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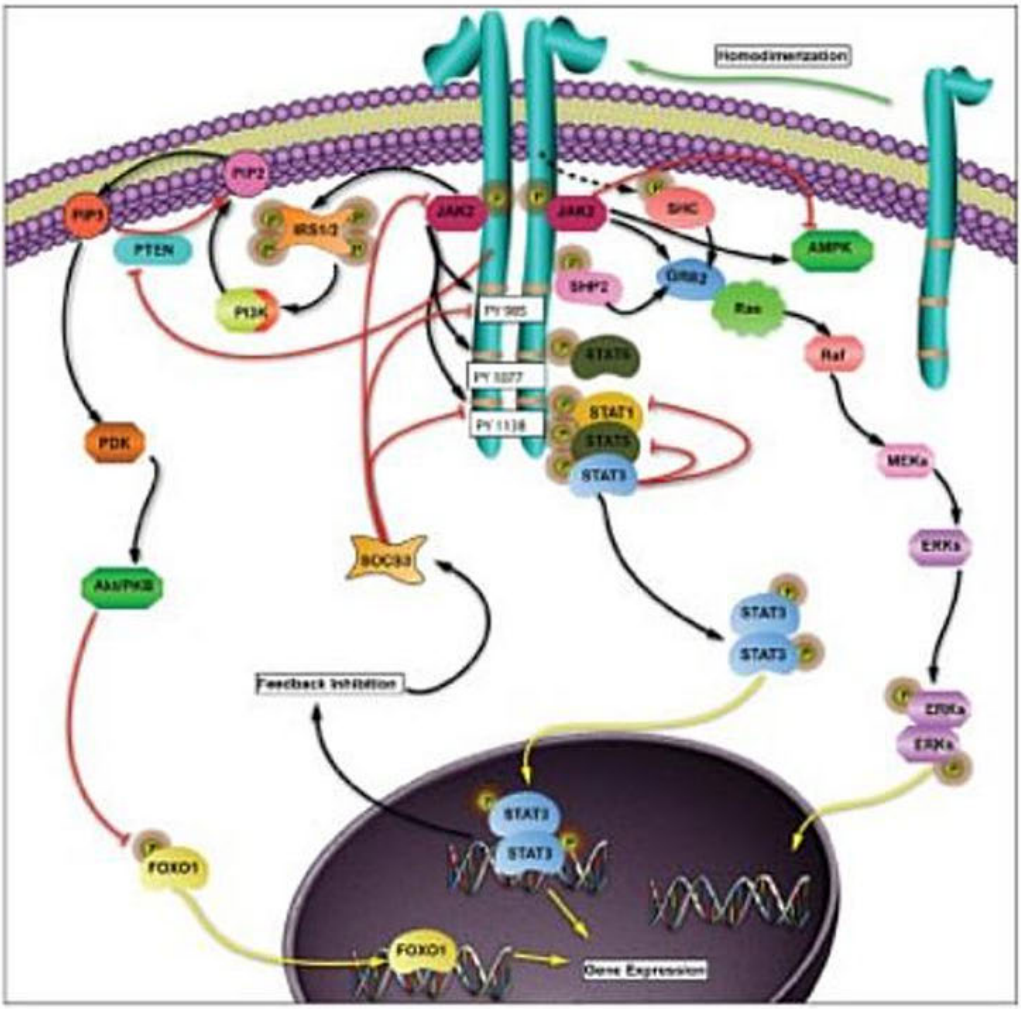
Edited by James D. Adams Jr. and Keith K. Parker

Extracellular and Intracellular Signaling



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Extracellular and Intracellular Signaling

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Preface

"The ten thousand things carry yin and embrace yang.
They achieve harmony by combining these forces."

Lao Tzu from the "Tao Te Ching"

Keith Parker and I were graduate students in the laboratory of Anthony Trevor at the University of California San Francisco. We studied Pharmacology diligently and were lucky enough to be taught by Dr. Trevor, who is one of the best teachers and a great mentor. The most important concept we learned from him was to be open minded. As we learned about diseases, I became aware that the causes of many diseases were not known, such as diabetes, arthritis, cardiovascular disease, congestive heart failure and other chronic diseases.

I had been fortunate to be taught the Science of Entomology by my father, James David Adams, PhD. On many hikes, he taught me to be open minded and to be ready to go where the data lead. It is after all the data that are important. The hypothesis must change as new data become available.

I married Linda Mei, who taught me to speak Cantonese. I learned about Chinese culture, history, language and medicine. Our son, Elliott Trevor Adams, has given me the opportunity to take him on hikes and explain science to him.

At the University of Southern California, I collaborate with Eric Lien, and am greatly expanding my understanding of Chinese medicine. It was due to his influence that I began to try to describe Chinese medical theory in terms of western scientific mechanisms.

I became the student of Cecilia Garcia, a Chumash Indian healer. She taught me that healing and disease prevention are the first priorities in medicine. I continue to be challenged to find scientific mechanisms to describe American Indian healing.

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This book demonstrates how much science has advanced in the understanding of the causes of diseases and how both intracellular and extracellular signaling are involved. A balance between these signaling cascades is required for health and disease prevention. The concept of balance in health is more than 2000 years old in Chinese medicine, and at least as old in American Indian medicine. This book attempts to give scientific mechanisms that explain the necessity of balance in health.

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This book is jointly edited by Jim Adams and myself, but there is no doubt that the book's inspiration came from Jim. I am most grateful to Jim for his invitation to join him in this endeavor, and his vision and drive to bring the book into reality has been instrumental to all of us as contributors! After stints in graduate school and post-doctoral research, I envisioned the move back to my home state of Montana in 1981. It was at this time that I began to appreciate the contributions to medicine and health made by native peoples. The holistic themes of this book resonate well with me in that context and, hopefully, such blending of multiple approaches will continue to increase.

As with any project, there are debts to many, and I hope to acknowledge a few of those here. My wife Julie's and the entire family's love, dedication and patience are without parallel. I cannot thank them enough. As Jim did, I would also like to thank Tony Trevor; his brilliance as a scientist and his personal confidence in us has been immensely influential. Other mentors that I would like to note are: Pierce Mullen of Montana State University for his continuing friendship; Frank Tikalsky formerly of the Los Alamos National Laboratory for his infinite wisdom and his persistence in showing the wonders of native cultures; Antonia Vernadakis, a true pioneer in the field of glial research; and Eric Wickstrom of the Thomas Jefferson Medical College, for his humanity and creativity. Of special note in this regard is Mike Norenberg of the University of Miami's Miller School of Medicine. Mike's willingness to write a chapter in the book is deeply appreciated and a unique plus for me and the readers. In these years back in Montana my colleagues throughout the state but especially at the Dillon (Western) and Missoula campuses of the University of Montana have been infinitely supportive and encouraging. In every sense, this book is truly a collaborative effort!

Keith Krom Parker
Missoula
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USA

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CHAPTER 1

Extracellular and Intracellular Signaling – a New Approach to Diseases and Treatments

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1.1 Introduction

1.1.1 Linear Model of Drug Receptor Interactions

The body and mind depend on a variety of receptors and endogenous, extracellular ligands in order to maintain health. In the past, a simplistic, linear model of ligand receptor binding has been used with great success for drug development.

$$Ligand + Receptor \rightarrow Ligand\text{-}Receptor\ complex \rightarrow \text{Effect} \qquad (1.1)$$

This model has led to a simplistic, linear model of disease where one aberrant gene produces one abnormal protein leading to the induction of one disease. It is now clear that the body does not function according to these linear models.

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Instead, the body makes use of a complex interacting system of intracellular matrices, called signal transduction networks, in order to maintain health. Proper health depends on maintaining the proper balance of these intracellular signal transduction networks. Most of us are born healthy and could stay healthy, if we knew how. Many economically important diseases are not caused by single gene aberrations, but are caused by lifestyle changes that alter many genes and the balance of signal transduction networks.

1.1.2 Matrix Model of Drug Receptor Interactions

$$\begin{gathered}\{\text{Calcium mechanisms}\} \\ \times \\ \{\text{Phospholipases}\} \times \textit{Ligand-Receptor complex} \div \{\text{Protein kinases}\} \rightarrow \textit{Effect} \\ \div \\ \{\text{NAD mechanisms}\}\end{gathered} \tag{1.2}$$

The model above is intended to show that matrix division and matrix multiplication lead to drug effects. For instance, phospholipase mechanisms liberate active lipids that may multiply the effects of a drug receptor interaction. This is synergism. However, protein kinase mechanisms that phosphorylate proteins, may decrease, or divide, the effects of a drug receptor interaction. This is dysynergism. For each ligand receptor interaction there may be synergism and dysynergism by many signal transduction networks (only four are shown) that are involved in processing the interaction and producing the effect. Equation (1.2) is simplified since many receptors can exist in active and inactive states, or states of altered activity. Ligand binding may have different effects depending on the state of the receptor at binding.

There are many endogenous, extracelllular ligands, made in the body, that produce effects upon receptor binding. These extracellular ligands, such as the adipokines and the cytokines, modify the balance of normal intracellular signal transduction networks in order to maintain health or cause disease. Drugs tend to mimic these endogenous ligands, in terms of chemical structure and receptor interactions. It is likely that only a minority of the endogenous ligands are currently known. Many more will be discovered in the future. Endogenous ligands include lipoxins, prostaglandins, endocannabinoids, enkephalins, endorphins, adipokines, cytokines, hormones, neurotransmitters and many others. Many of these endogenous ligands are produced locally, act locally and have short half-lives. This makes them superior to drugs that must penetrate to the site of action and persist until the effect is attained. Drugs are usually designed to have half-lives of about 24 hours so that patients can take them daily, or on a convenient schedule. The long residence time of drugs in the body increases the risk of toxicity.

In general, for each receptor in the body there is at least one endogenous agonist and one antagonist. Therefore, health depends on the balance of

extracellular antagonists and agonists as well as the balance of intracellular synergizing and dysynergizing signal transduction networks. Drugs can help restore the balance of endogenous agonist/antagonist activity. Drugs do not cure diseases, but facilitate the ability of the body to cure itself. This is similar to the American Indian theory of balance in health and the Chinese theory of health that depends on the proper balance of yin (cold) and yang (hot). Yin is also the female or inhibiting aspect of the body. Yang is the male or activating aspect of the body.

The receptor approach is further complicated by the following factors. Typical receptor-based research has reasonably focused on the ligand binding site of the receptor. As in enzymology, there may well be alternate sites on the receptor that are amenable to drug action.[1] An example in the GPCR realm is the interface between receptor and G protein.[2] Agonist or antagonist substances binding to any of a number of available regulatory sites between receptor and G protein could influence trafficking through the coupled signal transduction system.

Further downstream in the transducing system there could be drug targets that influence other enzymes or targets in the pathway, and in these targets a multiplicity of possible interactive locations, from ligand binding sites to allosteric sites, could occur. Specificity has long been a concern and goal of pharmacology, and for some disorders this approach is likely to be the best. In other cases, and migraine headache may be one of these, an agent or agents that hit in many locations may be preferable. Natural products like cannabis come to mind in this regard. Tetrahydrocannabinol (THC) or cannabidiol (CBD) alone or even THC and CBD in combination may not be the most effective therapeutic approach. Rather the combination of cannabinoids, terpenoids and other substances found in crude cannabis preparations may allow for the greatest coverage of useful target sites.

1.2 Experimental Approaches to Disease Treatment

The Chinese have experimentally found ways to stimulate yin and yang in order to restore balance between proinflammatory and anti-inflammatory factors and treat disease.[3] Too much yin can be caused by an insufficient flow of vital forces, such as blood, lymph and chi. Too much yang can be caused by excessive flow of vital forces. Chi is the source of life and is a life force carried in the body by the acupuncture channels. Chi is required to regulate the balance between yin and yang. It is possible that chi and the intracellular signal transduction networks accomplish the same purposes. Regulation of gene transcription is also involved in chi. It is also possible that endogenous extracellular ligands accomplish the same purposes as yin and yang.

The brain and nervous system depend on endogenous ligands and signal transduction networks, just like other organs. In addition, neurons modify each other's signals through synaptic interactions. Therefore, the brain has

intracellular signal transduction networks, extracellular ligands and synaptic interactions that are important in the production of neuronal impulses.

However, the brain also depends on perceptions that alter signal transduction networks. Perceptions come from a variety of stimuli outside or inside the body and are translated into neural signals and synaptic impulses in the brain. Perceptions are important in anxiety disorders, delusions, depression and other disorders. Even Parkinson's disease is more common in people who are more prone to anxiety. Unfortunately, very little is understood about how perceptions are involved in altering signal transduction networks and endogenous ligands in the brain. It is possible that a balance exists between perceptions of comfort and discomfort that is needed for proper brain health.

The involvement of perceptions in pain processing can be taken another step into the realm of an affective component of pain. In gate theory[4] once the original nociceptive signal is processed in a perceptual sense in the brain, an affective or emotional component of feedback is sent to the periphery to further gate in other words allow for increased or decreased movement of sensory information into the input pathway. Additionally, in this context, local signals in the periphery that involve non-pain input may further influence the strength of the pain signal. All of these aspects of pain processing suggest multiple sites for therapeutic intervention either alone or in some complex interacting fashion. These aspects are probably also important in the placebo effect that is prominent in pain treatment.

Before antibiotics were discovered, the treatment of infectious fevers involved aspirin and cold baths to bring down the fever. The patient usually died anyway because only the symptom was being treated, not the cause. This is true of many diseases today. Only the symptoms are known, not the causes. However, advances are being made that will be discussed in the current work. Diseases for which no cures (and sometimes no causes) are known include hypertension, heart disease, diabetes, arthritis, Alzheimer's disease, Parkinson's disease, migraine headache, neuropathic pain, fibromyalgia and others. Advances are being made with hypertension, heart disease, diabetes and arthritis. However, it is important to realize with all of these diseases that the most powerful drugs available to treat the symptoms do not cure the diseases.

1.3 Adipokines and Disease Causation

Recently, fat cells, especially visceral fat cells, have been found to secrete adipokines, such as visfatin, leptin, resistin, C-reactive protein, angiotensin II, tumor necrosis factor alpha, and interleukin 6, that are released into the blood and have effects throughout the body (proinflammatory factors). Macrophages, in obese patients, can also secrete adipokines. Obesity, caused by a sedentary and overindulgent lifestyle, produces more fat, more adipokines and more toxic lipids, such as ceramide and endocannabinoids. These adipokines and toxic lipids induce inflammation and alter the balance of signal

transduction networks necessary for normal health. In addition, many adipokines alter the secretion of other adipokines.

Interleukin-6, tumor necrosis factor alpha, resistin, C-reactive protein, angiotensin II, ceramide and other ligands are involved in altering the balance of signal transduction mechanisms that leads to heart disease.[5,6] Lipotoxicity, from ceramide, causes iNOS dysfunction resulting in oxygen radical production in the kidney leading to increased blood pressure. Adiponectin decreases due to increased endocannabinoids. Low adiponectin causes NO production to decrease. In endothelial cells, eNOS dysfunction leads to oxygen radical production. Angiotensin II and endothelin-1 increase, leading to increased peripheral resistance and more eNOS dysfunction. Resistin, TNFα and IL-6 decrease NO production. Wall defects develop in arteries, and are partially caused by leptin. Platelet, neutrophil and monocyte adhesion occurs due to increased production of adhesion proteins. TNFα, resistin and C-reactive protein may stimulate adhesion protein synthesis. Neutrophils invade arterial walls and start the inflammatory process. Monocytes and more neutrophils are activated and are stimulated to make oxygen radicals by visfatin and leptin. Oxidized LDL-C is taken up by macrophages making foam cells and plaque. Smooth muscle cell proliferation occurs due to the effects of PDGF, angiotensin II and heparin binding epidermal growth factor-like growth factor. Plaque instability occurs due to C-reactive protein induced matrix metalloproteinase activity in macrophages.

Hypertension is caused by angiotensin II, resistin, visfatin, C-reactive protein, tumor necrosis factor alpha and other endogenous ligands.[7,8] Vascular tone (blood pressure) is decreased by PGI_2, NO, acetylcholine and vagal nerve stimulation (decreased cardiac output). Blood pressure is increased by adrenergic nerve stimulation, endothelin, angiotensin, renin, aldosterone and other factors. Adiponectin levels decrease in obesity leading to impaired vasorelaxation, due to less NO production, and higher levels of endothelin-1. CRP decreases eNOS and prostacyclin synthase activities leading to increased blood pressure. TNFα decreases iNOS activity and NO production. Visfatin inhibits vasodilation through an unknown mechanism. Resistin inhibits vasodilation induced by bradykinin and induces endothelin-1 transcription. Angiotensinogen is secreted to some extent by visceral fat, increases angiotensin II and blood pressure.

Diabetes is caused by endocannabinoids, ceramide, resistin, visfatin, interleukin-6, tumor necrosis factor alpha and others.[9,10] Endocannabinoids decrease adiponectin production, and increase visfatin and TNFα production. Ceramide inhibits insulin receptor phosphorylation causing short-term insulin resistance. Ceramide also makes iNOS dysfunction, making oxygen radicals that destroy β cells. Resistin, RELMs and lipotoxicity cause short-term insulin resistance. Visfatin, IL-6 and TNFα are involved in long-term insulin receptor resistance.

Osteoarthritis is caused by endocannabinoids, leptin, resistin, TNFα, IL-6 and others.[8,11,12] Endocannabinoids decrease adiponectin production and increase the production of other adipokines. However, rheumatoid arthritis

patients have high adiponectin levels perhaps as a compensatory mechanism. Leptin levels increase and activate macrophages, T cells and chondrocytes, leading to increased production of oxygen radicals and cytokines that are inflammatory. Inflammation follows with macrophages and monocytes differentiating into osteoclasts. This differentiation is caused by colony stimulating factor-1 (CSF-1) from fibroblasts. TNFα is involved in the stimulation of CSF-1 production. Neutrophils are activated by leptin and visfatin and induce more inflammation. Macrophages secrete resistin that induces TNFα and IL-6 in a vicious cycle.

Clearly, a large portion of the economically important diseases in developed countries are caused by toxic lifestyles that produce toxic extracellular ligands that alter the normal balance of signal transduction networks in the body. It is also clear that a number of genes are involved in these disease processes to produce the adipokines and toxic lipids. Therefore, these diseases have a number of potential drug targets. In addition, it is clear that re-establishing a healthy lifestyle can alter the activities of many genes, leading to better disease treatment.

The current approach to many chronic diseases is to treat symptoms, such as hypercholesterolemia in heart disease, pain in arthritis and high blood glucose in diabetes. In Alzheimer's disease, billions of dollars are being spent to try to find ways to prevent or reverse plaque formation in the brain. Treating symptoms can sometimes slow down the disease process and may be of benefit to patients. However, the chronic disease is not cured or reversed. Lifestyle changes may be able to prevent or reverse chronic diseases, by re-establishing the proper balance between proinflammatory and anti-inflammatory factors.

1.4 Questions in Disease Treatment

A question that remains unanswered for many drugs is: Do drugs alter the activities of endogenous agonists and antagonists such that recovery from a disease is impeded? For instance, opioids cause receptor desensitization through receptor of G protein signaling (RGS) and other mechanisms. When patients try to stop taking opioids, the pain returns and is much worse than before. The pain is worse because the endogenous opioid agonists cannot function normally due to opioid receptor desensitization. Are there similar processes with other drugs, such that the normal balance of endogenous agonist and antagonist activity is so disturbed that proper health cannot be restored?

Despite recent advances, there is still much that is not known. For instance, muscles are vital to proper health. Do muscles secrete myokines that are involved in maintaining health? How does exercise promote the health of stem cells throughout the body? The guts produce hormones that are important for health. The guts are associated with a large array of immune tissue and nervous tissue. Are there unknown enterokines that are involved in the health of organs, the nervous system and the immune system? Similarly, it is not known if bone secretes osteokines that might maintain health in other organs. The brain,

ganglia and other nervous tissues secrete a variety of hormonal ligands. There are undoubtedly more neurokines still to be discovered. Immune cells secrete a huge array of cytokines with a variety of effects throughout the body. Many of these ligands are not fully understood. The kidneys, liver, pancreas, thyroid, adrenal glands, heart, vascular system, lymphatic system, reproductive organs, skin and other organs may also produce unknown extracellular ligands that are vital to proper health.

Drug development currently involves the carpenter approach: if the hammer does not work, get a bigger hammer. A good example of this approach is if aspirin does not work for rheumatoid arthritis, use methotrexate. Unfortunately, patients can die from improper use of methotrexate. A corollary of the carpenter approach is the search for more specific receptors. For instance, if antihistamines produce drowsiness by interacting with too many receptors (brain H_1 and H_2), find a more specific receptor (peripheral H_1) that will not cause drowsiness. Similarly, if non-steroidal anti-inflammatory agents cause ulcers by interacting with too many receptors (Cox-1 and Cox-2), find a drug for a more specific receptor (Cox-2) that will not lead to ulcers. Unfortunately, the more specific receptor approach has resulted in catastrophic events with many thousands of people suffering major toxic effects and death. Some of the peripheral H_1 blockers caused arrhythmias. Some of the Cox-2 inhibitors caused thrombosis leading to heart attacks and strokes. Despite all of the powerful drugs available, there are no drugs that cure hypertension, heart disease, diabetes, arthritis and other chronic conditions. Currently available drugs can only manage these diseases. It is possible that finding drugs to re-establish the proper balance of agonist/antagonist and signal transduction networks may be a better approach. Better yet, promoting healthy lifestyles may help maintain or re-establish good health.

1.5 Toxic Lifestyles and Disease Treatment

Healthcare in the future will depend on helping people maintain proper lifestyles that promote a healthy balance of extracellular agonists and antagonists as well as signal transduction networks. Lifestyles are far more powerful than most drugs in terms of maintaining health, since lifestyle can alter many endogenous ligands and signal transduction networks at once. Commonly prescribed drugs tend to be magic bullets that affect only one mechanism at a time. The magic bullet approach to drugs makes drugs far too specific and renders them useful only for managing one aspect at a time of a disease. However, drugs will always be needed to treat ill patients. Drugs that alter signal transduction pathways, such as protein kinase inhibitors, tend to have many effects throughout the body. Some of these effects are unwanted and therefore toxic. However, by using a model of matrix interactions that produces drug effects, as presented above, it is possible that ways of decreasing toxicity may be found. Another approach may be to find drugs that help re-establish normal endogenous ligand synthesis and release.

For instance, a drug that stimulates endorphin synthesis and release may be useful in pain therapy.

There are four statements that patients want to hear. 1. You do not have to lose weight and exercise, because we have powerful drugs to control blood cholesterol. 2. You do not have to lose weight and exercise, because we have powerful drugs to control blood pressure. 3. You do not have to lose weight and exercise, because we have powerful drugs to control blood glucose. 4. You should not exercise, because exercise, especially running, causes arthritis. All four of these statements are false. Drugs that control cholesterol slow down, but do not stop, atherosclerotic processes. Heart attacks and strokes are delayed by drug therapy, but not prevented. Drugs that control blood pressure delay, but do not prevent, the onset of congestive heart failure. Drugs that control blood glucose delay, but do not prevent, the loss of vision, loss of toes, loss of kidney function and other consequences of diabetes. Exercise, especially running, has been shown in several studies to slow down the progression of arthritis.[13–16]

Recommendations for lifestyle changes are not accepted readily by patients who are more interested in maintaining their toxic lifestyles, and adding drugs to manage their chronic conditions. In addition, healthcare professionals cannot make a living by teaching patients about healthy lifestyles. They make their livings by selling drugs and carrying out surgery. However, lifestyle changes are suggested for many chronic conditions, especially hypertension and diabetes. Patients are encouraged to lose weight, alter their diets, decrease sodium intake to no more than 2.4 g daily, stop smoking, decrease alcohol intake to no more than 15 ml (1 drink) daily for women or no more than 30 ml (2 drinks) daily for men, and to exercise. Among athletes, optimal body composition is about 4% body fat for men and 12% body fat for women. Women become amenorrheic and osteoporotic below 12% body fat. Optimal diets contain vegetables, whole grains and five servings of fruit daily. Recommended diets may have about 60% caloric intake from carbohydrate, 20% from protein and 20% from fat. Exercise should be moderate, not strenuous, and should be enjoyable. Walking, running, cycling, swimming and other forms of aerobic exercise are encouraged.[17] Yoga, tai chi, meditation and spiritual practices are becoming popular among health-conscious adults and seniors. Patients may start with about 20 minutes of physical exercise daily and should increase this over a year or more to about an hour each day. Spirituality and its health effects are poorly understood by science.[18] Yet many patients seem to benefit from increased spirituality during their recovery processes. As mentioned in the discussion on migraine headache later in the book, a form of homeostatic regulation introduced by Bruce McEwen, known as allostasis, fits nicely into the holistic concepts introduced in this opening chapter.[19] Daily physical exercise, meditation and spiritual practices should be maintained throughout life. The goals of lifestyle alterations are to maintain a productive personality, decrease abdominal fat, decrease osteoporosis, decrease inflammation of the joints, decrease blood cholesterol and glucose levels, decrease blood pressure and increase the exercise endurance of the heart.

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CHAPTER 2

Autocrine Effects in White Adipose Tissue and Pancreatic Islets: Emergent Roles in the Regulation of Adipocyte and Pancreatic β-cell Function

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2.1 Introduction

Diabetes mellitus (DM) is a metabolic abnormality characterized by abnormally high circulating concentrations of glucose (hyperglycaemia), together with dyslipidemia. Hyperglycemia and dyslipidemia arise from defects in insulin secretion and/or action (termed insulin resistance). The incidence of DM has grown rapidly into a global epidemic of enormous proportions that poses one of the most significant healthcare problems of the twenty-first century.[1] DM affects nearly 6% of the world's population, and it has been estimated that 200 million individuals suffer from diabetes worldwide.[2] Complications related to poorly controlled DM account for 4 million deaths every year and the costs arising from DM pose a significant burden to the healthcare systems of most countries.[3]

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Type 1 diabetes (T1DM) arises because of autoimmune destruction of the insulin-producing β cells of the endocrine pancreas.[4] This involves the participation of activated killer T cells,[5] cytotoxic T cells[6] and autoantigens; the latter include insulin, glutamic acid decarboxylase (GAD65) and IA-2, a protein tyrosine phosphatase-like molecule.[7–10] Approximately 60–80% of the pancreatic β cells are destroyed by the time clinical symptoms become apparent.[11] Both genetic[12–14] and environmental[15] factors contribute to the aetiology of T1DM.

Type 2 diabetes (T2DM) accounts for approximately 85% of cases of DM in Caucasian populations, and at least 95% of cases in other ethnic groups. In T2DM, impaired glucose-stimulated insulin secretion (GSIS) is coupled with insulin resistance. In predisposed individuals, there is an inability to secrete the increased amounts of insulin required to compensate for insulin resistance in order to maintain glucose and lipid homeostasis. Whilst the contribution of accelerating insulin resistance towards the development of T2DM is often highlighted, the concept that inadequate insulin secretory compensation by the β cells plays an important role is becoming appreciated.

The fact that only 15–20% of obese individuals with severe insulin resistance become diabetic, with β-cell compensation ensuring normal glycaemia in the others,[16,17] raises the question of why certain individuals with insulin resistance compensate with enhanced islet function and β-cell hyperplasia and remain healthy, whereas others develop β-cell failure, characterized by insulin secretory defects coupled with relative loss of β-cell mass. Glucolipotoxicity, resulting from exposure to a combination of high glucose and high lipid levels due to impaired metabolic control by insulin, describes deleterious or toxic effects on the β cell (deterioration of β-cell function and β-cell death)[18] and induces insulin secretory and cytotoxic lesions similar to those observed in T2DM.

In this chapter, we develop the hypothesis that T2DM may in part reflect altered autocrine interactions in the adipocytes and the β cells of the pancreatic islets of Langerhans, together with an impaired adipo-insular axis whereby molecules released from adipose tissue adversely impact β-cell function. Our discussion is not exhaustive. Our emphasis is placed on the roles of fatty acids (FA), derived from the breakdown of stored lipid, the adipokines leptin and adiponectin and the cytokine TNFα. In addition, as there is strong evidence that an inappropriate early life environment causes adaptations that confer an initial survival advantage in the fetus, but then increase the risk of T2DM in adulthood,[19,20] it is now accepted that an imbalance between prenatal (*e.g.* protein malnutrition) and postnatal (*e.g.* high-fat feeding) nutrition predisposes to obesity, insulin resistance and T2DM. We discuss the idea that an inappropriate early life environment may be an important factor in the metabolic maladaptations leading to T2DM *via* augmented FA release in combination with altered secretion or action of adipo/cytokines.

2.2 Heterogeneity of Adipose Tissue Composition in Relation to Adipokine and Cytokine Secretion

Both genetic and environmental influences determine the risk of developing T2DM. However, since the genetic background is unlikely to have altered over

a short timescale, the current world epidemic of T2DM is better explained by environmental influences or possibly epigenetic modification.[21] Epidemiological studies indicate that factors influencing the prevalence of T2DM include lifestyle (*e.g.* inactivity and dietary macronutrient composition) and obesity,[22–24] age and geographical area of residence. Of these factors obesity, defined as an excess of body fat that is deemed unhealthy for the individual and a body mass index (BMI) of over 30, is one of the most important.[25] Notably, both an enhanced β-cell secretory function and a compensatory increase in β-cell mass is seen in obese individuals who do not develop T2DM; these adaptations are able to counteract the increased metabolic load and obesity-associated insulin resistance.[26] In the subset of obese individuals who develop T2DM, a failure in β-cell adaptation takes place.[27] We hypothesize that failure of β-cell adaptation may, in part, reflect the changes in adipose composition and function that occur as it expands.

White adipose tissue comprises a range of different cell types: mature adipocytes (fat cells) themselves, their immediate precursors (preadipocytes) and stroma-vascular cells including fibroblastic connective tissue cells, leucocytes and macrophages.[28] In adult mammals, most of the adipose tissue mass comprises lipid-filled cells (the adipocytes) in a framework of collagen fibers. An increase in body fat is caused by a combination of increased differentiation of preadipocytes to mature adipocytes, together with hypertrophy of mature adipocytes mainly due to increased triglyceride (TAG) storage. Thus, white adipocytes vary greatly in size, and can change their diameters by 20-fold and their volumes by several-thousand-fold to accommodate lipid. T2DM, insulin resistance and the metabolic syndrome are characterized by increased circulating FA and by increased lipolysis (breakdown) of the expanded TAG stores.

The ability of adipocytes to store FA as TAG in the fed state provides animals with a fuel store for use in time of need; in addition, sequestration of excess dietary FA in adipose tissue can improve insulin action in liver and muscle by suppressing excess FA delivery to these tissues, which can cause insulin resistance and tissue dysfunction.[29] In the fed state, insulin increases adipocyte TAG storage by augmenting adipocyte glucose uptake (allowing the production of glycerol 3-phosphate for FA esterification to form TAG and the formation of FA *de novo*), by direct effects to stimulate enzymes in the pathway for esterification of incoming FA, mainly generated locally *via* adipocyte lipoprotein lipase (LPL), and by effects to block TAG lipolysis. Adipocyte glucose uptake and its conversion to glycerol 3-phosphate is also enhanced by activation of the peroxisome proliferator-activated receptor γ(PPARγ).[30,31] PPARγ, a "lipogenic" transcription factor, enhances the expression of genes involved in glucose uptake and conversion to TAG[32] and also stimulates glycerol transport, glyceroneogenesis and glycerol phosphorylation.[30,33]

In response to lipolytic stimulation, adipocytes hydrolyze stored TAG and release FA and glycerol into the circulation. FAs are used as substrates for ATP production in a range of oxidative tissues; glycerol is used for hepatic gluconeogenesis. The contribution of glycerol to glucose production depends on the nutritional state. This may vary from 5% postprandially in humans[34] and

approx. 50% in the post-absorptive state in rodents[35] to 20% in humans[34,36] and >90% in rodents after prolonged fasting.[35] The significance of glycerol as a gluconeogenic precursor is supported by the episodic hypoglycemia observed in patients with glycerol kinase deficiency.[37]

As well as serving as repositories for lipid, which can be mobilized when required, adipose tissue also releases adipokines involved in the regulation of normal physiological processes (*e.g.* the control of vascular homeostasis, blood pressure and regulation of energy balance)[26] as well as contributing to the pathology of some disease states. One adipokine, leptin, is secreted from adipose tissue in proportion to adipose mass. Leptin is thought to inhibit food intake and increase energy expenditure when healthy individuals experience chronic overnourishment. To achieve the former, leptin targets hypothalamic neurons in the arcuate and in paraventricular, dorsomedial and ventromedial nuclei. Leptin has opposing actions on two populations of arcuate neurons, one that synthesizes the appetite-promoting peptides neuropeptide Y and agouti-regulated peptide (AgRP) and a second that synthesizes anorexigenic peptides, *e.g.* POMC (the precursor of the appetite-suppressing peptide melanocyte stimulating hormone). Leptin stimulates POMC neurons whilst inhibiting NPY and AgRP neurons. Leptin also exerts direct effects on peripheral tissues (including liver, pancreatic islets, skeletal muscle), affecting peripheral energy partitioning, in particular the intracellular disposition of nutrients (storage *versus* degradation). The "energy-sensor" AMP-activated protein kinase (AMPK) orchestrates critical metabolic adaptations to reduced energy availability and is activated by leptin in peripheral tissues, in particular skeletal muscle and liver.[38] Obesity is characterized by leptin resistance.[39] Thus, while circulating leptin levels are elevated in obesity, its effects to limit food intake and promote energy expenditure are blunted due to the existence of leptin resistance. This may be due to leptin receptor signaling defects, for example elevated SOCS3 expression in leptin-responsive neurons of the arcuate nucleus,[39] and/or defects in leptin transport across the blood-brain barrier.[40]

Adiponectin is another important adipokine. Adiponectin administration ameliorates insulin resistance in mice,[41] whereas adiponectin-deficient mice exhibit insulin resistance[42] and diabetes. This insulin-sensitizing effect of adiponectin, like that of leptin, involves AMPK, the activation of which increases FA oxidation acutely,[43] and also augments signaling *via* the lipo-oxidative transcription factor PPARα.[44] PPARα increases the expression of genes involved in FA uptake and oxidation.[45] Enhanced insulin sensitivity in response to adiponectin arises because of increased lipid clearance from the tissue *via* oxidation. Low circulating levels of adiponectin and increased leptin and C-reactive protein (CRP) have recently emerged as novel diabetic risk factors.[46]

In obesity, there is progressive adipose-tissue infiltration by macrophages that secrete proinflammatory cytokines (*e.g.* tumor necrosis factor (TNF) α, IL (interleukin)-1β and IL-6). TNFα over-expression is observed in adipose tissue and muscle of obese humans[47] and in adipose tissue of genetically obese insulin resistant mice. Monocyte chemoattractant protein 1 (MCP-1) is mainly secreted by macrophages present in adipose tissue; however, adipocytes themselves

also release considerable amounts of MCP-1[48] and adipocyte MCP-1 may be the main chemokine that causes adipose tissue infiltration by macrophages within the context of obesity and insulin resistance. Mice over-expressing MCP-1 in adipose tissue using the adipocyte aP2 promoter (aP2-MCP-1 mice) are insulin resistant and have higher adipose-tissue macrophage accumulation than control mice.[49] It has been suggested that a paracrine loop exists between adipocytes and macrophages that aggravates inflammatory processes.[50] Coculture of differentiated 3T3-L1 adipocytes with RAW264 macrophages results in an upregulation of macrophage TNFα secretion and a downregulation of secretion of the insulin-sensitizing adipokine adiponectin.[50]

The chronic inflammatory state present locally within adipose tissue itself becomes systemic when inflammatory cytokines are released into the circulation. Thus obesity is characterized by increased circulating concentrations of pro-inflammatory cytokines[51–53] and acute phase proteins.[54,55] Circulating leptin concentrations are increased, probably reflecting leptin resistance, whereas adiponectin concentrations are decreased in obesity.[56] TNFα can impair insulin signaling and, in obese mice, lack of TNFα function improves insulin sensitivity and glucose homeostasis, confirming the critical role of TNFα in modulating insulin action.[57,58] Knowledge of the actions of the pro-atherogenic and inflammatory mediators (cytokines and chemokines) that are derived from adipose tissue, either from the adipocytes or from the infiltrating macrophages that are associated with increased adiposity,[59] may help gain a better understanding of the pathophysiology of the chronic proinflammatory state associated with obesity.

2.3 Feedback between FA and the Adipocyte

Evidence is now accumulating that FAs generated *via* adipocyte TAG lipolysis have pronounced direct feedback effects on adipocytes. Within the adipocyte itself, AMPK can be activated by cAMP-inducing agents.[60] This is proposed to be a consequence of stimulated lipolysis secondary to activation of cAMP-dependent protein kinase (PKA) and activation by phosphorylation of key components of the lipolytic machinery. It has been suggested that AMPK activation in adipocytes in this setting is caused by an increased AMP : ATP ratio that appears to be due, at least in part, to the re-acylation of FA released by lipolysis. AMPK activation has been suggested to be part of a mechanism to restrain the energy depletion and oxidative stress caused by excessive lipolysis.

Toll-like receptors (TLR; specifically TLR-2 and -4) constitute a molecular link between elevated circulating FA, as found in obesity, and inflammation in insulin-sensitive tissues including adipocytes.[61,62] TLR-2 and -4 bind lipid-based structures *via* their long-chain saturated FA moieties.[63] Saturated FA, but not unsaturated FA, induce the expression of cyclooxygenase-2 *via* TLR-4.[63,64] The adipocyte itself is capable of mounting a classical innate immune response initiated by ligand activation of TLR-4, followed by activation of nuclear factor-κB (NF-κB), increasing proinflammatory gene expression and

cytokine release.[65–67] Importantly, FA (released from hypertrophic adipocytes under conditions of lipolytic stimulation) may signal to macrophages through activation of TLR-4 and stimulation of MCP-1 release by mature adipocytes in a manner dependent on dose and class of FA.[68] The production of MCP-1 by adipocytes provides a potential explanation as to how macrophages are directed into the adipose tissue from the bloodstream within the context of obesity or insulin resistance (where plasma NEFA concentrations are elevated).

FAs, acting through the adipocyte TLR-4, also stimulate the release of TNFα[68,69] and, somewhat surprisingly, at physiological concentrations, influence the secretion of adiponectin. Exogenous FAs enhance the nuclear translocation of NF-κB p65 and NF-κB inhibition strongly antagonizes the proinflammatory effects of the FAs palmitic acid and stearic acid. It has therefore been argued that there might be a paracrine or autocrine role of FA in adipocyte biology, with a key involvement of NF-κB.[68]

2.4 Autocrine Effects of Leptin and Adiponectin in Adipocytes

Leptin's actions are initiated by its binding to a leptin receptor, encoded by the Lept gene, a member of the interleukin-6 receptor family of cytokine receptors. There are six different splice variants of the leptin receptor (Ob-R). Of these ObRb – the long isoform – is required for leptin's physiological actions. Leptin links metabolic and immune responses. Leptin levels are low in infection but high in autoimmunity, both systemically and at the site of inflammation. Immune abnormalities in obesity are reversed with energy restriction (which decreases leptin levels). Leptin levels correlate with energy status and with TNFα, which is also elevated in obesity and suppresses lymphocyte function.[70]

Peripheral and central responses to leptin can be intimately linked. Leptin can induce changes in the brain that, in turn, affect TAG homeostasis in adipose tissue. Leptin signaling *via* phosphoinositide 3-kinase (PI3K) in the hypothalamus engages the sympathetic nervous system (SNS), resulting in signaling back to the adipocytes through the endocannabinoid system.[71] Thus, infusion of leptin into the basal medial hypothalamus leads to a rapid switch from lipogenesis to lipolysis.[71] This change was reflected by suppression of sterol regulatory element binding protein 1 (SREBP1), together with marked downregulation of SREBP-1c targets, including the lipogenic enzymes acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD) 1.[71]

White adipocytes also express leptin receptors[72] allowing them to respond to their own secretions. Leptin can also exert an anti-lipogenic autocrine action on adipocytes *via* AMPK activation (a 7-fold higher ratio of phosphorylated to total AMPK). This depletes stored adipose-tissue TAG, an effect proposed to reflect oxidation of lipolytically generated FA *in situ*.[73] This effect is predicted not to be observed in adipose tissue of *db*/*db* mice. *db*/*db* mice are obese, insulin resistant and hyperleptinemic, a phenotype that develops as a result of leptin

receptor deficiency. Interestingly, OB-Rb mRNA levels in subcutaneous adipose tissue are lower in morbidly obese subjects compared with lean subjects,[72] suggesting that this may exacerbate accumulation of excess adipose-tissue TAG. Furthermore, the consequences of adipocyte-selective reduction of the leptin receptors (induced by antisense mRNA) include increased adiposity, dyslipidemia, and insulin resistance.[74]

Adiponectin is abundant in blood plasma relative to many other hormones, approximately 0.01% of all plasma proteins, at concentrations up to 10 μg/ml. Unlike most other adipokines, adiponectin's expression and secretion decrease significantly in obesity.[75] In adults, adiponectin levels are inversely correlated with percentage body fat. Adiponectin concentrations are increased in non-diabetic subjects compared to diabetic subjects. Plasma concentrations reveal gender dimorphism with higher circulating levels of adiponectin in females.

Adiponectin exists as both a full-length protein and a proteolytic cleavage fragment (globular adiponectin). Full-length adiponectin is a trimer (low molecular weight adiponectin) that forms hexamers (medium molecular weight adiponectin). These can further oligomerize to form polymers (high molecular weight form). Two adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) have been identified by expression cloning.[76] AdipoR1 activation stimulates AMPK, whereas AdipoR2 is linked to PPARα activation. AdipoR1 is highly expressed in skeletal muscle, whereas AdipoR2 is the major form in liver.[76] Adiponectin decreases insulin resistance by decreasing triglyceride content in muscle and liver in obese mice.[41] Adiponectin's action to enhance insulin sensitivity is mediated by activation of AMPK and PPARα, resulting in an increase in FA oxidation,[43,76,77] together with its ability to suppress hepatic glucose production.[78,79] Adiponectin increases fatty acid transport protein (FATP)-1 expression in muscle, whereas adiponectin-deficient mice are characterized by decreased FATP-1 in muscle, but not adipose tissue or liver, and impaired FA clearance.[80] Adiponectin reverses insulin resistance and diabetes in *db*/*db* and KKA mice, two different mouse models of T2DM diabetes characterized by obesity, hyperlipidemia, insulin resistance and hyperglycemia.[41] Conversely, heterozygous adiponectin-deficient (adipo$^{(+/-)}$) mice exhibit mild insulin resistance, while homozygous adiponectin-deficient (adipo$^{(-/-)}$) mice are moderately insulin resistant and glucose intolerant even though body weight gain is unaffected.[42] These results confirm that adiponectin regulation is an important factor influencing insulin sensitivity. Adiponectin also enhances AMPK activity in the arcuate hypothalamus (ARH) *via* AdipoR1 stimulating food intake.[81] ICV or intravenous administration of adiponectin increases energy expenditure and weight loss in obese (*ob*/*ob*) mice.[82] Adiponectin-deficient mice exhibit decreased AMPK phosphorylation in the ARC (arcurate nucleus of the hypothalamus) together with decreased food intake, andincreased energy expenditure, as well as resistance to high-fat-diet-induced obesity.[81] Administration of a high-fat/high-sucrose diet results in severe insulin resistance and increased weight gain in adiponectin-deficient mice.[80] Adipose tissue TNFα expression and circulating TNFα levels are increased in adiponectin-deficient mice, effects reversed by exogenous (adenovirus-mediated) adiponectin.[80] These data suggest that adiponectin and TNFα

act in a counter-regulatory manner. Under conditions of metabolic stress, such as diet-induced obesity, unopposed TNFα activity might lead to a shift towards insulin resistance.

Both AdipoR1 and AdipoR2 are expressed in adipocytes suggesting that adiponectin could exert an autocrine effect in adipose tissue. In support of this, transgenic mice with moderate expression of exogenous adiponectin targeted to adipose tissue displayed reduced adiponectin mRNA levels and protein in early life, together with decreased circulating adiponectin in adult mice.[83] As a result, the adult mice displayed glucose intolerance, insulin resistance and increased adiposity.[83] Reduced adiponectin expression in adipose tissue in these mice was also associated with decreased AdipoR2 and UCP2 expression and increased expression of TNFα.[83] Over-expression of adiponectin in 3T3-L1 fibroblasts accelerates adipogenesis, with more prolonged and robust gene expression for related transcriptional factors, CCAAT/enhancer binding protein alpha (C/EBP2), PPARγ, and adipocyte determination and differentiation factor 1; SREBP1c (also named ADD1) together with accelerated suppression of PPARγ co-activator-1α (PGC-1α).[84] These changes were associated with a greater number of larger lipid droplets in differentiated adipocytes.[84] However, over-expression of human adiponectin in the livers of mice maintained on a high-fat/high-sucrose diet impairs adipocyte differentiation and prevents excessive fat accumulation in both visceral and subcutaneous adipose tissues.[85] Macrophage infiltration in adipose tissue was also markedly suppressed in the transgenic mice.[85] Importantly, adiponectin antagonizes LPS-induced NF-κB activation,[66] together with production of IL-6[66] and TNFα.[86] This autocrine effect is observed in conjunction with increased PPARγ2 expression in adipocytes.[66]

A recent study analyzed AdipoR1 and AdipoR2 proteins in 3T3-L1 adipocytes and subcutaneous and visceral adipocytes/adipose tissue of humans and rats.[87] While differentiation of 3T3-L1 cells in the presence of the FA palmitate did not alter AdipoR1 and AdipoR2 expression, metformin (which acts *via* AMPK) and fenofibrate (a PPARα agonist) upregulated AdipoR2. Furthermore, in the rat, AdipoR2 protein expression is lower in obese diabetic animals compared with obese rats with normal glucose disposal.

2.5 Potential Effects of PPARα Deficiency on Autocrine Signaling in Adipose Tissue

PPARα has an established involvement in the metabolic adaptations to fasting, upregulating genes involved in FA uptake and oxidation.[88,89] PPARα null mice show an impaired response to starvation. In these mice, hypoketonemia is associated with elevated plasma FA and hepatic TAG accumulation, together with hypoglycemia.[90] Although highly expressed in the liver and other oxidative tissues (*e.g.* heart, skeletal muscle and endocrine pancreas), PPARα is also expressed (albeit to a lower extent) in adipose tissue.[91] Studies *in vivo* have shown altered regulation of lipogenic and cholesterogenic genes, together with increased lipogenesis *de novo* in adipose tissue of PPARα null mice.[91]

PPARα expression in white adipose tissue is enhanced in response to sustained intense hyperleptinaemia induced by adenoviral transfer of leptin cDNA.[92] PPARα signaling is required for the lipopenic action of hyperleptinemia on adipose tissue as adenovirus-induced hyperleptinemia does not cause fat loss in PPARα null mice.[93] The importance of autocrine effects of leptin, in addition to effects mediated by the hypothalamus, on the reduction of fat mass have been highlighted.[73]

Leptin secretion is positively correlated to adipocyte glucose handling.[94,95] Adipocyte glucose uptake is enhanced by PPARγ activation.[30,31,33,96] PPARγ is thus predicted to facilitate leptin secretion. PPARα null mice maintained on a high-fat diet become more obese than wild-type, despite elevated blood leptin levels.[97] Thus, PPARα deficiency uncouples the feedback control of fat mass by systemic leptin.

The augmented ability for storage of dietary-derived fat within the adipocyte, despite high leptin levels, protects PPARα null mice from the development of lipid-induced insulin resistance.[97] However, even when maintained on low-fat diet, post-absorptive PPARα null mice show increased whole-body glucose turnover, attributed to increased glucose uptake and phosphorylation by white adipose tissue *in vivo*.[98] Thus, in adipose tissue, PPARα deficiency induces a phenotype reminiscent of that evoked by PPARγ activation.

We investigated the involvement of PPARα in the regulation of leptin physiology using isolated adipocytes from PPARα null mice, demonstrating an effect of PPARα deficiency to increase adipocyte glucose uptake and conversion to TAG.[99] We also observed (unpublished observations) that under glucose plus insulin-stimulated conditions, total leptin increased significantly with PPARα null adipocytes and a lesser proportion of this total leptin was secreted. Our data highlight effects of PPARα, potentially important for obesity susceptibility, in the regulation of the balance between leptin synthesis and secretion. In these studies, adipose-tissue mass was not increased, but rather was lowered as a result of PPARα deficiency. This result is consistent with an increased autocrine effect of secreted leptin to lower TAG storage. In contrast to studies indicating a requirement for PPARα for the effects of adenovirus-induced hyperleptinemia, our study using PPARα null mice suggests that this loss of adiposity does not require the participation of PPARα. Our data instead support the concept that adipose tissue expandability may be limited by local leptin production due to increased leptin secretion. This could possibly be through a cycle of increased glucose uptake in adipocytes increasing leptin secretion, which exerts autocrine effects on the adipocyte to reduce TAG storage. In an independent study, Yessoufou *et al.*[100] observed a concomitant increase in both adiponectin and leptin mRNA expression in adipose tissue of PPARα null mice. While our mice, bred on an sv129 background, showed reduced adiposity, mice in this latter study, which were bred on to a different (C57BL/6J) background, had a higher adipose tissue mass compared to WT mice. These authors also quantified the mRNA of well-known markers of macrophages and observed that the expressions of mRNA of CD14 and CD68 (generally expressed in macrophages), but not of F4/80 (a robust macrophage

marker), were upregulated in the obese PPARα null mice. These observations seemed to suggest that PPARα deficiency causes adipocytes to assume a macrophage-like phenotype.

As noted previously, MCP-1 is secreted by adipose tissues and favors infiltration and differentiation of macrophages.[59] However, adipose tissue of PPARα-null mice expresses lower MCP-1 mRNA than that of WT animals.[100] Nevertheless, expression of both TLR-2 and -4 mRNA are upregulated in adipose tissue of PPARα-null mice compared with control.[100] This was suggested to be linked with higher leptin,[100] which induces the expression of TLR-1–9 in adipocytes.[101] We propose that it would also be predicted to increase responsiveness of the adipocyte to the effects of FA which are mediated *via* TLR-4, in particular promotion of MCP-1 secretion, leading to a proinflammatory adipose tissue phenotype under conditions causing adipocyte insulin resistance.

2.6 Metabolic Programming of Autocrine Signaling in Adipose Tissue

If the nutritional supply is intermittently poor and then ample, a selective advantage would be conferred if the organism were to anticipate increased nutrient availability by increasing the number of adipocytes or the capacity of the adipocyte to store excess nutrients as TAG, *i.e.* a thrifty phenotype. This could reflect altered TAG turnover, with relative suppression of lipolysis and/or more vigorous lipogenesis. An over-enthusiastic operation of such thrift would be predicted, ultimately, to lead to obesity if the nutrient supply was in excess of requirement.

The fetus receives from its mother a prediction of the nutritional conditions it will experience after birth, and it is now accepted that an imbalance between prenatal (*e.g.* protein malnutrition) and postnatal (*e.g.* high-fat feeding) nutrition predisposes to obesity. Numerous epidemiological studies have shown that a low birth-weight with relative thinness at birth (thought to reflect an adverse intrauterine environment) is associated with an increased prevalence of obesity and insulin resistance in later life.[19] The experimental evidence that adult obesity, insulin resistance and diabetes can be "programmed" in early life, *e.g.* by poor nutrition, is convincing,[20,102] but the underlying mechanisms have not been unraveled.

Maternal protein restriction during pregnancy and lactation (Maternal Low Protein, MLP) is well established as an animal model of early life programming of obesity and insulin resistance. The MLP protocol produces offspring that are small and more susceptible to the development of obesity, glucose intolerance and diabetes with aging or when given a high-energy diet.[103,104] The MLP model is considered, on the basis of striking parallels with the human situation, to be a suitable system to elucidate the molecular basis for increased susceptibility to the development of obesity, and has been used extensively to study programming of tissue function.[104–107] Prenatal exposure to a low-protein diet followed by rapid catch-up growth is also associated with a higher rate of

proliferation of preadipocytes isolated from weanling male pups (28 days of age).[108] Thus an increase in preadipocyte number (through preadipocyte proliferation) could be one determinant of the capacity of adipose tissue to store fat in MLP programmed obesity.

A previous study investigated the effect of MLP during pregnancy and lactation in rats on adipocyte functional properties and glucose tolerance[109] in young male offspring (6 weeks of age). The 6-week-old male offspring of mothers fed on a low-protein diet during pregnancy and lactation were significantly more glucose tolerant following intraperitoneal (i.p.) glucose challenge than controls. In addition, offspring of mothers fed on a low-protein diet during pregnancy and lactation had significantly smaller adipocytes than controls, suggesting altered TAG handling. Importantly, MLP programming was associated with increased basal and insulin-stimulated glucose uptake by isolated adipocytes[110] in conjunction with hyperleptinemia on insulin stimulation *in vivo*.[105] Inappropriately high circulating leptin levels in programmed offspring in adulthood[105] parallel findings in man.[111]

We therefore recently measured changes in leptin release, adipogenic gene expression and adipokine receptor expression in white adipose tissue from MLP programmed offspring (unpublished results). Adipocytes were challenged to secrete leptin by incubation with high glucose plus insulin. Leptin secretion was significantly higher with adipocytes from MLP offspring than with adipocytes from control offspring. Deficient PPARα signaling in adipose tissue could explain the hyperleptinemia seen in adults of low birth-weight. Taken together these data suggested that there might be enhanced autocrine signaling of leptin to the adipocyte, which would be predicted to deplete adipocyte TAG, in MLP programmed offspring. However, at the same time, there was a marked suppression of leptin receptor expression, which would be predicted to have the opposite effect.

2.7 Autocrine Effects on Adipose Tissue Could Modulate the Operation of the Adipocyte Circadian Clock

New data suggest that circadian rhythms within the adipocyte affect the regulation of adipocyte fat storage and consequently the risk of the development of adiposity. It has been noted that natural fluctuations in body weight are associated with seasonal changes in day length, suggesting a central role for the circadian clock mechanism in the control of adiposity. In rodents, decreasing the length of the dark (feeding/activity) phase (rodents are nocturnal) results in significant weight gain.[112] In healthy people, adipocyte lipolysis decreases in the afternoon and increases at night.[113] Shift work (which disturbs the normal synchrony between the light-dark phase and eating) has been associated with obesity,[114] and the duration of shift work associates with BMI and waist/hip ratio.[115] It seems likely that circadian disturbances precede the onset of obesity, since mutant mice with a ubiquitous loss of one of the core components of the circadian clock (CLOCK) develop obesity and are hyperphagic.[116]

The circadian gene expression is maintained through the coordinated action of bHLH-PAS domain proteins encoded by *Clock*, *Bmal1*, *Period* (*Per*) and *Cryptochrome* (*Cry*) genes). Clock heterodimerizes with Bmal1 to drive the rhythmic expression of *Per* and *Cry* and of circadian effector genes, such as that encoding the transcription factor Rev-Erbα.[117,118] *Rev-Erbα* is highly expressed in adipose tissue, with induction of its expression during adipocyte differentiation.[119] Expression of *Bmal1*, *Per*, *Cry1* and *Rev-Erbα* exhibits robust cycling in adipose tissue.[120,121] Rev-Erbα is activated by Clock : Bmal1 and repressed by Per : Cry complexes, and promotes adipogenesis by facilitating expression of PPARγ target genes, including ap2 and clebα.[122] Currently identified clock-regulated processes include rhythmic secretion of adipokines, including leptin, adiponectin (*apm1*) and visfatin (*pbef1*).[120,123–125] Given that both leptin and adiponectin exert autocrine effects on the adipocyte, as described above, it is tempting to speculate that periodicity of adipocyte function (*e.g.* of lipolysis, possibly extending to MCP-1 secretion) could be modulated through such feedback.

As the fetus receives from its mother a prediction of the nutritional conditions it will experience after birth, and an imbalance between prenatal and postnatal nutrition predisposes to obesity, we further hypothesize that intrauterine and early life nutritional adversity (*e.g.* because of maternal protein restriction) programs obesity *via* permanent resetting of the adipocyte circadian clock. In addition, abnormal autocrine feedback resulting in altered adipocyte clock function in metabolically programmed individuals could favor the development of obesity. Other options could include programmed changes in the levels of expression of Cry and Per such that they could either permanently repress Clock : Bmal1 or, alternatively, never achieve a level sufficient to repress Clock : Bmal1 resulting in an altered balance between diurnal adipocyte TAG storage (during feeding) and TAG lipolysis (between feeding). Alternatively, the duration of repression could be enhanced or impaired by early life events, disrupting the balance between phases of adipocyte TAG handling during the 24-hour feed-fast cycle. Similarly, if Rev-Erbα expression was programmed to be over-expressed, this would be predicted to favor adipogenesis and obesity.

2.8 Cell Heterogeneity in the Pancreatic Islet

The endocrine pancreas comprises approximately 1 million islets of Langerhans, comprising 2–3% of the total pancreatic mass. The islets of Langerhans are approximately 100–300 μm in diameter, containing about 2500 specialized epithelial cells secreting a variety of hormones. Major cell types are the β cells that secrete insulin, and the glucagon-secreting α cells. Other cell types include the somatostatin-secreting δ cells, the PP or F cells that secrete pancreatic polypeptide (PP) and adrenomedullin and the ε cells that secrete ghrelin. During differentiation, progenitor cells co-express these various endocrine hormones prior to differentiation into cells that express a single hormone.[126]

The anatomical arrangement of β cells within islets of Langerhans plays a major role in the secretion of appropriate and sufficient levels of insulin in healthy

individuals.[127] The integrated secretory responses of islet cells exceed those of segregated islet cell lines, thereby suggesting that interactions between the individual cells that comprise the islet are required for normal secretory function.[128–131] Distortions in islet architecture have been implicated in the development of T2DM in many animal models, including the Zucker diabetic fatty rat.[132] Altered islet morphology has also been reported in maturity onset diabetes of the young (MODY). There are, however, significant differences in cell composition and islet structure between species.[133] In rodent islets the different cell types are clearly segregated, with β cells (about 70%) clustered at the core surrounded by a mantle of α and δ cells.[134] Human islets contain fewer insulin-containing β cells (rodent *vs.* human: 77% *vs.* 55%) and more glucagon-containing α cells (18% *vs.* 38%). In addition, the insulin-containing cells are found interspersed with glucagon- and somatostatin-containing cells within the islet.[135]

Irrespective of the anatomical arrangement, it is emerging that the individual cells that comprise the islet may also modulate their actions *via* responses to their own secretions or by effects of peptides secreted from the other cell types. Autocrine effects of insulin on the β cell may be important within the context of islet compensation for insulin resistance.

2.9 Autocrine Effects of Insulin on the Pancreatic β Cell

Experimentally, increases in compensatory insulin secretion, in part due to increased islet mass, are observed in response to partial pancreatectomy,[136] continuous glucose infusion,[137] late pregnancy[138–143] and increased dietary saturated fat.[106,107] Hyperplasia and hypertrophy contribute to the increase in islet mass and hypertrophy occurs through both neogenesis from ductal cells and replication of differentiated β cells.[144] An autocrine effect of insulin has been implicated, particularly through the results of studies in mice in which insulin resistance was engineered by ablation of either the insulin receptor (IR), or insulin receptor substrate-1 (IRS-1), or both genes.[145–147] In these studies a 400-fold increase in plasma insulin levels was associated with a 40-fold increase in β-cell mass.

Insulin exerts its effects in target cells through binding to and activating its cell surface receptors. The IR consists of two α and two β subunits that are linked together by disulfide bonds to form a heterotetrameric complex. Insulin binding to the extracellular α subunits rapidly activates the tyrosine protein kinase activity of the intracellular β subunits, resulting in autophosphorylation of the β chains. This activation of the β-subunit tyrosine kinase activity results in phosphorylation of intracellular substrates including the IRS proteins on tyrosine residues.[148] This initiates a series of intracellular signaling events. IRS proteins contain multiple potential phosphorylation sites and, on tyrosine phosphorylation, they serve as multi-site docking molecules for other signaling molecules. In this way, the IRS proteins act as an interface between insulin receptors and signaling molecules further downstream.[149]

The finding that the insulin receptor and IRS proteins are both specifically expressed in rodent β cells[150,151] has led to the prospect of a potential role of the insulin signaling cascade in the regulation of β-cell function.[151] Data from studies using isolated islets and islet cell lines suggest that insulin itself governs its own gene transcription, thereby forming a functional autocrine loop. Other insulin-regulated processes in the pancreatic β cell have been reported to include gene transcription,[152] translation,[153] β-cell proliferation[154,155] and finally insulin secretion itself.[156–159] In accordance with this, a study determining the pattern of gene expression in single human α, δ and β cells demonstrated that the receptors and downstream elements that belong to the insulin receptor signaling pathway are expressed in human β cells at the mRNA level.[160] The major argument against autocrine insulin signaling is that β cells are exposed to so much insulin that the respective signal transduction pathways must be desensitized. However, this would be predicted to be the case only in situations of chronic stimulation to secrete insulin *e.g.* prolonged hyperglycemia. Relative hyperinsulinemia, but at low glucose levels, in the basal (*i.e.* non-stimulated, post-absorptive) state, as is observed in T2DM, could exert a long-term tonic effect on the islet itself.

Disruption of insulin signaling the level of the β-cell IR (the βIRKO mouse) does not cause a change in β-cell mass prenatally,[161–163] but reduces islet insulin content in the adult state, and the βIRKO mouse shows an age-dependent decrease in β-cell mass, with a selective loss of GSIS and development of a T2DM-like phenotype at the age of 8 weeks.[155,163] The IR exists in two isoforms as the result of alternative splicing of the 11th exon. The A type (IR-A) lacks the 12 amino acids encoded by exon 11, whereas the B type (IR-B) contains this sequence at the C terminus of the α subunit of the IR. Pancreatic β cells express both isoforms in an almost one-to-one ratio.[164] It appears that each IR isoform may have specific roles in mediating particular actions of insulin in the pancreatic β cell. Thus, it has been shown that over-expression of IR-A leads to a pronounced effect of insulin on insulin gene promoter activation, whereas over-expression of IR-B does not.[165,166] By contrast, selective blocking of IR-B using a blocking antibody raised against the 12 amino acids encoded by exon 11 abolishes insulin-stimulated upregulation of the glucokinase (GK) promoter (which would be predicted to modify islet glucose sensing), but has no effect on the insulin-stimulated upregulation of the insulin promoter, which would allow retention of normal insulin production.[165] Hence, within the context of compensatory insulin secretion, increased signaling *via* IR-B would be predicted to enhance islet glucose sensing and, therefore, GSIS. Of interest, a recent study has shown that chronic (48 h) exposure of insulin-secreting βTC6 cells to high glucose decreases insulin-induced tyrosine phosphorylation of the IR, in the absence of any change in expression of the IR as detected by immunoblotting.[167] This may explain why poor glycemic management in DM could ultimately lead to impaired β-cell adaptation to insulin resistance.

The IRS proteins are characterized by the presence of an NH_2-terminal PH domain adjacent to a PTB domain, and a variable-length COOH-terminal tail that contains several tyrosine and serine phosphorylation sites.[149] These proteins lack catalytic activity of their own, but they possess a number of interaction domains

and phosphorylation motifs that enable them to function as interface molecules in signaling between activated tyrosine kinase receptors and their downstream elements.[168] So far six members of this family have been identified (IRS1-6).[169] Specific interactions with the IR and IGF-1 receptor kinases are mediated by the PH and PTB domains on IRS proteins, while downstream signaling proteins are recruited by the phosphorylation of tyrosine sites in the COOH terminal end.[170] All four IRS proteins have been detected in pancreatic β cells.[154]

IRS-1 deficient islets and β-cell lines have decreased insulin content and glucose- or arginine-stimulated insulin secretion, which is partially restored by re-expression of IRS-1.[154] Signaling through IRS-1 in β cells appears to maintain intracellular insulin content and synthesis,[166] regulate calcium homeostasis[171] and promote insulin secretion.[156] However, despite whole-body insulin resistance, IRS-1 null mice do not develop diabetes because IRS-2 compensates for IRS-1 ablation by mediating β-cell growth and development and allowing compensatory insulin hypersecretion to overcome insulin resistance. This is demonstrated by β-cell hypoplasia and a corresponding decrease in insulin secretion in IRS-2 null mice.[155,172,173] In particular, global disruption of IRS-2 leads to lack of compensatory β-cell hyperplasia despite the development of hepatic insulin resistance,[173] and is ultimately fatal in young male mice.[155] Female mice are less affected but, nevertheless, die in middle age.[155] Histological analysis shows that β-cell mass is reduced compared with the wild-type – however, although hyperplasia is defective, individual β cells show normal or increased insulin secretion in response to glucose.[173]

Alterations in the insulin signaling pathways that might contribute to β-cell proliferation and/or apoptosis in IRS-1 knockout mice were identified using a transplantation approach to separate host insulin resistance from islet function. The results demonstrated that the IRS-1–/– mice displayed an 80% decrease in β-cell apoptosis as compared to wild-type, and this was accompanied by a substantial increase in IRS-2 expression.[146] These results indicate that the islet growth seen in IRS-1 deficient β cells is due to the compensatory increase in IRS-2 expression. Over-expression of IRS-2 in β cells was found to decrease apoptosis by more than 50% and promote β-cell proliferation.[146] In addition, an increased β-cell mass is seen in food-restricted pregnant rats which express increased level of IRS-2[174] and IRS2 is critical for β-cell hyperplasia in response to HF diet-induced insulin resistance.[175] In this study, an animal model for human T2DM (mice with β-cell-specific GK haploinsufficiency (Gck(+/) (–)) fed an HF diet) exhibited decreased β-cell proliferation which was reversed by over-expression of IRS2 in β cells.

During insulin stimulation, tyrosine phosphorylation sites in the IRS proteins bind specifically to SH2 domains in various downstream signaling molecules including phosphatidylinositide 3-kinase (PI3k), Grb2/son of seven-less (SOS) and SHP-2, with the Grb2/SOS complex mediating the activation of p21ras, thus triggering the ras/raf/mitogen-activated protein kinase kinase (MEK)/MAPK cascade. PI3Ks are a family of enzymes subdivided into three major classes (I, II and III) based on their structural homology, regulation and substrate specificity. PI3K class Ia is a heterodimeric enzyme that consists of an

adaptor protein, the p85 regulatory subunit (p85), that possesses two SH2 domains that interact with tyrosine phosphorylated IRS proteins. At least eight isoforms of p85 (α, β and others) have been identified. PI3K also contains a catalytic subunit (p110), of which several isoforms exist. The stoichiometric balance between p85α, p110 and the IRS proteins appears to be critical for signal transduction. One genetic model that has produced an interesting phenotype has emerged from KO of the p85α regulatory subunits of PI3K. p85α heterozygous KO mice exhibit improved insulin sensitivity.[176,177] It might be predicted (although it has not yet been shown) that the acute insulin response of the islet to glucose challenge in this KO might be correspondingly attenuated because the need to produce insulin is reduced. Thus increased p85α expression in the islet might be important for compensatory insulin secretion. Rothenberg *et al.*[159] and Velloso *et al.*[178] reported that insulin, secreted upon glucose stimulation, activated the β-cell IR, the downstream-located IRS and PI3K, and the PI3K downstream target Akt. Increased GSIS after inhibition of PI3K or with islets of p85 deficient mice[179–181] suggests that insulin may have an acute negative effect on β-cell stimulus-secretion coupling, directly modulating the islet response to glucose in addition to acting *via* changes in whole-body insulin sensitivity. In these reports, inhibition of islet PI3K led to an increase in the second phase of insulin release, which is often augmented in compensatory insulin secretion as a consequence of peripheral insulin resistance.

Use of pharmacological inhibitors (wortmannin, LY-294002) and the dominant negative mutant of p85 has demonstrated the involvement of PI3K class Ia in autocrine insulin feedback on glucose and/or insulin stimulated regulation of expression of the insulin gene itself.[152,165,166,182] By contrast, it appears that glucose and/or insulin-dependent transcription of the GK gene in pancreatic β cells requires insulin signaling *via* the class II PI3Ks (such as PI3K-C2α) and Akt.[165] Thus, Western blot analysis revealed the presence of PI3K-C2α in insulin-producing cells and coimmunoprecipitation of PI3K-C2α and IRS suggested a direct interaction.[183] The PI3K cascade also probably promotes survival of the β cells *via* Akt – either Akt 2[184] or Akt 1.[185]

Use of single-cell RT-PCR demonstrated that all the downstream elements which belong to the insulin receptor signaling pathway are expressed in human pancreatic β cells at the mRNA level.[160] These observations emphasize the possibility that the insulin signal transduction pathways that are responsible for mediating its action in peripheral target tissues might also be involved in β-cell autocrine regulation by insulin in man.

2.10 Is Early Life Programming of Insulin Resistance by Altered Insulin Signaling Accompanied by an Abnormal Autocrine Effect of Insulin on the Pancreatic β Cell?

