Irina Obrosova
Martin J. Stevens
Mark A. Yorek *Editors*

Studies in Diabetes

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
Donald Armstrong

Note from the Editor-in-Chief

All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

Donald Armstrong
Editor-in-Chief

Irina Obrosova • Martin J. Stevens
Mark A. Yorek

Editors

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Editors
Irina Obrosova (deceased)
Louisiana State University
Pennington Biomedical Research Center
Baton Rouge, LA, USA

Mark A. Yorek
Veterans Affairs Medical Center
University of Iowa
Iowa City, IA, USA

Martin J. Stevens
Centre for Endocrinology and Metabolism
School of Clinical and Experimental
   Medicine
University of Birmingham
Birmingham, UK

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Professor Irina Obrosova

A native of Ivanovo, Ukraine, Professor Irina Obrosova was born on 26 November 1956. After attending Central High School in Kiev, she joined Kiev State University to study Biology and qualified with the Highest Honors in Biology in 1979. She went on to do a higher degree in Biochemistry at Kiev State University and was awarded a Ph.D. in 1985. Following a couple of years as a

Junior Research Scientist, in 1987 she was appointed Senior Research Scientist in the Department of Diabetology, Institute of Endocrinology and Metabolism, Kiev. Her exceptional scientific qualities led her promotion to the position of Leading Research Scientist, Department of Diabetology, Institute of Endocrinology and Metabolism between 1991 and 1993.

Many of us first came to know Professor Irina Obrosova in 1991, when she gave an impressive oral presentation related to diabetic neuropathy at the European Association for the Study of Diabetes (EASD) meeting in Dublin, Ireland. Irina relocated to the USA in 1993 and had numerous interactions with peers at American and international meetings, including the Annual Meeting of the NEURODIAB (Diabetic Neuropathy Study Group of the EASD). Irina was an extremely bright and talented individual who has made an important contribution to the diabetes complications field. Her research on pathogenetic mechanisms of diabetic neuropathy has been excellent, and her findings have been reproduced by many leading investigators.

Irina moved to the University of Michigan in 1996, where she became a renowned expert in diabetic neuropathy and was soon promoted to a research faculty position. In Michigan, she conducted seminal studies demonstrating a key role for aldose reductase and oxidative stress in diabetic neuropathy, cataract formation as well as early diabetic retinopathy. Her excellent research and communication skills enabled her to obtain several research grants

including a career development award from the National Institute of Health/National Institute of Diabetes and Digestive Kidney Diseases (NIH/NIDDK). Recognition of her skills led her to secure a tenured position as an Associate Professor at Pennington Biomedical Research Center (PBRC). Further development of her career at PBRC and promotion to full professorship was a clear illustration of what can be achieved by a talented and dedicated researcher in an excellent scientific environment and with institutional support. While being at PBRC, Irina discovered several important mechanisms of diabetic neuropathy including nitrosative stress, PARP activation, and more recently, activations of 12/15-lipoxygenase and Na+/H+-exchanger-1. She also contributed important data describing a key role for PARP activation in diabetic cataract, nephropathy, and early retinopathy.

In her career, Irina received grant support from both federal and private organizations, including a research grant from Juvenile Diabetes Research Foundation International (JDRF), two research grants from the American Diabetes Association, two R21 grants, and, more recently, two RO1 grants from the NIH to study the role of Na+/H+-exchanger-1 in diabetic neuropathy and peroxynitrite as a clinical marker for progression of diabetic neuropathy. She has published many high quality papers in prestigious journals including Diabetes, the Federation of American Societies for Experimental Biology Journal, Diabetologia, and others. She has presented her work at national and international meetings and has

given invited lectures at the annual meetings of American Diabetes Association, EASD, Japan Diabetes Society, American Association of Vision and Eye Research, European Association for Vision and Eye Research, as well as invited seminars at several leading universities. She has also chaired many oral and poster sessions at international meetings, a clear testament for her international reputation in diabetes research. She was also a chartered member of the NIH Clinical Neuroplasticity and Neurotransmitters study section that reviews neuropathy-related grants, as well as a member of the ADA and Juvenile Diabetes Research Foundation grant review panels. She has reviewed grants for special emphasis panels at the NIH, as well as program project grants for the European Union and grants for American Institute of Biological Sciences, and several other associations.

She served as a reviewer for many leading journals including Nature Neuroscience, Nature Protocol, Diabetes, FASEB Journal, and others. In short, Professor Obrosova had an excellent reputation in diabetes complications research and as a result was highly respected by her peers in the diabetes research.

Irina was an outstanding scientist with an international reputation who had a clear evidence of an exceptional scholarly career by numerous, high quality scientific publications. She was highly articulate and unafraid to speak her mind, something that will be truly missed by her colleagues at future research meetings. She had a formidable intellect and could see through flaws in scientific methodology. At a personal level

she was a very loyal and supportive friend. She loved traveling around the world with her nephew Oleksandr and enthusiastically shared her experiences with her friends by bringing back treasures she had purchased, including novel paintings and jewelry. Unfortunately she was diagnosed with pancreatic cancer in early 2012 and passed away on 4 December 2012. Throughout her seven-month battle with cancer, she remained devoted to her work and was concerned about completing the projects she had going and for the people working for her. Her friends and colleagues were extremely sad at her untimely death, as she had so much more to offer. A highly intelligent, passionate, hard working and talented scientist, Irina will be greatly missed by her many friends in NEURODIAB and other institutions. Her strength of character in the face of adversity is an example to all of us. Her achievements in the field of diabetic complications will continue to inspire future young scientists. Stanley Arnold once remarked, "The greatest thing about life is to spend it for something that will outlast it." We think you will agree that Irina's achievements will stand the test of time. The Diabetes Complications field will be a poorer place without outstanding scientists like Irina.

Sheffield, UK   Solomon Tesfaye
Iowa City, IA, USA   Mark A. Yorek
Birmingham, UK   Martin J. Stevens

Contents

Contributors

Asad Ali, M.D., M.R.C.P. Department of Respiratory Medicine, University Hospital of Coventry and Warwickshire, Coventry, UK

Denise M. Arrick, B.S. Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center-Shreveport, School of Medicine in Shreveport, Shreveport, LA, USA

Trevor Askwith, Ph.D. Translational Drug Discovery Group, School of Life Sciences, University of Sussex, Brighton, UK

Jaya P. Buddenini, M.D. Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Kunal Chaudhary, M.D. Divisions of Clinical Medicine and Nephrology, Harry S. Truman Memorial Veterans' Hospital, Columbia, MO, USA

Triantafyllos Didangelos, M.D., Ph.D. Diabetes Center, First Propeudetic Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, American Hellenic Educational Progressive Association Hospital, Thessaloniki, Greece

John Doupis, M.D., Ph.D. Internal Medicine and Diabetes Department, Salamis Naval Hospital, Salamis, Greece

H. Fadavi, M.D. Division of Cardiovascular Medicine, Central Manchester Foundation Trust & University of Manchester, Manchester, UK

R.A. Malik, M.B., Ch.B., Ph.D., F.R.C.P. Division of Cardiovascular Medicine, Central Manchester Foundation Trust & University of Manchester, Manchester, UK

