-throughput screening in *C. elegans* has been elegantly demonstrated by researchers studying aging, in which large libraries (1,280–88,000) have been screened for compounds that can extend lifespan (Lucanic et al. 2016; Petrascheck et al. 2007, Ye et al. 2014; 2009), while other studies have screened (>1,200 compounds) for induction of mitochondrial *hsp-60* (Rauthan and Pilon 2015). More impressively, a 300,000-compound library was recently screened for antimicrobial activity in *C. elegans* infected with *Burkholderia thailandensis* (Lakshmanan et al. 2014), while a 364,000-compound library was screened for SKN-1 (NRF-2 homologue) inhibitors (Leung et al. 2011, 2013a). These studies have utilized lifespan and lethality assays, as well as various transgenic GFP reporter strains, further demonstrating the flexibility of the *C. elegans* model.

43.3 Limitations of *C. elegans* for Studying Mitochondrial Toxicity

43.3.1 Introduction

While there are numerous molecular and genetic parallels between *C. elegans* and higher-order organisms (Section 43.2), there are also differences that impose some limitations. These tend to be more significant at higher levels of biological organization (e.g., tissue and organ structure). Here, we discuss important differences that should be kept in mind when designing experiments in *C. elegans* to study disruption of mitochondrial functions by exposure to environmental contaminants. It is not within the scope of this chapter to review all known or possible limitations, but we highlight differences that may limit extrapolations from *C. elegans* to higher eukaryotes.

43.3.2 Genetic and Phylogenetic Differences

One of the most well-characterized and important classes of enzymes involved in the metabolism of xenobiotics is the CYP450 family (reviewed by (Coon (2005) and Guengerich (2008))). The *C. elegans* genome contains genes closely associated with mammalian genes for the

CYP2, *CYP3*, and *CYP4* enzyme families (Gotoh 1998). However, the genome appears to lack the *CYP1* gene family, which metabolizes classes of polycyclic aromatic hydrocarbons (PAHs) and aromatic amines and are under the transcriptional control of the AhR (Nebert et al. 2004). The lack of biologically active CYP1 enzymes has since been confirmed in *C. elegans* (Goldstone et al. 2007; Leung et al. 2010). This lack of CYP1 enzymes in *C. elegans* has direct implications for studying mitochondrial toxicity, since CYP1A1 and CYP1A2 localize to the mitochondrion in mouse (Senft et al. 2002), as does CYP1B1 (Dong et al. 2013). This limits the use of *C. elegans* for CYP1 enzymes and associated mitochondrial xenobiotic metabolism mechanisms, at least without the addition of transgenes. *C. elegans* is still potentially useful for other CYP classes within the CYP450 superfamily of enzymes, although not all CYP2, CYP3, and CYP4 enzymes are conserved; for example, CYP2E1 appears to be absent. In addition, some of the regulation of CYP as well as other xenobiotic metabolism genes is different in *C. elegans*; for example, while an AhR homologue is present, it does not appear to be a receptor for traditional xenobiotic ligands of the mammalian AhR (Powell-Coffman et al. 1998).

Mitochondrial fusion is an important function in maintaining mitochondrial networks. *C. elegans* mitochondrial fusion mutants (such as *fzo-1* and *eat-3*) provide a useful genetic background with which to study defects in mitochondrial fusion. However, there are fundamental differences in survival when homozygous mutations in mammalian mitofusins (Mfn1/Mfn2) and their homologue (*fzo-1*) in *C. elegans* are present: homozygous mutations in Mfn1/Mfn2 are embryonic lethal in mammals (Chen et al. 2003; Fyfe et al. 2011), but *C. elegans* survive throughout adulthood (Ichishita et al. 2008). These differences highlight roles of mitochondrial fusion mechanisms that are specifically essential for mammalian survival that are not represented in *C. elegans*. This is also true in an example of an enzyme important for mtDNA maintenance (Suetomi et al. 2013). Additionally, mtDNA biogenesis declines significantly during aging in *C. elegans* (Rooney et al. 2014), which would present challenges to studying mitochondrial-related aging mechanisms.

43.3.3 Biochemical Differences

C. elegans do not have a complete urea cycle (Falk et al. 2008) and excrete most ammonia via apical ammonia trapping and exocytosis (Adlimoghaddam et al. 2015), making it difficult to accurately study detoxification of hyperammonemic states caused by exposures in humans (e.g., occupational exposure to hydrazine (Zelnick et al. 2003)). The metabolism of certain antibiotics may also

be difficult to study in *C. elegans* in this regard; both hydrazine and ammonia are metabolites of isoniazid (metabolism and toxicity reviewed by Preziosi (2007)), and hydrazine itself has been shown to inhibit mitochondrial complex II activity in mammalian cells (Lee and Boelsterli 2014). The potential interactions between mitochondrial inhibition by hydrazine and urea cycle reactions housed within mammalian mitochondria are unclear. Thus, metabolism of a compound like hydrazine is an example of a drug metabolite/occupational exposure causing mitochondrial toxicity that may be difficult to study in *C. elegans* due to their differential detoxification of ammonia. Metabolic acidosis causes ammoniogenesis in isolated mitochondria, and the reaction is governed by phosphate-dependent glutaminase activity (Tannen and Kunin 1976). *C. elegans* contain an orthologue (*glna-2*) of the human mitochondrial glutaminase (GLS2) responsible for the observed ammoniogenesis (Hobert 2005); however, the specific activity of *glna-2* has yet to be determined. Thus, the detoxification mechanisms of environmental chemicals causing metabolic acidosis (and resultant mitochondrially induced hyperammonemia) may not be precisely modeled in *C. elegans*.

C. elegans lacks orthologues of heme synthesis enzymes (Jaffe 2003) and instead rely on nutritional sources of heme for biochemical processes (Rao et al. 2005). Because they do utilize heme for essential pathways, they may still provide a sound *in vivo* model for aspects of heme transport (Rajagopal et al. 2008), but not necessarily for chemical exposures that interfere with de novo heme synthesis, including both cytosolic and mitochondrially bound enzymes (Rao et al. 2005). For example, lead exposure in humans dramatically inhibits protoporphobilinogen synthase (PBGGS) (Chisolm et al. 1985; Hernberg et al. 1970; Jaffe et al. 1991), and circulating PBGGS activity is used as an early biomarker of acute lead exposure (Jaffe et al. 1991; Lawrence et al. 2013). Environmental and occupational exposures to contaminants other than lead including hexachlorobenzene (Schmid 1960), diazinon, dioxin, and arsenic (reviewed in Daniell et al. (1997)) can cause acute chemical-induced porphyria (a condition in which heme precursors are not converted to heme) in humans. Porphyria has recently been linked to mitochondrial respiratory failure in a rodent model of the condition (Homedan et al. 2014), demonstrating a link between heme synthesis mechanisms affected by common environmental contaminants and direct mitochondrial outcomes that could not be effectively investigated in *C. elegans*.

Additionally, *C. elegans* cannot synthesize sterols de novo from acetate (Rothstein 1968), and cholesterol itself does not appear to have an essential function in membrane structure in this organism (Kurzchalia and Ward 2003). In other organisms, including mammals and yeast,

sterols are important in providing rigidity to cellular membranes, and the relative sterol content in membranes influences cross-membrane transport and association with exogenous substances. The ratio of sterol content to other lipid content is low (1 : 30) for both inner and outer mitochondrial membranes (Bottema and Parks 1980), and increasing the cholesterol content in mitochondrial membranes is associated with membrane depolarization via cardiolipin peroxidation (Montero et al. 2010). Mitochondrial dysfunction associated with proteotoxicity is a well-documented intracellular pathology in neurodegenerative disease (reviewed by Hashimoto et al. (2003)), and sterol synthesis may play a role. In this way, *C. elegans* serves as a suitable model for investigation of some molecular pathways of neurodegenerative diseases that may result in part from environmental exposures, including Alzheimer's disease (AD) (reviewed by Alexander et al. (2014)) and Parkinson's disease (PD) (reviewed by Harrington et al. (2010)). However, more subtle mechanisms of disease development and mitochondrial toxicity in mammals may not be as readily accessible in *C. elegans*. For example, when sterol synthesis is inhibited in yeast, various splice isoforms of alpha-synuclein are more likely to associate within vesicles rather than on the plasma membrane, increasing its cellular entry and thus its relative downstream toxicity (Valastyan et al. 2014). Additionally, the presence of AD-associated amyloid- β 1-40 significantly decreases cholesterol synthesis *in vitro* (Koudinova et al. 1996). The demonstrated interaction of molecular hallmarks with AD and PD on endogenous sterol synthesis indicates that *C. elegans* may not be a suitable model for investigating environmental influences on these particular pathways of disease progression. This is especially important in terms of studying environmental exposures associated with neurodegenerative diseases, since only a small percentage of late-onset AD and PD cases are explained exclusively by genetics (Bertram and Tanzi 2005).

As an additional note, *C. elegans* exhibit high (relative to mammals) tolerance for both hypoxic (Powell-Coffman 2010) and hyperoxic (Yanase and Ishii 2008) conditions, which can potentially confound important oxygen-dependent mitochondrial mechanisms altered by environmental toxicants in this organism.

43.3.4 Physiological Limitations

While the use of *C. elegans* for examining important mitochondrial contributions to tissue-level toxicity research has expanded somewhat recently, it has been limited by species-specific differences in tissue composition and organization, cellular metabolic demands, and pharmacokinetic differences compared with more

complex mammalian species. A full comparison of mitochondrial differences in all cell types and tissues in *C. elegans* and humans is beyond the scope of this chapter. However, to illustrate ways in which tissue differences can limit applicability of the *C. elegans* model in some cases, we highlight specific characteristics of mitochondria in the brain, heart, and respiratory systems that may confer differential susceptibility to environmental mitotoxins. We focus on these tissues because of the particularly important role of mitochondrial function in these systems. Some key differences between mammals and *C. elegans* in cellular and tissue organization that may affect both routes of chemical uptake and target tissue effects are illustrated in Table 43.1. We note that this is not an exhaustive comparison; for example, differences in gut physiology and microbiome will affect uptake, and additional tissues (e.g., liver, kidney) are important targets of mitochondrial toxicity in humans, as evidenced by the fact that they are affected by mitochondrial diseases and mitotoxic drugs.

43.3.4.1 Brain Mitochondria

There is strong evidence for mitotoxicity as a mechanism of some forms of chemical-induced neurotoxicity and neurodegeneration (Farina et al. 2013; Kaminsky et al. 2015; Kubik and Philbert 2015). The brain is one of the most highly metabolic tissues in the body, accounting for 60% of the body's utilization of glucose in a resting state and simultaneously maintaining a very low metabolic reserve: if the glucose supply is exhausted, the brain relies on ketone bodies for energy (because unbound fatty acids do not cross the blood-brain barrier and are thus not used for energy substrates) (Berg et al. 2002). Energetic demands requiring proper mitochondrial function (and the subsequent mitochondrial responses to those demands) are not uniform between functionally and anatomically distinct brain regions (Kayser et al. 2016; Lee Do et al. 2013; Sundar Boyalla et al. 2011), nor between neural cell types (Motori et al. 2013; Sundar Boyalla et al. 2011; Voloboueva et al. 2007), and are temporally dynamic (Wirtz and Schuelke 2011). This is especially important during region-specific neural development in mammals, for example, as cerebellum-specific radial glial cells (Bergmann glial cells) provide an essential cellular scaffold utilized by Purkinje cells during migration, synaptogenesis, and neuronal maturation (reviewed by Yamada and Watanabe (2002)). Radial glial fibers extending from Bergmann glial cell bodies contain mitochondria that are metabolically independent from one fiber to the next and are essential in glial fiber-specific interactions with adjacent neurons (Grosche et al. 1999).

Differential regional and cellular responses in brain mitochondrial function exist in mammals in response to environmental exposures, such as those that cause

chemical hypoxia (Roemgens et al. 2011), naturally occurring elements such as manganese (Rao and Norenberg 2004; Sidoryk-Wegrzynowicz and Aschner 2013), nitrobenzene compounds (Kubik et al. 2015; Steiner and Philbert 2011), and polychlorinated biphenyls (PCBs) (Kodavanti et al. 1998). While *C. elegans* contain fully functional and well-characterized neuronal populations and subpopulations (GABAergic, dopaminergic, motor neurons, sensory neurons, etc.) and some glia (reviewed by Oikonomou and Shaham (2011)), they are far fewer in number, classification, and subsequent complexity in physiological connectivity compared with higher-order organisms (Table 43.1). The regional organization and cellular sophistication found in the mammalian brain and corresponding regional and cell-specific mitochondrial contributions to neural function that are impacted by environmental exposures are not physiologically parallel to the *C. elegans* neural system, rendering it an inadequate model for the study of such tissue-level relationships. However, our understanding of the role of glia in *C. elegans* may well keep expanding, and their relevance to human neural mitochondrial health may proportionally expand as a result.

43.3.4.2 Cardiac Mitochondria

Mitochondria within the heart are particularly important to discuss, as they represent a mitochondrial population within a highly metabolic tissue in mammals that is not present in *C. elegans*. There is evidence of cardiotoxicity mediated by mitochondrial effects of a wide range of chemicals (Finsterer and Ohnsorge 2013). Of note, there are developmental parallels between pharyngeal cells in *C. elegans* and vertebrate heart (Haun et al. 1998); however, mechanistic links between mitochondrial dysfunction in specific cellular subtypes in vertebrate heart and mitochondrial effects in *C. elegans* pharynx have not yet been directly established. Cardiomyocytes mainly utilize fatty acids rather than glucose as an energy source (Grynberg and Demaison 1996), which may fundamentally change their susceptibility to environmental exposures compared with other highly metabolic cell types that rely mostly on glucose. Cardiomyocytes are unique from other cell types in that the energy requirements are quite high, but mitochondria are largely fragmented (Kuznetsov and Margreiter 2009). This mitochondrial morphology is very tightly controlled in cardiomyocytes (Piquereau et al. 2010), as is mitochondrial movement, largely due to rigid and crowded cytoarchitecture (Vendelin et al. 2005). The physical hindrances preventing fusion and movement of mitochondria in cardiomyocytes may render the heart uniquely vulnerable to mitochondrial dysfunction as a result of environmental exposures. For example, classic mitotoxins such as acetaldehyde and ethanol inhibit mitochondrial

respiration in cardiomyocytes (Segel and Mason 1979). Emerging classes of environmental exposures exhibit mitochondrial toxicity within the heart as well: rodents exposed to mountaintop removal mining particulate matter exhibited decreased cardiac mitochondrial function and mitochondrially mediated induction of apoptosis associated with cardiac dysfunction (Nichols et al. 2015), and titanium dioxide nanoparticle exposure *in utero* negatively impacts mitochondrial respiration in hearts of the offspring in rodents (Stapleton et al. 2015). Whether these changes are due to the fragmented network of mitochondria within the heart is yet to be fully investigated. However, because *C. elegans* do not have cardiomyocytes, the nematode's utility for investigating hypotheses related to environmental mitotoxins and any resultant impacts on specialized mitochondria within these cells is unclear.

43.3.4.3 Lung Mitochondria

Lung mitochondria are involved in multiple responses to stress, including hypoxia sensing and immune cell signaling localized to the pulmonary system (Schumacker et al. 2014). A variety of chemical exposures can target mitochondria in lung cells (Brar et al. 2012; Xia et al. 2007; Yamada and Fukushima 1993), with well-studied pollutant exposures including paraquat and particulate matter. Inhalational routes of exposure in humans are not accurately modeled in *C. elegans*, as they do not have a respiratory system (Table 43.1). The tight regulation of oxygen exchange throughout the lung has major implications for specialized mitochondrial function. In mammalian lungs, mitochondrial density in type II pneumocytes (surfactant-producing cells), but not other lung cells, shows a linear correlation with total lung oxygen consumption (Massaro et al. 1975). Type II pneumocyte mitochondria are physically altered by environmental exposures, including lead ferricyanide, alcohol, and epoxypropane (Pattle et al. 1972). Exposure to acrolein causes a concentration-dependent inhibition of mitochondrial respiration in type II pneumocytes *in vitro* and a shift from glucose utilization likely to palmitate (essential for surfactant biosynthesis) utilization as an energetic substrate (Agarwal et al. 2013). This compensatory response to mitochondrial metabolism is likely not observed in other cell types due to substrate availability linked to surfactant production, making it physiologically infeasible to investigate in *C. elegans*.

43.3.5 Concluding Remarks on Limitations of *C. elegans* as a Model Organism

C. elegans is a powerful model organism for many aspects of mitochondrial research (Section 43.2) and provides a convenient *in vivo* system to measure mitochondrial

dynamics. However, the utility of *C. elegans* for the study of mitochondrial function is limited in some cases, including absence of specific biochemical pathways, cell types, and complex tissue organization. Mitochondrial researchers should consider and balance the benefits and limitations of the *C. elegans* model accordingly.

43.4 Methods for Assessing Mitochondrial Toxicity in *C. Elegans*

43.4.1 Introduction

As discussed earlier in this chapter, the nematode's small size, transparency, short developmental period and lifespan, high reproductive rate, and well-conserved mitochondrial function make it a valuable *in vivo* model for assessing both general and mitochondrial-specific chemical-induced toxicity (reviewed in Leung et al. (2008), Tejeda-Benitez and Olivero-Verbel (2016), and Hunt (2016)), and myriad toxicity assays that exploit nematode biology have been developed. Many of the genetic, biochemical, and molecular methods routinely used to assess mitochondrial health in cell culture have been adapted for use in *C. elegans* and are discussed in Table 43.3.

43.4.2 General Toxicity Assays

C. elegans has only been used as a toxicological model for a relatively short time. Initial pioneering work by Williams and colleagues (Williams and Dusenbery 1988) was eventually followed by myriad assays with medium- to high-throughput potential to monitor well-defined biological processes, including lethality and lifespan (Stroustrup et al. 2013), larval growth (Boyd et al. 2010b), reproduction (Boyd et al. 2010a; Ferreira et al. 2014), locomotion (Boyd et al. 2010b), germline function (Allard et al. 2013; Parodi et al. 2015), epigenetics (Lundby et al. 2016), and feeding (Boyd et al. 2007). Many of these assays utilize the COPAS Biosort (Pulak 2006), a large particle flow cytometer capable of dispensing and measuring thousands of nematodes per minute, making it amenable to high-throughput toxicity testing. Alternatively, lower-throughput variations of these assays that do not require costly COPAS equipment have been developed (reviewed in Tejeda-Benitez and Olivero-Verbel (2016)). Although growth, reproduction, locomotion, and feeding are energetically demanding processes that are dependent upon proper mitochondrial function, these assays do not specifically assess mitochondrial health. Nevertheless, when used in combination with RNAi or strains deficient in various mitochondrial stress response pathways, these assays

Table 43.3 Methods to detect mitochondrial toxicity in *C. elegans*.

Mitochondrial endpoint	Method	References ^a
Mitochondrial respiration	Clark-type electrode	Braeckman et al. (2002)
	Seahorse XF [®] 24	Luz et al. (2015a, b)
	Seahorse XF [®] 96	Andreux et al. (2014) and Koopman et al. (2016)
Steady-state ATP	Isolated mitochondria	Grad et al. (2007) and Kayser et al. 2004)
	ATP extraction	Brys et al. (2010) and Todt et al. (2016)
	<i>In vivo</i> ATP reporter strains (PE255 & PE327)	Lagido et al. (2008, 2015)
Mitochondrial morphology	Fluorogenic dyes and transgenic strains expressing fluorescent proteins in mitochondria	De Boer et al. (2015), Rolland (2014), and Smith et al. (2015)
Membrane potential	Potentiometric dyes (diS-C ₃ (3), TMRE, JC-1)	Gaffney et al. (2014) and Luz et al. (2016)
mtDNA damage and copy number	Real-time and long amplicon QPCR	Gonzalez-Hunt et al. (2016), Hunter et al. (2010), and Rooney et al. (2015).
	LC/MS	Arczewska et al. (2013) and Hunt et al. (2013)
Histochemical analysis	Carbohydrate staining (periodic acid Schiff's reagent)	Hench et al. (2011)
	Lipid staining (oil red O)	Soukas et al. (2009)
	ETC complexes I, II, and IV	Grad and Lemire (2004), Hench et al. (2011), and Sciacco and Bonilla (1996)

^a When available, detailed step-by-step protocols are referenced; however, primary research containing detailed method is also referenced when protocols are not available.

can provide evidence of mitochondrial toxicity or gene–environment interactions. However, mitochondrial toxicity must be confirmed using more direct measures of mitochondrial health (discussed in the succeeding text) as genes may have yet unrecognized biological functions unrelated to mitochondrial fitness, while RNAi and mutagenesis can cause compensatory changes that may alter the toxicant response. Finally, as for all such studies, it is critical to optimize and standardize assay conditions. Conditions that may be of particular importance with respect to mitochondrial function are nematode density in liquid medium or on plates, availability of food, viability of bacterial food (which may affect xenobiotic metabolism, among other parameters), temperature, oxygen availability, and shaking of liquid culture (Hanna et al. 2016; Hunt 2016; Maurer et al. 2015).

43.4.3 Mitochondrial Respiration

Historically, mitochondrial respiration has been measured polarographically in nematodes using Clark-type electrodes (Braeckman et al. 2002); however, Clark-type respirometers are relatively low throughput. The advent of the Seahorse extracellular flux analyzer (available in 24- and 96-well formats) and its subsequent optimization for use with *C. elegans* (Andreux et al. 2014; Luz et al. 2015a) has aided researchers' ability to rapidly measure mitochondrial respiration *in vivo*. Although the bioanalyzer is capable of simultaneously measuring basal oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) (a measure of glycolysis), ECAR does not appear to provide an accurate measure of glycolysis in nematodes (Luz et al. 2015a), likely due to differences in lactate excretion between cells in culture and nematodes. Finally, the ability to inject ETC inhibitors into Seahorse assays allows researchers to measure additional mitochondrial parameters, including ATP-linked OCR, maximal OCR, spare respiratory capacity, and proton leak. All of these parameters have been successfully measured in the Seahorse XF²⁴ (Luz et al. 2015a, b), while only basal, maximal, and spare respiratory capacity have been measured in the XF⁹⁶ (Andreux et al. 2014; Koopman et al. 2016). Although Seahorse technology is relatively novel to the *C. elegans* community, it has already been used to assess the effects of mutations (Luz et al., 2015a; Shen et al., 2016), pharmaceuticals (Andreux et al. 2014), and environmental toxicants (Caito and Aschner 2016; Luz et al. 2016) on mitochondrial function.

In addition to assaying whole nematodes, both Clark-type electrodes and the Seahorse bioanalyzer can be used to assay isolated mitochondria. Methods for isolating and purifying mitochondria from *C. elegans* are available (Brys et al. 2010; Grad et al. 2007; Kayser et al.

2004); of note, it was recently demonstrated that mitochondrial yield can be significantly increased (50–100%) by pretreating nematodes with collagenase 3, an enzyme that weakens the nematode cuticle, prior to homogenization (Daniele et al. 2016). Once isolated, mitochondrial ETC function can be assayed similarly to mitochondria isolated from cells or tissue (Grad et al. 2007; Kayser et al. 2004).