Correlations exist between a low birth-weight and deficient insulin release in several adult populations.[12–14,19,186–188] Thus, impaired pancreatic β-cell

development and function comprises one connection between poor early growth and type 2 DM. Use of a maternal low-protein diet during pregnancy and lactation (MLP model) leads to low birth-weight and a predisposition to the development of insulin resistance in later life in a rat model.[103] Dahri *et al*.[189] found a simultaneous reduction of cell proliferation, islet size, islet vascularization and insulin content in rat fetuses at E21.5, where pregnant mothers were subjected to a low-protein diet, which is consistent with either elevated fetal insulin levels (which are not observed) or abnormally high insulin signaling in the fetal pancreas during early development. These results would be consistent with altered IRS-2 expression and/or signaling in the islet itself, but this has not yet been investigated.

The relative weight of the pancreas at weaning is reduced and offspring of protein-restricted mothers show increased susceptibility to the development of insulin resistance with aging and in response to challenge to the endocrine pancreas such as the imposition of a cafeteria[190] or high-saturated-fat diet.[106,107] Early protein restriction also leads to a lower pancreatic insulin content in adulthood.[191] In addition, we have identified defects in compensatory GSIS *in vivo* when such offspring are challenged with a diabetogenic high-saturated-fat diet and gender-specific differences in compensatory GSIS in response to insulin resistance.[106,107,192] As islet IRS-2 deficiency impacts on compensatory insulin secretion and is gender related, it is again possible that islet signaling *via* IRS-2 in adulthood is modified by early life events. Evidence that maternal protein malnutrition can permanently impact on tissue IR expression and downstream components of the insulin signaling cascade in later life in non-islet tissues has been shown from studies of adipocytes and muscles of adult offspring of protein malnourished dams.[110,193,194] In particular, adipocytes isolated from early-protein-restricted rats show increased IR levels, increased basal and insulin-stimulated levels of IRS-1-associated PI3K activities and increased Akt activities.[193] In adipocytes, there are two major isoforms of the PI3K catalytic subunit (p110α and p110β). There is no change in p85 expression[56] or p110α protein expression[194] in adipocytes from early-protein-restricted rats compared with control, but adipocytes from early-protein-restricted rats have relatively low levels of p110β.[194] The association of p85 with p110α is similar in control and low-protein adipocytes; however, there is less p110β associated with p85.[110] These alterations in insulin signaling have functional implications as there are associated increases in basal and insulin-stimulated glucose uptake by adipocytes *in vitro*[194] and *in vivo* during euglycemic hyperinsulinemia in the intact animal.[195,196]

2.11 Effects of FA on the Pancreatic β cell

The FAs oleate (18:1), palmitate (16:0) and stearate (18:0) constitute 80% of the circulating FFA.[197] Oleate is the most abundant FFA in the human circulation,[197] whereas stearate is most abundant in the rodent circulation.[198] Exogenous FA can trigger insulin release from pancreatic β cells at basal

glucose.[199,200] A G protein-coupled seven-transmembrane-spanning receptor, termed GPR40, is implicated in the acute response of GSIS to exogenous FA.[201–203] Lack of sensitivity of FA-induced increases in Ca^{2+} to pertussis toxin pretreatment suggested that GPR40 is coupled to the G-protein subunit $G\alpha q/11$.[201]

Effects of FA on GSIS may also be linked to their metabolism. Their effects to stimulate insulin secretion are more marked at high glucose,[204] under which condition FA are re-esterified to TAG.[205] A non-metabolizable inhibitor of FA oxidation, 2-bromopalmitate, also activates insulin secretion.[206] The acute insulinotropic action of exogenous FAs depends on their chain length and degree of saturation (stearate > palmitate > oleate > linoleate > linolenate > octanoate). Thus the fold stimulation of insulin secretion is greater for saturated (*e.g.* stearate [18:0]) or palmitate [16:0] *versus* unsaturated (*e.g.* oleate [C18:1] or linoleate [C18:2] or palmitoleate [C16:1]) fat.[207] Epidemiological evidence suggests that insulin resistance in association with hyperinsulinemia is linked to the ingestion of saturated, rather than unsaturated, fat.[208–210] Thus, the ability of individual FAs to augment GSIS acutely parallels their ability to induce insulin resistance, but why their efficacy increases so profoundly with chain length and degree of saturation is not known.

Ectopic fat in the pancreas is characterized by adipocyte infiltration and altered lipid composition.[211] It is therefore possible that increased ectopic fat deposition in obesity could be linked to increased local release of FA. Islets of patients with T2DM display features of an inflammatory process including elevated levels of the cytokine IL-1β and macrophages and, as in adipose tissue, FAs induce a proinflammatory response in islets.[212] The IL-1R type I (IL-1RI) is part of the superfamily of TLR/IL-1R, defined by the presence of a common cytoplasmic signaling domain.[213,214] TLR and IL-1R couple to the same universal intracellular docking protein, Myd88, leading to upregulation of proinflammatory factors.[213]

Blocking the IL-1RI with the IL-1R antagonist strongly inhibited FA-mediated expression of proinflammatory factors in both human and mouse islets.[212] FA-induced IL-1β and KC expression in mouse islets was dependent on Myd88 and partly dependent on TLR-2 and -4. Activation of TLR-2 in purified human β cells and islets stimulated the expression of proinflammatory factors, and IL-1RI activity increased the TLR-2 response in human islets. It was concluded that FFA and TLR stimulation induce proinflammatory factors in islets and that IL-1RI engagement results in signal amplification. Within the clinical context, IL-1 receptor type I (IL-1RI) blockage improves glycemia and insulin secretion in humans with T2DM and in high-fat-fed mice, pointing to a pivotal role of IL-1RI activity in intra-islet inflammation.

2.12 Effects of Leptin and Adiponectin on the Pancreatic β Cell

Acute effects of leptin on GSIS have proved variable.[215] While it protects against fatty acid-induced apoptosis and stimulates β-cell proliferation, it

inhibits insulin gene expression and insulin secretion. The consensus view is that leptin directly suppresses insulin secretion. Acute physiological increases in serum leptin levels *in vivo* significantly reduces glucose-mediated insulin secretion in rats in a dose-dependent fashion.[216] Demonstration of acute glucose intolerance by intracerebroventricular administration of pharmacological doses of leptin in mice suggests that these effects could be *via* central actions,[217] possibly by increasing SNS activity.[218]

Partially pancreatectomized (Px) rats are a model of mild T2DM, in which insulin secretion is approximately halved but insulin resistance is increased.[219] A recent study showed that hypothalamic leptin modulates β-cell function and mass *via* the SNS in Px diabetic rats.[220] Acute ICV infusion of leptin suppressed first- and second-phase insulin secretion through the SNS, while long-term leptin infusion reduced second-phase insulin secretion only, and did not decrease β-cell mass.

Leptin signaling occurs typically through the JAK-STAT pathway resulting in induction of specific genes. Leptin can also induce β-cell apoptosis and impairs GSIS though JNK activation.[221] JAK2 has been identified in rat islets and INS-1 β cells and STAT5 translocates to the nucleus on activation.[222] In β cells, leptin activates STAT5,[223] which also mediates effects of prolactin and growth hormone to increase β-cell mass.[224] When subjected to a high-fat diet, transgenic mice expressing a dominant-negative mutant of STAT5a (RIP-DNSTAT5 mice) showed greater impairment of glucose tolerance than those expressing a constitutive active mutant of STAT5b and reduced β-cell proliferation.[225] STAT translocation is maximal within 30 min.[222] Interestingly, leptin modulates β-cell expression of IL-1 receptor antagonist and release of IL-1β in human islets,[226] thus at least two bioactive molecules released from adipose tissue could induce islet inflammation.

Both adiponectin receptors are expressed in the β cell. Two distinct regions of the adiponectin molecule, the globular domain and a small N-terminal region, have agonist properties. A recent study investigated the effects of two agonist regions of adiponectin on insulin secretion, gene expression, cell viability and cell signaling in the rat β-cell line BRIN-BD11. Both globular adiponectin and adiponectin (15-36) increased cell viability.[227] Adiponectin also reduces glucotoxicity-induced apoptosis of INS-1 rat insulin-secreting cells.[228] Leptin coincubation attenuated adiponectin (15-36) but not globular adiponectin induced cell viability. Globular adiponectin, but not adiponectin (15-36), caused a significant 450% increase in PDX-1 expression. AdipoR1 was expressed at a higher level than AdipoR2 and AdipoR mRNA levels were differentially regulated by NEFA and PPAR agonists. Treatment with the FAs oleate and palmitate and PPAR agonists WY14643 (PPARα agonist) and rosiglitazone (PPARγ agonist) were compared for effects on AdipoR1 and AdipoR2 expression. Twenty-four-hour exposure to oleate (a mono-unsaturated FA) significantly decreased AdR-1 mRNA expression, but AdR-2 mRNA levels were not significantly altered. A similar decrease in AdipoR1 mRNA expression was observed after treatment with WY14643, but this latter treatment also decreased AdR-2 mRNA expression. Palmitate (a saturated FA) decreased AdipoR2 mRNA expression, but not

that of AdipoR1, compared to control. Incubation with the PPARγ agonist rosiglitazone had no significant effect on expression of either receptor subtype.[227] These and other studies[229] suggest that AdipoR2 expression is regulated by saturated FA *via* PPARα.

2.13 Effects of TNFα on the β Cell

TNFα, the most well-established cytokine to participate in insulin resistance, is a major proinflammatory mediator, with an optional capacity to induce apoptosis. However, in some situations, TNFα shows functional duality, being engaged in both tissue regeneration/expansion and destruction, which makes it an attractive candidate cytokine for modulating β-cell mass expansion during islet compensation for insulin resistance. TNF-receptor R1 mediates actions of TNFα to activate proinflammatory transcription factors (such as NFκB), and JNK.[230]

In lipid-induced insulin resistance, serine phosphorylation of IRS-1 interferes with IRS tyrosine phosphorylation.[231] The kinases that serine phosphorylate IRS on exposure to lipids are those involved in cytokine signaling, including JNK and the inflammatory kinase IKK-β.[232] TNFα also targets insulin receptor signaling through serine phosphorylation of IRS-1.[233] Genetic JNK1 deficiency protects mice from obesity-induced JNK activation, IRS-1 phosphorylation and insulin resistance.[234,235] Should a similar action occur in β cells, it would be predicted to oppose β-cell proliferation in response to insulin resistance.

ER stress has been implicated in islet glucolipotoxity:[18,236] JNK-AP-1 and IKK-NFκB are linked to IRE-1 and PERK activation during ER stress.[237–239] IRE-1 interacts with IKK-β through TNF-receptor activated factor 2 (TRAF2) and PERK activation leads to degradation of IκB, facilitating the activity of NFκB.[238,239] Finally, in a study of human and rat primary β cells cultured for 24 hours with the inflammatory cytokine TNFα, impaired GSIS elicited by TNFα was associated with a decrease in insulin-stimulated phosphorylation of the IR in conjunction with markedly decreased IRS-2 protein expression.[240] Thus, a role for IRS-1 and IRS-2 is suggested in the regulation of β-cell mass and function of individual β cells and the onset of T2DM.

Cytokine-induced changes in gene expression are NFκB dependent in β cells.[241] IL-1β, like TNFα, activates MAPKs (ERK, p38 and JNK) and, in β cells, ERK may be essential for post-translational modification of the p65 subunit of NFκB.[242] Interfering with JNK suppresses cytokine-mediated cell death in human and rodent islets.[243,244] These kinases also promote inflammatory gene expression through activation of activator protein-1 (AP-1) complexes and NFκB.[245]

2.14 Is Programmed Obesity Associated with β-cell Inflammation?

The ability to survive starvation and to mount an effective immune response is critical to survival. A "thrifty phenotype" favoring the storage of excess

calories is predicted to be advantageous when access to food is limited or intermittent but, as discussed above, could set the stage for obesity in nutritional surplus. The selection of strong immune or inflammatory response is advantageous during periods of infection, but also requires prioritization of available nutrients should they be in short supply. The histology of islets from patients with T2DM displays an inflammatory process, characterized by the presence of cytokines, apoptotic cells and immune cell infiltration;[246] similarly, exposure to high glucose enhances IL-1β expression associated with severe impairment of insulin-mediated signal transduction in cultured, proliferating βTC-6 cells.[167]

Overfeeding postnatally causes increased adiposity and insulin resistance and, importantly, genes involved in inflammation (*e.g.* TNFα) are over-expressed in adipose tissue when the animals are placed on a high-fat diet.[247] We propose that a state of low-grade inflammation in the islet, induced by increased chronic exposure to adipocyte-derived cytokines such as TNFα, could predispose the β cell to react to nutrient stressors in an exaggerated fashion, so predisposing to gluco-lipotoxicity. This could arise because intracellular signaling pathways utilized by proinflammatory cytokines overlap with, or are identical to, those activated by lipids and high glucose. It is tempting to speculate that programming of obesity by early life events such as maternal protein restriction during gestation and lactation induces a propensity for adipocytes to respond to proinflammatory cytokines released by macrophages with augmented adipocytokine expression and secretion which, in turn, impacts β-cell function and compensation for insulin resistance.

2.15 Other Adipose-derived Factors that Could Contribute to the Adipoinsular Axis

Wnt proteins are a large family of secreted lipidated glycoproteins that regulate crucial aspects of development, including cell-fate specification, proliferation and survival. On presentation to its target cell, canonical Wnt signaling begins with Wnt proteins binding to a co-receptor complex that consists of frizzled (Fzd) family receptors with the lipoprotein receptor related 5/6 (Lrp5/6) proteins. In the presence of Wnt, the intracellular scaffolding protein Dishevelled (Dvl) associates with the transmembrane Wnt–Frizzled–LRP complex. Wnt receptor binding causes coordinate phosphorylation of Dvl and the intracellular domain of LRP. Activated Dvl inactivates a protein complex that includes the constitutively active serine-threonine kinase glycogen synthase kinase 3β (GSK3β) and the scaffolding proteins axin and adenomatosis polyposis coli (APC). A pool of β-catenin that is phosphorylated by GSK3 seems to be specifically involved in regulating gene expression. In the absence of ligand binding to both receptors, previously ''primed'' β-catenin (*i.e.* β-catenin phosphorylated by casein kinase 1 on Ser45) is phosphorylated by GSK3β at three further sites: Thr41, Ser37 and Ser33. This results in its ubiquination, which targets it for proteosomal degradation. GSK3 β inactivation by the

activated Wnt–Fzd–LRP complex prevents degradation of β-catenin, promoting its cytoplasmic accumulation. In a pancreatic β-cell line (INS-1 cells), which express a range of components of the Wnt pathway including β-catenin and GSK, the non-receptor protein tyrosine phosphatase-BL (ptpn13) PTP-BL opposes Wnt signaling as the increase in β-catenin seen in response to exogenous Wnt3a is prevented following induction of PTP-BL.[248]

Following its cytoplasmic accumulation, β-catenin translocates to the nucleus, where T-cell-specific transcription factor/lymphoid enhancer binding factor 1 (TCF/LEF1) family binding proteins, such as TCF7L2 (previously known as TCF4), are normally maintained in a repressed state by association with Groucho. β-catenin acts as a transcriptional co-activator by displacing Groucho and, with TCF/LEF1, forming a complex that activates transcription of canonical TCF/LEF1-regulated genes. A close association exists between poly-morphisms in the TCF7L2 gene and susceptibility toward type 2 diabetes in human populations. In Europeans, TCF7L2 is the most important locus predisposing to T2DM. Although originally reported to be non-detectable,[249] TCF7L2 is now known to be expressed in both islets and INS-1 cells.[250–252] Alterations in TCF7L2 expression confer risk for genotypes associated with impaired β-cell function.[253,254] TCF7L2 expression is increased 5-fold in type 2-diabetic pancreatic islets.[255] Thus understanding of how islet Wnt signaling is regulated and how it influences islet function is of immense potential importance for identifying means to decrease the risk of developing type 2 diabetes.

TCF7L2 expression is increased 5-fold in T2DM pancreatic islets and over-expressing TCF7L2 in human islets using an adenovirus system reduces insulin secretion but, paradoxically, insulin gene expression positively correlates with TCF7L2 gene expression.[255] These findings suggest that TCFL2 could be involved in regulating islet compensation for the insulin resistance that precedes and accompanies the development of T2DM. Consistent with this idea, older mice lacking LRP5, the Wnt coreceptor, have impaired β-cell function and impaired glucose tolerance when challenged with a high-fat diet.[256] Rat pancreatic islets express TCF7L2 and TCF7L2 gene expression is significantly elevated in islets from prediabetic Zucker fa/fa rats.[257] This indicates that islet TCF7L2 expression may be a patho/physiological variable. Taken together, these data strongly support the concept that altered Wnt signaling may be required to maintain islet functions in conditions demanding adaptive β-cell responses to insulin resistance.

Exposure of islets to exogenous Wnt-3a stimulates glucose-simulated insulin secretion (GSIS).[256] This is blocked by a soluble form of Wnt receptor, secreted Fzd-related (sFRP) protein-1.[256] Importantly, adipocyte-derived Wnt signaling molecules present in fat cell-conditioned media stimulate insulin secretion by primary islets, an action blocked by sFRP-1[258] indicating a specific effect of Wnt ligands. Treatment with human adipose-conditioned media increases the transcription of a TCF reporter gene in INS-1 cells and induces their proliferation.[258] Thus, as these authors suggest, the increased adipocyte mass in obesity (an insulin-resistant state) might be important to increase β-cell mass.

2.16 Concluding Remarks

It is increasingly apparent that the ability of white adipose tissue to secrete bioactive signaling molecules, adipokines, can underlie many of the pathophysiological adaptations of metabolism associated with endocrine disorders, including T2DM. In this chapter, we have attempted to discuss and develop a number of recent avenues of research that highlight the potential roles of adipokines in the development of T2DM. These include the potential for autocrine effects of adipokines, notably leptin and adiponectin, to influence the function of the adipocyte. We have also highlighted the potential role of the lipo-oxidative transcription factor PPARα in influencing the potential autocrine actions of leptin. Strong evidence exists to support the concept that an inappropriate early life environment can increase the risk of type 2 diabetes in adulthood. We have developed this concept to include a role for an inappropriate early life environment in orchestrating metabolic maladaptations leading to T2DM *via* augmented FA release in combination with altered secretion or action of adipo/cytokines. Circadian rhythms within the adipocyte influence adipocyte fat storage and consequently the risk of obesity. We have examined the evidence that circadian disturbances precede the onset of obesity. Adipose-tissue-generated products, namely FA and adipokines, as well as cytokines have a significant role in the regulation of pancreatic β-cell function, in particular insulin secretion, and are now recognized to play a crucial role in determining the ability of the β cell to secrete adequate insulin to compensate for a deterioration in insulin sensitivity. We have developed this to encompass a novel mechanism whereby a state of low-grade inflammation in the islet, induced by increased chronic exposure to adipocyte-derived cytokines such as TNFα, could predispose the β cell to react to nutrient stressors in an exaggerated fashion, so predisposing to gluco-lipotoxicity and T2DM. Finally, we have discussed the potential that Wnt signaling may influence islet function and may be involved in linking the increased adipocyte mass in obesity to increase β cell mass.

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CHAPTER 3

One Receptor for Multiple Pathways: Focus on Leptin Signaling

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3.1 Leptin

Leptin is a 16-kDa non-glycosylated polypeptide hormone encoded by the gene obese (ob), the murine homolog of the human gene LEP. From a structural viewpoint, leptin belongs to the type I cytokine superfamily with a four alpha helical bundle motif. It shares 84% homology between humans and mice.[1] It is mainly produced by adipocytes.[1,2] Although other cells or tissues produce leptin in a significant way, visceral and subcutaneous fat are responsible for more than 80% of total leptin production.[3,4] Circulating leptin levels are modulated by several factors such as food intake, sex, hormones and cytokines; however, leptin levels are directly proportional to the white adipose tissue (WAT) mass. Likewise, they are negatively correlated with glucocorticoid levels.[5–9]

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Leptin is involved in the control of the reproductive system,[10] vascular function,[11] glucose metabolism,[12] immune system and skeleton homeostasis.[13,14] Besides its pleiotropic role, leptin is a metabolic hormone that has an important function in the control of energy expenditure, food intake and glucose metabolism. At the hypothalamic level, leptin function as an anorexigenic peptide inducing the expression of specific factors such as cocaine-and-amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) and inhibits orexigenic neuropeptides such as neuropeptide Y (NPY), agouti-related peptide (AGRP) and orexin. In addition, regarding the straight correlation between circulating leptin levels and adipose tissue mass, leptin levels can be considered as a signal to the body of its energy reserves, and it is likely that the physiological relevance of leptin is based on the fact that low levels signal starvation.[15]

3.2 Leptin Receptors

Leptin exerts its biological actions through the activation of its cognate receptors, which are encoded by the diabetes (db) gene. These receptors belong to the class I cytokine receptor superfamily, which includes receptors for IL-6, LIF, CNTF, OSM, G-CSF and gp130.[16] Db gene alternative splicing gives rise to six receptor isoforms with cytoplasmic domains of different length, including one soluble, secreted form (Ob-Re), which lacks the transmembrane region, and four short forms (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf), which have slight differences in the length of their cytoplasmic domains and share the extracellular domain, the transmembrane domain and a small intracellular fragment. Finally, only the one long-functional isoform (Ob-Rb), which has the full intracellular domain with the typical signaling elements of cytokine receptors[17,18] is able to transduce the signal to the nucleus. Despite the fact that Ob-Ra is the most expressed form; it does not exhibit the ability to transduce properly the leptin signal. In contrast, the distribution of Ob-Rb, the functional form, correlates with leptin in proposed target tissues such as hypothalamus and peripheral tissues, like WAT.[19] However, it must be noted that, even though the short Ob-R forms (Ob-Ra and Ob-Rc) are devoid of full signaling, it has been described that they participate in leptin uptake and leptin availability for Ob-Rb.[20,21]

3.3 Leptin Receptor Signaling

3.3.1 The JAK2-STATs Routes

Leptin receptor (Ob-Rb), as a member of the class I cytokine receptor superfamily, does not have intrinsic tyrosine kinase activity. Due to this fact, it must recruit cytoplasmic kinases like the Janus kinase family JAK2 to achieve leptin signaling.[22] The activation of JAK2 kinase by the leptin receptor is supported by the constitutive interaction between JAK2 kinase and the leptin receptor.[23] In this situation, the ability of this receptor to form aggregates (homodimers),

in a ligand-independent way, facilitates the autophosphorylation of JAK2, eliciting leptin signaling.[24,25] In this sense, it has been shown that two JAK2 molecules are needed for the signaling pathway activation.[23] Once JAK2 is activated, it phosphorylates three tyrosine residues (Tyr985, Tyr1077, Tyr1138) of the murine leptin receptor.[24,26] These tyrosine residues are highly conserved, which means they are very important for leptin signaling. Accordingly, it has been determined that these conserved tyrosine residues behave as docking sites for proteins with SRC homology 2 domains, such as STATs (Signal Transducers and Activators of Transcription) proteins, which have high affinity for phosphotyrosine residues.[26,27]

Each tyrosine phophorylation site is involved with the recruitment of different signaling proteins.[27] Among these proteins there are STATs. These kinds of proteins are the most known targets of JAK activation. After binding to the phosphotyrosine residues of the leptin receptor, these signaling molecules are also phosphorylated by receptor associated JAK kinases (RAJK), and are involved in dimerization and translocation to the nucleus, where they function as transcription factors.[27]

One of the major STATs proteins involved in leptin signaling is STAT3. Activation of this STAT protein, after leptin binding to its receptor, has been found in multiple cell types such as hypothalamic neurons, adipocytes, immune cells and pancreatic cells.[28–31] Ob-Rb has a consensus binding site region for STAT3. This region, also named Box3, has the structure Tyr-X-X-Gln from position 1138 to 1141 and is essential for leptin signaling.[26] In fact, mutational analysis of box3 around Tyr1138 confirmed that this motif is necessary for STAT3 signaling in addition to other STATs.[26] In this sense, it has been described that leptin has the ability to signal through the recruitment and activation of other STATs different from STAT3. Among these STATs are STAT1, STAT5 and STAT6.[26,32] The exact role of these proteins in leptin signaling is still unclear. However, it is known that several residues of the leptin receptor are necessary for its recruitment, such as Met 1139 and Gln 1141.[26] So, leptin behaves as other members of the class I cytokine receptor superfamily such as IL-6,[33] inducing the activation of STAT1 and STAT3 through the above-mentioned conserved motif box3. According to this, it has been described that STAT1 signaling is involved in some leptin actions such as immune competence against microbial aggression.[34] Likewise, recently, it has been determined that STAT1 signaling is involved in leptin inhibition of peroxisome proliferator activated receptor gamma (PPARγ) expression.[35] Moreover, STAT1 is also involved in leptin inhibition of adipocyte differentiation and lipid accumulation in murine primary adipocytes.[35] On the other hand, it must be considered that interactions among different STATs proteins may also be at play since, in other members of the of the class I cytokine receptor superfamily, STAT1 signaling is possibly blunted by STAT3 activation.[36] Strengthening this idea is the fact that ASKO mice, a murine strain with an adipocyte specific disruption of the STAT3 gene, have a strong STAT1 signaling in comparison to WT littermates.[37]

In addition to STAT3 and STAT1, leptin signaling, through the activation of the leptin long form receptor, also involves other STATs proteins. It has been

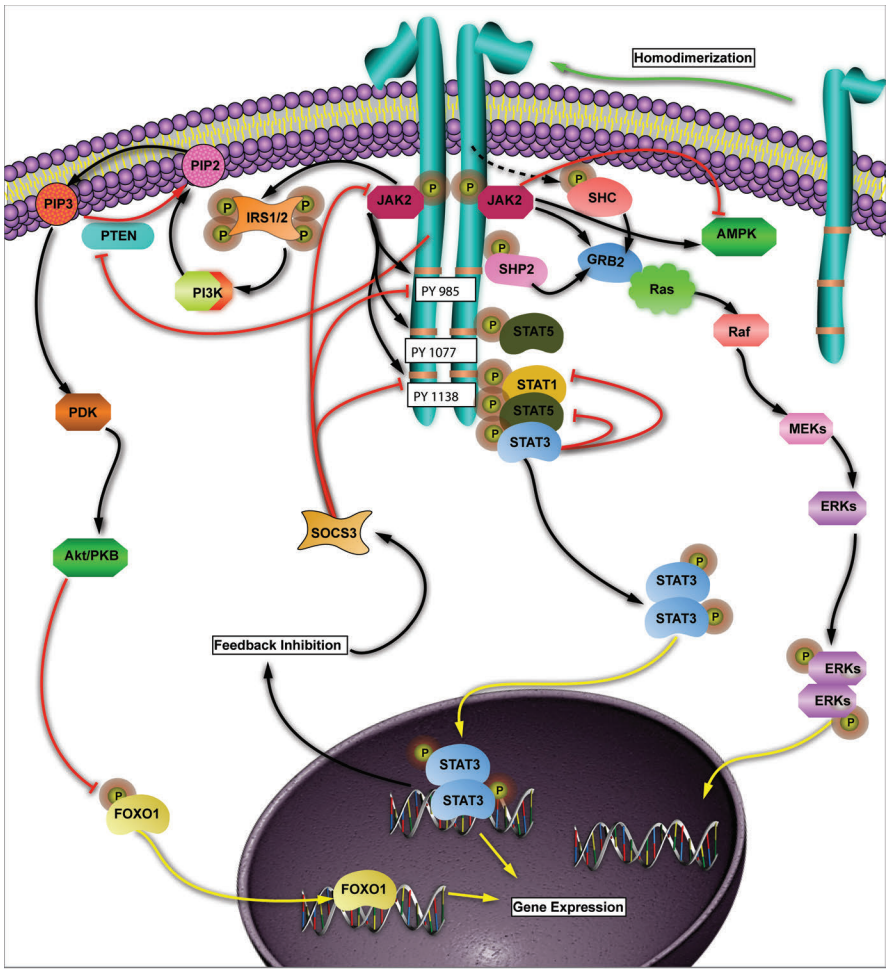

Figure 3.1 Schematic representation of leptin receptor signal transduction pathways. Upon leptin binding, leptin receptor initiates multiple intracellular signaling routes that in turn lead to the activation of ERK pathway, PI3/AKT pathway, AMPkinase pathway and STAT transcription factors.

described that the leptin receptor is able to recruit and activate STAT5 in two different phosphotyrosine residues, namely Tyr1077 and Tyr1138. As already mentioned above, it is noteworthy that the 1138 tyrosine residue is also considered part of the STAT3 recruiting mechanism.[26,38] Although the functional relevance of leptin-induced STAT5 activation still has to be determined, several studies point to a role for STAT5 in leptin action at the hypothalamic and pancreatic levels.[38–40] It is important to note that, as observed for STAT1 signaling, STAT5 signaling is also under the control of STAT3 activation. Indeed, Gong *et al*. described the existence of a Tyr1138/STAT3-mediated

feedback inhibition that attenuates STAT5-dependent transcription during chronic receptor activation.[38]

3.3.2 ERK1/2

Another important pathway elicited by leptin receptor activation is the ERK signaling pathway. Leptin has the ability to activate the extracellular signal regulated kinase (ERK) pathway[18,27,41] in multiple cellular systems. According to that, activation of the ERK pathway also has been reported for other members of the cytokine class I family of receptors.[42] The ability of the leptin receptor to activate the ERK pathway is mediated by the phosphotyrosine residue Tyr985 generated by JAK2 activity. This residue behaves as a binding site for an SH2 domain containing protein, namely SHP-2.[43] This interaction, in turn, induces firstly the activation of this protein and next the recruitment of growth factor receptor-bound protein 2 (GRB2).[27] Once this adaptor protein is recruited, the signaling pathway elicited by leptin receptor activation leads to the activation of ERK.

Bjorbaek *et al*. have demonstrated that two different pathways are critical for leptin-receptor-mediated ERK activation. In these studies, both dependent and independent Tyr985-mediated mechanisms for ERK activation have been demonstrated. Moreover, it has also been described that the SHP-2 protein is essential for both pathways.[44] According to this, it has been suggested that leptin also has the ability to induce ERK activation in a Tyr985-independent manner through a direct interaction between GRB2 and JAK2.[27]

As mentioned above, several activities of the leptin receptor are common among members of the cytokine class I family of receptors. In this sense, it has been reported that SHC, GRB2 and SHP-2 proteins directly bound to the murine granulocyte colony-stimulating factor receptor suggesting multiple routes for ERK activation.[45] According to this, Gualillo *et al*. have reported that after leptin receptor activation, SHC proteins were phosphorylated. This process induces the recruitment of GRB2 and the activation of the ERK pathway.[41]

After leptin receptor stimulation, the functional role of ERK activation has been related with the regulation of cell survival and to the control of linear growth in several different cell types.[28] According to these roles, very recently it has been reported that leptin protects neurons against glucose-oxygen-serum-deprivation (GOSD) injury through ERK activation.[46] Moreover, leptin-receptor-mediated activation of ERK also inhibited the apoptosis of trophoblastic cells.[47] In addition to this protective role, ERK activation has been involved in leptin-induced proliferation of hepatic, endometrial and renal cells.[48–50]

However, despite the above-described functions for leptin-mediated ERK activation, other functions have been described. Namely, hypothalamic ERK activation by leptin mediates the anorectic and thermogenic effects of leptin.[51] Another function mediated by leptin-induced ERK activation (through the phosphotyrosine residue Tyr985) is the phosphorylation of the ribosomal protein S6 kinase (RSK), which in turn leads to modulation of cap-dependent translation.[38]

3.3.3 PI3K/AKT

Another important signaling pathway elicited by leptin-receptor activation is the PI3K pathway. Supporting this idea is the fact that pharmacological blockade of PI3K activity blunted leptin anorectic effect.[52] This pathway is initiated by the JAK2 tyrosine phosphorylation of insulin receptor substrate 1/2 independently of leptin receptor phosphotyrosine residues.[52] The next step, downstream of IRS phosphorylation, is the recruitment and activation of PI3K, which in turn involves the accumulation of phosphatidylinositol 3,4,5-triphosphate (PI3P).[18,53] PIP3 activation leads to the subsequent activation of 3-phosphoinositide-dependent protein kinase1 (PDK1) and AKT. The last step in this signaling pathway is the repression of the transcription factor FOXO1, which is a member of the forkhead transcription-factor family and has been related with several activities of leptin signaling.[54]

In line with these facts, it has also been described that leptin signaling involves lipid phosphatase PTEN. This phosphatase plays a key role in PI3K activity since it has been reported that PTEN is able to inhibit PI3K signaling.[55] In this sense, Ning *et al.* reported that leptin induced the phosphorylation of PTEN, which blunted the inhibitory effect of this phosphatase.[55] In line with leptin-mediated PTEN inhibition, very recently it has been described that this phosphorylation mediated activation of ATP-sensitive K+ channels.[56] In agreement with these results, neurons of PTEN knockout mice showed increased ATP-sensitive K+ channel activity.[57]

Several other functions have been associated with PI3K activation upon leptin receptor stimulation, including control of energy homeostasis.[58] Regarding this function, very recently it has been reported that PI3K signaling, in the ventromedial hypothalamic nuclei, is required for the anorexigenic effects induced by exogenous leptin administration.[59] Moreover, this pathway has also been involved in the control of glucose metabolism. Actually, in POMC neurons, a decrease in PI3K signaling resulted in impaired glucose regulation.[60] In addition to these functions, leptin also controls cell fate through the PI3K signaling pathway. In this sense, it has been determined that leptin promoted cell proliferation and inhibited apoptosis in large B cell lymphoma and in papillary thyroid cancer cells.[61,62]

3.3.4 AMPK

In addition to all the above-mentioned signaling pathways elicited by the leptin receptor, it is noteworthy that leptin receptor activation has the ability to modulate the adenosine 5′ monophosphate-activated protein kinase (AMPK) signaling pathway. This kinase is a regulator of cellular and systemic energy homeostasis, although it has also been involved in the control of cell proliferation.[63,64]

The role of leptin on AMPK activation has a tissue specific pattern. Leptin induces AMPK activity in liver and muscle,[65,66] whereas it inhibits AMPK activation at the hypothalamic level.[67,68] Kola *et al.* have suggested that

different activities of leptin might be the result of differing patterns of AMPK subunit expression in the various tissues, or of differential expression of the AMPK activators LKB1, CaMKK or other possibilities.[63]

The mechanism by which leptin exerts its activities on AMPK activation are still unclear. However, several recent reports are uncovering these mechanisms. Uotani *et al*. have described that leptin activates AMPK in hepatic cells in a manner independent of phosphotyrosine residues of the long-form leptin receptor, whereas this mechanism involved a JAK2-dependent pathway.[69] It has been reported that the absence of the protein tyrosine phosphatase 1B (PTP1B) at the hypothalamic level, which regulates JAK2 phosphorylation,[70] increased leptin sensitivity by decreasing hypothalamic AMPK activity[71] and increasing peripheral AMPK activity. Very recently it has also been reported that the activity of skeletal muscle AMPK parallels hypothalamic leptin sensitivity and metabolic phenotype in transgenic mice over-expressing leptin. Moreover it was further indicated that the activation of skeletal muscle AMPK was mediated by the hypothalamic melanocortin pathway.[72] These results in turn revealed a complex mechanism for the regulation of peripheral leptin-mediated AMPK activities, establishing a link between central and peripheral actions of leptin AMPK-mediated effects.

3.3.5 SOCS3

Finally, leptin receptor activation also recruits the suppressor of cytokine signaling 3 (SOCS3), a signaling protein involved in a negative feedback of leptin signal transduction.[73] The SOCS3-mediated leptin signaling inhibition mechanism has been postulated by several studies. It was found that SOCS3 inhibits the leptin-induced tyrosine phosphorylation of JAK2 and co-precipitates with this protein in leptin-treated cell lysates.[74] Indeed, it was also reported that SOCS3 mediated the inhibition of leptin signaling at the phosphotyrosine residue Tyr985, where SOCS3 was bound and consequently dampened STAT3 signaling.[75] Also of note, SOCS3 expression is rapidly induced upon STAT3 activation, which is likely the main mechanism of leptin receptor self-regulation. Inhibition of phosphotyrosine residue Tyr1138, which is a binding site of the leptin receptor that elicits STAT3 recruitment, improved leptin receptor signaling inhibition caused by chronic stimulation.[76]

SOCS3 signaling has been related functionally to the establishment of leptin and insulin resistance.[58,73] It has been described that reducing SOCS3 activity, by neuron-specific knockout or by global knockout, resulted in enhanced STAT3 expression and leptin-mediated weight-reducing effects.[77,78] Likewise, It has been reported that SOCS3 inhibited insulin signaling in the adipose tissue of obese mice (Figure 3.1).[79]

3.4 Leptin Receptor Interactions

Apart from the above-mentioned signaling pathways elicited by the leptin receptor, it is noteworthy that the leptin receptor also interacts with other

proteins. These molecules may modulate leptin receptor signaling and introduce another piece of complexity in the generated signals.

3.4.1 Apolipoprotein D

Apolipoprotein D (Apo D) is widely expressed in mammalian tissues with a remarkable expression at the central level (brain). This apolipoprotein, from a structural point of view, does not have a relevant similitude with other apolipoproteins. By contrast, Apo D has been considered a lipocalin, due to its structural homology with lipocalins, a family of lipid-binding proteins involved in the transport of lipids and small hydrophobic molecules. The functional relevance of this lipocalin is supported by the fact that genetic variants of Apo D are associated with abnormal lipid metabolism and increased risk of developing the metabolic syndrome.[80] In line with this, it has been reported that Apo D and the leptin receptor are co-expressed in neurons of the hypothalamic arcuate and paraventricular nuclei, two brain areas that are known to be involved in food intake and body-weight regulation. Moreover, it has also been described, in the same study, that Apo D interacted specifically with the cytoplasmic portion of the long-form leptin receptor, but not with the short form Ob-Ra.[81] All together, these data suggested that Apo D may be involved in leptin receptor signaling and consequently in its functions such as body-weight regulation and energy homeostasis.

3.4.2 Sorting Nexin Molecules

The sorting nexins (SNXs) are a family of PX domain-containing proteins widely expressed in mammalian tissues. Despite their hydrophilic nature, the sorting nexins are found partially associated with cellular membranes. SNXs have been suggested to be involved in pro-degradative sorting, internalization, endosomal recycling, or simply in endosomal sorting.[82] Among the large family of sorting nexins, several sorting nexins, such as SNX1, SNX2, SNX4 and SNX6, have been co-immunoprecipitated with receptors with tyrosine kinase activity, like endothelial growth factor receptor EGFR, platelet-derived growth factor receptor and insulin receptor.[83,84] Intriguingly, these sorting nexins were also associated with the leptin receptor long form, but not with the short or medium isoforms.[83,84] As a whole, these data suggest that sorting nexins might control leptin receptor trafficking by means of its intracellular region and, consequently, this interaction could also control leptin receptor signaling.

3.4.3 Diacylglycerol Kinase Zeta

Diacylglycerol kinases (DGKs) are involved in the regulation of intracellular levels of diacylglycerol and phosphatidic acid, as well as in the synthesis of triacylglycerols.[85] Among these kinases there is diacylglycerol kinase zeta (DGKζ), which is characterized by its four C-terminal ankyrin repeats and is

found in both the cytosol and nucleus under the regulation of specific types of protein kinase C. DGKs are widely expressed in mammalian tissues including the brain, where DGKζ is highly expressed.[86] Accordingly, Liu *et al*. reported that DGKζ expression co-localizes with leptin receptor expression in hypothalamic neurons involved in energy homeostasis. Indeed, it has also been reported that DGKζ, through its ankyrin repeats, interacts with the long leptin receptor form but not with the short-form Ob-Ra. Moreover, this study also suggested that leptin receptor signaling modulates DGKζ expression since db/db mice have increased hypothalamic DGKζ mRNA compared to their wild-type littermates. All these data have prompted a suggestion that DGKζ modulation is a downstream consequence of leptin receptor signaling.[86]

3.4.4 Apolipoprotein J

Several plasma components can modulate leptin bioavailability. These compounds could modulate the transport, clearance and function of leptin. Among these compounds has been found apolipoprotein J (Apo J), also named clusterin or complement lysis factor. Apo J is involved in multiple processes such as lipoprotein transport, inhibition of complement-mediated lysis, regulation of sperm maturation or regulation of cell migration.[87] However, it has also been described that Apo J forms a binary complex with leptin, without affecting its ability to bind to the leptin receptor and elicit STAT3 signaling.[88] In addition, the fact that the Apo J-leptin complex has the ability to interact with members of the LDL receptor gene family suggests that Apo J might function as an hormone controller, by regulating the balance of bioavailable leptin.[88]

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CHAPTER 4

Cell Signaling Mechanisms Underlying the Cardiac Actions of Adipokines

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4.1 Introduction

The identification of adipokines as potent bioactive compounds has made a major impact on the area of endocrinology and physiology as it is now generally recognized that adipocytes represent endocrine organs secreting potent biologically active molecules producing a wide array of responses on different target tissues.[1] The present review centers primarily on adipokines that have been shown to modify cardiac function and that appear to play potentially important roles in pathology. Attention is particularly given to leptin and adiponectin since these compounds have been extensively studied, at least relative to other adipokines, in terms of their cardiac effects. Some of the latter such as resistin, apelin and visfatin are also discussed in this review, although their cardiac effects have been studied to a substantially lesser degree than either leptin or adiponectin.

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4.2 Leptin: A Brief Introduction

As noted above, among the adipokines primary attention has been directed towards leptin, a 16-kDa protein secreted primarily by adipocytes but also produced by many tissues including the heart.[2–4] The production of cardiomyocyte-derived leptin is increased by both endothelin-1 and angiotensin II suggesting a paracrine or autocrine role of leptin in the regulation of cardiac functions, particularly under pathological conditions.[3] Indeed, leptin mediates the prohypertrophic effect of both endothelin-1 and angiotensin II in cultured neonatal rat ventricular myocytes.[3] The primary cardiac response to leptin in terms of physiological function appears to be a negative inotropic response, which has been shown primarily in cardiomyocytes and which is mediated by endogenously produced nitric oxide.[4]

Circulating total leptin levels are generally positively related with body mass index and the degree of adiposity with plasma levels ranging from 5 to 15 ng/ml in non-obese individuals and greater than 100 ng/ml in very obese subjects.[5] Interestingly, circulating leptin exists primarily in the free form in obesity whereas in lean individuals leptin circulates primarily bound to plasma proteins.[5] This difference may be of biological importance since it suggests that, in obesity, substantially greater amounts of leptin are available to exert biological effects. The effects of leptin occur through leptin binding to its receptors, termed OBR, LEPR or LR, although the OBR designation will be used in this review for consistency. OBRs are expressed as splice variants classified as short (OBRa, c, d and f), secreted (OBRe) and long (OBRb) forms with OBRb generally considered as the primary functional isoform linked to full cell signaling processes.[6] These receptors are expressed abundantly in many different cells including cardiomyocytes and intact myocardium.[2,3] The intracellular domain of OBRb belongs to the Janus kinase signal transduction and translation system (Jak2/STAT3). As will be discussed below, it has been reported that leptin leads to the activation of various kinases in cardiomyocytes including RhoA/ROCK, ERK1/2, p38 MAPK, phosphoinositide 3-kinase (PI 3-kinase), Akt and protein kinase C.

4.3 Expression of Leptin Receptors in Cardiovascular Tissues

The first demonstration of the presence of OBR gene expression in cardiac tissue was reported in 1996 upon the discovery of the gene encoding the *db*/*db* mutation.[7] Further characterization of OBR isoforms indicated that cardiac tissue expressed OBRa, OBRb and OBRe.[8,9] Recent work from the authors' laboratory suggest that OBR gene expression in the heart differs in terms of regional distribution and is also affected by gender.[2] Semi-quantitative real-time polymerase chain reaction revealed that in both males and females all three isoforms investigated were expressed in both atria, left and right ventricular walls as the interventricular septum, although the greatest gene abundance was found in the atria. In terms of

gender differences, OBR expression was generally higher in tissues from female rats, especially in the right atria.[2]

The functions of each of the OBR isoforms in the heart, the relevance of regional distribution expression patterns or the influence of gender are currently unclear, although some potential functions for leptin signaling in the heart will be discussed later in this review. The identification of OBRe in cardiac tissue was of particular interest since this soluble receptor represents the primary binding protein for leptin in plasma and may thus dictate leptin availability to tissues. It is possible that the presence of OBRe in cardiac tissues is a consequence of proteolytic cleavage of the extracellular domains of one of the other isoforms.[10,11] Although the function of OBRe in the heart is currently unknown, it is interesting to speculate that its local tissue production serves to "fine tune" leptin concentrations in that specific tissue, which would be in keeping with its role as a clearance receptor, although evidence for this hypothesis needs to be obtained with further studies.

In addition to cardiac tissue, leptin receptors have also been identified in both cerebral and coronary vessels.[12,13] With respect to the latter it was proposed that OBR-mediated leptin-induced vasodilatation occurs through a nitric-oxide-dependent process and which was abolished by hyperleptinemia. This finding emphasizes the potential dual role of leptin on vascular tissue, a direct NO-dependent vasodilatation and vasoconstriction occurring secondarily to central stimulation of the sympathetic nervous system.

4.4 Effect of Leptin on Cardiomyocyte Function

Under *in vivo* conditions, the cardiovascular actions of leptin can be predicted based on the central sympathetic stimulatory effect of the polypeptide resulting in sympathetic nervous system-dependent effects such as elevations in blood pressure and positive inotropic and chronotropic effects. However, leptin can exert direct effects on both the heart and blood vessels through OBR-dependent cell signaling mechanisms. In isolated ventricular myocytes leptin produces a negative inotropic effect *via* a NO-dependent pathway as the effect was abrogated by NO synthase inhibition with L-NAME and associated with increased NO synthase activity.[4] The negative inotropism is also associated with both JAK-STAT and MAP kinase p38 activation.[8,14] Leptin has also been shown to stimulate fatty acid oxidation in working perfused rat hearts in the absence of any effect on glucose oxidation while lowering cardiac triglyceride content.[15]

4.5 Cardiomyocyte Hypertrophic Effects of Leptin

Evidence for leptin as a hypertrophic and pro-growth factor stems primarily from studies examining the direct effect of the polypeptide on myocyte preparations. For example, our laboratory reported that leptin produces marked hypertrophy in cultured neonatal rat ventricular myocytes as manifested by increased cells size, elevated protein synthesis and upregulation of a number of

genetic hypertrophic markers.[16] As summarized in Figure 4.1, the hypertrophic effect of leptin is likely associated with numerous cell signaling processes, some of which act in concert to modify transcriptional regulation resulting in the hypertrophic phenotype. For example, initial studies revealed that leptin-induced hypertrophy was associated with MAPK activation including both the p44/42 and p38 pathways whereas the hypertrophy was prevented only by p38 inhibition.[16] Xu and coworkers demonstrated that leptin-induced endothelin-1 release from neonatal rat ventricular myocytes results in activation of the endothelin-1 ETA receptor, which then stimulates production of reactive oxygen species, the latter inducing cardiomyocyte hypertrophy.[17] This study suggests that leptin does not induce hypertrophy directly *per se* but rather as a consequence of upregulation of other pro-hypertrophic factors. Accordingly, both ETA receptor blockade and catalase were effective in abrogating the hypertrophic response.[17] In view of the fact that endothelin-1 and other hypertrophic factors such as angiotensin II are upregulated in obesity,[18] this study describes an important potential synergistic relationship between various neurohumoral factors in the overall hypertrophic process. This relationship between leptin is further highlighted by evidence from our laboratory that leptin mediates the hypertrophic effects of endothelin-1 and angiotensin II in cultured myocytes.[3] In that study, the

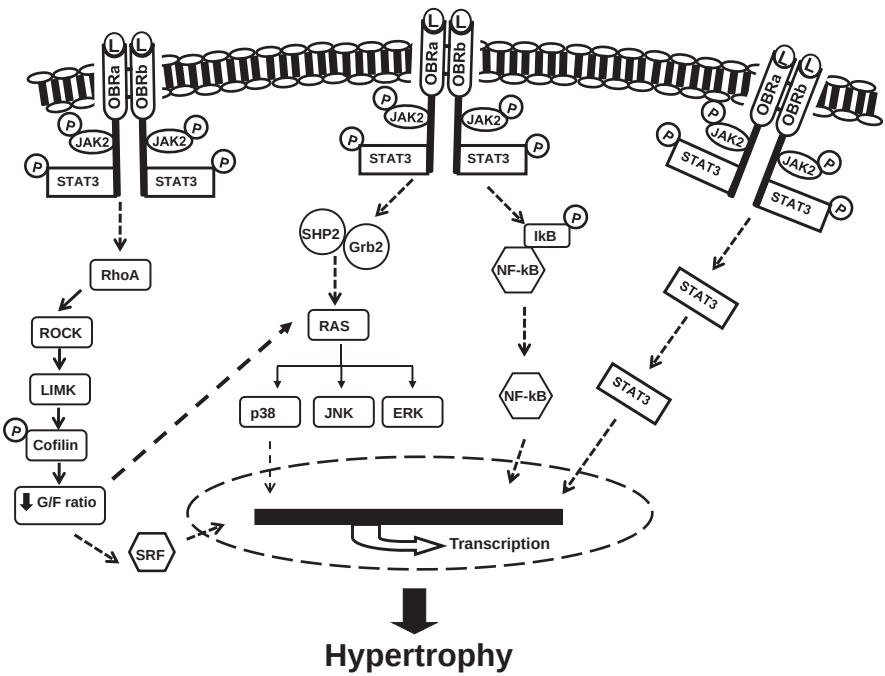

Figure 4.1 Summary of the multiplicity of cell signaling mechanisms underlying the hypertrophic effects of leptin in the cardiomyocyte. See text for detailed discussion.

hypertrophic effects of either endothelin-1 or angiotensin II were associated with increased OBR expression and release of leptin into the culture medium. Moreover, anti-OBR antibodies completely abrogated the hypertrophic responses to both endothelin-1 and angiotensin II.[3] These results need to be confirmed in other models but if validated they suggest that leptin plays a critical paracrine or autocrine obligatory role in mediating the hypertrophic to both endothelin-1 and angiotensin II and possibly other pro-hypertrophic factors.

Leptin has been shown to increase hyperplasia of the murine atrial HL-1 cell line as well as pediatric cardiomyocytes.[19] Activation of ERK and phosphatidylinositol 3-kinase was demonstrated and implicated in the increase in cell number. It should be noted that leptin-induced hypertrophy has also been shown in human pediatric ventricular myocytes, which was associated with increased ERK, p38 and JAK phosphorylation.[20]

As will be discussed later in this chapter, activation of the RhoA/ROCK pathway likely plays a unique and critical role in mediating the cardiomyocyte hypertrophic effect of leptin.

4.6 Post Receptor Leptin Signaling

In general, the complexity and diversity of leptin's effects are exemplified by its ability to activate several signal transduction pathways. It is beyond the scope of this review to comprehensively discuss leptin-mediated signaling in all tissues or organ systems. Instead, the discussion below focuses on signaling pathways that have been elucidated in cardiovascular tissue or that appear to be particularly relevant for understanding leptin-mediated cardiac signaling and its possible relationship to pathology, particularly in view of harnessing these pathways for cardiac therapeutics. For a more general treatise of this subject interested readers can consult various recent publications.[21–24] In terms of cardiac pathology it is important to consider that the status of the leptin cell signaling system is likely substantially altered in disease states. For example, the leptin system in the myocardium including leptin expression and expression of OBR is upregulated in heart failure.[25] Accordingly, the cell-signaling processes described below are most likely in a state of enhanced activity under pathological conditions.

4.6.1 JAK-STAT Pathway Activation

It is generally accepted that OBRb is the fully competent signal transduction isoform of the receptor, and that the short-form OBRs (a,c,d,f), while capable of signal transduction, do so to a lesser extent.[26,27] The major signaling pathway activated by leptin binding to OBRb is the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway,[26] although this pathway may also be stimulated secondary to activation of the short form of the OBR.[28] Upon binding of leptin to its receptor JAK1 and JAK2 are both capable of associating with the cytoplasmic domain of OBRb; however,

recently it has been demonstrated that JAK2 activation likely represents the physiologically relevant activated JAK during OBR signaling.[29] Activation of JAKs results in transphosphorylation of other JAK as well as phosphorylation of tyrosine residues of OBRb.[30] Recently, protein tyrosine phosphatase 1B (PTP1B) has been shown to be a negative regulator of JAK-STAT signaling.[31,32] PTP1B dephosphorylates the consensus recognition motif on JAK2, resulting in inactivation of downstream STAT proteins.[33] PTP1B knockout mice exhibit increased leptin sensitivity, STAT3 activation and decreased leptin to body-weight ratios.[33] Phosphorylation of the cytoplasmic domain of the receptor results in a docking site for STAT protein binding. STAT1, STAT3, STAT5 and STAT 6 have all been associated with leptin signaling *in vitro*.[34–36] Upon binding the receptor complex, STAT is phosphorylated by JAK where it dissociates from the receptor, forms a homo- or heterodimer and then translocates to the nucleus to act as a transcription factor.[34,35,37] STAT3 can be inhibited by PIAS3, an endogenous protein inhibitor of this transcriptional factor.[38]

Evidence for JAK-STAT-dependent signaling in cardiac tissue in terms of physiological effects is at present limited but recent evidence suggests that it may be involved in the negative inotropic effect of leptin in cardiomyocytes based on the ability of the JAK2 inhibitor AG-490 to abrogate these effects.[8] Interestingly, however, the effect of AG-490 was mimicked by the MAPkinase inhibitor SB203580 suggesting that leptin exerts its effects *via* multiple, and likely independent, cell signaling pathways.[8]

4.6.2 Mitogen Activated Protein Kinase Stimulation

Mitogen-activated protein kinase (MAPK) represents an additional target for leptin-mediated effects. In fact, OBRa has signal transduction capabilities through MAPK pathways both dependently and independently of JAK phosphorylation.[12,26,30] JAK2 phosphorylation of OBR tyrosine residue-985 (Tyr985) results in docking of an SH2-domain containing protein tyrosine phosphatase (SHP-2), which associates with an adaptor molecule, Grb-2, to activate extracellular regulated kinase (ERK) signaling.[30] Although ERK activation is possible in the absence of Tyr985, it still requires SHP-2 phosphatase activity.[30] SHP-2 activation by leptin-OBR interaction leads to ERK activation, possibly through MEK1, but this has not yet been confirmed.[22] Activation of ERK results in alterations in gene expression patterns for several genes including c-fos.[12] Another MAPK, p38, has not been studied as extensively as ERK, but has been shown to be activated by leptin in mononuclear cells.[39] In contrast, leptin was shown to reduce insulin-induced p38 activation, while having no effect on p38 activation on its own.[23] The role of leptin signaling through c-jun NH2-terminal protein kinase (JNK) has not been well characterized. However, there are two reports of leptin activating JNK in endothelial cells,[40] and in prostate cancer cells.[41]

In the cardiovascular system, leptin has been demonstrated to activate components of the MAPK pathways. In cultured neonatal myocytes, ERK1/2 and p38, but not JNK, were activated by leptin; inhibiting ERK had no effect, while inhibition of p38 completely inhibited leptin-induced cardiomyocyte

hypertrophy.[16] Leptin has also been shown to induce hyperplasia in the immortalized atrial HL-1 cell line *via* an ERK-dependent pathway.[19] The results from studies using the HL-1 cell line are difficult to compare to primary culture of ventricular myocytes since the two models would likely respond to stimuli differently in view of the fact that the primary response of HL-1 cells is hyperplasia, not hypertrophy. Recent evidence suggests that p38 activation (as well as activation of AMPK) mediates the anti-apoptotic effect of leptin in cultured myocytes,[42] although it should be added that STAT-3 activity has also been implicated in this phenomenon.[43] Moreover, leptin-induced cardiac fatty acid oxidation has recently been demonstrated to occur by a multiplicity of cell signaling transducers including STAT-3, NO and p38 MAPK activation.[44]

4.6.3 Pivotal Role for the RhoA/ROCK System in Mediating the Hypertrophic Effects of Leptin

Over the past number of years it has become apparent that the Rho/ROCK pathway, a downstream target protein of small GTP-binding protein Rho important for regulation of cell morphology, is likely also an important contributor to hypertrophy, although the mechanism leading to activation of Rho GTPases and subsequently to cardiac hypertrophy has not been well characterized.[45,46] RhoA activates several protein kinases, including Rho kinases (ROCK). This leads to the activation of LIM kinase-2 (LIMK2) resulting in phosphorylation (inactivation) of the actin binding protein cofilin, an important factor in the regulation of actin dynamics, which in turn leads to depletion of globular actin (G-actin) pool and enhanced actin polymerization (F-actin). Work from our laboratory has recently shown that leptin is a potent activator of the RhoA/ROCK pathway leading to a decrease in the G/F actin ratio.[47] The precise mechanism of how activation of this pathway leads to cardiac hypertrophy is not known with certainty. Interestingly, however, activation of RhoA/ROCK by leptin results in the selective translocation of p38, but not other MAPK isoforms, to the nucleus,[48] a finding in agreement with our initial observation that leptin-induced hypertrophy can be blocked by p38, but not by ERK inhibition.[16] Intact caveolae are also critical for both the activation of the RhoA pathway and the resultant p38 translocation and hypertrophy.[48] The role of caveolae in mediating the hypertrophic effects of leptin was supported by various lines of evidence.[48] Firstly, leptin significantly increased the number of caveolae as well as caveolin-3 protein expression in myocytes. Secondly, OBR were found to be colocalized with caveolae. Lastly, disruption of caveolae with the cholesterol-depleting agent methyl-beta-cyclodextrin was found to prevent leptin-induced hypertrophy, which was reversed by exogenous cholesterol repletion.

4.7 Adiponectin

Adiponectin is a 30-kDa protein secreted by adipose tissue that plays a critical role in differentiation of adipocytes. The peptide belongs to the complement 1 family and can exist as a monomer or high-molecular-weight multimers.[49]

Adiponectin can function as a full-length protein of 245 amino acids or a smaller globular fragment of 137 amino acids. The plasma adiponectin concentration in humans may range from 3 to 30 μg per ml and accounts for 0.01% of total plasma protein.[50] Adiponectin expression and subsequent release from adipocytes is stimulated by activation of peroxisome proliferators-activated receptor (PPAR)-γ, a key transcriptional factor involved in adipocyte differentiation.[51]

Two adiponectin receptors, termed as AdipoR1 (adiponectin receptor 1) having 375 amino acids and AdipoR2 (adiponectin receptor 2) with 311 amino acids, have been identified.[52] Structural analysis revealed that these receptors are integral membrane proteins containing conserved seven-transmembrane domains with internal N-terminus and external C-terminus.[52] Scatchard plot analysis demonstrated that AdipoR1 binds to globular adiponectin whereas AdipoR2 binds to full-length adiponectin.[52] AdipoR1 was shown to be expressed ubiquitously, while AdipoR2 expression is more restricted. In the heart, AdipoR1 is expressed in substantially greater abundance compared to AdipoR2.[52]

4.7.1 Adiponectin and Cardiovascular Disease

Adiponectin is the most abundant adipokine secreted by adipose tissue and has been suggested to be involved in various cardiovascular diseases.[53] Adiponectin levels are significantly reduced in obese subjects[54] and patients with type 2 diabetes.[55] The direct role of adiponectin in pathogenesis of cardiac disease still needs to be elucidated; however, it has been observed that increased plasma adiponectin levels are associated with a lower risk of myocardial infarction and coronary artery disease in men.[56,57] Adiponectin levels were shown to be reduced significantly in patients with coronary artery disease[58,59] as well as in patients with heart failure.[60] In addition an inverse correlation was reported between adiponectin levels and other cardiovascular risk factors such as hyperlipidemia,[50] hypertension[61] and C-reactive protein levels.[62] Adiponectin levels may also be a predictor of mortality in patients with chronic heart failure[63] and coronary artery disease.[64] A recent study showed a particularly strong relationship between elevated plasma adiponectin levels and mortality in patients with heart failure but an association was also present in patients without cardiovascular disease.[65] However, an association between plasma adiponectin levels and cardiovascular morbidity or mortality is not uniform as recent studies were unable to demonstrate any relationship between plasma adiponectin levels and the severity of coronary artery disease.[66–69] Such discrepant findings clearly support further research into the clinical relevance of adiponectin in cardiovascular disease. However, from a general perspective adiponectin exerts effects opposite to those manifested in response to leptin and as such exerts primarily beneficial effects in mitigating cardiac pathology.

4.7.2 Adiponectin and Experimental Cardiac Hypertrophy

Adiponectin knockout (ADN-KO) mice subjected to pressure overload by transverse aortic constriction (TAC) demonstrate elevated concentric

hypertrophy evidenced by increased left ventricular wall thickness as well as increased mortality after 7 days compared to wild-type animals. The adenoviral transfection of adiponectin (Ad-ADN) to ADN-KO mice 3 days prior to subjecting them to TAC attenuated the development of cardiac hypertrophy. In obese *db*/*db* mice, which lack the functional leptin receptor Ad-ADN, treatment abolished the TAC-induced increase in interventricular septum and left ventricular posterior wall thickness. In the presence of Ad-ADN, angiotensin II-induced cardiac hypertrophy was attenuated in both ADN-KO and wild-type mice.[69] These findings suggest that adiponectin over-expression can reverse the cardiac dysfunction induced by various pathological factors. For example, α-adrenergic receptor stimulation by norepinephrine increased cell surface area and protein synthesis in cardiomyocytes, which was attenuated in the presence of adiponectin.[69] Thus, adiponectin appears to be an endogenous anti-remodeling agent that may be beneficial in limiting heart failure.[70]

4.7.3 Cell Signaling Mechanisms Underlying Cardioprotective and Antihypertrophic Effects of Adiponectin

Adiponectin induces effects most likely *via* a multiplicity of cell-signaling mechanisms subsequent to adiponectin receptor (AdipoR1/AdipoR2) activation. For example, ERK1/2 MAPK activation was increased in ADN-KO mice subjected to TAC compared to wild-type. ERK1/2 activation induced by α-adrenergic agonist in cardiomyocytes was attenuated in presence of adiponectin or MEK inhibitor U0126.[69] Taken together, these studies suggest that the protective effect of adiponectin is partly mediated through inhibition of ERK1/2 MAPK.

AMPK modulation may also mediate some of the actions of adiponectin. In ADN-KO hearts AMPK phosphorylation at Thr 172 on α-subunit was suppressed compared to wild-type hearts.[69] Moreover, activation of AMPK has been proposed as a mechanism for the beneficial effects of adiponectin.[69] ADN-KO mice exhibit enhanced and accelerated myocardial remodeling following pressure overload, which is associated with reduced AMPK levels.[71,72] Hearts from ADN-KO mice also developed larger infarct area compared to wild-type after subjecting them to ischemia/reperfusion (IR). In the presence of exogenous adiponectin, both ADN-KO and wild-type hearts had reduced infarct size after IR, an effect associated with AMPK activation and suppression of TNFα production in myocardium.[73] A role for AMPK has also been demonstrated in a study implicating adiponectin as the underlying factor in mediating cardioprotection in mice subjected to a calorie-restricted diet.[74]

Adiponectin has also been shown to attenuate the increased gp91 protein in cardiac tissue subjected to IR and thus reduced oxidative stress-induced tissue injury.[75] Studies on the regulation of NO production from eNOS and iNOS by adiponectin demonstrated that in the hearts of ADN-KO mice subjected to IR eNOS phosphorylation is decreased and iNOS activity is increased compared to wild-type.[75] This may suggest that under physiological conditions adiponectin

increases the NO production from eNOS, which might contribute to its protective effects, whereas during cardiac pathology adiponectin inhibits iNOS activation and thus reduces the NO overproduction, which can contribute to increased cardiac injury.

Another potential mechanism underlying the cardioprotective effect of adiponectin may involve a stimulation of prostaglandin (PG) synthesis *via* the inducible cyclooxygenase-2 (COX-2) dependent pathway. In this regard, it was shown that adiponectin stimulates PGE2 synthesis and increases COX-2 expression in neonatal rat ventricular myocytes whereas a COX-2 inhibitor abrogated the infarct size sparing effect of adiponectin in mice subjected to 30 minutes coronary artery occlusion followed by 48 hours of reperfusion.[73] While the mechanism underlying the ability of adiponectin to upregulate COX-2 is not fully understood recent evidence suggests that this occurs subsequent to sphingosine kinase-1/sphingosine-1-phosphate receptor-dependent pathway.[76]

A recent study has also implicated PPARγ activation as mediating the anti-hypertrophic effects of adiponectin in a model of cardiac hypertrophy and remodeling produced by a high-fat diet,[77] although a PPARα-dependent effect has also been implicated at least with respect to angiotensin-2 induced cardiac fibrosis.[78] Lastly, it has recently been reported that the anti-hypertrophic effect of adiponectin in cardiomyocytes was associated with reduced heparin-binding epidermal growth factor (EGF) signaling, which included the downregulation of the EGF receptor.[79]

4.8 Resistin

A relatively new adipokine, resistin (for ''resistance to insulin''), was first identified in 2001 as a gene target of the insulin-sensitizing drugs thiazolidinediones (TDZs). Resistin is an adipose-derived secreted factor, produced almost exclusively in white adipose tissue. Resistin is a 12-kDa protein that circulates as either a trimer (monomeric form of the peptide hormone) or a hexamer (dimeric form of resistin). However, controversy remains as to which form, monomeric or dimeric, is responsible for the physiologic properties of the peptide.[1] The monomeric form was shown to impair hepatic insulin action more potently than the dimerized form.[80] In contrast, the dimerized form of resistin was shown to be more effective in antagonizing insulin-stimulated glucose uptake in adult murine cardiomyocytes.[81] Interestingly, the notion of differential regulation of hormone signaling based on oligerimization state is shared by another adipokine, adiponectin.[82,83] Resistin levels are elevated in patients with heart failure and may represent independent risk factors for heart failure development.[84,85] Indeed, a recent study reported that resistin is expressed in the cardiac cell[86] and its expression is increased by mechanical stretch, which occurs *via* a TNFα-dependent pathway involving MAPK and NF-κB activation.[87] Resistin is a prohypertrophic factor acting *via* MAPK activation as well as increased phosphorylation of the insulin receptor

substrate-1 (IRS-1).[86] Thus, the contribution of resistin to the etiology of heart disease in general and heart failure in particular and the underlying mechanistic bases for these effects is important to clarify particularly as targeting this cytokine may represent a possible useful therapeutic strategy.

4.8.1 Cardiac Actions of Resistin

Emerging evidence suggests that cardiovascular disease is accompanied by changes in resistin levels. For example, in women, plasma resistin levels are elevated in patients with coronary heart disease.[88] What role resistin plays in the disease process is not known, although in patients with atherothrombotic strokes, plasma resistin levels are associated with elevated risk of 5-year mortality.[89] Serum resistin concentrations have also been shown to be elevated in patients with heart failure with levels positively related to the severity of heart failure according to New York Heart Association functional classification.[90] Although these studies do not indicate cause-and-effect relationships, nonetheless increasing plasma resistin concentrations appear to be a predictor of poor prognosis in patients with cardiovascular disease.

4.8.1.1 Experimental Studies on the Cardiac Actions of Resistin

Although resistin cell receptors have yet to be identified, direct action of resistin in the heart and specifically on cardiomyocytes has been described. Mouse adult cardiomyocytes treated with resistin show a reduction in insulin-stimulated glucose uptake.[81] Furthermore, in contrast to liver, cardiomyocyte resistin signaling requires oligomerization of the ligand prior to receptor binding.[81] The precise mechanisms by which resistin exerts its effects on glucose transport is not completely understood but this appears to occur by impeding vesicular transport.[81]

The potential role of resistin in cardiac pathobiology has not been extensively studied, although a few studies have been carried out to assess the effect of resistin on the ischemic and reperfused heart with contradictory results. In one report, resistin depressed functional recovery from ischemia in isolated perfused rat hearts, an effect which appeared to be dependent on NF-κB activity.[91] In contrast, resistin reduced infarct size in mice subjected to coronary artery occlusion and reperfusion.[92] These authors also demonstrated that resistin improved functional recovery of isolated mouse hearts and reduced infarct size and proposed that the salutary effect of resistin occurs *via* a PI3K/Akt/PKC pathway. The obvious discrepancy between the two studies is difficult to explain at present but may reflect different concentrations of resistin, differences in experimental model or species diversity.

4.9 Apelin

Apelin is an adipokine that was found to be the endogenous ligand for the G protein-coupled APJ receptor. Apelin has been shown to exert potent

positive inotropic effects on both normal and failing myocardium.[93–97] These effects likely occur through multi-faceted cell-signaling mechanisms, which involve multiple kinases including protein kinases C as well as Na-H exchange activity.[93,97] The exact nature of these intracellular messengers, especially how they contribute to the effects of apelin, is uncertain but activation of Na-H exchange activity is likely of importance in mediating the ability of apelin to increase myofilamental sensitivity to calcium, thus producing a positive inotropic response.[97]

4.9.1 Apelin and Heart Disease

Apelin's role in heart disease is not well established. In experimental heart failure models, myocardial apelin expression has been reported to be decreased (Dahl sensitive rat)[99] or increased (ischemic heart failure),[99] whereas plasma apelin levels are decreased in patients with heart failure.[100,101] The human heart has been shown to produce apelin, although cardiac-derived apelin production is decreased in patients with heart failure.[102] Interestingly, but adding to the complexity, myocardial unloading in patients with heart failure with the use of a left ventricular assist device results in the upregulation of left ventricular apelin expression.[103] Thus, it appears the apelin response in heart failure is multi-faceted and complex.