William G. Mayhan, Ph.D. Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences Center-Shreveport, School of Medicine in Shreveport, Shreveport, LA, USA

Mitali Mehta University of Michigan Medical School, Ann Arbor, MI, USA

Christine L. Oltman, Ph.D. Iowa City Veteran Affairs Health Care System and Cardiovascular Diseases Division, Department of Internal Medicine, University of Iowa, Iowa City, IA, USA

Subramaniam Pennathur, M.D. Division of Nephrology, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Rodica Pop-Busui, M.D., Ph.D. Division of Metabolism Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

Jayadave Shakher, M.D., M.R.C.P., M.B.A. Department of Diabetes and Endocrinology, Heart of England National Health Service Foundation Trust, Birmingham, UK

Anders A. F. Sima, M.D., Ph.D., F.R.C.P.(C). Departments of Pathology and Neurology, Wayne State University School of Medicine and Detroit Medical Center, Detroit, MI, USA

William I. Sivitz, B.S., M.D. Department of Internal Medicine, University of Iowa, Iowa City, IA, USA

James R. Sowers, M.D., F.A.C.E., F.A.C.P., F.A.H.A. Thomas W. and Joan F. Burns Missouri Chair in Diabetes, Director of the MU Diabetes and Cardiovascular Center, Director, Division of Endocrinology, Diabetes Center, University Hospital, Columbia, MO, USA

Martin J. Stevens, M.D., F.R.C.P. Centre for Endocrinology and Metabolism, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, UK

Abd A. Tahrani, M.D., Ph.D. Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham, Birmingham, UK

M. Tavakoli, B.Sc. Optom (Hons), M.Sc., Ph.D., F.B.C.L.A., F.A.A.O. Division of Cardiovascular Medicine, Central Manchester Foundation Trust & University of Manchester, Manchester, UK

Aristidis Veves, D.S.C., M.D., Ph.D. Microcirculation Lab, Beth Israel Deaconess Medical Center, Boston, MA, USA

Adam Whaley-Connell, D.O., M.S.P.H. Harry S. Truman Memorial Veterans Hospital, Columbia, MO, USA

Division of Nephrology and Hypertension, University of Missouri-Columbia School of Medicine, Columbia, MO, USA

Mark A. Yorek Veterans Affairs Medical Center, University of Iowa, Iowa City, IA, USA

Abbreviations

4-HNE	4-Hydroxynonenal
8-epi-PGF$_2$	8-epi-prostaglandin
8-OHdG	8-Hydroxy deoxyguanosine
AASK	African American Study of Kidney Disease and Hypertension
AASM	American Academy of Sleep Medicine
ACE inhibitors	Angiotensin converting enzyme inhibitors
AChE	Acetylcholin esterase
ACL	Acetyl-L-carnitine
ACS	Acute coronary syndromes
AD	Alzheimer's disease
ADMA	Asymmetric dimethylarginine
ADDLs	Amyloid-beta-derived diffusible ligands
AGEs	Advanced glycosylation end products
AHI	Apnea–hypopnea index
AII	Angiotensin II
Akt	Protein kinase B
ALDH	Aldehyde dehydrogenase
AP-1	Activator protein 1
APP	Amyloid precursor protein
AR	Aldose reductase
ARBs	Angiotensin II receptor blockers
ASN	American Society of Nephrology
ATP	Adenosine 5′-triphosphate
BAE	Bovine aortic endothelial
BH$_2$	Dihydrobiopterin
BH$_4$	Tetrahydrobiopterin
BIM	Bisindolylmaleimide
BK	Large conductance calcium channel
BMI	Body mass index

BP	Blood pressure
BREC	Bovine retinal endothelial cells
CAN	Cardiovascular autonomic neuropathy
CAT	Catalase
CCB	Calcium channel blocker
CCCP	m-Chlorophenylhydrazone
CCM	Corneal confocal microscopy
cGMP	$3'5'$-cyclic monophosphate
CHOIR	Correction of hemoglobin and outcomes in renal disease
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
CML	Carboxymethyllysine
CNS	Central nervous system
CoPP	Cobalt protoporphyrin
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
CPAP	Continous positive airway pressure
CREB	Cyclic AMP response element-binding protein
CRP	C reactive protein
CSD	Cysteine sulphinate decarboxylase
CuZnSOD	Copper–zinc superoxide dismutase
CVD	Cardiovascular disease
DAG	Diacylglycerol
DCCT	Diabetes control and complications trial
DCF	$2',7'$-Dichlorodihydrofluorescein diacetate
DHE	Dihydroethidine
DHFR	Dihydrofolate reductase
DHPA	10-Acetyl-3,7-dihydroxyphenoxazine
DMPO	5,5-Dimethyl-l-pyrroline-N-oxide
DN	Diabetic neuropathy
DOQI	Dialysis outcome quality initiative
DPI	Diphenylene iodonium
DRg	Dorsal root ganglion
DSPN	Diabetic sensory-motor polyneuropathy
EC-SOD	Extracellular superoxide dismutase
EDCF	Endothelial derived contracting factors
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelial derived relaxing factors
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
ER	Endoplasmic reticulum
ESAs	Erythropoietin stimulating agents
ESRD	End stage renal disease
ET-1	Endothelin-1
FA	Fatty acid

FATP	Fatty acid transport protein
FFAs	Free fatty acids
FMD	Flow-mediated dilation
GABA	γ-Aminobutyric acid
GC	Gas-chromatography
GCP-2	Granulocyte chemotactic protein-2
GFAP	Glial fibrillary acid protein
GFR	Glomerular filtration rate
GK	Goto-Kakizaki
GLUT4	Glucose transporter 4
Gpx	Glutathione peroxidases
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HED	*meta*-hydroxyephedrine
HIF	Hypoxia-inducible factor
HMGcoA reductase	Hydroxymethylglutaryl coenzyme A reductase
HO-1	Heme oxygenase-1
HOCl	Hypochlorous acid
HOPE	Heart outcomes prevention evaluation study
HPLC	High pressure liquid chromatography
ICAM-1	Intercellular adhesion molecule 1
IDE	Insulin-degrading enzyme
IGF-1	Insulin like growth factor one
IL-6	Interleukin-6
IMT	Intima-media thickness
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate-1
JNC	Joint National Committee
JNK	c-Jun N-terminal kinase
KDEP	Kidney disease education program
KDOQI	Kidney disease outcomes quality initiative
KEEP	Kidney early evaluation program
Kir	Inward rectifier calcium channel
LDL	Low density lipoprotein
LEAAD	Lotrel and Enalapril in African Americans with diabetes
LNAME	L-NG-nitroarginine methyl ester
LVH	Left ventricular hypertrophy
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MDCK	Madin-Derby canine kidney
MDRD	Modification of diet in renal disease
MGO	Methylglyoxal
MI	Myocardial infarction
MMP	Metaloproteinase