43.4.4 Steady-State ATP Levels

ATP levels can be quantified in *C. elegans* using commercially available kits that measure luminescence produced by ATP-powered firefly luciferase in a microplate reader (Braeckman et al. 2002; Brys et al. 2010); however, extraction and purification of ATP is an expensive and time-consuming process. To overcome this, transgenic strains (PE255 and PE327, both available through CGC) stably expressing a firefly luciferase–GFP fusion protein were generated (Lagido et al. 2008). These transgenic strains bioluminesce when provided with exogenous D-luciferin, providing a relative *in vivo* measure of steady-state ATP. This transgenic approach offers a rapid, low-cost method of monitoring steady-state ATP levels and has been used by many researchers to investigate mitochondrial toxicity following pharmaceutical (Rooney et al. 2014) or toxicant (Andreux et al. 2014; Bishop and Guarente 2007; Culetto and Sattelle 2000; Lagido et al. 2001; Luz et al. 2015b; Sulston and Horvitz 1977; Sulston et al. 1983; Swierczek et al. 2011) exposure and has potential high-throughput applications for screening for mitochondrial toxicity (Lagido et al. 2015). Alternatively, Promega's ToxGlo™ assay was recently optimized for use with *C. elegans*, which offers researchers a non-transgenic approach for rapid steady-state ATP determination (Toldt et al. 2016).

43.4.5 Transcriptomics, Proteomics, and Metabolomics

To delineate molecular mechanisms of toxicity, *C. elegans* researchers have employed high-throughput -omics approaches. Changes in the nematode transcriptome have been investigated using DNA microarray and RNA-sequencing technology following mitochondrial toxicant exposure (Leung et al. 2013b; Sahu et al. 2013; Schmeisser et al. 2013; Zhao et al. 2016). Proteomics and metabolomics approaches have been used by *C. elegans* researchers to investigate the molecular changes that occur due to mutations in mitochondrial electron transport chain genes (Butler et al. 2010; Morgan et al. 2015; 2013); however, methodology for protein and metabolite extraction can easily be adapted for toxicity studies. Alternatively, non-omics approaches, such as real-time PCR, are also

frequently used to assess toxicant-induced alterations in various mitochondrial signaling pathways (biogenesis, stress response, etc.). These approaches are facilitated in *C. elegans* by the outstanding annotation of the *C. elegans* genome (Consortium 1998), which was the first-sequenced metazoan genome and remains one of the best annotated due to extensive, ongoing centralized curation at Worm Base (www.wormbase.org).

43.4.6 Enzyme Activity

The activities of numerous enzymes involved in intermediary metabolism have been measured in *C. elegans* following well-established biochemical principals (Ragan et al. 1987; Srere 1969; Trounce et al. 1996). Key metabolic enzymes that have been assayed in nematodes include fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (gluconeogenesis) (Castelein et al. 2008; O’Riordan and Burnell 1989), hexokinase, phosphofructokinase and pyruvate kinase (glycolysis) (Castelein et al. 2008; O’Riordan and Burnell 1989), aconitase, citrate synthase, fumarase, isocitrate dehydrogenase, malate dehydrogenase, pyruvate dehydrogenase and succinate dehydrogenase (Krebs cycle) (Brys et al. 2010; Castelein et al. 2008; Luz et al. 2016; O’Riordan and Burnell 1989), isocitrate lyase (glyoxylate cycle) (Castelein et al. 2008), and various complexes of the ETC (Kayser et al. 2001, 2004). Activity of many of these enzymes can easily be assayed in crude nematode lysate or isolated mitochondria using a microplate reader.

43.4.7 Mitochondrial Morphology

As with cells in culture, mitochondrial membrane potential-sensitive dyes, such as TMRE (Breckenridge et al. 2008; Rolland et al. 2009) and MitoTracker Red CMXRos (Kimura et al. 2007; Luz et al. 2015a), can be used to visualize mitochondrial morphology in nematodes. However, due to the nematode cuticle, uptake of dyes is slow, and a longer incubation period (hours vs. minutes) is required to stain mitochondria. Alternatively, transgenic strains (SJ4103, available through CGC) expressing mitochondrial-targeted GFP under the control of the *myo3* promoter, which results in GFP expression in body wall muscle cell mitochondria, have been generated (Benedetti et al. 2006). These transgenic lines have been used by numerous researchers to investigate the effects of genetic deficiencies and toxicants on mitochondrial morphology (Houtkooper et al. 2013; Palikaras et al. 2015b; Rooney et al. 2014) and offer the added advantage of tissue specificity, which in some cases is a major limitation of *C. elegans* (discussed further in the succeeding text). Finally, mitochondrial morphology can also be assessed using transmission electron microscopy (Frank et al. 2001).

Detailed protocols for assessing mitochondrial morphology in *C. elegans* have been published (De Boer et al. 2015; Rolland 2014; Smith et al. 2015).

43.4.8 Mitochondrial Membrane Potential

Mitochondrial membrane potential has been measured in *C. elegans* using the potentiometric dyes MitoTracker Red CMXRos (Gaffney et al. 2014), diS-C₃(3) (Gaskova et al. 2007; Lemire et al. 2009), TMRE (Palikaras et al. 2015b), and JC-1 (Du et al. 2015; Iser et al. 2005; Ji et al. 2012; Luz et al. 2016), with the mitochondrial uncouplers CCCP or FCCP being used as a positive control. The accumulation of potentiometric dyes in mitochondria is visualized via confocal microscopy and can then be quantified using ImageJ or other image processing software. Many of the advantages and limitations of these potentiometric dyes are discussed in Perry et al. (2011).

43.4.9 Stress Response

Myriad techniques used to assess oxidative stress in cell culture have been adapted for use in *C. elegans* (reviewed in Braeckman et al. (2016)). The fluorogenic dyes dihydroethidium and Amplex Red can be used to detect superoxide (Huang and Lemire 2009; Morcos et al. 2008) and hydrogen peroxide (Chávez et al. 2007; Zeitoun-Ghandour et al. 2011) production, respectively, while MitoTracker Red (CM-H2X ROS) can be used to detect mitochondrial-specific ROS production (Schmeisser et al. 2013). However, fluorogenic dyes must be used with caution, as they are highly reactive, and proper positive controls must always be used. Furthermore, ease of genetic manipulation has allowed researchers to generate transgenic strains expressing fluorescent biosensors, such as HyPer and GRX1-roGFP2, which allow for the *in vivo* detection of H₂O₂ production and redox potential (GSSG/GSH) (Back et al. 2012b). In addition, GFP expression driven by mitochondrial (*sod-3*)- or cytosolic (*sod-1*)-specific superoxide dismutase promoters can aid in the identification of the intracellular location of ROS production and provide an indirect measure of oxidative stress (Anbalagan et al. 2013; Curran and Ruvkun 2007), while direct oxidative damage of macromolecules, such as lipids and proteins, can be assessed in *C. elegans* using LC-MS/MS (Labuschagne et al. 2013) and Western blots (Adachi et al. 1998; Wang et al. 2010; Wu et al. 2011), respectively.

In addition to oxidative stress, GFP reporter strains have also been generated for the rapid assessment of proteotoxicity (mitochondrial and non-mitochondrial heat shock proteins), xenobiotic response (CYP450s and GSTs), metal response, and the general stress response transcription factor (*daf-16*), which allows researchers to

rapidly and affordably screen for induction of stress response pathways following toxicant exposure (Anbalagan et al. 2012, 2013).

43.4.10 Mitochondrial DNA Damage and Genome Copy Number

Following toxicant exposure, DNA damage can be detected in *C. elegans* using the comet assay (Sobkowiak and Lesicki 2009), GC/MS (Arczewska et al. 2013; Hunt et al. 2013), or the long amplicon quantitative PCR (LA-QPCR) assay. Certain DNA lesions can also be detected by antibody (Roerink et al. 2012). Most of these work poorly for mitochondrial DNA damage. One of the main advantages of using the primer-based LA-QPCR assay is that it allows both mitochondrial and nuclear DNA damage to be quantified and it allows for the simultaneous measurement of relative or absolute mitochondrial and nuclear genome copy number. Recently, the LA-QPCR assay was used to demonstrate for the first time that the environmental toxicants cadmium and paraquat preferentially damage mitochondrial DNA *in vivo* in *C. elegans* (Gonzalez-Hunt et al. 2014), demonstrating the importance of measuring DNA damage in both genomes. Further advantages and limitations of the LA-QPCR assay are discussed in Meyer (2010), while detailed protocols for measuring DNA damage and genome copy number can be found in Rooney et al. (2015); Gonzalez-Hunt et al. (2016), and Hunter et al. (2010).

43.4.11 Limitations

Although nematodes offer an *in vivo* advantage for studying mitochondrial toxicity, their small size and protective cuticle make it difficult to isolate biochemically relevant amounts of purified tissue; thus, mitochondrial function is typically assessed in whole nematodes or in whole nematode homogenates, preventing the investigation of tissue-specific mitochondrial dysfunction. However, well-established histochemical methods have been used to identify tissue-specific changes in morphology and metabolism (Hench et al. 2011; Tsang et al. 2001). For example, hematoxylin and eosin staining can be used to visualize morphological changes, oil red o (Soukas et al. 2009) and periodic acid Schiff's reagent (Hench et al. 2011) can be used to visualize changes in lipid and carbohydrate storage, and other histochemical stains can be used to measure the activity of electron transport chain complexes I, II, and IV in whole worms or in specific tissues (Grad and Lemire 2004; Hench et al. 2011; Sciacco and Bonilla 1996). Although these histochemical methods are time consuming, they do provide researchers with the ability to evaluate tissue-specific effects, as demonstrated by the identification of the

gonad as a highly sensitive target tissue of doxycycline and chloramphenicol toxicity (Tsang et al. 2001).

In addition to histochemical methods, transgenic strains expressing mitochondrially targeted fluorescent proteins or strains with tissue-specific (neurons, hypodermis, muscle, intestine) RNAi sensitivities provide researchers with alternative ways to investigate tissue-specific mitochondrial toxicity. For example, sensitivity to paraquat has been tested following tissue-specific knockdown of ETC CIV (Durieux et al. 2011), while mitochondrial-targeted fluorescent proteins (GFP, YFP, RFP) under the control of tissue-specific promoters allow tissue-specific subpopulations of mitochondria to be identifiable via flow cytometry (Daniele et al. 2016). Furthermore, the use of fluorogenic dyes to assess membrane potential or ROS may be used to assess isolated subpopulations of mitochondria for tissue-specific disruption of mitochondrial function (Daniele et al. 2016).

43.5 Environmental Mitotoxins and *C. Elegans*: Unique Discoveries and Emerging Roles

43.5.1 Introduction

There is a relative paucity of data on environmental contaminants that cause mitochondrial toxicity (reviewed by Meyer et al. (2013)), especially in comparison with what is known about drug-induced mitochondrial toxicity (reviewed by Varga et al. (2015), Begriche et al. (2011), Dykens and Will (2007), and Nadanaciva and Will (2011a, b)). Because of the sheer number and volume of chemicals produced annually, there is mounting concern regarding how to address toxicity resulting from exposure. *C. elegans* provides a medium- to high-throughput *in vivo* system with which to address these questions, combining the ease of use provided by an *in vitro* system with the physiological complexity of an intact organism. For these reasons, researchers across multiple sectors have begun to utilize *C. elegans* as a model system for studying mitochondrial toxicity. This section describes some of the primary contributions that *C. elegans* studies have made in understanding mitochondrial toxicity caused by environmental exposures, expanding our current comprehension of these mechanisms in higher organisms. However, these contributions are largely in the area of neurotoxicity and neurodegeneration. Furthermore, they are mostly relatively recent, both because the use of *C. elegans* for toxicology has been relatively limited until the last decade and because studies of environmental mitochondrial toxicity in any organism have only recently begun to take off. Therefore, we also highlight another related contribution from the *C. elegans* field: the role of mitochondria in

mediating lifespan-related stress responses, which has been extensively studied in *C. elegans*.

43.5.2 Contributions of *C. elegans* in Discovering Key Mitochondrial Roles in Neurotoxicity

Some of the most important unique mitochondrial toxicity discoveries using *C. elegans* are specific to neurotoxicity induced by environmental exposures or related to inherent age-related neurodegenerative processes. *C. elegans* is an excellent model for studying certain neurotoxicity endpoints, due to a well-characterized neural system that retains complex features of a mammalian brain (Bargmann 1998; Hobert et al. 2002) while also containing fewer cells and circuits, which permits simplicity of study (for caveats to this, please refer to Section 43.3). Neural cells are among the most metabolically active cells in an organism, making them vulnerable to imbalances in energy homeostasis, the maintenance of which is largely dependent upon proper mitochondrial function.

Environmental exposures are postulated to play a role in the onset or progression of neurodegeneration in mammals, including rotenone, aluminum, copper, zinc, lead, manganese, methylmercury, and others (reviewed by Cannon and Greenamyre (2011) and Shao et al. (2015)). Mitochondrial dysfunction is associated with exposure-induced neurodegeneration in *C. elegans* (Caito and Aschner 2016; Gonzalez-Hunt et al. 2014; Vanduyn and Nass 2014; Zhou et al. 2013), as well as mammalian models and humans (reviewed by Aschner et al. (2009)). Mitochondria exhibit a proclivity toward accumulating pollutants for a variety of reasons (Meyer et al. 2013), which is usually directly related to toxicity. Divalent metals accumulate in mitochondria via the calcium uniporter (Section 43.1), and recent evidence points to a potential mechanism for mitochondrial accumulation of metals in other valence states. The *C. elegans* homologue of divalent metal transporter 1 (DMT1), SMF3, is a novel aluminum (Al^{3+}) transporter *in vivo* and has been implicated as an essential component of neurodegeneration associated with Al^{3+} exposure (Vanduyn et al. 2013). DMT1 itself has since been found in the outer mitochondrial membrane (co-localizing with voltage-dependent anion channel 1, Tom6, and COXII) in mammalian cells and yeast (Wolff et al. 2014a, b), suggesting that DMT1 is involved in metal import to mitochondria in mammals. This discovery of DMT1 mitochondrial localization in mammals lends credence to the possibilities that Al^{3+} toxicity mediated by the DMT1 homologue in *C. elegans* is due at least in part to mitochondrial toxicity, and that *C. elegans* provides a model with which to study these mitochondrial components of exposure-induced neurotoxicity *in vivo*.

The *C. elegans* model brings to the table several particular strengths for these studies, including the ability to monitor neurodegeneration *in vivo*, the ability to test genetic sensitivity using mutants and RNAi knock-down, and the ability to screen a large number of individuals (and thus chemicals and doses and gene $\times$ environment interactions) quickly. This is important for neurodegeneration, as for mitochondrial toxicity, because the environmental contributions to neurodegeneration are as-yet poorly understood, and a relatively small number of chemicals have received most of the attention. Recent studies have identified novel candidate chemicals for dopaminergic neurodegeneration, including glyphosate (McVey et al. 2016; Negga et al. 2012), selected for testing based in part of volume of use and likelihood of exposure, and aflatoxin B₁ (Gonzalez-Hunt et al. 2014), selected for testing based on mechanism of action (generation of irreparable mitochondrial DNA damage). Similarly, recent work has demonstrated increased sensitivity of strains lacking mitochondrial homeostasis-related genes to pollutant-induced neurodegeneration, including paraquat and decreased neurite outgrowth in mitophagy-deficient *pink-1* mutants (Samann et al. 2009). Work demonstrating that agents that cause neurodegeneration in mammals do the same in *C. elegans* supports the possibility that novel findings in nematodes such as those described in this paragraph will be extrapolatable to mammals. However, given the decreased complexity of the nematode nervous system (Section 43.3), these findings will need to be tested in higher eukaryotes.

Environmental exposures include not only anthropogenic chemicals and their metabolites but also naturally derived compounds and their respective metabolites (i.e., essential metals, bacteria, etc.). A novel metabolite of *Streptomyces venezuelae* causes dopaminergic neurodegeneration in *C. elegans* associated with inhibition of complex I of the ETC, induction of the mitochondrial unfolded protein response (mtUPR), and decreased ATP (Ray et al. 2014). This bacterial metabolite-induced mitochondrial toxicity was directly tied to dopaminergic neurodegeneration, as pharmacologic rescue of complex I activity (by either riboflavin or D-beta-hydroxybutyrate) abrogated dopaminergic neurodegeneration in *C. elegans* (Ray et al. 2014). This group then showed, using *C. elegans* as a model for neurotoxicity, that this novel bacterial metabolite causes mitochondrial fragmentation by disrupting proteostasis through dysregulation of ubiquitin pathways, which are genetically regulated by loss of *pink-1* (the *C. elegans* homologue of PINK1) homeostasis mechanisms (Martinez et al. 2015). Further, the metabolite works synergistically with α -synuclein, amyloid-beta, and mutant huntingtin to cause neurodegeneration *in vivo* (Martinez et al. 2015), likely

mediated by defects in mitophagy. These findings provide direct evidence of a novel environmental mitotoxin mediating neurodegeneration *in vivo* and are especially striking in models of neurodegeneration, since most cases of neurodegenerative disease are idiopathic in nature and likely require some environmental component (reviewed by Cannon and Greenamyre (2011)).

43.5.3 General Stress Response Mechanisms Important for Mitigating Mitochondrial Toxicity and Promoting Healthspan: Discoveries in *C. elegans*

C. elegans has been instrumental in establishing mitochondrial function as a determinant of longevity *in vivo* (reviewed by Dancy et al. (2014), Kirstein-Miles and Morimoto (2010), and Wolff and Dillin (2006)). Many of the mitochondrial mechanisms probed in these longevity studies are important for mitigating mitochondrial toxicity and are conserved between *C. elegans* and mammals. Examples of these important mechanisms include the mtUPR (Haynes et al. 2007; Houtkooper et al. 2013; Nargund et al. 2012) and downstream effects of ETC inhibition (Durieux et al. 2011; Munkacsy et al. 2016). Many genetic manipulations that result in activation of the mtUPR also confer both stress resistance and extended lifespan (Tian et al. 2016), although these responses are to some extent nonmonotonic. For example, inhibition of ETC activity by RNAi, which can be titrated quantitatively, results at very low levels of inhibition in no effect on lifespan, then lifespan extension at slightly higher levels, and finally shortened lifespan at high levels (Rea et al. 2007).

Work in *C. elegans* has also helped to uncover the paradoxical nature of the role of mitochondrial ROS (mtROS) and total cellular ROS in aging and propagation of oxidative damage (reviewed by Back et al. (2012a)). A certain amount of mtROS is required for many signaling processes (reviewed by Shadel and Horvath (2015)) and appears to be part of an adaptive response to exposures (Wang and Xing 2010) and aging (Schaar et al. 2015). The specific effects of individual ROS and the threshold concentrations at which ROS move from essential to detrimental are difficult to untangle, but progress has been made in *C. elegans* models of mitochondrial mechanisms in this regard. Increasing mitochondrial superoxide levels (either by exposure to paraquat or by deletion of mitochondrial superoxide dismutase), at least up to certain levels, increases lifespan in *C. elegans*, whereas deletion of cytoplasmic superoxide dismutases significantly decreases lifespan (Schaar et al. 2015), suggesting divergent roles of intracellular compartment-specific levels of superoxide on lifespan. Repression of mitochondrial peroxiredoxin does not alter lifespan, but decreases ATP

levels, brood size, and motility in wild-type *C. elegans*, suggesting that a sufficient concentration of mitochondrial hydrogen peroxide is required to maintain these ATP-dependent mechanisms (Ranjan et al. 2013).

New areas of discovery in *C. elegans* aging, mtROS signaling, and thresholds of mtROS-mediated damage continue to emerge through the continued use of mutant backgrounds and exposures across the life course. However, from a toxicological perspective, some caution may be warranted in interpreting these results. For example, deletion of mitochondrial SODs also results in increased sensitivity to a variety of stressors. Furthermore, some of the same responses that result in stress resistance in some contexts, and lifespan extension, carry other fitness costs. For example, Rea et al. reported reduced fecundity and developmental rate in the same nematodes in which RNAi-based knockdown of ETC resulted in lifespan extension (Rea et al. 2007). *C. elegans* is an excellent model in which to explore the relationship between hormetic and toxic responses to environmental stressors, an emerging area of research interest for the toxicological community (Mattson 2008a, b) that may have important mitochondrial components (Schmeisser et al. 2013).

43.5.4 Emerging Roles for *C. elegans* in Investigating Environmental Mitotoxins

The utility of *C. elegans*, along with other simpler eukaryotes such as *Drosophila melanogaster* and *D. rerio*, is appreciated in current and upcoming phases of the Tox21 program (Merrick et al. 2015), a large-scale governmental effort to move toward higher-throughput alternatives to traditional animal models in toxicology. Aside from integrative systematic approaches to toxicology, *C. elegans* continues to be employed in academic institutions to investigate specific hypotheses across a wide range of mitotoxins. These discoveries are novel for a range of reasons, and include unexpected roles for mitochondrial modulation in phosphine exposure (Zuryn et al. 2008), inhibition of the UPR by mitochondrial enzyme-specific mitigation of hydrogen sulfide toxicity (Horsman and Miller 2016), and new mitochondria-specific interactions with proteotoxic products of aging (Cacho-Valadez et al. 2012). The advent of modeling sublethal mixture toxicity in *C. elegans* (Gomez-Eyles et al. 2009; Jager et al. 2014) provides for the opportunity to screen for synergistic effects of known and suspected mitotoxins. Discoveries in *C. elegans* bring the advantageous combination of medium- to high-throughput capacity for analysis and the physiological complexity of an *in vivo* system. These, and the availability of diverse and specific genetic backgrounds, are strengths of the *C. elegans* model for isolating complex mitochondrial mechanistic components of environmental exposures.

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Persistent Organic Pollutants, Mitochondrial Dysfunction, and Metabolic Syndrome

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CHAPTER MENU

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44.1 Introduction

Metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM) are worldwide public health problems. Currently, it is believed that high calorie diet and sedentary lifestyle are main causes of obesity and the interaction between genetic and environmental factors is associated in the onset of obesity, which are T2DM and MetS. However, 75–80% of obese people never develop T2DM (Gregg et al., 2007), so there may be other environmental factors. Recent epidemiological studies suggest a possible contribution of environmental chemicals to metabolic diseases such as MetS, T2DM (Lee et al., 2006a), and insulin resistance (IR), a prediabetic state (Lee et al., 2007a). More importantly, mitochondrial dysfunction was suggested to be an underlying cause of IR, MetS, and T2DM (Lee et al., 2010; Petersen et al., 2003, 2004).

Optimal mitochondrial function is central to cell physiology. A wide range of chemicals has been reported to disturb mitochondrial function and cause mitochondrial toxicities and diseases (Vuda and Kamath, 2016; Wagner et al., 2008). Additionally, a series of recent

studies demonstrated the connections between various environmental chemicals and metabolic disorders (Neel and Sargis, 2011). In this chapter, we will provide evidence that environmental chemicals may induce mitochondrial dysfunction resulting in IR, T2DM, and MetS.

44.2 Health Hazard of Environmental Chemicals: A Short History

Concerns about environmental chemicals began in 1962 after Rachel Carson published the monumental book entitled *Silent Spring* (Lytle, 2007). In this book, she claimed that the indiscriminate use of chemicals, especially pesticides, adversely affects the environment. For example, dichlorodiphenyltrichloroethane (DDT) was developed to prevent malaria epidemic by controlling mosquitoes, but its use is now prohibited due to causing human toxicity, cancer, and other illnesses. The US Environmental Protection Agency (EPA) and the United Nations Environment Programme (UNEP) were established in 1970 and 1972, respectively, to

pay closer attention to environmental chemicals and human health. UNEP proposed to control “persistent organic pollutants (POPs),” defined as “chemical substances that persist in the environment, bio-accumulate through the food chain, and pose a risk of adversely affecting human health and the environment.” Some POP chemicals such as pesticides, industrial chemicals, and by-products have been banned or are strictly controlled from use and production under the Stockholm Convention (<http://chm.pops.int/TheConvention/ThePOPs/tabid/673/Default.aspx>).