Overall, from experimental studies apelin appears to exert beneficial effects. For example, apelin has been shown to exert cardioprotective effects as demonstrated against both ischemia and reperfusion injury as well isoproterenol-induced cardiotoxicity.[104,105] Apelin knockout mice demonstrate enhanced cardiac dysfunction and enhanced myocardial remodeling in aging and in response to pressure overload, thus suggesting an important role for endogenous apelin in regulating cardiac function in response to insult.[106] Clinically, apelin has been shown to exert salutary effects in patients with chronic heart failure.[107] The mechanisms for the cardioprotective effects of apelin are not known, although pharmacological inhibitors of PI3K/Akt or P70S6 kinase were found to produce no effect on apelin-induced cardioprotection.[104]

Apelin has also been shown to modulate ion regulation in cardiac myocytes. For example, apelin increases sodium conductance in canine ventricular myocytes through a protein kinase C-dependent process; however, the relevance of this finding is not clear.[108] It is possible that apelin exerts a benefit in terms of anti-arrhythmic properties *via* this mechanism. In addition apelin has been reported to increase the intracellular calcium transient, also *via* a PKC-dependent pathway, which may contribute to the positive inotropic effect of the peptide.[109]

4.10 Visfatin

Visfatin is an adipokine that exerts insulin-like effects by binding to the insulin receptor, thus exerting a hypoglycemic effect.[110] Adipose tissue visfatin levels,

along with various other adipokines including leptin, were found to be elevated in patients with coronary artery disease, although adiponectin levels were reduced.[111] In that study, the effect was greater in abdominal adipose tissues, suggesting that changes in abdominal adiposity and the resultant influence on adipokine production exert a relatively greater influence on the etiology of coronary artery disease. It appears that visfatin is a cardioprotective agent[112,113] acting *via* PI3K and MEK1/2-dependent pathways and through a mechanism involving reducing opening of the mitochondrial permeability transition pore.

4.11 Other Novel Adipokines

A large number of other adipocyte-derived factors have also been identified recently, including vaspin, omentin and chemerin; however, these have not yet

Table 4.1 Various cell signaling mechanisms associated with cardiovascular effects of adipokines.

Adipokine	*Receptor*	*Signaling*	*Primary phenotypes*
Leptin	OBRb	MAPKs[16,19,20,48]	Hypertrophy
		RhoA/ROCK[47,48]	Negative inotropism
		ROS[17]	Antiapoptotic
		NO[4]	
		JAK2/STAT3[26–30]	
Adiponectin	AdipoR1	AMPK[8]	Antihypertrophic
	AdipoR2	ERK1/2 MAPK[69]	Cardioprotective
		NOS[75]	
		COX-2[69]	
Resistin	Toll-like receptors (TLR4)	P38MAPK[86]	Insulin resistance
		ERK1/2 MAPK[86]	Glucose uptake
		IRS-1[86]	Hypertrophic
		Ca^{2+} [86]	Positive inotropic
		PI3K[92]	Cardioprotection
		AKT[92]	
		PKC[92]	
Apelin	APJ	ROS[14]	Cardioprotection
		NHE-1[93]	Antihypertrophy
		NCX[93]	Positive inotropic
		PKC[92]	Vasodilator
			Antiapoptotic
Visfatin	Insulin receptor	PI3K[113]	Cardioprotection
		AKT[113]	
		MEK1/2[113]	
		ERK1/2[113]	
Vaspin	Unknown	Unknown	Unknown
Omentin	Unknown	Unknown	Unknown
Chimerin	Unknown	Unknown	Unknown

Abbreviations: MAPK: mitogen-activated protein kinase; NO: nitric oxide; AMPK: AMP-activated protein kinase; NOS: nitric oxide synthase; COX-2: cyclo oxygenase 2 isoform; IRS-1: insulin receptor substrate-1; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase; NCX: sodium-calcium exchanger; PKC: protein kinase C; MEK: mitogen-activated protein kinase B; ERK: extracellular signal regulated kinase.

been studied in terms of their ability to modulate cardiac function in either health or disease.

4.12 Summary, Conclusions and Future Directions

Since the initial identification of leptin numerous adipocyte-derived peptides have been discovered, which have been assigned the collective name of adipokines. As discussed in this chapter and as summarized in Table 4.1, the adipokines exert complex cardiac effects mediated by numerous effects on cardiac cell signaling processes. The importance of adipokines to cardiac function in either physiology or pathology is still emerging and studies addressing this question represent an exciting area of research not only with respect to obesity, since adipokine production is generally related to adiposity, but also with respect to the central issue of the potential role of adipose tissue as an endocrine organ-regulating cardiac function in health and disease. Of the adipokines currently identified, our primary understanding as related to cardiac actions and cellular mechanisms underlying these effects relate primarily to leptin and adiponectin, although the effects and the underlying cell signaling mechanisms of adipokines are steadily emerging. These adipokines appear to exert opposite effects and an emerging concept is that a balance between these adipokines influences the predisposition to heart disease. Thus, a major challenge for future research is to understand how the various adipokines interact with each other at the cellular and molecular levels since numerous adipokines with diverse biological properties can be released simultaneously and, as such, the net effect of increased adipokine production may not reflect the actions of a single individual substance.

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CHAPTER 5

Regulation of Muscle Proteostasis via Extramuscular Signals

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5.1 Basic Protein Synthesis

Muscle is a multi-functional tissue, the contractile function of which (*i.e.* locomotion) is well appreciated. This contractile function requires a substantial protein-based contractile apparatus and catabolism of energy which produces heat. Consequently, in normal healthy humans muscle constitutes the bulk of protein stores and is a major metabolic consumer and producer of heat. Presumably these interrelated functions of muscle have evolved over time such that each has partially independent regulation in various species. Here we consider the regulation of the synthesis and degradation of muscle proteins largely in isolation of the other intertwined functions.

In a normal healthy growth stable animal, protein synthesis and degradation exist in a dynamic equilibrium recently termed proteostasis.[1–3] When synthesis and degradation are precisely balanced, no net gain or loss of muscle mass is seen. Conversely if net synthesis exceeds net degradation a gain of mass is observed and if net degradation exceeds net synthesis then a loss of mass is observed. At the fundamental level, proteins are synthesized *via* translation of mRNA transcripts encoded within DNA based genes. These transcripts are then

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translated to produce unfolded peptides. The unfolded peptides are then subject to folding, trafficking and/or other post-translational modifications (*i.e.* glycosylation) to produce functional proteins.

For DNA transcription into mRNA, the bulk of genes are regulated by the binding of transcription factors to DNA elements and to the transcriptional machinery. The proper functioning of the transcriptional machinery is the key regulatory element during elongation, and the key regulatory element of termination appears linked to placing a polyadenylated tail on the mRNA transcript. The regulation of mRNA translation into peptides can be divided into three phases of initiation, elongation and termination. For the bulk of mRNA, initiation of translation involves engaging mRNA bound ribosomes into translation-competent protein/RNA complexes. The final step of translational initiation is regulated *via* placement of the first amino acid residue by methionyl tRNA. Peptide elongation occurs *via* delivery of anticodon aminoacyl tRNA to the ribosome and concurrent formation of peptide bonds. Stop codons signal the termination of mRNA translation by facilitating the binding of polypeptide release factor to the ribosome, thereby stimulating hydrolyzing of the final peptidyl transfer RNA linkage and releasing the completed polypeptide from the ribosome. Modulation of protein synthesis can occur at any or all of these stages.

Protein degradation, as we will consider it here, is simply the destruction of functional proteins. Degradation is achieved by cleavage of the peptide bonds that hold the protein together by catabolic enzymes collectively known as proteases. There are four major proteases that are currently considered to contribute to overall maintenance of muscle mass. The first is the proteasome, which degrades proteins that are trafficked to it from elsewhere in the cell *via* a polyubiquitin carrier system. The proteasome is currently thought to be the major regulator of muscle mass *via* its role in degradation. However, as it also participates in degrading improperly folded proteins during protein synthesis and at least 30% of bulk peptides do not complete synthesis as we define it here, the proteasome's role in regulating degradation *versus* synthesis currently remains open. The second major proteolytic system is the lysosome. Previously the lysosome was thought to be the major regulator of muscle mass *via* its role in degradation and this belief is currently returning and consistent with the notion that the lysosomal system is the major regulator of cell mass across cell types. Proteins destined for degradation are trafficked *via* autophagy in the lysosomes membrane enclosed acidic environment. In the case of both proteasome- and lysosome-mediated degradation the likely control points for modulating degradation are at the trafficking step. However, there is also substantial evidence for the regulation of the amount of protease *via* alterations in synthesis of the proteasome subunits and the lysosomal proteases (*i.e.* cathepsins). The final two proteases that are believed to contribute to the maintenance of muscle mass are the calpains and the caspases. In both cases these proteases are thought mainly to be regulated at the level of activation of the protease (*i.e.* cleavage events), which likely explains why both are also thought to have relatively specific and local effects within muscle. For example, both are thought to participate in disassembly of the complex actinomyosin

protein structures that make up the contractile apparatus prior to trafficking of the disassembled proteins to the proteasome or lysosome for final destruction.[4]

5.2 The Effects of Extramuscular Signals on Global Proteostasis in Muscle

A host of extramuscular signals that regulate proteostasis have been identified both in man and in biomedical models such as cultured muscles, rodents and invertebrates (Figure 5.1). The next sections will describe the role of some of these signals in the regulation of muscle proteostasis.[5,6] However, it is important to state that classifying extracellular signals as simply either anabolic or catabolic *per se* is an oversimplification since signals may be anabolic under some conditions and catabolic in others, probably as a result of crosstalk with other signals. Furthermore, detailed mechanisms by which many extracellular signals regulate muscle proteostasis are as yet unavailable, which highlights the paucity of research into this area. Nevertheless these signals still warrant highlighting as they are likely to emerge as important in the regulation of muscle proteostasis.

5.2.1 Hormones

One class of signals is hormones, which are produced by endocrine organs at a distal site from muscle. These are thought to generally promote catabolism (*e.g.* glucocorticoids, thyroid hormone (TH)) or anabolism (*e.g.* insulin, testosterone) and in many cases are directly responsible for muscle wasting in patients suffering from endocrine disorders.

Insulin has been suggested to control 50% of muscle size during development of rodents and therefore would be expected to play a major role in human beings. Indeed insulin increases muscle protein synthesis and suppresses degradation (see Section 5.2.2.1 for detailed discussion), demonstrating its importance in

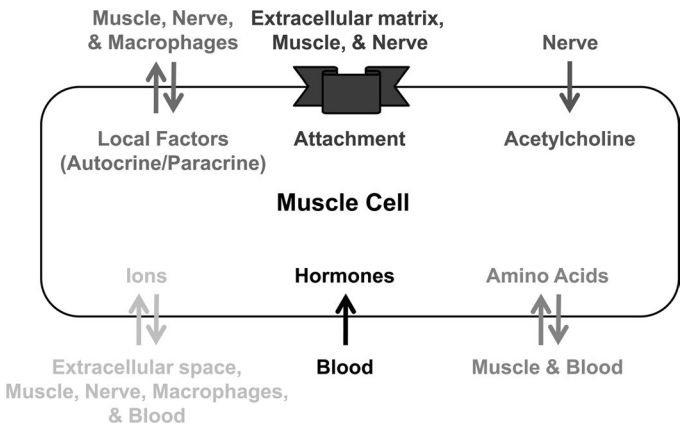

Figure 5.1 Extramuscular signals that muscle integrates to regulate proteostasis.

muscle proteostasis. In contrast, glucocorticoids (*i.e.* cortisol) decrease both protein synthesis and degradation, as does epinephrine, while TH appears to promote both processes. Presumably when both processes are inhibited or promoted the net effect will depend upon the quantitative extent of inhibition or activation. For example, TH promotes degradation to a larger extent than synthesis, which results in a net shift toward catabolism of muscle protein. Finally, despite its potent effects in developing/deficient states, growth hormone (GH) in healthy adults has no anabolic effects on skeletal muscle (despite increases in insulin-like growth factor (IGF-1) expression), instead increasing tendon collagen synthesis.

5.2.1.1 Mechanisms of Action: Glucocorticoids

Muscle wasting is observed in patients with excessive levels of glucocorticoids (*i.e.* Cushing's syndrome).[7] In addition, glucocorticoid concentrations are increased in cases of sepsis and burns when muscle is also undergoing atrophy. However, little is known of precisely how glucocorticoids, for example endogenous cortisol and clinically used dexamethasone, cause changes in proteostasis in humans. Glucocorticoids are traditionally thought of as binding to intracellular hormone receptors, which in turn bind to glucocorticoid response elements in DNA, thereby causing altered expression of mRNA. Indeed, current evidence suggests that glucocorticoids induce expression of a ubiquitin E3 ligase and proteasome activator subunit, which may contribute to increased degradation that is observed. On the other hand, glucocorticoid treatment also represses the expression of the myogenic transcription factors, myoD and myogenin, which presumably results in decreased expression of muscle-specific genes, including myosin. Additionally, glucocorticoids increase the expression of 4E binding protein 1 (4E-BP1), which is a translational repressor, thus reducing translational efficiency. Glucocorticoids also reduce the transcription of insulin receptor substrate (IRS), phosphoinositide 3 kinase (PI3K) and protein kinase B (Akt) (see Section 5.2.2.1 for more on these signals). Thus, glucocorticoids are capable of changing the responsiveness of muscle to other extramuscular signals known to regulate proteostasis. These observations demonstrate negative regulation over multiple levels of protein synthesis.

5.2.1.2 Mechanisms of Action: TH (T3)

Muscle wasting is observed in patients with excessive levels of TH (*i.e.* thyroid toxicosis).[8,9] Indeed, TH has a well-described catabolic effect on adult skeletal muscle and, as mentioned above, sustained elevations in blood thyroid hormone levels result in muscle weakness and wasting. Like glucocorticoids, TH is thought to influence muscle proteostasis *via* binding to intracellular hormone receptors (TH receptors), which in turn bind to TH response elements in DNA and thus cause changes in mRNA expression. TH exposure induces expression of the lysosomal enzyme cathepsin C and multiple subunits of the proteasome, which fits with its purported role in protein degradation. Similarly,

TH also increases the expression of the synthetic apparatus, notably increasing the expression of several transcription factors including myogenic factor 6 (Herculin), several ribosomal proteins and translation initiation factors. Notably, TH also increases the transcription of cellular receptors for muscle growth factors and also the transcription of intramuscular signaling proteins, thus TH is capable of changing the responsiveness of muscle to other extramuscular signals known to regulate proteostasis. Nevertheless, TH-induced catabolism must prevail over anabolism for muscle atrophy to occur (at least independent of other signal interactions).

5.2.1.3 Mechanisms of Action: Testosterone

Testosterone is a well-known anabolic hormone, the administration of which increases the basal rate of muscle synthesis in both healthy and hypogonadal young men.[10,11] Conversely, testosterone concentrations decline with aging, perhaps consonant with a role in sarcopenia (at least in men). Like glucocorticoids and TH, testosterone is thought to influence muscle proteostasis *via* binding to intracellular hormone receptors (androgen receptors), which in turn bind to androgen response elements in DNA and thus cause changes in mRNA expression. Notably IGF-1 expression increases in response to testosterone treatment, thus suggesting that the reason for similar effects of testosterone and IGF-1 on muscle proteostasis is that IGF-1 acts downstream of testosterone. In support of this, testosterone increases the phosphorylation of mTOR and its downstream targets 40S ribosomal protein S6 kinase 1 (p70S6K1) and 4E-BP1, which are parts of the IGF pathway, thereby increasing translational efficiency.

Additionally, testosterone has been show to bind to receptors at the muscle plasma membrane. These receptors are G-protein coupled receptors. Unlike insulin receptors, G-protein coupled receptors are not themselves kinases. However, like insulin receptors, G-protein coupled receptors work by recruiting other proteins, in this case G-proteins, to the receptor when bound to ligand. Upon activation of the receptor a G-protein in turn activates phospholipase C, which produces inositol trisphosphate (IP3), which then acts upon IP3 receptors located at the sarcoplasmic reticulum to stimulate release of intramuscular Ca^{2+} with an oscillatory pattern. The intramuscular Ca^{2+} can then, perhaps, act like Ca^{2+} release in response to contraction and also activate the Ras regulated protein kinase cascade, which can upregulate initiation/elongation factor expression and as such the capacity for protein synthesis. Alternatively or in addition, Ca^{2+} could activate the calmodulin-activated phosphatase calcineurin, which mediates hypertrophic response through regulation of downstream nuclear factor of activated T cells (NFAT) transcription factor and thus initiates a hypertrophic gene program.

5.2.1.4 Mechanisms of Action: Epinephrine

Adrenal-derived epinephrine is found elevated in a rare endocrine disorder associated with muscle wasting: pheochromocytoma.[12] More commonly,

epinephrine is thought of as anabolic since treatment of both animals and human beings with specific epinephrine-derived compounds (*i.e.* β2-adrenoceptor agonists) leads to muscle hypertrophy *via* stimulation of muscle protein synthesis and depression of muscle protein degradation. Moreover, infusions of epinephrine to humans suppresses release of phenylalanine from muscle suggesting an anti-catabolic effect. Indeed, epinephrine decreases expression of ubiquitin-conjugating enzymes, thus suggesting the mechanism by which epinephrine inhibits degradation in muscle (*i.e.* *via* decreasing proteasome-based degradation). This difficulty in classification highlights the complexity of assigning single actions to molecules which may act *via* several distinct mechanisms and may do so with different kinetics. Nevertheless, because of its role as a stress hormone, the actions of epinephrine are generally associated with the catabolic response to major illness. Epinephrine has been shown to bind to G-coupled receptors in the plasma membrane. In response to epinephrine binding G-proteins activate adenylate cyclase, which produces cAMP to activate protein kinase A (PKA). PKA is then able to phosphorylate and effect changes within muscle. One target of PKA is the cAMP response element binding protein (CREB), which stimulates the transcription of target genes. PKA can inhibit myogenic regulatory factors (MRFs) MyoD and Myf5, thus potentially accounting for the belief that epinephrine largely inhibits muscle protein synthesis. cAMP may also activate other transcription factors such as activator protein 2 (AP2) and NFκB, which can have potent catabolic effect on muscle (see Section 5.2.2.4).

5.2.2 Local Factors (Autocrine/Paracrine)

A second class of signals are "local" factors, produced in muscle. Local factors, like hormones, tend to be peptides but unlike hormones have a propensity to act in a paracrine or autocrine manner. However, it remains unclear if some or all of these factors are produced by other local cells (for example nerve and macrophages) to act upon muscle. Moreover, recently some "muscle" growth factors have been shown to be present also in the systemic circulation (for example Nerve Growth Factor (NGF)), suggesting that actions may not be exclusively local. This also supports the notion that contrary to popular belief, muscle is an endocrine organ and, as such, probably the largest in the body.

MechanoGrowth Factor (MGF) is a splice variant of IGF-1. Both MGF and IGF-1 act to promote synthesis and inhibit degradation and can be thought of as anabolic signals akin to the hormone insulin. MGF, as the name implies, is an autocrine signal released in response to mechanical load. Fibroblast growth factor (FGF) appears to be autocrine, and to promote both synthesis and degradation. Presumably the fact that both processes are promoted creates a situation similar to TH. Therefore, FGF may act as a catabolic mediator like TH when degradation is more strongly promoted, a condition shown in C. elegans mutants and possibly accounting for extreme wasting in a case report of a patient with facioscapulohumeral muscular dystrophy. Alternatively FGF may act as an anabolic factor, as the name implies, when synthesis is more

strongly promoted (*i.e.* similar to the anabolic effects of epinephrine-derived β2 agonists) and this may account for the notion that FGF largely works on muscle stem cells. Presumably, the fact that both synthesis and degradation are promoted by an autocrine signal creates a situation in which muscle poises itself to respond to other changes in the extramuscular environment *via* inhibition of either synthesis or degradation, a case of classical negative feedback regulation, which is widespread throughout physiologic systems. Indeed, at least for C. elegans, this is the case and insulin is the negative regulator.

Myostatin is an autocrine member of the Transforming Growth Factor beta (TGFβ) family of growth factors. Currently, myostatin is thought predominantly to inhibit protein synthesis. NGF and several other growth factors of the Neurotrophin family are also autocrine signals. NGF, cilliary neurotrophic factor (CNF) and neuregulin all appear to act to promote synthesis and inhibit degradation. Neurotrophins, akin to IGF/MGF, have been shown to be released by muscle in response to contractile activity. Unlike IGF/MGF, neurotrophins have also been shown to be released by neurons, thus raising the possibility that the neurotrophins act similarly to MGF in balancing against other autocrine signals such as FGF but also act distinctly to allow cross-tissue coordination, for example acetylcholine receptor (AChR) clustering between nerves and muscle.

Cytokines act in both an autocrine and a paracrine fashion between muscles but also allow for muscle-macrophage communication. The prevailing view is that tumor necrosis factor alpha (TNFα) inhibits synthesis and promotes degradation, thus acting similarly to catabolic hormones. Likewise, Interferon gamma (IFNγ) acts in a catabolic fashion by promoting degradation and also synthesis (*e.g.* akin to TH).

The remaining extramuscular signals can be broadly thought of as neurotransmitters (*i.e.* ACh), metabolites (*i.e.* amino acids), ions (*i.e.* Ca^{2+}) and extracellular matrix proteins. ACh has been shown to inhibit protein degradation in C. elegans and has a well-known myogenic effect in animals. Ca^{2+} acts to inhibit protein synthesis and increase breakdown but may also do the opposite. Diet-derived amino acids also strongly promote synthesis while inhibiting degradation. Lastly, attachment to the extracellular matrix has been linked to both modulation of synthesis and degradation. The current model is that stronger attachment leads to increased synthesis while weaker attachment leads to increased degradation.

5.2.2.1 Mechanisms of Action: Insulin/IGF Spliceoforms

IGF-1 is a hormone similar in molecular structure to insulin. Insulin and IGF-1 are growth factors generated at a distance from muscle (for example IGF-1Ea at the liver and insulin at the pancreas), and within muscle itself, for example MGF.[13,14] Insulin deficiency leads to muscle wasting when insulin-dependent diabetes is left untreated or is poorly controlled. Moreover, GH deficiency reduces circulating IGF-1 through its action as a secretagogue, and consequently attenuates both muscle and whole body growth. Insulin suppresses

protein breakdown (*i.e.* during postprandial periods; see Section 5.3.1) and stimulates protein synthesis, at least in animals. Although using different receptors, in general terms these hormones work in the same fashion by binding to insulin or IGF receptors at the plasma membrane. Since these receptors are themselves tyrosine kinases, growth-factor binding stimulates kinase activity, which permits the generation of phosphorylated binding sites on the receptor itself intracellularly. Various proteins are known to bind to these phosphorylated sites; however, there are currently two widely appreciated signaling systems that function downstream of insulin/IGF receptors. Both pathways involve the binding of an adaptor protein to the receptor followed by activation of the pathway. The first pathway utilizes Shc as an adaptor to recruit SOS and then the oncogenic guanosine triphosphate hydrolase (GTPase), Ras. Ras allows dimerization and thus activation of the protein kinase Raf, which subsequently activates other protein kinases such as mitogen-activated protein kinases (MAPKs) *via* a phosphorylation cascade. The second pathway utilizes insulin receptor substrate (IRS) as an adaptor, which recruits the oncogenic phosphoinositide 3-kinase (PI3K). PI3K similarly acts *via* phosphorylation cascades, which result in the activation of protein kinase B (Akt) and mammalian target of rapamycin complex 1 (mTORc1) among others (see Section 5.3.1.1 for more detailed discussion). Activation of MAPK downstream of Raf/Akt allows activation of transcription factors, which then translocate to the nucleus and upregulate the synthesis of specific transcripts, including initiation and elongation factors, thereby increasing translational capacity. At the same time activation of Akt allows activation of mTORc1, which activates ribosomal protein S6K1 (P70S6K1) and inhibits 4E-BP1, thereby driving global mRNA translation (*i.e.* increasing efficiency). Activation of Akt likewise inhibits the transcription factor Forkhead in human rhabdomyosarcoma (FKHR) thereby downregulating the synthesis of specific transcripts, including ubiquitin ligases. With E3 ligase enzymes such as muscle atrophy F-box (MAFbx) and muscle-ring finger 1 (MuRF1) repressed, ubiquitin proteasome based degradation goes down. While not yet demonstrated in humans, it has also recently been shown that mTORc1, which acts downstream of Akt also has the ability to inhibit autophagy, thereby driving down lysosome-based degradation. Thus, insulin/IGF appears able to coordinately regulate both synthesis and degradation and to do so *via* multiple mechanisms.

5.2.2.2 Mechanisms of Action: Fibroblast Growth Factor (FGF)

There are currently no definitive data on FGF being a causative/preventative factor of muscle disease in man, however we include it as it is a growth factor specifically identified for fibroblasts.[3] FGF, like insulin, binds to its receptor at the plasma membrane. Like the insulin receptor, the FGF receptor is itself a kinase. FGF has been shown to signal through the Ras kinase cascade to effect gene transcription in various, non-human, muscles. FGF has also been shown to signal through this pathway in C. elegans to stimulate lysosomal-based degradation. FGF has also been shown to signal *via* FGFR receptor in rodent

muscle to stimulate both muscle protein synthesis and proteasome-based degradation. While these signals may go through Ras/MAPK, the intracellular pathway(s) remain to be elucidated. Additionally, loss of FGF has been linked to worsening of muscular dystrophies in mice, effects theorized to be due to decreased muscle stem cells migration.

5.2.2.3 Mechanisms of Action: Myostatin

The circulating concentrations of myostatin protein are higher in patients with acquired immunodeficiency (HIV) syndrome than in healthy young men[15] and systemic administration of myostatin to rodents causes atrophy. On the other hand myostatin knockout mice are enormously muscular, due to both hypertrophy and hyperplasia.

Myostatin is a peptide hormone of the transforming growth factor beta (TGFβ) family. Myostatin binds to activin type II receptors, which recruits a type I TGFβ receptor to form a fully active receptor complex. Following activation the transcription factors small and mothers against decapentaplegic homolog 2/3 (Smad2/3) are phosphorylated and are then able to translocate to the nucleus to effect mRNA transcription. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NFκB-independent, FKHR-dependent mechanism. The decrease in Akt activity in response to myostatin treatment and resultant nuclear localization of the FKHR transcription factors, which regulate the expression of atrophy-related genes may explain this observation. Furthermore, the downregulation of Akt likely reduces translational efficiency through its diminished capacity to regulate downstream anabolic signaling (discussed in Section 5.3.1.1). As such myostatin is a potent negative regulator of muscle mass.

5.2.2.4 Mechanisms of Action: Cytokines

Cytokines have diverse functions in muscle but when present in excess they seem to have catabolic effects.[16] The two main cytokines that have been shown to regulate proteostasis in muscle are TNFα and IL-6. For example, TNFα has also been named cachectin because its administration causes cachexia in rodents. Similarly IL-6 can also induce protein degradation in myotubes *in vitro* and in muscles *in vivo*.

TNFα and IL-6 have been shown to be produced and secreted by muscle cells and as such have been termed "myokines". TNFα acts *via* TNF receptors (p55TNFR and p75TNFR) expressed on the surface of muscle fibers. Upon TNFα binding the receptors act to form signalosomes, which have the net effect of activating NFκB. One effect of the activation of NFκB is that it translocates to the nucleus where it can activate muscle gene expression. One set of genes that is upregulated in response to NFκB action are proteasome subunits and ubiquitin ligases, thus increasing the cellular proteolytic capacity. Activation of NFκB is also a major signaling hub for signaling pro-apoptotic events.

Importantly TNFα also stimulates IL-6 expression. IL-6 is a potent inducer of the acute phase response and is produced by T cells, macrophages, fibroblasts, endothelial and muscle cells. Homodimerization of gp130 triggers the Jak/STAT signaling cascade and the SHP2/Erk Map kinase cascade to regulate proinflammatory gene expression (including NFκB). IL-6 also induces a decrease in the phosphorylation of ribosomal S6 kinase (p70S6K1), suggesting that cytokines can also negatively regulate translational efficiency.

5.2.2.5 Mechanisms of Action: Neurotrophins

Neurotrophins are a family of growth factors first identified for the ability to induce neurogrowth in a trophic fashion.[17–19] They have recently emerged as regulators of muscle physiology. Nerve Growth Factor (NGF), Cilliary Neurotrophic Factor (CNF) and Neuregulin have all been shown to be produced in muscle and to act in an autocrine fashion. As a class they appear to promote synthesis while inhibiting degradation; however, the specific pathways remain to be established. Nonetheless, all bind to a receptor at the plasma membrane, which results in receptor dimerization (*e.g.* similar to myostatin). One member of the receptor complex is a protein tyrosine kinase family and is therefore able to signal *via* the same PI3K/Akt pathway used by insulin. These receptors also appear able to signal *via* protein kinase C (PKC), *via* activation of phospholipase C (PLC), which presumably also activates Ca^{2+} signaling within the muscle.

5.2.2.6 Mechanisms of Action: Acetylcholine (ACh)/Ca^{2+}

Neuronal input in the form of ACh is required for proper development and maintenance of muscle.[19–21] Indeed, denervation promotes rapid atrophy of skeletal muscles showing the critical importance of the nerve-muscle axis in the maintenance of muscle mass.

ACh acts to promote synthesis and inhibit degradation, although the precise mechanisms by which this is achieved are obscure. Presumably as ACh induces plasma membrane depolarization and Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum to regulate contraction, this same mechanism regulates proteostasis. Intramuscular calcium can regulate calcineurin, which has been shown to regulate the activity of NFAT. NFAT, when dephosphorylated by calcineurin, can bind other transcription factors, such as myogenic enhancer factor 2 (MEF2) and thereby promote synthesis of specific proteins. Importantly, over-expression of activated calcineurin has been shown to activate genes associated with type I, slow fibers, thus suggesting that muscle activity can induce specific fiber type transformation toward slow fiber formation. Consistent with this possibly being associated with neuronal input, endurance exercise (*e.g.* sustained muscle activity) also results in transformation toward a slow phenotype while denervation and disuse results in slow to fast fiber transformation. This suggests not only that ACh regulates specific muscle protein synthesis but also that ACh inputs must be maintained for the synthesis to be sustained. Intramuscular Ca^{2+} also can activate CaMK and PKC.

Both of these kinases have been shown to phosphorylate histone deacetylase (HDAC) proteins, which relieve HDAC inhibition of MEF and thus provide a second, complementary mechanism by which Ca^{2+} downstream of ACh can regulate specific muscle gene expression.

However, the role of Ca^{2+} is complex. Ca^{2+} concentrations are actually elevated in many situations of muscle-wasting including sepsis and denervation and, perhaps paradoxically, under these conditions, Ca^{2+}-dependent protein kinases act to suppress protein synthesis *via* eukaryotic elongation factor 2 (eEF2K) mediated mechanisms to suppress peptide elongation. Moreover there are Ca^{2+}-responsive proteases such as the calpains, which may explain increases in protein breakdown. Thus it may be speculated that the amplitude and frequency of Ca^{2+} currents are important in the outcome of proteostasis.

5.2.2.7 Mechanisms of Action: Extracellular Matrix

Physical attachment to the extracellular matrix is important to prevent muscle cell apoptosis and to allow for proper functioning of the muscle.[22,23] Several attachment complexes exist and thus several mechanisms for regulation of muscle proteostasis likely exist. However, to date the only evidence for modulation of muscle proteostasis in humans is *via* integrin-containing attachment complexes. These complexes contain an alpha and beta integrin and together they bind to components of the extracellular matrix. There are roughly 150 proteins thought to associate with these complexes in man and several cause limb girdle muscular dystrophy when mutant (myotilin LGMD1A, caveolin LGMD1C, calpain-3 LGMD2A, telethonin LGMD2G and titin LGMD2J). Importantly another of these proteins, focal adhesion kinase (FAK), has been shown to increase in amount and activity when muscle hypertrophies and decrease in amount and activity when muscle atrophies (both in response to use). Additional details of how FAK modulates proteostasis remain to be elucidated although interaction of the cell surface integrin receptors with extracellular matrix proteins results in the activation of intracellular signaling pathways, including p70S6K1, which positively affects mRNA translation. In addition it has been suggested that integrin complexes cluster insulin and other growth factor receptors together, presumably in an appropriate microdomain of the plasma membrane to allow them to be in the right location to receive signal from outside of the cell. Lastly, integrin complexes are well known in other cells to control the synthesis of the extracellular matrix thereby creating both the signal that activates them and a feedback mechanism, perhaps akin to the autocrine growth factors. It seems likely that other attachment complexes will emerge as key regulators of proteostasis.

5.2.2.8 Mechanisms of Action: Amino Acids (AA)

Dietary protein contains AA, which are key extracellular signals in the regulation of muscle proteostasis.[24] Importantly, AA act not only as substrates or

"building blocks" of muscle proteins, but also directly stimulate intracellular signaling pathways that regulate mRNA translation. Evidence for this is derived from studies in which the provision of single essential amino acids (EAA), such as leucine, is sufficient for the stimulation of muscle synthesis, even in the absence of other AA as substrate for peptide synthesis. Leucine, of all the EAA, seems to have the most potent capacity to stimulate muscle synthesis and inhibit lysosome-based muscle protein degradation. Precisely how the EAA stimulate proteostasis-regulating signals remains poorly understood. Unlike peptide hormones and local factors, there does not appear to be a receptor in the plasma membrane as genetic loss of the transporter system (for leucine: system L, a Na^+-independent, glutamine-dependent exchanger) abolishes intracellular signaling responses to EAA. Thus EAA presumably act like glucocorticoids, TH and testosterone by binding to proteins once inside the cell. However, there are no known transcription factors or EAA response elements within the DNA and it is not known whether leucine itself or metabolites generated during its catabolism are responsible for the effects. For example, it remains to be investigated whether deamination of leucine to αKIC *via* branch chain amino acid transferase or oxidative decarboxylation to isovaleryl CoA *via* branch chain keto-acid dehydrogenase is required for leucine-induced phosphoproteome activity. Currently, it is believed that AA promote synthesis while also inhibiting degradation, presumably *via* coordinated action of Akt/FKHR and mTORc1-mediated inhibition of autophagy (see Section 5.3.1.1 for more detail); though the mechanism is unknown and multiple mechanisms may exist as leucine and αKIC appear to inhibit lysosome-based degradation *via* distinct pathways.

5.3 Regulation of Muscle Proteostasis in Humans

Since the early 1980s, many of the advances made surrounding the regulation of muscle proteostasis in humans are a consequence of the development of methods for detecting stable isotopically labeled amino acid "tracers" by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS).[25] Constant improvements mean that these tracer approaches have extensive application even for studies of muscle proteostasis over periods as short as 30–60 min. Labeling amino acids such as the branch chain amino acid leucine with ^{13}C, for example [1,2-$^{13}C2$]leucine, allows measurement of fractional synthetic rates (muscle protein synthesis rates) of incorporation of these amino acids into functional muscle proteins from biopsy tissue. Increased sensitivity and precision of gas chromatography mass spectrometry (GC-MS) now allow measurement of the incorporation of deuterium labeled amino acids into protein *i.e.* d5 phenylalanine. Fractionation of proteins from these tissues further allows measurement of fractional synthetic rates of incorporation into different cellular compartments such as mitochondria, sarcoplasm and myofibers. Using these same tracers, the catabolism of the branch chain amino acids (BCAA), the only amino acids that may be oxidized in skeletal muscle, can also

be measured because the isotope label in the carboxyl position is released as CO_2 when leucine is irreversibly committed to oxidation with the decarboxylation of alpha-ketoisocaproate (αKIC). As such the appearance of the ^{13}C label in breath CO_2 provides a direct measure of leucine oxidation. Finally, when a tracer of an essential amino acid (EAA) such as phenylalanine is used that is neither synthesized nor oxidized (unlike the BCAAs) in muscle, net balance, uptake and dilution of the tracer can be equated not only to rates of muscle synthesis but also to breakdown. Thus, human muscle protein synthesis and breakdown can be simultaneously measured *in vivo*. It is precisely this difference between synthesis and breakdown (*i.e.* the net balance) that is the significant parameter relevant to the net gain (anabolism) or loss (catabolism) of muscle. When tracer methods are linked with sophisticated genetic or pharmacological approaches, and/or temporal observations of intramuscular signaling, we gain information on how signaling pathways and extracellular ligands are linked to alterations in human muscle proteostasis.

The mechanisms regulating changes in proteostasis in human aging, disease and trauma are complex and involve interplay between the systemic milieu (*i.e.* central hormones, Section 5.2.1), the immediate extracellular milieu (autocrine/paracrine signals, Section 5.2.2) and those within the cell (*i.e.* metabolites, 2nd messengers *etc.*). The rest of the chapter is devoted to discussing what is understood about the regulation of muscle proteostasis by the previously discussed extracellular and intracellular signals in humans. The main focus is upon the primary environmental factors long known to effect muscle size, nutrients and contraction, and on the regulation of muscle proteostasis in aging, disuse, disease and acute trauma.

5.3.1 Nutrients as Regulators of Muscle Proteostasis in Man

Starvation and chronic malnutrition have long been known to cause weight loss with associated muscle wasting, thus nutrients are widely appreciated as key extracellular signals in the regulation of muscle proteostasis.[26] In a healthy, weight-stable, weight-bearing human being the dynamic equilibrium between muscle protein synthesis and breakdown ensures that muscle mass remains constant. This occurs through two mechanisms. The first involves stimulation of muscle protein synthesis over basal rates for a period after feeding. Indeed, it was initially shown that muscle protein synthesis rates are increased about 2-fold above basal rates (which equate to about 0.05% h^{-1} in mixed human muscle) after feeding a mixed macronutrient meal (*i.e.* carbohydrate, fats, amino acids). Later the nutrients causing the stimulation were identified with AA (outlined in Section 5.2.2.8) now recognized as the nutritionally active constituents responsible for stimulating muscle synthesis. Because provision of EAA, those that cannot be synthesized *in vivo*, alone has equal efficacy in the stimulation of muscle synthesis to that of all AA or a mixed meal, we now recognize EAA as the active constituent. This key role of EAA in the regulation of proteostasis makes sense from an evolutionary perspective since the instruction to build muscle is received only when amino acids that cannot be

synthesized *in vivo* are ingested. Work from the authors' labs recently charted the time course of the response to AA. After feeding enough AA to maximally stimulate muscle synthesis (equating to about 20 g of EAA), the response switches off after about 1.5 h, despite continued AA availability. We have termed this the ''muscle full'' phenomenon on the basis that the increases in fractional synthesis rates of muscle protein are outlasted by substrate availability. The mechanism for this is not known, but it is likely that the muscle gauges its own amino acid requirements based upon losses incurred during fasting periods. The second route by which feeding stimulates muscle protein accretion is *via* reducing muscle protein breakdown. This is because both mixed meal and AA feeding stimulates insulin release from pancreatic β cells. Consequently, the reduction in protein breakdown after feeding (–50%) is at least in part due to the anti-proteolytic effects of insulin.

As a consequence, *in vivo* the two mechanisms combine such that food has anabolic effects on both arms of proteostasis *i.e.* substantial increases in muscle protein synthesis are observed in response to delivery of AA and moderate reductions in muscle protein breakdown are observed in response to delivery of insulin. As a result, negative net balance observed in the post-absorptive state (*i.e.* muscle protein breakdown exceeds synthesis) is transiently reversed. This small gain in muscle protein offsets loss during fasted periods. As such, if all else is equal muscle mass remains constant.

5.3.1.1 AA as Extracellular–Intracellular Signals

The intramuscular regulatory signals governing how AA and insulin stimulate muscle protein synthesis and reduce degradation have been the subject of much scrutiny.[27,28] With regard to protein synthesis, most work has centered on activities of phosphoproteins that, independent of cell type, (in)directly regulate the initiation and elongation phases of mRNA translation; so-called anabolic signals. Although the initiating events remain unknown, currently the most proximal step for sensing of AA involves stimulation of Ras-related guanosine triphosphate hydrolases (RAGs) and/or the class III PI3Ks, vacuolar protein sorting 34 (Vps34). These elements ultimately converge to increase signaling through the mammalian target of rapamycin complex 1 (mTORc1). The initiating events for insulin are better understood with insulin binding to its receptor initiating signaling *via* an insulin receptor/IRS pathway to Akt. Akt phosphorylates glycogen synthase kinase 3 beta (GSK3β), the key enzyme responsible for glycogen synthesis, and then GSK3β phosphorylates and inhibits eukaryotic initiation factor 2B (eIF2Bε) at serine 535. The net effect is a stimulation of eIF2B. The guanine nucleotide exchange factor activity of eIF2B serves a key role in translation by catalyzing the recycling of eIF2 methyl tRNA between consecutive rounds of peptide-chain initiation. Finally, Akt both directly (*via* phosphorylation) and indirectly (*via* tuberous sclerosis complex 1/2 and proline rich Akt substrate (PRAS40)) stimulates mTORc1 activity. Consequently, both AA and insulin signaling converge on mTORc1;

a heterotrimeric complex consisting of regulatory associated protein of TOR (raptor), PRAS40, mLST8 and mTOR and a central regulator of growth in all eukaryotic cells.

Activation of mTORc1 triggers a series of "anabolic" signaling events. The best characterized substrates of mTORc1 are 4E-BP1, whose phosphorylation promotes eukaryotic initiation factor 4F (eIF4F) complex assembly (an assumed requirement for cap-dependent translation) and p70S6K1, which phosphorylates, among other substrates, eukaryotic initiation factor 4B (eIF4B), which facilitates unwinding of tertiary mRNA structure and eukaryotic elongation factor 2 kinase (eEF2K), which promotes peptide elongation by recruiting charged tRNA to the ribosome. Though it is less well established how insulin suppresses protein degradation, Akt phosphorylates FKHR transcription factors and in doing so regulates their nuclear-cytoplasmic translocation thereby preventing transcription of pro-proteolytic genes. It is therefore assumed to be this mechanism by which insulin suppresses muscle proteolysis, though it may also be through stimulating the inhibitory effects of mTOR on autophagy.

Confirming the key role of mTORc1 in human muscle is the observation that Rapamycin, an immunosuppressant and potent mTORc1 inhibitor, robustly attenuates the stimulation of muscle protein synthesis and associated anabolic signaling in response to leucine. Thus it appears that AA and insulin action *via* mTORc1 principally serves to increase the efficiency of translation (*i.e.* number of mRNA translated per ribosome). Confirming distinct actions of AA and insulin upon muscle proteostasis is the observation that inhibiting postprandial increases in insulin does not suppress increases in muscle protein synthesis. Thus it appears that the physiological role of insulin is primarily in the suppression of protein breakdown in adult humans.

While the pathways described above are sufficient to account for the *in vivo* effects of AA and insulin upon muscle proteostasis, it is possible that there is a transcriptional component to the anabolic response to feeding. For example, performing euglycemic hyperinsulinemic clamps in humans acutely modulates ~800 transcripts in adult human muscle. Moreover, activation of mTORc1 by AA also modulates expression of rRNA and other transcripts. In support of this, there have been a number of reports of pro-anabolic transcriptional responses to feeding. Decreases in myostatin mRNA has been reported in humans after feeding, which could relieve inhibition of mTORc1 signaling. Furthermore, increases in AA transporter expression could facilitate influx of AA to intracellular pools. Nonetheless, early work using actinomycin D (a transcriptional inhibitor) failed to repress the acute synthesis response to leucine. Thus, both insulin and AA probably work to facilitate both short-term and long-term changes in muscle proteostasis. In the acute phase described above changes are largely *via* mTORc1 effects on translational efficiency and possibly protein degradation. In the longer term, changes are due to increased transcription of AA transporters, intramuscular 2nd messenger signals and perhaps the transcriptional/translational machinery (*i.e.* preserving anabolic capacity).

5.3.2 Muscular Activity (*i.e.* Exercise) as a Regulator of Muscle Proteostasis

Repeated muscle use, in the form of manual labor, and lack of muscle use, in response to cessation of work or convalescence, have long been known to affect muscle size; increasing and decreasing it respectively.[29] We now appreciate that some form of muscular activity, even weight bearing *per se*, is essential for the maintenance of muscle mass. Indeed, it has become apparent that aside from nutrients the most potent regulators of proteostasis in postnatal skeletal muscle are Newton's gravity and active muscle contraction (NB: in modern Western society this is largely only exercise). As examples, significant loss of soleus muscle cross-sectional area (CSA) of about 15–26%, depending on fiber type, is evident following removal of Newton's force in the form of 17 days in Low Earth Orbit onboard a NASA Space Shuttle. Conversely, a 5% increase in muscle CSA is evident in leg muscles after fewer than ten individual bouts of heavy resistance exercise. These observations detail the importance of ambulatory activity in the maintenance of muscle mass and also demonstrate chronic high-force contractions stimulate muscle growth. Although muscle mass is ultimately regulated by the balance of protein synthesis and degradation, the prevailing view is that changes in protein synthesis are most critical to development of atrophy and hypertrophy since, when exposed to spaceflight or exercise, the magnitude of changes in synthesis are much larger than those of degradation. Consequently, the consensus is that changes in protein synthesis are facilitative, while changes in breakdown are adaptive.

Resistance exercise, in which each effort is performed against a specific opposing force, is singly the most potent hypertrophic stimulus in adult human muscle. This is evidenced by the gross musculature achieved in bodybuilders engaged in routinely lifting heavy weights. The mechanism by which resistance training promotes muscle hypertrophy is chiefly through inducing transient increases in muscle protein synthesis after each exercise bout. The amplitude and duration of increases in muscle synthesis after exercise ranges $\sim$50–300% and lasts $\sim$4–48 h. Though largely unexplored, such a wide range in responsiveness is likely the function of exercise protocol (*e.g.* intensity/duration), nutritional status (*e.g.* post-absorptive/fed), subject characteristics (*e.g.* age, sex), training status and measurement duration. The crucial role for the stimulation of muscle synthesis by exercise in regulating adaptation is demonstrated by data that show that the amplitude of post-exercise increases in muscle synthesis are qualitatively predictive of long-term adaptation (*i.e.* muscle hypertrophy). After resistance exercise, increases in muscle protein breakdown are also observed and these can exceed the magnitude of increases in synthesis. Consequently, resistance exercise in post-absorptive conditions creates a net catabolic state despite increases in synthesis. Importantly this catabolic state is prevented when AA are ingested in close proximity to exercise. This incapacity to stimulate muscle growth without exogenous AA makes sense because one cannot achieve muscle growth without sufficient building materials. This also highlights the important interaction between exogenous AA availability and muscular activity in the regulation of muscle proteostasis.

Interestingly, it seems that muscle protein synthesis is increased only in the "remodeling period" after resistance exercise (as described above) but is actually suppressed during muscular activity. It is likely this suppression is a direct result of cellular energy stress (*i.e.* ATP turnover) because the degree of suppression relates directly to the contraction duty-cycle. Consequently, the adaptive cellular response is to switch off ATP consuming processes such as mRNA translation (which requires 4 ATP per peptide bond) and synchronously switch on catabolic pathways (ATP creating pathways). This highlights the close links between cellular energy status and muscle proteostasis.

5.3.2.1 Extracellular–Intracellular Signaling and Muscular Activity

The molecular mechanism(s) by which contractile activity modulates proteostasis is poorly defined.[30] Most of the various intramuscular signaling molecules have been reported to be activated in response to exercise in some form or other. However, most recent work supports the notion that ostensibly anabolic hormones (*i.e.* testosterone, growth hormone and insulin-like growth factor 1) do not play a significant role, at least in alterations in short-term anabolic signaling and muscle proteostasis after muscular activity. There are several examples of this. Firstly, when the acute responses of muscle protein synthesis to resistance exercise are compared in arm muscles under conditions that either do generate increases in systemic hormones (*i.e.* previous intense exercise of large muscle groups) or do not (*i.e.* no prior exercise) the synthetic responses are identical. This is in spite of gross differences in systemic concentrations of anabolic hormones such as testosterone and growth hormone. Furthermore, muscle hypertrophy after chronic exercise training under these conditions is identical and thus adaptation is independent of the systemic environment. Secondly, when both legs of the same person are trained, one using a resistance training protocol and one using an endurance protocol, both adapt distinctly. That is, the resistance-exercised leg hypertrophies while the endurance trained leg does not, but instead becomes fatigue resistant. Thus the molecular mechanism involved appears to be largely within a given muscle rather than from the systemic milieu (*i.e.* hormones). It also appears that known local factors (autocrine/paracrine) may not play a significant role in the acute responses in muscle proteostasis to contractile activity. For example, while changes in mTORc1 signaling (associated with a variety of peptide growth factors, such as IGFs; see Section 5.2.2.1) are observed in response to contraction, these changes are extremely fast, detectable within seconds, and are PI3K-independent (*i.e.* not going through receptor -> PI3K -> Akt -> mTORc1 pathways). This observation almost certainly excludes short-term receptor-mediated IGF-1/MGF inputs. Perhaps even more strikingly, a functional IGF-1 receptor is not necessary for load-induced skeletal muscle hypertrophy. Further evidence excludes other local factors as having a role because the medium in which stretched muscle is bathed (*i.e.* conditioned medium) does not induce the same anabolic signaling changes as stretch, for example

mTORc1 changes. Collectively, these data show that while signaling through these anabolic pathways is largely linear (*i.e.* ligand receptor → kinase-kinase-kinase → kinase-kinase → kinase → effect) during development or specific disease states, complex non-linear signaling and cross-talk between what were previously thought of as distinct pathways occurs in adults subject to exercise.

Currently it seems that muscles control proteostasis in response to muscular activity largely from within. From the limited data available, it seems likely that physical deformation of the cell (*i.e.* mechanotransduction) and biochemical perturbations (*i.e.* chemotransduction) are key upstream regulators of the phosphoproteins regulating muscle proteostasis. Mechanotransduction is probably sensed though physical transmembrane links (*i.e.* the attachment complex) between the extracellular matrix and the actin cytoskeleton to which many of the anabolic signals may be physically tethered. However, titin, a major structural component of the contractile apparatus, has also been shown to be a stretch-activated kinase. Following stretch activation of the kinase substrates such as nbr1 and p62 are phosphorylated to create binding sites. Notably NFκB signaling can be activated *via* p62, although the relevance of this in human muscle remains to be established. The E3 ligase MuRF2 can also be activated by p62 and upon activation causes serum response factor (SRF) to translocate to the sarcoplasm from the nucleus. SRF acts as a transcription factor and participates in the expression of muscle genes, thus titin may modulate long-term muscle gene expression *via* modulation of SRF, which likely accounts for the presence of hereditary myopathy with early respiratory failure in individuals with mutations in titin kinase. Despite the promise of titin as a mechanosensor within muscle, this mechanism does not currently explain the activation of known anabolic signals in response to exercise.

On the basis of chemotransduction, muscular activity has been associated with many metabolic changes. As examples, muscular activity generates alterations in ion movements *via* opening of stretch-activated channels (SAC) in the plasma membrane and fluctuations in SR-derived $[Ca^{2+}]i$ to facilitate contraction. Also increased ATP turnover during contraction produces metabolites, which are also able to modulate cellular signaling processes. Furthermore, as a by-product of increased ATP turnover, reactive oxygen species (ROS) are generated, which function to activate redox-sensitive pathways such as NFκB (see Section 5.2.2.4). Finally, production of lipid second messengers derived from the plasma membrane, which can be damaged during contraction, regulates cell signaling processes.

While the precise control of muscle proteostasis in human muscle subject to exercise remains elusive, there are several lines of evidence from *in vitro* studies that the anabolic signals observed are mechano-/chemosensitive (see Figure 5.2 for summary scheme). For example, inhibition of extracellular Ca^{2+} influx through SAC inhibits mechanical stretch-dependent signaling to p70S6K1, suggesting that extracellular Ca^{2+} is required for signaling to mTORc1. Likewise, disruption of the actin cytoskeleton using cytochalasin D suppresses stretch-induced signaling to p70S6K1 *via* altering the dynamics of the actin cytoskeleton. Indeed, a direct link from the cytoskeleton to signaling activity

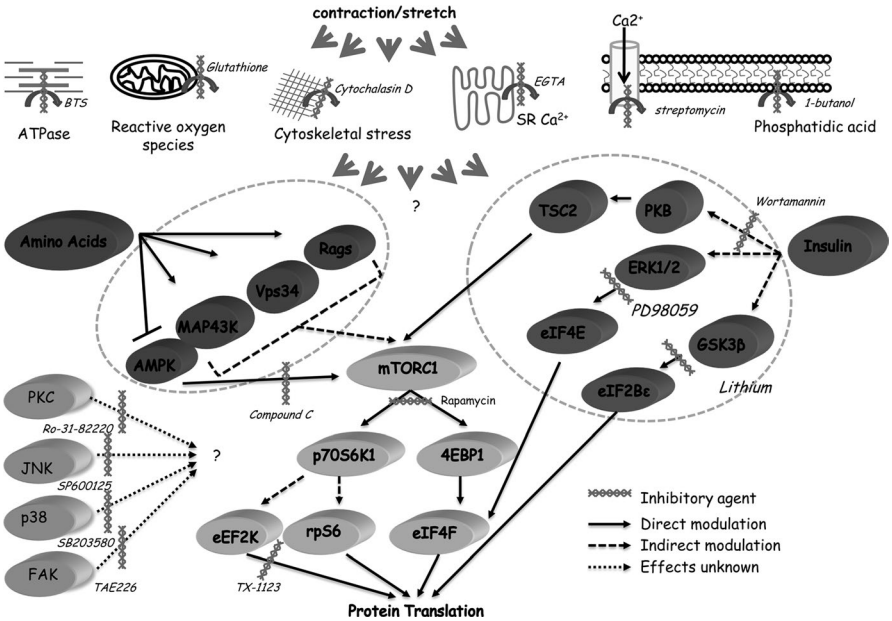

Figure 5.2 Extracellular/intracellular signaling in the regulation of muscle proteostasis by muscular activity and nutrients.

may be in part because some signaling proteins are physically immobilized to the cytoskeleton and are thus sensitive to alterations in cytoskeletal dynamics. In addition, inhibition of contraction induced phospholipase D (PLD1) activity reduces production of the lipid second messenger, phopsphatidic acid (PA) thereby attenuating signaling to p70S6K1 and muscle protein synthesis. On the other hand, inhibiting ATPase activity during contraction with *N*-benzyl-p-toluenesulfonamide suppresses Ca^{2+}-mediated increases in eEF2 phosphorylation (which normally acts to suppress protein synthesis) and in doing so attenuates the blunting of protein synthesis normally seen during muscle contraction. Also, increases in cellular AMP : ATP ratios during contraction promotes activation of AMPK, which can have a negative influence on protein synthesis. Unfortunately, although the potential exists for the influence of other factors generated during contraction such as ROS and other purinergic signals (UTP, ADP *etc.*) to modulate muscle proteostasis this remains to be functionally confirmed. Nevertheless, once again these findings again highlight the close relationship between energy and protein metabolism.

5.4 Conditions Associated with Alterations in Muscle Proteostasis in Humans

Amino acids are released from muscle protein during wasting conditions such as aging, starvation, sepsis, chronic obstructive pulmonary disease (COPD), thermal

Table 5.1 Alterations in proteostasis under conditions of muscle wasting in humans.

Condition	*Postabsorptive synthesis*	*Postabsorptive breakdown*	*Anabolic response to feeding*
Starvation	↓	↓ (↑)?	?
Trauma/sepsis	↓ or ↑	↑	?
Burns	↓	↑	?
Cancer cachexia	↓	↓(↑)?	↓
Muscular dystrophy	↓	↓	?
Sarcopenia	=	=	↓
Resistance exercise	↑	=or ↑	?

injury (burns), acute trauma, HIV/AIDS, diabetes, renal failure and some aggressive forms of cancer (*i.e.* pancreatic). Under these circumstances muscle wasting may be extremely rapid *i.e.* noticeable within days. On the other hand, loss of muscle protein with aging as discussed below is a slow, incipient process. Depending on the catabolic insult and its severity, loss of muscle mass results from decreased, normal or even increased protein synthesis, which in the latter case remains insufficient to compensate for higher proteolysis. Changes in proteostasis that occur in a variety of muscle wasting conditions are illustrated in Table 5.1.

Is there a physiological foundation for muscle breakdown under pathological circumstances? In short the answer is yes, because release of amino acids from muscle provides substrate for hepatic gluconeogenesis, supports acute phase protein synthesis and the immune system (*i.e.* high rate of glutamine utilization in lymphocytes, macrophages and neutrophils), and is an important energy source to enterocytes. As such, at least in the short term, this is an adaptive response that is beneficial to support the function of certain vital tissues, for example during the acute phase of sepsis.

However, during situations of sustained muscle wasting such as severe protracted sepsis, cancer cachexia or burn injury, the benefits of muscle loss will be outweighed by the costs. This is for several reasons. Firstly, as a consequence of muscle weakness, ambulation is delayed and as such this increases thromboembolic episodes. Moreover, forced inactivity due to muscle weakness (*i.e.* disuse atrophy) further exacerbates muscle wasting by superimposing onto the initial cause of muscle loss. Muscle wasting of respiratory muscles also increases reliance upon ventilatory support, which leads to increased weaning difficulties and, consequently, morbidity and mortality. Therefore, muscle wasting exacerbates clinical outcome. In fact, loss of ~30% of muscle mass results in death. An extensive discussion of the regulation of atrophy in all situations of muscle loss is not possible due to the large numbers of pathological situations associated with muscle wasting; as such only a select number are discussed.

5.4.1 Effects of Aging on Muscle Proteostasis

Aging is accompanied by a loss of skeletal muscle mass, which is defined as sarcopenia, a word adopted from Greek roots *i.e.* "*sarx*" for flesh and "*penia*"

for loss. Although the cause(s) of sarcopenia are unknown, unlike with disuse atrophy (as will be discussed later; Section 5.4.2), aging is not associated with gross changes in protein turnover during fasted/post-absorptive periods (*i.e.* depressions in synthesis or increases in breakdown).[26,31,32] Rather, it has recently come to light that aging is associated with anabolic resistance to feeding. In a nutshell this means that when exposed to equivalent amounts of AA or insulin, the capacity to increase protein synthesis and reduce protein breakdown, respectively, are diminished in elderly individuals.

The evidence for this is as follows. As previously discussed, EAA are the major drivers of muscle synthesis and the first demonstration of a decreased sensitivity and capacity for increasing muscle synthesis was made during oral feeding of EAA to men aged 65–75 years when compared to young men. For example at doses of EAA of 10 and 20 g the elderly individual's increases in synthesis were blunted by about 50%. A possible explanation for this was reduced concentration and phosphorylation (*i.e.* capacity and efficiency) of the mTORc1 substrates, p70S6K1 and 4E-BP1. More recently we also identified that this blunting is not restricted to the muscle protein synthesis (MPS) arm of turnover. This is because in older individuals, the $\sim$50% inhibition of muscle breakdown in response to a modest rise in insulin availability is also blunted. As such there is anabolic resistance in both arms of protein turnover after feeding, which we propose contribute to or even cause sarcopenia. As was mentioned earlier, with aging, there are no major alterations in muscle proteostasis under post-absorptive conditions to explain sarcopenia (*i.e.* breakdown > synthesis). Perhaps it makes sense that small reductions in protein accretion after feeding may instead regulate sarcopenia in view of the slow, incipient wasting with which it is associated. Indeed, because protein turnover under post-absorptive conditions predominates a diurnal cycle ($\sim$19 *vs.* $\sim$5 h) sarcopenia should be much more rapid if differences in post-absorptive turnover were apparent.

Significantly it was recently shown that, as for feeding, protein synthesis responses to resistance exercise are lower in older than younger men across a range of intensities when matched for work. This likely explains the observation that muscle hypertrophy is less after training in old *versus* young humans. These observations further add weight to the concept of anabolic blunting being key in the regulation of age-related muscle wasting.

5.4.2 Disuse Atrophy

Skeletal muscles house some 40% of all protein in the body of a healthy human but this store is depleted when habitual mechanical input (*i.e.* standing, walking *etc.*) is removed.[33,34] The loss of muscle associated with muscular inactivity is collectively known as disuse atrophy and is the product of a reduction in muscle CSA and length. Disuse atrophy occurs under many ground-based situations of reduced neural input such as whole-leg casting after fractures/breaks, chronic bed-rest during hospitalization, denervation due to spinal cord injury, chronic sedentarism in aging populations, but also during space-flight.

As a consequence of muscle atrophy, there is loss of strength and reduced capacities of whole-body glucose storage and metabolism, which contribute to insulin resistance. Rates of muscle loss in all models of disuse are fastest within the first 30 days with a mean loss in muscle CSA of ~0.6% per day.

Muscle atrophy in disuse and indeed any other wasting situation (*e.g.* aging, cancer, sepsis *etc.*) must ultimately be regulated by changes in proteostasis that favor a net loss of tissue (*i.e.* muscle protein synthesis is exceeded by muscle protein breakdown). The prevailing view is that reductions in synthesis, not increases in breakdown, cause disuse atrophy in humans (though the latter has not yet been measured). This is because impairments in muscle synthesis are likely sufficient to explain the observed muscle loss without the need for substantial increases in muscle breakdown. For example, unlike muscle loss with aging, disuse is associated with reductions in muscle synthesis under post-absorptive conditions. Indeed, the first demonstration of this was a reported decrease of ~25% in post-absorptive rates of muscle synthesis during leg-cast immobilization. Since then, these findings have been substantiated in other immobilization studies with the most recent study showing ~50% reductions in synthesis of quadriceps muscle both 10 and 21 days after unilateral leg immobilization. Furthermore, the blunting of MPS is not restricted to post-absorptive periods. Indeed, as with aging, increases in muscle synthesis during infusion of amino acid infusions are severely blunted in immobilized human legs. Therefore, when coupled to reductions in post-absorptive muscle synthesis, this would strengthen the evidence for impairments in muscle synthesis being the key cause of human disuse atrophy. Furthermore, these findings are yet another example of anabolic blunting, adding to what was previously reported in aging and which likely contributes to atrophy in other conditions (*i.e.* aging, chronic obstructive pulmonary disease, cancer, type 2 diabetes *etc.*).

What causes blunted muscle synthesis in disuse under post-absorptive conditions or after feeding is not known. Unlike in aging, those signals typically associated with the acute upregulation of MPS after increasing AA availability to muscle (*i.e.* Akt/mTOR signaling) are neither suppressed under post-absorptive conditions nor blunted in fed conditions when comparisons are made between immobilized and non-immobilized legs at 14 days. Large-scale gene expression analyses (microarrays) gathered from these studies indicate that the largest downregulation of functional gene sets at both 2 and 14 days were those encoding for proteins representing all facets of mitochondrial function, including the key mitochondrial gene transcriptional co-activator peroxisome proliferator co-activator 1 (PGC1α). Since over-expression of PGC1α protects against disuse atrophy in rodents (and sarcopenia) it is plausible that sustaining mitochondrial volume prevents decreases in muscle synthesis. The second notable down-regulation was in genes encoding for RNA/proteins regulating the capacity for muscle synthesis (*i.e.* initiation factors, ribosomal units). Thus it may be that a reduction in translational capacity rather than efficiency (*i.e.* phosphorylation) regulates muscle loss in response to disuse.

Whether or not increases in muscle protein breakdown contribute to atrophy in human disuse remains contentious. Calculations based upon the blunting of

synthesis over a diurnal cycle suggest that increases in breakdown are not required to explain the observed muscle loss. For example, normal turnover is 0.05% h^{-1} or 1.2% d^{-1} where synthesis and breakdown are equal and opposite. Based on data showing that muscle synthesis increases 2.5-fold after a maximally effective feed with the increase only lasting 1.5 h, it is likely that $\leq$5 h/day is spent in ''fed'' periods where muscle is gaining protein (assuming three good meals). Thus, based on conservative assumptions from previous findings in disuse in which muscle synthesis is suppressed $\sim$50% in both post-absorptive and fed periods then diurnal protein accretion would be: $(0.025 \times 19) + (0.025 \times 1.25 \times 5) = 0.63\%$ per day. If muscle protein breakdown remained constant then muscle would be lost at a rate of: $1.2 - 0.63 = 0.57\%$ per day. This is indistinguishable from the typically measured $\sim$0.6% per day over the first 30 days, which suggests that increases in muscle breakdown are not necessary to explain human disuse atrophy. Nevertheless, some work has provided some secondary evidence from static markers (*e.g.* increased expression of proteasomal subunits) for short-term increases in degradation. For example, increases in interstitial 3-methylhistidine (a marker of myofibrillar breakdown) was reported to occur 3 days after immobilization as was upregulation of total ubiquitinylation and mRNA for the E3 ubiquitin ligases and so-called ''atrogenes'', muscle ring-finger 1 (MuRF1) and muscle atrophy F box (MAFBx), which have been suggested as a common mechanism for muscle wasting (see ref. 35 for further detail). Additionally, other workers have shown that during short-term immobilization, for 5 days and after spinal cord transection in patients, there are increased amounts of markers of increased protein breakdown, in terms of both mRNA and protein. Furthermore, in animals, there is also evidence that acute disuse may cause damage to mitochondria, leading to the generation of ROS and oxidative stress, which themselves have been purported to increase apoptotic and proteolytic processes in muscle. One possibility that has been overlooked is that such increases in markers of breakdown are not necessarily regulating ''bulk'' increases in protein breakdown, but rather targeted degradation. For example, MAFbx targets the eukaryotic initiation factor 3 subunit 5 (eIF3-f) for ubiquitination and degradation by the proteasome and since mTOR and p70S6K1 interact directly with eIF3-f to mediate assembly of the translation pre-initiation complex this could be the explanation for reduced muscle synthesis, rather than proposed increases in protein breakdown.

5.4.3 Sepsis

Sepsis due to infection induces whole body inflammation and is a major cause of comorbidity and mortality in critically ill patients and is associated with and exacerbates acute trauma and thermal injury.[36,37] Wasting of body muscles is associated with sepsis and this can directly contribute to the morbidity and mortality, particularly when the respiratory muscles are involved. While sepsis has negative consequences and is associated with illness, the induction of the inflammatory state is presumably adaptive in as much as liberation of energy

from various tissues can assist in healing. As such, there is a large induction of glucocorticoids, proinflammatory cytokines and increases in cellular Ca^{2+} in sepsis. Collectively, this results in increased protein degradation. This increase is predominantly *via* proteasome-mediated degradation and is associated with upregulation of MURF-1 and MAFbx mRNA, likely due to a downregulation in PI3K/Akt signaling. However, both the calpains and caspases are activated and thought to initiate breakdown of myofibers with final degradation occurring *via* the proteasome. As cytokines all promote degradation *via* the proteasome, these factors are presumably extramuscular signals responsible for triggering muscle degradation in septic patients. However, decreased protein synthesis and mitochondrial dysfunction have been observed in septic patients. Presumably the decreased protein synthesis could be the result of elevated cortisol, TNFα and/or Ca^{2+} in septic patients as a result of degradation of key pieces of the transcriptional and translational machinery (for example transcription factors, initiation and/or elongation factors). Indeed, both glucocorticoid and Ca^{2+} antagonists (RU38486 and dantrolene, respectively) have efficacy in reducing muscle wasting in experimental sepsis. Similarly, mitochondrial dysfunction and altered plasma membrane conductance could be due to increased membrane and/or protein damage arising from the increased ROS being present in inflamed tissue as the result of release from inflammatory cells (for example macrophages and neutrophils).

5.4.4 Burns

Burn patients often enter a hypermetabolic state where energy is used in healing the wound and raising core body temperature.[38] Typically this hypermetabolic state is observed for burns covering 10% or more of the total body surface area and there is a proportional relationship between size of the burn and the resting metabolic rate. When left untreated the muscle wasting associated with burn contributes to both comorbidity, for example infection, and mortality, particularly in patients with larger burn surface areas. Successful treatment modalities point to the complexity of the muscle wasting observed.