MMSE	Mini mental state examination
MnSOD	Manganese superoxide dismutase
MPO	Myeloperoxidas
MRS	Magnetic resonance spectroscopy
NAD	Nicotinamide adenine dinucleotide
NC	Nerve conduction
NDS	Neuropathy disability score
NGF	Nerve growth factor
NHANES	National Health and Nutrition Examination Survey
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIH	National Institutes of Health
NKDEP	National Kidney Disease Educational Program
NKF	National Kidney Foundation
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOD	Non-obese diabetic
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NS	Nitrosative stress
O_2^-	Superoxide
OGTT	Oral glucose tolerance test
OH^-	Hydroxyl radical
OLETF	Otsuka Long-Evans Tokushima Fatty
$ONOO^-$	Peroxynitrite
OS	Oxidative stress
OSA	Obstructive sleep apnoea
PAI-1	Plasminogen activator inhibitor-1
PARPS	Poly(ADP-ribose) polymerases
PBMC	Peripheral blood mononucleocytes
PET	Positron emission tomography
PIV	Pressure induced vasodilation
PKA	Protein kinase A
PKC	Protein kinase C
PREP	Pain related evoked potentials
PUFA	Polyunsaturated fatty acids
QSART	Quantitative sudomotor axon reflex testing
QST	Quantitative sensory testing
RAAS	Renin-angiotensin-aldosterone system
RAGE	Receptor for advanced glycation endproducts
RCS	Reactive chlorine species
RDI	Respiratory disturbance index
RhoA	Rho kinase rMC-1 transformed retinal cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPE	Retinal pigment epithelial

sCr   Serum creatinine
SFN   Small fiber neuropathy
SOD   Superoxide dismutase
SSR   Sympathetic skin response
STZ   Streptozotocin
T1DM   Type 1 diabetes mellitus
T2DM   Type 2 diabetes mellitus
TauT   Taurine transporter
TBARS   Thiobarbituric acid reactive substances
TCA   Tricarboxylic acid
TCAC   Tricarboxylic acid cycle
TMRM   Tetramethylrhodamine methyl ester
TNF-α   Tumor necrosis factor-α
TonE   Tonicity response element
TREAT   Trial to Reduce Cardiovascular Events with Aranesp Therapy
TTFA   Thenoyltrifluoroacetone
UCP1   Uncoupling protein-1
UCP2   Uncoupling protein 2
UCP3   Uncoupling protein-3
UKPDS   United Kingdom Prospective Diabetes Study
USRDS   United States Renal Data System
VACM   Vascular cellular adhesion molecule
VEGF   Vascular endothelial growth factor
vWF   von Willebrand factor

Chapter 1
Oxidative Stress and Diabetes-Induced Vascular Dysfunction: Role in Diabetic Neuropathy

Mark A. Yorek

1.1 Introduction

Oxidative stress is an important component of diabetes and its complications [1–15]. Studies showing that treatment with antioxidants prevents diabetes- and hyperglycemia-induced impairment of endothelium-dependent relaxation suggest that oxidative stress is a major factor in the development of diabetic vascular disease [7, 16–20]. In addition, treatment of streptozotocin-induced diabetic rats with anti-oxidants has demonstrated that oxidative stress and vascular dysfunction may be a major factor in the development of diabetic neuropathy [5, 6, 8, 21–23]. In this chapter I will present past studies from my laboratory that have focused on the effect of streptozotocin-diabetes-induced oxidative stress on vascular reactivity of epineurial arterioles and neural function.

1.2 Diabetes-Induced Oxidative Stress and Vascular Dysfunction in Epineurial Arterioles

My laboratory has for many years focused on the effect of diabetes on vascular and neural dysfunction. Our studies first demonstrated that vascular impairment of epineurial arterioles, blood vessels that provide circulation to the sciatic nerve, and reduced endoneurial blood flow precede neural dysfunction as determined by slowing of motor nerve conduction velocity (see Fig. 1.1 [24]). Our studies demonstrated that one week after the induction of diabetes using streptozotocin, that

M.A. Yorek (✉)
Veterans Affairs Medical Center, University of Iowa,
Iowa City, IA 52246, USA
e-mail: Mark-yorek@uiowa.edu
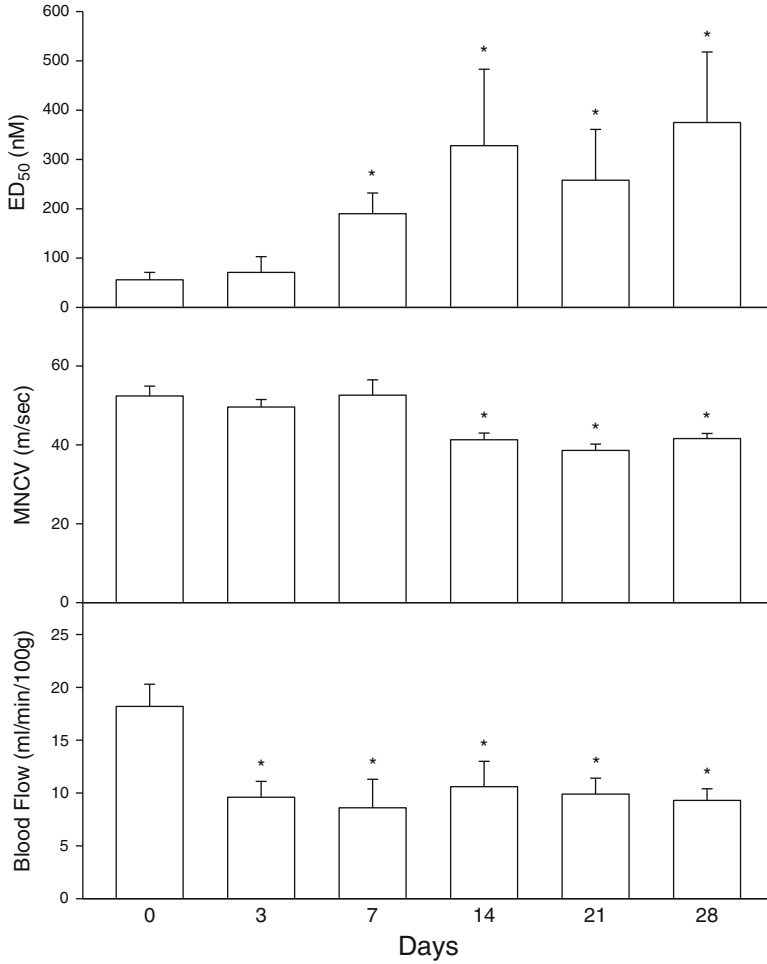
Fig. 1.1 The effect of streptozotocin-diabetes in the development of vascular and neural dysfunction. Vascular reactivity to acetylcholine by epineurial arterioles (section A ED_{50}), endoneurial blood flow (section B), and motor nerve conduction velocity (section C) was examined in control (0) and streptozotocin-induced diabetic rats following 6–30 days of diabetes. Data is presented as the mean ± SEM. *$P < 0.05$ compared to control (0)

vasodilation in response to a low dose of acetylcholine was significantly impaired and after two weeks of diabetes, maximum impairment in acetylcholine-mediated vascular relaxation was observed [24]. During this time period endoneurial blood flow of the sciatic nerve was also reduced. Impairment in motor nerve conduction velocity was not observed until after two weeks of diabetes suggesting that vascular dysfunction may be an early development in diabetes and a major factor contributing to diabetic neuropathy.