In 2009, the Endocrine Society published the landmark paper “Endocrine Disrupting Chemicals (EDC): An Endocrine Society Scientific Statement,” known as “EDC-1” (Diamanti-Kandarakis et al., 2009). Then, UNEP and the World Health Organization (WHO) copublished another landmark book, *State of the Science of Endocrine Disrupting Chemicals in 2012*. In 2015, the Endocrine Society published *EDC-2, The Endocrine Society’s Second Scientific Statement on Endocrine-Disrupting Chemicals* (Gore et al., 2015). EDC-2 described the underlying mechanisms by which exposures during development in animals and humans lay the foundation for diseases later in life. Importantly, it provided a simplified operational working definition of EDCs: an exogenous chemical or mixture of chemicals that interferes with endogenous hormonal axes and induces metabolic disruption *in vitro* and *in vivo*. Unlike the early studies of EDCs, which focused on identifying chemicals to modulate the signal transduction of sex steroid and thyroid hormones, emerging data strongly suggest that EDCs disturb the signaling pathways critical for energy homeostasis, mitochondrial activities, and insulin signaling.

44.3 Low-Level Exposure to Multiple Chemicals

Humans are exposed to a complex mixture of chemicals from various sources in everyday life. The exposure levels of individual environmental chemicals to the general population are far lower than in the case of environmental disasters such as chemical plant explosion, military personnel exposures, and occupational exposures. However, even low-level exposure over decades to a complex cocktail of pollutants in air, water, food, consumer products, and buildings can have a significant effect on the health status of citizens (Committee, 2009). In other words, mixtures of dissimilarly acting chemicals may not be safe even if individual constituent chemicals are at levels below no observed adverse effect levels (NOAELs) or lowest observed adverse effect levels (LOAELs) (Kortenkamp et al., 2007). We refer to this

additive enhancement of chemical actions as “mixture effect” or “cocktail effect.”

Many of the synthetic organic chemicals are better known for causing weight loss at high levels of exposure, but much lower concentrations of these same chemicals have powerful weight-gaining actions. For many years, the mechanisms by which some environmental chemicals acted at low doses were not well understood. There are two major concepts in EDC studies: low-dose and nonmonotonicity. Low-dose effects are defined by the National Toxicology Program (NTP) as those that occur in the range of human exposures or effects observed at doses below those used for traditional toxicological studies. Nonmonotonic dose response is a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined (Vandenberg et al., 2012), suggesting that low-dose effects may be different, and sometimes even opposite, from high-dose effects. Nonmonotonic responses and low-dose effects are remarkably common in studies of natural hormones and EDCs.

Hormones produced by the endocrine system are functioning at extremely low concentrations, comparable with the levels of EDCs. Natural hormone-mimicking EDCs at low doses exert similar biological effects to hormones because they share the same receptor. Circulating natural hormones are present in three different forms: free (the active form of the hormone), bioavailable (bound weakly to proteins such as albumin), and inactive (bound with high affinity to proteins such as hormone binding proteins) (Vandenberg et al., 2012). Protein-bound hormones act as a natural buffering system, allowing the hormone to be accessible in the blood, but preventing large doses of physiologically active hormone from circulating. In contrast, the majority of a circulating EDC can be physiologically active because EDC-specific binding protein is not present. Therefore, even a low concentration of an EDC can disrupt the natural balance of endogenous hormones in the circulation. Therefore, traditional criteria for chemical testing and safety determination need to be changed to protect human health.

44.4 POPs and Obesity Paradox

POPs are lipophilic chemicals that accumulate in adipose tissue and are hard to eliminate from contaminated environments and human bodies. POPs of concern at present are polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyl congeners (PCBs), organochlorine pesticides (OCPs), and brominated flame retardants (BFR). Simultaneous exposure to various POPs may cause obesity and diabetes as a mixture effect in the general population

(Lee et al., 2006b, 2011), which posited environmental “obesogen hypothesis.” This hypothesis postulates that certain EDCs or POPs that interfere with the body’s adipose tissue biology, endocrine hormone systems, or central hypothalamic–pituitary–adrenal axis are derailing the weight control homeostasis (Grun and Blumberg, 2009; Heindel et al., 2015). It is important to note that POPs in fat tissue, not obesity itself, may contribute to the development of IR and T2DM. An “obesity paradox” has been documented that the overweight and obese elderly have better prognosis than those with normal body weight. This may reflect the relative safety of storing the harmful lipophilic POPs in adipose tissue rather than in bloodstream, which damage other critical organs (Hong et al., 2012).

44.5 Body Burden of Chemicals

More than 800 chemicals may contain endocrine-disrupting activity (Gore et al., 2015). Table 44.1 summarized the nature and health hazards of individual POPs and/or EDCs. We did not include heavy metals such as arsenic and cadmium in the table although they are known to be toxic to mitochondria (Prakash et al., 2016) and are causally associated with diabetes and cardiovascular diseases (Kuo et al., 2015; Moon et al., 2013). For better understanding the nature and distribution of EDCs, we categorized them into two groups: nonpersistent and persistent chemicals according to half-life. Each group is then subdivided by usage or source.

Nonpersistent chemicals, such as bisphenol A (BPA) and phthalates, released from widely used plastics and epoxy resins, are commonly detected in most populations. The herbicide atrazine and the fungicide vinclozolin are of great importance in agriculture and are easily excreted. Diethylstilbestrol (DES) and ethinyl estradiol (EE) are synthetic estrogen-like compounds, which are used as drugs and are involved in carcinogenesis. Without accumulation in the body, nonpersistent EDCs still enhance the risk of diseases such as cancer, liver damages, and/or neurological disorders (Gore et al., 2015; Heindel et al., 2015). Owing to the short biological half-life of nonpersistent EDCs, human epidemiological studies did not clearly show an association of the plasma or urine levels of EDCs with incidence of obesity and diabetes (James-Todd et al., 2016; Lind et al., 2012; Sun et al., 2014). But several animal studies demonstrated that exposure to environmentally relevant doses of these compounds increased body weight, suggesting they are obesogens (Angle et al., 2013; Lim et al., 2009). Estrogen receptor has been thought to mediate their obesogenic activities, while adipocyte differentiation and adipogenesis might also be involved via

various mechanisms (Biemann et al., 2012; Chamorro-Garcia et al., 2012; Heindel et al., 2015).

In contrast, persistent EDCs such as dioxins, OCPs, and other POPs are accumulated and stored in adipose tissue. The classification by persistency may help understand the “body burden,” which is defined as the total amount of environmental chemicals that are present in the human body at a given point in time. Body burden tests analyze the concentration of environmental chemicals in blood, urine, breast milk, and other fluids and tissues. This biomonitoring test tells us about the extent of our exposure to various substances, but not necessarily what the effects of this “burden” will be. Because of lipophilicity, a large amount of POPs can be stored in fat tissue depot that may sequester those toxic persistent chemicals to protect vital organs. Upon lipolysis of stored fat, the lipophilic chemicals can also be released into blood from the storage. Therefore, weight loss in the obese elderly with high serum concentrations of POPs may carry some risk of damage on vital organs by the released chemicals although weight loss may be beneficial among the obese elderly with low POP concentrations. To prevent the surge of persistent chemicals into blood, we believe that the biomonitoring test for the chemicals should be performed during weight control or health checkup processes. At the same time, sampling of blood or urine should be done at certain fixed times of the day, like early in the morning without stress, for consistent and reliable monitoring.

This classification may also be useful to locate EDCs geographically. Some insecticides are used mostly in rural agricultural areas, while many other POPs are associated with industrial activities.

44.6 Diabetes Mellitus, Insulin Resistance, and Metabolic Syndrome

Diabetes is defined by high blood glucose level. In normal physiology, high blood glucose should only occur with low level of insulin, the most important glucose-lowering hormone. However, subjects with T2DM frequently have high blood glucose levels with rather high serum insulin levels, suggesting a development of IR in the body. Current paradigm understanding of diabetes is that it occurs when insulin production cannot overcome IR induced by high fat diet and inactivity. Therefore, diabetes is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.

MetS is a cluster of the most dangerous cardiovascular risk factors: hyperglycemia, prediabetes (IR), abdominal obesity, hypertension, and dyslipidemia (Huang, 2009). Reaven named the clustered state of risk factors as syndrome X in 1993 (Reaven, 1988, 1993). In 2001, the Adult

Table 44.1 The nature of common EDCs or POPs and their known health hazards.

Class	Chemicals	Nature	Known health hazards
Nonpersistent	Bisphenol A	Used in plastics and resins	Estrogenic, obesogenic, neurological effects, adverse thyroid hormone action, reproductive and developmental effects
	Phthalates	Plasticizers	Carcinogen, liver damage, reproductive and developmental effects, asthma, obesogen
	Atrazine	Chlorotriazine herbicide	Endocrine, respiratory, and nervous system targets, liver damage, obesogenic; increases aromatase expression; antiandrogenic
	Vinclozolin	Dicarboximide fungicide	Antiandrogenic activity, male reproductive and neurological effects, transgenerational reproductive effects, potential carcinogen
	Diethylstilbestrol (DES)	Used as drugs; nonsteroidal synthetic estrogen	Transplacental carcinogen, teratogen
	Ethinyl estradiol	Used as drugs; synthetic derivative of 17 β -estradiol	Cardiovascular disease, cerebrovascular disease, thromboembolic disease, gallbladder disease, carcinogenic
Persistent	Methoxychlor	Organochlorine insecticide	Central nervous system depression, damage to liver and kidney, developmental and reproductive effects in animals, transgenerational kidney and ovary disease, obesogen; binds ER
	DDT, DDD, and DDE	Organochlorine insecticides and its metabolite	Carcinogen, central nervous system, kidney, liver and peripheral nervous system effects
	TCDD or dioxin	Released from industries; most toxic among dioxins or PCDDs	Liver damage, weight loss, atrophy of thymus gland, immunosuppression, reproductive effects, and cancer
	PCDFs	Released from industries; organochlorines	Developmental teratogen and carcinogen
	PCBs	Released from industries; organochlorines; 209 possible congeners	Carcinogen, chloracne, stomach and liver damage, reproductive and nervous system effects and thyroid injury, stimulation of fat production; impairs glutamate pathways, mimics estrogen, antiestrogenic activity, and so on
	PBDEs	Released from industries; pentaBDE, octaBDE, decaBDE	Alters TH synthesis
	PFOA and PFOS	Released from industries; perfluorochemical	Liver and mammary gland developmental and immune system toxicant, carcinogen
	Aldrin/dieldrin; chlordane and related compounds; endrin; mirex; heptachlor; hexachlorobenzene; toxaphene	Rarely detected	Estrogenic activity; binds ER; sexually dimorphic behavior Stimulates glucocorticoid receptor; decreases testosterone levels; induces testosterone hydroxylases; modulates binding of ligand to TRE; weakly binds AhR

DDD, dichloro-diphenyldichloro-ethane; DDE, dichloro-diphenyldichloro-ethylene; DDT, *p,p'*-dichloro-diphenyltrichloro-ethane; DES, diethylstilbestrol; PBDEs, polybrominated diphenyl ethers; PCBs, polychlorinated biphenyls; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDFs, polychlorinated dibenzo-*p*-furans; PFOA, perfluorooctanoic acid; PFOS, perfluoro-octane yl sulfonic acid; TCDD, tetrachloro-dibenzo-*p*-dioxin.

Treatment Panel III (ATP III) of the National Cholesterol Education Program of the United States made different diagnostic criteria (Table 44.2), which are now widely used (Expert Panel on Detection and Treatment of High Blood Cholesterol in 2001). It should be noted that the ATP III was created to define MetS for clinical purpose.

Although the MetS is frequently considered as a disease entity, it is a *diagnostic category*, identifying individuals to initiate lifestyle changes with the goal of decreasing risks of cardiovascular disease, the most important cause of death (Mortality and Causes of Death 2015). IR is a pathophysiological state in which body and cells fail to

respond normally to insulin. But IR is found not only in diabetes but also in many other disease states, such as dyslipidemia, hypertension, and neurodegenerative diseases (Reaven, 1988; Shen et al., 1970), suggesting that IR may be a critical contributor in developing these diseases.

The primary value of the concept of IR is that it provides a framework for placing a substantial number of apparently unrelated biological events into a *pathophysiological construct* (Reaven, 2005). This point is crucial in understanding the association between exposure to environmental chemicals and IR or MetS, because high exposure to environmental chemicals frequently occurs together with IR.

Table 44.2 ATP III criteria for diagnosing the metabolic syndrome.

MetS criteria	Any three or more of the five criteria are met
Abdominal obesity	Waist circumference (WC) Men: WC > 40 inches (102 cm) Women: WC > 35 inches (89 cm)
Hyperglycemia	Fasting glucose >110–126 mg/dL (6.1–7.0 mmol/L)
Dyslipidemia	Triglycerides (TG) >150 mg/dL (1.7 mmol/L)
Dyslipidemia	High-density lipoprotein cholesterol (HDL-C) Men: <40 mg/dL (1.04 mmol/L) Women: <50 mg/dL (1.3 mmol/L)
Hypertension	Blood pressure >130/80 mm Hg

44.7 Association of POPs with Diabetes and Metabolic Syndrome

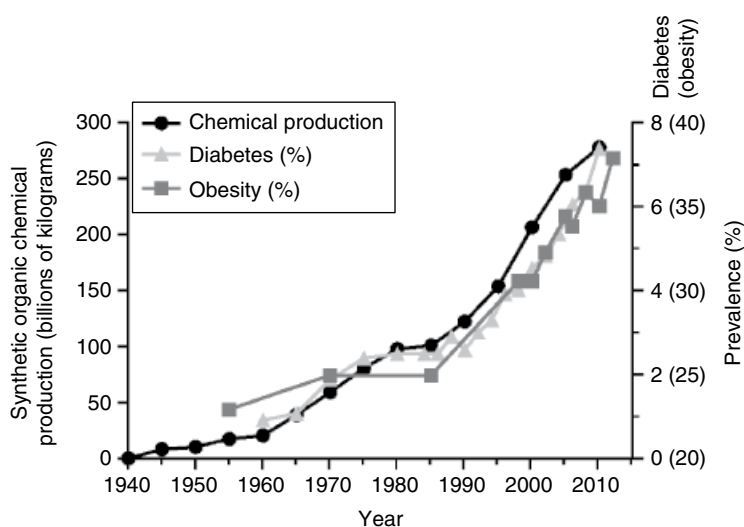
44.7.1 Ecological Studies

In 2002, Baillie-Hamilton hypothesized that the toxic chemicals might be obesogenic, based on an observation that there is a parallel increase of chemical toxin production and incidence of obesity (Baillie-Hamilton, 2002). The parallelism was firmly established with diabetes in 2011 by Neel and Sargis (2011). Figure 44.1 demonstrated that obesity and diabetes epidemic in United States might be closely related to the increasing production and usage of synthetic organic chemicals. These data suggest that the human exposure to the chemical compounds is strongly suspected as a causal factor in developing the diseases (Heindel et al., 2017).

44.7.2 Epidemiologic Studies on the Association between POPs and T2DM

Prior to Baillie-Hamilton's report, there have been many anecdotal reports linking exposure to POPs and development of diabetes. However, epidemiologic studies began only recently to prove the association between serum levels of POPs and T2DM. Lee et al. (2006b, 2007a, b) analyzed US NHANES 1999–2002 database and provided critical evidence for the involvement of serum concentrations of POPs in obesity or diabetes. They showed that the sum of 6 most frequently detected POPs (2,2',4,4',5,5'-hexachlorobiphenyl, 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin, 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin, oxychlorodane, *p,p'*-dichlorodiphenyltrichloroethane, and

Figure 44.1 US synthetic chemical production and prevalence (%) of diabetes of total population and obesity (BMI > 30). Linear regression analysis of overlapping data from 1989 to 1994 show correlation coefficient $r^2 = 0.948$. Adapted from Baillie-Hamilton (2002) and Neel and Sargis (2011).



trans-nonachlor) among US citizens was clearly associated with prevalence of diabetes (Lee et al., 2006b, 2014), which made quite a stir to the scientific community. Cross-sectional and case-control studies have reported strong associations of T2DM and MetS with OCPs, dioxins, PCBs, and/or dichlorodiphenyldichloroethylene (DDE) (Taylor et al., 2013). There is less indication of an association between T2DM and other non-organochlorine POPs, such as perfluoroalkyl acids and brominated compounds. There are more than 80 epidemiological studies, showing that POPs are related to altered glucose homeostasis, IR, and/or MetS. As this chapter aims to introduce this field, authors recommend other review articles (Gore et al., 2015; Lee et al., 2014; Taylor et al., 2013) for further details, such as EDC-2 (Gore et al., 2015) and the NTP workshop review (Taylor et al., 2013; Thayer et al., 2012), which was organized by US NTP in 2011. In summary, epidemiologic studies have provided sufficient overall evidence for proving a positive association between some organochlorine POPs, BPA, phthalates, organotins, and arsenics with T2DM.

44.7.3 Animal Experiments: Low-Dose Exposure to Chemicals and Development of Diabetes

Experimental data are needed to confirm the causality of low-level exposure to POPs, which will shed new light on the pathogenesis of diabetes. Lim et al. (2009) reported that atrazine, a chlorotriazine herbicide when given at low concentration, caused IR and induced mitochondrial dysfunction in skeletal muscle in rats, when fed with high fat diet for 2 months. Ruzzin et al. (2010) found that feeding contaminated Atlantic salmon oil to rats for 28 days induced IR and fatty liver. In addition, it induced downregulation of two master regulators of lipid homeostasis, insulin-induced gene-1 and Lpin1, as well as genes related to mitochondrial function, including peroxisome proliferator-activated receptor- γ (PPAR γ) coactivator-1 α (PGC-1 α), citrate synthase, succinate dehydrogenase A, and medium-chain acyl-CoA dehydrogenase, in the liver of rats.

In addition, various EDCs at low doses were studied for their diabetogenic or obesogenic effects (Heindel et al., 2017; Vandenberg et al., 2012). For example, BPA alone or in combination with DES induced either diabetes or obesity in animal studies. DDT, phthalate metabolites, PCBs (-77, -126, -153), perfluorooctanoic acid (PFOA), perfluoro-octane yl sulfonic acid (PFOS), tributyltin (TBT), and tetrachlorodibenzo-*p*-dioxin (TCDD), alone or in combinations, are also listed as causative agents for obesity and diabetes (Heindel et al., 2015; Ngwa et al., 2015; 2016). In fact, there are lots of EDCs for which high-dose toxicology studies have been performed

and the NOAEL has been derived, but no animal studies in the low-dose range. Also, several hundred additional EDCs where no significant high- or low-dose testing have been performed. Table 44.3 summarizes a limited selection of the environmental chemicals that are related with obesity and T2DM, nuclear receptors involved in function, and the reported effects on mitochondria.

44.7.4 Cause-Effect Relationship between Exposure to POPs and the Onset of T2DM or MetS

Despite many reports, there is no direct evidence that the exposure to POPs is the cause of T2DM or MetS in humans. There are several reasons. Traditionally, a cause of a disease is established when the suspected causative agent meets Koch's three principles: (i) that agent is always present in the persons with a specific disease, (ii) that agent could be isolated in pure form, and (iii) that transfer of that pure agent reproduces disease in susceptible animals. These principles are not applicable here, because environmental pollutants are enormously diverse chemicals with varying toxicities and the diseases they cause are not specific, but complex. POPs are present in the environment as mixtures of chemicals, some with known toxicities, but potentially others with many unknown ones; hence, they cannot qualify as a specific "causative agent." It is estimated that about 10,000 new chemicals are synthesized and released to our environment every year without evaluation of their toxicity in humans. Also, there is no good method of evaluating biological or toxicological effects of mixtures of POPs, to which people are exposed.

T2DM itself, a component disease of MetS, is a "complex disease," meaning that T2DM may have many "causes." It is reasonable to hypothesize that clustering of complex diseases in a person may be a result of introducing a mixture of POPs into our body.

44.8 Toxic and Biological Effects of Some POPs via AhR

Some POPs are known to induce adverse effects on various biological systems via binding to aryl hydrocarbon receptor (AhR), a cytosolic nuclear receptor present in most vertebrate tissues (Schleizinger et al., 2010). AhR is a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH)/Per-Arnt-Sim family (Beischlag et al., 2008). Ligand binding to AhR activates the transcription of multiple genes including cytochrome P450 (*CYP*) members, CYP1A1/2 and CYP1B1, and ligand-metabolizing enzymes. Induction of *CYP* enzymes enhances the metabolism and clearance of AhR ligands

Table 44.3 Environmental chemicals associated with T2DM and obesity.

Chemical	Examples	Type or source	Human epidemiological studies	Potential mechanism: nuclear receptor	Animal studies	Mitochondrial dysfunction
PCDD, PCDF	POPs/210 dioxins and furans, 17 highly toxic (e.g., TCDD)	In food, air pollution, incomplete combustion of fossil fuels	MetS, obesity, and T2DM	AhR, PPAR γ , ERs	Affects energy metabolism, accumulation of visceral fat, inflammation	Mitochondrial fragmentation, decreased OCR, ROS generation, decreased membrane potential, ATP level
PCBs	POPs/209 possible congeners (e.g., PCB126, PCB169)	Coolants, plasticizers, and flame retardants	MetS, obesity, T2DM, childhood obesity	AhR, PPAR γ , ERs	Altered thyroid function, Altered metabolism, bioaccumulation in fat cells	ROS generation, decreased membrane potential, ATP level. Inhibition of OXPHOS complexes I, II, and IV
HCB	POPs	Fungicide, a byproduct of the industrial chemicals (e.g., CCl ₄)	Overweight. Obesity in offspring	AhR, TH-responsive genes	Obesity in infancy and childhood	NA
OCPs	POPs/DDT, DDE, endrin, heptachlor, mirex, toxaphene	Pesticides, insecticides	MetS, obesity, T2DM, and childhood obesity	AhR, PPAR γ , ERs	Glucose intolerance, hyperinsulinemia, dyslipidemia, altered bile acid metabolism (rat), gender differences	Reduced energy expenditure, impaired thermogenesis
BFR	PBB, PBDE	Chemicals applied to furniture and electronics	Associated with MetS, obesity, and T2DM	PXR, ERs, TR	Alters lipolysis and glucose oxidation (rat)	Impaired mitochondrial bioenergetics, membrane potential, OCR, calcium release, mitochondrial swelling, and ATP levels
PFCs	PFOA, PFOS	Components of lubricants, nonstick coatings, and stain-resistant compound	Increased cholesterol level, offspring obesity	ERs, AR, PPARs	Weight gain and increased serum insulin and leptin levels (mice), increased adipocyte differentiation	Alteration in energy metabolism genes, ATPase, OXPHOS complexes 1 and IV, ROS generation
Phthalates	DEHP, DBP, DEP, MEHP	Plasticizers, adhesives, and personal care products	Obesity, insulin resistance	PPARs, CAR/PXR, GR	Reduced plasma insulin and leptin levels (mice), increased rate of adipocyte differentiation	Uncoupler, mitochondria swelling, inhibiting succinate dehydrogenase
BPA	BPA	Plastics and epoxy resins	Diabetes, insulin resistance, childhood obesity, liver abnormalities	ERs, AR, TR, GR	Increased body weight (mice, rat), Induced adipogenesis	Altered morphology, membrane potential, mass, etc. ROS generation
Organotins	TBT, TPT, TPTO	In food. Fungicide in paints and heat stabilizer in polyvinyl chlorides, pesticide, wood preservation	Obesity	PPAR γ , RXR	Induced adipogenesis (mice), increased fat cell differentiation, increased lipid accumulation	NA

AhR, aryl hydrocarbon receptor; AR, androgen receptor; BFR, brominated flame retardant; BPA, bisphenol A; CAR, constitutive androstane receptor; DBP, dibutyl phthalate; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; DEHP, diethylhexyl phthalate; DEP, diethyl phthalate; ER, estrogen receptor; GR, glucocorticoid receptor; HCB, hexachlorobenzene; MEHP, bis(2-ethylhexyl) phthalate; MetS, metabolic syndrome; OCPs, organochlorine pesticides; OCR, oxygen consumption rate; PBDE, polybrominated diphenyl ether; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxins; PCDF, polychlorinated dibenzofurans; PFC, polyfluoroalkyl compound; PFOA, perfluorooctanoate; PFOS, perfluorooctane yl sulfonic acid; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RXR, retinoid X receptor; T2DM, type 2 diabetes; TBT, tributyltin chloride; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPTO, bis(triphenyltin) oxide; TR, thyroid hormone receptor.

in liver. Since 2,3,7,8-TCDD is the most potent AhR ligand with high affinity, AhR is called the dioxin receptor. AhR is able to bind a wide range of structurally divergent chemicals, suggesting that AhR contains promiscuous ligand binding site (Denison et al., 2011). Dennison et al. (Denison and Nagy 2003; Denison et al., 2002) reported exogenous AhR ligands covering diverse synthetic environmental chemicals or naturally occurring compounds such as indole-3-carbinol (I3C) and flavonoids using AhR-mediated reporter gene assays. I3C dietary AhR ligand provided from vegetables protects the host by an induction of active immunologic tolerance in the intestine (Li et al., 2011). A tryptophan catabolite, kynurenine, has been identified as an endogenous AhR ligand that is constitutively generated in human brain tumor cells (Opitz et al., 2011).