Muscle wasting appears to occur *via* at least four distinct mechanisms. Firstly, in the immediate post-burn period there is a hypercatabolic state, which includes increased muscle protein degradation. The initial hypercatabolic state can be eased by excision and closure of the burn, for example a 40% reduction for large burn surface areas that are excised and covered after two to three days *versus* after one week. This suggests that at least part of the catabolic state is directly tied to the increased requirement for heat production. While muscle is the major metabolically active tissue in the body and therefore the major producer of heat due to inefficiency of running oxidative phosphorylation, the notion that human muscle functions to produce heat is controversial and therefore the mechanisms controlling increased heat production are currently largely unknown. Presumably increased mitochondrial uncoupling occurs in muscle *via* increased expression of uncoupling proteins. Thyroid hormone and epinephrine which, as stress-induced hormones, are likely both elevated

immediately post-burn are both capable of inducing uncoupling protein expression in muscle. These hormones presumably also trigger a net negative protein balance (see Sections 5.2.1.2 and 5.2.1.4 for putative mechanisms) with the energy liberated being used to produce heat *via* less efficient oxidative phosphorylation. The ability to attenuate the hypermetabolic state immediately post-burn by use of beta blockers highlights the role of epinephrine as a key inducer of the hypermetabolic state immediately post-burn. Secondly, increased caloric intake can maintain lean body mass while loss of lean body mass results in delayed healing time. This suggests that at least part of the catabolic state is directly tied to increased caloric demand associated with the healing process. Presumably the signals regulating entry into the catabolic state due to nutritional insufficiency are both decreased plasma AA levels and, consequently, decreased insulin levels (see Section 5.3.1 for details of signals which are presumably lacking). Thirdly, sepsis is quite common after thermal injury and can elevate the metabolic rate by an additional 40% (mechanisms and interventions for sepsis are discussed in Section 5.4.3 above). Fourthly, once patients have recovered from the initial burn injury and/or sepsis stages, resistance exercise training and hormone management have both been shown to improve muscle mass (see Section 5.3.2 for putative mechanisms underlying exercise actions on muscle mass). Because insulin and testosterone levels often drop in response to the burn and recombinant growth hormone (stimulates IGF-1 production), IGF-1, insulin and testosterone (or oxandrolone, which has much less potent androgenic effects) all can counteract muscle wasting, loss of inhibition of protein degradation by insulin/IGF-1 seems a likely contributor to longer-term muscle loss post-burn (see Sections 5.2.2.1 and 5.2.1.3 for insulin/IGF-1 and testosterone mechanisms, respectively).

5.4.5 Cancer Cachexia

Patients with pancreatic or gastric cancer have the highest frequency of weight loss, while patients with non-Hodgkin's lymphoma, breast cancer, acute non-lymphocytic leukemia and sarcomas have the lowest frequency.[39,40] Myosin heavy chain is selectively degraded by the ubiquitin proteasome pathway in the cachectic state, while other core myofibrillar proteins including troponin T, tropomyosin (α- and β-forms) and sarcomeric actin remain unchanged. As with thermal injury and sepsis, glucocorticoids may play a role in the development of cancer cachexia, although adrenalectomy has been shown not to alter the course of cachexia in other animal models, which argues against this. In addition, there is considerable evidence from animal studies that TNFα and IL-6 play a role in muscle loss in cancer cachexia, although its role in the human condition may be more questionable. Further discussion of these is unwarranted as their upregulation likely follows a similar track to sepsis and burns. On the other hand, highly specific to tumors is the production of proteolysis-inducing factor (PIF), a 24-kDa molecular mass sulfated glycoprotein, originally isolated from the cachexia-inducing MAC16 tumor. PIF has also been shown to inhibit protein synthesis and stimulate protein degradation

directly in isolated murine myotubes. Moreover, urinary PIF excretion is related to weight loss in patients with prostatic and primary gastrointestinal tumors. Mechanistically, PIF expression leads to increased proteasome expression (20S proteasome subunits, MSS1 and p42, another ATPase subunit of the 19S regulator, as well as an increased chymotrypsin-like enzyme activity of the β5 subunits of the proteasome) and activity is likely *via* inducing NFκB in a ROS-dependent manner. Indeed, studies in animal models of cancer cachexia, as well as in cancer patients, suggest that the ubiquitin proteasome pathway plays the predominant role in the degradation of myofibrillar proteins, particularly in patients with a weight loss of $>10\%$.

Depressed protein synthesis during cancer cachexia in skeletal muscle is related not only to anorexia, as protein synthesis is also depressed in other animal models of cachexia where anorexia is absent. This suggests an underlying defect in the protein synthetic machinery. For example, gastrocnemius muscles from mice bearing the cachexia-inducing tumor MAC16 show activation of protein kinase R (PKR) when the weight loss is $>16\%$ and a corresponding increase in phosphorylation of eIF2α (an inhibitor of eIF2Bε), which would suppress mRNA translation. Weight loss in mice bearing the MAC16 tumor is also associated with an increased amount of eIF4E bound to 4E-BP1 in gastrocnemius muscle, due to hypophosphorylation of 4E-BP1, resulting in a progressive decrease in the concentration of the active eIF4G-eIF4E complex. This would also contribute to a depression in protein synthesis, as would also a decrease in phosphorylation of mTOR and p70S6K1. There is also an increase in the phosphorylation of eEF2, which would also decrease protein synthesis through a decrease in translation elongation.

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CHAPTER 6

Contact Normalization: Mechanisms and Pathways to Biomarkers and Chemotherapeutic Targets

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6.1 Introduction

About 12% of American women are likely to be diagnosed with breast cancer within their lifetimes.[1] However, studies of healthy women, without any apparent risk for breast cancer, find that nearly half of them harbor genotypically abnormal and possibly premalignant mammary epithelial cells.[2] Other studies have identified microadenomas in intestines[3] and skin[4,5] from significant numbers of healthy humans and animals. Interestingly, most microadenomas do not progress into macroscopic tumors. Histological studies have found that microadenomas surrounded by normal cells retain their transformed genotype, but assume a normal morphology.[6–8] This phenomenon, first reported in polyoma transformed cells by Stoker *et al*. in 1966, is known as contact

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normalization.[9] It is the process by which tumor cell growth is normalized by contact with neighboring non-transformed cells.

6.2 Contact Normalization

Contact normalization is a powerful process. To become malignant and metastatic tumor cells must overcome inhibition by contact normalization. Cells transformed by a variety of chemicals,[10] viral agents[9] and oncogenes including Src[11–15] can be normalized by contact with non-transformed cells. This process is clearly exemplified by malignant tumor cells that form normal adult organs when injected into mouse blastocysts.[16] Demonstrations that a population of tumor cells can be normalized by as little as three times as many non-transformed cells illustrate the power of this process.[17,18]

Contact normalization is an important process *in vivo*. Genetically transformed cells can assume a normal morphology and reside in many organs including skin,[4,5,19] breast[2,20] and intestine.[3] Moreover, since these "occult tumor" cells are phenotypically normal, they tend to resist chemotherapy.[21–23] As stated above, contact normalization is a powerful process; transformed keratinocytes that comprise up to 4% of epidermal volume can be controlled in human skin for decades.[24]

Taken together, a number of studies indicate that contact normalization is mediated through direct contact between transformed and non-transformed cells.[9,15,25–29] Direct contact between cells is mediated by intercellular junctions such as adherens and gap junctions. Though much remains to be elucidated, progress has been made in identifying mediators involved in this process. While not essential, gap junctions appear to facilitate the process of contact normalization.[7,8,15,29] In addition, a number of genes associated with contact normalization have been identified.[29–33]

6.3 Cadherins

Cadherins form intercellular junctions that are required to maintain normal cell architecture. Cadherins are tethered by catenins to the actin cytoskeleton. Cadherin junctions are disrupted by tyrosine phosphorylation of β-catenin. In addition to disturbing cell morphology, disruption of cadherin junctions allows β-catenin to enter the nucleus and participate in mitogenic transcriptional signaling events.[34,35]

Cadherins are tumor suppressors. Aberrant cadherin expression is often found in tumor cells.[36,37] Interestingly, loss of cadherin expression was found necessary, but not sufficient, for tumor cell invasion in genetic screens for metastatic behavior of tumor suppressor genes in a *Drosophila* model.[38] As expected, expression of wild-type cadherins can suppress transformed cell growth,[39] while expression of dominant negative cadherins can enhance cell invasion.[40]

Epithelial-mesenchymal transition (EMT) is a characteristic event seen in many epithelial malignant tumors. The loss or decrease of E-cadherin expression results in the dedifferentiation, loss of epithelial morphology and increase of cellular motility. In the process of EMT, differentiated epithelial cells acquire attributes that are similar to embryonic mesenchymal cells.[41]

Like E-cadherin, N-cadherin is also involved in cell adhesion, differentiation and invasion.[42] As with other "classic" cadherins, protein kinase, exemplified by Src, can phosphorylate β-catenin to break N-cadherin junctions with adjacent cells.[43–45] In addition, tumor promoting proteases can disrupt N-cadherin junctions to induce β-catenin nuclear signaling.[46] It should be noted that N-cadherin can promote the growth of some epithelial tumor cells.[42] However, N-cadherin can suppress the growth of many other tumor cells including osteosarcoma,[47] ovarian carcinoma,[48] glioblastoma and astrocytoma.[49–51]

6.4 Gap Junctions

Gap junctions form aqueous channels that connect the cytoplasm of adjacent cells. These channels are formed by integral membrane proteins called connexins. Connexins have evolved into a family of at least 20 mammalian members, which are commonly named by their predicted molecular weights.[52–54] Gap junctions allow adjacent cells to share intracellular signals and function in a coordinated fashion.[55–57]

Evidence indicates that connexins play an important role in cell growth control.[58] Like cadherins, experiments have identified connexins as tumor suppressor genes.[59–61] In general, gap junctional communication is blocked between transformed cells.[62,63] For example, Cx43 expression is robust in normal glial and mammary epithelial cells, but repressed in some human glioma and mammary carcinoma cells. Moreover, restoration of Cx43 expression can normalize the growth of human glioma and mammary carcinoma cells.[61,64,65]

Results from experiments with connexin knockout cells and chemical blockers indicate that gap junctional communication is not required for contact normalization.[29] This is consistent with previous reports of contact normalization in the absence of dye transfer between transformed and non-transformed cells.[14,66] However, while gap junctions are not absolutely *required* for contact normalization, many reports suggest that gap junctional communication *augments* the ability of normal cells to control the growth of neighboring tumor cells.[15,26,64,67,68] This has been demonstrated for cells transformed by a variety of agents.[12–15]

Cx43 can augment the contact normalization of some transformed cells. We have previously shown that inhibiting gap junctional communication with a Cx43 anti-sense construct curtails the ability of non-transformed cells to normalize Src transformed cells in coculture.[15] We have also shown that non-transformed cells normalize gap junctional communication with adjacent Src transformed cells.[15,69] Thus, signals passed between transformed and non-transformed cells may help normalize the growth of coupled cells.

For example, we have shown that Cx43 can effectively equilibrate levels of the high-energy metabolite ATP throughout a population of cells. In this way, gap junctional communication could prevent an individual tumor cell from acquiring a level of metabolic energy required to undergo mitosis.[55,56]

Src phosphorylates Connexin43, but may require downstream events to block gap junctional communication. Like Cas and β-catenin, Cx43 is a functionally relevant Src substrate. Src phosphorylates Cx43 on critical tyrosine residues, and this event can reduce intercellular communication.[70–73] However, modification of tyrosine residues 247 and 265 to glutamate does not affect channel function.[74] It has become apparent that Src requires other factors to block gap junctional communication mediated by Cx43. For example, MAPK acts downstream of Src to phosphorylate Cx43 and actually close the gap junction channel. In addition, other components may be involved since potential SH3 binding domains on Cx43 are required for channel closure in Src transformed cells.[75] Src and the focal adhesion adaptor protein Cas both possess SH3 domains.[76] In addition to Src, Cas associates with Cx43, and Src utilizes Cas to block gap junctional communication between transformed cells.[77,78]

As described above, junctions formed by Cx43 and cadherins are disrupted by oncogenic protein kinases in transformed cells. In addition, tumor promoters such as TPA also disrupt Cx43 and cadherin junctions.[79] Interestingly, cadherins are required for Cx43 assembly and function in some cells. Disruption of junctions formed by N-cadherin can block gap junctional communication.[80] Moreover, induction of N-cadherin has been shown to increase Cx43 expression while inhibiting tumor cell growth.[81]

6.5 Contact Normalization and Tumor Suppressors

Comprehensive analysis of gene expression has found that less than about 0.01% of the transcriptome is affected by contact normalization. The expression of most of these gene products is inhibited in transformed cells and induced during contact normalization. Some of these genes can act as tumor suppressors.[29,30]

Fhl1 and Sdpr provide examples of tumor suppressors that are induced by contact normalization. Fhl1 consists of four-and-a-half LIM domains. Fhl1 can move between intercellular junctions,[82] focal adhesions and the nucleus,[83] to affect gene expression.[84,85] For example, Fhl1 associates with the RBP-J DNA binding protein to modulate gene transcription.[84,85] Sdpr is a phosphatidylserine-binding protein[86] that is induced during growth arrest by serum deprivation of non-transformed cells, but not transformed cells.[87] Fhl1 and Sdpr expression is suppressed in some human tumors including those of the breast, kidney and prostate.[32,33] Fhl1 is a functionally relevant protein that inhibits anchorage-independent growth and migration of transformed cells.[33] Interestingly, in addition to blocking gap junctional communication,[77,78] Src utilizes the Cas adaptor protein to suppress Fhl1 expression.[33] This relationship

bears special significance in light of the fact that Src phosphorylates Cas to promote tumor cell growth and migration.[88–94] Cas is an important component of the focal adhesion complex signaling network,[95] which also includes FAK, Grb2, Shc and paxillin.[96,97] After phosphorylation by Src, Cas can bind to other proteins including Crk, PI-3-kinase, Nck and PLCγ.[98–100]

In addition to genes transcribed into mRNA, alterations in miRNA expression have also been associated with contact normalization. These miRNAs have the ability to downregulate the expression of target genes by binding to the 3′ untranslated region of mRNA.[101] Some miRNA species, called "oncomirs", can affect tumor formation by targeting gene products that affect cell growth and migration.[102,103] Analysis of miRNA expression in transformed, non-transformed and contact normalized cells suggests that approximately 1% of these oncomirs can be affected by contact normalization.[31]

miR-126 provides an example of an oncomir affected during contact normalization. Interestingly, miR-126 can suppress the expression of Crk, which is a focal adhesion adaptor protein that cooperates with Cas to promote non-anchored tumor cell growth and migration. Some transformed cells express lower levels of miR-126 and higher levels of Crk than non-transformed or contact normalized cells. Moreover, miR-126 expression suppresses Crk expression and inhibits transformed cell growth and migration. Evidently, contact normalization induces miR-126 to target Crk production and inhibit the migration of neighboring transformed cells.[31]

6.6 Contact Normalization and Tumor Promoters

In contrast to tumor suppressors, non-transformed cells can inhibit the expression of specific tumor promoters, including Pdpn, Vegfr2 and Tmem163, in neighboring cancer cells. For example, we have recently reported that Src utilizes Pdpn to promote tumor cell migration, and that Pdpn expression is inhibited in contact normalized cells.

Tmem163 is predicted to form (Figure 6.1) an integral membrane protein with six transmembrane helices. Interestingly, elevated Tmem163 mRNA expression has also been found in papillary thyroid carcinoma (GEO DataSet Browser: GDS1732/1552626_a_at/Tmem163/homo sapiens) and nodular lymphocyte-predominant Hodgkin's lymphoma.[104] Moreover, recent experiments indicate that Tmem163 can significantly increase cell growth and migration.[30]

Vegfr2, also called kinase insert domain receptor (Kdr), clearly promotes tumor cell migration and angiogenesis, and is already a prime target for chemotherapy.[105,106] For example, inhibitors have been generated to target in order to prevent VEGF signaling and suppress angiogenesis required for malignant tumor growth.[107]

Pdpn belongs to the type-I transmembrane sialo-mucin-like glycoprotein, which consists of an ectodomain having abundant O-glycosylation sites, a highly hydrophobic membrane spanning domain and a short intracellular domain of nine amino acids.[108,109] Increased Pdpn expression has been

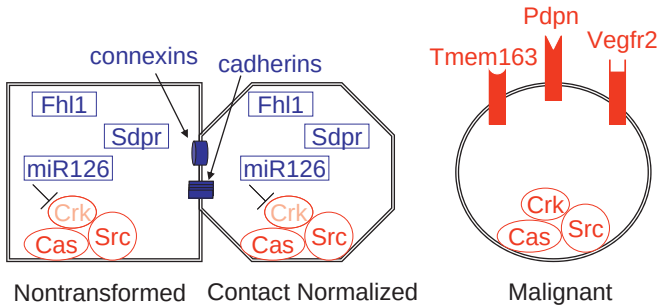

Figure 6.1 Schematic of some effectors of contact normalization. Non-transformed cells utilize junctional communication to induce the expression of tumor suppressors (*e.g.* miR-126, Fhl1 and Sdpr) and normalize the growth and morphology of neighboring transformed cells. Tumor cells must escape this form of growth control to express tumor promoters (*e.g.* Tmem163, Pdpn and Vegfr2) and realize their malignant growth potential.

reported in several human cancers including angiosarcoma, mesothelioma, squamous cell carcinoma of oral cavity, lung, skin, head and neck, glioma and gastrointestinal stromal tumors.[108,110–114]

Like Vegfr2, Pdpn also holds promise as a chemotherapeutic target. Antibodies against Pdpn and a potential Pdpn ligand (tetraspanin CD9) can inhibit lung metastasis of Chinese hamster ovary and human sarcoma cells transfected with human podoplanin.[115,116] Another strategy may be to block glycosylation-dependent interactions of podoplanin with ligands like Galectin-8.[117]

6.7 Conclusions

A major problem with most current cancer treatments is that they are not very specific for cancer cells. Anthracyclines, taxanes and nucleotide analogs (such as doxorubicin, taxol and fluorouracil) target dividing cells. This general toxicity doxorubicin, taxol and fluorouracil causes significant damage to many organs including those of the digestive and immune systems.[118–120] The success of more targeted therapies demonstrates the utility of targeting specific proteins expressed by malignant cells. These reagents include monoclonal antibodies that target Vegf and other oncogenic kinase activities.[105,106,121,122] Understanding how contact normalization operates activities can provide us with helpful information on how tumors first form. Identifying key players in this process could provide more specific biomarkers and chemotherapeutic targets for malignant and metastatic cancers.

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CHAPTER 7

Involvement of Adipokines in Migraine Headache

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7.1 Introduction

In the context of the holistic themes of this book, migraine headache has a very fine fit. Historically, it has been immensely misunderstood, in terms of both its biological underpinnings and the psychological and societal contexts of the disorders that, in combination, are classified as migraine headaches. Attempts at identifying singular triggers, psychological parameters and biological targets of drug therapy have largely been frustrating. Despite some profound successes in the last two decades for acute migraine pharmacotherapy, there is no question that new, creative approaches are necessary to find efficacious, new drugs, especially prophylactics.[1]

While we are far from understanding all the interrelationships that play out to cause real pain in migraine sufferers, it is increasingly clear that complex and decidedly non-linear processes, in a mathematical sense, combine to give the known subtypes of the headache. Regulatory events within cells and control pathways between cells that have grown pathological underlie these disorders. In this chapter, we will explore what is known, and what needs to be known about these sophisticated interrelationships.

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7.2 Background on Migraine Headache

Migraine headache is a spectrum of cerebrovascular disorders that result in widespread suffering and long has been a challenge to medical therapeutics.[2,3] If the pain and distress itself is not enough, in the United States alone the economic impact for lost work productivity and treatment is estimated to be billions of dollars annually.[4] While for many years the genesis of migraine pathophysiology was largely unknown, by the 1960s, mostly due to the presence of anti-migraine ergot alkaloids (*e.g.* ergotamine, ergonovine, methysergide and dihydro-ergotamine) with complicated tryptamine structures, serotonin (5-hydroxytryptamine; 5HT) began to take a lead in mechanistic speculation.[5] Although 5HT may now be recognized more in a modulatory role rather than a central position in the pathogenesis of migraine headache, in the context of multi-faceted signaling networks, which are the theme of this chapter, 5HT is involved in a number of the pathways common to migraine and other related disorders. 5HT may be a crucial integrative factor with respect to these diverse networks.

Serotonin is widely active in nature as a regulator and modulator of physiological systems *via* nervous, hormonal and autacoidal means.[6–10] Of the seven known classes of 5HT receptors (R), receptors from G protein-coupled categories (5HT1 and 7; especially 5T1a, 1d, 1e and 1f; and 5HT2; especially 5HT2a) have been particularly prominent in ideas underlying headache disorders and in drug development.[11] G protein coupling of these receptors is an important characteristic in that these transducers are common to many other non-serotonin receptors and thus keys to shared communication. To date, the lone category of 5HTR ionophores (5HT3) has not been implicated other than in the nausea and vomiting that often accompanies migraine, but that issue will be addressed later in this discussion (see astrocyte section 7.4; 5HT3R is mentioned there in the context of cultured astrocytes).

Development of the triptan 5HT1d receptor agonists (such as sumatriptan) from knowledge of the actions of the semi-synthetic ergot derivative methysergide[5] led to substantial improvements in treatment of acute migraine. Although drugs like methysergide (a 5HT2aR antagonist) have considerable efficacy as migraine prophylactics, toxicity is a definite concern, and preventative treatment remains problematic. A serious problem with the ergot drugs, especially in the past, when they were used much more in migraine than they are today, was the inability of some patients to adhere to dose and time constraints considering the drugs' toxicities (this was especially true of methysergide in which "drug vacations" were absolutely necessary for safe utilization of the drug for long-term use).

Ethnopharmacologic approaches to migraine and related headaches have been employed both culturally and in a more systematic and recent scientific vein.[12] An example would be the migraine prophylactic, feverfew, which has been studied *in vitro*[13] as well as in clinical studies that support its efficacy. While the overall scientific vigor of studies analyzing alternative *versus* "conventional" approaches to migraine therapy is still controversial,[14] the use of

multi-faceted (a crude or semi-crude mixture of compounds from a natural product) herbal drugs to treat interconnected regulatory pathways in headache is appealing, and, at the very least, justifies considerably more research effort to validate the putative efficacies (see the brief discussion of this topic using cannabis as an example in the introductory chapter of this book).

An interesting development in this regard is increasing evidence that serotonin[15] as well as dopamine (DA) plays a role in the brain's reward system. This may be at least partially explanatory for abuse potential of the ergot drugs, which interact with multiple receptors, including those for both 5HT and DA. The observation that 5HT is involved with reward may play a role in future drug development. Possibly related to this reward effect is the demonstration that corticotrophin releasing factor (CRF; CRH) applied to the dorsal raphe nuclei differentially dose-regulates serotonin in the nucleus accumbens (a brain area suggested to be of great importance to reward events and drug dependency).[16] This stress-related connection is a potential example of how looking at uncommonly considered networks could produce unexpected results in chronic treatment of migraine (which is the area of greatest need in migraine drug development), much as it has in previously non-responsive disorders such as obesity.[17] Similar thoughts on interacting signaling pathways in schizophrenia are now under consideration in developing new anti-psychotics.[18]

While many hypotheses have been advanced as to migraine pathophysiology, the only firmly established mechanism is for the rare form of headache known as familial hemiplegic migraine.[19] In this case, a mutation results in a defective calcium channel, and much mechanistic speculation currently surrounds so-called channelopathies.[2] The mechanistic theme of channelopathies goes far beyond migraine to a number of neurological disorders, some of which are mentioned in relation to migraine in this chapter.

Amongst the many ideas that have been advanced to explain migraine occurrence, some of the more interesting and diverse examples include: migraine as dysfunction of the autonomic nervous system[20] and migraine as a disorder of mitochondria.[21] Recently, it has also been speculated that migraine pathophysiology may be associated with energy balance metabolism. There is a high rate of comorbidity between migraine and the eating disorders anorexia nervosa and bulimia nervosa.[22] Interestingly, there is a correlation between serotonergic regulation and hypothalamic physiology and pathology.[23] Specifically, the adipocyte hormone, leptin, influences melanocyte-stimulating hormone (MSH) in the hypothalamic arcuate nucleus. Further, the MSH system is under serotonergic control. Beyond these considerations, neuroendocrine dys-regulation, energy intake and the sleep disorder known as night eating syndrome are correlated.[24] Another hypothalamic-based connection that is worth exploring in the migraine area is adiponectin.[25] Adiponectin receptor type 1 (AdipoR1) in the hypothalamus triggers the insulin (see discussion under the section 7.5: Adipokine signaling) and leptin (see the next paragraph) pathways following activation by adiponectin.

Migraine is known to be triggered by a wide variety of factors, and the observation that hypothalamic involvement, such as *via* autonomic

regulation, may be one of an interrelated set of triggers that may fit the adipokine signaling pathways emphasized in this chapter.[26] Specific adipokines that are known to exert effects because of interactions with discrete hypothalamic nuclei include: leptin (arcuate nucleus; and after NPY release, the paraventricular nucleus) and ghrelin (arcuate nucleus); and both ghrelin and the orexins regulate growth hormone release.[27] There may be an interesting connection between ghrelin, metabolic dysfunction and the brain's reward system (noted earlier with respect to serotonin). Another neurotransmitter/neuropeptide connection that could have relevance to migraine is the synergistic and complementary role that the orexins and histamine have in weight regulation and sleep.[28] These hypothalamic effects may tie in to migraine, as we will discuss later in the chapter, *via* a larger theme of sleep, metabolic regulation and migraine pathogenesis.

Ghrelin binds to and activates receptors in the substantia nigra, ultimately increasing dopamine in the corpus striatum.[29] This neuroprotective effect is most directly related to neurodegenerative disorders such as Parkinson's disease; however, the potential to re-regulate metabolic problems in the neurodegenerative setting may have relevance to metabolic dys-regulation in migraine and perhaps also to related dopamine reward pathways, although this is an area that requires research exploration.

Clearly, adipokines such as leptin and visfatin have been shown to play a role in the pathophysiology of diabetes and obesity.[30] It is noteworthy that in this diabetic/obesity realm, adipokines may be secreted not only by adipocytes but also by macrophages that have moved into adipose tissue. This inflammatory connection could be of relevance to migraine headache, in which there are known inflammatory components which are at least modestly accessible in a pharmacotherapeutic sense by NSAIDs such as ibuprofen and aspirin. Additionally, the hypothalamic hormones, Orexin A and B, mentioned above as appetite regulators, have been directly implicated in pain processing, including that found in migraine.[3]

7.3 Migraine and Neuropathic Pain

Broadly speaking, there are some common characteristics[31] between certain types of migraine and another highly refractory disorder known as neuropathic pain.[32] In migraine, the fifth cranial nerve system (trigeminal) has come under increasing scrutiny as being central to the sensory and pain processing phenomena leading to migraine headache. Painful stimuli associated with trigeminal pathology may produce pain *via* the nearby superior sagittal sinus.[3] Overall, "normal" stimuli such as light and sound may produce pain much as in general neuropathic pain; that is, there is pain production that exceeds the amount expected from the sensory input. Additionally, the long-recognized phenomenon of cortical-spreading depression (CSD) may trigger trigeminal dys-regulation, and the complicated factors leading to CSD may be related to other upstream signaling complications, only now being identified as the initial causes of migraine.

One interesting example that could have parallels to headache pain involves injury to the mouse sciatic nerve (SCN). Such injury activates macrophages in the vicinity, which results from upregulation of leptin in adipocytes.[33] This type of mechanism has potential for broad spread implications. Leptin is over-produced in obesity-related metabolic disorders like diabetes. Accordingly, metabolic pathology may leave subjects prone to neuropathic pain. This spectrum of dys-regulatory events may be reversible *via* weight control and subsequent lowering of leptin production, and fits the lifestyle themes promoted in this book.

Seemingly, these unsuspected metabolic signaling connections may have similar applications to other disorders such as headache. Adipoonectin has also been linked to migraine,[34] and it is relevant to note that abnormalities in feeding behavior (both over- and under-eating may be relevant) suggest that metabolic dys-regulation itself may be the trigger. Both anorexia nervosa and bulimia nervosa are potentially related to migraine *via* biogenic amine action and metabolism,[35] and the trace amines tyramine, octopamine and synephrine may tie this association to the neuroendocrine system. Trace amine associated receptors are found in particular brain regions regulating hormones such as the hypothalamus and amygdala.[36]

Traditional difficulties with reducing neuropathic pain have slowly been addressed in recent years.[37] Systematic and highly organized schema have been developed and tested for guiding both conceptualization as well as therapeutic strategies for handling this puzzling set of disorders. From a pharmacotherapeutic standpoint, current state-of-the-art treatment utilizes a diverse array of drugs, either alone or more likely in combination, for reducing the severity and frequency of neuropathic pain. Specific calcium channel inhibitors, anti-depressants, GABA agents, anti-seizure agents, opioids and cannabinoids, amongst others, constitute the effective list. As of yet, the exact mechanisms for efficacy are not known or at best are incompletely understood. Despite this ignorance, the multiplicity of useful drugs suggests involvement of many underlying hormonal, neurotransmitter and ionic systems, and very likely complex interactive signaling networks, much as is suspected in migraine headache.

One historical problem in understanding migraine pathophysiology and appropriately targeted therapy is that the label migraine represents a number of related, yet unique, disorders. Thus, at least some of the underlying mechanisms should differ from one migraine subtype to another. Nevertheless, whether considering subtypes or the general disorder, there is substantial evidence to support multiple interacting mechanisms. For example, migraine and depression are comorbid, and the presence of pain during pharmacological treatment of depression has been recognized as an interfering factor.[38]

Additionally, fibromyalgia and chronic headache are comorbid,[39] and in both cases disruption of sleep mechanisms is associated with the disorder. Since adipokines under consideration in this chapter are so highly correlated with sleep behavior, it is tempting to hypothesize that adipokine signaling provides an underlying template for these related disorders. Attempts to study this

complex situation of dynamic signaling, with both intra- and extracellular components, should be fruitful in ultimate targeting of therapy.

7.4 Role of Astrocytes in Pain

Brain-based changes relevant to migraine development and progress may not be totally neuronal. Astrocytes are now thought to play a substantial role in calcium signaling.[40] Additionally, as noted later in this communication, astrocyte regulation of calcium metabolism is also a part of blood flow regulation (a matter significant to migraine headache) and brain metabolism.[41] Leptin and other adipokine receptors are known to occur in astrocytes.[42–44,74]

Indeed, glial cells generally, and astrocytes in particular, are increasingly being viewed as active partners with neurons and other cellular elements in neurobiology.[45] There is an expanding literature pointing to pathogenesis associated with astrocytes in a large number of brain-related disorders.[46] Astrocyte serotonin receptors are expressed both *in vivo*[57] and in cell culture.[47–51] Serotonin receptors known to occur in primary astrocyte cultures from rat include the 5HT1a, 5HT2a, 5HT3 and 5HT7 receptors. While the 5HT2a receptor seems to be the dominant receptor in primary culture and occurs without differentiation, it appears that the remaining minor receptor subtypes require differentiation with dibutyril cAMP for expression. A complete understanding of functionality for these receptors is yet to be examined, and represents a tremendous unmet need.

Astrocytes regulate cerebral blood flow[52] and cellular volume.[53] A number of investigations have highlighted distinct relationships between astrocyte/neuronal signaling and brain metabolism.[41] These findings, though far from specific mechanistically, are potentially meaningful to migraine headache because the changes are also correlated with changes in vascular regulation and blood flow. Astrocytes play a role in sleep regulation,[54] an observation that is potentially relevant to migraine headache, as both sleep (Willie (2001) discusses sleep in the context of metabolic regulation)[55] and serotonin are known parameters in migraine pathogenesis. There is also a potential tie-in between medullary astrocytes and neuropathic pain.[56] When fifth cranial nerve injury occurs in rats, astroglial cells have been shown to enhance nocioception. It is interesting to note that astrocytes both receive neurotransmitter signaling and secrete transmitter/hormonal signals themselves.

The 5HT1a receptor plays a part in astrocyte regulation although much remains to be explored in this area.[57] As a note on integrative themes, control of this receptor may not be totally under the grip of serotonin. The cannabinoid, cannabidiol, is an agonist at the 5HT1a receptor both in culture[58] and physiologically.[77] The 5HT2a receptor in primary astrocyte culture is a regulatory factor for glycogenolysis *via* glycogen synthetase.[48,59] The 5HT2c receptor has low agonistic affinity for the SSRI transport inhibitor fluoxetine.[60] This may have relevance to the potential connection between metabolic dysfunction and migraine since this agonistic activity releases arachidonic acid,

which in turn increases glucose metabolism in astrocytic culture. Arachidonic acid itself very likely plays a role in the inflammatory aspects of migraine headache.

7.5 Adipokines and Related Extracellular Signalling

The role of serotonergic systems in glucose metabolism is complex and controversial.[61] As pointed out above, there are at least two examples where serotonin is regulatory in metabolically related subsystems: in the hypothalamic arcuate nucleus where MSH is controlled by serotonin and leptin and in astrocyte culture where glycogen metabolism is controlled by serotonin *via* the 5HT2a receptor. In the latter case, there may be homeostatic feedback at the level of synapses, altering neuronal serotonin release. So, while glucose metabolism may regulate serotonin, serotonin in return may regulate glucose disposition.

There is contradicting evidence about glucose dynamics: serotonin has been demonstrated both to drive down and to elevate glucose levels. The preponderant evidence suggests that the hypoglycemic effect may be the most prominent. This change may be the result of the 5HT2a receptor's influence on the surface glucose transporter. There is evidence to suggest that serotonin levels are abnormal, at least in some diabetics. Nevertheless, there is little suggestion of a direct effect on insulin. The incredibly intertwining and complex pathways of glucose metabolism and the multiple roles of insulin are being unraveled in the highly controllable fruit fly (*Drosophila*) model system.[62] It is remarkable how much conservation of function there has been from this organism to humans and other vertebrates. Further, the unexpected overlapping nature and redundancy of multiple hormonal systems, such as growth hormone and the estrogenic and androgenic steroids,[63] is testimony to the intercellular nature of signaling. Such expansion of thought beyond the expected bounds of single hormonal control is significant with respect to a globally complex syndrome such as migraine headache.

A most interesting example of potential intercellular signaling involving astroglial-neuronal interactions is demonstrated by properties of the new antimigraine drug, tonabersat.[64] In the trigeminal ganglion, neurons are surrounded by a type of astrocyte known as satellite glia. These cells make multiple contacts with themselves and neurons *via* gap junctions, composed primarily of the connexion family of proteins. The hot pepper natural product, capsaicin, increases signaling in this communication network, and this type of function may be related to the inappropriate sensory processing and pain of migraine headache and related phenomena such as infection and allergic response in the nearby sinuses. Of potentially great significance to better understanding the patho-physiology of migraine and effective treatment, tonabersat blocks the action of capsaicin and reduces the number of connexions at the gap junctions. This seems like an excellent example of external signaling in migraine.

As we have been exploring, numerous neurotransmitters, autacoids, hormones and ions have been put forward as etiological factors or at least correlated parameters for genesis and progress of migraine headache. Some of these include (and many have been at least mentioned earlier in this communication): serotonin,[76] norepinephrine, dopamine, acetylcholine[20] glutamate, endogenous opioids and cannabinoids, histamine, estrogens, progestins, the orexins[75] and leptins,[76] calcium[19] and magnesium. Some of these biological factors, such as calcium, may be primary in terms of headache initiation. Other factors, such as serotonin, may be secondary or even tertiary to primary changes, or may be regulatory factors in which the system is attempting to re-establish homeostasis following changes in the primary factor.

It is possible that considering the tenets of this book, the term allostasis, as advanced by Bruce McEwen, is a more relevant term[65] than homeostasis. In this concept, a more dynamic adaptation is envisioned, with respect to individual interacting systems as part of a larger whole that may stray substantially from the norm at times to address stressful situations. In other words, for certain parameters, it could be homeostatic to stray from the norm (*i.e.* to be imbalanced), at least temporarily, to be adaptive to a presenting stress. However, this brief, useful adaptation can become non-adaptive and pathological as it becomes chronic and especially in the absence of the stressful situation. So, how are these ideas relevant to the previously mentioned molecules and, at whatever level of headache development, what could these varied molecules have in common, if anything?

Interesting parallels may exist between hormonal modulation of neurotransmitter signaling in lipid metabolism and signal transduction in headache. For example, insulin has been shown to regulate beta adrenergic receptor (BAR) activity in controlling lipolysis.[66] In long-term over-excretion of insulin, BARs link to a discrete group of PKAs (protein kinase A); this change seems to be the result of an actual physical change *via* architectural proteins that couple the receptors to the transducing enzymes. Since BARs are in turn involved in regulating hormones such as leptin, there are major possibilities for cascading effects and interacting feedback loops for homeostasis (or allostasis). Perhaps a fascinating example of these connections is the recently discovered regulatory loop (LMB) featuring leptin, melanocortin (alpha MSH) and BAR3.[67] While far from established yet in the realm of migraine, the components are there, and it is exciting to propose experiments that could test these ideas in headache. Provocatively, the LMB is tied to sympathetic nervous system functioning, further implicating possible involvement in migraine.

As mentioned earlier, there is considerable evidence *in vivo* and in culture[40,45] that astrocytes are involved in regulation of calcium metabolism in neural systems. In culture, astrocytes secrete calcium in response to neuronal transmitters, and calcium waves propogate from astrocyte to astrocyte. It is controversial, however, as to whether these changes in extracellular calcium are in turn involved in regulating neurotransmitter release from neurons (such as glutamate). Nevertheless, changes in intracellular and extracellular calcium

concentrations may be vital to processes such as cell volume control,[51,53] general osmotic regulation and the regulation of a variety of calcium-dependent enzymes and proteins.

While primary astrocytes are known to release calcium, the receptors or other macromolecular systems responsible for these changes are not completely known. With rat astrocytes containing four subtypes of 5HTR (see earlier section 7.4 on astrocytes), a good candidate for involvement in calcium changes is 5HT2aR, which is Gq coupled. Gq is directly linked to calcium homeostasis in most systems.[9,10] It is possible that other 5HTR are less directly linked to calcium homeostasis. One possibility is the 5HT1aR that can influence ionic disposition *via* effects downstream from changes in intracellular cAMP.[6,9,10,58] Phosphodisterases (PDEs) are important factors in controlling cAMP concentrations (a matter relevant to multiple, potentially overlapping transmitter systems) and are themselves major regulatory molecules in nervous system integrity, and particularly relevant to migraine, vascular physiology and pathology.[68] Thus, these enzymes have long been appealing subjects for intracellular and extracellular connectivity. Since both 5HT1a and -2a receptors have been implicated in migraine pathophysiology,[11] it is possible that the connection is related to these calcium processes mediated by astrocytes.

Additionally, a staggering list of environmental trigger factors are known: light, sound, hydration status, sleep, toxins, drugs and their metabolites, to name a few. Again, what might these triggers have in common, and what relationships might they have to the above-mentioned transmitter, hormone and ionic components? Light has been, perhaps, historically the most commonly identified migraine trigger. There have been recent experimental developments in this regard, which bear analysis in terms of connectivity to other variables such as those under discussion here. In a rat model, neurons in the posterior thalamus are regulated by cells in the retinal ganglion.[69] These cells are not part of image formation as blind individuals are also subject to this effect!

If the classification of migraine as a neurovascular disorder is accurate, and if the neurovascular dys-regulation is fundamentally a problem of ionic homeostasis, then disturbances of metabolism, the most basic of cellular processes, may be influential in creating or maintaining the ionic problems. It is within this context that adipokine regulators of metabolism such as with the leptins may tie all of this together. Perhaps it is time to revisit thermodynamic neurochemistry as the platform in which the widespread and diverse changes of migraine headache unfold. That is, dynamic control of energetic processes is natural to all physical, chemical and biological processes.[70] When things go badly in this regard (loss of allostasis?), many individual components may be perturbed (such as seen in migraine). Re-establishing this energetic balance may be more primary to migraine headache than some of the more modulatory approaches (such as neurotransmitter control) that many of us have previously envisioned.

Migraine, of course, occurs in all ages of humans and, in a fascinating study with children treated for four months with traditional anti-migraine prophylactic drugs,[71] drug effectiveness correlated with decreases in cytokine

levels. For two of the drugs, the 5HT2aR antagonist, cyproheptadine (it also is a histamine H1 antagonist, and it is worth noting that histamine is an oft-neglected factor in migraine) and the calcium channel antagonist, flunarizine, a positive therapeutic outcome resulted in increased leptin levels. Although it is not clear whether this result extends to adults, it is suggestive of adipokine involvement in migraine pathology and establishes a clearly testable hypothesis. In an indication that adipokine signaling in the metabolic context may require complex combinations of regulatory substances, addition of the beta cell pancreatic hormone amylin to leptin in both rodent models and humans leads to markedly greater weight loss than to either agent or its antagonistic analog alone.[17] Migraine susceptibility also changes with age, particularly in women, leading to the thought that neuroendocrine and metabolic pathways, both of which also change in the geriatric context, bear examination in migraine.

7.6 The Future of Signaling Research to Migraine

There are still many unexplored targets for potential anti-migraine drugs. In fact, it seems likely that the number of yet-to-be-explored sites greatly exceeds those already explored. One possibility that is already under active investigation is alternate locations (such as allosteric sites) on targets already considered. A good example would be the GPCR, where there are multiple regulatory interfaces on the receptors, G proteins, downstream effectors and additional interacting molecules[72,73] (also see the introductory chapter). It is tempting to suggest that the most far-reaching future research in the realm of migraine headache will be in the areas of integrative metabolism discussed in this chapter. Even should these adipokine/metabolic ideas not turn out to be the key to unraveling the mysteries of migraine headache, the approach that has been used so profitably in diabetes/metabolic research, where multiple interacting, natural substances are operative,[17,78] is likely to be a fruitful strategy in migraine research.

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CHAPTER 8

Adipokines and Alzheimer's Disease

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8.1 Alzheimer's Disease

Alzheimer's disease (AD), first described in 1907, is a progressive neurodegenerative disorder clinically characterized by an increasingly severe cognitive decline. With age representing the main risk factor, the already considerable socioeconomic impact of the disease is destined only to increase with the progressive aging of the world population. For this reason, the efforts of researchers in the last few years have been devoted to the more detailed understanding of the genetic and biochemical bases of AD, a necessary step towards the definition of disease-modifying strategies for both diagnosis and therapy.[1]

AD is a complex multi-factorial disease influenced by a combination of environmental and genetic factors. According to the age of onset, AD has been classified into two forms. A rare familial form of AD (FAD), accounting for about 5% of cases, is characterized by early onset (45–60 years of age) and is linked to causative genetic mutation.[2,3] Sporadic AD accounts for the remaining 95% of AD cases and is characterized by late onset (>65 years). This form has not been associated with specific gene mutations, but with genetic risk factors that seem to underlie an increased chance to develop the disease.[3]

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8.1.1 β-Amyloid and Tau

The pathological hallmarks of AD are represented by extracellular senile plaques and intracellular neurofibrillary tangles, which are always present in the brains of AD patients. Both plaques and tangles are the result of aberrant protein accumulation and aggregation, respectively of amyloid β protein (Aβ) and of hyperphosphorylated tau protein.[4] In addition, a third distinctive feature of the AD brain is a chronic inflammatory status.

Microtubule-associated protein tau is involved in the regulation of microtubule assembly and disassembly, and its activation is dependent on phosphorylation at different sites. In AD, tau is in a hyperphosphorylated state that increases its potential to aggregate into filaments that accumulate inside the cell, disrupting the cytoskeleton.[5] In addition, the protein undergoes multiple truncations that influence its conformation and ability to polymerize.[6] Although much debated, tauopathy is considered to be secondary to Aβ dysregulation. In support of this, it is not strictly related to AD but it is common to other forms of dementia.[5]

Classically, the amyloid cascade hypothesis is the more widely accepted mechanism proposed for AD pathogenesis.[7,8] Aβ is a short peptide (39–42 amino acids) derived from the proteolytic cleavage of the amyloid precursor protein (APP). APP is endowed with a single transmembrane domain and is cleaved at different sites by transmembrane proteolytic complexes known as secretases. Secretase activity yields peptides of different lengths and with different tendencies to aggregate. According to the amyloid theory, an imbalance leading to an over-production of the highly aggregation-prone Aβ 42 species triggers its accumulation and aggregation first into low-molecular-weight oligomers, then into fibrils and finally into plaques, in specific brain regions. Plaques have long been considered the main cause of AD. Plaques contain Aβ as well as degenerating neurites, and evoke strong local inflammatory responses. Activated microglia and astrocytes are attracted to the area around the plaque and release a number of cytokines, chemokines and complement components. Even though microglia and astrocytes are involved in Aβ removal mechanisms, the real significance of inflammation around plaques still needs clarification, since it is not yet evident if it indeed represents a protective mechanism against neurodegeneration or rather it is responsible for increased neuronal damage in the areas surrounding the plaques.[9,10] In a more recent interpretation of the amyloid hypothesis, based also on the observation that plaque load does not effectively correlate with cognitive decline, oligomers have been recognized possibly to represent the driving cause of the disease. Recent findings show oligomers to be highly synaptotoxic species able to cause neuronal synaptic dysfunction and degeneration.[7,11,12]

APP processing implies the alternative activation of three identified secretase complexes, α, β and γ as reviewed recently[7,13] γ-Secretase includes at least five different proteins, Presenilin (PS) 1 and 2, APH1, PEN-2 and Nicastrin, and performs a C-terminal cut inside the lipid bilayer of the plasma membrane. Such C-terminal cleavage is coupled to cleavage in the N-terminal ectodomain,

alternatively performed by α or β secretases. α-Secretase activity has been attributed to metalloproteases belonging to the family of A Dysintegrin and Metalloproteinase (ADAM). Three main ADAMs have been assigned a role in APP processing, ADAM10, 9 and 17, with ADAM10 apparently being the main player. α-Secretase activity is considered to be favorable in pathologic conditions, for its site of cleavage is located inside the Aβ 42 sequence, thereby precluding the formation of the amyloidogenic isoforms. Release of the Aβ peptides depends instead on the coupling of γ-secretase with β-secretase activity. β-site APP-cleaving enzyme 1 (BACE1) is the main β-secretase, and its action is the rate-limiting step in Aβ 42 formation. It is, however, the precise site of cleavage by γ-secretase that determines the length of the amyloid species produced, which ranges from 39 to 42 amino acids, with Aβ 40 and Aβ 42 being the most represented. As mentioned, Aβ 42 is the most insoluble species and has a greater tendency to aggregate compared to Aβ 40.

Interestingly, all identified mutations (more than 200) linked to FAD are located in the APP or PS genes and are responsible for the increased ratio of Aβ 42 *vs.* Aβ 40.[7,14,15] Also, a variety of mouse models of AD have been obtained by inserting into their genomes the human APP and PS1 genes carrying one or multiple mutations. Although these animals do not fully reproduce the human pathology, they are characterized by abnormal production of Aβ 42, which aggregates in hippocampal and cortical plaques.[16,17]

Sporadic AD is associated with dysregulation of Aβ synthesis, leading to accumulation of Aβ 42. The ε4 allele of the apoE gene is the prevalent risk factor for this form of the disease, although its exact role remains to be elucidated. ApoE is a lipid carrier protein that binds to amyloid peptides and influences transport, aggregation and metabolism of Aβ.[18,19] The apoEε4 isoform has been persistently associated with increased cerebral levels of Aβ.[19]

Another factor influencing Aβ synthesis is linked to trafficking of APP and secretases, all transmembrane proteins. Trafficking represents a crucial regulatory event that may affect their location and interaction, and thus the chance of Aβ formation. In this regard, lipoprotein receptors have been shown to play a role in regulating APP endocytic trafficking.[20,21]

Recently, an important role has emerged also for intracellular accumulation of Aβ, linked to development of synaptic pathology. Aβ has been recently shown to be taken up and concentrated inside acidic intracellular compartments such as lysosomes, where it is cleared. In pathologic conditions, the load of intracellular Aβ 42 likely becomes excessive. In these conditions, it begins to accumulate and aggregate inside the cell, eventually leading to neuritic degeneration. This causes aggregated Aβ to be released in the extracellular space, where it may in turn give rise to plaque formation.[15,22,23]

Although attention has been primarily focused on the biochemistry of Aβ production, the importance of Aβ clearance mechanisms has recently emerged. The brain is endowed with Aβ-degrading systems mainly related to the activation of microglial and astroglial cells. In addition, recent evidence suggests the possibility that circulating monocytes could infiltrate the AD brain and contribute to Aβ degradation with a distinct functional role.[24] It has been shown

that astrocytes are in a reactive state in the areas surrounding plaques, where they release Aβ-degrading enzymes.[25–27] Several proteases have been identified with Aβ-degrading action. The most efficient has been shown to be neprylisin,[28] belonging to a family of zinc-metallopeptidase that includes at least two other enzymes endowed with Aβ-degrading activity, endothelin-converting enzymes 1 and 2.[29] Matrix metalloproteinases (MMP), mainly MMP2 and MMP9, have been demonstrated to possess the same ability.[30] Notably, the insulin-degrading enzyme (IDE) is another primary enzyme responsible for Aβ degradation,[31] and accordingly insulin signaling has been shown to be involved in APP processing and Aβ clearance. While hyperinsulinemia has been indicated as a risk factor for AD, levels of insulin in the central nervous system (CNS) are inversely related to the levels of Aβ 42 accumulation.

8.1.2 Target for AD Therapy

Current therapy for AD and related dementias includes only symptomatic treatments that delay progression of the disease during its early stages (Table 8.1). Based on pre-clinical studies and on the available knowledge of AD pathogenesis, alternative approaches should be used only at very early stages of the disease, even when no clinical evidence is present, in order to realize a preventive strategy. According to current approaches, research is now trying to prove the effectiveness of β- and γ-secretase inhibitors[32,33] or alternatively α-secretase activators. This kind of intervention would affect the processing of APP leading to Aβ generation. It is also possible to modulate the activity of γ-secretase, thus reducing the production of Aβ 1–42 in favor of shorter, less toxic fragments.[34] Other potential interventions are aimed at inhibiting plaque formation or favoring plaque removal. In this regard, major attention should be reserved to Aβ passive immunotherapy that is at present being actively tested in humans and appears promising, despite some serious adverse effects that have

Table 8.1 AD therapy.

Current treatments
Acetylcholinesterase inhibitors
Donepezil
Rivastigmine
Galantamine
NMDA antagonist
Memantine
Potential future pharmacological targets
α-, β-, γ-secretases
Tau kinase
Tau clearance
Aβ and tau aggregation
Immunotherapy

emerged in some cases.[35] Tau is considered today a novel attractive target in AD.[36,37] While targeting tau production and aggregation is more theoretical, the possibility to affect tau phosphorylation has been tested using GSK3β and other tau kinase inhibitors. Compounds able to regulate tau aggregation and folding, to stimulate its clearance and to stabilize microtubules are also being explored as therapeutic agents. Finally, tau immunotherapy has recently been considered and preclinical studies have provided some comforting data.[35]

Although much is on the way, available treatments are still poor and, in some cases, initial results from early phase clinical trials have been discouraging. Furthermore, there is no absolute certainty that the course of the disease can be modified simply by altering Aβ concentrations and/or that results obtained in transgenic animal models of overproduction of Aβ can be reproduced in patients with AD. In addition, as mentioned, early intervention may be critical for efficacy of treatment. This requires the development and identification of biomarkers that can be monitored even before the onset of clinical signs of AD. In this respect, according to Montine and co-authors,[38] it appears necessary to broaden our current view of potential therapeutic/preventive approaches to AD and cognitive impairments to include selection of specific biomarkers and a multi-faceted approach to these conditions. In this line, the possibility to place AD in a more global scenario and/or to relate it to metabolic disorders such as diabetes, hyperinsulinemia, insulin resistance and obesity has to be taken into account.[39,40]

8.2 AD and Metabolic Dysfunction

8.2.1 Impaired Glucose Metabolism

Although still debated, an association between diabetes and the risk of AD has recently found solid support from data provided by several studies with longer follow-up. The association based on several epidemiological studies mainly relates hyperinsulinemia and increased risk of AD. Hence, peripheral hyperinsulinemia and insulin resistance represent the key elements of the increased risk of AD reported in patients with diabetes mellitus.[39–43] Available epidemiological and clinical data have provided a solid support in this sense, underlying the potential connection between a peripheral metabolic disorder and a disease of the CNS. Under conditions of hyperinsulinemia, in fact, brain insulin levels are decreased due to the impaired transport of peripheral insulin across the blood-brain barrier (BBB).[39] The concentrations of insulin in the cerebrospinal fluid are also low in AD patients and this correlates with a lower expression of insulin receptors and insulin receptor substrate (IRS). At the CNS, insulin is known to exert a physiological role in Aβ production and clearance. Hence, low brain insulin concentrations result in Aβ accumulation since low insulin and impaired insulin signaling affect APP processing. At the same time, in the CNS, insulin becomes a substrate for insulin degrading enzyme (IDE), thus competing with Aβ and decreasing its

clearance.[31] Changes in Aβ levels under these conditions are accompanied by increased tau phosphorylation due to reduced phosphatidylinositol-3-kinase (PI3K)/AKT signaling and increased glycogen synthase kinase 3β (GSK3β) activity. All these events contribute to AD pathogenesis. To confirm this hypothesis, increases in peripheral insulin levels produce a rise of Aβ 1–42 in cerebrospinal fluid, and administration of intranasal insulin that reaches the CNS yields beneficial effect in AD.

8.2.2 Lipid Disorders

More recently, the view of AD in a context of metabolic disorders has been broadened including as a background scenario different aspects of disturbances in lipid metabolism. The first association in this regard comes from the demonstrated link between AD and apoE that, as mentioned, represents the main risk factor for the late onset form of the disease. In particular, the presence of two e4 alleles increases the risk of AD by approximately 12-fold and lowers the age of onset of the disease by about 15 years.[19] ApoE is a 35 kDa protein that exists in three isoforms, E2, E3 and E4, differing in only one amino acid. Cholesterol metabolism in the brain is regulated independently of peripheral cholesterol. It is synthesized mainly by astrocytes and microglia or recycled from degenerating neurons.[44] Its transport in the brain depends on lipoproteins that are similar to HDL and contain mainly ApoE4 and to a lesser extent ApoE1.[45] ApoE in brain is expressed in all types of glial cells, astrocytes, microglia and oligodendrocytes, and under conditions of neuronal damage its main role is the transport of cholesterol and phospholipids from glia to neurons where regeneration and remyelination occur.[46,47] Although an exact correlation between the ε4 allele and the risk of AD has not been clarified, ApoE is known to play a major role in modulating Aβ production and clearance together with its receptors, LDL receptor (LDLR) and LDL receptor related protein (LRP). Accordingly, increased amyloid deposition and impaired cognitive behavior have been reported in transgenic mice lacking LDLR.[48] ApoE co-localizes with amyloid deposits in the brain[49] and its lipidation state increases its ability to bind Aβ.[50] ApoE4 is more efficient in inducing Aβ synthesis and fibrillogenesis[19,51,52] and affects Aβ clearance though several mechanisms. It causes retention of Aβ within the brain, impeding its crossing the blood-brain barrier, thus reducing its trafficking toward the periphery.[53,54] A correlation between the E4 isoform and increased tau hyperphosphorylation as well as the formation of neurofibrillary tangles has also been demonstrated.[55] Although apoE4 has been mainly related to changes in Aβ metabolism, the possibility that this isoform differently affects neuroinflammatory processes or altered brain blood flow and metabolic function that accompany AD have also to be considered.[19]

Although brain cholesterol is recognized to be regulated independently of the periphery, hyperlipidemia and midlife elevation of serum cholesterol (<6.5 mmol/l) significantly augment the risk of AD.[56–58] Accordingly, studies

support the use of statins, fibrates and other lipid-lowering agents to diminish the risk of AD.[59] In the brain, cholesterol concentrates in lipid rafts where it affects the activity of several enzymes.[60] In fact the activity of β- and γ-secretases and of α-secretase are respectively positively and negatively modulated by high cholesterol concentrations.[61–63] The opposite, *i.e.* a decrease of Aβ formation, occurs when cholesterol levels are lowered.[64] Hence high cholesterol seems to positively correlate to Aβ synthesis in a direct way. However, evidence also exists to support a more indirect link between cholesterol homeostasis and Aβ production. More precisely, inhibition of acyl CoA cholesterol transferase (ACAT), responsible for cholesterol esterification, reduces Aβ production in cultured cells[65] and amyloid pathology in Alzheimer transgenic mice.[66] Moreover, the ATP-binding cassette (ABC) transporter A1, which in the CNS prevents cholesterol accumulation by facilitating its flux toward the periphery, is known to have a role in reducing Aβ accumulation in the brain.[67] Reduction or knockout of ABCA1 results in increased Aβ plaque load,[68–70] whereas ABCA1 over-expression increases amyloid accumulation in AD transgenic mice.[71]

Dyslipidemia accompanies hyperinsulinemia and insulin resistance. Under these conditions, in fact, free fatty acids levels rise, causing an elevation of very low density lipoproteins (VLDL), which results in postprandial hyperlipidemia. Increased VLDL has been associated with increased Aβ deposition in the brain[72] and with elevated risk of AD.[73]

8.2.3 Obesity

One of the main causes of insulin resistance is obesity, with over 80% of obese people being insulin resistant.[74] Combination of these two conditions is responsible for persistent free fatty acid (FFA) elevation. FFAs inhibit IDE, the metalloprotease that participates not only in insulin signaling but also in Aβ clearance.[75] FFAs favor also aggregation of Aβ and tau *in vitro*.[76,77] A strong correlation has been demonstrated between obesity and AD risk. More specifically, midlife obesity is considered a risk factor for dementia,[57,78,79] whereas later in life a low[80] and high body mass index have been related to higher and lower AD risk, respectively.[81] To explain this apparent paradox, weight loss often precedes dementia onset[82] and may precede diagnosis by more than ten years.[83] Interestingly, distribution of adiposity seems to play a role in the risk of dementia as already reported for cardiovascular diseases and diabetes.[84] In particular, central distribution of fat, known as visceral adiposity, can be especially dangerous, even for patients who are not overweight, increasing the risk of dementia.[85] This suggests that there might be something intrinsic to the condition of central adiposity that increases this risk.

8.3 Adipokines

Emerging evidence is accumulating on the relevance of adipokines, biologically active substances released by adipose tissue, that make this tissue actively

participate in metabolic functions. Adipokines can be synthesized by fat as well as non-fat cells of the adipose tissue but they can also be synthesized at different sites and exert independent actions. Many of them exert proinflammatory effects, including leptin, tumor necrosis factor-α (TNFα), interleukin-6 (IL-6) angiotensinogen, resistin, plasminogen activator inhibitor (PAI), whereas a few others exert mainly anti-inflammatory activity. Among the latter are adiponectin and transforming growth factor β (TGFβ). Most of the adipokines identified to date have high circulating plasma levels in obese subjects. Some of them reflect the mild inflammatory condition that characterizes obesity in its initial phases, others have more inflammatory features and their origin is debated.[86] Additional adipokines may be endowed with anti-inflammatory effect and be at higher circulating levels in obesity because of a compensatory response to inflammatory mediators. IL-10 as well as IL-6 may be considered in this context.[87,88] As a whole, adipokines are considered fundamental regulators of carbohydrate and lipid metabolism. This gains importance in terms of deposition and mobilization of fatty acids and regulation of obesity-related comorbidities.[89] However, the effect of adipokines is not restricted to the periphery since some of them can cross the BBB or modulate the function of endothelial cells.[90]

8.3.1 Leptin

The more well known and characterized adipokine, leptin, which has allowed the identification of this large family of proteins all related to lipid metabolism, was initially recognized because of its anorexigenic and appetite suppressive effects. Both effects are exerted within the brain. Since then, interest in the effects of adipokines in the brain has increased and knowledge of this specific issue has broadened. Leptin is a 16-kDa peptide that exerts its biological actions by binding to Lep-Rb (ObR, a–f), all members of the IL-6 receptor family of the class I cytokine receptor superfamily, and divided into long, short and soluble forms.[91] Six different isoforms of the ObR have been described. The long form ObRb seems to mediate most of the actions of leptin. Short forms of the receptor such as ObRa and ObRc are abundantly expressed in cerebral vessels of the BBB suggesting a role for these short forms of receptor in the transport of leptin from the periphery into the brain. Interestingly, leptin binds also megalin/LRP2, a multi-ligand receptor expressed in choroid plexus epithelial cells that is known to participate also in Aβ clearance from the brain to the blood.[92,93] Transport across the BBB seems to be crucial although leptin is also known to be produced in very low amounts within the brain. ObRb is devoid of autophosphorylation and signals mainly through JAK2 responsible for phosphorylation of STAT, above all STAT3, that dimerizes and translocates to the nucleus to control the expression of a series of genes. Alternatively JAK2 activates the SH2/SH3 domain-containing adaptor protein growth factor receptor-bound protein 2 (GRB2), responsible for downstream involvement of the mitogen-activated

kinase (MAPK) pathway. The latter is the only signaling cascade activated by the short forms of ObR.[94]

Leptin *via* its central actions does not control exclusively food intake, but recognizes receptors also in the hippocampus where it controls cognitive processes. It in fact facilitates learning and memory events, increases long-term potentiation (LTP)[95] and enhances synaptic density.[96] Accordingly, impairment of LTP is reported in db/db mice, which carry a leptin receptor mutation and do not express functional leptin receptors,[97] whereas leptin treatment increases hippocampal concentrations of synaptic proteins including synapsin 2A and synaptophysin.[98] Leptin is also endowed with neuroprotective effects as shown by decreased neuronal death following either serum or neurotrophic factor deprivation.[99] In addition, leptin has been proven to counteract the loss of dopaminergic neurons observed in experimental models of Parkinson's disease, to be neuroprotective against ischemic stroke and to exert anticonvulsant properties in most seizure models.[100]

Among various adipokines, leptin is the one that has been more convincingly related to AD. In fact, observational studies have demonstrated an inverse relationship between blood leptin levels on one side and human cognitive impairment and AD development on the other side.[101] Conversely, high peripheral leptin concentrations correlate to a low incidence of dementia and AD.[102] Leptin can affect AD pathogenesis by acting at multiple sites (Figure 8.1). Evidence exists in fact to suggest that it affects not only Aβ but also NFT formation. In particular, due to its lypolitic effect, leptin modifies lipid raft composition and affects indirectly BACE activity, thus decreasing Aβ formation.[103] It increases also Aβ clearance by facilitating ApoE-dependent Aβ uptake through LRP1.[103] These *in vitro* data find support in animal studies reporting the ability of leptin to reduce Aβ load in a transgenic model of AD[103,104] and to improve cognitive performance in AD mice.[105] The effects of leptin are not limited to modulation of Aβ synthesis and degradation as leptin decreases also tau phosphorylation in neurons,[106] through modulation

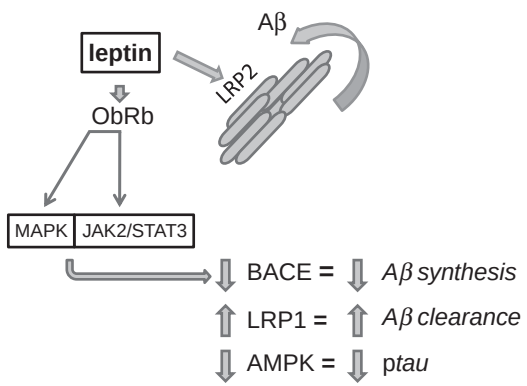

Figure 8.1 Mechanisms involving leptin in the pathogenesis of AD.

of AMP-activated protein kinase (AMPK)[107] and ensuing inactivation of GSK-3β,[108] which is known to be involved with tau hyperphosphorylation and neurofibrillary tangle formation. Of note, AMPK seems to mediate not only the effect of leptin on tau phosphorylation, but also the reducing action of leptin on Aβ production. Moreover, the effects of leptin, through AMPK activation, involve two independent downstream signaling pathways, with peroxisome proliferator-activated receptor-γ (PPAR-γ) affecting Aβ formation through BACE and GSK3β modifying tau phosphorylation.[107] All these experimental data have identified leptin as a novel potential therapeutic tool in AD[104] and have also indicated leptin-induced intracellular signals as a potential target in the optic of novel treatments of AD.

8.3.2 Adiponectin

Adiponectin is a 224 amino acid protein whose main function is decreasing glucose synthesis in the liver and enhancement of fatty acid oxidation, thus exerting in anti-atherogenic, anti-diabetic and insulin-sensitizing properties.[109] Accordingly, decreased serum concentrations of adiponectin correlate with obesity, type 2 diabetes and insulin resistance. Adiponectin binds two different receptors, ADIPOR1 and ADIPOR2. Stimulation of both receptors leads to subsequent activation of AMPK, PPAR-α and PPAR-γ. An action of adiponectin at the CNS has not been clearly demonstrated as it does not cross the BBB. However, an upregulation of adiponectin receptors has been observed in brain endothelium during fasting.[110] Thus adiponectin seems to exert only indirect effects in the CNS, such as reduced release of IL-6 observed in brain endothelial cells following treatment with adiponectin.[111] To date, the ability of adiponectin to modify IL-6 (or other proinflammatory cytokines) in the brain represents the only link between this adipokine and AD. However, the possibility that adiponectin plays an indirect role by reducing insulin resistance, diabetes, obesity, all conditions considered main risk factors for AD, has also to be taken into account.

8.3.3 Resistin

Resistin is a member of the resistin-like molecule (RELM) family of cysteine-rich proteins, which appears to increase in obesity, type 2 diabetes and insulin resistance, although some controversies regarding its role in humans still exist.[112] It is clear that resistin is upregulated in inflammatory processes and a reciprocal control exists between resistin, TNFα and IL-6. Thus, resistin has been suggested to be involved in several inflammatory conditions including inflammatory bowel disease, coronary artery disease and rheumatoid arthritis.[112] Resistin is present in the brain and its expression increases under conditions of hypoxia/ischemia, traumatic brain injury or cerebral LPS injection.[113] Although leptin and resistin synergize to control glucose homeostasis in diabetes, they affect STAT3 and AMPK in an opposite way. Thus, resistin counteracts central leptin action and nullifies its signaling.[114]

Although direct information is missing, what is known to date, which includes the indirect proinflammatory action exerted through upregulation of inflammatory cytokines, increased expression under several inflammatory brain states and the antagonism of leptin effects in the CNS, seems to indicate an unfavorable action of resistin in AD.

8.3.4 Visfatin

Visfatin, also known as pre-B cell colony enhancing factor (PBEF) and nicotinamide phosphoribosyl transferase (Nampt), in most species, is a 491 amino acids protein with several functions including enhancement of cell proliferation, maturation of B cells, biosynthesis of nicotinamide mono- and di-nucleotide and hypoglycemic effects derived from reduction of glucose release from liver and stimulation of glucose utilization in adipocytes and myocytes. Serum levels of visfatin are elevated in obesity and type 2 diabetes[115,116] and visfatin mimics insulin binding to its receptor at a site different from that of insulin.

Visfatin is recognized as a proinflammatory agent, stimulating inflammatory cytokine expression, such as TNFα, IL-6 and promoting smooth muscle cell maturation. High visfatin serum levels have been associated with inflammatory conditions and ischemic stroke,[117] but a protective effect of visfatin in cerebral ischemia has also been shown.[118] One interesting aspect that may help in identifying a specific role for visfatin in AD resides in its nicotinamide phosphoribosyl transferase activity. NAD originates in fact from nicotinamide, substrate for visfatin, which is converted to nicotinamide mononucleotide (NMN) and then to NAD, and further reduced to NADH. The latter is a substrate for NADH oxidase that forms superoxides. Aβ seems to be endowed with NADH oxidase activity to form oxygen radicals from extracellular NADH. Hence, an enhanced production of visfatin, in the presence of Aβ, results in increased formation of free oxygen species that can contribute to an increased damage to neurons and the BBB.[119] It is suggested that visfatin represents the central factor of a vicious cycle in which enhanced oxygen radicals cause damage to the brain vasculature, increasing chemotaxis of blood cells that contribute to produce visfatin with ensuing proinflammatory cytokines. As visfatin levels may increase with age, an excessive accumulation, with other concomitant factors, may be responsible for a cumulative brain damage that characterizes AD or other neurodegenerative conditions.

8.3.5 Plasminogen Activator Inhibitor

Plasminogen activator inhibitors (PAI-1 and -2) are protease inhibitors belonging to the serpin family. PAI regulates the plasminogen activator (PA)/plasmin system that is involved in a variety of functions including cell migration, invasive growth, neuronal migration and plasticity. Overexpression of PAI causes adipocyte hypotrophy whereas mice deficient in

PAI-1 have faster weight gain in diet-induced obesity indicating a protective role of PAI-1 in this condition.[120] However, increased PAI-1 concentrations are considered a risk factor for thrombotic diseases. Elevated PAI levels correlate also with the metabolic syndrome and insulin resistance. PAI-1 is positively controlled by TGF-β both in the periphery and in the CNS where TGF-β exerts its neuroprotective effect by stimulating production of PAI-1 in astrocytes.