Acetylcholine-induced vasodilation of epineurial arterioles is mediated by two mechanisms involving the production of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) [25]. This is important since one mechanism by which superoxide/oxidative stress can cause vascular dysfunction is by quenching the bioactivity of NO (see below). A primary factor contributing to diabetes-/hyperglycemia-induced impairment in vascular relaxation in epineurial arterioles is increased oxidative stress [26, 27]. Oxidative stress occurs when the balance between the production of oxidation products and the ability of antioxidant mechanisms to neutralize these products is shifted in the favor of formation/accumulation of oxidative stress products.

It is widely known that diabetes causes an increase in the production of reactive oxygen species [18, 28–30]. The most common forms are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$) [31]. There are many potential sources for production of these compounds. Superoxide can be produced by the electron transport chain of the mitochondria, NADH oxidase, NAD(P)H oxidase, xanthine oxidase, nitric oxide synthases, cyclooxygenase, lipoxygenase, and cytochrome P-450 [31]. Superoxide can spontaneously acquire an electron to form hydrogen peroxide. Hydrogen peroxide can also be formed from superoxide via superoxide dismutase (SOD), of which there are three isoforms: manganese (Mn)-SOD, which is located in the mitochondria, and two isoforms of copper and zinc (Cu, Zn)-SOD, which are located in the cytosol or extracellularly, respectively [31]. Hydrogen peroxide can be converted to water by the action of catalase or by glutathione peroxidase in the presence of reduced glutathione [31]. However, in the presence of trace metals such as iron (Fe), hydrogen peroxide can form OH^- via a process known as the Fenton reaction [31]. The formation of peroxynitrite is also important pathologically and occurs by the reaction of O_2^- and NO [30, 31]. We have demonstrated that superoxide and peroxynitrite, as indicated by the presence of nitrotyrosine staining, formation is increased in epineurial arterioles from diabetic rats (see Fig. 1.2 [26, 27]).

In a hallmark study Brownlee et al. [32, 33] presented a unifying hypothesis that increased production of superoxide by the mitochondrial chain is a causal link between elevated glucose and three of the main biochemical pathways (glucose-induced activation of protein kinase C, increased formation of glucose-derived advanced glycation end products, and increased glucose flux through the aldose reductase pathway) responsible for diabetes/hyperglycemia complications [32, 33]. Our studies have indicated that in epineurial arterioles from diabetic rats, the increased formation of superoxide seems to be primarily derived from the mitochondria [34]. We had previously demonstrated that reducing superoxide formation and oxidative stress in diabetic rats by treatment with several different types of antioxidants improved vasodilation by acetylcholine in epineurial arterioles of the sciatic nerve [26, 27]. In studies designed to investigate the source of superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats, we demonstrated that antioxidants were capable of preventing superoxide formation and
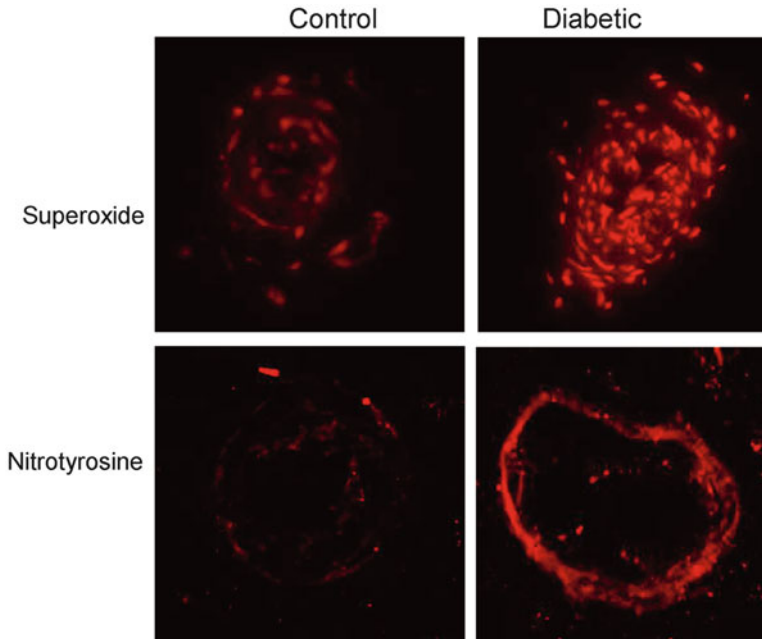
Fig. 1.2 Representative images for superoxide and nitrotyrosine staining in epineurial arterioles from control and streptozotocin-induced diabetic rats. Duration of diabetes was 8 weeks

reversing diabetes-induced vascular impairment in vitro. Dihydrolipoic acid and to a lesser extent α-lipoic acid were effective in decreasing superoxide formation and restoring acetylcholine-mediated vasodilation to arterioles from diabetic rats. α-Lipoic acid is capable of scavenging hydroxyl radicals, hypochlorous acid, and singlet oxygen, but not superoxide or peroxyl radicals [35, 36]. α-Lipoic acid is also effective at chelating transition metals. In contrast, in its reduced form as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation [35, 36].

In vivo α-lipoic acid can be converted into dihydrolipoic acid [35]. In addition, both α-lipoic acid and dihydrolipoic acid can regenerate other cellular antioxidants including dehydroascorbate, ubiquinol, oxidized glutathione, and, indirectly, the tocopherols [35]. The combination of these properties was likely responsible for the effectiveness of α-lipoic acid and dihydrolipoic acid in decreasing superoxide formation [34]. Tempol, a superoxide dismutase mimetic, also reversed the diabetes-induced impairment of acetylcholine-mediated vasodilation and increased superoxide formation in epineurial arterioles [37]. This is in agreement with other studies, which demonstrated that tempol or M40403, another superoxide dismutase mimetic, restores diabetes-induced endothelial dysfunction [27, 37, 38]. The decrease in superoxide formation by α-lipoic acid, dihydrolipoic acid, or tempol

and the reversal of the diabetes-induced impairment in vasodilation suggest that the increased formation of superoxide and perhaps scavenging of nitric oxide is responsible in part for the reduced vascular response to acetylcholine in epineurial arterioles from diabetic rats. This is supported by our previous studies demonstrating the formation of superoxide and/or peroxynitrite by epineurial arterioles of the sciatic nerve from diabetic rats causes vascular dysfunction that is prevented with treatment by antioxidants in vivo [26, 27]. This was further supported by studies demonstrating that pretreatment with L-arginine in vitro improved acetylcholine-mediated vasodilation in epineurial arterioles from diabetic rats without decreasing the formation of superoxide by these vessels. Acute pretreatment with L-arginine of vessels from diabetic rats as well as L-arginine treatment of diabetic animal models and humans has led to the suggestion that reduced availability of nitric oxide during periods of hyperglycemia may be responsible for impaired vascular relaxation [39–43]. This may be due to a limitation in arginine as a substrate for nitric oxide synthase in diabetes or an increase in scavenging of nitric oxide by superoxide [26, 27, 44]. Our studies would support the latter conclusion. We have demonstrated increased superoxide and peroxynitrite formation in epineurial arterioles of diabetic rats and impairment in endothelium-dependent vascular relaxation that is prevented by antioxidant treatment [26, 27].