AhR ligands mediate a wide range of biological and toxic effects, including tumor promotion; teratogenicity; immuno-, hepato-, cardio-, and dermal toxicity; wasting; lethality; and alteration of endocrine homeostasis (Denison et al., 2011). The molecular mechanisms underlying AhR ligand action have been studied with multiple molecules interacting with AhR (Denison et al., 2011). In classical mechanism of AhR-dependent gene activation, the cytosolic AhR forms a complex with HBV X-associated protein 2 (XAP2), Hsp90, and p23 (Shetty et al., 2003), which are displaced by Arnt after binding to the ligand. The resulting AhR/Arnt dimer binds to a drug response element (DRE) (5'-TNGCGTG-3') on the promoters of target genes such as CYPs, aldehyde dehydrogenase 3 (ALDH3), AhR repressor (AhRR), p27kip1, and so on. AhRR is another bHLH/Per-Arnt-Sim transcription factor, which is also induced by AhR/Arnt complex upon binding on the DRE of AhRR promoter. Unlike other target genes, the transactivation domain-deficient AhRR functions as a naturally occurring dominant negative factor by competing with AhR for heterodimer formation with Arnt (Evans et al., 2008). Therefore, AhRR and AhR constitute a regulatory loop affecting each other's transcriptions to maintain a homeostasis. In various human malignant tissues, AhRR mRNA is downregulated due to methylation of DRE sites of the promoter, suggesting that AhRR might play a role as a tumor suppressor (Zudaire et al., 2008). Despite accumulation of evidence showing that many environmental chemicals are AhR ligands and destroy mitochondrial activities, it is not clearly understood whether their toxic effects are linked to mitochondrial damages. TCDD increases mitochondrial ROS production, which was observed in liver mitochondria of CYP1A1 null mice, but not in those of AhR null mice (Senft et al., 2002). The AhR-dependent oxidative stress in mitochondria mediated the concomitant mitochondrial DNA (mtDNA) cleavage (Shen et al., 2005). Proteomic analysis of AhR-interacting proteins showed that ligand-absent AhR was interacting with ATP5 α , a subunit of

adenosine triphosphate (ATP) synthase in mitochondria and this interaction was lost upon 10nM TCDD treatment (Tappenden et al., 2011). Recently, it was found that AhR was present in the intermembrane space of mitochondria. TCDD exposure at 10nM induced a degradation of mitochondrial AhR, reduction of respiratory capacity, and alteration of mitochondrial proteome in an AhR-dependent manner (Hwang et al., 2016). The AhR was also suggested as a regulator of the influx of electrons into electron transfer chain to maintain cellular respiratory capacity. A study using C2C12 skeletal myoblast cells demonstrated that nongenomic AhR signaling generated TCDD-induced mitochondrial toxicities (Biswas et al., 2008). Therefore, both genomic and nongenomic AhR signaling would affect mitochondrial toxicities by prolonged TCDD exposure as the TCDD-induced transcription-independent retrograde signaling from the mitochondria to the nucleus.

44.9 Insulin Resistance and Mitochondrial Dysfunction

Impaired mitochondrial function is linked with aging as well as many pathological states including IR, T2DM, MetS, obesity, and cancer (Petersen et al., 2003; Vuda and Kamath, 2016). Mitochondria are locations for metabolism of glucose, fatty acid, and protein and calcium homeostasis and are the control tower for cell death. When mitochondrial oxygen consumption is decreased due to altered metabolism, there is an increase in reactive oxygen species (ROS) that can impair different types of molecules and cells. Reversely, insulin is able to stimulate mitochondrial function by enhancing mitochondrial dynamics via the Akt-mTOR-NF κ B-Opa-1 signaling pathway (Parra et al., 2014).

Several human studies suggest that mild mtDNA variants, haplogroup or single-nucleotide polymorphism, may be associated with aging or diseases although mechanistic evidence at the molecular level is lacking. Some human mtDNA variations are associated with IR. Several population studies showed that mitochondrial haplogroup N9a confers resistance against T2DM, MetS (Fuku et al., 2007; Hwang et al., 2011; Tanaka et al., 2007), and diabetic complications (Niu et al., 2015). Conplastic animals with the same nuclear genome but with different mtDNAs show profound transcriptomic, proteomic, and metabolomic differences (Latorre-Pellicer et al., 2016). These mtDNA variations were linked to risk factors for T2DM, reduced mitochondrial oxidative phosphorylation (OXPHOS) enzyme levels, IR, cardiac hypertrophy, and systolic dysfunction (Houstek et al., 2014; Pravenec et al., 2007). Recent systematic characterization of conplastic mice throughout their lifespan showed that the mtDNA haplotype profoundly influences mitochondrial

proteostasis and ROS generation, insulin signaling, obesity, and aging parameters and mitochondrial dysfunction (Latorre-Pellicer et al., 2016). In fact, blood mtDNA content was decreased and mitochondrial function in skeletal muscle was reduced in subjects with IR, offsprings of T2DM, or prediabetes (Petersen et al., 2003; Shoar et al., 2016). When mtDNA was artificially depleted using ethidium bromide, cellular glucose uptake and glucose metabolism were critically impaired (Park et al., 2001). Therefore, we believe that mitochondrial function needs to be maintained during lifetime to ensure healthy status (Lee et al., 2010).

44.9.1 Mitochondrial Damages Induced by Environmental Chemicals

There are many different ways to damage mitochondria. Environmental chemicals in serum may damage mitochondria of insulin-sensitive organs such as the adipose

tissue, pancreas, liver, and muscles, which fail to function properly. It is not clear how environmental chemicals mediate mitochondrial dysfunction and IR although many environmental chemicals have been identified as AhR ligands.

There are only a handful studies on how POPs damage mitochondria. In addition to the results described in Section 44.7.3, TCDD also induced oxidative stress (Shen et al., 2005), mitochondrial pathways of apoptosis (Camacho et al., 2005), damaged mitochondrial OXPHOS and ATP production, and disrupted cristae structure in mitochondria in cardiomyocytes (Neri et al., 2011). We also demonstrated that TCDD and diabetic serum suppressed the basal oxygen consumption rate and ATP turnover rate of mitochondria (Park et al., 2013). TCDD at pM concentrations induced mitochondrial fragmentation and dysfunction measured by intracellular ATP concentration by luciferase–luciferin reaction and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate,

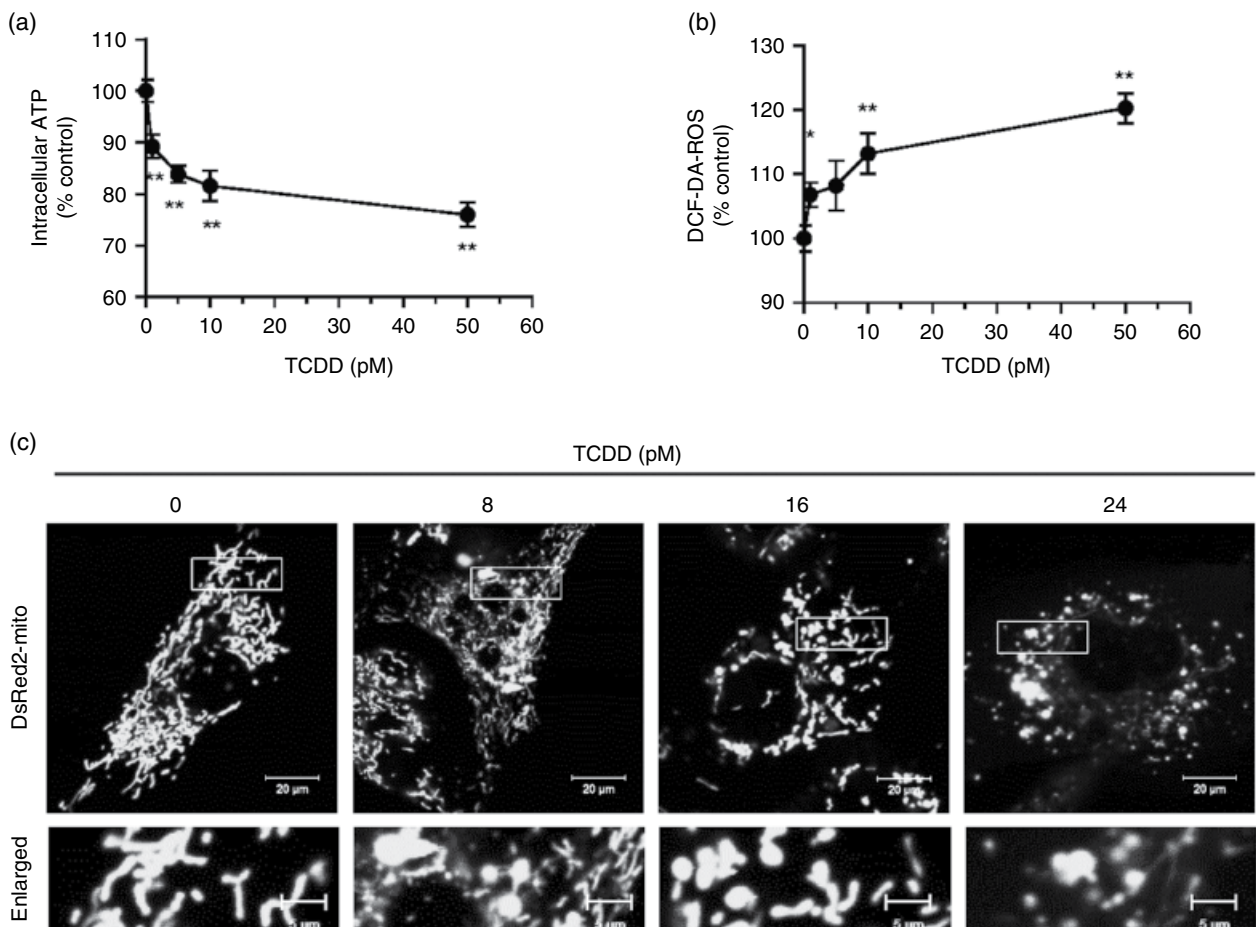


Figure 44.2 TCDD treatment-induced mitochondrial damages. C2C12 cells were treated with various concentrations of TCDD for 72 h. Intracellular ATP contents (a) and ROS generation (b) were determined (* $p < 0.05$, ** $p < 0.01$ vs. DMSO). (c) The DsRed2-mito-transfected SK-Hep1 cells were treated with TCDD as indicated and fixed. The mitochondrial morphology was visualized by confocal microscopy ($\times 400$ magnification, Scale bar = 20 μm). White box of each image was enlarged to view the morphology of mitochondria, reticular or fragmented (scale bar = 10 μm).

acetyl ester (CM-H₂DCFDA)-stained cellular ROS generation (Figure 44.2). The list of environmental chemicals that have shown to induce mitochondrial dysfunction is summarized in Table 44.3.

It should be noted that the glucose-induced insulin secretion is mediated by mitochondrial ATP production through glucose metabolism of glycolysis and the TCA cycle. Increase in blood glucose stimulates glucose uptake into pancreatic β -cells through glucose transporter (GLUT), where it undergoes glycolysis and mitochondrial TCA cycle to produce ATP. The increase of ATP level closes plasma membrane ATP-sensitive K⁺ channels (K_{ATP} channel) that are responsible for the resting membrane potential ($\Delta\Psi$). The cellular depolarization opens up the voltage-dependent calcium channel (VDCC) and increases intracellular calcium level, ultimately resulting in insulin secretion (Figure 44.3). Therefore, if ATP production in mitochondrial is decreased, insulin secretion would also decrease.

On the other hand, insulin signaling pathway could be disrupted by mitochondrial dysfunction. Insulin binding to insulin receptor initiates the signaling pathway, leading to glucose uptake, glycogen synthesis, lipogenesis, protein synthesis, decrease of gluconeogenesis, and lipolysis (Figure 44.4). Hub molecule of this pathway is Akt. Various mitochondrial stress-induced damages decreased Akt phosphorylation as well as serine phosphorylation of insulin-related substrate (IRS). Consequently, environmental chemical-induced mitochondrial damages can shut down most insulin-mediated responses. Recently, a whole issue of BBA Molecular Basis of Disease (Vol 1862, Issue 3, 2016) was devoted to

studies on connections between mitochondrial dysfunction and MetS. We refer an article by Jha et al. (2016) and some of key evidences listed in Table 44.4.

44.9.2 Scaling Law and Mitochondria

Cells are the basic unit of life. Each cell has a certain number of mitochondria, the content of which varies according to the energy requirements of tissue or organ. Mitochondria need to produce as much energy as the cell need for proper functioning. Our body is a complex energy-dissipating network system that evolves to withstand the destructive forces of the environment physically and chemically using energy and resources with extraordinary efficiency. Individual organism needs to repair damaged parts and the repair process requires energy. If mitochondria cannot supply enough energy (ATP) to repair damaged parts completely, a vicious cycle of energy deficiency and tissue damage will follow. From this point of view, mitochondria play a central role in increasing the entropy of our body, ultimately leading to death. Mitochondrial function shows a complex quantitative relationship to body size, as revealed by its scaling relationship to metabolic rate or energy use (West, 2012). Ultimately, we hypothesized that this scaling relationship could explain the associations between IR and mitochondrial dysfunction.

This concept can be extended to include the atherosclerotic process, a complication of T2DM and MetS. In this scheme, atherosclerosis is considered an adaptation of the vasculature to reduced energy demand, diminished mitochondrial function, and the consequently reduced nutrient needs of organs or tissues (Lee and Shim, 2013).

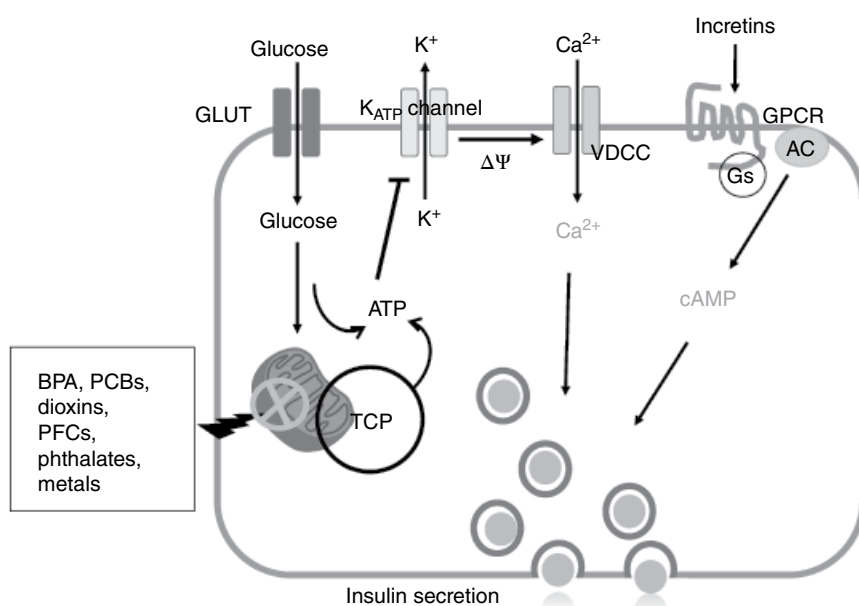


Figure 44.3 Role of mitochondria on insulin secretion in pancreatic β -cells. Environmental chemical-induced mitochondrial damages decreased ATP production, leading to closure of K_{ATP} channel and depolarization of membrane potential. Consequently, insulin vesicles do not undergo exocytosis because there is no calcium ion influx into cytoplasm through voltage-dependent calcium channel (VDCC). Incretins activating G-coupled receptor enhance cAMP for insulin secretion.

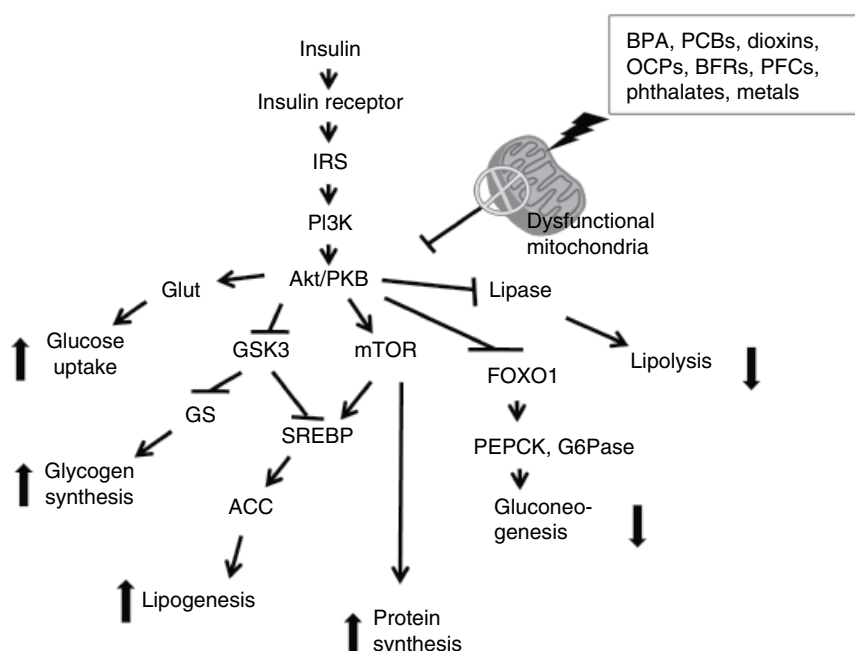


Figure 44.4 Role of mitochondria on insulin signaling pathway in insulin-responding cells, such as liver, muscle, fat, and brain cells. Environmental chemical-induced mitochondrial damages decreased Akt phosphorylation, leading to failure on insulin responses. Insulin-stimulated Akt activation stimulates activities of glucose transporter (Glut), glycogen synthase (GS), sterol response element-binding protein (SREBP), acyl-CoA carboxylase (ACC), and mTOR. Insulin also represses activities of lipase, phosphoenolpyruvate carboxykinase (PEPCK), and glucose 6-phosphatase (G6Pase).

Table 44.4 Metabolic disruptors, disrupted metabolisms mitochondrial dysfunction.

Chemicals	Obesity	T2DM	Lipid disorders	Mitochondrial toxicity
Bisphenol A	***	***	***	***
DEHP	***	***	***	***
DDT/DDE	***	**	*	***
PBDE			*	***
PFOA	**		***	***
PFOS		*	***	***
TBT	***	***	***	***
PAHs				**
PCBs	*	***	***	***
TCDD		**	***	***
Atrazine		*	**	*
HCB	*			**
Triflurazole	*			*
Benzo(a)pyrene			**	***
Tolylfluanid	*	*		*
Cadmium		*	**	***
Arsenic	**	***	***	***

Adapted from Heindel et al. (2016). Reproduced with permission of Elsevier.

The number of asterisk is higher when the strength of evidence is high.

BPA, bisphenol A; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; DEHP, diethylhexyl phthalate; HCB, hexachlorobenzene; PAH, polyaromatic hydrocarbon; PBDE, polybrominated diphenyl ether; PCB, polychlorinated biphenyl; PFOA, perfluorooctanoate; PFOS, perfluoro-octane yl sulfonic acid; TBT, tributyltin chloride; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

44.10 Measurement of POPs

44.10.1 Instrumental Analysis for POPs

POPs are quantified by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) method. This conventional analytical method determines the concentrations of individual POP congeners by combining organic solvent extraction of samples and quantification of internal dioxin standards (Bourdon et al., 2010; Kim et al., 2010). Toxic effect of POPs is expressed as toxicity equivalence (TEQ) value, which is calculated from the concentration of individual POPs (pg/g lipid) multiplied by their toxic equivalency factor (TEF) values (Van den Berg et al., 2006). The TEF value is a relation of the AhR-inducing potential of a single compound to that of 2,3,7,8-TCDD, which has a TEF of 1. The fact that this instrumental analysis requires large amount of serum or plasma samples, organic solvent extraction, lipid normalization, specialized facilities, and expertise as well as its high expense has prevented its application in large-scale clinical or cohort studies. In addition, various POPs in the serum might not be detectable by the HRGC/HRMS because their concentrations are lower than the detection limit (Rajapakse et al., 2002). However, these non-detectable POPs may be key contributors to the disease. In addition, the EQ method does not account for the antagonistic activity of POPs. Nevertheless, most epidemiological studies have used data generated by GC/MS for specific POPs of interest, even if they may not necessarily be biologically active.

44.10.2 Cell-Based Assays for POPs

Many POPs bind to the AhR, leading to transcriptional activation of multiple genes through binding to dioxin-responsive element (DRE) of their promoters (Denison and Nagy, 2003). The well-known chemically activated luciferase gene expression (CALUX) assay is the first cell-based measurement of AhR ligand mixtures (Schleizinger et al., 2010; Ziccardi et al., 2000). The CALUX assay has been used primarily to screen for the presence of POPs rather than to show their possible associations with adverse health effects because it also used the solvent extracted serum (Medehouenou et al., 2010). Later, CALUX-like methods were adapted to use with unmanipulated neat sera without extraction (Schleizinger et al., 2010). The attraction of this approach lies in its potential to assess the cumulative biological effects of a mixture of extractable AhR agonist and antagonist.

Independently, we modified the CALUX assay and established cell-based AhR ligand activity (CALA) assay. The CALA assay allows us to quantify total AhR-dependent

Table 44.5 Correlations between phenotype variables and serum AhR ligand activity by CALA assay among subjects who came to Eulji Hospital for health checkup.