PAI is expressed in the human brain in both neurons and astrocytes[121] and has been reported to exert anti-apoptotic and neurotrophic activities in the CNS.[122] Evidence also exists to suggest a role for PAI-1 in AD. Plasmin in fact contributes to Aβ clearance, cleaving both monomeric and fibrillar Aβ and its protein levels are reduced in AD.[123] In addition PA activity in the frontal cortex of AD patients is dramatically reduced, although PAI-1 concentrations are not changed.[124] These data confirm results obtained in animal models of AD in which elevated Aβ correlates with inhibition of PA/plasmin system and upregulation of PAI-1. Moreover, in mice lacking PA or plasminogen, but not in wild-type mice, injection of Aβ causes PAI expression and neuronal damage.[125] Available data seem to suggest that inhibition of the PA/plasmin system by PAI-1 contrasts with clearance of Aβ favoring its accumulation. However, controversies still exist in this regard as the increased PAI-1 levels observed in the AD mouse model have also been considered neuroprotective against Aβ-induced neuronal damage.[122]

8.3.6 Interleukin-6

IL-6 is a pleiotropic proinflammatory cytokine produced by adipocytes whose plasma levels correlate with insulin resistance and obesity. In obese patients about 30% of total IL-6 can originate from adipocytes. Although IL-6 is produced both in the periphery and centrally, its levels have been reported to be elevated in plasma, CSF and brains of AD patients.[126,127] As cerebral inflammatory processes may play a main role in AD, several inflammatory molecules including IL-6, either produced by resident cells surrounding the plaque, such as microglia, or periphery-derived cells, are elevated in AD patients.[128] However, although peripheral IL-6 can cross the BBB, its real contribution to the cerebral inflammatory process is not clear. Despite this, peripheral blood levels of IL-6 have been suggested as potential biomarkers of AD severity.[129] Although the upregulation of IL-6 in the AD brain might suggest a detrimental role on neuronal viability, a recent report very elegantly supports a protective function of IL-6 with reduction of Aβ deposition consequent to enhanced Aβ clearance.[130]

8.3.7 Transforming Growth Factor-β1

TGF-β1, a 25-kDa protein, is a potent anti-inflammatory molecule produced by adipocytes as well as other tissues. The ratio of TGF-β1 mRNA produced in

fat cells to that in non-fat cells is, however, very low and this, together with its low ability to cross the BBB, makes it difficult to look at TGF-β1 as an adipokine of fat-cell origin acting in the CNS. TGF-β1 is instead produced in the CNS mainly by astrocytes and microglia in response to injury or during aging. TGF-β1 is elevated in the CSF, but not in plasma, of AD patients whereas a defect in TGF-β1 signaling facilitates Aβ deposition and reduces neuronal viability in a mouse model of AD and negatively correlates with neurofibrillary tangle formation in humans.[131,132] Among the mechanisms involved in the neuroprotective actions of TGF-β1 in AD it is plausible to consider the release of neurotrophic factors as well as the reduction of microglia activation with ensuing lower release of the inflammatory cytokines IL-1β, TNFα, IL-6 and enhanced degradation of Aβ.[133]

8.4 Conclusions

In summary, interest in adipokines cannot remain restricted to their functions in adipose tissue or in metabolic disturbances. This actively growing family of proteins is in fact produced in different tissues exerting various actions. It remains to be established whether actions in the CNS, or specifically in AD, can be reconnected to their peripheral origin or should be considered absolutely independent of the adipose tissue. It has to be remembered in this regard that some of them do not cross or barely cross the BBB. On the other hand, some adipokines may represent promising molecules as biomarkers to follow the disease state. Unfortunately, no data are available to correlate with the actions of most adipokines in AD pathogenesis or to understand their real roles in the synthesis, action and degradation of Aβ or hyperphosphorylation of tau. When more profound information is provided, it is hard to believe that a direct connection exists between adipose tissue and CNS. This, for instance, can be the case of TGF-β1. Much has to be done to clarify these aspects.

However, in a broader view of AD on a background scenario of metabolic disorders that include also lipid dysregulation and glucose homeostasis dysfunction, a profound characterization of the role of adipokines in the pathogenesis of AD or their consideration as target of focused interventions in the treatment of the disease certainly deserves great attention and may provide useful tools to help relate AD to main metabolic disorders. Leptin may represent the leading molecule in this sense but several other adipokines seem promising and require further investigation.

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CHAPTER 9

Astrocyte Signaling in Neurological Disorders

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9.1 Introduction

Astrocytes are neural (glial) cells with numerous functions in the central nervous system (CNS). Astrocytes become activated in response to CNS injury and undergo hypertrophy, a phenomenon referred to as reactive astrocytosis (astrogliosis). Whether this response is beneficial or detrimental remains controversial. Cell swelling is another common astrocytic response that contributes to brain edema and increased intracranial pressure in various neurological conditions. This chapter will review general features of these astrocytic responses and then discuss signaling mechanisms associated with these astrocytic alterations.

9.1.1 Structure and Function of Astrocytes

9.1.1.1 Morphology

Astrocytes are the most abundant cells in the central nervous system (CNS), and constitute about 30–50% of the mammalian brain volume, depending on

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the brain region. Individual astrocytes possess non-overlapping domains,[1,2] wherein in humans they associate with approximately 2,000,000 synapses.[3] Astrocytes are, in a sense, the "epithelial" cells of the CNS in that they line key surfaces of the brain and spinal cord (subpial, subependymal, pericapillary and perisynaptic areas). Like epithelial cells they are enriched with gap junctions allowing them to communicate with adjacent astrocytes, resulting in a complex syncitium that encompass the entire CNS. This configuration allows them to interact with all major systems of the CNS, including neurons, other glial cells (microglia, oligodendrocytes) and capillaries.

Three major types of astrocytes are recognized in the CNS. Protoplasmic astrocytes are only found in grey matter, where they display numerous, highly branched, short cellular processes. Fibrous astrocytes are the dominant form in the white matter. These cells have sparsely branched but elongated cellular processes. Fibrous astrocytes are the cells that possess a star-like appearance (hence the term "astrocyte"). Radial glial cells are generally observed only during development, where they assist neurons in their migration. Modified forms of astrocytes are present in the cerebellum (Bergmann glia) and Müller cells in the retina. The most commonly used marker for identifying astrocytes is the intermediate filament, glial fibrillary acidic protein (GFAP).

9.1.1.2 Astrocyte Functions

While in the past astrocytes were traditionally viewed as only "supportive" cells, it is now clear that they play active and important roles in the CNS, including regulation of synaptogenesis,[4,5] synaptic function,[6,7] modulation of excitatory and inhibitory neurotransmission,[8,9] pH and ion homoeostasis,[10–12] regulation of energy metabolism[13–15] and the detoxification of ammonia, metals and free radicals.[16–19] Astrocytes are also involved in the provision of nutrients to the neurons[20] and formation and maintenance of the blood-brain barrier,[21–23] assist oligodendrocytes in the process of myelination,[24–26] repair and scarring processes in CNS[27,28] and also play a significant role in inflammation.[29–31]

Astrocytes are well known to release many trophic factors, including glial cell line-derived neurotrophic factor (GDNF),[32,33] brain-derived neurotrophic factor (BDNF),[32,33] vascular endothelial growth factor (VEGF),[34,35] nerve growth factor (NGF),[33] transforming growth factor beta (TGF-β),[36,37] ciliary neurotrophic factor (CNTF),[38] basic fibroblast growth factor (bFGF)[39] and platelet-derived growth factor (PDGF).[40] Astrocytes likewise secrete the nuclear protein high-mobility group box 1 (HMGB1), a cytokine-like factor that promotes inflammation, whose release has been shown to be protective against neuronal injury after ischemia.[41,42]

The glutamate-glutamine cycle is an important process in the regulation of the principal CNS neurotransmitters, glutamate and GABA. Under normal conditions, glutamate released from neurons is taken up by astrocytes and then converted into glutamine by the enzyme glutamine synthetase. Astrocytes then release glutamine, which is subsequently taken up by neurons and metabolized to glutamate by phosphate-activated glutaminase, and to GABA by glutamate

decarboxylase.[43–45] In conditions such as ischemia, trauma, neurodegenerative disorders and hepatic encephalopathy, astrocytic glutamate uptake is impaired, potentially resulting in a disturbance of the glutamate-glutamine cycle, as well as an elevation in extracellular glutamate concentration that may lead to excitotoxic neuronal injury.[46,47]

9.1.2 Responses of Astrocytes to Injury

There are three prototypical responses of astrocytes to CNS injury: reactive astrocytosis (astrogliosis), Alzheimer type II change and cell swelling. Reactive astrocytosis is the response of astrocytes to destructive injury, whereas the Alzheimer type II change is typically observed following metabolic/physiologic disorders. Cell swelling is an acute change that occurs in many forms of CNS insults and reflects a degenerative change resulting from impaired cell volume regulation.

9.1.2.1 Reactive Astrocytosis

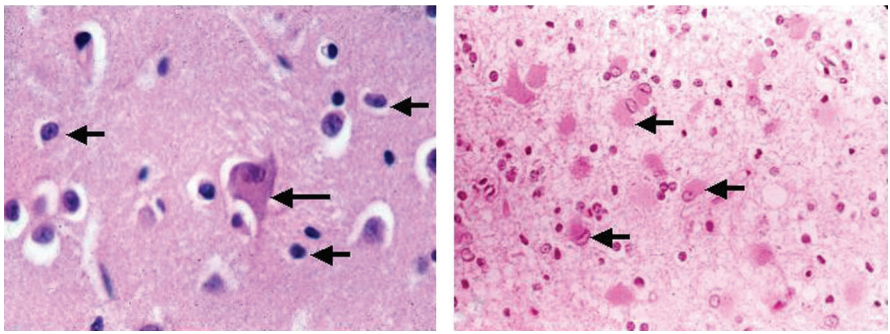
This response of astrocytes to destructive injury occurs robustly in acute CNS disorders such as stroke and trauma. Morphologically, reactive astrocytes display cytoplasmic enlargement, associated with the development of numerous thickened cytoplasmic processes[48] (Figure 9.1). The nuclei are also enlarged, hyperchromatic and occasionally multi-nucleated. By electron microscopy the Golgi complexes are enlarged and increased numbers of mitochondria, ribosomes and glycogen granules are observed. Increased amounts of intermediate filaments (GFAP, vimentin and nestin) are a prominent feature of reactive astrocytes (Figure 9.2). All of these features are characteristic of metabolically activated cells. These changes are initially subtle at 3–4 days following injury and achieve their full expression at 2–3 weeks.[48] A cluster of reactive astrocytes possessing numerous intertwining processes is often referred to as a ''glial scar'', although this scar bears no resemblance to the connective tissue scar seen in the rest of the body.

Reactive astrocytosis may also evolve in a more protracted fashion. This is the typical response in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease and others. In contrast to the acute response, the reactive astrocytosis in these conditions tends to be less prominent.

a) Triggering Factors

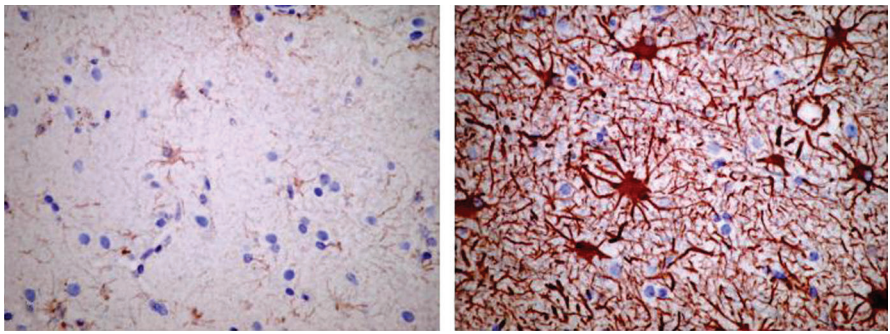
While the precise factors responsible for the induction of reactive astrocytosis are unclear, the release of proinflammatory cytokines such as IL-1, IL-2, IL-6, TNFα and IFN-γ, likely from activated microglia, has been implicated.[49–51] Elevated brain levels of these cytokines have been detected following CNS injuries,[52] along with an over-expression of their receptors on astrocytes.[53,54] Infusion of cytokines in brain has been shown to induce astrocytosis.[49,50] Conversely, anti-inflammatory cytokines were shown to prevent astrocytosis.[55]

As noted above, astrocytes are well known to release many growth-promoting trophic factors, all of which have also been shown to stimulate astrocytosis,



A B

Figure 9.1 A Appearance of normal astrocytes (short arrows) from the striatum stained with hematoxylin and eosin (H&E). This figure discloses only the nuclei and little to no cytoplasm is evident. The large cell in the center of the field is a normal medium spiny neuron (long arrow). B Section of striatum from an individual who died with Huntington's disease. Note the presence of numerous reactive astrocytes characterized by cells with abundant eosinophilic cytoplasm and nuclei that are larger and paler than normal (arrows).



A B

Figure 9.2 A GFAP-immunostained section of normal human cortex. Slight staining is observed in astrocytes. B GFAP-immunostained section of reactive astrocytes displays enhanced intensity of staining and additionally illustrates the increase in cytoplasmic volume and the extent and length of cytoplasmic processes. Both photographs were obtained from the same slide. The slide B image was just adjacent to a primary sarcoma of the brain, while the slide A image was approximately 1.5 cm away from the tumor where the tissue appeared normal.

possibly through autocrine effect.[56,57] Additionally, infusion of thrombin in brain or its addition to cultured astrocytes have also been shown to potently stimulate reactive astrocytosis.[58,59]

In addition to cytokines and thrombin, ATP also has been implicated as a stimulant for reactive astrocytosis. The P2Y receptor agonist ATP analog

2-MeSATP has been shown to cause reactive astrocytosis.[60–61] Microinfusion of 2-MeSATP *in situ* into the rat nucleus accumbens was shown to cause reactive astrocytosis, as demonstrated by upregulation of GFAP-immunoreactivity, cytoplasmic hypertrophy as well as an increase in the number of astrocytes.[60] Reactive astrocytosis was also documented after injection of 2-MeSATP in the optic nerves of rats.[61] However, as suggested by Franke *et al.* (1999),[60] ATP may initially act on microglia resulting in their release of factors that ultimately cause reactive astrocytosis.

b) Factors Produced by Reactive Astrocytes

Reactive astrocytes are known to express and/or release macrophage antigen complex-1 (mac-1), growth factors (GDNF, BDNF, VEGF, NGF, CNF, bFGF, TGF-β) and antioxidant enzymes (superoxide dismutase, catalase and glutathione-s-transferase).[48,62] Reactive astrocytes also increase the production of adhesion molecules, cytokines, chemokines and proteoglycans.[63] Likewise, reactive astrocytes have also been shown to over-express glutamate transporters GLAST[64] and GLT1,[65–67] the glucose transporter[68,69] and gap junctional proteins.[70–72] All of these factors provide the means for reducing oxidant injury, promote repair and diminish the extent of excitotoxic neuronal/oligodendroglial injury.

Upregulation of ion channels such as the inward rectifier K^+ channel Kir2.3 and L-type Ca^{2+} channels were also observed in reactive astrocytes in different neurological conditions.[73–75] While the significance of the upregulation of these ion channels is unclear, it has been proposed that they may contribute to the maintenance of ion homeostasis and to enhance the release of neurotrophic factors.[74,76]

c) Functions of Reactive Astrocytes

The functional significance of reactive astrocytosis remains unclear. As noted above, it has been proposed that reactive astrocytes are involved in restoring the integrity of the blood-brain barrier, maintaining appropriate extracellular glutamate levels, supporting neurite outgrowth and providing neurotropic factors (NGF, CNTF, bFGF).[48,77]

While beneficial functions have been proposed for reactive astrocytes, these cells have also been implicated in detrimental outcomes. Thus, glial scar formation may be an impediment to axonal regeneration.[78] This aspect will be elaborated upon below.

It should be emphasized that reactive astrocytes may be heterogeneous depending on the age of the disease process (acute or chronic), location of the astrocytes relative to the core of the lesion and the nature of the disease process the astrocyte is responding to. This heterogeneity needs to be considered when interpreting conflicting views on the significance of the reactive astrocytes following CNS injury. Additionally, many conclusions regarding their significance have been reached based on cell culture models, whose fidelity to the *in vivo* condition is frequently uncertain.

9.1.2.2 Cell Swelling

Astrocyte swelling is a common response to CNS injury. It is often observed after various intoxications (*e.g.* dinitrophenol, triethyltin, hexachlorophene, isoniazid),[79] Reye's syndrome,[80–82] acute hepatic encephalopathy (acute HE),[83–84] as well as in the early phase of ischemic stroke[85–87] and traumatic brain injury (TBI).[88–89] A major consequence of such cell swelling is the development of cytotoxic edema, a life-threatening process that may lead to increased intracranial pressure and brain herniation. Swollen astrocytes often display morphologic abnormalities (Figure 9.3), suggesting that such swelling may also lead to astrocyte dysfunction and an inability of these cells to carry out their vital functions.

Two forms of brain edema are recognized: cytotoxic and vasogenic.[90] Cytotoxic edema is largely due to astrocyte swelling, and the signaling systems involved in such swelling will be discussed below. Vasogenic edema is due to an extracellular fluid accumulation due to a breakdown of the blood-brain barrier. A discussion of signaling systems involved in vasogenic edema is beyond the scope of this review.

9.1.2.3 Alzheimer Type II Astrocytosis

In chronic metabolic disorders such as hepatic encephalopathy, hyperammonemia, Wilson's disease, renal failure, endocrine disorders and other conditions, astrocyte nuclei become enlarged and pale and no cytoplasmic abnormalities are observed by light microscopy, except for the presence of lipofuscin pigment (often a marker of oxidative stress) (Figure 9.4). Such cells

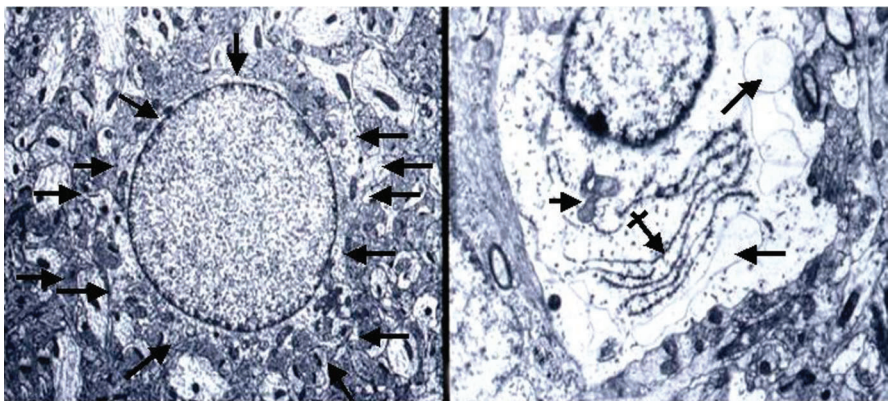


Figure 9.3 A Electron micrograph of a cortical astrocyte from a normal rat. The cytoplasm is barely discernable in this figure (the margins of the cytoplasm are outlined by arrows). B Astrocyte from a rat with acute hepatic encephalopathy showing a marked expansion of the cytoplasm which is clear (''watery'') and displays several vacuoles (long arrows), slightly disrupted endoplasmic reticulum (crossed arrow) and an irregular-appearing mitochondrion (short arrow).

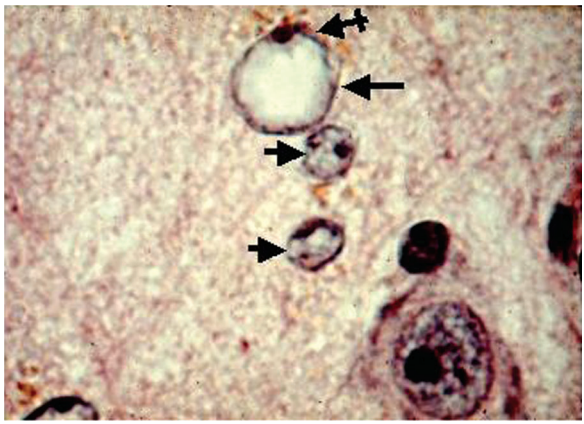


Figure 9.4 At the top of the figure is an Alzheimer type II astrocyte (long arrow) displaying an enlarged pale nucleus that contains a prominent nucleolus attached to the nuclear membrane (top of the nucleus). No well-defined cytoplasm is evident, except for the presence of lipofuscin pigment granules (crossed arrow). Relatively normal astrocytes are seen below (short arrows) for comparison.

are referred to as Alzheimer type II astrocytes. This astrocyte response has also been observed in the very early phase of ischemia, trauma and other acute injuries. The precise significance of this change is not known. By electron microscopy, the cytoplasm is slightly swollen and alterations in organelles have been described.[91] It is likely that these swollen cells are dysfunctional and that such dysfunction represents a major mechanism by which metabolic conditions bring about a disorder of the CNS.[92] As signaling systems involved in Alzheimer type II astrocytosis are unknown, this change will not be further considered in this chapter. For additional information on the Alzheimer type II astrocyte response, see references 93–94.

9.2 Intracellular Signaling System in Reactive Astrocytes

As noted above, cytokines, ATP, thrombin and various trophic factors are considered to be potent triggering agents for reactive astrocytosis. These factors stimulate various signaling systems that have been implicated in the development of reactive astrocytosis. This section will discuss the role of oxidative/nitrosative stress, mitogen-activated protein kinases, protein kinase C, phosphatidylinositol 3-kinase, STAT3 and NF-κB in the evolution of reactive astrocytosis.

9.2.1 Oxidative/Nitrosative Stress (ONS)

ONS is perhaps the earliest and most important factor that triggers various downstream signaling systems leading to reactive astrocytosis. ONS has been

considered as a major pathogenic factor in ischemic stroke, traumatic brain injury and acute hepatic encephalopathy (acute HE), as well as in most neurodegenerative disorders. Astrocytes are known to have potent antioxidant defense systems as they possess high concentrations of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. Nevertheless, astrocytes are also vulnerable to ONS.[95–96]

Free radicals have been shown to induce reactive astrocytosis as demonstrated by increased levels of GFAP as well as by morphological characterization (stellate appearance).[97–99] Similarly, oxidative stress following ischemic insult was reported to result in reactive astrocytosis in cultured astrocytes[100] and inhibition of ONS was shown to reduce astrogliosis in culture.[101,102] On the other hand, several reports indicate that reactive astrocytes can also produce free radicals, possibly by enhancing the production of proinflammatory cytokines and reactive oxygen/nitrogen species.[103]

9.2.2 Protein Kinase C (PKC)

PKC is involved in controlling the function of various proteins through the phosphorylation of serine and threonine amino acid residues on these proteins.[104,105] Upon phosphorylation (activation) by Ca^{2+}- and ONS-mediated pathways,[106] PKC is translocated to the plasma membrane where it is involved in proliferation, differentiation, apoptosis, receptor desensitization, plasma membrane modulation and cell growth.[107] Transforming growth factor-beta 1-induced activation of PKC in cultured astrocytes has been implicated in reactive astrocytosis.[108,109] Additionally, increased GFAP mRNA levels and PKC activation was observed in astrocytes over-expressing the HIV-1 envelope protein gp120 in mice.[110] Further, exposure of astrocyte cultures to soluble gp120 led to the activation of PKC and an increase in GFAP mRNA levels, while inhibition of PKC prevented the rise in GFAP mRNA levels, as well as the development of reactive astrocytosis.[110]

9.2.3 Phosphatidylinositol 3-Kinases (PI3K)

PI3K is an intracellular signaling kinase involved in cell growth and proliferation, differentiation, motility, survival and intracellular trafficking.[111,112] Astrocytes express PI3K, and its activation was implicated in the formation of reactive astrocytes after transient forebrain ischemia.[113] Further, purinergic receptor-mediated reactive astrogliosis was shown to occur through stimulation of PI3K signaling in cultured astrocytes.[114]

9.2.4 Mitogen-activated Protein Kinases (MAPKs)

One important consequence of oxidative stress is the activation of mitogen-activated protein kinases (MAPKs), including p38MAPK, c-Jun N-terminal kinase (JNK) and the extracellular signal-regulated kinase (ERK).[115] MAPKs

are serine/threonine-specific protein kinases that regulate gene expression, differentiation and proliferation as well as cell survival.

Activation of MAPK represents a major signal transduction pathway in reactive astrocytes. Activation of ERK/MAPK was observed in reactive astrocytes associated with various human conditions (trauma, chronic epilepsy, progressive multi-focal leukoencephalopathy).[116] Sustained ERK/MAPK activation was observed in reactive astrocytes following a forebrain stab lesion in mice.[117] Activation of ERK was also found in reactive astrocytes induced by a mechanical injury in cultured astrocytes.[118]

Increased activation of MAPK was detected in penumbral reactive astrocytes after middle cerebral artery occlusion in rats,[119,120] and in cultured astrocytes after an ischemic insult.[121] Activation of MAPKs was also implicated in reactive astrocytosis after focal mechanical injury in cultured astrocytes.[118]

Increased levels of ERK immunoreactivity were observed in reactive astrocytes in brain areas prone to neurofibrillary tangle formation (CAl/subiculum) in patients with Alzheimer's disease.[122] Likewise, activation of MAPKs was observed in cultured astrocytes after exposure to amyloid precursor protein (a key protein in the pathogenesis of Alzheimer's disease), and such activation-mediated reactive astrocytosis as demonstrated by increased GFAP expression and a stellate morphology.[101]

Activated ERK1/2 was identified in reactive astrocytes following injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice (model of Parkinson's disease),[123,124] after kainic acid-induced seizures in mice,[125,126] and in scrapie agent infected sheep and hamsters.[127–129]

Reactive astrocytosis, as demonstrated by increased GFAP levels, was also shown in astrocyte cultures exposed to stromal-derived cell factor-1 alpha (SDF-1 alpha; CXCL12) and cysteinyl-leukotriene receptor 1 (cys-LT1), agents known to activate ERK1/2.[130,131] Additionally, purines were shown to cause reactive astrocytosis in cultured astrocytes by their activation of ERK/MAPK.[132] Activation of JNK signaling was also reported to be involved in the astrogliosis associated with amyotrophic lateral sclerosis.[133]

9.2.5 Signal Transducer and Activator of Transcription 3 (STAT3)

One key signaling molecule that regulates GFAP expression is the transcription factor STAT3. Ciliary neurotrophic factor or cytokine induced STAT3 activation was shown to induce reactive astrocytosis.[134–136] Additionally, LPS and other inflammatory mediators such as meteorin, oncostatin M (a member of the IL-6 subfamily of cytokines, likely derived from activated microglia) and neuropoietin (a recently discovered cytokine of the gp130 family that shares functional and structural features with CNTF), were all shown to induce STAT3 activation *in vivo* or *in vitro*, and such activation led to reactive astrocytosis.[137–139] Conversely, use of a conditional gene deletion strategy that targets STAT3 in astrocytes in mice, or pharmacological inhibition of STAT3

with AG490 in cultured astrocytes, was shown to inhibit reactive astrocytosis.[140] A similar reduction of astrogliosis was observed in STAT3 deleted astrocytes in mice after spinal cord injury.[141,142]

9.2.6 Nuclear Factor Kappa B (NF-κB)

NF-κB is a protein complex that controls the transcription of many genes, especially those involved in immune responses, inflammation and cell proliferation.[143] NF-κB is normally present in the cytoplasm in an inactive form where it is bound to a member of the IκB family of inhibitory proteins. Upon activation by extracellular stimuli (*e.g.* cytokines, free radicals and intracellular signaling kinases), IκB inhibitors are modified through site-specific phosphorylation thereby allowing NF-κB to translocate to the nucleus and bind to target DNA elements where it activates the transcription of many genes.

Increased NF-κB was first identified in reactive astrocytes after kainic acid-induced seizures in rats,[144] and has subsequently been found in reactive astrocytes in scrapie-infected mice.[145] Amyloid precursor protein stimulates NF-κB activation in cultured astrocytes and such activation was shown to promote reactive astrocytosis.[101] Recent studies have also shown that cultured astrocytes exposed to a mixture of gangliosides (inducers of inflammatory processes in astrocytes[146]) develop reactive astrocytosis as demonstrated by increased GFAP expression. A ganglioside mixture also increased iNOS expression and NF-κB activation in cultured astrocytes, and these changes were blocked by inhibiting NF-κB.[146]

Increased GFAP, as well as activation of astrocytic NF-κB, was observed in rat brain after cerebral ischemia induced by bilateral common carotid artery occlusion, and inhibition of NF-κB prevented the formation of astrogliosis.[147] Ischemia-like injury induced by the alpha(2)-adrenoceptor agonist clonidine, or by adenosine A2a receptor activation in cultured astrocytes, was shown to induce astrogliosis,[148,149] and such an effect was partly mediated by activation of NF-κB.[150]

While the precise means by which activation of NF-κB contributes to increased GFAP expression and subsequent astrocytosis is unclear, it has been demonstrated that inhibition of NF-κB reduced levels of GFAP mRNA and protein in cultured human astrocytes.[150]

In summary, the above findings suggest that ONS and activation of signaling kinases, including PKC, PI3K and MAPKs, as well as the activation of the transcription factors STAT3 and NF-κB contributes to the development of reactive astrocytosis. A schematic diagram illustrating signaling systems involved in reactive astrocytosis is shown in Figure 9.5.

9.3 Signaling Systems in Astrocyte Swelling

As noted above, astrocyte swelling represents an important component of the cytotoxic brain edema following various forms of intoxication

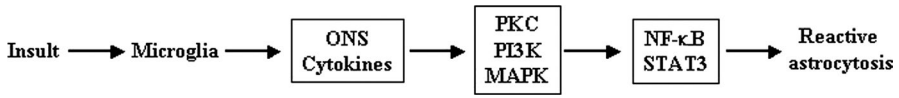


Figure 9.5 Proposed pathogenetic scheme whereby various signaling factors lead to reactive astrocytosis. Cytokines and ONS from microglial sources activate intracellular signaling kinases (PKC, PI3K and MAPKs), and such kinases further stimulate transcription factors (NF-κB and STAT3) resulting in reactive astrocytosis.

(*e.g.* dinitrophenol, triethyl tin, hexachlorophene, isoniazid), severe hypothermia, Reye's Syndrome, acute hepatic encephalopathy (acute HE), as well as in the early phase of ischemic stroke and traumatic brain injury (TBI). The signaling pathways involved in such swelling process are presented below.

9.3.1 Oxidative/Nitrosative Stress (ONS)

As noted above, ONS is an important early signaling event in many neurological disorders. ONS has been viewed as a particularly important signaling event in hyperammonemia/acute hepatic encephalopathy.[151,152] Increased free-radical formation, including nitric oxide, was observed in hyperammonemic rat brain,[153,154] as well as in ammonia-treated astrocyte cultures.[155] Cultured astrocytes exposed to ammonia induce lipid peroxidation and oxidation of cellular proteins.[156–158] A variety of morphological abnormalities are produced in astrocyte cultures exposed to ammonia,[91] and such effects are attenuated by treatment of cultures with antioxidants.[159]

Free radicals have been shown to cause cell swelling in brain slices,[160–162] as well as in cultured astrocytes.[163–166] We recently demonstrated that astrocyte cultures exposed to oxidants/nitric oxide donors or to a pathophysiological concentration of ammonia (known to induce free radicals) resulted in cell swelling, and such swelling was significantly attenuated by antioxidants or NOS inhibitors.[167,168]

Increased free radical production was also observed in brain after ischemia, as well as in cultured astrocytes after ischmic insult,[169,170] and trauma.[171–174] Inhibition of ONS significantly reduced cell swelling in cultured astrocytes after ischemia and trauma.[171,173,174] These studies strongly suggest that ONS is an important early event in cell swelling.

9.3.2 Cytokines

Proinflammatory cytokines, likely derived from activated microglia, have been considered as a major driving force in cell swelling/brain edema after ischemia and acute HE. Various cytokines have been shown to be synthesized in brain after injury.[175] Recombinant human IL-1-β infusion in brain was reported to aggravate the cytotoxic brain edema after middle cerebral artery occlusion.[176] Elevated levels of TNFα, IL-1β and IL-6 were found in blood of patients with

acute HE,[177–179] and induction of endotoxemia was shown to exacerbate cytotoxic brain edema in an experimental model of hyperammonemia.[180] Additionally, it has recently been shown that cultured astrocyte exposed to cytokines (TNFα, IL-1β, IL-6 and IFN-γ) stimulate cell swelling.[181] Taken together, these studies suggest an important role of cytokines in the development of astrocyte swelling/brain edema in various neurological conditions. While the means by which cytokines cause astrocyte swelling/brain edema is unclear, cytokines are well known to induce ONS as well as to activate signaling kinases and transcription factors (*e.g.* MAPKs and NF-κB). It is likely that the activation of these factors contributes to the cell swelling/brain edema in these conditions.

Signaling Kinases

9.3.3 Protein Kinase C (PKC)

Activation of PKC has been shown to facilitate the swelling induced by hypoosmotic stress in astrocyte cultures.[182] Cultured astrocytes exposed to arachidonic acid (a well-known free radical inducer) were also shown to induce cell swelling, and such cell swelling was mediated, at least in part, through activation of PKC.[183] Additionally, ATP and thrombin-induced PKC activation were shown to contribute to cell swelling in cultured astrocytes.[184,185] Activation of PKC and the subsequent increase in astrocyte swelling were also observed in a rat model of acute hemophilus influenzae meningitis.[186] While these studies support the involvement of PKC in cell swelling mechanisms, some reports indicate that activation of PKC decreases brain edema in rats after middle cerebral artery occlusion followed by reperfusion,[187] and decreases cell swelling in cultured astrocytes after cerebral ischemia/reoxygenation.[188] These contrasting results may reflect differences in timing and the experimental models employed.

9.3.4 Phosphatidylinositol 3-Kinase (PI3K)

Activation of PI3Ks was demonstrated in astrocytes after middle cerebral artery occlusion in transgenic mice expressing the human vascular endothelial growth factor, and it was shown that inhibition of PI3K significantly reduced cytotoxic brain edema.[189] Activation of PI3K was also documented in perivascular astrocytes after cerebral ischemia in mice and such activation was shown to contribute to cytotoxic brain edema.[190]

9.3.5 Protein Kinase G (PKG)

PKG, also known as cGMP-dependent protein kinase, is a serine/threonine kinase that phosphorylates other proteins known to be involved in cell cycle regulation.[244] Activation of PKG has recently been shown to be involved in cell swelling of cultured astrocytes after ammonia treatment, and inhibition of such activation blocked ammonia-induced astrocyte swelling.[191]

9.3.6 Mitogen-activated Protein Kinases (MAPKs)

Activation of MAPKs was observed in brains after acute HE.[192,193] Additionally, cultured astrocytes exposed to ammonia have been shown to increase the phosphorylation (activation) of ERK1/2, p38 and JNK,[192–194] and inhibition of ERK1/2, p38 and JNK activity significantly blocked ammonia-induced astrocyte swelling.[167]

Activation of ERK1/2, p38 and JNK was also observed in rat brain after TBI,[195] as well as in cultured astrocytes after *in vitro* trauma,[174] and inhibition of such activation reduced the trauma-induced swelling in cultured astrocytes.[174] Similar findings were also observed in rats subjected to middle cerebral artery occlusion.[196] Activation of MAPKs was also reported after hypoosmotic swelling in cultured astrocytes.[197]

While the means by which MAPKs mediate astrocyte swelling is unclear, recent studies have suggested that they are involved in the activation of ion transporting systems (ITSs), *i.e.* NKCC1,[198,199] NHE1[200–202] and NCX.[203,204] These ITSs have been implicated in the astrocyte swelling/brain edema associated with ischemia,[205] trauma[206,207] and ammonia neurotoxicity.[208] The involvement of ITSs in astrocyte swelling is elaborated in greater detail below.

Transcription Factors

9.3.7 Signal Transducer and Activator of Transcription 3 (STAT3)

Sinn *et al.* (2007)[209] found decreased STAT3 phosphorylation and increased brain edema following intracerebral hemorrhage in rats. Conversely, increased STAT3 phosphorylation and decreased brain edema were observed when rats were treated with geranylgeranylacetone, an activator of STAT3. We recently found that STAT3 is inactivated in ammonia-treated astrocyte cultures and that over-expression of STAT3 in cultures treated with the phophatase inhibitor sodium orthovanadate increased STAT3 phosphorylation as well as reduced cell swelling after ammonia treatment (unpublished observations). It is noteworthy that factors known to induce cell swelling (ONS, activation of MAPKs, NF-κB and p53) become activated after STAT3 inactivation.[210–212] These findings suggest the interesting possibility that STAT3 inactivation may be a crucial event in the evolution of astrocyte swelling/brain edema in various neurological conditions.

9.3.8 Nuclear Factor Kappa B (NF-κB)

Activation of NF-κB has been implicated in the formation of brain edema after ischemia.[213] We recently documented the activation of NF-κB in ammonia-treated astrocyte cultures, as well as in the cerebral cortex of mice with acute HE.[214,215] Such activation was mediated, at least in part, through the activation

of MAPKs.[214] Additionally, treatment of astrocyte cultures with BAY 11-7082, an inhibitor of NF-κB, reduced cell swelling.[214] Similar findings were also observed in astrocyte cultures derived from transgenic mice that have a functional inactivation of astrocytic NF-κB.[215] We also found that transgenic mice having a functional inactivation of astrocytic NF-κB did not exhibit brain edema after thioacetamide-induced acute HE.[215]

While the means by which activation of NF-κB contributes to cell swelling remains to be determined, it is known that inhibition of NF-κB significantly reduces the activity of inducible nitric oxide synthase,[214,216,217] an enzyme implicated in cell swelling mechanisms.[214,218,219] Additionally, NF-κB was found to increase the activity of cyclooxygenase 2 (COX2), and the inhibition of COX2 was shown to reduce cell swelling in cultured retinal Müller cells.[220] Inhibition of NF-κB significantly reduced COX2 activity as well as ammonia-induced astrocyte swelling.[218]

9.3.9 p53

p53 is a tumor suppressor protein and transcription factor that is a key mediator of stress responses.[221,222] Activated p53 triggers a number of signaling pathways that may lead to cell cycle arrest, apoptosis and DNA repair.[223,224] It also mediates mitochondrial dysfunction, including decreases in the mitochondrial membrane potential and reduction in complex IV activity.[225–227] Increased p53 expression was observed in rat brain cytoplasmic extract after acute ammonia administration.[228] It was recently found that astrocyte cultures exposed to ammonia stimulate p53 (phospho-p53^{ser392}) and that treatment of cultures with pifithrin-α (PFT), an inhibitor of p53, attenuated the ammonia-induced astrocyte swelling.[229,230] Additionally, Yan *et al.* (2008)[231] reported that rats treated with PFT had less brain edema after subarachnoid hemorrhage.

Swelling Effectors

Activation of the above-noted signaling factors ultimately converge on systems that immediately bring about cell swelling. These include various ion channels, exchangers and transporters, as well as the water channel protein aquaporin-4 (AQP-4).

9.3.10 Ion Channels/Transporters/Exchangers

Ion channels, exchangers and transporters (ion transporting systems, ITSs) are key factors involved in the maintenance of ionic balance in cells.[232–235] These ITSs include the Na–K–Cl cotransporter (NKCC), volume-sensitive osmolyte anion channels, Na^+/Ca^{2+} exchanger, the Na^+/H^+ exchanger, the SUR1-regulated non-selective cation channel ($NC_{Ca\text{-}ATP}$) and the transient receptor

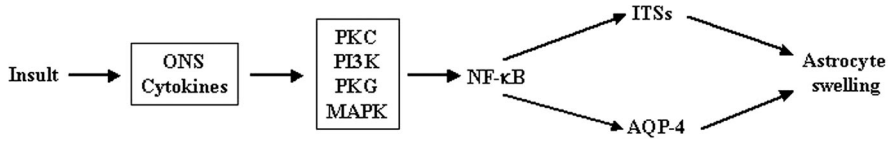


Figure 9.6 Schematic representation of signaling pathways in astrocyte swelling in different neurological conditoins. Cytokines and ONS from microglial or peripheral sources activate intracellular signaling kinases (PKC, PI3K, PKG and MAPKs), which then stimulate various transcription factors (NF-κB and p53). Activation of these signaling factors subsequently converge to activate ion transporting systems (ITSs) as well as AQP4 ultimately to result in cell swelling.

potential (TRP) ion channels.[232–235] A disturbance in one or more of these systems may result in loss of ion homeostasis and cell swelling. NKCC1, in particular, plays an important role in the maintenance of intracellular levels of Na^+, K^+ and Cl^-, and a defect in this system results in astrocyte swelling in many neurological disorders including ischemic stroke, TBI and acute HE.[89,205,236] Additionally, activation of NCX, NHE and $NC_{Ca\text{-}ATP}$ was also shown to induce cell swelling/brain edema after ischemia and TBI.[205,234]

9.3.11 Aquaporin-4 (AQP-4)

Aquaporins are integral membrane proteins that form pores and regulate water movement across biological membranes.[237,238] AQP-4 is the predominant water channel in brain,[239] and it is particularly enriched in astrocytes. AQP-4 in astrocytes has been shown to play a prominent role in the development of cell swelling/brain edema following ischemia, TBI and acute HE.[240,241] Conversely, AQP-4-null mice have been shown to be more resistant to cytotoxic brain edema.[242,243] A schematic diagram illustrating the involvement of signaling systems in cell swelling is shown in Figure 9.6.

9.4 Conclusions and Perspectives

A growing body of evidence indicates that astrocytes play crucial roles in the normal function of the CNS, including the regulation of synaptic activity, modulation of excitatory and inhibitory neurotransmission, support of energy metabolism, maintenance of extracellular pH and ion homeostasis, detoxification processes and maintenance of blood-brain barrier (BBB) integrity. Astrocytes are also key players in almost all pathological processes affecting the CNS. Their dramatic transformation into hypertrophic cells, a process referred to as reactive astrocytosis (astrogliosis), constitutes an event that follows all destructive lesions in the CNS. These activated cells play vital roles in restoring the composition of the extracellular milieu relative to appropriate levels of excitatory and inhibitory amino acid neurotransmitters, ions and pH, as well

as eliminating free radicals, generating growth factors necessary for repair, improving the operation of the BBB and providing a barrier to confine the lesion.

However, reactive astrocytes have also been implicated in deleterious consequences following CNS injury, in particular, by creating an obstacle to axonal regeneration. While there is evidence to support this claim, the issue remains unsettled. It should be recalled that impediments to axonal regeneration are found in oligodendrocytes, and when lesions occur in the spinal cord, they are often invaded by meningeal-derived cells that do indeed provide a barrier to regeneration.[48] The so-called glial scar also contains microglia and macrophages, which are capable of generating toxic cytokines and free radicals. It remains to be proven whether the astrocytic glial "scar" alone is capable of obstructing the process of regeneration.

Thus, whether reactive astrocytosis is beneficial or detrimental remains a controversial issue. In the authors' opinion, the beneficial effects far outweigh the potential detrimental effects of astrogliosis. We propose that reactive astrocytosis is a critical and necessary response in the early stages of destructive CNS processes. However, it may be harmful in latter stages by possibly contributing to an inhibition of axonal regeneration.

While the signaling pathways involved in reactive astrocytosis remain incompletely understood, it appears that the release of factors derived largely from microglia (cytokines, free radicals, ATP and others) can activate a number of signaling pathways in astrocytes culminating in astrogliosis. These include the signaling kinases, PKC, PI3K and MAPKs, as well as the activation of the transcription factors STAT3 and NF-κB.

The other astrocytic alteration following many CNS injuries is cell swelling. No beneficial consequences have ever been proposed for this astroglial response as it always appears to have detrimental effects, in particular by contributing to the formation of cytotoxic brain edema and increased intracranial pressure. Additionally, swollen astrocytes display degenerative ultrastructural changes that may result in defective astroglial functions.

Many of the identified signaling pathways that contribute to reactive astrocytosis are also involved in the mechanism of cell swelling. These include PKC, PI3K and MAPKs, as well as the transcription factor NF-κB. On the other hand, STAT3, which is activated in reactive astrocytes, appears to be inactivated in swollen astrocytes. Additionally, activation of p53 and PKG has been shown to contribute to cell swelling/brain edema in different neurological conditions, although their role in reactive astrogliosis has not been investigated.

The aim of this article was to review normal and pathological properties of astrocytes, focusing on the signaling mechanisms involved in the formation of reactive astrocytes and cell swelling. It is clear that much work is still necessary to achieve a more comprehensive assessment of signaling pathways involved in these critical astrocytic responses to CNS injury. A fuller understanding of the signaling mechanisms involved in these responses may provide novel therapeutic targets for ameliorating the ravages brought about by neurologic disease.

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CHAPTER 10

DNA, Nuclear Cell Signaling and Neurodegeneration

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10.1 Adipokines, Toxic Lipids and the Aging Brain

Most people are born with healthy brains and could keep their brains healthy if they knew how. But many aging people become less active and adopt toxic lifestyles that alter normal physiology and muscle mass. As the body ages muscle tissue is lost and fat tends to accumulate, sometimes with the development of the metabolic syndrome. At the same time, neurons and other brain cells accumulate more fat.[1,2] As ectopic and visceral fat accumulate, adipokines are secreted into the blood, including visfatin, leptin, resistin, TNFα, IL-6 and others.[3] At the same time, adiponectin, the protective adipokine, decreases.[3] Adipokines are secreted by visceral fat cells, macrophages and perhaps other cells. What are the consequences in the brain of these changes to the body?

Health care for stroke and Alzheimer's disease patients is very limited and has not greatly improved for more than 40 years. The typical care for a stroke patient is to observe them in the emergency room six hours after they suffered a stroke. This is because six hours is required for the brain lesion to stabilize. Many patients are then released to return home. For most other patients, health care is supportive. For 10% or fewer patients, a blood-clot-dissolving factor may be given, which can provide a small benefit to them. Alzheimer's disease care is

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limited to supportive care for many patients. Ginkgo or cholinesterase inhibitors can be used with small benefit to the patients.

10.1.1 Toxic Lifestyles, Adipokines and Toxic Lipids

Considerable controversy exists about lifestyle and obesity as causes of some forms of neurodegeneration. However, the most common cause of stroke is thrombosis lodged in the middle cerebral artery. Thrombosis frequently is caused by atherosclerosis, that is associated with toxic lifestyles, including alcohol consumption, smoking, the metabolic syndrome and lack of exercise.[4,5] It is clear that stopping toxic lifestyles and increasing exercise can have an impact on adipokines and the metabolic syndrome.[5,6] Lifestyle improvements, in clinical trials, lead to decreased leptin levels,[7] increased adiponectin levels,[8,9] decreased TNFα levels[9] and decreased C-peptide levels.[10]

Alzheimer's disease appears to be associated with, and perhaps caused by, changes in lipid metabolism in the brain, especially ceramide. Cholesterol and lipid metabolism are altered in Alzheimer's disease, which results in lipid accumulation in the brain and other cells.[2,11–14] Ceramide increases in the brain as an early event in the pathogenesis of Alzheimer's disease.[2,15–17] Astrocytes and cerebral cortical cells are the primary sites of increase.[18–20] Ceramide accumulation clearly is caused, in part, by sphingomyelin catabolism. However, excessive triglyceride accumulation can also lead to switches in cellular biochemistry that cause an increase in ceramide synthesis as an alternative fat storage mechanism.

Apolipoprotein E isoforms appear to be risk factors for Alzheimer's disease,[20,21] and are involved in triglyceride transport and uptake. This implies that the uptake of triglycerides into the brain may be involved in the development of Alzheimer's disease. This leads, in part, to ceramide accumulation.

A lifestyle that is good for the heart is also good for the brain. This is clearly demonstrated in the WHIMS trial where postmenopausal women with hypertension were at increased risk of developing dementia and white matter lesions.[22] Another risk factor for Alzheimer's disease is decreased muscle mass.[23] Exercise is good for the muscles, heart and perhaps the brain. Diets low in fat and high in fruit and vegetables are good for the heart and also decrease the risk of Alzheimer's disease.[24,25]

What causes the changes in lipid metabolism in Alzheimer's disease? Leptin levels may be low in Alzheimer's disease patients,[26] leading to decreased fatty acid catabolism and increased ceramide accumulation. It is not clear why leptin levels are lower in Alzheimer's disease patients compared to non-demented, age-matched controls. Is it possible that Alzheimer's disease patients are less obese than controls? Or is it possible that another factor causes leptin levels to decrease in people at risk of developing Alzheimer's disease?

The effects of lifestyle and toxic lipids are not well examined in other forms of neurodegeneration. However, half of the patients who have suffered from Parkinson's disease for more than a few years develop Alzheimer's disease. This suggests some commonality between the causes of Alzheimer's disease and Parkinson's disease.

10.1.2 Ceramide Toxicity in the Brain

During aging the brain switches from using tropomyosin related kinase A (TrkA) to p75 neurotrophin receptor (p75NTR) as the main receptor for nerve growth factor (NGF).[27] Both NGF receptors regulate the processing of APP (amyloid precursor protein) in the brain. p75NTR is involved in the activation of the beta cleavage of APP. APP is an integral membrane protein that is found abundantly in synapses. Ceramide accumulation increases the interaction of p75NTR, NGF and ceramide, which activates the pathway for beta scission of APP.[27] This produces amyloidβ in the brain that forms extracellular plaques and deposits around arteries and arterioles. All people accumulate amyloidβ plaques to a certain extent during normal aging. Alzheimer's disease patients frequently accumulate more plaques than normal. Amyloidβ causes ceramide to accumulate in the brain, in a vicious cycle, by stimulation of sphingomyelinase activity.[2,28]

Ceramide induces both the inducible and endothelial forms of nitric oxide synthase (iNOS and eNOS).[29,30] Ceramide also causes these enzymes to dysfunction, which produces peroxynitrite, superoxide and oxygen radicals rather than the normal nitrous oxide (NO). Superoxide and oxygen radical formation lead to dismutation and hydrogen peroxide generation. NO is usually protective of endothelial cells and causes vasodilation. However, peroxynitrite, superoxide and hydrogen peroxide damage endothelial cells and astrocytes in the brain.[31,32] This leads to a leaky blood-brain barrier that may attract monocytes and neutrophils.[33] Monocytes are known to penetrate the blood-brain barrier in Alzheimer's disease.[34]

Ceramide production in the brain is a vicious cycle in which initial formation of ceramide causes more ceramide accumulation.[35] Ceramide activates amyloidβ formation that induces NADPH oxidase (NOX) activity, which forms extracellular hydrogen peroxide.[35] Hydrogen peroxide can cross cell membranes and activates neutral sphingomyelinase that forms ceramide.[35] NOX is primarily a transmembrane enzyme found in macrophages, monocytes and neutrophils. NOX is discussed in more detail below.

Ceramide decreases glycolysis in the brain.[36] Alzheimer's disease is associated with decreased glucose uptake/metabolism in the brain.[37] This may involve amyloidβ competition with insulin for binding to the neuronal insulin receptor, thereby causing neuronal hypoglycemia and potential neuronal death.[38] This could be a critical mechanism for some populations of neurons that may rely on insulin or insulin-like growth factor to stimulate glucose uptake and use.[36] However, ceramide induced decreases in glycolysis will lead to less use of glucose and perhaps more use of fats as energy sources in the brain.

10.1.3 Endocannabinoids, Ceramide and Amyloidβ

Endocannabinoids, 2-arachidonyl glycerol and anandamide are involved in the regulation of energy metabolism in the body, and stimulate eating.[39] They are synthesized on demand and are released from neurons where they function

as neurotransmitters.[39] Endocannabinoids increase during the metabolic syndrome and when lipids accumulate in cells.[39] A cannabinoid receptor, transient receptor potential cation channel vanilloid 1 (TRPV1), may be involved in inflammation in the brain.[40] Amyloidβ increases 2-arachidonyl glycerol levels and decreases anandamide levels.[40] This may produce a vicious cycle in which endocannabinoids stimulate more eating, leading to more ceramide and amyloidβ, which increases 2-arachidonyl glycerol levels.

10.2 The Blood-Brain Barrier as a Target for Neurodegenerative Conditions

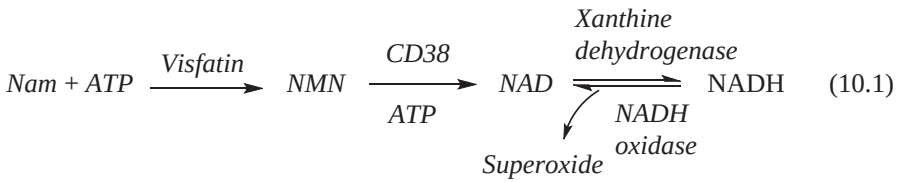
If adipokines are involved in neurodegeneration, it is logical that they might act at the blood-brain barrier. Adipokines are proteins that do not readily cross the blood-brain barrier. Damage to the blood-brain barrier might allow amyloid to penetrate into the brain, accumulate around blood vessels and form plaques in the brain. A recent paper suggests that protection of the blood-brain barrier may be useful in Alzheimer's disease.[41] Monocytes and other white blood cells penetrate the blood-brain barrier and infiltrate into brain tissue in Alzheimer's disease.[42] The adipokine monocyte chemoattractant protein-1 (MCP-1) causes monocytes to stick to brain endothelial cells which increases blood-brain barrier permeability.[43] Monocytes induce endothelial cells to make tissue-type plasminogen activator that allows the monocytes to penetrate through endothelial tight junctions.[44] How does the blood-brain barrier become damaged such that monocytes begin to stick?

10.2.1 Visfatin and the Blood-Brain Barrier

Visfatin is secreted by visceral adipocytes and macrophages, and has insulin mimetic activity such that it binds to and activates the insulin receptor.[45] Visfatin is also known as pre-B cell colony enhancing factor and is important in the maturation of B cells.[40] Visfatin is also nicotinamide phosphoribosyl transferase and makes nicotinamide mononucleotide (NMN) from nicotinamide and ATP.[46] ATP is found normally in plasma,[47] as is nicotinamide. Plasma NMN is made into NAD by CD38, NADH pyrophosphatase and other extracellular enzymes located on lymphocytes and other cells.[48] CD38 normally breaks down NAD. However, when NAD levels are very low, such as in normal plasma, CD38 operates in the reverse direction and synthesizes NAD. NAD is reduced to NADH by xanthine oxidoreductase and other extracellular dehydrogenases.[49] NADH is a substrate for NADH oxidase, an ecto-enzyme that is found on endothelial cells, monocytes, macrophages, neuronal plasma membranes and other cells.[50–54] An NADH oxidase located on white blood cells increases in activity during aging.[55]

NADH oxidase differs from NOX.[56] NOX transfers electrons from cytoplasmic NADPH through the transmembrane enzyme to extracellular oxygen, forming extracellular superoxide.[57] NADH oxidase is an extracellular,

membrane-bound enzyme that transfers electrons from extracellular NADH to extracellular oxygen, making extracellular superoxide.[50] NADH oxidase is the major source of superoxide in endothelial cell preparations.[50] Amyloidβ has NADH oxidase activity and forms extracellular oxygen radicals from extracellular NADH.[58] Therefore, NADH generated in the blood of aging patients may enter into a vicious cycle with the generation of oxygen radicals that may damage the vasculature and neurons.



(10.1)

Nicotinamide (Nam) has significant pharmacological activity that is distinct from niacin.[59] Nam is a powerful neuroprotective agent that has been in use in clinics to save the lives of thousands of patients with pellagra-induced dementia and brain damage since the 1930s.[59] Nam is a precursor for NAD and an inhibitor of poly(ADP-ribose) polymerase (PARP). NAD is primarily involved in the repair of DNA damage in the nucleus since it is a substrate for PARP.[59] Of course, NAD and NADH are involved in cellular energy metabolism, especially in mitochondria. The pharmacology of NMN is poorly described. Several compounds similar to NMN, such as cyclic ADP-ribose, ADP-ribose and NaADP (nicotinic acid ADP), are calcium-mobilizing agents.[60] NMN can inhibit the enzymatic degradation of NAD by glycohydrolase.[61]

Visfatin is secreted by visceral adipocytes and macrophages.[62] Therefore, damage to the blood-brain barrier by ceramide may attract monocytes/macrophages that locally produce visfatin. Visfatin induces the formation of TNFα, IL-6 and other cytokines in monocytes and other cells.[63] TNFα stimulates the secretion of adhesion molecules that cause the chemotaxis of white blood cells, such as more monocytes.[64–66] Il-6 stimulates the activation of monocytes.[64–66] Visfatin then establishes a redox cycle that depends on extracellular NADH and results in much more damage to the blood-brain barrier. It may be that ceramide and visfatin work together to damage the blood-brain barrier and attract macrophages.[67]

10.3 Oxygen Radicals, Hydrogen Peroxide and Cell Death

Ceramide and visfatin appear to induce the formation of oxygen radicals including peroxynitrite and hydrogen peroxide. Peroxynitrite is charged and does not normally cross cell membranes. It is a powerful protein-nitrating agent that nitrates protein phosphatase type 2A.[68] This nitration inhibits the normal

phosphorylation that controls the enzyme. The nitrated enzyme, in endothelial cells, is dysfunctional and causes the blood-brain barrier to leak.

Hydrogen peroxide quickly passes through membranes and within minutes causes DNA to fragment (Figure 10.1). Hydrogen peroxide may form a hydroxyl radical that rapidly breaks DNA. DNA fragmentation occurs through three mechanisms: cleavage of the deoxyribose radical, Criegee rearrangement and peroxide migration. Radical cleavage involves cleavage of a negatively charged phosphate moiety leaving a positively charged deoxyribose radical. Criegee rearrangement involves hydroxide attack of the deoxyribose with elimination of a negatively charged phosphate moiety. Peroxide migration involves elimination of an uncharged phosphate moiety with ring opening of the deoxyribose. Of course, radical oxidation of DNA bases can also occur. DNA peroxidation and cleavage leads to activation of PARP that activates

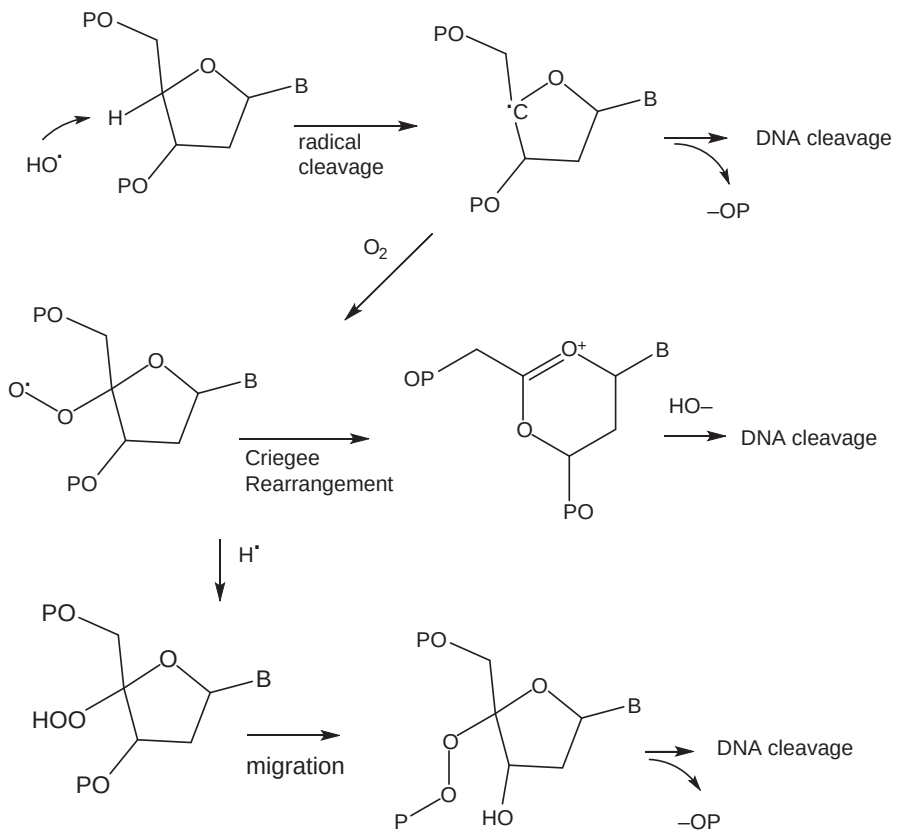


Figure 10.1 DNA peroxidation and cleavage by oxygen radicals. B is any DNA base. PO is phosphate in the DNA structure. DNA cleavage occurs through three mechanisms: cleavage of the deoxyribose radical, Criegee rearrangement and peroxide migration.

DNA repair enzymes.[69] PARP uses NAD as an energy source and as a substrate to poly(ADP-ribosylate) itself and several nuclear enzymes. This poly(ADP-ribosylation) alters the activities of many enzymes.

Neuronal DNA is much more than just an archive of genetic information. It is very actively involved in producing proteins for neurotransmitter synthesis, release, reuptake and other neuronal functions. Damage to neuronal DNA very extensively affects the ability of neurons to function normally, including maintaining neurotransmitters.[70]

PARP may ADP-ribosylate various transcription factors that regulate gene transcription.[71,72] PARP is a component of positive cofactor 1 activity that regulates class II gene transcription.[70] When DNA is damaged, PARP is activated, which inhibits class II gene transcription regulated by RNA polymerase II.[73] PARP is also important in the action of p53, the tumor suppressor protein. Both PARP and p53 bind to DNA breaks. PARP can form complexes with p53 that may alter the activity of p53.[74] Interestingly, p53 is involved in the inhibition of RNA polymerase III dependent gene transcription.[75]

It is important to recognize that the PARP referred to above is PARP-1. There are several enzymes with PARP activity.[76] It is not known if the other enzymes, PARP-2, PARP-3, tankyrase and V-PARP, can fill in for PARP-1 when PARP inhibitors are used. Clearly, in PARP-1 knockout mice, the other PARP enzymes are still functional and can protect DNA and synthesize poly(ADP-ribose). It is also not known if PARP inhibitors are specific for PARP-1 or can inhibit all forms of PARP.

The energetic consequences of DNA damage and PARP activation are enormous (Figure 10.2). PARP activation rapidly depletes NAD and ATP levels in the cell, leaving the cell depleted of energy sources.[69,77] NADPH depletion also occurs.[77] Glutathione oxidizes. Glycolysis, the pentose phosphate pathway and mitochondrial energetics are affected.

Recent research indicates that the secondary brain injury associated with stroke is induced by inflammatory processes. Nicotinamide can decrease the recruitment of neutrophils to potential sites of inflammation by inhibiting PARP in neutrophils and other cells.[78] In fact, nicotinamide has been recommended for the treatment of arthritic patients since the 1940s. It was found in pilot trails that nicotinamide improved joint mobility and decreased the need for anti-inflammatory medication in arthritic cases.[79] In the process of inflammation, the genes for intercellular adhesion molecule 1 and collagenase in neutrophils are activated. Neutrophils are recruited to sites of inflammation. Nitric oxide synthase is activated and oxygen radicals, hydrogen peroxide and nitric oxide are released. These reactive species can damage cellular DNA in the area of inflammation, resulting in apoptosis, necrosis and more serious inflammation.[80] PARP has a number of functions in inflammation due to its ability to regulate gene expression.[80] Inhibition by nicotinamide of PARP leads to decreased expression of these genes and decreases the extent and severity of inflammation. Nicotinamide also decreases the induction of iNOS, thereby decreasing damage to the blood-brain barrier.[81]

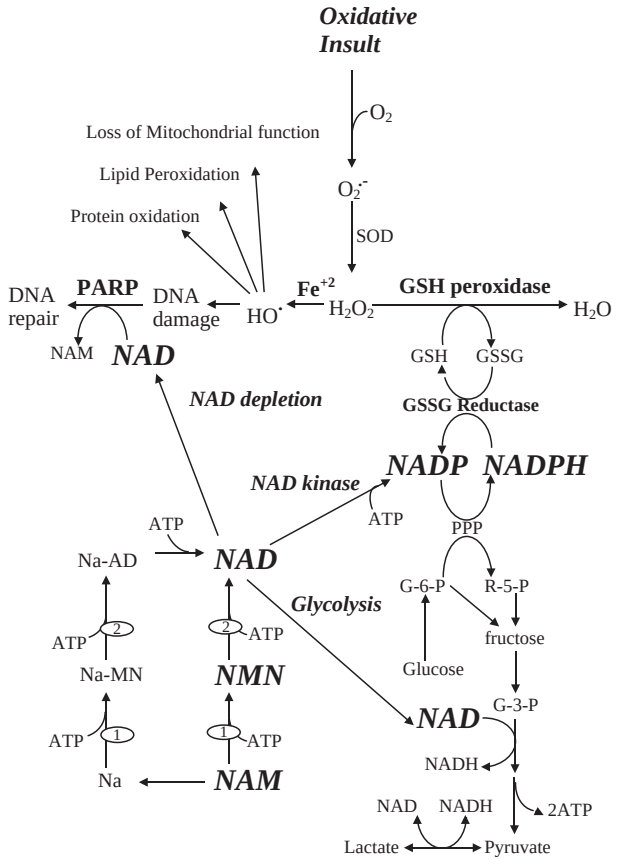


Figure 10.2 The energetic consequences of DNA damage caused by oxidative stress. PARP is poly(ADP-ribose) polymerase. NAM is nicotinamide. NMN is nicotinamide mononucleotide. Na is niacin. Na-MN is niacin mononucleotide. Na-AD is niacin adenine dinucleotide. 1 is nicotinamide phosphoribosyl transferase, which requires ATP. 2 is NMN adenyl transferase, which requires ATP. PPP is the pentose phosphate pathway. G-6-P is glucose-6-phosphate. R-5-P is ribose-5-phosphate. G-3-P is 3-phosphoglycerate.

DNA damage leads to cell death. A large amount of DNA fragmentation causes rapid necrosis, with cell swelling and rupture, nuclear swelling and rupture and cytoplasmic vacuole formation. A smaller amount of DNA fragmentation causes apoptosis with cell shrinkage, nuclear condensation and fragmentation, large vacuole formation in the cytoplasm and cell fragmentation forming apoptotic bodies.[82] Necrosis is a rapid process that does not require energy. Apoptosis is a delayed process that requires ATP. The center of a brain infarction is typically made up of necrotic cell debris.[83] The limit area surrounding the core contains necrotic, apoptotic and normal cells.[83]

10.4 Gene Transcription and DNA Damage

A very useful model for DNA damage in the brain is the tertiary-butylhydroperoxide (tBuOOH) model.[84,85] Following intracerebroventricular injection of tBuOOH, DNA fragments within minutes followed by apoptosis and necrosis of many cells in the brain.[70] DNA fragmentation and PARP activation lead to changes in gene expression within 1 hour or more (Table 10.1).[86] Most of the genes are involved in apoptosis. Reactive oxygen species, such as hydrogen peroxide, at the cell surface activate TNFR that activates caspases 3, 6, 7 and 8. Hydrogen peroxide penetrates into the cell and inactivates various cytoplasmic IAPs. Hydrogen peroxide also penetrates into the nucleus, fragments DNA, activates PARP and increases the transcription of genes for caspases 3, 6 and 7. Hydrogen peroxide penetrates into the endoplasmic reticulum and increases the release of active caspase 4. All of these events are critical to the induction of apoptosis. Of course, apoptosis is a cascade of several events for which the full sequence is not completely understood. However, if enough pro-apoptotic events are activated and enough anti-apoptotic events are inhibited, apoptosis occurs. Nicotinamide can prevent apoptosis by interfering with the cascade of apoptotic events.[86]

As mentioned above, PARP and p53 bind to DNA breaks and become activated. Another protein that is activated and involved in altering gene transcription during DNA damage is cohesin.[87] Cohesin is required to hold together the chromatid pairs. There is a variety of p53-mediated alterations in gene expression following DNA damage. These alterations involve activation of the forkhead transcription factor (Foxp3).[88] DNA damage leads to histone activation and neovascularization, that may be critical in stroke.[89] Neovascularization helps restore blood flow to brain areas that are recovering from stroke. It is not clear if neovascularization is helpful or harmful in Alzheimer's

Table 10.1 List of genes observed to be significantly differently expressed between t-BuOOH treated and control HCN2 cells 1 to 6 hrs following analysis.

Name of gene	*Gene ID*
Bcl2 like 11	NM_006538
TNF (ligand) superfamily, member 9	NM_003811
Caspase 4	NM_001225
Nucleolar protein 3	NM_003946
Lymphotoxin beta receptor (TNFR superfamily, member 3)	NM_002342
Myeloid cell leukemia sequence (mcl-1)	NM_021960
Bacculoviral IAP containing repeat 6	NM_016252
cIAP1	NM_001166
cIAP2	NM_001165
Mdm-2	NM_002392
TNFR2	NM_001066

Bcl2 like 11 is the B-cell lymphoma 2 like 11 gene also called apoptosis facilitator. TNFR is tumor necrosis factor receptor. IAP is inhibitor of apoptosis proteins. Mdm is murine double minute oncogene, a suppressor of P53 expression.

disease and Parkinson's disease. Neovascularization can produce arterioles that leak until they become well established and form tight junctions between endothelial cells. Leaky arterioles may produce more damage in stroke, Alzheimer's disease and Parkinson's disease.