In studies to investigate the possible sources of superoxide formation in epineurial arterioles of diabetic rats, we found that increased formation of superoxide by epineurial arterioles was attenuated by preincubation with rotenone but not *m-chlorophenylhydrazone* (CCCP) or thenoyltrifluoroacetone (TTFA) [34]. Rotenone is an inhibitor of complex I of the mitochondrial electron transport chain, TTFA is an inhibitor of complex II, and CCCP is an uncoupler of oxidative phosphorylation. We are unsure why CCCP was less effective than rotenone in reducing superoxide formation by epineurial arterioles of the sciatic nerve of diabetic rats. It is possible that CCCP did not penetrate the vascular wall under the incubation conditions. Nonetheless, this study implicated complex I of the mitochondrial electron transport chain in the production of superoxide by epineurial arterioles of the sciatic nerve of the diabetic rat. In our studies increased formation of superoxide by epineurial arterioles from diabetic rats was also partially decreased by diphenylene iodonium (DPI). DPI has been used for many years as a NAD(P)H oxidase inhibitor [45]. Therefore, our studies at first would suggest that NAD(P)H oxidase may also be a source for the production of superoxide by epineurial arterioles of the diabetic rat. However, Li and Trush have reported in studies with monocytes that DPI at concentrations that inhibit NAD(P)H oxidase diminished the production of superoxide by mitochondrial respiration [46]. They found that DPI was as potent as rotenone in inhibiting the production of superoxide by the mitochondria, likely by complex I. If the studies by Li and Trush are correct, we cannot unequivocally state that NAD(P)H oxidase is a source of superoxide formation by epineurial arterioles of the sciatic nerve.

1.3   Neural Dysfunction

In two separate studies we examined the effect of treating streptozotocin-diabetic rats with α-lipoic acid or M40403 on vascular dysfunction, endoneurial blood flow, and nerve activity, as determined by measuring motor nerve conduction velocity [26, 27]. These studies demonstrated that treating diabetic rats using a prevention protocol with α-lipoic acid or M40403 prevented the diabetes-induced decrease in motor nerve conduction velocity and endoneurial blood flow (Fig. 1.3) and impairment of acetylcholine-mediated vascular relaxation by epineurial arterioles (Fig. 1.4). These treatments generally improved markers of oxidative stress including serum thiobarbituric acid reactive substance and superoxide and nitrotyrosine staining of epineurial arterioles [26, 27]. These studies imply that diabetes causes the increased production of superoxide and peroxynitrite in neural microvascular tissue and this is responsible for impaired vascular function. Moreover, improving vascular function in diabetes by use of antioxidants also restores endoneurial blood flow and neural activity.

Diabetes has been shown to cause an increase flux of glucose through the aldose reductase pathway that leads to the accumulation of sorbitol by nerve and other tissues [33]. Numerous investigators have demonstrated that treating diabetic rats with an aldose reductase inhibitor improves nerve function, and we have shown that treatment with an aldose reductase inhibitor also improves vascular dysfunction in epineurial arterioles [47]. The mechanism responsible for improving diabetes impaired vascular and nerve function by aldose reductase inhibitor treatment is
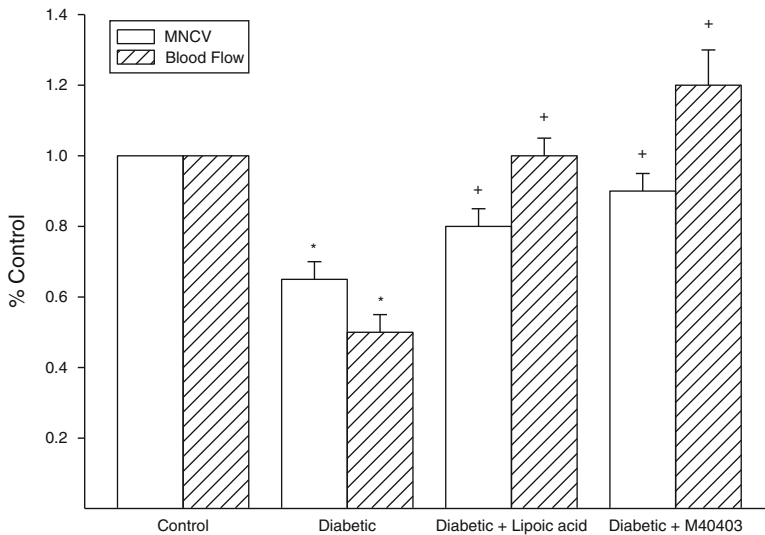

Fig. 1.3 Effect of treatment of streptozotocin-induced diabetic rats with 0.5 % α-lipoic acid or M40403 on motor nerve conduction velocity and endoneurial blood flow. Data is presented as the mean ± SEM % of control. *$P < 0.05$ compared to control; $^{+}P < 0.05$ compared to diabetic
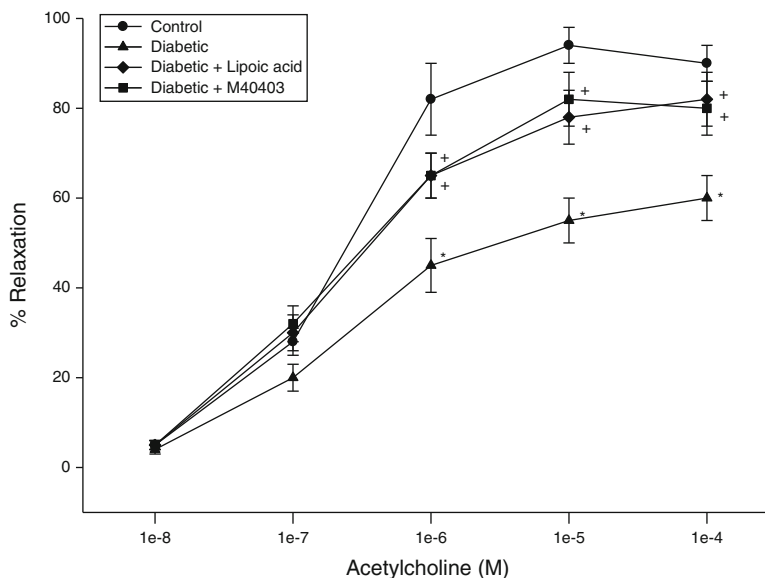
Fig. 1.4 Effect of treatment of streptozotocin-induced diabetic rats with 0.5 % α-lipoic acid or M40403 on acetylcholine-mediated vascular relaxation by epineurial arterioles. Data is presented as the mean±SEM % of control. *$P < 0.05$ compared to control; +$P < 0.05$ compared to diabetic