Phenotype variables	<i>r</i>	<i>P</i> value
Body mass index	0.402	<0.0001
Body weight	0.329	<0.001
Waist circumference	0.420	<0.0001
Systolic blood pressure	0.316	0.0016
Diastolic blood pressure	0.270	0.0076
Fasting blood glucose	0.379	0.0002
Triglyceride	0.251	0.0132
Homeostasis model assessment of insulin resistance	0.238	0.0257

Park et al. (2013). Reproduced with permission of John Wiley & Sons.

transcriptional activity of circulating AhR ligand mixture in 10 μ l heat-inactivated serum samples without solvent extraction. The CALA-determined serum AhR ligand level was linearly correlated with TEQ values calculated from serum concentrations of 7 dioxins and 10 furans measured by HRGC/HRMS (Park et al., 2013). The linear relationship between calculated TEQ and serum AhR ligand activity was reproduced when TEQ was deduced from serum concentration of 7 dioxin-like PCBs in Swedish elderly cohort study (unpublished data). The results indicate that serum AhR ligand level determined by CALA reflects indeed the biologically active contents of POPs on AhR activation.

Using this CALA assay, we measured serum AhR ligand activities in a cross-sectional case-control study, consisting of normal fasting glucose, impaired fasting glucose, and T2DM (Park et al., 2013). Serum AhR ligand level was elevated by 40–50% in subjects with T2DM and impaired glucose tolerance and was correlated with BMI, waist-hip ratio, blood pressure, fasting blood glucose, serum triglyceride, IR score (Table 44.5), and adiponectin (Roh et al., 2015). Similarly, T2DM patients with diabetic nephropathy complication also showed the increased serum level of AhR ligands compared with those without nephropathy (Kim et al., 2014).

44.10.3 Association between CALA-Determined Serum POP Levels and Mitochondria Inhibitor Activity

With cell-based assay similar to CALA, effects of the serum samples on mitochondrial function of cultured cells can be determined by analyzing intracellular ATP

content and DCF-DA-stained ROS. ATP contents of 10 μ l human serum sample-treated cells were inversely related with CALA-determined AhR ligand levels. In contrast, the DCF-DA-stained ROS levels and AhR ligands showed a positive linear correlation (Park et al., 2013). However, there were some portions of outliers in this linear relationship. It suggests that some POPs may damage mitochondrial ATP production without binding to AhR. Reversely, it is possible that some serum AhR ligands might not affect mitochondrial function.

It should be noted that diabetic patient serum was as toxic as TCDD to mitochondria when incubated with skeletal muscle cells: T2DM serum decreased the ATP content by 32% and increased ROS level by 12% compared with normal serum. T2DM serum also suppressed oxygen consumption rate and mitochondrial ATP-generating ability. When mitochondrial morphology was visualized by confocal images, treatment of T2DM serum fragmented mitochondria of dsRed2-mito-transfected cells. The T2DM-induced morphology of mitochondria was similar to that shown in the TCDD-treated mitochondria (Figure 44.2). The experimental results support that serum AhR ligands may directly reduce mitochondrial function in tissues, leading to obesity, IR, and MetS.

44.11 Summary

There is a parallel increase of industrial chemical production and the incidence of obesity, T2DM, and MetS. IR and mitochondrial dysfunction emerged as the common pathophysiologic mechanism of these disorders, and mitochondrial dysfunction became known induced by environmental factors. Both nuclear and mtDNA variations also confer genetic susceptibility. In this chapter, we introduced environmental pollutants, which are discussed under the different names such as EDCs, metabolic disrupting chemicals (MDCs), and POPs, depending on their effects on human and environment. We summarized epidemiological and experimental evidence, showing that T2DM and MetS may result from low-dose exposure to various environmental chemicals via mitochondrial dysfunction. Because the composition and amount of the chemicals are enormously diverse, vary greatly over time, and exert additive or synergistic (mixture) effects, establishing the cause effect relationship was difficult. Furthermore, the molecular mechanism of the environmental chemical-induced mitochondrial dysfunction is not clearly established. However, the cell-based assays for AhR ligand and mitochondria function provided strong evidences for the environmental pollutants cause IR, MetS, and diabetes by damaging mitochondria.

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Cigarette Smoke and Mitochondrial Damage

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CHAPTER MENU

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45.1 Introduction

Tobacco is produced from the leaves of the tobacco plant by curing them. The plant is a member of the genus *Nicotiana* and of the *Solanaceae* family. Seventy species of tobacco are recognized and the main commercial crop is *N. tabacum*. The more powerful variant *N. rustica* is also utilized around the world. Every year, about 6.7 million tons of tobacco is prepared throughout the world. The top producers of tobacco are China (39.6%), India (8.3%), Brazil (7.0%), and the United States (4.6%) (FTDWebMaster 2005). There are an estimated 1.3 billion tobacco smokers worldwide, and it is estimated that this figure will be 1.7 billion by 2025 (World Health Organization 2003, pp. 83–102).

Tobacco contains a stimulant alkaloid called nicotine. The primary use of dried tobacco leaves is for smoking as cigarettes, cigars, pipe tobacco, and flavored shisha tobacco. These leaves can be also used as snuff, chewing tobacco, and dipping tobacco and for sniffing. Tobacco smoke has many chemicals that cause the harmful effect in both smokers and nonsmokers. Breathing even a little tobacco smoke can be deleterious (U.S. Department of Health and Human Services 2004, 2006, 2014; Centers for Disease Control and Prevention 2010). Among more than 7000 chemicals in tobacco smoke, at least 250

are recognized to be harmful, including hydrogen cyanide, carbon monoxide, and ammonia (Centers for Disease Control and Prevention 2010; U.S. Department of Health and Human Services 2014), and there are about 60 known carcinogens. There is no threshold level for exposure to cigarette smoke (CS), but conclusive evidence exists that long-term (years) smoking considerably raises the possibility of developing numerous fatal conditions.

Cigarette smoking, the major preventable cause of illness and death in a developed country, is a primary contributor to mortality of various malignancies such as cardiovascular and other respiratory disease.

45.2 Cigarette Smoke Components and Mitochondrial Toxicity

The special concern about health risks of CS is related to following six toxins: acetaldehyde, acrolein, benzene, 1,3-butadiene, acrylonitrile, and formaldehyde (Nazaroff and Singer 2004). Among them, acrolein has a high hazard index, induces oxidative stress through binding to sulfhydryl groups, and is more toxic (about 10–1000 times) than acetaldehyde, formaldehyde, and 4-hydroxynonenal (Nguyen and Picklo 2003). Acrolein, an important fraction of CS gas phase and also a product of lipid peroxidation *in vivo*, has been demonstrated to be a

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mitochondrial toxicant that evokes mitochondrial impairment in isolated liver mitochondria (Sun, Luo et al. 2006; Naserzadeh et al. 2015a). The inhibitory effects of acrolein on mitochondrial function have been proposed by Hirose Zollner et al. before 1973 (Zollner 1973). The toxic influences of acrolein on mitochondrial function have been studied *in vitro* in cultured cells such as PC12 cells (Luo and Shi 2005) and CHO cells (Tanel and Averill-Bates 2005) or in isolated mitochondria from the brain and spinal cord (Luo and Shi 2004), the lung (Liu and Tai 1985), the heart (Biagini, Toraason et al. 1990), and also with pure mitochondrial enzyme pyruvate dehydrogenase (PDH) and alpha-ketoglutarate dehydrogenase (alpha-KGDH) (Pocernich and Butterfield 2003). Hepatotoxicity of acrolein and its precursor, allyl alcohol, has been reported *in vitro* and *in vivo*, and alteration of some liver mitochondrial parameters has been reported in an *in vivo* investigation by administration of acrolein to rats for 45 days (Arumugam, Thanissar et al. 1999). It was also found that in primary hRPE and ARPE-19 cells, acrolein induces significant decrease in cell viability, oxidative damage, and mitochondrial impairment (i.e., membrane potential collapse, decrease in mitochondrial complexes activities, decrease in oxygen consumption and factors for mitochondrial biogenesis, and an increase in calcium) (Jia, Liu et al. 2007; Liu, Sun et al. 2007; Maliakel, Kagiya et al. 2008). In isolated mitochondria from rat liver, the toxic effects of acrolein on mitochondrial parameters such as protein oxidation, mitochondrial respiration, mitochondrial permeability transition, mitochondrial complexes I, II, III, IV, and V, PDH, alpha-KGDH, and malate dehydrogenase have been reported (Sun, Luo et al. 2006).

Current studies demonstrated that acrolein could stimulate mitochondrial oxidative stress. It has also shown that mitochondrial exposure with acrolein results in reactive oxygen species (ROS) formation and decreases in glutathione (GSH) content and aconitase activity. This effect is not accompanied by mitochondrial calcium influx or mitochondrial permeability transition; instead, it affects mitochondria by impairment of electron transport system functionality. The investigation also showed that acrolein could significantly inhibit mitochondrial adenine nucleotide translocase (ANT). This probably contributes to acrolein-induced ROS formation, since atractyloside, a specific ANT inhibitor, causes a tremendous increase of ROS (Luo and Shi 2005).

The effects of acrolein on mitochondria also invoke neurodegenerative diseases. This could be the inhibitory effect on state 3 of respiration; however, there is no any remarkable alteration in complex IV activity. Investigations showed that the respiratory inhibition was preventable by GSH and *N*-acetylcysteine. Although acrolein promotes many changes in mitochondria, there is not any modifi-

cation in calcium transporter activity or induction of cytochrome c release (Picklo and Montine 2001).

The study of acrolein and methylvinylketone impacts on some mitochondrial processes showed that the respiration with various substrates is inhibited. This investigation suggested that acrolein acts on three different sites: Pi transport, glutamate transport, and succinate dehydrogenase.

The effect on Pi transport is competitive. Methylvinyl ketone also shows the similar characteristics as acrolein though, with a smaller effect (Zollner 1973). These results show that acrolein is a powerful inhibitor of some mitochondrial processes.

Low amount of acetaldehyde could inhibit mitochondrial respiration. A site that is more sensitive to acetaldehyde is thought to be localized before the NADH-ubiquinone oxidoreductase compartment of the respiratory chain. Acetaldehyde can also inhibit energy production through the mitochondria. In response to the substrate and adenosine triphosphate (ATP) decline, energy utilization is also inhibited-supported reduction of Ca^{2+} -stimulated oxygen uptake and ATPase activity. The malate-aspartate, α -glycerophosphate, and fatty acid shift for oxidation by mitochondria are extremely sensitive to acetaldehyde. Acetaldehyde also inhibited the uptake of specific anions that are involved in the shuttles. The inhibition is evidently caused by NAD^{+} -dependent state 3 respirations, anion entry, and efflux interference (Cederbaum, Lieber et al. 1974).

Acetaldehyde could also inhibit the oxidation of fatty acids through mitochondrial pathway. Oxygen uptake that is stimulated by ADP is more sensitive to acetaldehyde inhibition than uncoupler-stimulated oxygen uptake. This suggests that acetaldehyde has an effect on the electron transport phosphorylation system, and it is significantly more than acetate. A decline in the respiratory control ratio connects to fatty acid oxidation. On the other hand, acetaldehyde depresses ketone body production. Some experiments proved that acetate is not accused for the inhibition. The depression of the end products of fatty acid oxidation (CO_2 , ketones, acetyl-CoA) proposes β -oxidation, citric acid cycle activity, and the respiratory phosphorylation chain inhibition by acetaldehyde (Cederbaum, Lieber et al. 1975).

Acetaldehyde (Ac) is an important element of tobacco smoke, being primarily generated through the burning of (poly)saccharides. Ac can impair mitochondria through a procedure that involves oxidative stress (Olivares, Bucio et al. 1997). There is a report (Lluis, Colell et al. 2003) that Ac impairs GSH transport in the mitochondria. The proposed mechanism is endoplasmic reticulum stress, which could promote oxidative stress. On the other hand, metabolism of Ac leads to excess NADH production that is oxidized by complex I in the mitochondrial respiratory chain and could finally generate superoxide

anion and oxidative stress. Although mitochondria are the primary source of free radicals, they are also one of the primary targets of Ac in inducing apoptosis (Colell, García-Ruiz et al. 2004) or being removed by mitoptosis (Lyamzaev, Nepryakhina et al. 2008).

In mitochondria isolated from Wistar rats, Ac reduced 50% of respiratory activities that involved a collapse in ATP content (28.5%). These results proposed that Ac could be causing alterations in cell redox status. Following measurement of protein oxidation, GSH ratio, and superoxide dismutase (SOD) activity, it has been shown that Ac caused an increased oxidation of proteins and a reduction in SOD activity (90%) and GSH/oxidized GSH ratio (36%). The data demonstrated that Ac-induced oxidative stress mediated by mitochondrial dysfunction and that resulted in cell sensitization to a second oxidative challenge (Labonne, Gutiérrez et al. 2009).

Pyridines are the important component of CS, and 2-ethylpyridine (2-EP) is one of its most toxic derivatives. Biochemically, addition of methyl or ethyl groups significantly promotes the toxicity of pyridine. Toxicity of pyridine derivatives toward cells is through different mechanisms. Mitochondria are primary organelles for energy generation so their integrity is very critical for cell survival, yet several measurements demonstrated that considerable mitochondrial impairment occurs after 2-EP exposure. The 2-EP-exposed ARPE-19 cells had promoted ROS/RNS concentrations, which show the important role of oxidative stress in its cytotoxicity (Ji, Melkonian et al. 2002; Melkonian, Eckelhoefer et al. 2003). ROS generation may cause further damage to the mitochondria, thereby resulting in vicious cycle(s) of ROS-related damage within cells (Tritschler, Packer et al. 1994). Some studies have reported that many mitochondria molecules are susceptible to ROS damage, such as lipids, proteins, and mitochondrial DNA (mtDNA). Interestingly, investigation shows that mtDNA is especially vulnerable to damage from oxidation, partly because it lacks histones or suitable process to repair damaged mtDNA (Yakes and Van Houten 1997). Thirteen proteins that are critical for oxidative phosphorylation (OXPHOS) encode by 37 genes of mtDNA. Following mtDNA damage, dysfunction of OXPHOS and increased leakage of electrons through the electron transport chain result in promoted endogenous ROS formation from the mitochondria. Lasting of this vicious cycle causes the higher ratio of deletions and/or rearrangements within the mtDNA, such as those reported in AMD retinas (Karunadharma, Nordgaard et al. 2010), which in turn can again reduce energy generation levels (Melov, Coskun et al. 1999).

Benzene is one of the notable carcinogens in CS. Investigations on isolated rat mitochondria showed that benzene could inhibit mitochondrial RNA synthesis in a

dose-dependent manner. The fact that the inhibition of mitochondrial RNA synthesis requires the exogenous NADPH suggests that benzene must be bio activated within this organelle.

It is noteworthy that the equivalent concentrations of toluene exhibited no inhibition and its coexistent with benzene caused the significant protection against benzene inhibition. Resembling results was obtained with mitoplasts, which are the mitochondria, stripped of their outer membrane. These results support the idea that inhibition by benzene is the result of solvent effects not only on the membranes but also on the benzene bioactivation that occurs within the organelle. Researches showed that when mitochondria incubated with benzene, it could activate this substance to a metabolite that covalently binds to guanine residues in mtDNA. Mitochondrial translation can also inhibit by benzene. This occurs because of transcription inhibition that results in a lack of mRNA and the disaggregation of polysomes (Kalf, Rushmore et al. 1982).

45.3 Health Problems Caused by Cigarette Smoking

Tobacco smoke causes heart disease, aortic aneurysm (a balloon-like bulge in an artery in the chest), rheumatoid arthritis, diabetes, osteoporosis, age-related macular degeneration, stroke, cataracts, and chronic obstructive pulmonary disease (COPD) (chronic bronchitis and emphysema) and worsens asthma symptoms in adults. It also causes a high risk of pneumonia, tuberculosis, and other airway infections in heavy smokers (U.S. Department of Health and Human Services 2004, 2014; Centers for Disease Control and Prevention 2010). Furthermore, smoking damages the immune system that leads to inflammation (U.S. Department of Health and Human Services 2014).

High incidence of respiratory carcinoma in human is associated with chronic use of nicotine products (Weitzman and Gordon 1990). A variety of secondary amines produced as a result of nicotine nitrosation; among these amines NNN (nitrosamines *N'*-nitrosonor-nicotine) and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) have the highest concentration in CS (Hoffmann, Adams et al. 1979). As a result of variation in metabolism of TSNA in different tissue and species, NNK induces lung, nasal cavity, and liver tumors in F344 rats and the lung, trachea, and nasal cavity in hamsters (Hecht and Hoffmann 1988), while NNN induces tumors of esophageal and nasal in F344 rats (Hoffmann, Castonguay et al. 1981). Polycyclic aromatic hydrocarbons (PAHs) induce an array of genes involved in a wide range of tissue pathologies, including inflammation,

immune response, drug toxicity, and cancer that are predominantly elicited through activation of the aryl hydrocarbon receptor (AHR). AHR is activated by PAHs, polychlorinated biphenyls, and heterocyclic amines/amides present in the CS and environmental pollution. Risk factors for AMD include smoking, nutrition, genetics, and higher body mass index (Cai, Nelson et al. 2000). Among these, cigarette smoking is one of the strongest agents correlated with inducing the most severe types of AMD (Klein, Klein et al. 1993; Cai, Nelson et al. 2000). Current smokers have a 45% chance of developing early AMD and show increased disease progression compared with nonsmokers (Schmidt, Hauser et al. 2006). *In vitro* investigations demonstrate that cigarette smoke extract (CSE) may cause human RPE cell death and changes in extracellular matrix synthesis (Dunaief, Dentichev et al. 2002). Addicted smokers have sperm count 13% lower than that of nonsmokers (Melkonian, Eckelhoefer et al. 2003). This is due to the 7000 components, including 69 documented carcinogens, of CS (Feher, Kovacs et al. 2006) collected in the systemic circulation and seminal plasma and irreversibly disrupts both the quality and quantity of human spermatozoa (Practice Committee of the American Society for Reproductive Medicine 2012). Chronic exposure to CS is toxic to germ cells and leads to excessive production of free oxygen radicals (Rodgman and Perfetti 2013). Thus, the spermatozoa of cigarette smokers have increased the incidence of oxidative DNA damage and adducts, oxidized unsaturated fatty acids, and chromosomal abnormalities (Ramla-Hansen, Thulstrup et al. 2007). Cigarette smoke condensate (CSC), or tar, includes mainly of halogenated and non-halogenated PAHs, dioxins, and prooxidants that powerfully prevent meiotic progression of spermatocytes and stimulate genes participated in the metabolism of PAHs (Vicizian 1968). Saleh et al. (Saleh, Agarwal et al. 2002) proposed that PAHs could disrupt gametogenesis by AHR-mediated inhibition of meiosis. Furthermore, it was displayed that *in vivo* exposure to CSC leads to spermatocyte cell death and seminiferous tubule impairment (Mostafa 2010). Smoking is the main risk factor correlated with the prevalence and the occurrence of neovascular macular degeneration and geographic atrophy (Khan, Thurlby et al. 2006). This relation between smoking and age-induced macular degeneration (Hoffmann, Zarrintan et al. 2013) has recently been confirmed by three large epidemiologic researches, including the Age-Related Eye Disease Study (AREDS) (Hansen, Esakky et al. 2014). Smoking is a source of serious oxidative stress, because of the high levels of aldehydes and NOX in CS, which significantly use up ascorbic acid concentration and protein sulfhydryl levels and cause oxidation of lipids and proteins (Esakky, Hansen et al. 2012). CS is an environmental toxicant that influences mitochondrial

function. Some proved influences induced by CSEs consist of mitochondrial fragmentation in bronchial epithelial cells (Feher, Kovacs et al. 2006) and change of mitochondrial function and structure in airway epithelium (Hara, Araya et al. 2013). In buccal cells of smokers that used at least 10 cigarettes/day for at least 6 months, mtDNA mutations, including point mutations and insertions/deletions in the DNA sequence, have been demonstrated (Hoffmann, Zarrintan et al. 2013). Low concentration of CS also induces an alteration in the morphology of mitochondria and causes the hyperfused networks that are believed to be an adaptive reaction to low levels of stress (Tan, Goerlitz et al. 2008).

It is harder for smoking women to get pregnant. A pregnant smoker is at higher risk of miscarriage, having her baby born with a cleft lip and/or cleft palate, having an ectopic pregnancy, and having her baby born too early and with an abnormally low birth weight (U.S. Department of Health and Human Services 2014). Smoking during pregnancy increases the infant's risk of death from Sudden Infant Death Syndrome (SIDS) (U.S. Department of Health and Human Services 2004; Centers for Disease Control and Prevention 2010). Erectile dysfunction is more common in smoking men (Austoni, Mirone et al. 2005; U.S. Department of Health and Human Services 2014).

45.4 Cigarette Smoke and Mitochondrial Damage in Different Disease

45.4.1 Cardiovascular Disease

It has been well documented that smoking has adverse effects on the cardiovascular system. Cigarette smoking is a major risk factor of atherosclerotic disease and is regarded as one of the major cause for coronary heart disease (CHD), along with lipid disorders and hypertension. Cardiovascular diseases (CVDs) such as many diseases of the blood vessels and the heart, peripheral vascular disease, cerebrovascular disease including CHD, and aortic aneurysm are all related to smoking. In younger men and in young and middle-aged women, cigarette smoking has been particularly connected with acute myocardial infarction (Barbash, White et al. 1995). The connection between smoking and cerebral atherosclerosis was also reported in autopsy studies and in noninvasive studies, which showed more severe atherosclerosis in the carotid and cranial arteries of smokers, as characterized by thickening of the wall and by narrowing of the arterial lumen (Tell, Polak et al. 1994). CS reduces cerebral flow, while smoking termination promotes cerebral perfusion. Progression of the atherosclerotic

Table 45.1 Effect of cigarette smoke on cardiovascular disease.

Pathologic effects	• Atherosclerosis initiation • Smooth muscle cell proliferation • Vascular intimal injury
Hemodynamic effects	Increased • Heart rate • Blood pressure • Cardiac output
Metabolic effects	Increased • VLDL • LDL • Insulin resistance • Glucose
Hematologic effects	Increased • Thromboxane release • Platelet aggregation • Platelet adhesion • Plasma viscosity Decrease • Prostacyclin release • Platelet survival • Bleeding time

lesions associates many metabolic and physiologic procedures, most of which are increased by cigarette smoking (Table 45.1). Smoking induces endothelial damage, which is regarded as the antecedent to atherosclerosis (Ross 1986). It has been shown that nicotine has a desquamating influence on the endothelium, possibly by augmented shear stress from increased blood viscosity and the rise in blood pressure, vasoconstriction, heart rate, and cardiac output induced by smoking (U.S. Department of Health and Human Services 2004). In addition to this endothelial injury by mechanical factors, chemical damage to the endothelium is induced by PAHs in CS. Smoking produces profound changes in the hemostatic system, which contributes to the progression of the atherosclerotic lesions and to the development of thrombosis, which is responsible for acute cardiovascular events. Platelet adhesiveness and agreeability are increased, and platelet survival is shortened in smokers (Nowak, Murray et al. 1987). Smoking inhibits prostacyclin and increases thromboxane synthesis; these changes favor vasoconstriction and platelet aggregation. Smoking increases the viscosity of the blood and elevates the levels of fibrinogen and factor VII and reduces bleeding time (Meade, Imeson et al. 1987). Smoking reduces acutely coronary vasodilatory capacity, and this could lower the ischemic threshold in smokers with CHD and contribute to the increased risk for sudden death (Czernin, Sun et al. 1995). Cigarette smoking increased the risk for reinfarction from

6.3% in nonsmokers to 12.5% in smoking patients (Rivers, White et al. 1990). During the 15 years of follow-up of patients who underwent coronary bypass surgery, increased risks for myocardial infarction, recurrence of angina pectoris, and recurrent bypass surgery were documented in patients who continued smoking or started to smoke again after the operation, compared with patients who stopped smoking since surgery and patients who did not smoke (Voors, van Brussel et al. 1996).