10.5 Conclusions

Neurodegeneration may be caused by or increased by toxic lifestyles. Diet and exercise improvements could be encouraged to prevent or perhaps control neurodegenerative diseases. Toxic lifestyles lead to adipokine elevations in the blood and oxygen radical formation, possibly through visfatin, CD38 and NADH oxidase mechanisms. Hydrogen peroxide formed during this process crosses cell membranes and damages DNA. The cells first damaged in this process are probably the endothelial cells of the blood-brain barrier. Adipokines cause white blood cells and platelets to stick to the damaged blood-brain barrier. This sets up an inflammatory process that produces more damage. The leaky blood-brain barrier allows the infiltration of inflammatory cells into the brain parenchyma. These inflammatory cells produce oxygen radicals that damage the DNA in neurons and other brain cells leading to brain lesions. In Alzheimer's disease these lesions are most important in the hippocampus, cerebral cortex and other areas. In Parkinson's disease these lesions are critical in the dopaminergic neurons of the substantia nigra, dopaminergic neuronal projections in the basal ganglia and other regions.

Stroke is a rapid process that also involves toxic lifestyles. Adipokines damage arteries in the body. Clots and thrombi are produced that lodge in the middle cerebral artery and other locations to produce ischemia, followed later by reperfusion as the clot or thrombus is removed or resolves. Ischemia and reperfusion produce oxygen radicals and hydrogen peroxide that damage cellular DNA leading to cell death through apoptosis and necrosis. Brain lesions are typically seen in the basal ganglia, hippocampus, cerebral cortex and other regions.

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CHAPTER 11

G Protein-Coupled Receptors: Conformational "Gatekeepers" of Transmembrane Signal Transduction and Diversification

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11.1 Introduction

Cells are the fundamental units of all known life forms. This central role requires the cells to sense and interact with their environment or other cells and produce a physiological response vital to the proper functioning of the cell. Cellular signaling machinery is comprised of different proteins that enable and drive the amazing plethora of functions necessary for growth, survival and eventual death at the cellular level and in turn at the organism level (for multi-cellular life).

Cells need to process signals produced both inside and outside the cell. The extracellular signals can originate from other cells in the same organism (*e.g.* adrenaline), other organisms (*e.g.* pheromones) or the environment (*e.g.* photons, tastants, *etc.*). Cellular signaling proteins have evolved to sense this diverse set of signals either *via* direct contact with the signals

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(*e.g.* binding to the signaling molecule) or *via* binding to other proteins that are in direct contact with the signals. Different cells are also programmed to sense different signals depending on the proteins expressed in their plasma membrane. These membrane proteins not only enable signal transmission, but also signal modulation and diversification by undergoing conformational changes and/or through the specificity of protein-protein interactions.

Transmembrane (TM) signal transduction by membrane proteins is the dominant component of cellular signaling, as it enables a cell to convert an extracellular signal into one or more intracellular signals or responses. There are three main classes of membrane proteins that enable TM signal transduction: ion-channel receptors, enzyme-linked receptors and G protein-coupled receptors (GPCRs), with GPCRs playing the most prominent role.

In this chapter, we will focus on GPCRs and use current structural insights from experiments and computational predictions to describe the spectacular role played by conformationally malleable GPCRs in sensing extracellular signals, which cover a broad spectrum from photons (for vision), chemosensory ligands (*e.g.* for taste and smell) and neurotransmitters/hormones (*e.g.* dopamine, serotonin, adrenaline, acetylcholine) to peptides (*e.g.* cytokines) and larger proteins. Being receptors for neurotransmitters, hormones and cytokines, GPCRs have been implicated in a majority of disease processes, which has made them a very attractive target for therapeutic applications, as blocking or activating these receptors by antagonists or agonists respectively blocks or activates a whole cascade of signaling events. However, different subtypes of a GPCR (capable of sensing the same agonist) might induce a different or even opposite response to the signal depending on the cell type and the location in the organism. This is one of the many reasons that most drugs have side-effects and the lack of complete knowledge about the signaling pathways being manipulated by drugs makes it difficult to ascertain whether some of the side-effects are caused by off-target proteins (other proteins or other subtypes of the target protein) or by the target protein itself. Designing GPCR drugs with no or minimal side-effects is one of the biggest challenges in the rational design of GPCR targeting therapeutics.

This chapter contains a brief section on cellular signaling to provide some background and context for GPCR-mediated signal transduction followed by a detailed section on GPCRs, which is divided into subsections on their structure, signal-induced conformation driven signal transduction (amplification and diversification), biased signaling induced by extracellular ligands and challenges in rational design of GPCR targeting drugs. The focus of the chapter is the processing and modulation of extracellular signals by GPCRs, so discussion on intracellular signaling pathways will be limited and will only focus on their direct connection to GPCR activation. The chapter concludes with a brief section on the discoveries and challenges that lie ahead for GPCR-mediated signaling.

11.2 Cellular Signaling

Life of an organism at the biochemical level can be thought of as a collection of biological events, some occurring sequentially in time and some in parallel. Each of the biological events can, in turn, be broken down into one or more signaling cascades that usually consist of multiple signaling processes separated in space and time. How a specific cell in an organism will behave depends critically on this spatio-temporal separation of signaling processes. Cellular signaling broadly refers to these highly evolved networks of signaling events and cascades that allow a cell to function.

Cellular signaling has been studied for more than 100 years now and our knowledge of its complexity at multiple levels has been greatly enhanced through advances in many different areas of biology.[1–3] It still appears that we may have barely opened the "Pandora's box" as the current knowledge seems unable to explain the beautiful richness of the complexity of life observed on land and especially in the oceans. One of the many great examples of signaling complexity manifested in nature is the dynamic camouflage ability of cuttlefish, where highly coupled signaling cascades enable these mollusks to replicate not only the color of their environment but also its visual pattern and texture (depth) onto their skin to blend in with that environment.[4,5]

11.2.1 Types of Signaling

Any signaling network or cascade is a series of biochemical processes, where each process is initiated by the appearance of a signal which is followed by its sensing, processing and transmission as another signal or signals for the next downstream process in the signaling cascade. The signal may appear either inside or outside the cell for processing. Extracellular signals are usually sensed and processed by plasma membrane proteins. Intracellular signals are processed by soluble proteins or membrane proteins in the plasma membrane or those on the surface or cell organelles.

The spatio-temporal separation of signaling processes and cascades mentioned earlier allows one to classify signaling processes into the following types that depend on the spatial origin of the signal in an organism and its reach within the organism:

a) *Endocrine signaling:* In this long-range signaling, signal molecules such as hormones are released by a cell and travel long distances (*via* bloodstream in animals or vascular system in plants) to cause an effect in a different part of the organism. Processing of sensory signals like light, taste and smell can also be considered endocrine.
b) *Paracrine signaling:* This is a short-range version of endocrine signaling, where the signal produced by a cell is sensed locally, *e.g.* neurotransmitters that are processed by proximal neurons.
c) *Juxtacrine signaling:* In this signaling process, the signal is membrane bound on one cell and is sensed by a receptor on the adjacent cell,

e.g. membrane proteins on a cell membrane can be sensed by a Notch protein on the neighboring cell.

d) *Autocrine signaling:* In this signaling process, cells release a signal molecule outside the cell, which is sensed by a membrane protein on the same cell leading to self-stimulation, *e.g.* breast cancer cells release transforming growth factor alpha (TGF-α) that interact with its epidermal growth factor (EGF) receptor.
e) *Intracrine signaling:* In this signaling process, the signal molecule is generated inside the cell and sensed by another receptor from inside the cell.
f) *Electrical signaling:* This specialized signaling process propagates an electrical potential along the length of the cell and occurs on a long spatial scale. The cells that use this process are the neurons of the animal nervous system, which are unusually long cells.

Any signaling cascade may be made up of one or more of the above-mentioned signaling processes.

11.2.2 Membrane Proteins in Signaling

The diversity of signals is immense. Chemical signals are molecules ranging greatly in size from the very small (like oxygen molecule, adrenaline, *etc.*), to peptides (like cytokines) and large proteins. Non-chemical signals include photons that are absorbed by cis-retinal-rhodopsin complex in the retina and initiate a cascade of processes that start in the cell and end in the brain with the perception of vision. Different proteins have evolved along with the signaling processes to sense this broad spectrum of signals.

Spatial separation of signaling cascades in an organism is achieved by cells expressing different receptors on their surface as well as inside the cell. Cell surface receptors (membrane proteins) enable signal transduction across the plasma membrane by converting an extracellular signal into one or more intracellular signaling cascades. Three main classes of membrane proteins dominate TM signal transduction:[6]

a) *Ion-channel receptors (ICRs):* These proteins are responsible for sensing neurotransmitter molecules or voltage gradients across the membrane, as upon binding to the signal molecules or sensing the membrane potential these receptors undergo a conformational change that opens or closes a channel and allows specific ions to cross the plasma membrane.[7]
b) *Enzyme-linked receptors (ELRs):* These are a diverse class of single-pass TM proteins that contain an extracellular ligand binding site and an intracellular catalytic/enzyme-binding site with a guanylyl cyclase, phosphatase, serine/threonine kinase or tyrosine kinase activity. Receptor tyrosine kinases dominate this class.[8]
c) *G protein-coupled receptors (GPCRs):* These form the largest superfamily of membrane proteins that undergo "signal-specific" conformational changes upon activation by a diverse set of extracellular signals. These

conformational changes in the receptor are transmitted to cytoplasmic G proteins and β-arrestins for downstream signal transmission and potential diversification for a physiological response as will be discussed later.

GPCRs are integral membrane proteins with an extracellular N-terminus and seven TM helices connected by loop regions. They use their N-terminus, extracellular loops and extracellular facing TM portions to sense their signals. As a single protein family, they interact with the most diverse set of signals from sensory signals (vision, taste, smell, pheromones, *etc*.) to large signal molecules (other proteins).

GPCRs are the focus of this chapter because being embedded in the plasma membrane they sit at the top of complex signaling cascades as gatekeepers and use their conformational flexibility to amplify, diversify and select downstream signaling pathways inside the cell with amazing specificity. This role has also implicated them in almost all disease mechanisms[9] and about 30% of the approved drugs use them as targets to block or activate a whole signaling pathways in cells.[10]

In the next section we will describe what is known about GPCRs, both experimental and computational efforts to determine their structures, biochemical studies probing their downstream signaling effects, structural as well functional implications of their signal sensing (ligand binding) properties and challenges in drug design aimed at GPCR targets.

11.3 G Protein-Coupled Receptors

GPCRs are integral membrane proteins with seven TM helices connected by three extracellular loops (ECLs) and three intracellular loops (ICLs). They form the largest superfamily in the human genome with ~800 GPCRs identified, including ~370 non-sensory receptors.[11] A variety of bioactive molecules, including biogenic amines, peptides, lipids, nucleotides, hormones and proteins modulate GPCR activity to effect regulation of essential physiological processes (*e.g*. neurotransmission, cellular metabolism, secretion, cell growth, immune defense and differentiation). Thus, many important cell recognition and communication processes involve GPCRs. Due to mediating numerous critical physiological functions, GPCRs are involved in all major disease areas including cardiovascular, metabolic, neurodegenerative, psychiatric, cancer and infectious diseases.[9] GPCRs represent 30–50% of the current drug targets for activation (by agonist drugs) or inhibition (by antagonists or inverse agonists). It is estimated that the ~80 GPCR-targeting drugs currently marketed account for ~$50 billion annual sales. Many of these drugs have annual sales >$2 billion. Target evaluation, lead identification and optimization of GPCR assays have accelerated progress in identifying multiple subtypes for many GPCRs with specific cell and tissue functions. A detailed structural understanding of their function (activation) will have a tremendous and broad impact in many areas.[12]

11.3.1 Structure of GPCRs

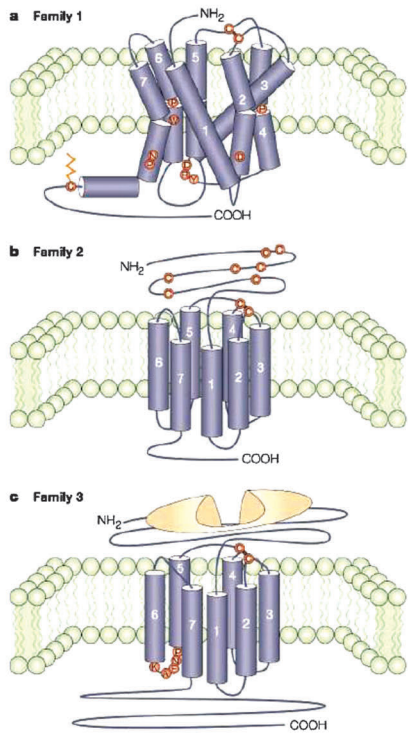
GPCRs can be organized into six families (acronymed GRAFTS, a minor variation on the original GRAFS classification proposed[13] based on the phylogenetic criteria): glutamate, rhodopsin, adhesion, frizzled, taste2 and secretin.

a) *Rhodopsin family (also called Class A or Family 1):* This diverse family dominates the human GPCRs with ~670 members (out of ~800 total). The family is further divided into four subfamilies – α, β, γ, δ. The α subfamily includes light-sensing rhodopsin receptor, biogenic amine (dopamine, serotonin, histamine, muscarinic) receptors as well as cannabinoid and prostanoid receptors among others. The β subfamily mainly consists of peptide-binding proteins. The γ subfamily receptors bind to peptides or lipid-like molecules, some examples being chemokine, angiotensin, somatostatin and opiod receptors. The δ subfamily is dominated by olfactory receptors (~388 out of ~670 total in rhodopsin family) and also contains purinergic and glycoprotein-binding receptors. The vomeronasal pheromone receptors putatively also belong to the rhodopsin family. Being the largest family, it is not surprising that this family is targeted by the majority of GPCR drugs.
b) *Secretin/Adhesion family (also called Class B or Family 2):* The secretin receptors of this family bind peptide hormones, whereas Adhesion receptors bind to extracellular matrix molecules based on the knowledge of receptors de-orphaned so far.[13]
c) *Glutamate family (Class C or Family 3):* This family consists of metabotropic glutamate receptors, γ-aminobutyric acid B ($GABA_B$) receptors, sweet and umami (due to glutamate in monosodium glutamate or MSG, a food additive) taste receptors and calcium-sensing receptor. One of the two taste receptor monomers (T1R1, T1R2) combines with a third monomer (T1R3) to form functional heterodimers for sweet taste (T1R2 + T1R3) or umami taste (T1R1 + T1R3).[14]
d) *Frizzled family:* This family consists of ~10 frizzled receptors (which bind to Wnt glycoproteins) and a smoothened receptor (which appears to function without binding to any ligand).
e) *Taste2 family:* This family exclusively consists of ~25 bitter taste receptors,[15–16] which share the sensing of different bitter tastants with a different subset of receptors. These taste receptors have recently been found in the gastrointestinal (GI) tract as well.[17] Their function in the gut is not known but their activation (in mice) has been shown to activate gut hormonal receptors (cholecystokinin or CCK and peptide YY or PYY),[18] which are coupled to the glucagon-like peptide 1 (GLP-1) and other glucose metabolism pathways.

The structural topology of the receptors within each of the families mentioned above appears to be similar based on structural and sequence analysis as shown in Box 11.1 (for Family 1, 2 and 3). GPCRs in general are not

Box 1 | **Classification of G-protein-coupled receptors**

G-protein-coupled receptors (GPCRs) can be divided phylogenetically into six families (see the GPCR database online). Schematic representations of receptor monomers showing some key structural aspects of the three main families are shown. Family 1 (panel a; also referred to as family A or the rhodopsin-like family) is by far the largest subgroup and contains receptors for odorants, small molecules such as the catecholamines and amines, some peptides and glycoprotein hormones. Receptors of family 1 are characterized by several highly conserved amino acids (some of which are indicated in the diagram by red circles) and a disulfide bridge that connects the first and second extracellular loops (ECLs). Most of these receptors also have a PALMITOYLATED cysteine in the carboxy-terminal tail. The recent determination of the crystal structure of rhodopsin has indicated that the transmembrane (TM) domains of family 1 receptors are 'tilted' and 'kinked' as shown. Family 2 or family B GPCRs (panel b) are characterized by a relatively long amino terminus that contains several cysteines, which presumably form a network of disulfide bridges. Their morphology is similar to some family 1 receptors, but they do not share any sequence homology. For example, the family 2 receptors also contain a disulfide bridge that connects ECL1 and ECL2, but the palmitoylation site is missing, the conserved prolines are different from the conserved prolines in the family 1 receptors and the DRY (aspartic acid, arginine, tyrosine) motif adjacent to TM3 is absent. Little is known about the orientation of the TM domains, but — given the divergence in amino-acid sequence — it is probably quite dissimilar from that of rhodopsin. Ligands for family 2 GPCRs include hormones, such as glucagon, gonadotropin-releasing hormone and parathyroid hormone. Family 3 (panel c) contains the metabotropic glutamate, the Ca^{2+}-sensing and the γ-aminobutyric acid $(GABA)_B$ receptors. These receptors are characterized by a long amino terminus and carboxyl tail. The ligand-binding domain is located in the amino terminus, which is often described as being like a 'Venus fly trap'. Except for two cysteines in ECL1 and ECL2 that form a putative disulfide bridge, the family 3 receptors do not have any of the key features that characterize family 1 and 2 receptors. A unique characteristic of the family 3 receptors is that the third intracellular loop is short and highly conserved. Although the structure of the amino terminus is well characterized, similar to the family 2 receptors, little is known about the orientation of the TM domains.



Box 11.1 Reprinted by permission from Macmillan Publishing Ltd.: George *et al.*, *Nat. Rev. Drug Discov.*, **1**(10), 808–820. Copyright 2002.

homologous to each other unless they bind to the same ligands and, apart from the seven-TM helix topology, nothing appears to be common across all receptors.

Rhodopsin (Family 1) receptors share some common sequence motifs like D(E)RY at the bottom of TM3, WXPFF motif in TM6, NPXXY motif in TM7 and some conserved prolines usually in the middle of many TMs that produce kinks in their helices. Small molecule ligands typically bind in the extracellular facing half of the TM regions and peptides/proteins bind mainly to the extracellular loops and N-terminus. There is a highly conserved disulfide bridge between cysteines in ECL2 and top of TM3.

Secretin/Adhesion (Family 2) receptors have a long N-terminal ectodomain that binds to ligands and contains many conserved cysteines, which can help the long N-terminus to form a stable tertiary structure (see Box 11.1). These receptors don't share any sequence motifs with Family 1 receptors even in the TM regions, so it is not obvious if they will have the same TM bundle topology of Family 1 receptors.

Glutamate (Family 3) receptors have a long N-terminus and a long C-terminus as well. Most receptors use their long N-terminus to bind to their

endogenous ligands and the binding pocket is sometimes referred to as the *venus fly trap* (or VFT; see Box 11.1).

Next we will describe the structure determination efforts aimed at GPCRs and what we have learnt from the available structures generated by these efforts and functional studies of GPCRs.

11.3.1.1 Structure Determination

The experimental structure determination of GPCRs had been quite slow until recently relative to other membrane proteins (and obviously soluble proteins) despite intense efforts by many protein crystallography and NMR groups (currently, six GPCR structures present in the PDB out of more than 65,000 structures). Until 2007, crystal structure was available only for bovine rhodopsin.[19,20] This lack of structures was due to various factors including poor protein expression levels, difficulties in large-scale receptor purification, the insolubility in media-lacking phospholipids and other difficulties in crystallization. Significant technological advances in GPCR crystallization techniques have been made in the last few years that include emergence of lipidic cubic phase crystallization[21] and its coupling to the protein fusion methodology[22] that replaces a disordered region of protein structure with T4-lysozyme to increase the surface area potential for crystal contacts.

These advances have resulted in the availability of crystal structures[23] of two human GPCRs: β2 adrenergic receptor (β2AR) bound to a partial inverse agonist[24] and adenosine A_{2A} receptor ($A_{2A}R$) bound to an antagonist.[25] Other advances include increasing GPCR thermal stability by systematic mutagenesis (which led to the structure of turkey β1 adrenergic receptor (β1AR),[26] and optimization of receptor purification (which led to the structure of activated ligand-free bovine opsin structure by itself[27] and in association with a carboxyl-terminal peptide fragment of its G_α subunit transducin).[28] Figure 11.1

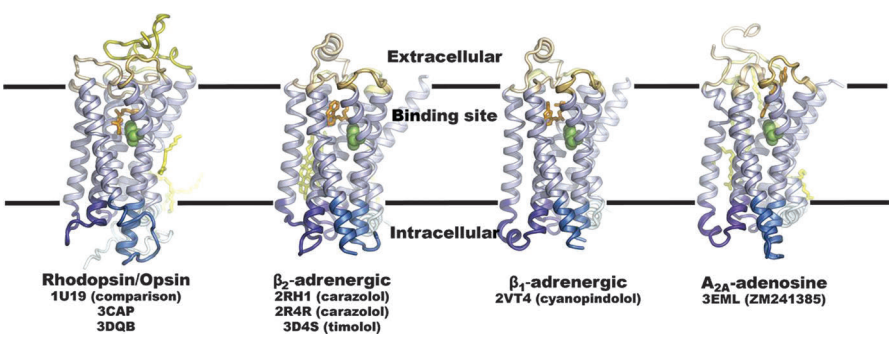


Figure 11.1 Four representative crystallized GPCRs solved to date. Reprinted from structure **17**(1), Hanson and Stevens, Discovery of New GPCR Biology: One receptor Structure at a Time, 8–14, Copyright 2009 with permission from Elsevier.

shows the structures of bovine rhodopsin, human β2 adrenergic, turkey β1 adrenergic and human adenosine A_{2A} receptors.[23] They all share the same TM topology (relative positions of TM helices), but can differ (sometimes significantly) in helix tilts and rotations as will be discussed in the next section.

An invertebrate GPCR (squid rhodopsin) has also been crystallized, which showed unusually long TM regions 5 and 6.[29] At least two more human GPCR structures are expected this year (dopamine D3 and chemokine CXCR4 receptors) and significantly more in the next decade. Progress is also being made in developing solid-state NMR techniques[30] for GPCR structure determination. This rapid growth in GPCR crystal structures since 2007 is beginning to provide insight into the structural biology of these proteins,[23,31] however, the progress is expected to remain slow due to intrinsic flexibility of these versatile receptors, which prevents them from packing into ordered crystals.

11.3.1.2 Structural Diversity of Current GPCR Structures

The seven-TM helix topology of GPCRs presents unique advantages and challenges for the quantification of sequence-structure relationships. Many comparative modeling programs can predict structures of globular proteins (with 30% or higher sequence identity to a crystallized protein) to a reasonable accuracy, as the belief is that a major fraction of structural folds is now known for globular proteins. The same cannot be said for membrane proteins in general. GPCRs, however, can be thought of as having one structural fold, consisting of seven TM helices interconnected by intracellular and extracellular loops. The TM helices display high sequence conservation as compared to the loop regions as expected (see Table 11.1).

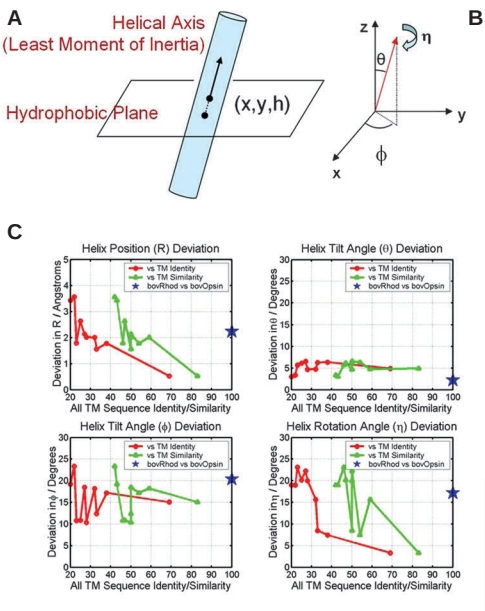
The table shows the sequence identity (Table 11.1A) and sequence similarity (Table 11.1B) (similarity using BLOSUM62,[32] where two residues are considered similar if the corresponding substitution element in the BLOSUM62 matrix is > 0) for the five GPCR sequences that have been crystallized.

To quantify the relationship between sequence and structure for GPCRs, we need to characterize the known structures using some standard geometrical parameters. As crystal structures don't provide absolute membrane orientation of GPCRs, we use their orientation as predicted by the OPM (Orientation of Proteins in Membrane) database,[33] which aligns each newly deposited membrane protein structure to an implicit membrane maximizing the free energy of membrane insertion. The middle of the membrane corresponds to the $z = 0$ plane or the hydrophobic plane. Each GPCR structure can then be characterized by the six orientation parameters of the seven helices relative to this plane. Figure 11.2A shows how the helix position and tilt are defined. Helix position (R) on the hydrophobic plane is then given by x and y. Value h corresponds to the hydrophobic center residue from the helix that will be

Table 11.1 Sequence comparison across five GPCR sequences that have been crystallized so far, with first number for the whole sequence and the second number for all TMs: **A** Sequence Identity; **B** Sequence Similarity (using the Blosum62 matrix).[32]

A	% Sequence Identity (Full_Seq / Only_TMs)				
	bovRhod	humBeta2	turBeta1	humA2A	squRhod
bovRhod	100 / 100	14 / 20	14 / 22	14 / 25	21 / 32
humBeta2	14 / 20	100 / 100	43 / 69	21 / 33	17 / 28
turBeta1	14 / 22	43 / 69	100 / 100	22 / 38	16 / 27
humA2A	14 / 25	21 / 33	22 / 38	100 / 100	18 / 23
squRhod	21 / 32	17 / 28	16 / 27	18 / 23	100 / 100

B	% Sequence Similarity (Full_Seq / Only_TMs)				
	bovRhod	humBeta2	turBeta1	humA2A	squRhod
bovRhod	100 / 100	31 / 43	26 / 42	27 / 47	38 / 59
humBeta2	31 / 43	100 / 100	55 / 83	33 / 50	33 / 50
turBeta1	26 / 42	55 / 83	100 / 100	34 / 54	31 / 50
humA2A	27 / 47	33 / 50	34 / 54	100 / 100	32 / 46
squRhod	38 / 59	33 / 50	31 / 50	32 / 46	100 / 100



B

GPCR2-GPCR1	TMH	Δx	Δy	Δθ	ΔΦ	Δη
Beta1-Beta2	TMH1	-0.2	1.2	-6	-3	3
Beta1-Beta2	TMH2	-0.1	0.0	-7	-8	-6
Beta1-Beta2	TMH3	0.0	0.0	-4	2	0
Beta1-Beta2	TMH4	0.1	-0.1	2	-35	4
Beta1-Beta2	TMH5	0.0	0.2	1	13	1
Beta1-Beta2	TMH6	0.3	0.0	4	-4	-3
Beta1-Beta2	TMH7	0.5	0.3	7	-12	4
A2A-Beta2	TMH1	-2.2	-1.5	-6	-24	7
A2A-Beta2	TMH2	-0.3	0.0	-9	-9	-16
A2A-Beta2	TMH3	0.0	0.0	-7	-4	-10
A2A-Beta2	TMH4	2.3	1.1	5	-7	-6
A2A-Beta2	TMH5	-0.2	1.1	0	-13	3
A2A-Beta2	TMH6	-1.0	0.3	-9	9	4
A2A-Beta2	TMH7	0.1	-1.1	-3	-10	6
SquRh-Beta2	TMH1	-3.6	-0.7	-8	-19	1
SquRh-Beta2	TMH2	0.2	0.0	0	5	31
SquRh-Beta2	TMH3	0.0	0.0	0	5	1
SquRh-Beta2	TMH4	-0.7	-0.2	-5	7	-26
SquRh-Beta2	TMH5	-2.5	2.2	3	-2	32
SquRh-Beta2	TMH6	-1.3	0.8	-8	-4	12
SquRh-Beta2	TMH7	-1.1	-0.2	0	-16	-7
BovRh-Beta2	TMH1	-3.6	-1.6	-3	-36	17
BovRh-Beta2	TMH2	-0.2	0.0	-2	-11	10
BovRh-Beta2	TMH3	0.0	0.0	-2	-24	11
BovRh-Beta2	TMH4	2.1	1.1	-1	-9	-2
BovRh-Beta2	TMH5	-3.7	5.0	0	-23	43
BovRh-Beta2	TMH6	-3.1	2.1	-1	-1	9
BovRh-Beta2	TMH7	-2.9	0.6	7	-1	7

GPCR2-GPCR1	TMH	Δx	Δy	Δθ	ΔΦ	Δη
Rhodopsin-Opsin	TMH1	-0.4	-0.3	3	-6	5
Rhodopsin-Opsin	TMH2	0.1	0.0	-1	-5	-7
Rhodopsin-Opsin	TMH3	0.0	0.0	-1	-14	28
Rhodopsin-Opsin	TMH4	2.1	1.4	0	-6	16
Rhodopsin-Opsin	TMH5	-1.1	3.6	-1	-26	6
Rhodopsin-Opsin	TMH6	0.4	3.7	3	43	31
Rhodopsin-Opsin	TMH7	0.0	0.8	4	-10	2

Figure 11.2 **A** Definition of the helical axis; **B** Table showing relative orientation parameters for the GPCRs with crystal structures; **C** Correlation of deviation in orientation parameters with sequence identity/similarity.

positioned on the hydrophobic plane. Two angles, θ and ϕ, specify the tilt angles of the helix and the angle η corresponds to the helix rotation angle about its axis. The two tilt angles (θ,ϕ) and the rotation angle (η) require a definition of the helical axis, which needs to account for the reality of bent helices as prolines are commonly found in the TM helices. We use a helical axis that corresponds to the lowest moment of inertia vector for the helix obtained by diagonalizing the moment of inertia matrix for the helix using only heavy backbone atoms.

We rotate the membrane-aligned GPCRs from the OPM database in the x–y plane such that the helical axis of TM helix (TMH) 3 goes through the origin, and that of TMH 2 intersects the x-axis. Figure 11.2B shows the relative six orientation parameters for all seven helices for the crystallized GPCRs relative to β2 adrenergic receptor and of bovine rhodopsin (*cis*-retinal bound form) relative to bovine opsin (the retinal free form) that is considered a conformation along the activation pathway of rhodopsin.

In order to correlate the sequence variability of these GPCRs with their helix geometries, we calculated all-to-all (across these systems) RMS (root-mean-squared) deviations in position R (x,y position in the $z = 0$ plane), and angles θ, ϕ, η averaged over all helices and plotted them against the corresponding sequence identity and similarity. The equations used for the deviations between

a GPCR i and a GPCR j are:

$$R_{RMSD}^{i,j} = \sqrt{\frac{1}{7}\sum_{k=1}^{7}\left[\left(x_k^i - x_k^j\right)^2 + \left(y_k^i - y_k^j\right)^2\right]}$$
$$\alpha_{RMSD}^{i,j} = \sqrt{\frac{1}{7}\sum_{k=1}^{7}\left(\alpha_k^i - \alpha_k^j\right)^2}, \alpha = \theta, \phi, \text{or } \eta \quad (11.1)$$

The sum over index k is for the seven TM helices. Figure 11.2C shows all-to-all deviations (for all i–j pairs of GPCRs) as a function of identity or similarity between GPCR i and GPCR j, to highlight the variability seen in these five systems. In addition, the deviation of bovine rhodopsin and opsin is plotted (as a blue star symbol) to show the variability in functional conformations originating from a single GPCR sequence. At least across the GPCRs with known structure, we see that the deviations in helix position (**R**) and helix rotations (η) are inversely correlated to the closeness (identity or similarity) between sequences. The corresponding deviations in helix tilts (θ,ϕ) appear to be independent of the sequence identity or similarity, with bigger deviations in the ϕ tilt angle. Absolute deviations of these tilt angles across the GPCRs can be inferred from Figure 11.2B and are roughly in the range $\pm 10^\circ$ for θ and $\pm 45^\circ$ for ϕ. The deviation of bovine rhodopsin and opsin is at par with that of weakly related sequences as seen from the blue star symbol in Figure 11.2C. Overall, the deviations in helix orientation appear large except for receptors in the same family (β1 and β2) and any structural prediction method should be able to sample these deviations in a complete way in order to identify all low-energy conformations, including the active conformations. Except for the differences between bovine rhodopsin and opsin, a clear structural view of the conformational changes that occur upon GPCR activation is still lacking. It is critical to understand these activation-related conformational changes because it will not only shed light on the function of GPCRs but will also provide a unique structural handle on designing better drugs through direct modulation of GPCR function.

11.3.1.3 Prediction of GPCR Structure and Ligand Binding

In the absence of experimental structure information for almost all GPCRs, protein structure prediction and modeling is playing an increasingly important role in providing detailed structural information that is relevant to their activation and ligand binding. Membrane proteins and their environment have been the focus of structure prediction and dynamics simulations for some years now.[34] The interaction of these proteins with their lipid environment is considered critical to their *in vivo* folding and many recent studies have attempted to quantify this interaction on an absolute thermodynamic basis[35] by providing, for example, thermodynamic costs for the insertion of

amino acids (that make up the TM helices) into the lipid bilayer.[36] An implicit membrane potential of mean force has also been obtained recently for each amino acid as a function of the membrane normal using experimental structures of α-helical membrane proteins.[37] The 3D-structure of these α-helical membrane proteins, to which GPCRs belong, is strongly affected by interhelical interactions (mainly H-bonds and salt-bridges).[38] An accurate structure-prediction methodology needs to be able to sample and describe these interhelical interactions very thoroughly.

Availability of a good structure (from experiment or modeling) allows for the molecular dynamics (MD) simulations to be performed on these proteins in their native lipid environment under ambient conditions. MD simulations of biomolecular systems have come of age and are contributing enormously to the understanding of their dynamical behavior. With more affordable and more powerful computers, the dynamics of these membrane proteins can be followed in their explicit lipid environment for hundreds of nanoseconds or more. This situation is only going to improve with time, allowing for dynamics over even longer timescales of the order of microseconds. However, the conformational changes that accompany GPCR activation are known to occur on the millisecond or higher timescales, suggesting that explicit all-atom dynamics will not be able to describe these large conformational changes for some years; coarse grained simulations may do it sooner.

Many methods have been used to obtain model structures for membrane proteins due to their pharmacological importance. These methods have been reviewed elsewhere.[34,39] For GPCRs the main approach has been homology modeling (using the X-ray structure of bovine rhodopsin as a reference until 2007, and others more recently). Because of their low homology to other GPCRs of pharmacological interest, most studies have used constraints based on mutation and binding experiments coupled to the homologous rhodopsin structure to guide additional mutation experiments. These structures have not generally been sufficiently accurate for predicting binding sites of ligands. Methods are also available for predicting structures of membrane proteins in general.[40]

Our group has been developing *de novo* computational approaches (not based on homology) such as MembStruk and HierDock,[41] for predicting the 3D structure of a GPCR, and its ligand binding sites. MembStruk method involved prediction of the TM regions, helix optimization based on TM regions, placement of optimized helices in a template (rhodopsin), followed by a local optimization of the helix rotations. These methods have been applied successfully to dopamine,[42] adrenaline,[43] muscarine,[44] chemokine,[45] prostaglandin DP[46] and serotonin[47] receptors. In all these cases, MembStruk was used to generate an ensemble of GPCR structures, out of which only one structure was carried forward for docking. After predicting the best structure for the GPCR, we used the HierDock procedure to locate the binding region and to predict the binding configuration in this region. Then we compared the predicted structures for the ligand-GPCR complex with experimental binding and mutation data and in some cases with experimental Structure-Activity-Relationship (SAR) data.

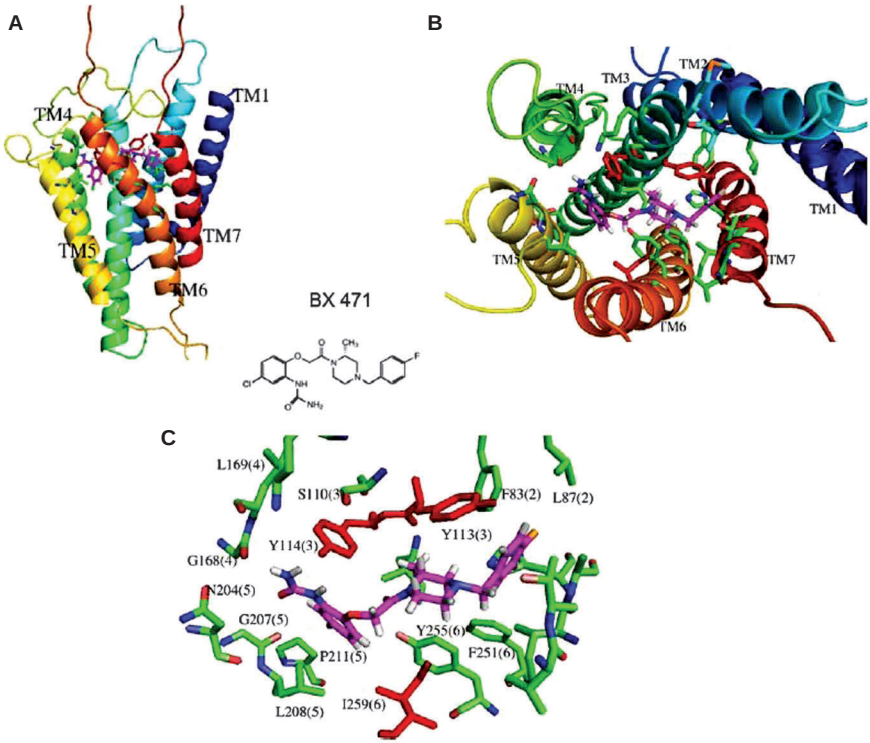


Figure 11.3 The predicted structure of human CCR1 bound to BX471 (from ref. 45). **A** Side view. **B** Top view. **C** Detailed binding site view.

As reviewed recently,[48] this led to excellent results for modest-sized ligands such as dopamine and epinephrine and even for ligands such as haloperidol and domperidone. For example, Figure 11.3 shows the predicted structure for human CCR1 along with the binding site for its antagonist BX471.[45] In this GPCR system, the mutagenesis studies were performed after the predictions, providing confidence in these prediction methods.

We have recently replaced the MembStruk method with the GEnSeMBLE (GPCR Ensemble of Structures in Membrane BiLayer Environment) method, which besides implementing an improved TM prediction step (PredicTM) contains a helix rotation optimization step (BiHelix) based on pairwise helix interactions that performs complete and thorough sampling of $\sim$1 billion conformations in a highly efficient way (see Figure 11.4). This method was recently applied to predict the structure of human adenosine A_{2A} receptor and its ligand binding site.[49] The structural comparison of predicted and crystal structure is shown in Figure 11.5. The method was able to predict the ligand to within 2.8 Å of the crystal pose and also identified 9 out of 12 protein residues in the binding site.

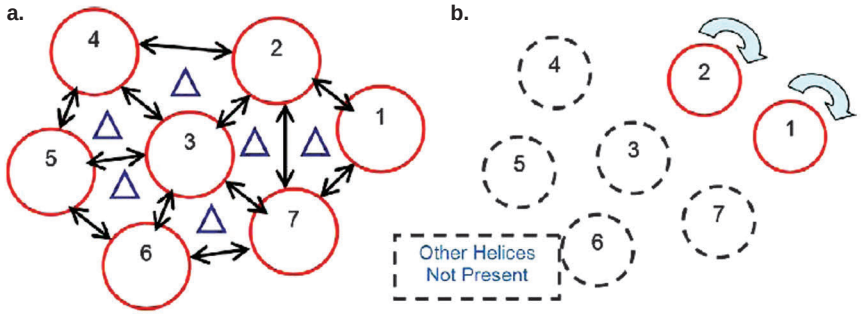


Figure 11.4 BiHelix sampling scheme, where sampling is done two helices at a time for all interacting helix pairs. **a**. All interacting helix pairs shown with a double arrow. **b**. Helix1-Helix2 optimization shown in the absence of other helices.

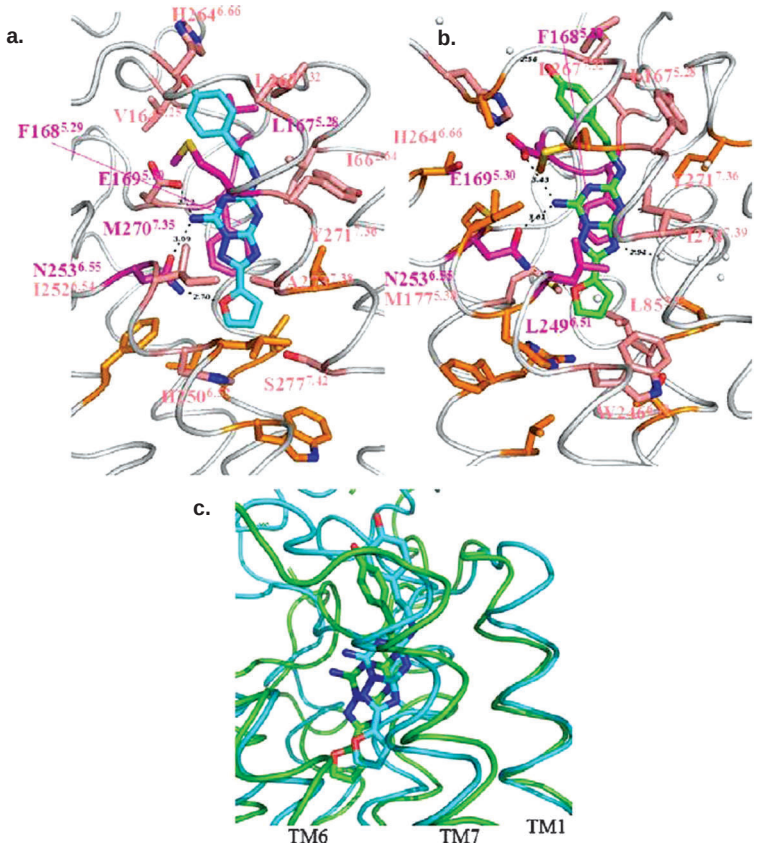


Figure 11.5 Predicted binding site of ZM241385 for human A_{2A} adenosine receptor. **a**. Predicted pose. **b**. Crystal pose. **c**. Predicted and crystal poses overlaid. Reprinted with permission from Elsevier from Goddard *et al*., *J. Struct. Biol*., **170**(1), 10–20. Copyright 2010.

11.3.2 GPCR Activation: Conformation Driven Functional Selectivity

GPCRs undergo activation in response to an extracellular signal (*e.g.* an agonist ligand) and that information is relayed inside the cell through the coupling of the activated GPCR with cytoplasmic G proteins, which is normally followed by its coupling to β-arrestins that eventually ends in the internalization of the GPCR embedded into endosomes inside the cell. In that respect they can be called nature's allosteric robots that enable transduction of extracellular signals across the plasma membrane.

Proteins by their very nature are dynamic macromolecules and under physiological conditions exist in an ensemble of conformations. This dynamic motion can be considered molecular "breathing"[50] and is supported by NMR data as well as computer simulations. This motion can be visualized as that of a golf ball (protein) rolling on a golf course (the potential energy surface) being randomly kicked around (due to thermal fluctuations) by a force (the temperature, solvent, *etc.*). The bottom of the protein's multi-dimensional potential energy surface (corresponding to troughs on the golf course), called an energy well, can accommodate a micro-ensemble of isoenergetic and similar conformations. The greater the depth of an energy well, the more time the protein will spend in the conformations corresponding to that well. Favorable interaction with another molecule (*e.g.* a ligand or another protein) will change the character of the protein's potential energy surface and the energy wells resulting in a different ensemble of protein conformations that would prefer binding to that other molecule. Now we will see how these ideas can be applied to GPCRs.

11.3.2.1 Multi-Conformational View of GPCRs

One of the fundamental challenges in structure determination of GPCRs is their conformational flexibility. The Kobilka group has shown evidence for multiple conformational states in β2 adrenergic receptor even in the presence of a single ligand,[51] consistent with the energy landscape idea presented earlier. Debra Kendall's group has shown convincingly that a single mutation in the CB1 receptor can change the constitutively active receptor into the inactive form or the active form.[52] This strongly suggests that even in the absence of any ligand, GPCRs are capable of major conformational changes with different functional outcomes.

These observations present a special problem in understanding structure and function of GPCRs: conformational changes in the protein structure are an essential aspect of its function. Thus, to understand GPCR activation we need to consider the multiple conformational states that the receptor can have under physiological conditions and we must consider the changes in these populations as the ligand interacts with them. This presents an enormous challenge to purely experimental structure determinations since the crystal must have all ligand-protein complexes identical, as evidenced by the fact that all currently available GPCR structures are in their inactive form (except opsin).

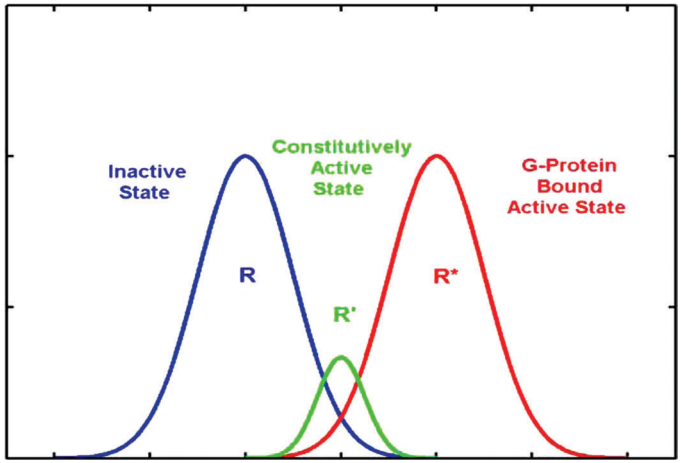


Figure 11.6 Functional ensemble view of GPCR conformations (adapted from ref. 53).

Kenakin provides a nice ensemble framework to think about multiple conformations of GPCRs.[53] Let us call **R** the set or ensemble of conformations that correspond to the inactive state of a GPCR. Similarly, let us call **R*** the set of active conformations. GPCRs commonly show constitutive activity, which can have a phenotype distinct from an active state or an inactive state, so let us call **R′** the set of conformations which are constitutively active. In general, the **R** and **R*** sets could overlap in the region of constitutive activity, so the functional ensemble picture of GPCR dynamics can be represented by Figure 11.6. Also, a constitutively active conformation from R′ can easily convert into an inactive (**R**) or an active (**R***) conformation depending on the conditions.

A picture is slowly emerging of the dynamic role played by these GPCR conformations in modulating and diversifying an extracellular signal inside the cell. Kenakin and Miller have aptly labeled these receptors as "shape-shifting" proteins to capture this conformational dynamism.[54] In their words, these receptors are "pleiotropic" in terms of the multiple intracellular signaling cascades they can affect upon binding to an agonist (*e.g.* multiple G protein-coupled and β-arrestin coupled pathways). This opens the possibility of different agonists affecting the multiple intracellular signaling cascades differently, which appears to be the norm for these receptors.[55] This not only turns the classical receptor theory for the relative efficacy of agonists on its head but also leads to diabolical ligand classifications as will be seen later in the chapter.

11.3.2.2 Ligand or Mutation Stabilized Ensemble of GPCR Conformations

Most GPCRs in the apo (ligand-free) form are capable of displaying constitutive (also called basal) activity. This state would correspond to the **R′** set of conformations shown in Figure 11.6. GPCRs exist in this state ready to

be converted to their active or inactive forms. They putatively go through a series of conformations during these conversions, where an agonist will stabilize conformations along the active pathways, and an inverse agonist will stabilize conformations moving towards the inactive form as shown in Figure 11.7.[56] Even receptors like rhodopsin with no basal activity undergo transformations during activation from an inactive conformation to an active conformation through multiple intermediates.[57]

The Kobilka group has shown that in the presence of a single ligand (norepinephrine), the β2 adrenergic receptor can exist in multiple conformational states[51,58] as shown in Figure 11.8. They have also linked different ligands to specific conformational switches for the β2 adrenergic receptor[59] and have very recently shown conformational coupling between the extracellular surface (ECS) and orthosteric binding site in the TM region that can stabilize different conformations of a GPCR.[60] Recent computational studies

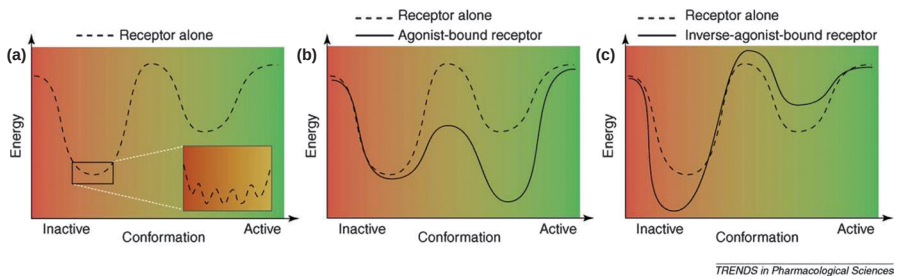


Figure 11.7 Energy landscape of the inactive and active GPCR conformations. Reprinted with permission from Elsevier from Kobilka and Deupi, *Trends Pharmacol. Sci.*, **28**(8), 397–406. Copyright 2007.

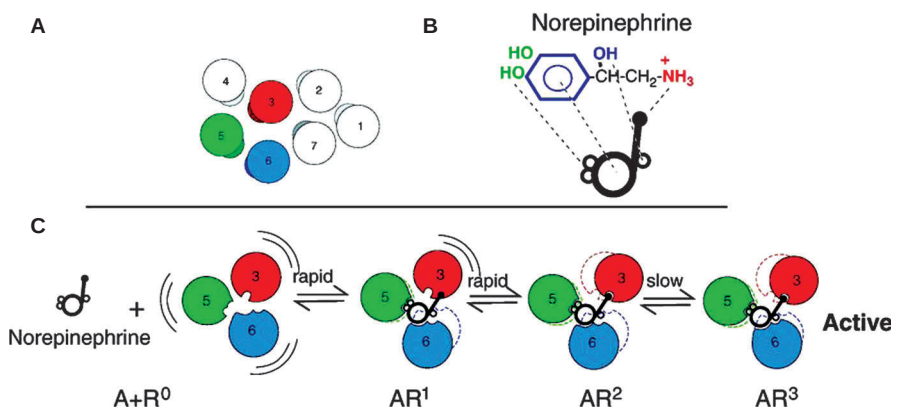


Figure 11.8 Sequential binding of norepinephrine to multiple β2 adrenergic receptor conformations. Adapted from *J. Biol. Chem.*, **279**, 686. Copyright 2004, American Society for Biochemistry and Molecular Biology, Inc.

have been inspired by these experiments and starting from inactive structures (from crystals) provided a more detailed view of these ligand-stabilized conformations,[61,62] which is consistent with experimental observations. These observations of ligand-stabilized conformational states of a GPCR open the possibility of different agonists stabilizing different active conformations with different functional roles as will be seen later.

The seven-helix topology of GPCRs also enables a mutation to have a significant effect on their conformations accessible under physiological conditions. The Kendall group was able to show using two mutations at the same position in the cannabinoid CB1 receptor that the receptor can be switched to the constitutively active form (**R***) or into the inactive form (**R**) with no basal activity.[52] They found that the T210I mutant converts the CB1 receptor into a more fully constitutively active state (**R***), showing enhanced binding efficacy for agonists and diminished binding efficacy for inverse agonists. They also find that the T210A mutant is constitutively inactive, *i.e.* it converts the CB1 receptor into a fully inactive state (**R**), which shows enhanced binding efficacy for inverse agonists and weaker binding for agonists. This strongly suggests that even in the absence of any ligand, GPCRs are capable of major conformational changes with potentially different functional outcomes.

We have recently predicted an ensemble of structures (conformations) for the CCR5 chemokine receptor that is a co-receptor for HIV entry using our GEnSeMBLE method for the efficient optimization of helix tilt angles (θ, ϕ) and rotation angle (η) that were defined in Figure 11.2A. The top lowest energy predicted conformations are shown in Table 11.2 and how they differ from each other in terms of structure and energies.

We have also discovered that mutations can have significant impact on the ensemble of conformations accessible by the apo proteins. Table 11.3 and Figure 11.8 illustrate this using CCR5 mutants as examples. The W86A CCR5 mutant conformations show that the wild-type #30 (wt30) structure now ranks at #4 (Table 11.3). Also, Figure 11.9 shows that this Trp86 residue has a steric clash with helix 7 in the wild-type structure, which disappears in the W86A mutant increasing its stability. The table also shows that for the A90H mutant, the wt1 structure disappears from the top conformations, in this case due to steric clash of His90 with helix 7.

This phenomenon is not fully appreciated for GPCRs, but has the potential to explain the origin of the effect of mutations on ligand binding, whether the mutation affected the protein structure or directly affected ligand binding. We have docked multiple ligands to wild-type CCR5 and mutant ensemble of conformations. The results for binding of Maraviroc (the only drug in the market aimed at CCR5) are shown in Table 11.4, which shows the different protein conformations preferred by the wt and mutant proteins in apo and Maraviroc bound forms. We also find that different ligands (Maraviroc, PF-232798, Aplaviroc) prefer different CCR5 conformations, which is consistent with observations that these ligands bind to the same site but show different interaction profiles in mutagenesis studies[63,64] and in effects on antibody binding.[65]

Table 11.2 Top predicted conformations for human CCR5 chemokine receptor.

Theta	H1	H2	H3	H4	H5	H6	H7	Phi	H1	H2	H3	H4	H5	H6	H7	Eta	H1	H2	H3	H4	H5	H6	H7	AvgRank	CCR5	Energy
Theta	-10	-10	-10	0	0	0	0	Phi	-15	0	0	-15	-15	-15	-15	Eta	0	240	0	0	15	0	0	12.8	wt1	0 ± 3
Theta	0	-10	-10	0	0	0	0	Phi	-15	0	-15	0	0	0	0	Eta	345	225	0	0	345	0	0	14.5	wt2	
Theta	0	-10	-10	0	0	0	0	Phi	-15	0	-15	-15	0	0	0	Eta	345	225	0	0	345	0	0	21.3	wt3	
Theta	-10	-10	-10	0	0	0	0	Phi	-15	15	-15	-15	0	-15	-15	Eta	345	225	0	15	345	0	0	71.3	wt4	15 ± 3
Theta	0	-10	-10	-10	0	0	0	Phi	-15	0	0	15	0	0	0	Eta	345	225	0	15	345	0	0	89.8	wt5	
Theta	-10	-10	-10	10	0	0	0	Phi	-15	-15	15	-15	-15	0	0	Eta	15	0	0	15	30	0	0	98.5	wt6	
Theta	-10	-10	0	0	10	0	0	Phi	-15	-15	15	-15	-15	-15	0	Eta	15	0	0	105	15	0	0	100.3	wt7	
Theta	-10	-10	-10	0	0	0	0	Phi	-15	0	-15	-15	0	-15	-15	Eta	0	225	0	15	345	0	0	108.5	wt9	
Theta	0	-10	-10	-10	0	0	0	Phi	-15	0	0	15	-15	0	0	Eta	345	225	0	15	15	0	0	109.8	wt10	18 ± 4
Theta	-10	-10	-10	10	0	0	0	Phi	-15	-15	0	0	-15	15	0	Eta	15	15	0	15	15	0	0	111.0	wt11	
Theta	0	-10	-10	0	0	0	0	Phi	-15	0	-15	-15	0	-15	0	Eta	330	225	0	15	345	0	0	122.0	wt13	
Theta	-10	-10	-10	10	0	0	0	Phi	-15	-15	0	-15	0	0	0	Eta	15	0	0	105	345	0	0	126.3	wt14	
Theta	0	-10	-10	0	0	0	0	Phi	-15	0	-15	-15	0	-15	-15	Eta	345	225	0	0	345	0	0	143.5	wt15	
Theta	0	-10	-10	-10	0	0	0	Phi	-15	0	0	0	-15	0	0	Eta	345	225	0	15	0	0	0	145.5	wt16	20 ± 4
Theta	0	0	-10	10	0	0	0	Phi	-15	-15	-15	-15	0	0	0	Eta	0	0	0	120	345	0	90	145.8	wt17	
Theta	-10	0	-10	10	0	0	0	Phi	-15	-15	-15	-15	0	0	15	Eta	0	15	0	105	345	0	0	146.5	wt18	
Theta	-10	-10	-10	10	0	0	0	Phi	-15	-15	0	-15	0	15	0	Eta	15	15	0	120	75	0	0	147.0	wt19	
																									:	:
																									wt50	27 ± 3

Table 11.3 Reordering of CCR5 wild-type conformations for mutants W86A, A90H and T105A.

W86A	AvgRank
wt1	8.0
wt3	11.8
wt2	15.0
wt30	16.0
wt6	20.8
wt17	26.0
wt7	28.3
wt8	29.3
wt4	30.0
wt18	30.3
wt11	30.3
wt48	31.0
wt20	31.3
wt12	31.3
wt19	34.8
wt14	34.8
wt9	35.8
wt27	36.0
wt34	37.0
wt13	38.0

Wildtype #30 becomes W86A #4

A90H	AvgRank
wt3	3.0
wt2	6.0
wt4	15.3
wt5	16.3
wt13	18.5
wt10	21.0
wt9	22.5
wt27	22.8
wt14	23.8
wt32	24.8
wt64	25.0
wt15	25.0
wt16	25.8
wt26	27.0
wt28	27.5
wt31	28.5
wt17	29.3
wt33	30.3
wt11	32.0
wt74	32.5

wt1 not in top 20?

T105A	AvgRank
wt1	4.0
wt3	7.8
wt2	10.0
wt11	28.3
wt9	28.8
wt12	29.3
wt18	30.5
wt4	30.5
wt27	30.8
wt6	31.0
wt7	31.3
wt20	31.5
wt8	31.8
wt14	31.8
wt13	34.0
wt15	36.0
wt5	36.0
wt64	37.3
wt32	38.0
wt30	42.5

40

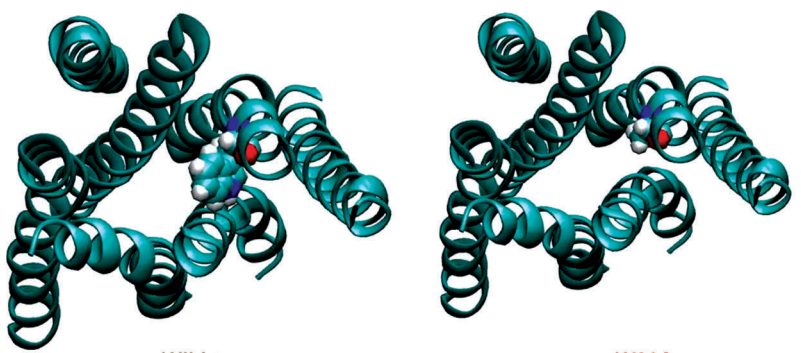


Figure 11.9 Structural basis for mutation effect on conformations (see text).

Next, we will describe the known intracellular consequences of GPCR activation and how the sensing of a single signal by the receptor can turn on a very wide range of intracellular signaling cascades.

Table 11.4 Reordering of wild-type CCR5 conformations for various mutants and the conformation preferred by Maraviroc for each of the mutants.

Mutations	CCR5 Confs	
	Apo	Mara
Wild-Type	wt1	wt7
F109Y	wt3	wt5
F112A	wt1	wt5
Q194A	wt1	wt5
Y251F	wt1	wt2
D276A	wt1	wt2
Q277A	wt1	wt5
W86A	wt1	wt3
A90H	wt3	wt10
T105A	wt1	wt2
Y108A	wt3	wt2
F109A	wt1	wt5
I198A	wt1	wt5
Y251A	wt2	wt3
Q280A	wt3	wt5
E283A	wt3	wt3

11.3.2.3 Intracellular Signal Diversification and GPCR Regulation

GPCR activation can lead to a diverse set of signaling events inside the cell. So, these receptors can be called pleiotropic. These signaling events are mainly transduced through multiple G proteins and arrestins (Figure 11.10). GPCR coupling to G proteins (which gave these receptors the name "GPCR") enables signal diversification inside the cell as these G proteins control a spectrum of downstream signaling pathways. These pathways respond within seconds to minutes of the sensing of the signal by the GPCR. Arrestins appear to be playing a dual role of signal diversification as well as GPCR regulation (receptor desensitization and internalization). Arrestin-mediated signaling responses have a slower onset and are sustained over minutes to hours. As will be shown later, the ability of a single receptor to activate multiple pathways inside the cell can have important therapeutic consequences, as the G protein-mediated pathway may have a therapeutic benefit and the arrestin-mediated pathway may have unintended side-effects or *vice versa*. Next we will briefly describe the G protein-mediated and arrestin-mediated pathways and how their actions define GPCR function.

11.3.2.3.1 G Protein Mediated Signal Diversification. GPCRs got their name due to coupling to G proteins, which are heterotrimeric proteins with

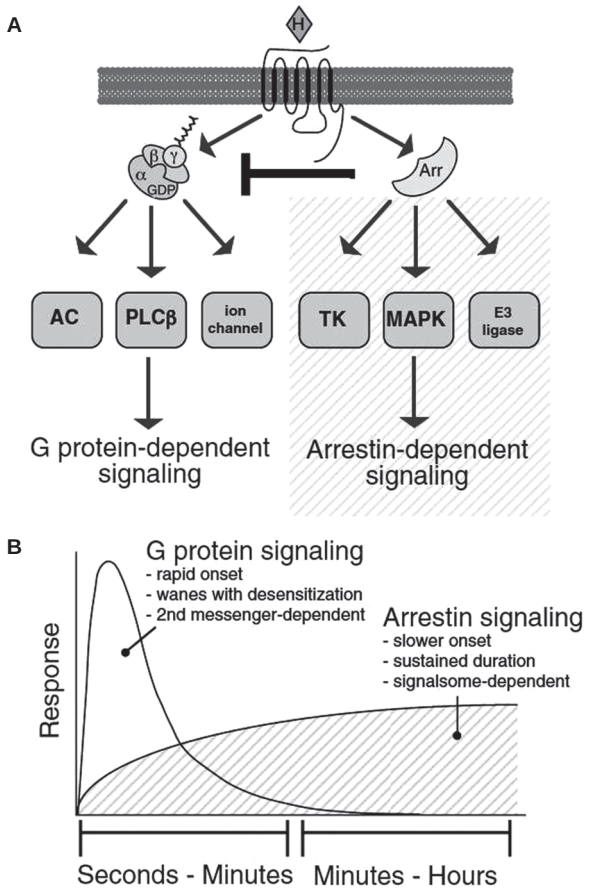


Figure 11.10 **A** G protein and arrestin-dependent signaling pathways. **B** Difference in response times from different pathways. Reproduced with permission from American Society for Pharmacology and Experimental Therapeutics from Luttrell and Gesty-Palmer, *Pharmacol. Rev.*, **62**, 305–330. Copyright 2010.

Gα, Gβ and Gγ subunits. The Gα subunit is made up of a ras-like GTPase domain and a helical domain. The guanine nucleotide GDP is sandwiched between these two domains. There are 23 known Gα proteins that can be assigned to four major classes represented by $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ proteins. The Gβ subunit forms a bridge between Gα and Gγ subunits, which don't appear to have a close contact as shown in Figure 11.11. There are 5 known human Gβ and 12 human Gγ subunits, which can lead to a large number of possible Gβγ dimers that are coupled to many downstream signaling pathways[66,67] as will be discussed below.

The inactive G protein exists in the heterotrimeric form bound to ADP. The activation of a GPCR upon agonist binding leads to a series of conformational

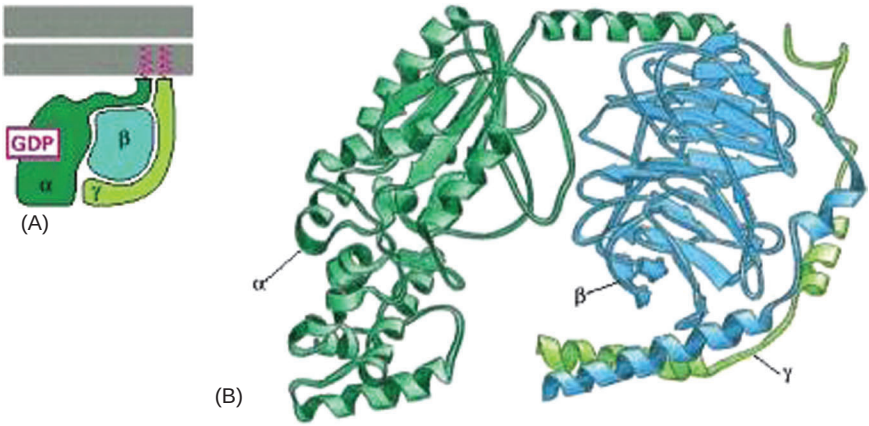


Figure 11.11 The architecture of the heterotrimeric G protein.

changes, which recruit binding to the G protein using the receptor's intracellular loops and the C-terminus residues interacting mainly with the Gα subunit. This association leads to the dissociation of the G protein into the Gα subunit (which undergoes a conformational change coupled to the displacement of ADP by ATP) and the heterodimer complex Gβγ as shown in the left panel of Figure 11.12 that doesn't undergo any major conformational change. Each of these units, the Gα subunit and the Gβγ complex, activates specific signaling pathways inside the cell. A recent crystal structure of Opsin (ligand-free form of rhodopsin) bound to the carboxy terminus of the Gα subunit[28] has shed light on the detailed molecular level contacts present between GPCRs and G proteins as shown in the right panels of Figure 11.12, which highlights through the middle panel the conformational change necessary upon activation to accommodate binding to the Gα subunit.

The Gα subunit controls many downstream signaling cascades through different effector molecules. The $G\alpha_s$ class of proteins stimulate adenylyl cyclase (AC), which converts ATP into cyclic AMP (cAMP), whereas $G\alpha_i$ class inhibits AC thereby opposing the effect of $G\alpha_s$ class. This suggests how a single GPCR activated by the same agonist can have opposite effects inside the cell just by coupling to different G proteins. The $G\alpha_q$ class of subunits activate phospho-inositide-specific phospholipase C (PI-PLC) isoenzymes, which generate second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 activates Ca^{2+} channels leading to the increase in calcium concentration, which facilitates protein kinase C (PKC) activation by DAG. Besides this critical function that controls many cellular functions through phosphorylation events caused by PKC, DAG also acts as a source of prostaglandins and as a precursor of the endocannabinoid 2-arachidonoylglycerol among its other functions.

The Gβγ complex activates its own set of effectors and the first ones identified were G protein-regulated inward-rectifier K^+ channels (GIRKs).

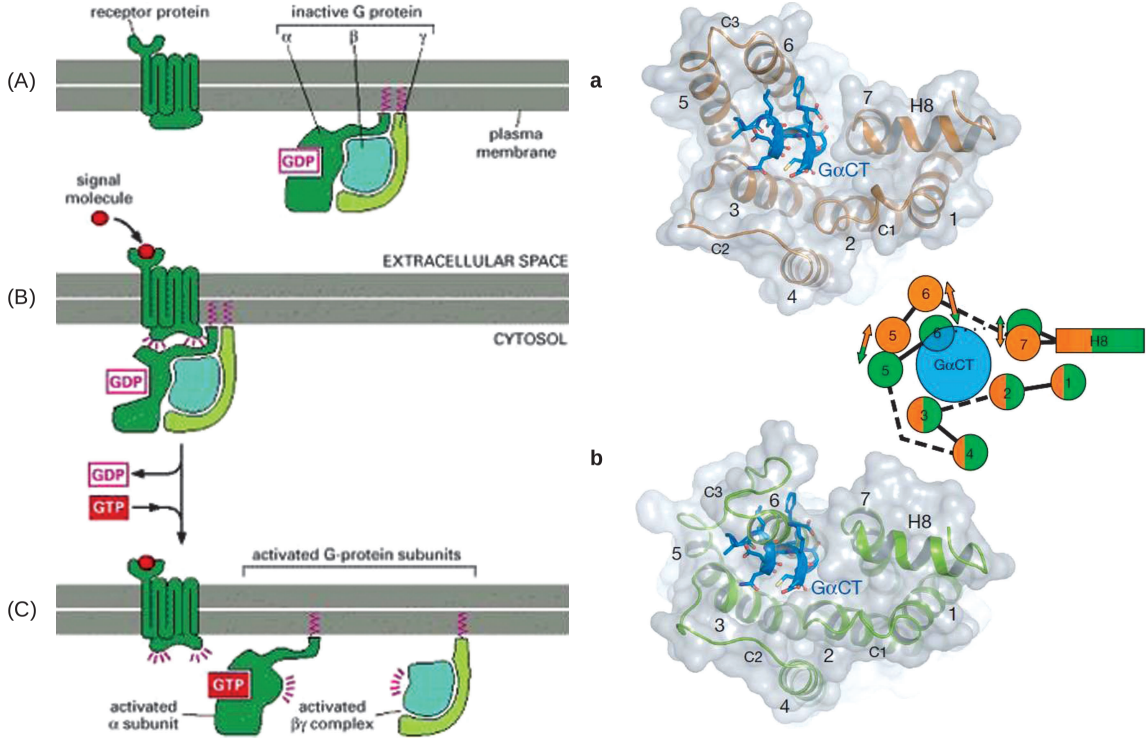


Figure 11.12 Left panel shows the breakup of G protein heterotrimer upon GPCR activation. The right panel shows molecular level details of the Gα-GPCR contact based on the recent crystal structure of opsin. Reprinted by permission from Macmillan Publishers Ltd.: Scheerer *et al.*, *Nature*, **455**(7212), 497–502. Copyright 2008.