unclear but in part may be due to reducing oxidative stress [47]. Previously we had reported that treating streptozotocin-induced diabetic rats with 0.5 % α-lipoic acid (see above) provides maximum protection against diabetes-induced oxidative stress and the development of vascular and neural dysfunction [26]. We have also reported that sorbinil, an aldose reductase inhibitor, partially prevented the development of diabetes-induced vascular and neural defects but were not as efficacious as antioxidant therapies [26, 47]. We next sought to determine whether combining these therapies at lower doses may be synergistic [48]. We found that the combination of 0.25 % α-lipoic acid and fidarestat (3 mg/kg B.W.), an aldose reductase inhibitor, completely prevented the diabetes-induced impairment of acetylcholine-mediated vascular relaxation in epineurial arterioles of the sciatic nerve (Fig. 1.5) and that this combination was more effective in preventing diabetes-induced vascular dysfunction than monotherapy of either compound. Our explanation for these results was that treatment of diabetic rats with fidarestat in combination with α-lipoic acid favored the formation of dihydrolipoic acid. α-Lipoic acid is a good metal chelator and is capable of scavenging hydroxyl radicals, hypochlorous acid, and singlet oxygen, but not superoxide or peroxyl radicals [35, 36, 49, 50]. However, in its reduced form, as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation [35, 36, 49, 50]. In vivo, the conversion of α-lipoic acid to dihydrolipoic acid requires either NADH or NADPH [49, 51]. In the mitochondria, preferentially R(+)-α-lipoic acid is converted to dihydrolipoic acid by the action of dihydrolipoamide dehydrogenase which requires NADH [50, 51].
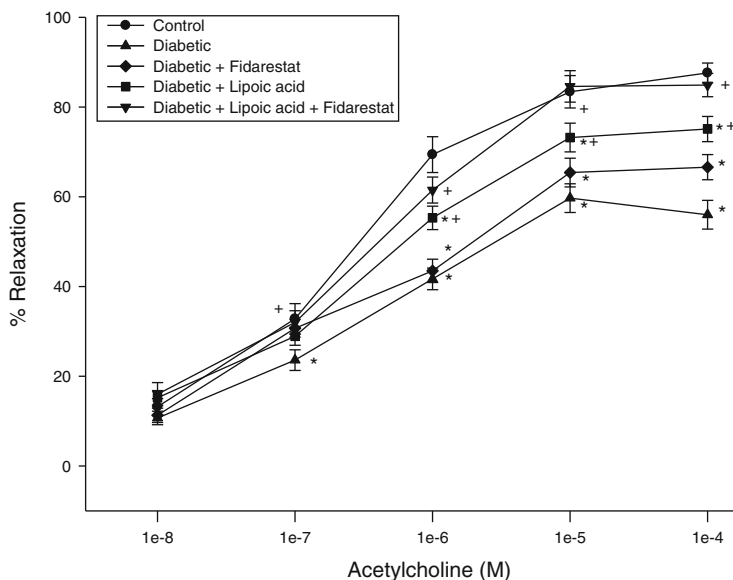
Fig. 1.5 Effect of treatment of streptozotocin-induced diabetic rats with 3 mg/kg fidarestat and/or 0.25 % α-lipoic acid on acetylcholine-mediated vascular relaxation by epineurial arterioles. Data is presented as the mean ± SEM % of control. *$P < 0.05$ compared to control; +$P < 0.05$ compared to diabetic

Both stereo-isoforms of α-lipoic acid can be reduced in the cytosol by glutathione reductase or thioredoxin reductase, both require NADPH [50–52]. In neutrophils, as well as rat heart, kidney, and brain, NADH-dependent reduction of α-lipoic acid is prominent, whereas with rat liver, NADH- and NADPH-dependent pathways were about equally active [50, 52]. In erythrocytes and endothelial cells, NADPH is the primary reducing cofactor for α-lipoic acid [50, 53]. In diabetes, NADPH levels are reduced due to the increased flux of glucose through the aldose reductase pathway [40, 41]. Therefore, blocking the aldose reductase pathway with an aldose reductase inhibitor such as fidarestat likely protects cellular NADPH levels permitting the formation of dihydrolipoic acid. This explanation is supported by our studies demonstrating that serum dihydrolipoic acid levels are increased in diabetic rats treated with α-lipoic acid and fidarestat [48]. These studies suggest that in addition to protecting glutathione production, treatment of diabetic rats with an aldose reductase inhibitor may promote the formation of dihydrolipoic acid. This result may explain the antioxidant properties of aldose reductase inhibitors [54]. In these studies there was a synergistic effect on improving lens glutathione levels when treating diabetic rats with the combination of α-lipoic acid and fidarestat. Treatment of diabetic rats with fidarestat alone independently improved endoneurial blood flow and motor nerve conduction velocity, by 50 and 60 %, respectively, and reduced superoxide formation in the aorta. Furthermore, treating diabetic rats with 3 or 15 mg/kg body weight of fidarestat had a concentration-dependent effect on improving endoneurial

blood flow, motor nerve conduction velocity, and acetylcholine-mediated vasodilation in epineurial arterioles. Taken together our results imply that some markers of oxidative stress and neural function are significantly improved by monotherapy using α-lipoic acid; however, the greatest beneficial effects were observed on all markers of oxidative stress and vascular function when the combination treatment consisting of α-lipoic acid and fidarestat was used.

In summary, diabetic neuropathy is a multifactorial disorder and vascular dysfunction in part due to an increase in oxidative stress is a contributing factor. Since diabetic vascular and neural disease is multifactorial, combination therapy may be the best approach for an effective treatment. The studies presented above suggest that an effective combination therapy should include an antioxidant such as α-lipoic acid and an aldose reductase inhibitor.

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Chapter 2
Cerebrovascular Disease in Type 1 Diabetes: Role of Oxidative Stress

Denise M. Arrick and William G. Mayhan

2.1 Introduction

Diabetes mellitus is a group of metabolic diseases that produces an increase in blood glucose as a result of inadequate production/release of insulin by the beta cells of the pancreas (type 1 diabetes mellitus, insulin-dependent diabetes, juvenile-onset diabetes) or as a result of inadequate responses of cells to insulin that is produced/released by the pancreas (type 2 diabetes mellitus, non-insulin-dependent diabetes, adult-onset diabetes). Estimates suggest that there are about 26 million children and adults (over 8 % of the population) that have been diagnosed with diabetes, about 7 million individuals that have diabetes but have not been diagnosed, and about 79 million people that are prediabetic. The cost of diabetes has been estimated to be over $180 billion per year (disability, work loss, and premature mortality). The complications from diabetes include, but are not limited to, hypertension, neuropathy, nephropathy, blindness, peripheral vascular disease, inflammation, heart disease, and stroke. Thus, diabetes contributes to an increase in morbidity and mortality in children, adolescents, adults, and the elderly. It remains critical to define mechanisms by which diabetes contributes to dysfunction of many organ systems in order to provide new therapeutic approaches for the prevention of diabetes-induced disease states. In this chapter, we will focus on mechanisms by which type 1 diabetes (T1D) may contribute to an increase in oxidative stress in the brain and how this increase in oxidative stress may contribute to cerebrovascular dysfunction, brain injury, cognitive dysfunction, and perhaps stroke.