The activity of the myocardial cytochrome oxidase (complex IV) declines by 25% following a single 30 min exposure to secondhand smoke in rats, and the activity continues to decrease with prolonged exposures (Gvozđjak, Gvozđjakova et al. 1986). The effects of CS on OXPHOS of isolated rabbit myocardial mitochondria were investigated in three experimental models: after a single exposure lasting 30 min and after 2 and 8 weeks of twice-daily exposures. A considerable reduction in respiration as well as in the phosphorylation rate of mitochondria was shown, whereas the respiratory control index and coefficient of OXPHOS did not change. It is known that both carbon monoxide and nicotine of CS play important roles in the damage of mitochondria, and the previously mentioned studies indicate that cigarette smoking worsens metabolic and morphological changes in the heart muscle over time, contributing to smoke cardiomyopathy.

Similarly, CS seems to prevent mitochondrial OXPHOS in platelets, resulting in enhanced production of mitochondrial RS (Davis, Shelton et al. 1989). Rats exposed to passive CS from two cigarettes per day for 2 months have severely injured myocardial OXPHOS function during reperfusion injury (Van Jaarsveld, Kuyl et al. 1992). CS exposure also enhances sensitivity to heart ischemia/reperfusion damage in rats (Van Jaarsveld, Kuyl et al. 1992). Levels of CoQ are reduced as well (Gvozđjakova, Bada et al. 1984; Gvozđjaková, Šimko et al. 1999). Secondhand smoke exposure in mice leads to extensive aortic mtDNA damage, increased nitration and inactivation of SOD2, decreased ANT activity, and mitochondrial impairment when compared with unexposed mice (Knight-Lozano, Young et al. 2002). When added to other CVD risk factors (e.g., hypercholesterolemia), secondhand tobacco smoke synergistically hastens both mitochondrial damage and atherogenesis (Knight-Lozano, Young et al. 2002). Treatment of vascular smooth muscle cells (VSMC) with benzo-a-pyrene (BaP), an ingredient of CS, causes non-shivering thermogenesis in cockerels (Penn, Chen et al. 1994), does not appear to cause any stress response or collapse of mitochondrial membrane potential (MMP), but does cause cell death by necrosis (Vayssier-Taussat, Camilli et al. 2001). More recently, it has been demonstrated that rats treated with BaP, another chemical found in CS, showed an atherogenic phenotype with increased expression of

mtDNA transcripts. Cultured cells (myocytes? fibroblasts?) from these rats have increased growth rates and, significantly, proliferation in response to serum mitogens (Lu, Alejandro et al. 2002). Incubation of human monocytes and vascular smooth muscle cells with tobacco smoke filtrate results in the reduction of MMP and increased apoptosis and necrosis that are diminished by n-acetylcysteine treatment (Vayssier-Taussat, Camilli et al. 2001). Similarly, SOD2 overexpression in mouse fibroblasts considerably decreases the cytotoxic effect of CS (St. Clair, Jordan et al. 1994). The CVD commonly initiates well before the initial clinical manifestations. However, most investigations examining the effects of tobacco on CVD development have been carried out in adult animals/humans, and many are retrospective. One study in adults demonstrated that childhood smoking is a risk factor for reduced carotid artery elasticity in adulthood (Li, Chen et al. 2004). Others have demonstrated that CS exposure in younger adults, 15–34 years old, enhances atherosclerotic lesion development, as found by autopsies (McGill, McMahan et al. 2000). These studies propose that childhood exposure and/or circumstances within the fetal environment may affect disease development (Naserzadeh, Hosseini et al. 2015b). Indeed, some e-studies of gestational CS exposure demonstrated that umbilical cords from exposed children have an enhanced degree of atherogenic pathology (Asmussen and Kjeldsen 1975). More recently, it has been reported that children and young adults exposed to CS also have enhanced vascular wall thickening and atherosclerotic lesion development (McGill, McMahan et al. 2000). Similarly, atherosclerotic lesions were observed in children 1–36 months old who are exposed to secondhand smoke via parental smoking (Matturri, Ottaviani et al. 2005). Such neonatal and *in utero* exposure to CS enhances susceptibility of aortic rings to phenylephrine-produced vasoconstriction, decreases the endothelium-dependent relaxation by acetylcholine, and increases the EC₅₀ dose in both treatments. *In utero* ETS exposure also elevated the EC₅₀ of nitroglycerin in endothelium-independent vasodilation (Hutchison, Glantz et al. 1998). Neonatal ETS exposure leads to increased levels of endothelin-1, which can cause growth factor release from vascular smooth muscle cells and ROS generation by macrophages, and induces the expression of adhesion molecules in endothelial cells (Michael, Markewitz et al. 1997). Prenatal ETS exposure has also been correlated with larger myocardial infarct volumes after ischemia–reperfusion (Zhu, Sun et al. 1997). Results of investigation that assessed the influence of gestational contact to CS in mice have demonstrated that *in utero* exposure to CS is correlated with elevated concentrations of atherogenic cytokines, including MCP-1 and TNF- in adult mice. Similarly, it has been

found that childhood exposure to CS associates increased MCP-1 concentrations, a monocyte chemoattractant (Berrahmoune, Lamont et al. 2006). The effect of *in utero* or childhood CS exposure on the mitochondrion is investigated in the relative new field of research. It was shown that *in utero* ETS exposure is correlated with enhanced atherogenesis, oxidative stress, and mtDNA damage in mice and an increased susceptibility to complex IV inhibitors. Similarly, it has been found that smoking reduces complex III activity in placenta and decreases mtDNA levels, demonstrating that smoking is correlated with placental mitochondrial impairment. This could be the possible mechanism related to the placental transfer of components of tobacco smoke to fetal tissues (Lu, Disher et al. 1986). *In vivo* researches have demonstrated that components of tobacco smoke target the mtDNA (Bandy and Davison 1990). Carbon monoxide directly prevents OXPHOS by decreasing the amount of available oxygen (by generating carboxyhemoglobin (COHb)) to the mitochondrion (Couch 1986). Moreover, •NO control of respiration may aggravate the influences of hypoxia due to ETS-induced inflammation and induction of iNOS. Hence, vascular mitochondrial damage sustained *in utero* could impair energetic function and/or mitochondrial-dependent signaling in vascular cells postnatally, thereby raising the risk for atherosclerotic lesion development. Hence, these studies propose that fetal exposure to CVD risk factors maybe induce mitochondrial damage that contributes to the earlier onset of disease in adults.

45.4.1.1 Endothelial Superoxide Anion

Endothelial dysfunction is an initial manifestation of CS toxicity (Feher, Kovacs et al. 2006). It has been previously demonstrated that CS has a negative effect on nitric oxide (Ide, Tsutsui et al. 2000)-mediated endothelial function via increased generation of superoxide anion (O₂^{•−}). One investigation studied whether stable compounds present in CS activate specific pathways responsible for the increased endothelial O₂^{•−} generation. It was demonstrated that thiol-reactive stable metabolites in CS can activate NADPH oxidase and raise endothelial O₂^{•−} generation, thereby decreasing NO bioactivity, leading to endothelial dysfunction.

45.4.2 Brain Related Diseases

CSE and nicotine toxicity were assessed on mouse brain mitochondria *in vitro*, and the protective effect of vitamin C was studied. Mouse brain mitochondria were treated with CSE or nicotine in the absence or presence of vitamin C for 60 min *in vitro*, and the alterations of mitochondrial structure and function were assessed. CSE caused inhibition of mitochondrial ATPase and

cytochrome C oxidase activities in a dose-dependent manner that were diminished by vitamin C. The CSE treatment also yielded mitochondrial inner membrane injury. The toxic effect of CSE on brain mitochondria may be due to its direct influences on enzymatic activity rather than by the oxygen free radical itself. The main component responsible for the toxicity of CSE toward brain mitochondria is not nicotine (Yang and Liu 2003; Hosseini, Naserzadeh et al. 2013).

45.4.3 Respiratory System-Related Diseases

Smoking is the primary reason of COPD. In many other pulmonary diseases such as asthma, pneumonia, and even influenza, cigarette smoking is accused. The imbalance between proteolytic and antiproteolytic enzymes can lead to impairment of lung tissue. CS may intensify the activity of proteolytic enzymes and decrease antiprotease activity through smoke-induced oxidants in the lung. It has been revealed that heightened airway responsiveness in smokers is collaborated to the decline of pulmonary function. In addition, cigarette smoking is also connected to morbidity increasing from acute respiratory disease. The risk of lower respiratory tract illness and the duration of cough are increase in smokers. Oxidative stress induced by CS that results in airway inflammation has been proposed to be directly associated with tissue damage and cell death in COPD. Increased mortality from COPD remarkably higher between smoker than nonsmokers and also a dose-response relationship between COPD death and smoking is observed (Sherman 1992).

Demolition of bronchiolar alveoli, which found in smokers' lung, may impart to elasticity frailer of recoil and lead to emphysema. Chronic smokers suffer from lung problem and its result in three serious dysfunctions of pulmonary system: first, decrease of maximum expiratory volume; second, diminish plateau phase of FEV1; and third, accelerated decline of FEV1 (Samet and Lange 1996).

45.4.3.1 Cigarette Smoke-Induced Mitochondrial Damage in Airway Smooth Muscle

There is increasing interest in the non-bioenergetic roles of mitochondria and the potential role of mitochondrial dysfunction in disease. For example, mitochondria act as cytoplasmic Ca^{2+} buffers (Rizzuto and Pozzan 2006; Shkryl and Shirokova 2006) and modulate cell fate (proliferation vs. apoptosis) (Youle and Karbowski 2005; Liesa, Palacín et al. 2009). Under normal conditions, mitochondria exhibit tubular, reticular, or networked morphology, which is regulated by dynamic remodeling via the balance between mitochondrial fission versus fusion, which shifts in response to changes in the envi-

ronment (Chan 2006; Liesa, Palacín et al. 2009; Youle and van der Blik 2012). The equilibrium between fission and fusion processes initially thought to be exclusively morphology importance is now widely appreciated for its multiple and far-reaching implications, such as the content exchange between mitochondria, protection of mtDNA stability, respiratory functions, cell fate determination, and adaptation to cellular stress (Chan 2012).

Mitochondrial morphology fluctuates between two configurations of fused and highly fragmented mitochondria. In all cells, mitochondria continually experience fission–fusion cycle, and both features are controlled by fission versus fusion proteins. Mfn-1/-2 and Opa1 have critical roles in mitochondrial fusion, while the Drp1 and Fis1 seem to mediate fission (Youle and van der Blik 2012). There are some new evidence that revealed that Bax and Bak, which regulate apoptosis, are downstream of the mitochondrial fission/fusion proteins (Karbowski, Lee et al. 2002). CS can disrupt human airway cells by modifying Drp1 and Mfn1 expression and changes in mitochondrial function and structure.

Maintenance of the fission/fusion is mandatory for mitochondrial normal operation, accordingly for cell survival and growth. Intensified fission (fragmentation) results in impaired Ca^{2+} buffering impaired energetics, ROS generation, and finally greater apoptosis. In return, increased Ca^{2+} and ROS affect mitochondrial fission. Disturbance in mitochondrial fusion can cause MMP decline, deficient cell growth, and increased susceptibility to cell death. Inappropriate fusion can impair cellular energetics and proliferation, as well as the imbalance between fission/fusion, could alter mitochondrial function and tissue instability. Based on current knowledge, CS not only shifts mitochondrial balance toward enhanced fission but also disables fusion in pulmonary cells (Frank, Gaume et al. 2001).

Proteins involved in the regulation of mitochondrial morphology and the maintenance of the delicate fission–fusion equilibrium are essential for overall cellular health (Chan 2006; Liesa, Palacín et al. 2009; Youle and van der Blik 2012). Of particular interest is the fission protein dynamin-related protein 1 (Drp1), a master regulator of fission and a member of the dynamin family of GTPases, as well as other fusion proteins of the mitofusin family. Drp1 translocates from the cytoplasm and polymerizes into spirals around specific mitochondrial sites, constricting and breaking mitochondrial membranes at these sites, thus bringing about fission (Smirnova, Griparic et al. 2001). Mitofusins 1 and 2 (Mfn1 and Mfn2, respectively), on the other hand, are evolutionarily conserved GTPases on the mitochondrial outer membrane and are indispensable for mitochondrial fusion (Eura, Ishihara et al. 2006). As vital determinants of fission–fusion balance,

Drp1 and Mfn2 likely share a reciprocal relationship, and factors perturbing this balance are likely to also skew the stoichiometric relationship between these two proteins. There is currently no information on mitochondrial fission/fusion dynamics in airway smooth muscle (ASM) or its importance in asthma or CS effects. The clinical relevance of these processes lies in their potential contributions to altered airway structure and function that are keys to the pathophysiology of asthma and COPD. The latest studies of COPD patients have demonstrated that chronic exposure to CS causes mitochondrial fragmentation and increases expression of proteins in fission/fusion pathway in airway epithelial cells. The epithelium is the initial tissue that is exposed to CS so that the impact of volatile or the other permeable agents on airway smooth muscle cannot be ignored (Barbera, Peinado et al. 2003).

CS could also alter the production of ROS through mitochondrial pathways and cause severe cellular oxidative stress (Montuschi, Collins et al. 2000). Depending on tissue, it is thought that mitochondria produce a basal level (1–3% of oxygen consumed) of ROS under normal conditions; however such production increases when mitochondria have exposed a variety of cellular stressors. CS has both gas and tar phases, and both contain high levels of oxygen free radicals (Cueto 1990; Cueto and Pryor 1994). Accordingly, CS would be expected to have a variety of deleterious effects on mitochondrial structure and function. Many studies have shown that after CS exposure mitochondria in airway smooth muscle bear considerable changes compared with undamaged healthy mitochondria (Aguilera-Aguirre, Bacsí et al. 2009). CS causes changes not only in the morphology of mitochondria but also in the expression of some regulatory proteins like Drp1 and Mfn2. Moreover, fragmentation could be observed in mitochondria following exposure to CS in ASM cells (Aguilera-Aguirre, Bacsí et al. 2009). Mitochondrial fragmentation has also a direct relationship with ROS generation and is linked to the expression of the regulatory proteins Drp1 and Mfn2. Changes in these proteins result in perturbation of pathways in which ERK, PI3K/Akt, PKC, NF- κ B, and Nrf2 have important roles. To conclude, exposure to CS can modulate structure and normal function of mitochondria and also alter the fission–fusion process in damaged ones (Lee, Johnson et al. 2001).

As mentioned earlier airway smooth muscles are more vulnerable to CS especially in asthmatic patients that have more fragmented mitochondria in their airway muscles. It is important to note that inhibition of fission protein expression can reverse CS-imposed damage in smooth muscle mitochondria and disease symptoms.

45.4.3.2 Effects of Cigarette Smoke Extract on Alveolar Epithelial Cells

COPD is one of the universal main causes of death, which is characterized by progressive obstruction of airflow. Exposure to CS is the leading cause of COPD in Western countries. Recent studies showed that CS, due to its complex mixture of chemicals and reactive oxidants, causes alternations in mitochondria (elongation or fragmentation) in lung epithelial cells. Such changes in airway epithelial cells are followed by upregulation of Drp1 and downregulation of Mfn2, mitochondrial fission, and fusion protein (Karbowski, Lee et al. 2002).

Another study showed that CS influences mitochondrial morphology in alveolar epithelial cells. Exposure of these cells with nontoxic concentrations of CSE resulted in mitochondrial hyperfusion of and augmentation of Mfn2. These results are accompanied by increases in metabolic activity and MMP (de Brito and Scorrano 2009).

Various investigations showed that chronic exposure to CSE, induced vigorous and long-lasting effects in mitochondria in human bronchial epithelial cells, such as increasing the density of the matrix, increasing fragmentation and branching, and also diminishing numbers of cristae (Lamb and Reid 2001).

All mentioned changes, excluding fragmentation, are persistent even after depletion of CSE, indicating that these are interminable. In accordance with these observations, a significant rise in expression of OPA1 mRNA, which is a key regulator of cristae formation, and fission/fusion was observed (Duvezin-Caubet, Jagasia et al. 2006).

Additionally, it revealed that the level of oxidative stress marker is increased in addition to the OXPHOS components and antioxidant Mn-SOD increases, which indicate the augmentation of oxidative stress and energy demand. These mitochondrial alternations are associated with higher levels of pro-inflammatory cytokines such as IL-1 β , IL-6, and IL-8 in cells chronically exposed to CSE (Moodie, Marwick et al. 2004). Many studies also proposed that CSE is capable to damage mtDNA that bring cells to the premature aging. These functional and morphological changes may be useful as a biomarker for mitochondrial damage in different conditions (Ballinger, Boudier et al. 1996; Tan, Goerlitz et al. 2008).

45.4.3.3 Cigarette Smoke Effect on Mitochondrial Respiratory Chain

CS can induce necrosis and apoptosis of lung epithelial cells by obstruction of the mitochondrial respiratory chain (Hosseini et al. 2013a). Thus, mitochondrial dysfunction and resultant loss capacity ATP production are critically involved in pulmonary disease. Studies on isolated mitochondria from bronchial epithelial cells demonstrate that CS decreases ATP to a critical point, leading

damaged cells to the necrosis and inflammatory malignancies (van der Toorn, Slebos et al. 2007).

Comprehensive inquiry showed that CSE exposure inhibited both complexes I and II correspondingly decreasing oxygen consumption, MMP, and ATP production. On the other hand, CSE inhibited caspase-3 and -7 activities switching cell apoptosis into necrosis, suggesting that CS-induced cellular necrosis instead of apoptosis could be the etiology of COPD (Wickenden, Clarke et al. 2003).

45.4.3.4 Cigarette Smoke Effects on Mitochondrial in Alveolar Epithelial Cells

CSE could perturbed mitochondrial function in alveolar epithelial cells.

CS is capable of inducing the initial adaptive response in exposed cells such as increasing oxidative stress and inflammatory responses. Almost always, these conditions alter mitochondrial functions according to its severity, which means mild stress causes mitochondrial hyperfusion, whereas severe stress results in mitophagy. Hyperfused mitochondria make cells more susceptible to additional stress, such as chronic exposure to CS (Ballweg, Mutze et al. 2014).

One of the main species in CS that increases mitochondrial ROS generation is lipid-soluble components. These compounds besides ROS exacerbate bronchi-alveolar disease, while ROS can barely penetrate airway epithelial cells and definitely not enter into circulation.

It was assumed that lipophilic components can reach to airway epithelial cells and intensify mitochondrial dysfunction and intracellular ROS production (van der Toorn, Rezayat et al. 2009).

45.4.3.5 Aryl Hydrocarbon Receptor and Cigarette Smoke-Induced Mitochondrial Dysfunction

Fibroblasts and epithelial cells are susceptible to the oxidative components of CS, and loss of these cells leads to the development of the pulmonary disease. Fibroblasts express the AHR, which can diminish pulmonary inflammation that is suspected to prevent apoptosis caused by CS. It was reported that AHR can regulate fibroblast expansion by preventing morphological changes like blebbing and chromatin condensation induced by CS.

Repression of AHR expression invokes PARP cleavage, cytochrome c release, and activation of caspase-3 that initiates the intrinsic apoptosis pathway (de Souza, Zago et al. 2011). Recent studies revealed that fibroblasts with low expression of AHR contain inadequate amounts of antioxidant enzymes (Mn-SOD and CuZn-SOD). According to new investigations, the capability of the AHR to forestall apoptosis is observed in lung epithelial cells as well as fibroblasts. The presence of this type of

receptor in lung cells is the reason for their vulnerability to CS (Sarill, Zago et al. 2015).

Apart from the energetic role in cells, mitochondria have a crucial role in certain diseases, due to their ability in modulating cells between proliferation and apoptosis (Liesa, Palacín et al. 2009) and their response to environmental changes by remodeling their morphology between networked, tubular, or reticular morphology through fission/fusion (Youle and van der Bliek 2012).

So far, it has been thought that fission and fusion just act as morphology oriented process; however, it is now widely accepted that these changes can protect mtDNA and respiratory functions and determine cell fate and response to cellular stress (Chan 2011).

Proteins associated with the mitochondrial morphology management and maintenance of the fission–fusion balance is fundamental for overall cellular functions (Chan 2006).

In addition to supplying cellular energy, mitochondria buffer cytoplasmic Ca^{2+} and are key regulators of cell death and proliferation (Hajnoczky, Csordás et al. 2002). Ca^{2+} can increase intracellular ROS generation and in turn, ROS may control intracellular Ca^{2+} concentration and cell expansion.

45.4.4 Cigarette Smoke Damage on Mitochondria in the Retinal Cells

One of the major toxicants in CS that can induce ocular injury is acrolein. Research showed that retinal pigment epithelial (RPE) cells exposed to acute acrolein exposure exhibited toxicity, including a decline in cell viability, MMP, antioxidant capacity, GSH, and Nrf2 expression. Acute exposure to acrolein also increases oxidant production, protein carbonylation and (cytosolic and/or mitochondrial?) calcium levels, and antioxidants have been shown to diminish these effects (Jia, Liu et al. 2007).

45.4.5 Cigarette Smoke Induce Mitochondrial Damage in Blood Cells

Some investigations on human peripheral lymphocytes showed that CS is able to affect mitochondria through induction of oxidative stress. As mentioned earlier, mitochondria are the main source of ROS, and CS exposure exacerbates ROS production yielding a wide variety of oxidative damage including membrane lipid peroxidation. Indeed, a smoker's lymphocytes show high levels of peroxidation. Complex IV is the most inhibited component of the respiratory chain in circulating lymphocytes in smokers. Such mitochondrial dysfunction in smokers is thought to contribute to the carcinogenicity of CS (Miró, Alonso et al. 1999).

45.4.6 Mitochondrial Damage by Cigarette Smoke Results in Cancer

Cigarette smoking is the most important cause of cancer around the world. Death rates of smokers from cancer are totally two times higher than those of nonsmokers, and these rates are elevated near fourfold in heavy smokers (Doll 1996).

Lung cancer is the most common cause of cancer death in both sexes among smokers. According to researches on tobacco smoke in 1985, International Agency for Research on Cancer (IARC) stated cigarette smoking was carcinogenic to humans. Smoking was convinced to be the main cause of eight divers' cancers: pancreas, bladder, renal pelvis, lung, larynx, and esophagus. Cigarette smoking is also suspected to be a cause of abdominal cancers such as the stomach, renal body, liver, and colon as well as bone marrow problem such as myeloid leukemia (Brownson, Novotny et al. 1993). Many epidemiological studies provided beneficial evidences in support of relation between cigarette smoking and cancer. The information obtained through these studies are very persuading by their consistency, by the quality of the association, as measured by the ratio of rates of disease between smokers and nonsmokers, or dose-response relationship, which is obvious for all cancers. Epidemiologic investigations also exhibit that the risks of nearly all smoking-related cancers reduce by quitting smoking.