They stimulate many kinases like extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinases (MAPKs). The Gβγ complex can directly modulate Ca^{2+} channels and also upregulate or downregulate AC as well as PLC-β activity. This range of effects allows the Gβγ complex to stimulate multiple signaling pathways inside the cell and to cross-talk with pathways activated by the Gα subunit as well. The Gβγ complex also recruits G protein-receptor kinases (GRK2 and GRK3) to the plasma membrane, where they phosphorylate agonist-bound GPCR conformations at the Ser and Thr positions in the intracellular loops or the C-terminus, preparing the receptor for subsequent binding to arrestins for further signaling and desensitization. However, this phosphorylation is not necessary in some cases to enable arrestin binding.

The intracellular signaling *via* G proteins can diversify depending on the type of coupling between GPCRs and G proteins.[69] Four scenarios arise (as shown in Figure 11.13):

a) An agonist activates different GPCR subtypes that display highly specific G protein couplings, which as will be shown below can lead to very different downstream responses. This is most commonly observed in biogenic amine receptors for which multiple subtypes exist, *e.g.* there are nine adrenergic receptors, five dopamine receptors and four histamine receptors (Figure 11.13A).
b) An agonist activates a single GPCR, which activates the G protein and initiates multiple signaling cascades inside the cell. As mentioned above, upon activation G protein breaks up into a Gα subunit and Gβγ complex, both of which can promote signaling *via* the same effectors, or acting antagonistically, or cooperatively modulating one signaling

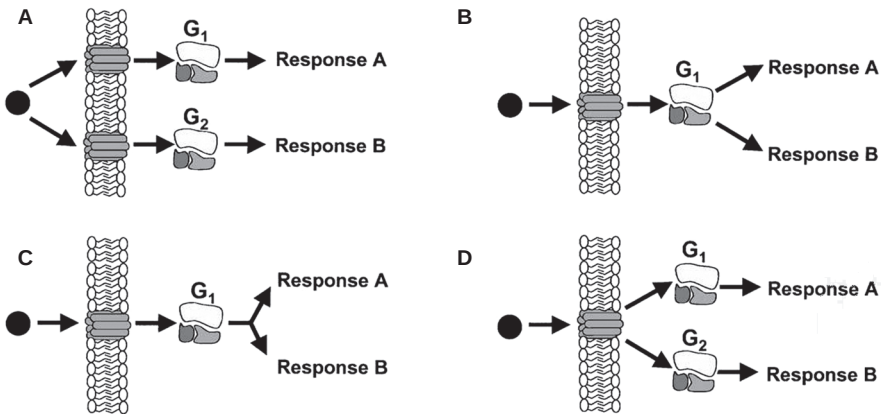


Figure 11.13 Different mechanisms of intracellular signaling divergence. Reprinted with permission from Elsevier from *Pharmacol. Therap.*, **99**(1), 25–44. Copyright 2003.

pathway, or *via* different effectors leading to multiple signaling pathways (Figure 11.13B).

c) Agonist binding to GPCR activates a G protein leading to a single signaling pathway inside the cell, which diverges downstream (Figure 11.13C).
d) GPCR activation allows it to couple to multiple G proteins inside the cell, which can activate many signaling pathways (Figure 11.13D).

These different scenarios are enabled due to conformational flexibility of activated GPCRs, which allows them a range of controls over intracellular signaling pathways. Next, we will describe the interaction of GPCRs with arrestins, which is another mode by which they activate various pathways inside the cell.

11.3.2.3.2 Arrestin Mediated Signaling and GPCR Regulation. β-Arrestins were originally thought to be only responsible for desensitization of activated GPCRs followed by internalization and recycling or degradation. As will be shown below, this view has changed dramatically and arrestins can activate multiple signaling pathways independent of any G protein induced signaling. The arrestin family comprises four proteins in vertebrates: visual arrestins (arrestins 1 and 4) as well as β-arrestins 1 and 2 (arrestins 2 and 3).[68]

Desensitization of GPCRs (or loss of G protein-coupled response in the presence of an agonist) has been reviewed elsewhere[70] and lucidly depicted (along with the endocytosis mechanisms) in Figure 11.14. A succinct picture of classical desensitization involves receptor phosphorylation by second messenger-stimulated protein kinases (like PKA and PKC) or by specific GRKs followed by arrestin binding to the intracellular receptor site that sterically hinders any further G protein coupling. In addition, two new roles have emerged for β-arrestins in desensitization: a) degradation of second messenger molecules like cAMP[71] and diacylglycerol (DAG);[72] and b) limiting or reducing the generation of second messenger molecules as shown in a study[73] that β-arrestin promotes the switching from G_s to G_i coupling, which further limits the production of cAMP. The class A GPCRs show greater affinity for β-arrestin 2, whereas class B GPCRs bind to β-arrestin 1 and 2 with equal affinity.

The receptor internalization (or endocytosis) is mediated by β-arrestin binding to clathrin *via* the adaptor protein AP-2, which targets the GPCRs to clathrin-coated pits (CCPs). These CCPs are pinched off from the plasma membrane by a large GTPase dynamin (see Figure 11.14) into endosomal vesicles, which either recycle the receptor to the plasma membrane or take the receptor through degradation pathways. In the vesicle, the agonists are released from the receptor due to the acidic environment of the vesicle and the receptor is dephosphorylated in preparation for recycling back to the plasma membrane (or resensitization).

One of the important functions of GPCRs is the regulation of cell proliferation and differentiation, *via* the activation of mitogenic pathways. The

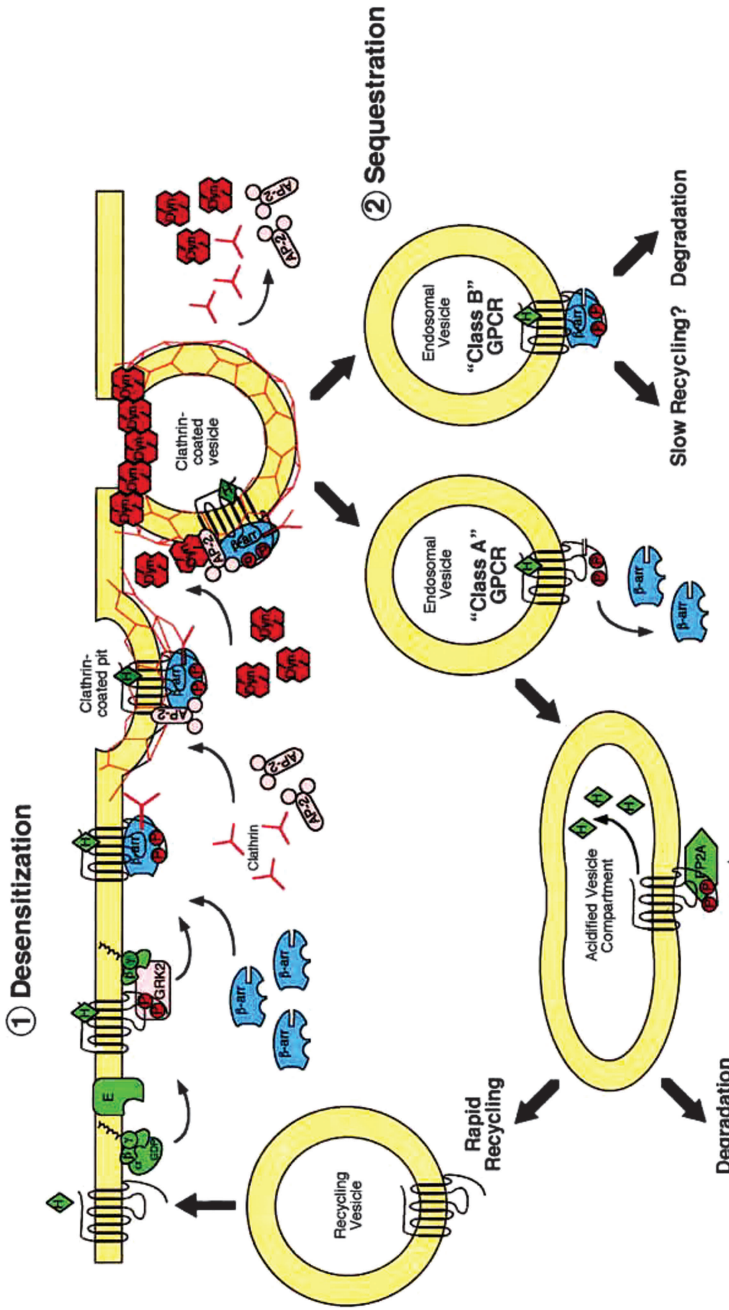


Figure 11.14 Global view of the GPCR lifecycle. Reproduced with permission from *J. Cell Sci.* (ref. 70).

β-arrestins play a vital role in the promotion of the formation of multi-protein signaling complexes with ERK and various tyrosine kinases involved in these mitogenic pathways independent of G protein activation. Specific GRKs can induce receptor phosphorylation that can lead to agonist-stimulated ERK activation in the absence of G protein activation. The angiotensin II activation of angiotensin II type 1 receptor (AT1R) with a mutation in the conserved DRY motif (at the intracellular end of TM3) doesn't cause any G protein-mediated signaling, but maintains β-arrestin recruitment and ERK activation.[74,75] In addition, the use of siRNA against β-arrestin 2 blocks the angiotensin-stimulated ERK activation.[76] This elucidates that GPCRs can signal exclusively through β-arrestins even in the absence of G protein coupling. This appears to be a more general feature of GPCR signaling as the same has been shown for another rhodopsin family receptor β2AR[77] and also for a secretin family receptor type 1 PTH/PTH-related peptide receptor.[78] A general view of this biased signaling is shown in Figure 11.15, where either a ligand could bias the receptor towards β-arrestin pathway, or a protein might be innately biased towards β-arrestin pathway as shown recently for the CXCR7 receptor.[79]

11.3.2.4 GPCR Dimers and Interaction with Other Proteins

All prior discussion in the chapter implicitly assumed that GPCRs couple to G proteins as monomers. Any functional association of a GPCR with its copy, or a different GPCR, or another protein only increases the GPCR repertoire of signaling mechanisms for selective functional control within the cell. Early evidence of GPCR dimerization was observed in recombinant cell systems that over-expressed these receptors, which raised doubts about their functional significance.[80] There is now strong functional evidence based on experiments

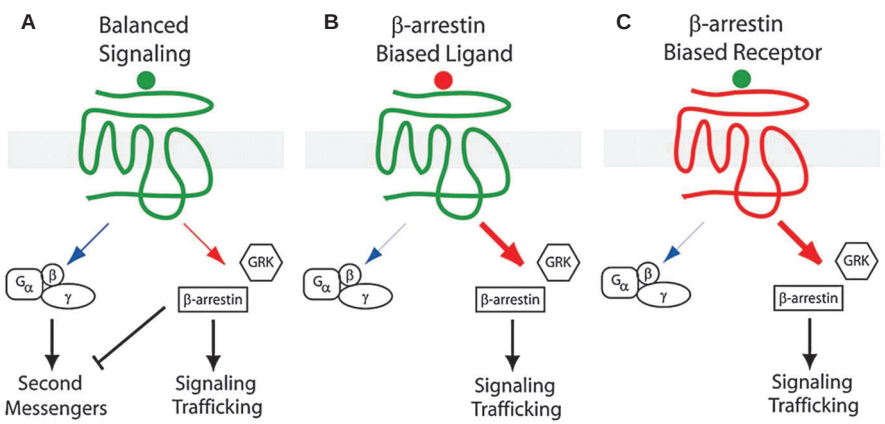


Figure 11.15 Balanced and biased signaling by GPCRs. Reproduced with permission from Rajagopal *et al.*, *Proc. Nat. Acad. Sci. U. S. A.*, **107**(2), 628. Copyright 2010.

involving native tissues that some GPCRs form homodimers and some form heterodimers in their functional forms.

A GPCR class C receptor mGlu5 has been shown to be a homodimer stabilized by a disulfide bridge in the extracellular domain.[81] The extracellular VFT domain of mGlu1 receptor has been crystallized as a dimer with a disulfide bridge in different relative orientations of the monomers depending on the presence or absence of the agonist glutamate,[82,83] suggesting a possible mechanism of activation involving the relative change in the orientation of the monomeric TM bundles in the homodimer.[84]

Another class C GPCR provided the first conclusive evidence of a functional heterodimer. The γ-aminobutyric acid-binding ($GABA_B$) receptor is only functional as a heterodimer between $GABA_{B1}$ and $GABA_{B2}$ monomeric subunits, where each subunit plays a distinct role: $GABA_{B1}$ subunit binds to the agonists and $GABA_{B2}$ subunit couples to the G protein.[85] Sweet and umami taste receptors provide other class C examples of heterodimerization. Of the three genes encoding these receptors (T1R1, T1R2 and T1R3), T1R2-T1R3 heterodimer results in a sweet receptor and the T1R1-T1R3 heterodimer results in an umami receptor.[14] The three monomeric receptors don't display the functional behavior if they are expressed alone.

There is also plenty of evidence now in favor of the presence of functional homodimers and heterodimers in class A GPCRs.[80] Here we will mention a more recent example involving the dopamine D2 receptor, which has been shown to form homodimers out of monomers in functionally different states; maximal activation was observed when one monomer was bound to the agonist (was active) and the other monomer was bound to an inverse agonist (was inactive).[86]

Interaction of GPCRs with other membrane and cytoplasmic proteins has been known for a while and has also been reviewed.[87] The physiological implications of these interactions are slowly being uncovered as only some of these interactions have been amenable to detailed experimental investigations. A discussion of these interactions is beyond the scope of this chapter.

11.3.3 Functional Control of GPCRs by Ligands

As mentioned earlier, GPCRs are pleiotropic in terms of the multiple intracellular signaling cascades they can affect upon binding to an agonist through both G protein-coupled and β-arrestin coupled pathways. Experimentally, a single functional assay (usually by definition) cannot see all the signaling effects of a ligand. It is now evident that agonist-bound GPCRs exist in multiple distinct conformations, where each conformation can potentially activate a different signaling pathway. From a therapeutic perspective, this may not be desirable if, of the multiple signaling pathways activated by a drug-molecule, one pathway may be mainly responsible for the desirable therapeutic benefit and another may be causing unwanted side-effects. This opens at least two distinct possibilities of controlling the functional consequences of GPCR activation: biased agonists and allosteric modulators. Each of these possibilities will be

described below along with a few representative examples. As will be seen, some of these ligands defy the classical definitions of agonists and antagonists.

11.3.3.1 Biased Agonism

Ligands and especially agonists can induce multiple GPCR conformations upon binding. As has been mentioned before, GPCRs interact with cytosolic G proteins and β-arrestins. Different ligands can induce a different ensemble of GPCR conformations, which will have a different range of interactions with G proteins and/or β-arrestins and hence induce different intracellular signaling pathways in a ligand-dependent fashion. As shown in Figure 11.16, agonist

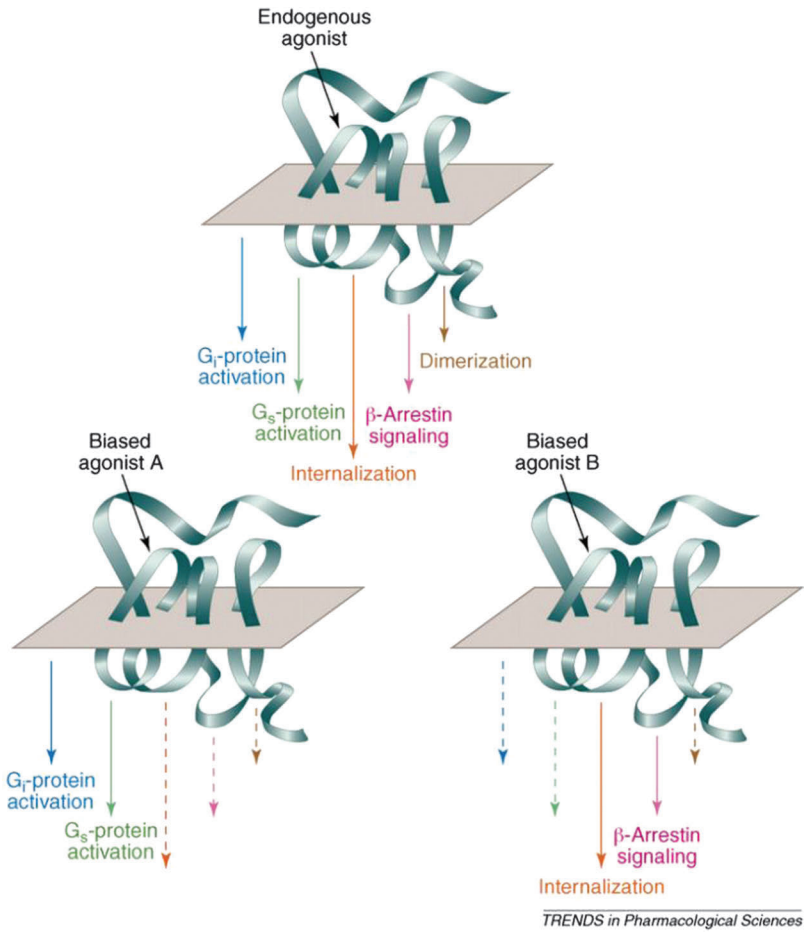


Figure 11.16 Multiple pathways that can be initiated by GPCR activation and biased by ligands. Reprinted with permission from Elsevier from Kenakin, *Trends Pharmacol. Sci.*, **28**(8) 407–415. Copyright 2007.

binding to GPCRs can induce a cascade of processes, some through G protein coupling and some through β-arrestin coupling. As shown before, β-arrestin coupling can lead to specific signaling events and receptor endocytosis, which can further lead to either recycling of the receptor or its degradation. Figure 11.16 also shows the effect of biased agonists A and B that can either exist or be designed to activate a subset of possible signaling pathways.

Classically, the GPCR agonists have been characterized as such by their effect on G protein-coupled pathways. Experiments measuring β-arrestin signaling are becoming more commonplace so the effects of classical agonists need to be evaluated and should lead to a detailed characterization of these ligands. The parathyroid hormone (PTH), for example, can activate extracellular signal-related kinase using distinct G protein-dependent and G protein-independent pathways. The PTH analogs, however, can use the same PTH receptor and separately use either G protein-dependent or -independent pathways. An example is that [Trp^1]PTHrp-(1-36) stimulates ERK1/2 *via* G protein pathway, whereas PTH-1A [[D-Trp12,Tyr34]PTH-(7-34)] stimulates the same *via* β-arrestin pathway in a G protein-independent manner.[78] This can have direct therapeutic consequences because PTH regulates calcium homeostasis as well as bone metabolism and utilization of β-arrestin 2 pathway is critical for this benefit,[88] so β-arrestin biased PTH analogs mentioned above provide potentially improved therapy for osteoporosis. Another example is that nicotinic acid is therapeutically very beneficial as an anti-lypolytic agent (*via* G protein-mediated pathways), but causes cutaneous flushing as a major side-effect, which has been directly linked to the activation of β-arrestin 1 pathways.[89] An analog of this molecule that doesn't affect the G protein pathways but blocks the β-arrestin 1 pathways will be highly desirable. This also necessitates new characterization of classical agonists, *e.g.* a ligand that blocks G protein pathways but uses β-arrestin pathways may have been classified before as an antagonist (or inverse agonist) but now should be more accurately described as a β-arrestin biased agonist.

These studies are also increasing our understanding of the relationship between various signaling pathways and previously unexplained side-effects of drug molecules. The use of knowledge about biased signaling during the drug design phase has the potential to generate multiple novel ways to control and hopefully cure many ailments with minimal side-effects.

11.3.3.2 Allosteric Ligands and Signal Modulation

Orthosteric ligands bind to GPCRs in regions that fully or partially overlap with that of the endogenous ligand(s), thereby sterically excluding the possibility of both occupying the GPCR at the same time. Allosteric ligands bind to GPCRs in regions that don't overlap with the endogenous ligand binding site, so both can occupy the receptor at the same time. This can have important signaling consequences because, as mentioned before, agonists induce an ensemble of GPCR conformations with a range of functional implications and allosteric ligands can dramatically modulate those conformations (*e.g.* by

stabilizing a subset), which can lead to modulation of signaling and hence function. Allosterism provides a powerful natural tool for modulating signaling cascades, but not many natural modulators are known, probably because of the difficulty in identifying these ligands which are structurally dissimilar to endogenous ligands. One of the examples is the unnatural D-amino acid D-serine formed in the brain, which is a strong allosteric modulator of the *N*-methyl-D-aspartate (NMDA) receptor.[90]

An allosteric modulator can bind to a unique ensemble of GPCR conformations and have three modulatory effects on GPCR activation: allosteric antagonism (including allosteric inverse agonism), allosteric agonism and allosteric partial antagonism. In allosteric antagonism, the modulator stabilizes more inactive conformations or destabilizes more active conformations, resulting in the net reduction of GPCR activation relating signaling. These are usually referred to as negative allosteric modulators (NAMs). In allosteric agonism, two scenarios arise where the modulator either enhances the effect of the orthosteric agonist by stabilizing the more active receptor conformations or directly causes the GPCR activation in the absence of the orthosteric agonist. The former kind are referred to as positive allosteric modulators (PAMs), some of which are capable of directly agonizing the receptor in the absence of the orthosteric agonist.[91] In allosteric partial antagonism, the modulator (also called biased antagonist) selectively blocks only a subset of the activation related pathways as shown in Figure 11.17. The figure shows that Postaglandin D_2 normally activates G protein as well as β-arrestin pathways for its CRTH2 receptors, where both of these pathways can be blocked by an orthosteric

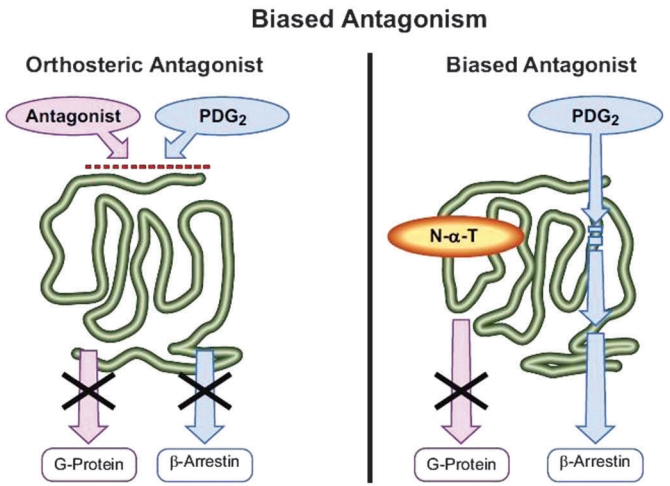


Figure 11.17 Biased antagonism, which can be called allosteric partial antagonism. Reproduced with permission from American Society for Pharmacology and Experimental Therapeutics from Kenakin and Miller, *Pharmacol. Rev.*, **62**, 265–304. Copyright 2010.

antagonist. The biased antagonist N-α-tosyltryptophan (N-α-T) selectively blocks the G protein coupled pathways allowing the agonist PGD2 to continue to activate the β-arrestin pathways.[92]

There is plenty of evidence in favor of different allosteric modulators stabilizing a different subset of GPCR conformations, one being antibody binding profiles of Ab45531 and Ab45523 for the chemokine receptor CCR5, which is a coreceptor for HIV-1 entry. These antibody binding profiles differ in the presence of allosteric HIV-1 entry inhibitors like aplaviroc, TAK-779 and SCH-C.[65]

The muscarinic acetylcholine receptors (mAChRs) are class A GPCRs that provide a good pharmacological example for the need of selective allosteric modulators.[93,94] There are five mAChR subtypes implicated in many physiological pathways. The acetylcholine binding site residues are highly conserved across these five receptor subtypes, making it difficult to design orthosteric agonists or antagonists. It has been shown that these receptors have one or two allosteric binding sites available for allosteric modulators to regulate the effect of orthosteric ligands.[93] This provides for a general strategy for therapies targeting receptor subtype selectivity as the allosteric sites will be much less conserved than the orthosteric sites. In addition, a new class of modulators has emerged called "bitopic" that interact with both the allosteric and the orthosteric sites for self-modulation of their activity.[95,96]

The studies of these modulators really adds to the diverse ways in which GPCR conformational flexibility can be exploited for innate signaling as well as for therapeutic applications where target receptor subtype selectivity is highly desirable or activation of specific pathways causes undesirable side-effects.

11.3.4 Challenges in GPCR Targeted Drug Design

The lack of 3D structures for most human GPCRs (none were available until 2007) has led to the almost exclusive use of high-throughput screening (HTS) and virtual ligand screening (VLS) techniques in drug design, which do not require protein structures as input. Even though the number of compounds being screened by HTS and VLS techniques is higher than ever, the number of new approved drugs is on a decline. A large proportion (43%) of drug candidates in clinical trials fail due to lack of efficacy and a significant one-third fail due to toxicity and side-effects.[97] Some of these undesirable effects result from drugs hitting other GPCRs or even other subtypes of the same receptor, *e.g.* many dopamine D2 receptor agonists used for treating Parkinson's disease produce behavioral side-effects (*e.g.* compulsive gambling even in non-gamblers)[98] because they actually bind with higher affinity to the dopamine D3 receptor which has been associated with emotion, reward and addiction. Such side-effects can be effectively minimized by designing D2 selective agonists, which requires atomic-level structures for both D2 and D3 receptors. As the theme of this chapter suggests that multiple GPCR conformations and their signaling consequences need to be accounted for, it is a daunting challenge. Concerted efforts are underway to crystallize a number of GPCRs with agonists, antagonists and inverse agonists, which will slowly provide valuable

information. Parallel to these efforts, validated structure prediction methods that can generate an ensemble of GPCR structures that can be validated by mutagenesis as well as functional/binding assays and provide avenues for rational structure-based drug design are highly desirable.

Identification of the molecular basis of toxicity and other side-effects has been possible for a very few cases, some of which have been mentioned in this chapter. A recent pertinent example is the anti-obesity target, the cannabinoid CB1 receptor, which is a GPCR stimulated by *cannabis*, generating feelings of euphoria. Application of two promising anti-obesity CB1 antagonists, rimonabant (marketed, and then pulled from the European Union) and taranabant, resulted in adverse psychiatric symptoms like depression, anxiety and suicidal ideation. This has led many big pharmaceutical companies to abandon drug development programs on CB1 target, as it is not clear whether side-effects are due to off-target interactions (*i.e.* not related to CB1) or due to CB1 antagonism.

This leads to two possible scenarios. Firstly, if the side-effects are confirmed to be off-target and it becomes known which receptors need to be avoided (hereafter called anti-targets), the structure-based rational drug design process can potentially use the structures of the target receptor and all the anti-target receptors to tailor the drug towards the target and away from the anti-targets, taking into account any information about signaling pathways that might be available. Secondly, if the side-effects are confirmed to be due to the target receptor, then it is most likely due to one of the multiple signaling pathways activated by the receptor. This will require a detailed therapeutic characterization of conformation-specific signaling pathways for the target receptor, so that biased agonists or allosteric modulators can be rationally designed using the therapeutically beneficial pathways.

Identification of off-targets for side-effects of drugs is an area of active research. A recent study looked at 3665 FDA approved and investigational drugs and their chemical similarities against 200,000 ligands that have been organized into families based on the proteins they target.[99] This identified thousands of unanticipated off-targets for the approved drugs. Some of these are shown in Figure 11.18 using red arrows. Based on these predictions, 30 off-target associations were tested experimentally and 23 were confirmed, 5 of which were potent (< 100 nM). Key unknown associations uncovered were that transporter inhibitor Prozac antagonizes the β1 adrenergic receptor, the ion-channel drug Vadilex inhibits the 5-hydroxytryptamine (5-HT) transporter and the enzyme inhibitor Rescriptor antagonizes the histamine H_4 receptor. Prozac's adverse effects in terms of SSRI-discontinuation syndrome and sexual dysfunction can be explained by its β1 blocking ability as β blockers have those effects. Vadilex and Rescriptor are displaying polypharmacology as they are hitting very unrelated receptors, confirming that structurally diverse proteins can bind to structurally similar ligands. Such analysis needs to become routine to help minimize potential side-effects of the drugs being developed.

Experimental studies are uncovering new signaling mechanisms and computational studies can complement them through their use in rational drug

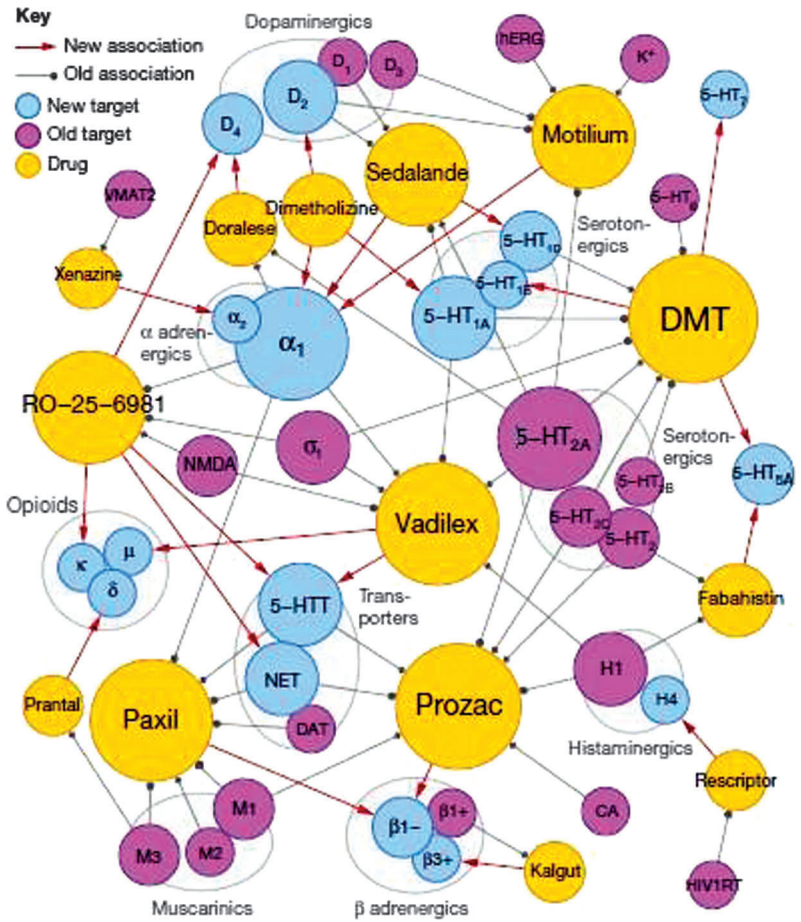


Figure 11.18 Off-target associations predicted for known drugs. Reprinted by permission from Macmillan Publishers Ltd.: Keiser *et al.*, *Nature*, **462**(7270), 175. Copyright 2009.

design approaches and in finding new off-targets. The complexity of GPCR signaling that underlies therapeutic applications requires a synergistic role for experimental and computational methods in producing novel therapies with minimal side-effects.

11.4 Summary and Looking Ahead

Significant efforts aimed at understanding the signaling from the protein level to the cellular level have uncovered multiple layers of complexity that would have been difficult to imagine even if the structures for all proteins involved were known. GPCRs have evolved into a nearly "perfect" receptor capable of activating and controlling multiple intracellular signals through its variety of

conformational states, each with potentially different function. Ligands capable of stabilizing these distinct GPCR conformations have the potential to control GPCR signaling with high selectivity but with different functionality, as discussed in terms of biased agonists and allosteric modulators. As more signaling pathways are discovered downstream of GPCR activation, the need for such biased ligands is likely to increase.

Tremendous progress has been made over the last 20 years on GPCR function, broadening our perspective on the diverse capabilities of these receptors. In the last few years long-term GPCR crystallization efforts are finally bearing fruit, providing rapid progress in structural information. Complementing these experimental advances are dramatic improvements in the GPCR structure prediction methods aimed at identifying the multiple conformational states that appear sometimes to lead to different functions. As these predictions are validated with biological and X-ray experiments, we can expect new, more refined predictions on dynamical processes that are difficult to follow experimentally. We can expect that this will provide useful structural and functional data for GPCRs not yet crystallized and for orphan GPCRs whose functions are unknown. These theoretical and computational studies complemented with experiment are providing a basis for understanding the mechanism of activation so important for many diseases. The theory also has the potential to suggest ligands and/or mutations that would stabilize the active conformations of GPCRs so that they can be crystallized in their active form, the next "holy-grail" for GPCR structure determination.

We can expect the 3D structures and function of many GPCRs to be determined experimentally and computationally over the next decade or two, providing the potential for an enormous increase in our understanding of the various signaling processes in which they are involved.

The advances in our understanding of GPCR structure and function have resulted from recent breakthroughs in uncovering new signaling pathways. Molecular biology, GPCR crystallization and computational modeling have established a strong basis for proceeding to develop targeted subtype specific therapeutics that will likely help increase the efficacy while helping reduce the side-effects associated with current drugs ranging from Parkinson's to schizophrenia and from depression to obesity.

Acknowledgements

Our development of predictive methods for structures and function of GPCRs started around 1998 with an ARO-MURI grant oriented toward olfaction, a time when no 3D structures were available. The early advances were led by Dr. N. Vaidehi (now Professor of Immunology at City of Hope, Duarte, California) and Wely Floriano (now Associate Professor of Chemistry at Lakehead University, Ontario, Canada). Other important early contributions were made by Deqiang Zhang, Spencer Hall, Peter Freddolino, Eun Jung Choi, Georgios Zamanakos, Peter Kekenes-Huskey, Peter Freddolino, Yashar Kalani, Rene Trabanino, Victor Kam, Art Cho, John Wendel, P. Hummel, Peter Spijker;

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CHAPTER 12

Phytochemicals as Modulators of Signaling in Inflammation

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12.1 Introduction

The triggering of inflammatory pathways *via* signaling mechanisms underlies many disease conditions. Inflammation involves activation of immune cells by molecules such as cytokines or toll-like receptor ligands at the cell surface receptor. This leads to propagation of a signal transduction pathway, involving NF-κB (nuclear factor kappa-light chain-enhancer of activated B cells). NF-κB induces the transcription of inflammatory products, such as cyclooxygenase-2 (COX-2). The upregulation of COX-2 escalates the inflammatory cascade. The activation of NF-κB also leads to the expression of prostaglandin E2, iNOS (inducible nitric oxide synthase), ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1), IL-1β (interleukin-1β) and TNFα (tumor necrosis factor α). If left uncontrolled, this cascade may result in chronic inflammation associated with many disease processes, and result ultimately in bystander damage to healthy tissues by free radical damage or direct chronic inflammation in affected tissues.

Phytochemicals and natural products can act as modulators to influence disease progression, either as ligands or as inhibitory agents of signal transduction in inflammation. Many medicinal herbs are used to dampen

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inflammation in chronic conditions (for reviews see[1–4]). Many of the mechanisms of phytochemical suppression of inflammation involve blocking NF-κB-induced gene expression of proinflammatory mediators. Examples include plant polyphenols or flavonols. Other actions of phytochemicals involve stimulation of the transcription factors, peroxisome proliferator-activated receptor γ (PPARγ) and liver-X-receptor (LXR), which indirectly repress NF-κB, such as plant-derived fatty acids or plant sterols.

This chapter focuses mainly on the influence of some of the more common natural products on transcription factors, which are the most promising targets for designing new active drugs against inflammation. Alternatively current herbal therapeutic remedies have been recognized for centuries. Ultimately the question may revolve around the choice between dampening inflammation with long-term, but possibly less potent dietary ligands *versus* more potent synthetic analogs under acute conditions, but which may have uncertain toxicity.

12.2 Overview of the Inflammatory Cascade

The process of inflammation involved in all tissues can be initiated by cells such as macrophages and dendritic cells. These cells can undergo activation, whereupon they release inflammatory mediators responsible for propagating an inflammatory cascade. TNFα and interleukin-1 (IL-1) are cytokines produced primarily in macrophages, to induce fever, production of more cytokines, endothelial regulation, chemotaxis and leukocyte adherence.

If inflammatory mechanisms are not downregulated appropriately, conditions such as rheumatoid arthritis may develop, among other conditions. Production of TNFα, IL-6, IL-8, histamine and other inflammatory mediators by activated mast cells are believed to drive synovitis in rheumatoid arthritis.

It is now known that innate immune mechanisms become amplified in acute coronary syndromes. In atherosclerotic lesions, platelets become incorporated into growing thrombi, which results in the surface expression of P-selectin. This binds to leukocytes, thereby mediating monocyte and granulocyte activation and thrombus adhesion. Trapped platelets and leucocytes within a thrombus secrete RANTES (regulated upon activation, normal T-cell expressed and secreted) and other inflammatory mediators resulting in activation of other immune pathways and thrombotic mediators, such as the release of TNFα and tissue factors, thereby escalating tissue injury.

Many neurodegenerative conditions also involve inflammatory mechanisms that may be causative in etiology or amplify the condition, such as multiple sclerosis. Multiple sclerosis is known to involve T-cell entry into the brain across the blood-brain barrier. T-cells then recognize myelin as an antigen and trigger inflammatory pathways and antibodies toward myelin. This stimulates increased levels of cytokines, swelling, activation of macrophages and microglia and more pathology.

Alzheimer's disease also seems to have an inflammatory component, whether it is a primary event in its etiology or a secondary event, involved in a later

process.[5] Alzheimer's disease also involves the accumulation of amyloid beta (Aβ) peptide deposits and phosphorylated tau, which appear to promote neurofibrillary tangles and plaques in the brain. However, bystander damage from inflammation appears to at least exacerbate pathology, as the upregulation of complement, cytokines, acute phase reactants and other inflammatory mediators are present in affected tissues.

12.3 Overview of NF-κB

When immune cells are activated by cytokines such as TNFα, IL-1 or toll-like receptor activators binding to their receptor targets, NF-κB is also activated in a complex set of pathways. NF-κB is a ubiquitous transcription factor that controls the expression of genes involved in inflammatory responses. Increased over-activation of NF-κB may cause inflammatory and autoimmune diseases, or other chronic conditions. Five mammalian NF-κB family members have been identified:

- NF-κB1 (also known as p50)
- NF-κB2 (also known as p52)
- RelA (also known as p65)
- RelB
- RelC

They all share a highly conserved Rel homology domain, responsible for their dimerization and binding to DNA and IκB (inhibitor of NF-κB). The transcription factor NF-κB works only when two members form a dimer. The most abundant activated form consists of a p50 or p52 subunit and a p65 subunit.

As shown in Figure 12.1, NF-κB is located in the cytosol, in an inactive state, complexed with the inhibitory protein, IκBα. Extracellular signals such as cytokines activate the enzyme IκB kinase (IKK). IKK then phosphorylates the IκBα protein, which becomes ubiquitinated. This results in the dissociation of IκBα from NF-κB and degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus, where it binds to response elements in the promoter region of DNA in order to initiate transcription and be translated into proteins that magnify the inflammatory response.

The surface receptors (with associated ligands) that activate NF-κB are: RANK (Receptor activator of NF-κB, found on the surface of stromal cells, osteoblasts, T-cells and dendritic cells), TNFR (tumor necrosis factor receptor or CD120), lyphotoxin beta receptor, BAFF-R (B-cell activation receptor or CD40), tumor necrosis factor receptor superfamily, member 8 (TNFRSF8 or CD30, found on activated B or T-cells). In addition, bacterial products activate NF-κB, through toll-like receptors, such as TLR4, which is activated by LPS in Gram-negative bacteria. IL-1R interleukin-1 receptor (IL-1R) also activates NF-κB through the binding of its ligand, IL-1.

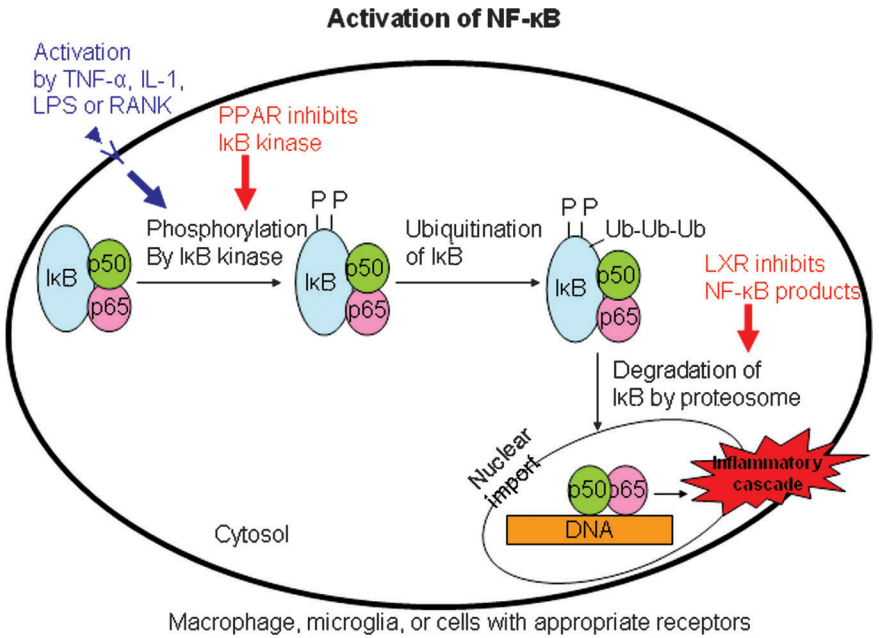

Figure 12.1 Overview of NF-κB signal transduction pathway.

12.4 PPARγ and LXRs Regulate NF-κB

NF-κB can be negatively regulated by PPARγ, LXR, glucocorticoid receptors, estrogen receptors, progesterone receptors and the androgen receptor. PPARγ and LXR may present desirable strategies for altering NF-κB and the inflammatory cascade, since nearly all cells have a form of PPAR and LXR.

This chapter focuses on direct NF-κB inhibitory mechanisms (and indirect PPAR agonist–NF-κB inhibitory pathways) as having the potential for limiting inflammatory pathways, while avoiding the profound and undesirable effects of hormones such as glucocorticoids. For instance, glucocorticoids not only directly inhibit NF-κB pathways, but also bind to glucocorticoid receptors that produce undesirable side-effects, such as: suppression of calcium absorption in bone, hyperglycemia, muscle weakness and pubertal delay.

Similarly, steroid hormones such as estrogen inhibit NF-κB pathways, but also have multiple undesirable effects, such as mitogenic effects and the potential for tumor proliferation. PPAR agonists, in contrast, may be relatively benign.

PPARγ is a transcription factor, which, when bound to the retinoic acid receptor (RXR), promotes transcription of proteins involved in fatty acid metabolism. It is activated by ligands such as fatty acids, oxLDL and thiazolidinediones. However, with PPARγ a trans-repression mechanism occurs, such that when PPARγ is inhibited NF-κB is activated promoting inflammation. Conversely, when PPARγ is activated, NF-κB and hence inflammation are

inhibited. Thiazolidinediones are common PPARγ agonists used in diabetes therapy, insulin resistance and obesity.

The three PPAR subtypes, α, γ and δ, regulate different lipoproteins and lipid homeostasis depending on the tissue. PPARα potentiates fatty-acid catabolism in the liver. However, PPARγ is involved in adipocyte differentiation and insulin sensitizing. PPARγ also regulates insulin responsive genes involved in glucose production, transport and utilization. PPARα is expressed in liver, kidney, heart, muscle and adipose tissue. PPARβ and PPARδ are expressed in high levels in brain, adipose tissue and skin. PPARγ is expressed in all tissues.

In macrophages, PPARγ is believed to coordinate a complex response to oxLDL that involves increased scavenging ability and increased lipid efflux. One important role of PPARγ in macrophages may be the removal of oxLDL from the artery wall and promoting cholesterol efflux through upregulation of a cholesterol transport pathway.[6] Activation of PPARγ reduces blood levels of triglycerides. Therefore inhibition of PPARγ may result in elevated cholesterol and triglycerides while NF-κB-mediated inflammatory pathways are increased.

Another transcription factor involved in NF-κB regulation is LXR, so named because it is present in high levels in the liver, but is ubiquitous in all tissues. LXR and PPAR are nuclear receptor proteins of the same family, which, when activated by their ligands, become transcription factors for metabolic pathways. Similar to PPAR, LXRs are important regulators of cholesterol, fatty-acid metabolism and glucose homeostasis.[7] Their activating ligands consist of oxysterols. LXR binds to RXR before transcription can occur. In addition, LXR acts as a trans-repressor of NF-κB analogous to PPAR.[7] LXR agonists also inhibit expression of the protein products of NF-κB, such as COX-2, IL-1β, iNOS and TNFα. Therefore activators of LXR and PPAR also inhibit NF-κB-mediated inflammatory pathways. However, PPAR is more involved in fatty-acid metabolism, whereas LXR is more involved in cholesterol and oxysterol metabolism.

PPARγ and LXR are of particular relevance to inflammatory pathways in macrophages. Since macrophages (and microglia) are primary generators of inflammatory cytokines and chronic immune responses, the modifying effects of the PPARγ and LXR transcription factors can dramatically affect the immune response. For instance, in atherosclerotic lesions, macrophages are recruited to atherosclerotic plaques. If inhibition of PPARγ or LXR occurs, lipid and cholesterol homeostasis is disturbed within the macrophage. This leads to inhibited efflux of lipid and cholesterol from the cell, in the form of HDL (in order for transport to the liver). This consequently results in the conversion of macrophages into foam cells, which are unable to phagocytize oxidized cholesterol properly. This also leads to the activation of NF-κB with increased local inflammation and pathology during a cardiovascular incident.[8–11] Therefore, the inhibition of PPARγ or LXR within macrophages means that they are not only failing to phagocytize apoptotic debris, pathogens or oxLDL, but they are promoting increased inflammation, oxidative stress and pathology.

This is why synthetic agonists of PPARγ, such as thiazolidinediones, decrease cytokine production in activated macrophages and provide beneficial

effects in cardiovascular disease.[12,13] However, questions have arisen recently, regarding the safety and efficacy of PPARγ agonists in cardiovascular disease.[14,15] Although they are effective, they may also have their own associated toxicity in susceptible individuals.

Another study implicated LXR in the immune response, showing that the downregulation of LXR led directly to decreased phagocytosis of Aβ peptides, and increased inflammation in the brains of mice in Alzheimer disease models.[16] The same study showed that LXR agonists can increase Aβ phagocytosis, while decreasing inflammatory pathways. In addition, thiazolidinediones are known for improving outcomes in Alzheimer's disease,[17–19] diabetes[20] and other chronic inflammatory conditions. Here, phytochemicals and natural product inhibitors of NF-κB will be considered, as well as PPARγ agonists and LXR agonists as alternatives to the current synthetic pharmacologic strategies.

12.5 Natural Products and Phytochemical Inhibitors of NF-κB

How do phytochemicals interact with NF-κB? Many of the phytochemicals discussed below intervene at the stage of IκB kinase to inhibit the action of this enzyme. Other phytochemicals intervene upstream or downstream of IκB kinase. Still others intervene indirectly, such as the PPAR or LXR agonists, which, when stimulated, have an indirect but dramatic inhibitory effect on NF-κB pathways. Most of these polyphenol compounds are considered to be poorly absorbed and rapidly eliminated. However, human plasma levels of these agents can reach μM levels in normal dietary regimens, thereby approaching levels that may inhibit IκB kinase. The following descriptions of phytochemicals are a small sampling of common inhibitors of inflammation, their mechanisms of action with NF-κB and data as to their potential bioavailability.

12.5.1 Anthocyanins

Some of the more powerful inhibitors of NF-κB-mediated inflammatory pathways are anthocyanins. Anthocyanins are glucosides of anthocyanidins (Figure 12.2). Anthocyanins are often responsible for the blue, red or purple pigment found in fruits and plants and belong to the class of flavonoid molecules. They also protect cells by absorbing blue-green and UV light. Plants that contain high amounts of anthocyanins are blueberry, cranberry, bilberry,

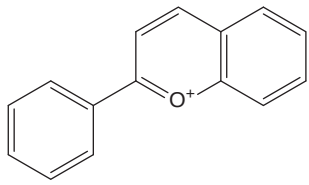

Figure 12.2 Basic structure of an anthocyanin.

raspberry, blackberry, blackcurrant, cherry, grape, red cabbage and eggplant peel. Although they are considered powerful antioxidants, their anti-inflammatory properties in signal transduction mechanisms are emphasized here.

Pomegranate fruit extracts contain anthocyanins and tannins, which inhibit NF-κB-dependent inflammatory pathways.[21] This study exposed mast cells and basophils to the inflammatory stimuli, phorbol-12-myristate 13-acetate and calcium inophore A23187, and found that pomegranate extracts, in concentrations of 20–100 μg/ml, can decrease the levels of inflammatory cytokines IL-6 and IL-8 promoted under these conditions. These pomegranate extracts contained ellagitannins, punicalagins, punicalins, ellagic acid and gallic acid. The anti-inflammatory effect was found to be c-jun N-terminal kinase (JNK) and extracellular regulated kinase (ERK) dependent. NF-κB activation was also inhibited by pomegranate extracts by inhibiting IκB-degradation in basophils.

Another study looked at pomegranate extracts and found that a prostate cell cancer line, dependent on activation of TNFα and NF-κB for cell growth, was inhibited by these extracts in a dose-dependent fashion.[22] Under their conditions, TNFα decreased IκBα protein levels and increased NF-κB activity directly, but pomegranate extracts were able to block the degradation of IκBα, thereby inhibiting NF-κB. In this particular study, the authors then went on to examine the effects in an *in vivo* mouse model. Murine xeonografts of LAPC4 tumor cells, with known endogenous NF-κB activity, were placed in immunodeficient mice. Pomegranate extracts were able to reduce tumor size to about one-third compared to controls with no treatment. The amount of ellagitannins and punicalagins in a single serving of pomegranate juice is 80 mg. Ten times this dose was used and adjusted according to mouse body weight to make 0.8 mg of pomegranate extracts.

In addition, anthocyanins appear to be bioavailable. For instance, one report documented that individuals drinking 480 ml of cranberry juice had plasma concentrations of various anthocyanins that ranged from 0.56–4.64 nM after four hours.[23]

12.5.2 Gallates

Gallates (gallic acid esters) are found in wine, red tea and green tea and have been found to inhibit NF-κB pathways in human umbilical vein endothelial cells (HUVEC).[24] Treatment of HUVEC cells with cytokines promotes inflammation. However, pre-treatment with ethyl gallate in 10-μM concentrations inhibited NF-κB activation, which resulted in suppression of IL-1α, TNFα, VCAM-1, ICAM-1 and E-selectin. This results in decreased adhesion of leukocytes to HUVEC cells. Suppression of NF-κB occurred through the blocking of NF-κB–p65 translocation into the nucleus and not by binding to the promoter region of NF-κB.[24]

Galloyl compounds are often found in plants in the form of gallic acid, alkyl esters (methyl gallate or ethyl gallate) and galloyl tannins (galloyl glucose, epicatechin gallate and procyanidin gallate). One study found that the ingestion

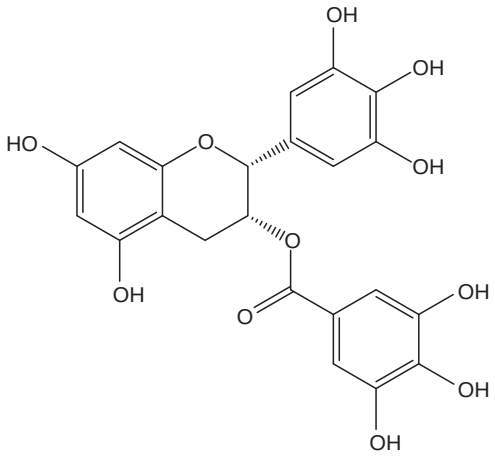

Figure 12.3 Epigallocatechin gallate.

by human subjects of 200 mg of epicatechin gallate (Figure 12.3) resulted in a plasma concentration of 0.15 μM epicatechin gallate two hours after ingestion.[25]

12.5.3 Quercetin

Quercetin is a plant-derived flavonoid found in onions, shallots, garlic, leeks, black and green tea, capers, apples and various berries. Quercetin (Figure 12.4) has been found to be a direct inhibitor of IκB Kinase.[26] The cell lines used in this study were histiocytic lymphoma (U-937), HeLa or T (Jurkat) cells. These cells were treated with 80 μg/ml of quercetin for one hour followed by TNFα. This concentration of quercetin completely abolished TNFα activation of NF-κB-mediated inflammation. This study also observed by Western blot that quercetin abolished IκBα degradation, thereby inhibiting inflammation.

Another study looked at the direct inhibition by enzyme kinetics studies.[27] They found that IκB kinase and activation of NF-κB-mediated inflammation is strongly inhibited by quercetin. This study found that IκB Kinase α and β were inhibited with apparent Ki values of 11 and 4 μM.

Quercetin has reasonable bioavailability in humans. In one study, healthy humans ingested 50, 100 or 150 mg/day of quercetin for two weeks. After 150 mg/day of quercetin, plasma levels of quercetin were observed to be 0.43 μM.[28]

12.5.4 Isoflavones

Genistein is an isoflavone found in soy and fava beans. It has been demonstrated to be an inhibitor of IκB kinase.[26] This study (Figure 12.5) compared inhibition of IκB kinase by quercetin or genistein, and found that 40 μg/ml of genistein was enough to abolish TNFα activation of NF-κB. Downstream

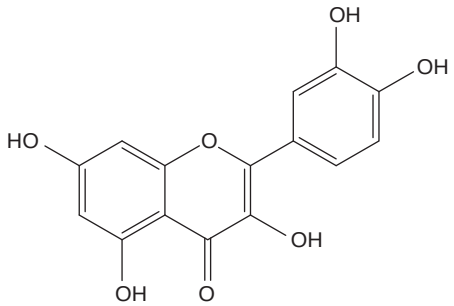

Figure 12.4 Quercetin.

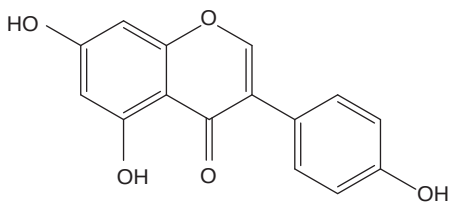

Figure 12.5 Genistein.

effects were found such as that genistein inhibits TNFα induced adhesion of neutrophils to endothelial cells and inhibits the expression of cell adhesion molecules ICAM-1, VCAM-1 and E-selectin. Genistein has other useful purposes such as inhibiting parasitic infection.

Bioavailability studies have been performed using genistein contained in soy foods. It was found that 96 mg of isoflavones/day contained in soy products resulted in plasma levels of 4 μM.[29]

12.5.5 Piperine

Piperine, the active ingredient in black pepper (Figure 12.6), has been shown to block the translocation of NF-κB by blocking the degradation of its inhibitory protein, IκBα, and, therefore, transport into the nucleus in endothelial cells.[30] In this study, endothelial cells were stimulated by TNFα, which resulted in increased IκB kinase activity. However, pretreatment of cells with piperine before TNFα inhibited IκB kinase activity, with maximal inhibition of activity occurring at 40 μg/ml.[30]

Piperine is known for increasing bioavailabilty in the gut of many other medicinal compounds. Piperine itself, after being given in one 50 mg dose, after one hour resulted in peak concentrations of 1 μg/ml in healthy human males, and is highly bioavailable.[31]

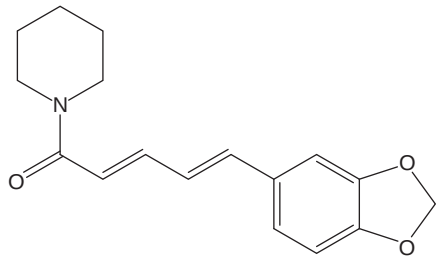

Figure 12.6 Piperine.

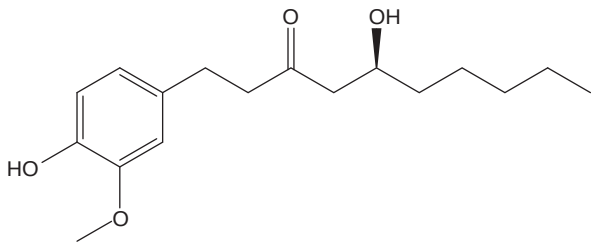

Figure 12.7 Gingerol.

12.5.6 Gingerol

Gingerol is another phytochemical, known to inhibit NF-κB-mediated inflammation (Figure 12.7). It is the pungent constituent of ginger. Gingerol has also been found to inhibit IκB kinase activity.[32] In this study, macrophages (RAW 264 cells) were stimulated by LPS, which activated the cells and initiated an inflammatory cascade with NF-κB stimulation. It was found that an 80-μM concentration of gingerol can inhibit 70% of the NF-κB activation by LPS. The bioavailability of gingerol is not high, with peak concentrations of metabolites being 0.1 μg/ml to 1.7 μg/ml in human plasma after a 1 g dose.[33]

12.5.7 Curcumin

Curcumin is the main ingredient found in the curry spice tumeric (Figure 12.8). Curcumin has been found to inhibit NF-κB. One study found that 10–20 μM amounts of curcumin can inhibit NF-κB in MyD88 and RAW264.7 cells activated by LPS.[34] This was shown to occur through blocking the dimerization of TLR-4 and by inhibiting IκB kinase. Curcumin inhibits numerous inflammatory pathways upstream and downstream of NF-κB, such as mitogenic pathways, AP-1 transcription and expression of COX-2, anti-apoptotic proteins and growth factors.[35,36] Curcumin was confirmed to inhibit IκB kinase activity.[36] The concentration needed to inhibit NF-κB from IL-1 activation in U937 cells was 50 μM.

However, curcumin has very poor bioavailability. The concentrations found in human plasma were 0.4–3.6 μM after the ingestion of 4–8 g of

curcumin.[37] The bioavailability is greatly improved with the addition of piperine.

12.5.8 Guggulsterone

Guggulsterone has been used for centuries in Ayurvedic medicine for its anti-inflammatory activity in arthritis, cardiovascular disease, obesity and bone fractures[38] (Figure 12.9). It is found in the guggul plant in northern India. Guggulsterone has been found to inhibit NF-κB activation by RANKL (TNFα cytokine family) in RAW-269.7 cells.[38] This study demonstrated that guggulsterone was a direct inhibitor of IκB kinase. The doses found to inhibit NF-κB entirely were 10 μM in this cell system. Another study similarly found that NF-κB activation by IL-1β could also be abolished at 10 μM concentrations of guggulsterone in fibroblast-like synoviocytes, a cell model for arthritis,[39] by blocking the degradation of IκBα.

It is known that μM amounts of guggulsterone inhibit inflammation *in vivo*, and guggulsterone has numerous beneficial effects *in vivo*,[40] such as in the cardiovascular system in humans. However, there are few data as to the bioavailability of this agent.

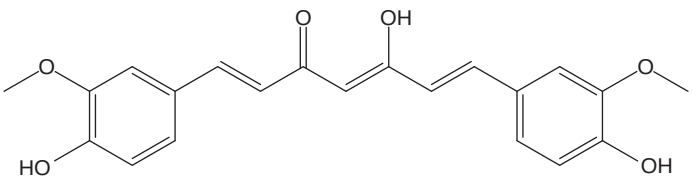

Figure 12.8 Curcumin.

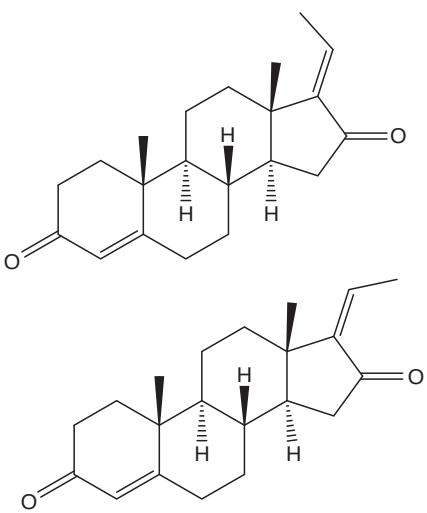

Figure 12.9 *E*- and *Z*-Guggulsterone.

12.6 Agonists of PPARγ that Reciprocally Inhibit NF-κB

In general, many of the natural agonists of PPARγ are analogs of fatty acids, lipid molecules, cholesterol and other terpenoids.[41] Terpenoids can be found in eucalyptus, cinnamon, cloves, ginger, citral, menthol, camphor and many plants. The following are a few examples of common natural product PPARγ agonists.

12.6.1 Phytanic Acid

Phytanic acid is an example of a terpenoid derived from chlorophyll in plant extracts. It is a derivative of the phytol side chain of chlorophyll. In one study, phytanic acid (Figure 12.10) was able to stimulate PPARγ activation in hepatocytes in a dose-dependent manner from 10–100 μM, in doses comparable to thiazolidinedione derivatives.[42] Some of the original studies on phytanic acid demonstrated that human plasma contains μM levels of this compound.[43]

12.6.2 Dehydroabietic Acid

Dehydroabietic acid is a diterpenoid found in pine tree resins. It is also a potent PPARγ agonist.[44] In this study, dehydroabietic acid (Figure 12.11) was found to inhibit the production of MCP-1, TNFα and NO in LPS-activated macrophages at a 40 μM concentration. However, no bioavailability data or toxicity studies are yet available for this agent.

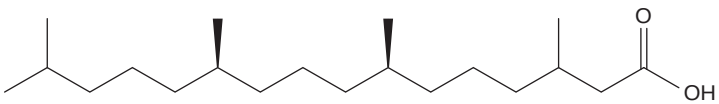

Figure 12.10 Phytanic acid.

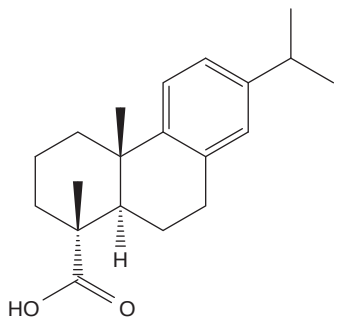

Figure 12.11 Dehydroabietic acid.

12.6.3 Geraniol

Geraniol, farnesol and geranylgeraniol are another class of PPARγ agonists that stimulate PPARγ.[45] The geraniols (Figure 12.12) are found in rose oil, palmarosa oil, citronella oil and in geranium, lemon and other essential oils. Geraniols have been documented to inhibit LPS-induced stimulation of COX-2, iNOS and NF-κB in μM concentrations.[46] However, bioavailability data are unavailable for geraniol at this time.

12.7 Agonists of LXR that Reciprocally Inhibit NF-κB

Many of the natural product agonists of LXR resemble cholesterol-like molecules such as plant sterols. Many cholesterol intermediates are also agonists for LXR. The following are a few examples of common natural product LXR agonists. However, there are few human bioavailability data available.

12.7.1 Stigmasterol

Stigmasterol is a plant-derived sterol and a potent agonist of LXR (Figure 12.13). It is found in lipid-rich areas of plants or oils of soybean, calabar bean, rape seed and Chinese herbs such as ginseng. The addition of stigmasterol to CHO-7 cells was able to increase expression of many genes downstream of LXR, such as the ATP binding cassette transporter (ABCA1), which promotes the efflux of cholesterol.[47]

Stigmasterol is also known for its anti-inflammatory activity.[48] In osteoarthritis cell culture models stimulated by IL-1, stigmasterol in a 20 μg/ml concentration was able to inhibit NF-κB-induced expression of IL-6 and MMP-3.

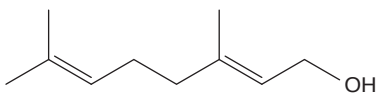

Figure 12.12 Geraniol.

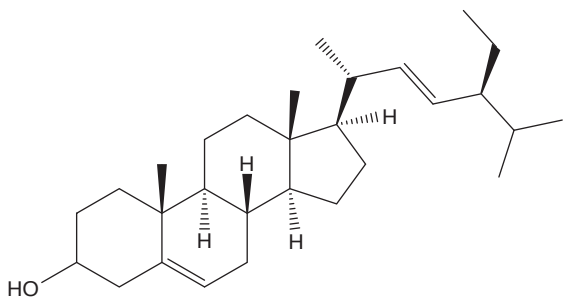

Figure 12.13 Stigmasterol.

12.7.2 β-Sitosterol

β-sitosterol is another plant-derived sterol found in black cumin seed, pecans, saw palmetto, avocados, pumpkin seed, cashew, rice brain, corn oil and soybeans. It is considered to be a potent LXR agonist and anti-inflammatory agent[49] (Figure 12.14). In cell culture models of osteoarthritis, β-sitosterol was able to decrease proinflammatory pathways.

12.7.3 Ergosterol

Ergosterol is another LXR agonist, found in fungal membranes, and is considered to be a very potent LXR agonist. In fact, some of the ergosterol derivatives found in nature activate LXR (Figure 12.15) much more effectively than the known cholesterol agonists.[50] Ergosterol has been found to have anti-inflammatory properties as well.[51]

12.8 Conclusion

Numerous phytochemicals and natural products can intervene in signal transduction mechanisms to attenuate NF-κB-mediated inflammatory

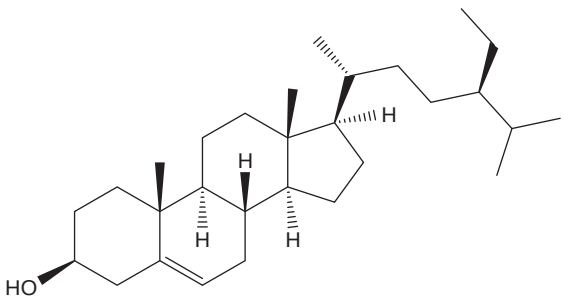

Figure 12.14 β-Sitosterol.

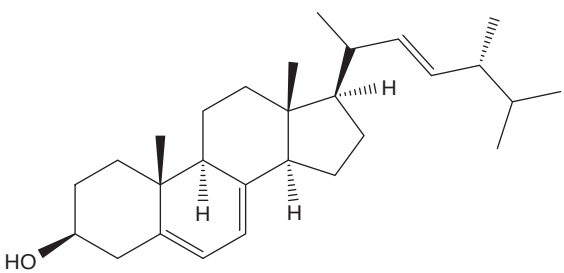

Figure 12.15 Ergosterol.

pathways. However, much remains to be elucidated regarding the pharmacology, toxicology and peak concentrations of these natural products that can be achieved in human plasma. Transcriptional regulators in inflammation present a powerful strategy to target chronic inflammatory diseases. It may be that the life-long prophylactic use of these natural ligands may preempt the use of stronger synthetic derivatives later on.

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CHAPTER 13

Intracellular Signaling Pathways in Parkinson's Disease

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13.1 Introduction

Parkinson's disease (PD) is a neurodegenerative condition characterized by insidious onset of bradykinesia, rigidity and resting tremor that respond to a dopamine mimetic agent.[1] Clinical manifestations are correlated to a progressive degeneration of dopaminergic neurons in the pars compacta region of the substantia nigra (SN) and the resulting loss of striatal dopamine.[2]

The etiology of PD appears to be multi-factorial involving a chronic, progressive process that ultimately leads to dopaminergic neuronal death. The initiating event or events that trigger the beginning of the disease remain unknown. However, most data support a shared role for environmental and genetic factors. The events involved throughout the initiation of the disease until the selective dopaminergic neuronal death occurs are diverse, involving damage to cell components, abnormal organelle function and activation or inhibition of signaling pathways. Research done in this area has used different approaches including cell tissue exposure protocols and animal models as well as human genetics. The variation in experimental conditions and inconsistent results when comparing studies adds complexity to our understanding

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of the disease. The role of some signaling pathways and signaling components in the neurodegenerative process have been clearly defined and are consistently reported, while the role(s) of others are still to be clarified. Additionally, it has been suggested that some signaling pathways experience cross-talking as well as non-exclusive activation and an overlapping of functions. Studying these signaling processes involved in dopaminergic neuronal death is important in order to elucidate how PD starts and how it progresses as well as to develop therapeutic interventions.

13.2 Selective Dopaminergic Neuronal Death

Neuronal cells, particularly dopaminergic neurons, are generally at a higher risk of injury and death than cells in other tissues. Dopaminergic neurons and some other neuronal populations in the central nervous system (CNS) show a differential sensitivity to stress.[3] Some findings offer an explanation to this particular susceptibility. It has been proposed that dopaminergic neurons in the SN need to have a rigorous control of the balance between their high metabolic needs and their high production of metabolic by-products. Additional metabolic loads, genetic deficiencies and aging may affect this balance leading to these cells becoming less tolerant and consequently premature cell death.

A significant loss of mitochondrial complex I activity is found in PD patients and it has been proposed to result in a lowered threshold for oxidative damage[4] (see Figure 13.1). In support of this, animal and cellular models generated by exposure to exogenous mitochondrial complex I inhibitors such as 1-methyl-4-phenylpyridinium (MPP^+), Paraquat and Rotenone induce phenotypes resembling those of typical PD, including selective dopaminergic neuron death. Since mitochondrial complex I impacts respiratory chain function, its inhibition induces superoxide radical leakage from the mitochondria into the cytosol as well as decreases NADH and ATP production. As a mechanism for buffering the superoxide radicals from the cytosol, superoxide dismutases catalyze the dismutation of superoxide radical to hydrogen peroxide, which can be converted to the highly reactive hydroxyl radical.[5] Reactions involving transition metal ions, such as the Fenton reaction, could be facilitated in dopaminergic neurons given the high levels of free iron and copper in the SN.[6] Superoxide anion, hydrogen peroxide and hydroxyl radical are all reactive oxygen species (ROS), which induce a variety of oxidative lesions when they react with cell components. Under excessive production of ROS, there is a greater chance that these species react with cell components giving rise to oxidative stress.