D.M. Arrick • W.G. Mayhan (✉)
Department of Cellular Biology and Anatomy, Louisiana State University Health Sciences
Center-Shreveport, School of Medicine in Shreveport, 1501 Kings Highway,
Shreveport, LA 71130-3932, USA
e-mail: wmayha@lsuhsc.edu

2.2   Oxidant Pathways in Diabetes

T1D impairs nitric oxide synthase (NOS)-dependent responses of large and small peripheral and cerebral blood vessels. Mechanisms responsible for T1D-induced impairment in vascular function appear to be related to the generation of reactive oxygen species (ROS) through a variety of cellular pathways. This increase in oxidative stress during T1D can occur from many cell types (endothelium, vascular smooth muscle, neurons, glia, astrocytes) and represents an imbalance between the production of ROS by oxidizing enzymes and the scavenging of these ROS by antioxidant defense enzymes, which serve to interfere with the downstream signaling events triggered by these ROS. In T1D, the activity of ROS-producing enzymes is increased, while antioxidant defense enzymes appear to be unaltered or decreased, shifting the balance in favor of ROS production. There are several oxidant-producing and antioxidant-protecting pathways that are altered by T1D in peripheral and cerebral blood vessels. In the following sections, we will outline some of the key aspects of these pathways.

2.2.1   Cyclooxygenase Pathway

The cyclooxygenase pathway has been implicated in synthesis of ROS for many years and has been thought to be a contributor to the formation of ROS during diabetes [30, 154, 156]. Early studies by Kontos and colleagues [88, 90, 91, 173] found that application of arachidonate to the cerebral microcirculation could produce dilation of large and small cerebral arterioles. This dilation could be inhibited by a combination of superoxide dismutase (SOD) and catalase, thus implicating a role for superoxide anion, hydrogen peroxide, and hydroxyl radical [90, 173]. It is now becoming apparent that hydrogen peroxide may be acting as an endothelium-derived hyperpolarizing factor in the brain and other vascular organs [89, 92, 104, 167]. Support for the production of ROS by the cyclooxygenase pathway during diabetes can be found in early studies by Pieper et al. [127, 129] and Tesfamariam et al. [155]. Pieper et al. [127] found that oxygen radicals, generated via xanthine plus xanthine oxidase, could impair relaxation of the thoracic aorta in nondiabetic and diabetic rats and that catalase and SOD could enhance relaxation of the thoracic aorta in diabetic rats [129]. Tesfamariam et al. [155] found that indomethacin could restore impaired relaxation of the thoracic aorta in diabetic rats to that observed in nondiabetic rats. Thus, it appeared that ROS generated via the activation of the cyclooxygenase pathway could contribute to impaired vascular function of peripheral blood vessels during T1D. With regard to cerebral vessels, we [108] found that treatment with indomethacin or the thromboxane A2/prostaglandin H2 receptor (SQ 29548) improved impaired endothelial NOS (eNOS)-dependent responses of cerebral arterioles in diabetic rats. In addition, others [79] have reported that indomethacin can restore impaired cerebrovascular reactivity to insulin in

Fig. 2.1 Hyperglycemia-induced activation of NADPH oxidase. Activation of NADPH oxidase isoforms via an increase in cellular levels of glucose (hyperglycemia) can increase the formation of superoxide (O_2^-) from numerous cellular sources. Superoxide can then combine with nitric oxide (NO), forming peroxynitrite (ONOO$^-$), which can then reduce NO bioavailability, leading to cerebrovascular dysfunction
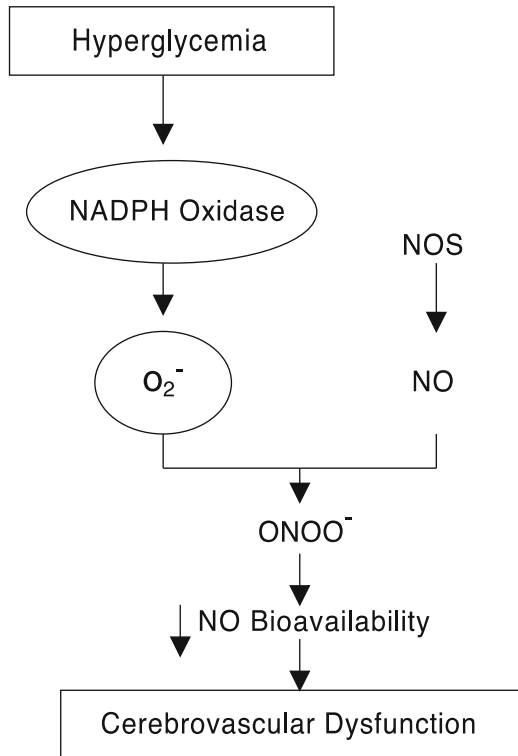
insulin-resistant obese rats. Taken together, these findings suggest that the production of a cyclooxygenase constrictor substance and/or the production of ROS via the cyclooxygenase pathway may contribute to impaired eNOS-dependent responses of cerebral arterioles during T1D.

2.2.2 NADPH Oxidase

NADPH oxidases are a primary source of ROS in the vascular system and are active in all cell types within the walls of blood vessels [66, 162]. NADPH oxidases are comprised of two membrane bound subunits (Nox and p22phox), up to three cytoplasmic subunits (p67phox, p47phox, and p40phox), and a G protein (Rac1/Rac2). Several NADPH oxidases have been identified (Nox1, Nox2, Nox4, and Nox5), and these are a primary source of ROS in the vasculature [13, 17, 30, 156]. Since the formation of ROS (presumably via an increase in cellular levels of glucose) appears to be of primary importance in vascular dysfunction during T1D, compounds that inhibit Nox activity may offer therapeutic benefits in T1D-induced cerebrovascular dysfunction (Fig. 2.1). In fact, investigators have shown that inhibition of Nox with