CS consists of more than 4000 compounds. Many of these are distinguish to be toxic, carcinogenic, and mutagenic. Two critical steps are supposed to be included in carcinogenesis: DNA damage, which is an initiation irreversible phase, and promotion phase, in which infected cells are transformed to the malignant phenotype. In the particulate matter of CS tar, polyaromatic hydrocarbon is the major tumor initiator. This compound causes tumors in animals in direct dose and time-dependent manner (Tang, Morris et al. 1995). The effect of these initiators is increased by promoters or other cocarcinogens exist in the weakly acidic and neutral parts of tobacco smoke condensates (Hoffmann, Wynder et al. 1987). The existence of reversible promoters in CS is supported by a fact that the risk for all smoking-related cancers is reduced following cessation of smoking. The immense carcinogenic effect of cigarette is on directly exposed tissues, such as the airway system. However active constituents and organ-specific carcinogens of the smoke affect also distant organs. For example, 2-naphthylamine that concentrates in urine is directly related to bladder carcinoma. Lung cancer is the main cause of cigarette-related cancer death and is attributable to 90% of lung cancer in men and 79% among women. Male smokers have 22-fold increased risk, whereas female

smokers have 12-fold increased risk for lung cancer. It is obvious that there is a dose-response association between CS and the risk of lung cancer in both sexes, so people who smoke 40 cigarettes per day have twice the risk comparison to those who smoke 20 cigarettes a day. Smoking multiplies the risk for all main types of lung cancer squamous cell, small cell, large cell, and adenocarcinoma. It is notable that male smokers have a heightened proportion of squamous cell carcinoma and female smokers have a higher proportion of small-cell carcinoma (Anton-Culver, Culver et al. 1988). Other organs directly in contact with smoke like the oral cavity, larynx, and esophagus are also at risk of different problems. Therefore, laryngeal cancer, with a 10-fold increased risk in male smokers, as well as oral and esophageal cancer is expected (Augustine, Hebert et al. 1988). Organs far apart from CS are also at risk, for example, the risk of kidney and bladder cancer is two to three times higher in smokers (Naserzadeh and Pourahmad 2013b). In addition, CS may interact with occupational chemicals and produce kidney and bladder cancers. Most of the time workers in rubber, paint, and leather industries demonstrate these different kinds of cancers (Boyko, Cartwright et al. 1985). About 30% of deaths from pancreatic cancer are connected to CS, and heavy smokers have a fivefold increase in risk for this tumor over that of nonsmokers. Furthermore, there is an increased risk of gastric carcinoma in smokers compared with nonsmokers. Many studies revealed that cervical carcinoma is also related to CS exposure. Moreover, CS is composed of two important leukemogenic compounds—benzene and polonium isotopes—and a relationship between myeloid leukemia and smoking in men are observed, so CS is the most important avoidable cause of myeloid leukemia.

CS can increase the risk for liver, colon, and prostate cancer as well. To conclude, cigarette smoking has been recognized as a clear cause of cancer in many organs in direct or indirect contact. These data suggest that smoking may have a harmful effect and diminish survival in patients with each kind of cancer (Rodniguez, Tatham et al. 1997).

Cigarette smoking is the most important cause of cancer around the world. Death rates from cancer are totally two times higher in smokers than nonsmokers, and these rates increase nearly fourfold in heavy smokers (Doll 1996).

Investigation in cigarette-related cancers showed that mtDNA damage plays a prime role. These studies found that mtDNA had multiple point mutations and some deletions/insertions, especially in buccal cells. Another study showed an increase in mtDNA content and decrease in mitochondrial function in response to CS exposure that is positively associated with smoking duration and dose (Tan, Goerlitz et al. 2008).

45.5 Summary

Tobacco plant, a member of the genus *Nicotiana*, is the initial material from which tobacco is produced. This product contains nicotine that is a stimulant alkaloid. There are various applications for dried tobacco leaves such as smoking cigarettes, cigars, and pipe tobacco.

In developed countries, cigarette smoking is one of the preventable factors that cause severe illness and even death. The special concern about health risks of CS related to following six toxins: acetaldehyde, acrolein, benzene, 1,3-butadiene, acrylonitrile, and formaldehyde. It has been proved that smoking has detrimental impacts on several parts of the body; among them cardiovascular and respiratory systems are in concern. Cigarette smoking is the major causes of COPD and also suspected to cause many other pulmonary diseases such as asthma, pneumonia, and even influenza.

The correlation between smoking cigarette and many kinds of cancer has been pronounced via several investigations. It is well documented that the mortality rates from cancer among smokers is nearly two times higher than those of nonsmokers and is elevated four times higher between heavy smokers.

Modification in lipoprotein metabolism has also reported by smoking the cigarette. Cigarette compounds have significant cardiovascular impacts like elevation in triglycerides, VLDL, LDL, and apolipoprotein B levels and a decrease in HDL levels and oxidative modification of LDL.

Mitochondria, cell energy generator, are involved in many diseases and malignancies. Mitochondrial dysfunctions can be caused by mutations in mtDNA that

lead to dysfunction of the respiratory chain, decrease in ATP production, increase in free radicals generation, and alter cellular calcium handling. CS causes mitochondrial respiratory dysfunction, which results in MMP collapse and reduces ATP generation.

CS can obstruct mitochondrial respiratory chain and induce necrosis in lung epithelial cells. Thus, mitochondrial dysfunction and alteration in the ATP synthesis are critically involved in pulmonary disease. Researches on isolated mitochondria from bronchial epithelial cells demonstrated that decreasing cellular ATP down to the critical point leads damaged cells to the necrosis and inflammatory malignancies.

Some investigation on human peripheral lymphocytes showed that CS is able to affect mitochondria through induction of oxidative stress. As mentioned before mitochondria are the main source of ROS in cells, but CS exposure can exacerbate ROS production and induce oxidative damage to the biological membrane.

Investigation in cigarette-related cancer showed that mtDNA damage can be the main culprit. In these researches, it was clear that mtDNA experience several point mutations and some deletions/insertions, especially in buccal cells. Another study also showed an increase in mtDNA content and decrease in mitochondrial function in response to CS exposure that is positively associated with smoking duration and dose.

Based on current knowledge, CS not only shifts the mitochondrial balance toward enhanced fission but also disables fusion in pulmonary cells that attenuates apoptosis signaling and makes an unhindered avenue for progressing cancer.

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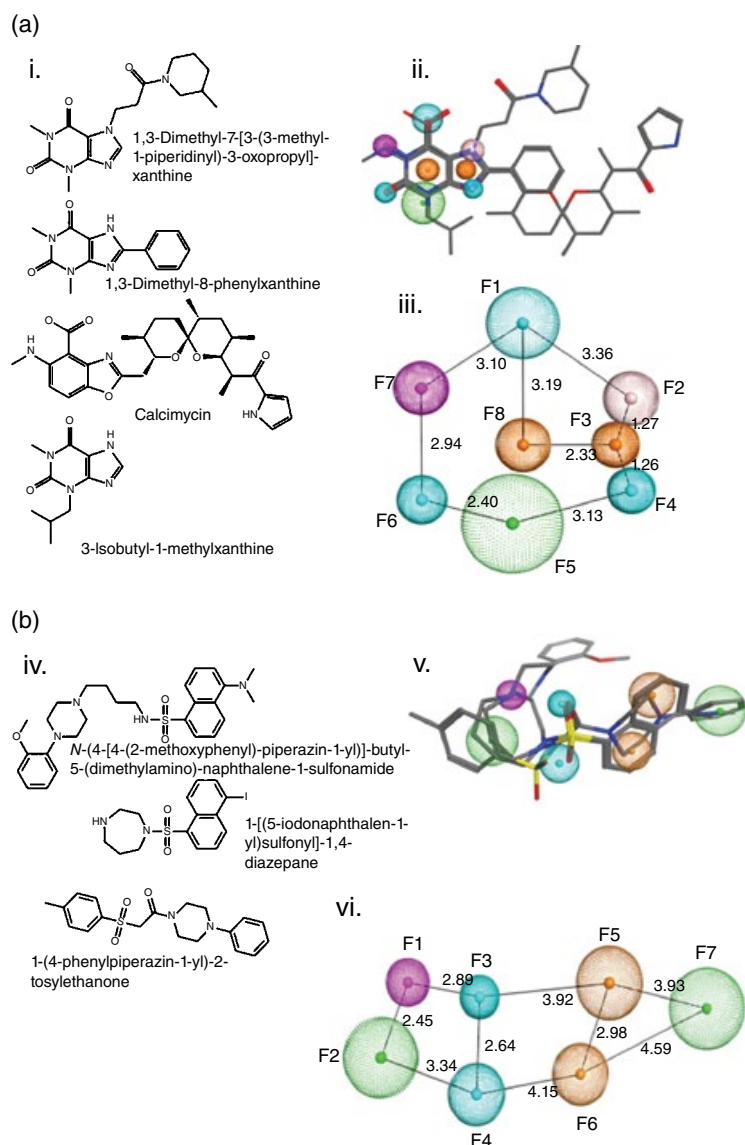


Figure 12.2 Developing toxicophore models from known toxicants. (a) Highly similar compounds with a shared chemical scaffold (i) are rigidly aligned (ii) to develop a toxicophore model (iii). This strategy is particularly useful for identifying functional groups to exclude from consideration during the optimization of lead compounds. (b) Compounds with moderate structural similarity but a dissimilar chemical scaffold (iv) are flexibly aligned (v) to generate a toxicophore model (vi). This strategy is particularly useful for the identification of potential toxicants during initial screening of large chemical libraries. Adapted from Wills et al. (2013). Reproduced with Permission of Elsevier.

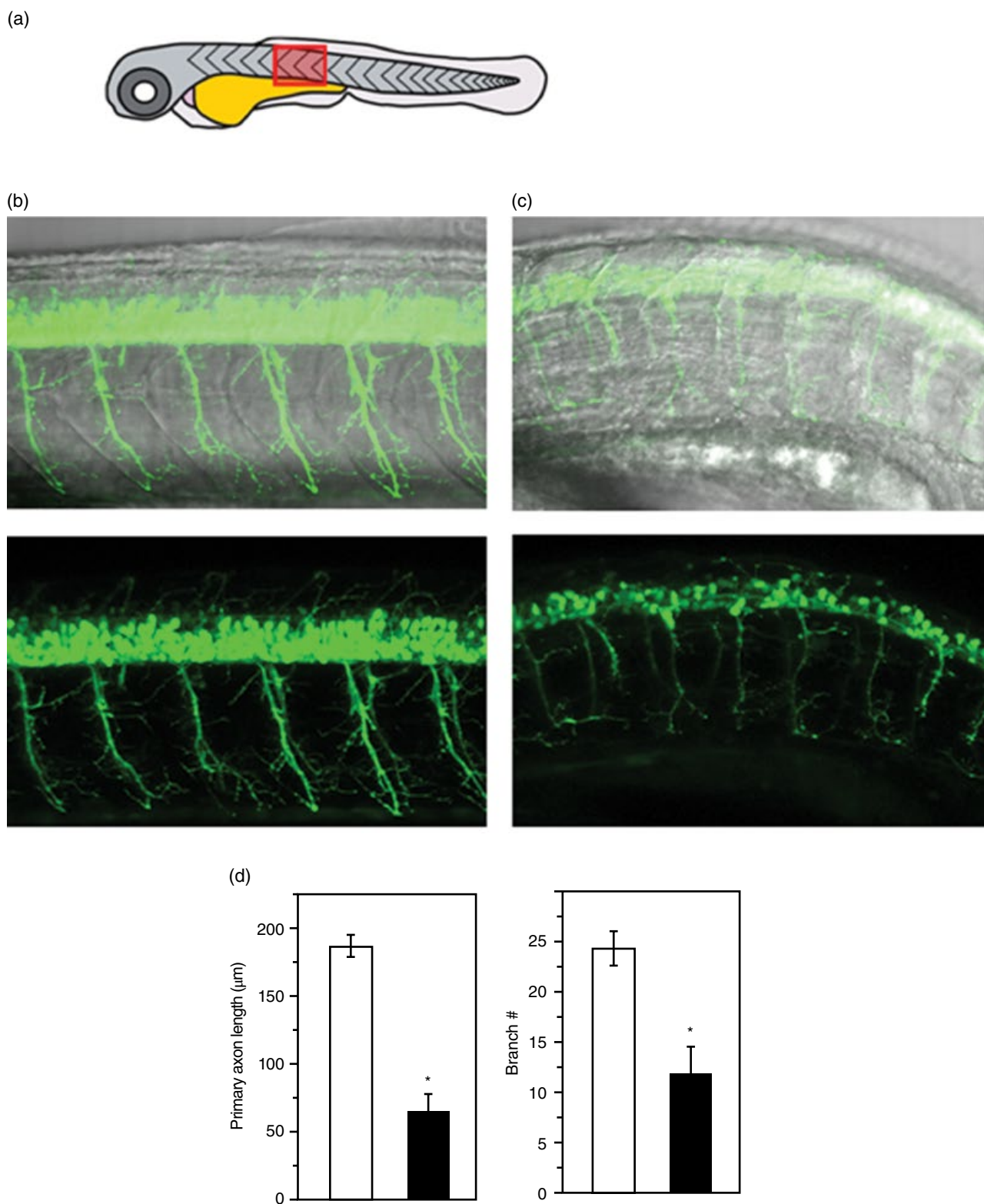


Figure 21.1 Motor neuron abnormalities are observed in Peo1 (mtDNA helicase) knockdown (KD) embryos. (a) Diagram of zebrafish embryo with red box indicating the approximate position of (b and c). Projections of 150 μm two-photon stacks of control (b) and Peo1 knockdown (c) embryos at 53 hpf. Peo1 KD embryos have altered caudal primary (CaP) motor neuron axonal branch patterns that fail to extend. Growth of CaP motor axon arbors is significantly inhibited in Peo1 KD embryos: total axon branch lengths were decreased as were the length of the primary axon, average sidebranch lengths, and branch numbers. * Indicates significant difference ($p < 0.05$) from control levels.

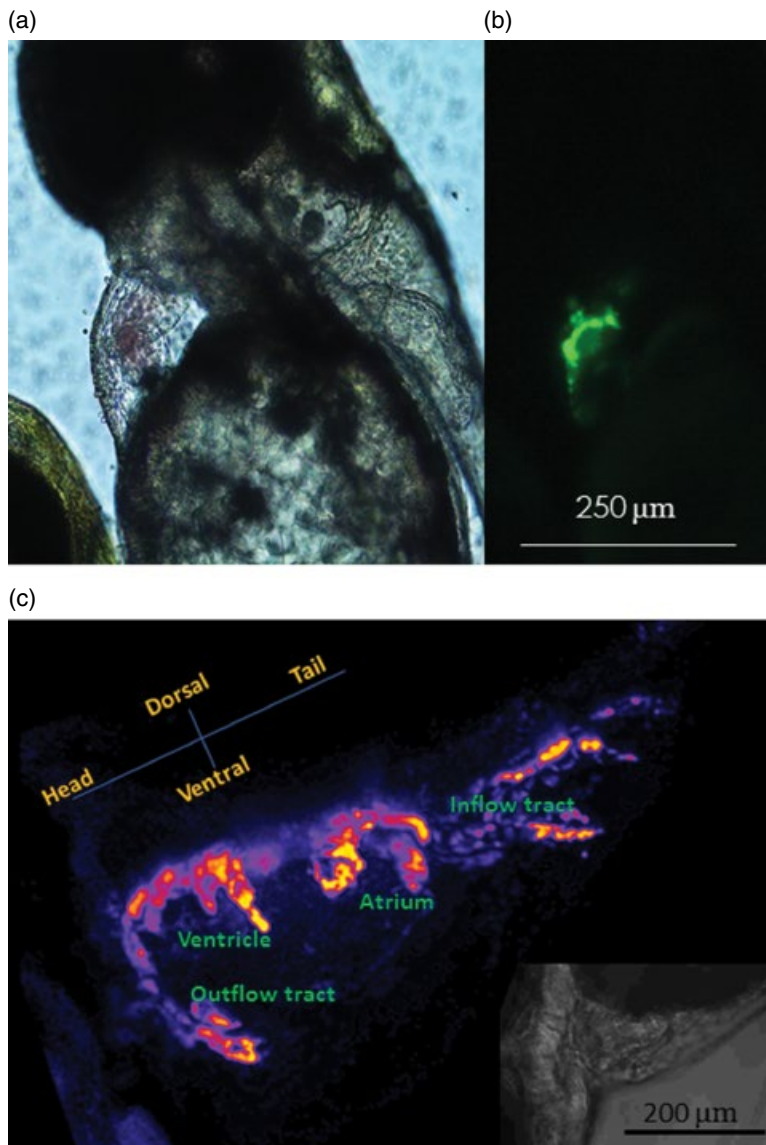


Figure 21.2 Mitochondrial GFP targeted to cardiac tissue in zebrafish. The plasmid construct used contains the *cmlc2* promoter, a mitochondrial leader sequence, and green fluorescent protein (GFP) sequence, flanked by Tol2 sites. Plasmid and Tol2 mRNA are injected into embryos at the 2-cell stage. (a) Bright-field image of a 72 hpf zebrafish at 100× magnification. (b) Fluorescent mitochondria in the heart of the individual from A. Viewed using FITC filter on an inverted Zeiss microscope (excitation, 450–490 nm; emission, 515–545 nm). (c) 72 hpf WT zebrafish injected with the plasmid construct mentioned earlier. Zebrafish were fixed in 4% paraformaldehyde for 5 h at room temperature and washed three times for 10 min in PBS before mounting in agarose. This confocal image was taken on the Olympus FV1200 MPE intravital microscope at 30× magnification and 1.5 μm slice interval. Presented as a heat map of mitochondrial density in a 3D projection.

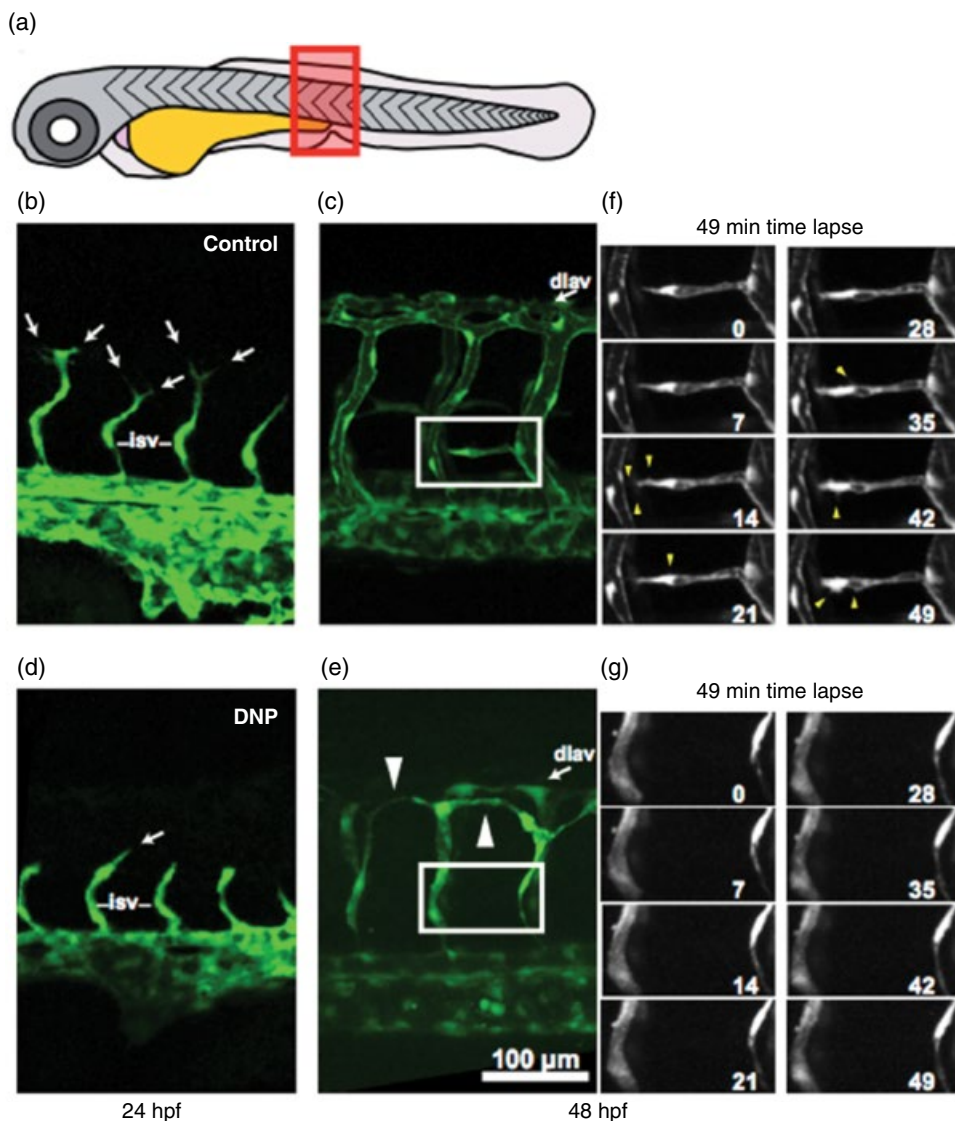


Figure 21.3 DNP exposure disrupts blood vessel development. *In vivo* 2-photon stacks acquired through the trunks of 24 and 48 hpf flil1: eGFP zebrafish reveal the developing blood and lymph vessels. (a) Diagram of zebrafish embryo with red box indicating the approximate position of (b–e). At 24 hpf, both control (b) and DNP-treated (d) zebrafish have dorsally growing intersomitic vessels (isv), but those of DNP-treated fish end in fewer filopodial projections (arrows, (b, d)), indicating that DNP inhibits angiogenesis. At 48 hpf, the dorsal longitudinal anastomotic vessel (dlav) of control fish has a fully formed lumen able to pass blood cells; it remains constricted in the DNP-treated fish (e) (arrowheads). Boxed regions in (c) and (e) are magnified and the time lapse frames reveal the filopodial projections (yellow arrowheads) on the growing tip of the parachordal chain in controls (f), which is absent in the DNP-treated animal (g).