In addition to the impairment of complex I function, different processes that take place in dopaminergic cells can lead to oxidative stress. Endogenous dopamine and its oxidation may be causal factors in the pathogenesis of PD[7] (see Figure 13.2). Dopamine is easily auto-oxidized at physiological pH. Therefore, oxidation is normally prevented through storage in synaptic vesicles where acidic conditions are maintained. During stressful conditions, excessive dopamine turnover can result in extravesicular pools that are oxidized or

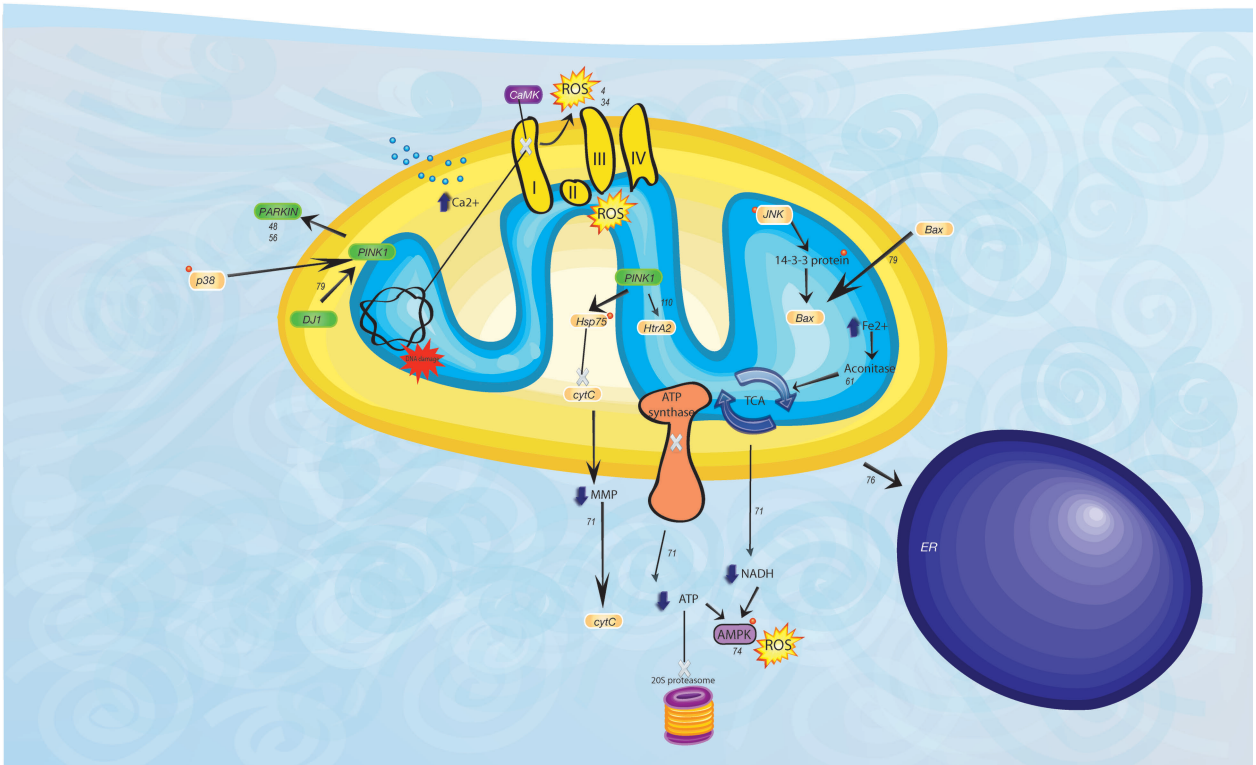
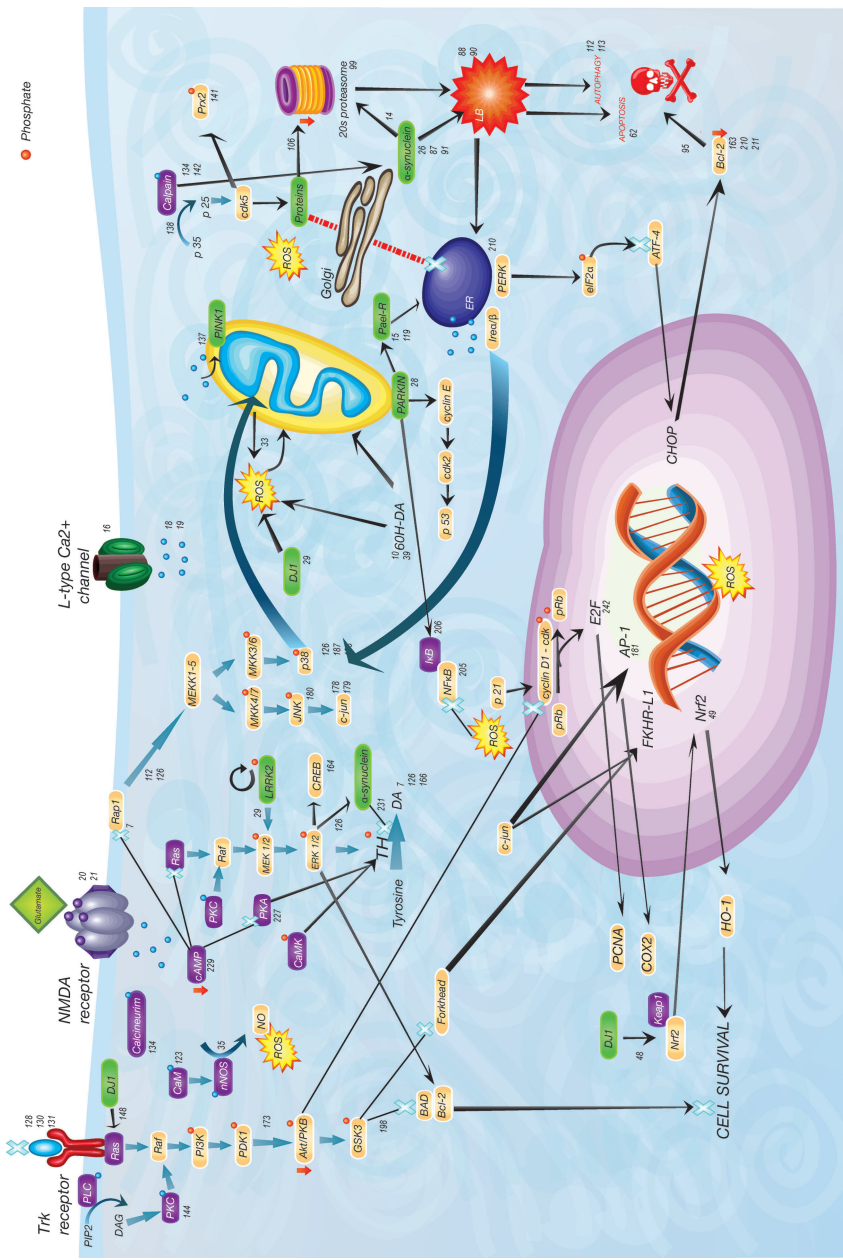


Figure 13.1 Graphic representation of pathological events in the mitochondria in PD and PD models. *ROS* = reactive oxygen species. *I*, *II*, *III*, *IV* = components of the respiratory chain complex, *MMP* = mitochondrial membrane potential, *CytC* = cytochrome C, *ER* = endoplasmic reticulum.

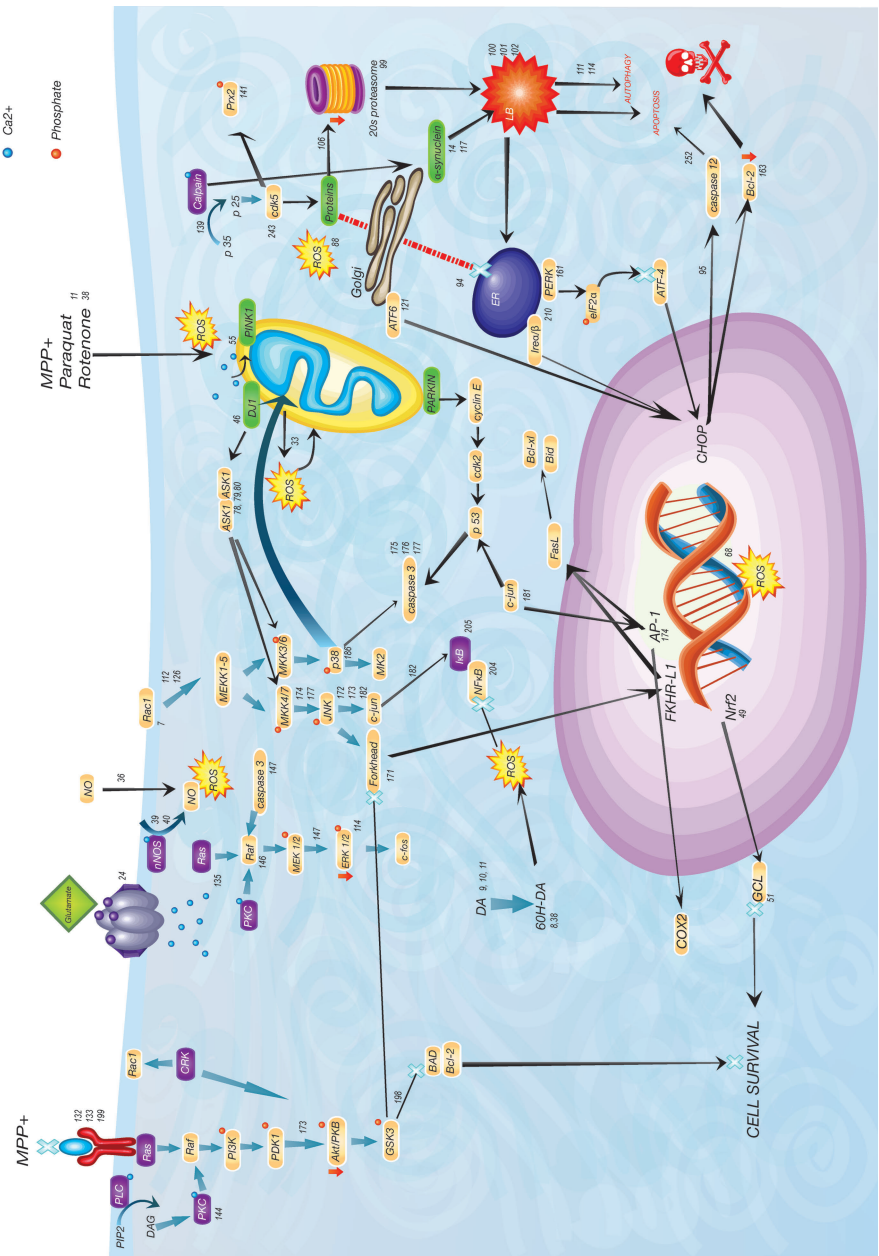

Ca2+
Phosphate
Trk receptor
PLC
PIP2
DAG
PKC
Raf
Ras
PI3K
PDK1
Akt/PKB
GSK3
DJ1
Calcineurin
CaM
nNOS
NO
ROS
NMDA receptor
Glutamate
L-type Ca2+ channel
Rap1
MEKK1-5
MKK4/7
MKK3/6
p38
JNK
c-jun
cAMP
PKC
Ras
Raf
MEK 1/2
ERK 1/2
PKA
CaMK
LRRK2
CREB
α-synuclein
TH
DA
Tyrosine
Forkhead
BAD
Bcl-2
IκB
NFκB
p 21
ROS
cyclin D1 - cdk
pRb
c-jun
E2F
AP-1
FKHR-L1
PCNA
COX2
DJ1
Keap1
Nrf2
Nrf2
HO-1
CELL SURVIVAL
CHOP
ATF-4
eIF2α
PERK
ER
Golgi
Rtec/β
Pael-R
PARKIN
cyclin E
cdk2
p 53
60H-DA
DJ1
PINK1
ROS
ROS
Calpain
p 35
p 25
cdk5
Proteins
Prx2
20s proteasome
α-synuclein
LB
AUTOPHAGY
APOPTOSIS
Bcl-2

metabolized.[8] Also mutations in PD-related genes interfere with normal incorporation of dopamine into synaptic vesicles.[9] Oxidation of dopamine causes the production of ROS and oxidized intermediates such as 6-hydroxydopamine (6-OHDA)[10] and leukoaminochrome-o-semiquinone radical.[11] Used as a PD model, exposure to 6-OHDA causes cell death of dopaminergic neurons by an increase in intracellular oxidants,[7,12] while another intermediate, neuromelanin, may have a protective role in the disease.[8,13] Increased extravesicular dopamine induces activity of monoamine oxidase (MAO), an enzyme important for metabolizing monoamines. The activity of MAO is coupled to the production of hydrogen peroxide at the mitochondrial outer membrane.[4] Despite the evidence supporting dopamine auto-oxidation as a causal factor, dopaminergic neurons in the ventral tegmental area (VTA), also an important dopaminergic cell population in the midbrain, do not show the same vulnerability as cells in the SN. Nigral dopaminergic neurons show a differential proteomic profiling compared to VTA dopaminergic neurons such as a higher expression of antioxidant genes, suggesting a high intrinsic oxidative stress status.[3]

Loss of balance in other processes in dopaminergic neurons may overwhelm normal cell function and result in cell stress and death. Abnormal protein processing is a significant observation in PD. Lewy bodies (LBs), a pathologic hallmark in PD, are intracytoplasmatic formations composed mainly of ubiquitinated proteins and α-synuclein. MPP^+ and 6-OHDA can induce abnormal processing of α-synuclein, proteasomal dysfunction and cell death[14] (see Figure 13.3). In a juvenile autosomal recessive form of PD, LBs are not found. Alternatively, it has been described as an accumulation of a different protein, Pael-R, which implicates a susceptibility to abnormal protein processing in these patients. Interestingly, there is an increased expression of Pael-R gene in dopaminergic neurons in the SN.[15]

Dopaminergic cells are at a higher risk of Ca^{2+} imbalance, which results in activation of signaling pathways, increased DA synthesis and production of ROS. Increased intracellular Ca^{2+} levels could be the result of increased extracellular Ca^{2+} influx, decreased Ca^{2+} excretion from the cell or deregulated Ca^{2+} transport through organelle membranes. Evidence has been presented showing that Ca^{2+} influx into the neuron can be responsible for their high susceptibility to cell death. Two Ca^{2+} channels have been associated with high

Figure 13.2 Graphic representation of pathological events in PD. Findings from PD affected individuals and from *in vitro* and *in vivo* studies on PD-related genes are included. *ROS* = reactive oxygen species, *NMDA receptor* = *N*-methyl D-aspartate glutamate receptor, *Nrf2* = nuclear factor erythroid 2-related factor, *HO-1* = hemeoxygenase, *Pael-R* = Parkin-associated endothelin-receptor-like receptor, *CaM* = calmodulin, *CaMK* = Ca^{2+}-calmodulin-dependent protein kinase, *PKC* = protein kinase C, *PLC* = phospholipase C, *DAG* = diacylglycerol, PIP_2 = phosphatidyl inositol-bisphosphate, *PI3K* = phosphatidylinositol 3-kinase, *Akt* = v-akt murine thymoma viral oncogene homologue or protein kinase B, *PDK1* = phosphoinositide-dependent kinase 1, *TH* = tyrosine hydroxylase, DA = dopamine, *6OH-DA* = 6-hydroxy-dopamine, *PKA* = protein kinase A, *cdk* = cyclin-dependent kinases, *pRb* = retinoblastoma protein, *ER* = endoplasmic reticulum, *LB* = Lewy body.


Ca2+
Phosphate
MPP+
Paraquat
Rotenone
MPP+
Glutamate
NO
nNOS
Rac1
Ras
Raf
PI3K
PDK1
Akt/PKB
GSK3
BAD
Bcl-2
CRK
PLC
PIP2
DAG
PKC
PKC
Rac1
MEKK1-5
MKK4/7
MKK3/6
p38
JNK
MK2
c-jun
Forkhead
c-fos
Raf
MEK 1/2
ERK 1/2
caspase 3
ROS
ROS
ROS
ROS
ROS
ROS
ROS
ASK1
PINK1
DJ1
PARKIN
cyclin E
cdk2
p 53
caspase 3
c-jun
FasL
Bcl-xl
Bid
IκB
NFκB
DA
6OH-DA
Golgi
ER
PERK
eIF2α
ATF-4
ATF6
Ire1β
CHOP
caspase 12
Bcl-2
Calpain
p 35
p 25
cdk5
Proteins
Prx2
20s proteasome
α-synuclein
LB
AUTOPHAGY
APOPTOSIS
AP-1
FKHR-L1
Nrf2
GCL
COX2
CELL SURVIVAL

dopaminergic susceptibility, L-type Ca^{2+} channels and glutamate receptors. L-type Ca^{2+} channels are voltage-gated ion channels involved in the autonomous pacemaker activity in dopaminergic neurons in the SN.[16] Even though this pacemaker activity involves the function of multiple ion channels, it is the L-type Ca^{2+} channel activation that involves frequently elevated intracellular Ca^{2+} levels[17–19] (Figure 13.1). *N*-methyl D-aspartate (NMDA) glutamate receptors are both ligand-gated and voltage-gated receptors. NMDA receptors are non-specific cationic channels that allow the passage of Ca^{2+} into the cell in response to glutamate binding. Dopaminergic neurons in the SN could be more vulnerable to glutamate stimulation through NMDA receptors as their subunit composition is predominantly NR1/NR2B/NR2D.[20,21] NR2D subunits are characteristic of dopaminergic neurons of the SN pars compacta and mainly determine the functional diversity of NMDA receptors.[22] Upon glutamate stimulation, individual NR2D subunits activate intracellular pathways that are not activated in non-dopaminergic neurons. Additionally, during the course of PD, decreased inhibition from nigral inputs to the striatum may induce an over-stimulating striatal feedback on nigral dopaminergic cells. Continuous exposure to low concentrations of glutamate through striatal inputs decreases the number of viable dopaminergic neurons in rat-derived mesencephalic neurons.[7] The finding that inhibition of glutamate transporters and the resulting increase in extracellular glutamate have toxic effects on dopaminergic neurons[23] and the protective effects of NMDA receptor antagonists to MPTP and 6-OHDA[24] further supports the role of glutamate in selective dopaminergic neuronal toxicity.

The previously described mechanisms account in part for the inherent susceptibility of dopaminergic cells to death; however, genetic and epigenetic factors may explain inter-individual and phenotypic variations of the disease. Genetic mutations associated to familiar PD could mediate the susceptibility of these cells to damage. Mutations and gene variations are the cause of familial PD and explain up to 10% of the cases.[25] Mutations in α-synuclein gene (PARK1) have been linked to autosomal dominant PD[26,27] and are associated with abnormal α-synuclein processing (Figure 13.2). Mutations in parkin gene (PARK2) result in loss of proteasomal function and are found in autosomal recessive early-onset cases.[28] Mutations in DJ1 (PARK7) and PINK1 (PARK6) genes are associated with early-onset familial PD and have been linked to a higher susceptibility to

Figure 13.3 Graphic representation of pathological events in toxicant-based PD models. Findings from *in vitro* and *in vivo* PD models using MPTP, Paraquat, Rotenone and 6OH-DA are included. *ROS* = reactive oxygen species, *NMDA receptor* = *N*-methyl D-aspartate glutamate receptor, *Nrf2* = nuclear factor erythroid 2-related factor, *GCL* = glutamate cysteine ligase, *PKC* = protein kinase C, *PLC* = phospholipase C, *DAG* = diacylglycerol, PIP_2 = phosphatidyl inositol-bisphosphate, *PI3K* = phosphatidylinositol 3-kinase, *Akt* = v-akt murine thymoma viral oncogene homologue or protein kinase B, *PDK1* = phosphoinositide-dependent kinase 1, DA = dopamine, *6OH-DA* = 6-hydroxy-dopamine, *PKA* = protein kinase A, *cdk* = cyclin-dependent kinases, *pRb* = retinoblastoma protein, *ER* = endoplasmic reticulum, *LB* = Lewy body.

oxidative stress.[29] Mutations in LRRK2 gene (PARK 8) cause autosomal dominant PD and have been found with high frequency in sporadic PD cases.[30,31] Recently, epigenetic factors have been shown to be involved in the susceptibility of dopaminergic cells to death. Early changes in epigenetic regulation of gene expression may be responsible for epistatic genetic effects by affecting several genes or can exert additive effects on gene mutation in genes associated to PD.[32]

13.3 Signaling Pathways Involved in Selective Dopaminergic Neuronal Death

As previously mentioned, mechanisms involved in selective loss of dopaminergic neurons in the SN in PD include oxidative stress, calcium imbalance, glutamatergic overstimulation and abnormal protein processing. In the next section, it will be discussed how these proposed initiators originate intracellular signals and the pathways involved in the signal transduction that result in selective dopaminergic neuronal death in PD and PD models.

13.3.1 Initiators and Signaling Molecules

Proposed mechanisms by which progressive damage and cell death occur in PD may include cell autonomous and non-autonomous factors or the interplay between the two. For instance, genomic mutations result in an abnormal protein structure that creates a toxic intracellular environment, whereas environmental toxicants require entering dopaminergic cells by transporters or membrane receptors in order to exert their toxic effects. Causative factors act as intracellular or extracellular signals to initiate the transduction of a message using intracellular pathways. The initiation of signal transduction involves highly specific processes such as binding to membrane receptors and inducing molecular modifications that are able to change physiological cell conditions and turn on signaling molecules.

13.3.1.1 Response to Oxidative and Nitrosative Stress

Increased production of ROS and reactive nitrogen species (RNS) has been consistently found in PD and in cellular and animal PD models. ROS and RNS are produced as by-products of normal cell respiration, metabolism and aging; however, it has been suggested that dopaminergic neurons in the SN in PD could generate more ROS and RNS as a result of their impaired respiratory chain function.[33] Similarly, in PD models, MPP^+ has a direct inhibitory effect on complex I.[34] As a result of complex I dysfunction, increased amounts of superoxide anion and hydrogen peroxide leak into the cytosol increasing the production of hydroxyl radical, nitric oxide (NO) and peroxynitrite and turning the redox balance towards oxidative stress.[3,4] Additionally, NO is also produced by the induction of nitric oxide synthase (NOS) activity by glutamate[35] as well as MPP^+ (Figures 13.2 and 13.3).[36,37] Neurotoxins such as Paraquat and Rotenone also have an inhibitory effect on complex I inhibition.

Additionally, Paraquat induces ROS production *via* reaction with molecular oxygen[38] and Rotenone directly oxidizes endogenous dopamine[11] affecting redox cycling (Figure 13.3). *In vitro*[7,12] and *in vivo*[39] response to exposure to 6-OHDA involves an early increase in intracellular oxidants through auto-oxidation to aminochrome (Figure 13.3), however, the relevance of 6-OHDA as an endogenous toxic oxidized dopamine intermediate in PD patients remains unknown.

To prevent the damage caused by ROS and RNS, cells have antioxidant systems that protect them from specific reactive species. High levels of superoxide in the cytosol are dismutated to hydrogen peroxide by superoxide dismutases (SOD). Hydrogen peroxide is cleared out by peroxidases or catalases preventing formation of hydroxyl radicals and peroxynitrite.[40,41] There are three types of peroxidases: peroxiredoxins (Prxs), catalases and glutathione peroxidases (GPxs). GPxs are involved in the reduction of hydrogen peroxide mediated by glutathione (GSH). GSH is a major antioxidant system in neuronal cells that is involved in both enzymatic reductions as well as non-enzymatic redox regulation. Studies on antioxidant systems in PD are inconclusive. Peroxidase and catalase levels have not been found altered, whereas superoxide has been found increased and GSH decreased.[42,43] Antioxidant systems in PD models such as MPP^+ toxicity have been found to be important toxicity mediators. Transgenic mice over-expressing Cu-Zn SOD (SOD1) have been shown to be resistant to MPTP neurotoxicity.[44] It should also be noted that MPP^+ redox cycles with the production of oxygen radicals.[45]

Abnormal function of PD-related proteins, such as DJ1, PINK1 and parkin, has been linked to deficient antioxidant systems. DJ1 has been proposed as a redox responsive chaperone that, when oxidized, can translocate from the mitochondrial matrix and inter-membrane space into the outer mitochondrial membrane.[46] Also, DJ1 can undergo self-oxidation and act as an atypical mitochondrial "peroxiredoxin-like peroxidase", decreasing levels of hydrogen peroxide.[47] Indirect antioxidant effects of DJ1 include the formation of nuclear complexes with RNA-binding proteins and DNA-binding proteins that regulate gene transcription and stabilization of the antioxidant regulator nuclear factor erythroid 2-related factor (Nrf2)[48] (Figure 13.2). This occurs by preventing Nrf2 association to its inhibitor, Keap1, and therefore Nrf2 ubiquitination.[49] Nrf2 binding to antioxidant response element (ARE) gene regulatory regions activates transcription of neuroprotective genes such as heme oxygenease (HO-1) and glutamate cysteine ligase (GCL). HO-1 has also been shown involved in cell survival processes and its over-activity is coupled to excessive levels of heme-derived free iron and carbon monoxide.[50] GCL is the rate-limiting enzyme in the GSH synthesis; both its mRNA expression and activity have failed to be induced in response to oxidative stress in rat dopaminergic cells.[51] By inducing gene expression changes, DJ1 may contribute to cell survival and prevent apoptotic cell death under oxidative stress conditions.[52]

PINK1 is a ubiquitously expressed protein located in the inner mitochondrial membrane with its C-terminus exposed to the intermembrane space[53] (Figure 13.1). PINK1 regulates Ca^{2+} efflux from the mitochondria. PINK1 deficiency or expression of disease-associated mutated PINK1 is associated with

mitochondrial Ca^{2+} overload, decrease in mitochondrial complex I activity and an increase of levels of ROS production.[54] PINK1 deficient cells are more susceptible to die after exposure to MPP^+ or Rotenone.[55] A collaborative function for PINK1 with parkin and DJ1 in protecting from oxidants has been suggested; this is supported by the susceptibility of PINK1 deficient cells to die, which can be rescued by over-expressing parkin, implicating that PINK1 is upstream on a linear pathway.[56] This also indicates that DJ1 stabilizes PINK1 allowing for their binding to have a synergistic action protecting from MPP^+ exposure.[57]

In PD etiology, genetic deficiencies or environmental insults generate toxic cell conditions that affect the balance between production of reactive species and antioxidant defenses giving rise to oxidative and nitrosative stress. ROS and RNS react rapidly with cell components and are responsible for nitration and oxidation to proteins, lipids and/or nucleic acids. Protein nitration, oxidation and carbonylation are increased in PD.[58–60] Specifically, PD-related proteins are oxidatively damaged, including α-synuclein oxidation and nitration and parkin nitrosilation. Additionally, in PD more ubiquitous enzymes are found to be oxidatively modified affecting central cell pathways. Aconitase, an iron-sulfur enzyme, is inactivated by superoxide-induced loss of the labile Fe^{2+} atom, which affects the tricarboxylic acid cycle and consequently cellular energy metabolism (Figure 13.1).[61] Lipid peroxidation is increased in PD as high levels of lipid hydroperoxides and 4-hydroxynonenal (HNE) are found in the SN.[62,63] HNE is found as part of LBs and also forming adducts with nucleophilic groups on proteins, such as dopamine transporter.[64] Lipid peroxidation in PD has been associated with apoptosis, PARP-induced cleavage, decreased GSH levels and inhibited mitochondrial complexes I and II.[62] Lastly, oxidative stress in PD produces several oxidative lesions to genomic and mitochondrial DNA. Mitochondrial DNA (mDNA) is particularly susceptible given its proximity to the mitochondria, an important ROS production site. A higher number of deletions in mDNA are found in PD patients as a result of oxidative damage[48] and missense mutations affect respiratory chain genes[65] (Figure 13.1). Additionally, loss of function of PINK1 has resulted in decreased mDNA synthesis.[66] Levels of 8-hydroxyguanine and 8-hydroxy-2-deoxyguanosine are increased in PD,[67] whereas exposure to 6-OHDA induces an increase of double strand breaks.[68] Severely damaged nucleic acids could affect gene expression and lead to apoptotic cell death in PD. Given that neuronal cells are post-mitotic and DNA does not benefit from turnover, DNA repair systems are critical to defend against oxidative lesions in neuronal cells. The efficiency of DNA repair systems may influence aging in response to oxidative stress.[69]

Damage to macromolecules due to oxidative and nitrosative stress results in cell components dysfunction. Two of the most affected organelles in PD are mitochondria and endoplasmic reticulum (ER). Mitochondrial dysfunction in PD is likely the result of complex I inhibition, increased ROS production and Ca^{2+} overload.[70] Under these conditions, mitochondria lose their control over Ca^{2+} levels, mitochondrial membrane potential (MMP) and generation of NADH and ATP. Mitochondrial Ca^{2+} overload stimulates mitochondrial

permeability transition pore (mPTP) opening, which results in mitochondrial membrane depolarization and mitochondrial swelling. As a result, there is loss of GSH and NAD(P)H from the mitochondria and release of cytochrome c to the cytosol.[71] Under physiological conditions, mitochondrial Ca^{2+} stimulates tricarboxylic acid cycle and oxidative phosphorylation, however, it is uncertain how this stimulation changes under Ca^{2+} overload conditions.[71] Decreased MMP affects normal mitochondrial processes such as fusion and fission[72] and mitochondrial trafficking.[73] By nature, the main function of mitochondria is to supply energy to maintain physiological cell processes, and impaired ATP production directly affects the function of ATP-dependent processes such as maintenance of plasma membrane potential, glucose transport into the mitochondria, ubiquitination and the proteasome function. Additionally, master cell energy regulators, such as AMP-activated protein kinase (AMPK), respond to changes in AMP/ATP ratio, Ca^{2+} levels and ROS initiating the activation of an intracellular signaling pathway that prevents cell death.[74]

PD-related proteins that are localized in the mitochondria, such as DJ1, PINK1 and parkin, can show differences in their functions under mitochondrial dysfunction.[75] Parkin redistribution from the mitochondria into the cytosol occurs in response to inhibitors of respiratory chain activity and cell cycle blockers.[48] Oxidized DJ1 changes its distribution from being diffused throughout the cytoplasm to the proximity of the mitochondria. Additionally, mitochondrial dysfunction could induce ER stress due to their adjacency in the cytoplasm. ER stress is found in PD and it is accompanied by the unfolded protein response (UPR) and protein aggregation (discussed in the next section). It has been proposed that a cross-talk between the mitochondria and ER could initiate a signaling pathway resulting in apoptosis in dopaminergic cells.[76]

Although believed to be only damaging, a new role for ROS in normal intracellular signaling has emerged. Such function in signaling has especially been applied to hydrogen peroxide since its half-life and specificity allows it to fit criteria for being a second messenger.[77] Thiols in proteins can be oxidized to sulfenic acid residues, glutathionylated residues or to the formation of intramolecular disulfide bonds in the presence of hydrogen peroxide. These protein modifications determine the origin of cell messages. In the 6-OHDA model, oxidation of thioredoxins (Trx), such as Trx1, allows apoptosis signal-regulated kinase1 (ASK1) to dimerize and activate the apoptosis-inducing pathways, p38 and JNK intracellular pathway.[78,79] Using a similar mechanism, in cells treated with hydrogen peroxide, oxidized DJ1 converts Cys-106 to cysteine sulfinic acid (Cys-SO2H), and binds to ASK1 inducing cytoprotection.[80] The activation of another cell survival intracellular signaling cascade, the Akt pathway, has been found to be facilitated by DJ1.[81] Additionally, the "floodgate hypothesis" proposes that, under oxidative stress conditions, hydrogen peroxide oxidizes peroxydases, thus making them inactive.[77] Peroxidase inactivation, specifically Prx1, has been described to induce cell-cycle arrest mediated by the activation of p38 and caspase-3.[82] Some promising examples of hydrogen peroxide-mediated signaling include oxidative modifications in AP-1[83] and PTEN.[84]

13.3.1.2 Response to Altered Proteostasis

Proteostasis refers to a complex regulation of protein synthesis, folding, quality control, transport and degradation.[85] This regulation is important in order to keep the normal protein structure and function and to prevent abnormal protein accumulation. The identification of LBs as hallmarks of PD has raised the interest in the role of alterations of proteostasis in PD. LBs, found in most degenerating dopaminergic neurons in PD patients, are mainly proteinaceous inclusions formed by overlapping steps of protein aggregation, protein post-translational modifications and proteolysis.[86] α-Synuclein is the main filamentous component of LBs;[87] it is natively unfolded and has a structural trend to aggregate due to its hydrophobic non-amyloid beta domain.[25] In the formation of LBs, α-Synuclein monomers aggregate to form fibrils, which have been shown as being favored by oxidative modifications.[88] The role of α-Synuclein as a causal factor of PD is supported by the findings that mutations and gene variations of PARK 1 increase the aggregation propensity of α-Synuclein,[89,90] and that α-Synuclein over-expression is a causative factor in familial cases of PD.[26] Therefore, the pathogenic mechanisms of α-Synuclein in PD include self-aggregation and decreased ability of cells to eliminate α-synuclein before it reaches critical intracellular concentrations to aggregate.[91] Ubiquitinated proteins, parkin and chaperones Hsp70/Hsp90, as well as mitochondrial proteins such as cytochrome c, also colocalize in LBs.[92] Some other of the familial PD-associated mutations and gene variations have been related to changes in protein structure that affect hydrophobicity, protein-protein interaction and degradation, such as mutations in DJ1 that make its protein product easily degraded.[93]

Different cellular systems participate in proteostasis, including the ER, the ubiquitin-proteasome system (UPS), ubiquitin-independent proteases and autophagy. The ER has a function in folding and post-translational modification of proteins after they are synthesized. Abnormal proteins, such as truncated proteins, are not folded properly and are not transported out of the ER, which favors their subsequent accumulation. Protein accumulation into the ER interferes with protein traffic from ER to the Golgi and induces ER stress.[94] ER stress induces a compensatory response cascade, the unfolded protein response (UPR), which involves an increased expression of ER chaperones and ER-associated degradation (ERAD)-associated molecules to increase the cellular folding capacity and the translocation of unfolded proteins from the ER into the cytosol. Some of these ER chaperones include Hsc/Hsp70 and Hsp40, which re-fold unfolded or misfolded proteins. In the cytosol, the ERAD directs unfolded proteins to be degradated by the proteasome. ER stress also induces an ER Ca^{2+} imbalance and persistent protein accumulation resulting in caspase-12 and ER-specific apoptosis activation.[95]

If proteins are normally synthesized and folded, molecular crowding can account for an abnormal protein aggregation. The neuronal cytoplasm is a crowded environment, mostly occupied by macromolecules, that offers a limited access to proteins. Molecular crowding is non-specific and can fluctuate as cell

volume changes with age or disease. Under the molecular crowding scenario, protein folding and protein-protein interaction equilibrium is driven towards the lower volume (globular/oligomeric) species as well as an entropic driving force that compensates the decreased entropy of fibril-forming protein for the high entropy of other proteins.[96] The failure to properly dispose proteins and the over-expression of PD-related proteins lead to excessive cytoplasmatic protein content, molecular crowding and spontaneous oligomerization.[91]

The UPS is the major system responsible for degradation of cytosolic abnormal proteins. UPS involves degradation of damaged proteins as part of the physiological protein turn-over through a sequential ATP-dependent processing by the ubiquitin-activating (E1), -conjugating (E2) and -ligating (E3) enzymes. These enzymes function in degradation of proteins by adding a ubiquitin tag to the substrate protein to be recognized by the proteasome.[97] E1 activates ubiquitin in its glycine 76, E2 transfers the activated ubiquitin from E1 to E3, which binds covalently to the substrate protein in a substrate-specific manner. Following this first ubiquitination, a polyubiquitin chain is added to the substrate protein making it a target for degradation by the 26S proteasome. The 26S proteasome is assembled by a catalytic core 20S proteasome and two regulatory subunits, 19S and 11S. The 20S proteasome hydrolyzes peptide bonds by proteolysis.[98] The UPS has been shown to be affected in PD[99] as well as proteasome function has been shown to be inhibited leading to LBs-like intraneuronal inclusions and selective degeneration of dopaminergic neurons[100–102] (Figure 13.2). The pathogenic processes responsible for UPS and proteasome dysfunction may involve α-Synuclein and parkin. Specifically, an α-Synuclein alternatively spliced form, the 112-aa form, has been shown to induce proteasome disfunction[14] whereas E3-ubiquitin ligase function of parkin may be affected by mutations in PARK2 and S-nytrosylation.[103] Parkin-associated endothelin-receptor-like receptor (Pael-R) is a substrate for parkin and has been shown to form insoluble aggregates in PD patients carrying mutations in PARK2.[15] Accumulation of Pael-R results in ER stress and neuronal death, while PARK2 over-expression protects against ER stress induced by unfolded proteins[15] (Figure 13.2). An alternative proteolytic processing for unfolded Pael-R requires its binding to Hsp70 and ubiquitination by an E4 reaction.[104] Programmed cell death-2 (PDCD2) protein and CDCrel-1 are potential parkin substrates. PDCD2 is highly homologous to Rp-8, a protein associated with apoptosis, inflammation and cell proliferation in rodent brain.[28] CDCrel-1 is a synaptic protein predominantly expressed in the nervous system and involved in cytokinesis.[105] In addition, a mutation in ubiquitin carboxy terminal hydrolase L1 gene (UCH-L1), involved in autosomal dominant PD cases, can be related to altered UPS function since it has been shown to have a function in cleaving polymeric ubiquitin.[106] The impact of abnormal function in these PD-related genes on UPS and proteasome function have been involved in the resulting ER stress, UPR and apoptosis in PD.[107]

When the action of chaperones is overcome, other mechanisms are used by the cellular quality control machinery to control protein accumulation.[108] Ubiquitin-independent protein degradation constitutes a mitochondrial system

of ATP-dependent proteases to monitor protein quality in the mitochondrial matrix. HtrA2 is a mitochondrial serine protease that can interact with and is regulated by PINK1 (Figure 13.1).[109] HtrA2 has a dual role in neuronal death: it activates proapoptotic proteins upon release into the cytosol and induces apoptotic death upon its inactivation.[110] Autophagy is another protein quality control in cells and it is considered a last line of defense against damage. Autophagy is initiated by the UPR to compensate UPS dysfunction and high protein accumulation states. Autophagy involves the removal of damaged or redundant cell components utilizing the lysosomes. MPP^+, mutations in the PD-related gene leucine rich repeat kinase 2 (LRRK2), neurotoxic doses of dopamine, as well as 6-OHDA induce autophagy.[111–114]

Although the link between oxidative stress and alteration of proteostasis is not completely clear, proteasome dysfunction is likely a result of an overload of unprocessed oxidized proteins and the limited availability of ATP for ATP-dependent peptidases required for proteasome function.[115] In some cases, oxidatively damaged proteins affect proteostasis. α-Synuclein oxidation and nitration cause its misfolding and decreased ubiquitination.[88] Oxidative modifications in parkin affect its E3 function.[103,116] Paraquat toxicity, which is associated with oxidative stress, upregulates and induces aggregation of α-Synuclein.[117] DJ1, which may have antioxidant properties, reduces aggregation of α-Synuclein and Pael-R preventing the apoptotic cell death induced by their toxic accumulation.[118,119] Some of the PD-related proteins that are involved in oxidative stress such as PINK1 and UCH-L1, as well as oxidized proteins and HNE, have been found colocalized to aggrosomes and LBs.[120] The effect of oxidative stress on proteostasis may be mediated by different mechanisms depending upon the initiator stimulus. A comparison between the response to 6-OHDA and MPP^+ shows that 6-OHDA activates the expression of genes involved in UPR and translation, whereas MPP^+ activation is restricted to genes involved in translation.[121] Both 6-OHDA and MPP^+ induce CHOP,[121] a stress-induced transcription factor involved in apoptosis (Figure 13.3).[122]

13.3.1.3 Response to Glutamate

Some dopaminergic neurons in the SN pars compacta receive moderate excitatory glutamatergic input from the subthalamic nucleus in the striatum. As the cell density progressively decreases in the SN in PD, dopamine levels in the striatum are progressively decreased causing reduction in the inhibition and over-activity of the internal globus pallidus and subthalamic nucleus resulting in overstimulation of dopaminergic nigral cells by glutamate. This excessive stimulation by glutamate causes an increased influx of Ca^{2+} into the cell through NMDA receptors (Figure 13.2).[123] Their characteristic NMDA NR2 subunit contents may make these neurons more susceptible to damage due to their direct interaction with specific small GTP-binding proteins, such as Ras and Rap.[124,125] As it will be discussed below, activation of small GTP-binding

proteins starts signaling pathways involved in cell survival and synaptic plasticity. Additionally, exposure to glutamate facilitates *de novo* synthesis of dopamine in dopaminergic neurons and changes dopamine distribution from vesicles to the cytosol,[126] which provides increased levels of free dopamine that can be oxidized.

13.3.1.4 Other Initiators

Most mechanisms of signaling initiation discussed above involve the effect of noxious stimulus to the dopaminergic cell; however, selective dopaminergic cell death may involve the lack of or deficient signaling through cell survival pathways. Neuroprotective factors activate pro-survival pathways or block pro-apoptotic signaling pathways resulting in cell death. However, there are not clearly identified neuroprotective factors against PD; some putative neuroprotective factors have been described including neurotrophic factors, dopamine receptor agonists, Na^+ channel blockers with anti-glutamatergic activity, anti-inflammatory agents and the long-term treatment with L-DOPA.[127] Neuroprotective properties of neurotrophic factors have been extensively studied and are the primary target for gene therapy for PD. Neurotrophic factors act *via* two classes of receptors: tyrosine kinase receptors (Trk) and $P75^{NTR}$. Upon Trk receptor binding, growth factors induce receptor dimerization and trans-autophosphorylation of tyrosine residues in the cytoplasmic domains. Adaptor proteins and kinases then associate with the activated receptors to form a signaling complex that will activate downstream pro-survival pathways[128] (Figure 13.2). $P75^{NTR}$ activates pro-survival as well as pro-apoptotic signals depending on the cellular context and the ligand.[129] Specifically, glial cell line-derived neurotrophic factor (GDNF), brain-derived growth factor (BDNF) and basic fibroblast growth factor (bFGF) have been shown to protect dopaminergic neurons against PD.[128] BDNF, bFGF and nerve growth factor (NGF) levels are found to be decreased in PD.[130,131] NGF is found to be low in MPTP-treated mice[132] and after serum deprivation in cell cultures.[133]

13.3.2 Signal Transducers, Intracellular Messengers and Upstream Elements

In response to an initial stimulus, specific molecular switches are turned on to transduce the message downstream using intracellular signaling pathways and effectors depending on the cellular context.

13.3.2.1 Ca^{2+}

Increased intracellular Ca^{2+} levels could be an initial event in PD. An excessive Ca^{2+} influx through NMDA receptors from glutamate overstimulation and a constant influx through L-type Ca^{2+} channels during pacemaking function

could be contributing factors to Ca^{2+} imbalance in dopaminergic neurons. Cybrids containing dysfunctional mitochondria from PD patients have been shown to increase intracellular Ca^{2+} levels correlating with increased Calpain activation (Figure 13.2).[134] Intracellular Ca^{2+} levels are selectively increased by exposure to MPP^+.[135] Supporting the role of increased intracellular Ca^{2+} in PD, midbrain cells containing calbindin, a Ca^{2+} binding protein, are spared from degeneration in PD patients and MPTP-induced parkinsonism.[136] The resulting Ca^{2+} imbalance is associated with altered membrane permeability, abnormal microtubule function and activation of Ca^{2+}-dependent enzymes.

In the mitochondria, loss of PINK1 function affects Ca^{2+} efflux resulting in increased mitochondrial Ca^{2+} levels and ROS production (Figure 13.1).[137] Elevated intracellular Ca^{2+} levels arrest microtubule-based mitochondrial movement seemingly by affecting formation of kinesin-Miro-Milton complex.[72] As an intracellular signaling messenger, Ca^{2+} can activate calmodulin/Ca^{2+}-calmodulin-dependent protein kinase (CaM/CaMKII), calpain and protein kinase C (PKC) in the cytosol (Figure 13.2). Upon CaM activation by Ca^{2+}, CaMKII is activated. CaMKII activation is associated with increased mitochondrial ROS production either by activation of Ca^{2+}-dependent dehydrogenases that stimulate oxidative phosphorylation or *via* activation of downstream targets such as MAO. Also, CaM functions as a co-activator of nNOS. nNOS is involved in production of NO from L-arginine. NO is an unconventional neurotransmitter and is important in messenger functions during inflammatory responses in the SN and in signaling pathways in striatal neurons. Elevated nNOS activity could contribute to dopaminergic cell death in SN by increasing NO available for peroxynitrite production.

Calpains are cystein proteases. Calpains have been found to cleave several substrates including cytoskeletal components, proteases and cell signaling molecules. Calpain activity is increased in PD patients as well as in the MPTP and Rotenone rodent model.[138,139] Calpain has been involved in the induction of apoptosis through conversion of p35 to p25 (Figures 13.2 and 13.3).[140] p25 causes cdk5 to be constitutively activated and mislocated. Increased p25/cdk5 activity cause degeneration and apoptotic cell death, suggestively mediated by phosphorylation of Prx2, a Prx located in the cytoplasm of neurons.[141] Calpain has shown to cleave α-synuclein *in vitro*, which differentially affects α-synuclein aggregation because calpain cleavage of α-synuclein monomers prevents further oligomerization, while cleavage of α-synuclein fibrils promotes further co-assembly.[142]

PKC is an important initiator kinase in neurodegeneration. PKC is a family of serine/threonine kinases divided into four groups: the conventional, the novel PKCs, the atypical PKC and a PKN subfamily. The conventional PKCs comprise PKCα, PKCβ and PKCγ, which are activated by a combination of diacylglycerol (DAG) and phospholipid, and Ca^{2+}-dependent phospholipid binding (Figures 13.2 and 13.3). The novel PKCs include PKCδ, PKCε, PKCθ and PKCη and differ in that they do not respond to Ca^{2+}. The atypical PKC and the PKN subfamily do not depend on Ca^{2+} or DAG for activation but are allosterically activated.[143] Given their activation under elevated Ca^{2+} levels,

conventional PKC have been activated in MPP^+ toxicity and upon glutamatergic stimulation (Figure 13.3).[144] The activation of PKC requires the function of phospholipase C (PLC), which is also activated by Ca^{2+}. PLC is a membrane-bound enzyme that produces DAG from hydrolysis of phosphatidyl inositol-bisphosphate (PIP_2). PIP_2, Ca^{2+} and DAG activate PKC and induce its recruitment to the plasma membrane. Importantly, PKC can be activated depending on its spatial and temporal localization, as the binding partners needed for activation are also spatial and temporal specific.[145]

13.3.2.2 Small GTPases

Typically, small GTPases such as Ras and Rap transduce an extracellular signal into intracellular pathway activation. These small GTPases are activated by guanine nucleotide exchange factors (GEF), which put on guanosine triphosphate (GTP) and take away a guanosine diphosphate (GDP). GEF are directly activated by the second messenger cAMP, which is in turn provided by the activation of G proteins. The compartmentalization and controlled activation of small GTPases are crucial and controlled by ubiquitination. Ras and Rap activation determines downstream MAPK cascades. Ras activation induces Raf activation and the PI3K pathway activation. Blocking of Ras activation protects nigral cells from MPTP-induced death.[146] Raf protein has been found to be caspase-3 degraded as a result of apoptotic induction mediated by MPTP.[147] Described first as an oncongene, DJ1 was shown to act cooperatively with Ras and c-myc inducing tumoral cell transformation in response to growth factors,[148] but the significance of these findings to PD is uncertain.

13.3.3 Intracellular Signaling Cascades

As was mentioned earlier, different initiators activate intracellular signaling pathways involved in both cell toxicity and protection in dopaminergic neuronal cells.[149,150]

13.3.3.1 Mitogen Activated Protein Kinases (MAPK) Pathway

The MAPK pathway is activated by a protein kinase cascade. A first upstream kinase, MAPKKK, is phosphorylated by small GTPases or PKC. Raf and MEKK1-5 are specific MAPKKKs involved in PD (Figures 13.2 and 13.3).[151] MAPKKKs in turn phosphorylate the MAPKKs MEK 1/2, MKK4/7 and MKK3/6, followed by phosphorylation of the last kinases, MAPKs: the extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38. Activation of these three MAPKs determines different intracellular signaling pathways and has been implicated in selective induction of dopaminergic neuronal apoptosis in PD.[152,153] Two of these pathways are ROS-dependent whereas the third one (p38) is not. It has been proposed that induction of the three pro-apoptotic pathways is required in order to drive the

cells to cell death. In the 6OHDA model, ERK, JNK and p38 activation is necessary for inducing apoptosis because blocking either of them is sufficient to keep the cells alive. Additionally, the three cascade activator kinases are found phosphorylated after exposure of mesencephalic cultures to glutamate.[126] In contrast, dopaminergic neurons also activate two intracellular pathways that compete with cell death pathways including ERK[154] and phosphoinositide 3-kinase (PI3K)/Akt,[155,156] thus promoting cell protection and neuronal survival.[157]

The role of ERK pathway has been found to be highly dependent on the experimental paradigm tested. In some cases its activation has a protective role,[158,159] while in other cases it mediates neurotoxicity.[160–162] ERK pathway has been found to be activated in response to neurotrophic factors stimulating the activity and/or expression of anti-apoptotic proteins, including BCL2,[163] and the transcription factor cyclic AMP responsive element-binding protein (CREB, Figures 13.2 and 13.3).[164] In support of a neuroprotective role for ERK pathway activation in PD, ERK phosphorylation is reduced in SH-SY5Y cells after treatment with MPP^+.[147] In contrast, a neurotoxic effect is also supported by the findings that ERK and also p38 pathway are found to be highly activated after exposure of mesencephalic cultures to glutamate[126] and that ERK phosphorylation status is required for regulating the splicing of α-synuclein[14] (Figure 13.2). Moreover, some studies have shown no relation of ERK pathway activation with PD.[165]

JNK is one of the molecules reportedly activated by oxidative stress,[166,167] and it is considered an essential molecule in neurodegeneration.[168] JNK and downstream c-jun are activated by Ire α/β in response to ER stress resulting in ER-specific apoptosis.[169] The JNK activator MKK7 (Erk kinase kinase-1/MAPK kinase 4 and MAPK kinase 7) is phosphorylated by the intracellular serine/threonine kinase MLK3 (ASK1 and mixed-lineage kinase 3), and is therefore considered to be a mitogen-activated protein kinase kinase kinase (MAPKKK).[170] JNK1 and JNK2 isoforms have a broad tissue distribution, while JNK3 is predominantly found in brain and is specifically related to neuronal death.[171] JNK, and particularly JNK3, have shown to increase and activate its target c-jun after 6-OHDA treatment.[172,173] MPP^+ neurotoxicity is dependent upon JNK and c-jun activation.[173] JNK activation is involved in apoptosis induced *in vitro* by several oxidants.[79,175–177] Also, JNK is involved in dopamine-induced neuronal death in the striatum[166] and hydrogen peroxide-induced toxicity of primary cortical neurons.[178] JNK is found to be activated after oxidative stress in *in vivo* experimental models[179] and in PD patients.[180] c-jun activates AP-1 causing elevated genetic expression of Fas ligand (FasL)[181] and cycloxigenase 2 (COX2), which are proposed as final mediators in activation of JNK by MPTP.[174] In the 6-OHDA model, JNK activates the NFκB cascade[182] and JNK2 traslocates to the mitochondia and phosphorylates 14-3-3 protein, which facilitates translocation of Bax to the mitochondria where it generates cytochrome c release[79] (Figure 13.1). JNK is also phosphorylated, as well as BH3-only members of the Bcl-2 family, *via* $P75^{NTR}$ in an alternate pro-apoptotic function of neurotrophic factors.[183]

p38 has been reported to be activated in oxidant-induced apoptosis[184] and, conversely, not involved oxidant-induced toxicity.[185] After treatment with 6-OHDA, activation of p38 and its direct target MAPK-activated protein kinase 2 (MK2) (MAPKAPK-2)[186] are maintained over time in both dopaminergic and non-dopaminergic cells.[172] Additionally, p38 has been shown to mediate neuronal cell death in *in vivo* experimental models of other neurodegenerative diseases.[187,188]

13.3.3.2 PI3K/Akt Pathway

PI3K/Akt pathway is initiated by the binding of neurotrophic factors or hormones to Trk receptors inducing the phosphorylation of tyrosine residues and activation of adaptor proteins (Figures 13.2 and 13.3). At the plasma membrane, phosphatidylinositol 3-kinases (PI3Ks) are activated by Ras and phosphorylate phosphatidylinositol lipids turning them into binding sites for signaling proteins including Akt (v-akt murine thymoma viral oncogene homologue or protein kinase B) and PDK1 (phosphoinositide-dependent kinase 1). The proximity of Akt and PDK1 at the membrane facilitates phosphorylation of Akt by PDK1.[189] Akt is a serine-threonine protein kinase that is found to be activated in pro-survival intracellular signaling pathways in neuronal cells.[190–193] PI3K/Akt pathway has been shown to mediate neuronal survival in multiple paradigms including resistance against oxidative insults in the brain.[194–196] However, 6-OHDA treatment decreases Akt phosphorylation, which is not affected by antioxidant treatment indicating that Akt pathway is not directly activated by oxidative stress.[172] Pro-survival effects of PI3K/Akt pathway activation are the result of inhibition of the apoptotic activities of Forkhead[197] and BCL2 (b-cell leukemia/lymphoma 2)-associated death protein (Bad).[198] PI3K/Akt pathway associated to Ras activation is activated by rasagiline, a selective inhibitor of MAO-B, in the MPTP model, emphasizing the importance of this pathway as a therapeutic target.[199] An effect of the PD-related proteins, PINK1, DJ1 and parkin, has been suggested but remains to be defined. The tumor suppressor, exogenous phosphatase and tensin homolog (PTEN), has been proposed to be a regulator of the PI3K/Akt pathway.[200] Although PINK1 expression is induced by PTEN in cancer cells, PINK1 function in PI3K pathway is unknown. Similarly, DJ1 promotion effects on the PI3K/Akt pathway by inhibition of PTEN have been shown in cancer cells but not in dopaminergic cells. Using a proteasome-independent mechanism, parkin regulates epidermal growth factor receptor (EGFR) endocytosis and EGF-induced Akt signaling by ubiquitination of Eps15, a phosphorylation target of EGFR that functions as an endocytic accessory protein.[201]

13.3.3.3 NFκB Signaling Cascade

NFκB is a nuclear transcription factor that in its inactive form is located in the cytosol bound to IκB. NFκB activation is induced by the IκB kinase (IKK) complex consisting of two catalytic (IKKα and IKKβ) and one regulatory

subunit (IKKγ/NEMO).[202] Degradation of IκB results in translocation of NFκB to the nucleus, where it induces gene expression. NFκB pathway activation is related to anti-apoptotic and cell survival effects.[203] NFκB pathway is activated during MPP^+ toxicity followed by p53 activation and caspase 3 activation as well as increased expression of the antioxidant defenses Mn-SOD[147] and nNOS.[204] In PD patients, nuclear translocation of NFκB is greater and NFκB pathway has been proposed to be an oxidative stress-mediated apoptosis signal as it has been found to be inhibited by HNE.[205] Parkin has been shown to interact with and promote degradation-independent ubiquitination of the upstream IKK complex regulators: IKKγ/NEMO and tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2).[206]

13.3.3.4 Unfolded Protein Response (UPR)

This multi-dimensional signaling cascade is activated in response to accumulation of misfolded proteins and ER stress. UPR comprises a series of precisely intertwined pathways that cells activate independently,[85] beginning with three main pathways activating Ire α/β, PERK and ATF6.[207] Ire α/β activation induces caspase 12 and JNK pathway activation.[208] PERK activates eIF2α and results in inhibition of translation and activation of ATF4, subsequently causing activation of the encoding of the C/EBP homologous protein-10 (CHOP). CHOP is a nuclear protein that is tightly regulated by stress and forms stable heterodimers with C/EBP family members capable of recognizing novel DNA target sequences.[122] CHOP enhances cell death *via* mechanisms that include downregulation of Bcl-2 expression.[209] Bcl-2 protein is found to be increased in PD patients, involving UPR-induced apoptosis.[210,211] The third UPR pathway activator, ATF6, induces the expression of CHOP, Xbp1, chaperones and proteins involved in protein folding, trafficking and degradation.[169] In response to 6-OHDA, the three pathways are activated resulting in the dual over-expression of CHOP by PERK and ATF6. Conversely, during MPP^+ exposure, PERK pathway is activated singularly and to a lower magnitude than when activated by 6-OHDA exposure.[121]

13.3.4 Potentially Involved Intracellular Signaling Components

There is little evidence supporting the role of some other intracellular signaling components; however, because they are relevant in the context of PD a brief description will be discussed.

13.3.4.1 AMP-Activated Protein Kinase (AMPK)

AMPK is an important regulator in adult cells supporting a high metabolism, such as skeletal muscle and myocardium and in development. AMPK is activated by kinases such as CaMK. AMPK activation is regulated by AMP/ATP ratio, Ca^{2+} concentration and ROS (Figure 13.1). AMPK can potentially be

involved in PD given that in other tissues AMPK is activated in response to energy imbalance and genotoxic stress.[74]

13.3.4.2 Leucine-Rich Repeat Kinase 2 (LRRK2)

LRRK2 is a promising initiator of intracellular signaling pathways. LRRK2 has a kinase domain that may activate cell death pathways and contribute to protein aggregation[29] (Figure 13.2). Also, LRRK2 has a Roc (Ras of complex proteins) that is a GTPase domain with high sequence similarity to Ras and other related small GTPases.[212] The phosphorylation state of LRRK2 is proportional to its toxic effects and LRRK2 autophosphorylates its own ROC as well as its kinase domains.[213] Moesin, Ezrin and Radixin have been found to be substrates for LRRK2 phosphorylation; however, their relevance in PD remains to be elucidated.[214] PARK8 mutations are found in a high proportion of sporadic cases of PD and in some familial cases, which likely supports a connection between genetic and environmental factors. Loss of function of LRRK2 has been implicated in the pathogenesis of PD as a result of decreased expression of its gene in nigral neuronal processes in PD brains.[215]

13.3.4.3 PINK1

PINK1 has a kinase domain that functions in auto-phosphorylation and phosphorylation of other substrates,[53] such as HtrA2, a protein that is released from the mitochondria into the cytosol after permeabilization of the mitochondrial membrane and binds to inhibitors of apoptosis[109] (Figure 13.1). PINK1 may phosphorylate mitochondrial proteins in response to stress and protect against mitochondrial dysfunction.[216] PINK1 phosphorylates the mitochondrial chaperone TRAP1/heat shock protein 75 (Hsp75), which prevents cytochrome c release and apoptosis (Figure 13.1).[217]

13.3.5 Effector Pathways and Final Effects

Dopaminergic neurons in the SN respond to the damage caused by exogenous agents, toxic intracellular environment or the lack of neurotrophic support by activating signaling cascades that lead to apoptosis or cell survival. In the chronic course of PD, such sustained response may show to be defective or successful. Cell death, most likely apoptotic, is the final outcome leading to PD and reflecting a defective response against the damage; indeed, a classification has been suggested for the pathogenic mechanisms leading to cell death in PD according to their likelihood of inducing cell death.[209] Nevertheless, no more than 50% of the dopaminergic cell population in the SN die as a result of PD,[218] which indicates that, at least temporarily, dopaminergic neuronal cells survive by developing adaptive responses. With regard to pro-apoptotic pathways, it is noteworthy to say that the flow of signaling is not unidirectional as multiple signaling pathways may be activated at the same time and also may have modulator functions on each other. For apoptosis to be inevitable,

however, activation of JNK, p53, Rb, cdk-5 or pro-apoptotic members of the Bcl-2 family is almost always required.

13.3.5.1 Inflammatory Response

Inflammation could be a final result or a contributing factor in PD. Different glia populations may participate in the pathologic process. Reactive gliosis is found in the surrounding tissue in the SN in PD. Activated microglia, found in these sites, releases proinflammatory factors such as interleukin-1b, interleukin-6 and tumor necrosis factor-α (TNFα), which are involved in generation of oxidative stress and neurotoxicity[219] as well as correlated to upregulation of inducible NOS (iNOS).[36] Microglial-related inflammatory events have shown to be important in MPTP neurotoxicity, whereas ablation of iNOS attenuates toxicity.[220,221] An inverse correlation is described between astrocytic reactivity and dopaminergic cell loss. Astrocytes surrounding surviving dopaminergic neurons in PD express high levels of GPx[222] while levels of this enzyme are reduced in homogenates of SN in PD.[43] Additionally, PD-related proteins may have a role in inflammation. It has been shown that over-expression of α-synuclein can facilitate cell migration and recruitment of microglia to the site of gliosis,[223] whereas extracellular α-synuclein induces glial cells to an inflammatory state by activation of all three MAPK pathways ERK, JNK and p38.[224] DJ1 expression is predominant in glial cells in PD brains.[225] COX-2, the rate-limiting enzyme in prostaglandin E2 (a pyretic agent) synthesis, is upregulated in dopaminergic cells in PD and in the MPTP model through a JNK/c-Jun-dependent mechanism.[226]

13.3.5.2 Dopamine Metabolism

Dopamine production is increased by the phosphorylation of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, thus enhancing its enzymatic activity.[227] A number of studies have suggested that CaMKII, ERK and protein kinase A (PKA) are responsible for the phosphorylation of Ser19, Ser31 and Ser40, respectively.[228] ERK, specifically ERK1/2 members, phosphorylates TH at Ser31 while JNK and p38 pathways have no effect on either glutamate-induced phosphorylation of ERK or TH. After exposure to glutamate, phosphorylation of TH at Ser19 and 31 promptly increases and elicits the release of dopamine not at all changing dopamine amounts intracellularly. The phosphorylation at Ser19 has no direct effect on TH activity, but can potentiate the phosphorylation at Ser40 and subsequent activation of TH.[228] Lindgren *et al.*[229] have suggested that NMDA decreases cyclic AMP production, counteracting the phosphorylation at Ser40 by PKA. The phosphorylation at Ser31 directly results in approximate two-fold activation of TH, but to a lesser extent than that at Ser40.[230] In glutamate-stimulated neurons, blocking p38 and JNK pathways and inhibiting TH activity are additive and fully prevent the dopaminergic neurotoxicity.[7] TH is also a target for α-synuclein. α-synuclein

has the ability to bind and inhibit TH phosphorylation and activity, resulting in a decrease of dopamine synthesis.[231]

13.3.5.3 Cell Cycle

Re-entry of post-mitotic neurons to the cell cycle has been proposed as the cause of cell death in several acute or chronic neurodegenerative disorders,[232] including PD. Cell cycle progression is regulated by cyclins, which bind to and activate specific serine-threonine kinases called cyclin-dependent kinases (cdk). Cyclins are important regulators of the cell cycle and have been shown to inhibit the start of DNA replication. p21 allows cyclin-cdk complexes to phosphorylate their target proteins.[233] Importantly, cyclin D1 and p21 are increased and arrest the cell cycle in post-mitotic cells[173] and in neuron-like PC12 cells.[234] Intracellular redox states can regulate cell cycle progression.[235] Both cyclin D1 and p21 have been found to decrease after exposure to intracellular oxidants[236–238] although several antioxidants and inhibitors of cell proliferation only partially prevent apoptosis.[239]

In addition to changes in cyclin and p21, cdk5 and retinoblastoma protein (pRb) have an important role in cell cycle regulation. cdk5 is a cyclin-dependent kinase predominantly associated with post-mitotic neurons. cdk5 expression and activity are found to be increased in PD patients, specifically in LBs.[165] pRb is a specific target of cyclin D1-cdk complexes during G1/S transition. Rb hyperphosphorylation (P-pRb) decreases pRb-E2F binding, allowing E2F to trigger transcription of S phase-related genes such as the PCNA, a nuclear protein involved in DNA replication,[240] and avoids the repression of apoptosis-related genes silenced by such complexes[241] (Figure 13.2). PD patients have increased expression of the P-pRb in the nucleus of dopaminergic neurons of the SN and P-pRb staining also co-localizes with LBs.[242] In the MPTP model, cdk5 has been found to be increased whereas the cyclin D1/cdk4/6/Rb pathway is not induced.[243]

13.3.5.4 Autophagy

Autophagy involves degradation of cell components by the lysosomes and can be done in three ways: macroautophagy, chaperone-mediated and microautophagy. Macroautophagy involves degradation of large debris and organelles, such as mitochondria (mitophagy). Chaperone-mediated autophagy involves direct lysosomal targeting and degradation of proteins that have a specific target sequence. Microautophagy is poorly understood and involves uptake of nutrients into lysosomes. Macroautophagy and chaperone-mediated autophagy are related to PD pathogenic effects.[209] Mitophagy is increased in PINK1-deficient cells[244] while chaperone-mediated autophagy is involved in α-synuclein altered turn-over.[245] In physiological conditions a cell induces autophagy in response to low levels of protein intake. In PD, autophagy has been found to be activated in response to abnormal intracellular protein accumulation and induces cell death. As protein degradation systems are interlinked to autophagy, there is a proposed inter-play between ER stress

and autophagy. Initiators of autophagy in PD include the NFκB pathway, Ca^{2+}-dependent and redox sensitive pathways. Induction of autophagy by MPP^+ or 6OHDA is mediated by ERK2[114] and LRRK2-induced autophagy can be blocked by MEK inhibitor.[112]

13.3.5.5 Apoptosis

Most of the evidence about dopaminergic cell death supports apoptosis as the primary mechanism of death involved in neurodegeneration. Apoptosis is an active process that takes place in response to severe cell damage. Apoptosis initiation uses either extrinsic (receptor-mediated) or intrinsic (mitochondrially mediated) pathways. Intrinsic apoptotic pathway involves *de novo* protein expression of Bcl2 family member proteins. Extrinsic pathway involves ligand-mediated death receptor multimerization, adaptor proteins such as FAD and autoproteolysis of caspases 8 and 10.[246] Apoptosis is controlled by changes in the levels of anti-apoptotic and pro-apoptotic factors such as Bcl2, caspases and Apaf-1.[247] Oxidative stress may induce changes in anti-apoptotic and pro-apoptotic levels leading to apoptosis. For instance, caspase 3, an execution-phase caspase, is activated by superoxide anion and NO.[248] The specific mechanism for caspase 3 by oxidative stress involves neither the intrinsic nor the extrinsic apoptosis pathways, since 6-OHDA exposure does not affect BH3-only family proteins levels[249] and over-expression of Bcl-2 as well as deletion of Bax did not protect against 6-OHDA-induced cell death.[121,250,251] Similarly, 6-OHDA-induced apoptosis requires protein synthesis and does not involve death-inducing ligands, death receptors (Fas, TNR-R1, TNF-related apoptosis-inducing ligand receptor I and II) or Fas-associated death domain. Taken together, the findings on the 6OHDA model suggest that apoptosis is mediated by a different mechanisms, most likely by ER stress-pathway requiring caspase 12 and 9.[252] Apoptosis is also induced by the lack of pro-survival signaling and prevented by UPR. Specifically, the decrease of PI3K/Akt signaling pathway activation allows Bad to bind to Bcl-2 family members (*i.e.* Bcl-2) preventing them from mediating cell survival responses. Bcl-2 expression is increased by UPR, which favors cell survival. DJ1 has been shown to bind to Daxx sequestering it in the nucleus and preventing its interaction and activation of ASK1. Daxx is an adaptor protein of the intracellular death receptor Fas. When activated, Daxx in not bound to DJ1 and activates Fas as well as ASK1.[253] Activated ASK1 dimerizes and signals through p38 and JNK. Although cell studies have shown apoptotic cell death in PD models, it remains controversial whether apoptosis is involved *in vivo* and in PD cases. In tissue culture studies, MPP^+ does not induce apoptosis.[254,255]

13.4 Conclusions

Intracellular signaling in PD has been shown to be important for identifying a missing link between insults to dopaminergic neurons in the SN and their

selective death. Studies in this area have contributed to the knowledge of the possible pathways involved in this process. Studies in PD patients provide an approach to involved signaling processes with a disadvantage of having limited intervention to the disease. Animal, cell and toxicant-based models allow testing of interventions but they do not fit the complete disease phenotype. In spite of limitations of the experimental approaches, components of the intracellular signaling pathways in PD patients and animal models have been shown to be consistent. Toxicant-induced model systems have provided complementary information about intracellular signaling and selective dopaminergic neuronal death. However, some components found in toxicant-induced models are not found to be involved in PD patients or animal models.

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