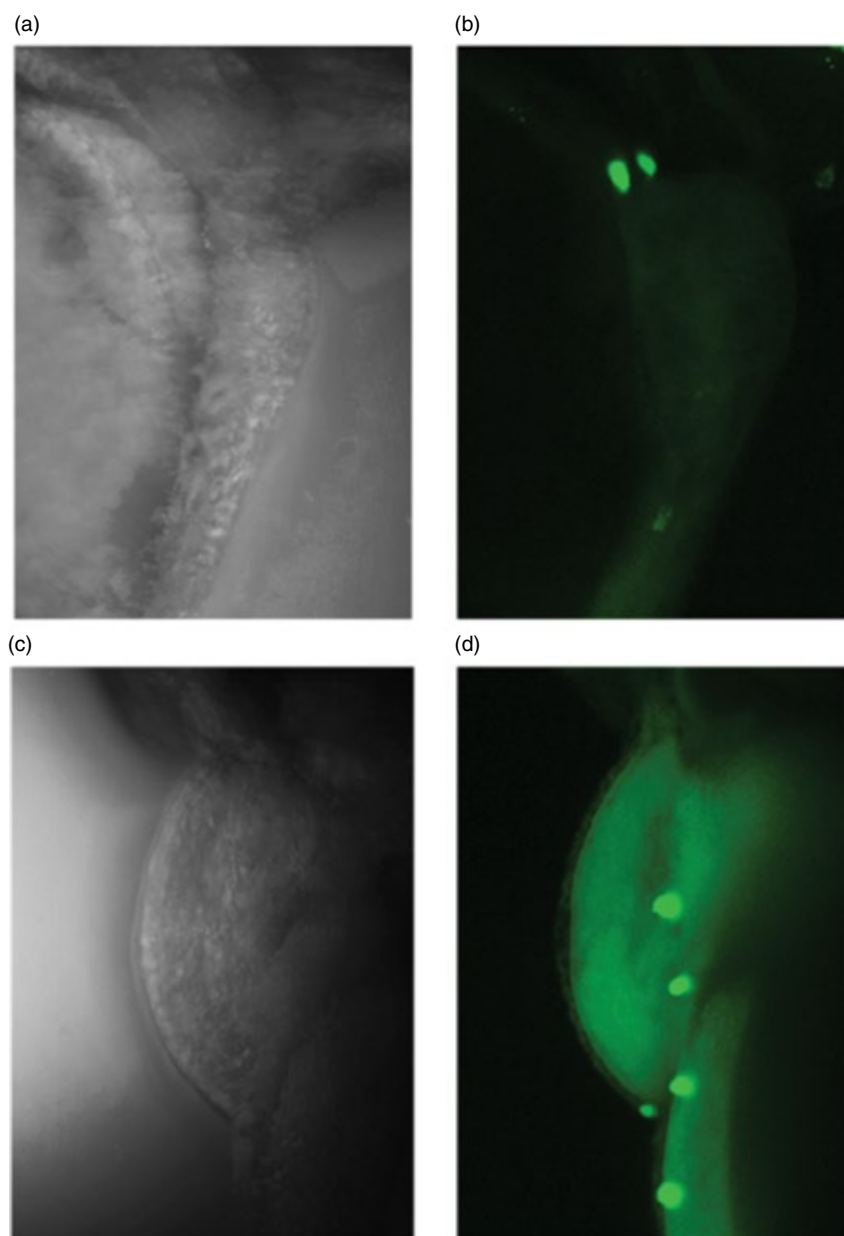


Figure 21.6 Increased reactive oxygen species observed in Peo1 knockdown embryos at 3 days' post-fertilization. Representative 2-photon image projections of H₂DCFDA fluorescence from mismatch control (a (brightfield) and b (fluorescence)) and Peo1 knockdown (c (brightfield) and d (fluorescence)) embryos. Peo1 knockdown hearts appears to have more H₂DCFDA fluorescent product than controls. The bright spots in both panels are epidermal cells (melanocytes) and are excluded from analyses.

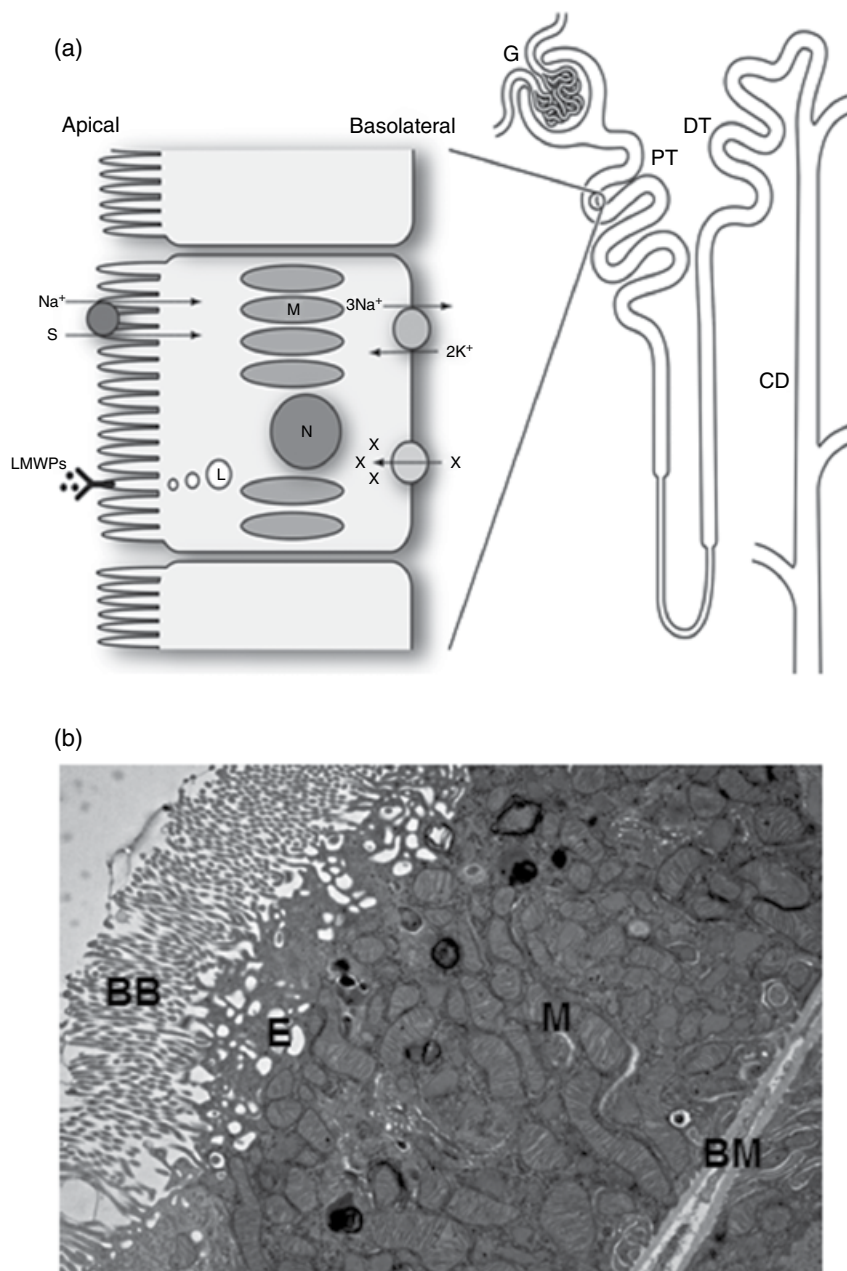


Figure 27.1 Solute handling and uptake of drugs in the nephron. (a) The majority of fluid filtered by the glomerulus (G) is reabsorbed along the proximal tubule (PT). Further reabsorption takes place in the distal tubule (DT) and collecting duct (CD). Various solutes (S) are cotransported across the PT apical membrane with Na^+ , while low molecular weight proteins (LMWPs) are taken up by receptor-mediated endocytosis into endosomes and eventually lysosomes (L). Sodium transport is driven by the basolateral Na^+/K^+ -ATPase, which requires ATP generated by mitochondria (M) located in close proximity (N, nucleus). A number of toxic drugs (X) can rapidly accumulate into PT cells via transporters expressed in the basolateral membrane and induce mitochondrial dysfunction and acute kidney injury. (b) Electron micrograph of a mouse PT showing the characteristic morphological features, including a highly developed apical membrane brush border (BB), numerous subapical endosomes (E), and a high density of elongated mitochondria (M) lying in a striated distribution close to the basolateral membrane (BM). Part A: Hall et al. (2014). Reproduced with permission of Oxford University Press.

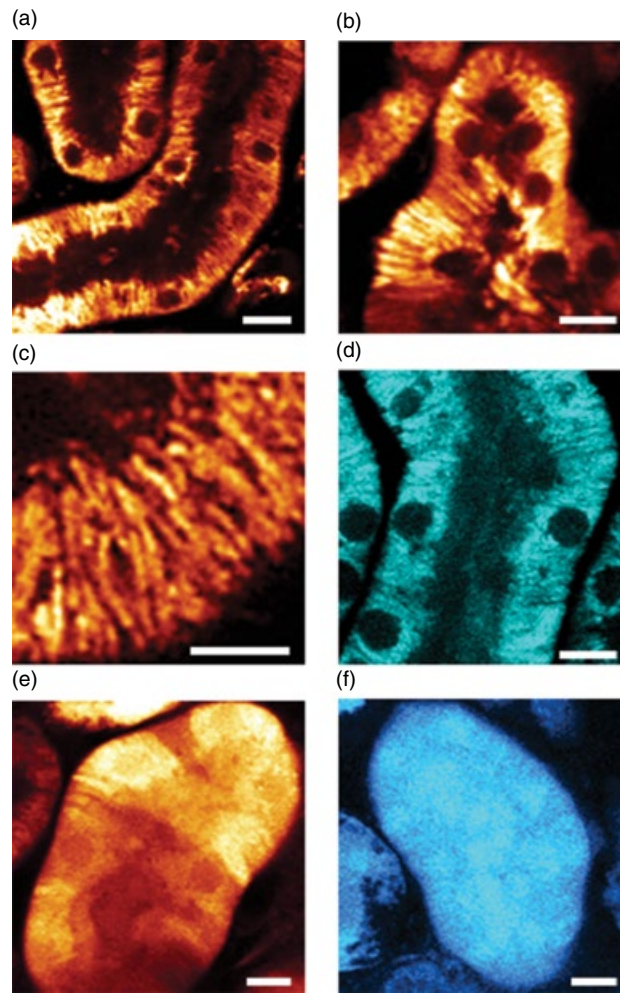


Figure 27.2 Multiphoton imaging of mitochondria in live mouse kidney cortex slices. A variety of aspects of mitochondrial function can be imaged in different nephron sections. (a–c) Mitochondrial membrane potential (measured using TMRM, excited at 850 nm) in proximal tubules (PTs) (a) and in a distal tubule (DT) (b). High resolution imaging of mitochondria in the PT, demonstrating the typical elongated morphology (c). (d–f) Mitochondrial NADH (excited at 720 nm) (d), mitochondrial pH (measured with SNARF, excited at 800 nm) (e), and the antioxidant glutathione (measured using monochlorobimane, excited at 720 nm) (f) in the PT. Scale bars = 10 μm in (a and b) and (d–f) and 5 μm in (c).

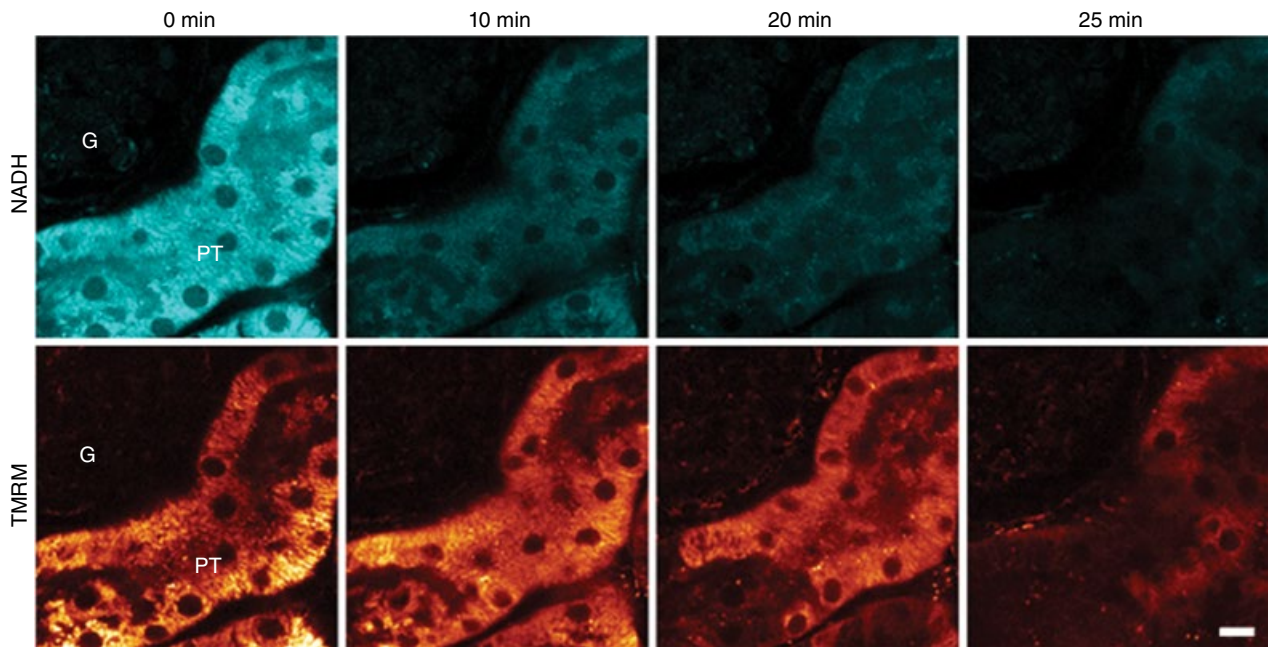


Figure 27.3 Multiphoton imaging of mitochondrial toxicity in live mouse kidney cortex. By adding the drug under investigation to the tissue perfusate, toxic effects on mitochondria in different nephron segments can be visualized in real time. In the example shown, the effects of the established nephrotoxin sodium maleate (20 mg/mL) are depicted on NADH (excited at 720 nm) and membrane potential (measured using TMRM, excited at 850 nm), two important readouts of mitochondrial function. Maleate causes an acute decrease in NADH signal in the proximal tubule (PT), which is followed by progressive loss of potential. G, glomerulus. Scale bar = 10 μ m.

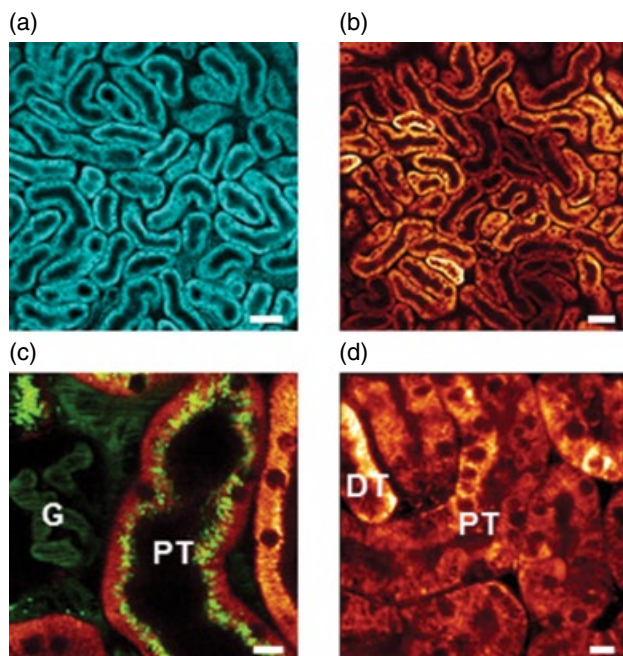


Figure 27.4 Intravital multiphoton imaging of mitochondria in the mouse kidney. (a and b) Mitochondrial NADH (excited at 720 nm) (a) and membrane potential (measured using TMRM, injected intravenously, and excited at 850 nm) (b) in tubules of the outer kidney cortex. (c) Mitochondrial signals in the proximal tubule (PT) can be co-imaged with real-time uptake of filtered solutes across the apical membrane. In the example depicted, fluorescently labeled albumin injected intravenously can be visualized in the capillaries of the glomerulus (G) and in subapical endosomes of the PT. (d) Example image from a mouse injected with TMRM, acquired 48 h postinjection of cisplatin (30 mg/kg bodyweight), showing mitochondrial damage in PTs, but a normal appearance in the DT. Scale bars = 50 μ m in (a and b) and 10 μ m in (c and d).

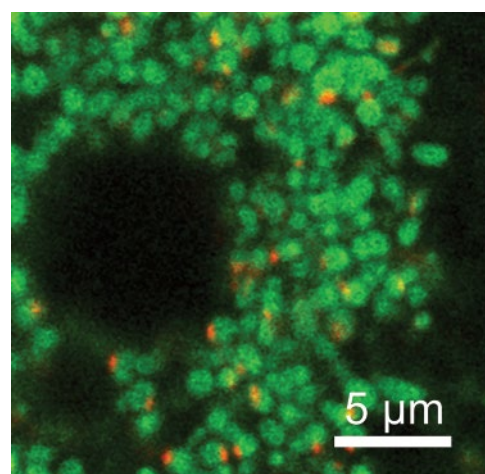


Figure 28.2 Red and green mitochondrial fluorescence after loading with JC-1. A cultured mouse hepatocyte was loaded with 100 nM JC-1 for 30 min in Krebs-Ringer-Hepes (KRH) buffer at 37°C, and green and red fluorescence was imaged by multitrack confocal microscopy using 488 and 543 nm excitation light, respectively. Red inclusions within green fluorescing mitochondria are JC-1 J-aggregates.

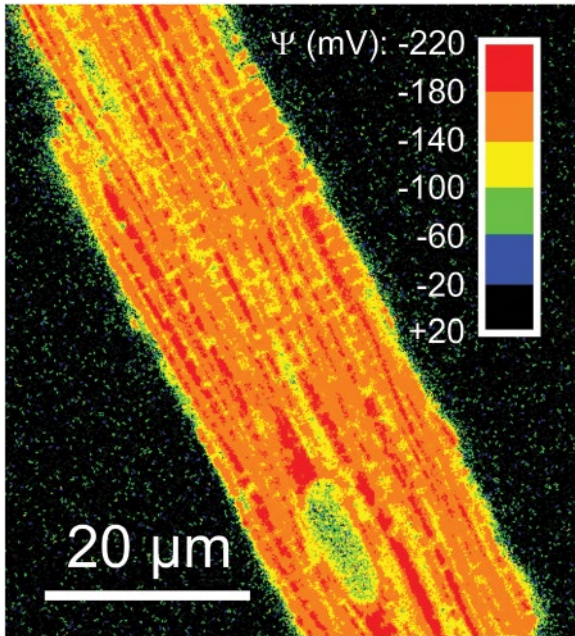


Figure 28.3 Distribution of electrical potential in a cardiac myocyte. An adult feline cardiac myocyte was loaded with 200 nM TMRM for 20 min at 37°C, and TMRM fluorescence was imaged by confocal microscopy using 543 nm excitation and a 565–615 nm emission filter. The distribution of Ψ is displayed in pseudocolor, as described in the text.

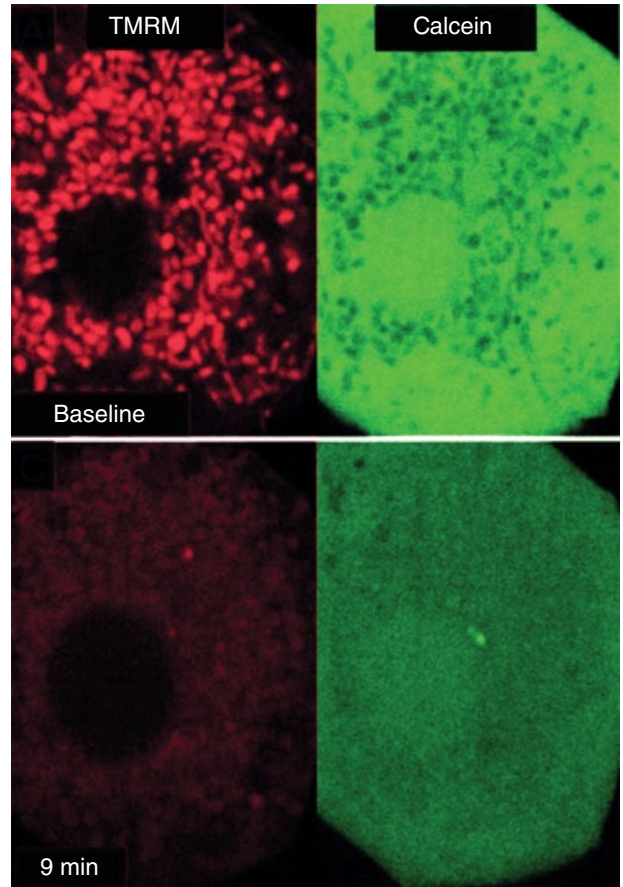


Figure 28.6 Increased mitochondrial inner membrane permeability in a rat hepatocyte induced by *tert*-butylhydroperoxide. A cultured rat hepatocyte was loaded with TMRM (left panel) and calcein (right panel). Note that dark round voids in the green calcein fluorescence coincide with red TMRM labeling of mitochondria. After 9 min exposure to $100 \mu\text{M}$ *tert*-butylhydroperoxide, dark mitochondrial voids filled with green calcein fluorescence. Simultaneously, mitochondrial release red TMRM fluorescence. These events signified onset of the MPT. Adapted from Nieminen et al. (1995). Reproduced with permission of Portland Press.

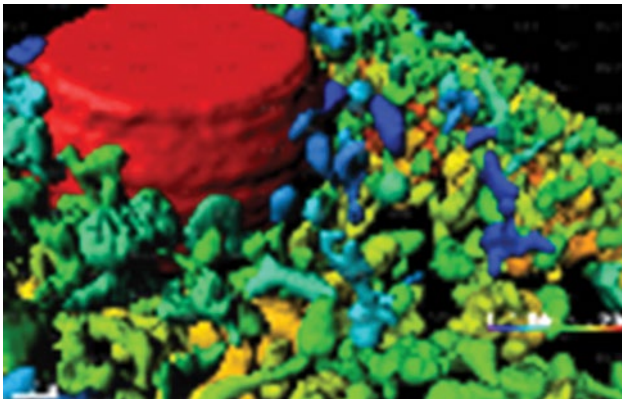


Figure 28.4 Airyscan super-resolution volume rendering of TMRM-labeled mitochondria in a primary rat hepatocyte. Mitochondrial color represents median TMRM fluorescence intensity from low (blue to green) to high (yellow to red). The nucleus is segmented based on PicoGreen labeling and is colored red.

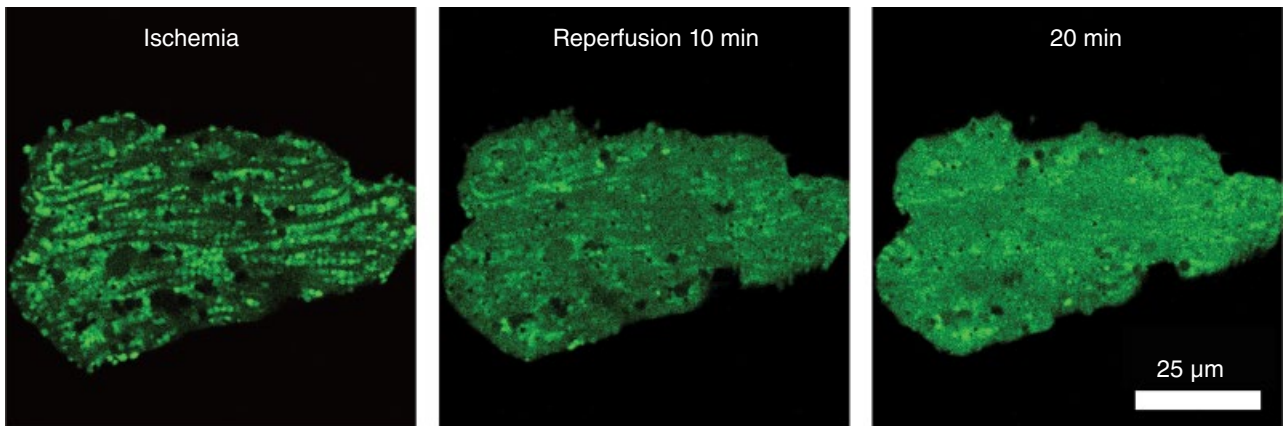


Figure 28.7 Inner membrane permeabilization after ischemia/reperfusion in rat myocytes visualized by mitochondrial calcein release after cold ester loading/warm incubation. An adult rat cardiac myocyte was cold-loaded with calcein AM and subjected to 3 h of simulated ischemia at 37°C at pH 6.2 followed by reperfusion at pH 7.4 for 10 and 20 min. Green calcein fluorescence was retained by mitochondria at the end of ischemia. After reperfusion, mitochondria began to release calcein, signifying inner membrane permeabilization. For experimental details, see Kim et al. (2006). Reproduced with permission of American Physical Society.