Fig. 2.2 Role for angiotensin II in mediating hyperglycemia-induced activation of NADPH oxidase. Angiotensin, acting via AT-1 receptors, can activate NADPH oxidase, lead to a decrease in NOS activity (and subsequent formation of NO), and/or increase the synthesis/release of endothelin-1 (ET-1). These actions can lead to a decrease in NO bioavailability and cerebrovascular dysfunction
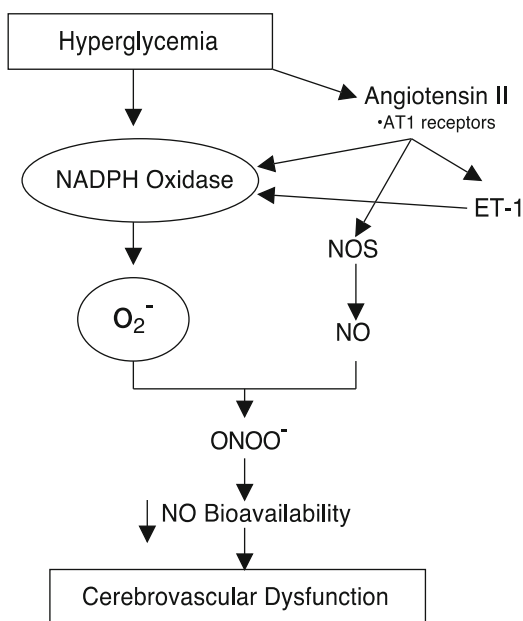
apocynin reversed upregulation of Nox enzymes, improved nitric oxide function, and reduced vascular dysfunction of peripheral blood vessels in diabetic animals [8, 15, 56, 124]. While studies have shown that apocynin can influence the pathogenesis of stroke [153] and can improve impaired cerebrovascular function during hyperhomocysteinemia [33], few studies have examined the role of Nox enzymes in impaired responses of cerebral blood vessels during T1D. In a previous study, we found that T1D increased superoxide levels in brain tissue and increased the protein expression of various subunits of Nox in brain tissue and cerebral blood vessels [105]. Further, we found that chronic treatment of diabetic rats with apocynin could reverse the increase in superoxide levels in brain tissue and also could reverse impaired eNOS-dependent responses of cerebral arterioles [105]. Although studies have shown that Nox may be of benefit during diabetes, some have questioned the specificity, potency, and toxicity of this type of treatment and how it may translate to treatment of humans with diabetes or, in fact, with other disease states [73, 161]. Thus, while there may be a significant role for Nox enzymes in the generation of ROS, there may not be enough definitive evidence to determine which isoform of Nox may be most important in cerebral vessels during T1D.

The precise cellular pathway underlying increased Nox expression/activity in T1D remains unclear. One possibility is that angiotensin II plays a critical role (Fig. 2.2). Stimulation of vascular smooth muscle cells with angiotensin II, thrombin, lipopolysaccharide, and cytokines increases the activity of NADPH oxidase, vascular p47phox expression, and production of ROS [1, 21, 54, 65, 66, 93]. Since tissue and plasma levels of angiotensin-converting enzyme, and thus angiotensin II, are elevated in diabetics [42, 97, 138] and since angiotensin II has been shown to

activate NADPH oxidase (presumably Nox2) via stimulation of AT-1 receptors [65, 132, 177], it seems reasonable to suggest that the formation of superoxide during T1D may be related to angiotensin II-induced stimulation of Nox. Support for this concept can be found in studies that report treatment of diabetic subjects with angiotensin-converting enzyme inhibitors improves impaired NOS-dependent responses of large peripheral blood vessels [24, 116]. Given that angiotensin II can influence the brain via the circulation and via local production, it is not surprising that the cerebral circulation is also quite sensitive to angiotensin II. Investigators have shown that angiotensin II can produce endothelial dysfunction, impair neuro-vascular coupling, and alter the transport properties of the blood–brain barrier [6, 35, 48, 62, 67, 81, 112, 137]. With regard to T1D, we have reported that treatment of diabetic rats with enalapril [163] or losartan [6] can alleviate impaired eNOS-dependent responses of cerebral arterioles [163]. Although most studies have suggested that angiotensin II promotes endothelial dysfunction largely due to activation of NADPH oxidase and the subsequent formation of superoxide, additional mechanisms may also account for angiotensin II-induced vascular dysfunction. For example, angiotensin II can limit the production of nitric oxide [61, 100], can lead to the formation of an endothelium-derived contracting factor [38, 102, 172], and can lead to an increase in the synthesis/release of endothelin-1 [130, 179]. Impaired responses of cerebral arterioles during T1D have also been implicated to be related to alterations in nitric oxide production [79, 84], the production of a cyclooxygenase constrictor substance [79, 108], and/or the increased synthesis of endothelin-1 [4]. Thus, future studies will be required to determine the mechanism underlying the role for angiotensin II in cerebrovascular dysfunction during T1D.

2.2.3 Mitochondria

The mitochondria are a key source of ROS in cells as a result of an imbalance in the electron transport chain. Since oxidative stress is now widely accepted to play a key role in vascular dysfunction in a variety of disease states, including T1D, it has become apparent that the mitochondria might be a major contributor to this increase in oxidative stress (Fig. 2.3). The production of ROS by the mitochondria is a very complex process that involves oxidative phosphorylation across the electron transport chain; for review see [135]. Although mitochondrial complexes I and III may be mainly responsible for the generation of ROS, complexes II and IV may also result in the production of ROS [25, 115]. The mechanism by which hyperglycemia can lead to an increase in the synthesis/release of ROS by mitochondria is not entirely clear, but appears to involve an increase in electron donors (NADH and FADH2) through the electron transport chain. The role of mitochondria in impaired vascular function during T1D has not been extensively examined, but investigators have shown that inhibition of the electron transport chain can reduce oxidative stress in the heart [25, 98]. In addition, rotenone, an inhibitor of complex I, has been shown to decrease the levels of hydrogen peroxide in the posterior cerebral artery of
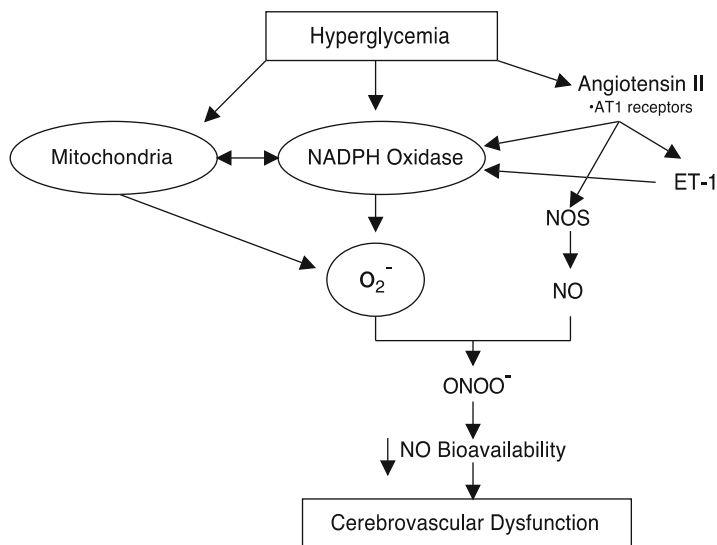
Fig. 2.3 The role of the mitochondria. Increases in cellular levels of glucose can stimulate the mitochondria to release ROS via activation of NADPH oxidase and through the electron transport chain. Once formed, ROS can then produce a decrease in NO bioavailability and cerebrovascular dysfunction

diabetic mice and partially reverse decreased calcium currents in smooth muscle cells during T1D [40]. Thus, we speculate that inhibition of the electron transport chain may have important implications for cerebrovascular dysfunction in T1D. Support for this concept may come from studies of type 2 diabetic patients. One of the more common treatments of type 2 diabetes is metformin. One mechanism of metformin is the ability to inhibit complex I of the electron transport chain [20]. Therefore, it is conceivable that inhibition of the mitochondrial electron transport chain also may have important clinical applications to T1D.

2.2.4   Endothelial NOS

eNOS is modulated by many mechanisms including enzyme phosphorylation, interactions with various proteins, several transcription factors, levels of substrate, and the availability of critical cofactors. In addition, there are various downstream regulators of cellular signaling pathways that are able to modulate eNOS function including Rho kinase (RhoA) [140]. With regard to cofactors for eNOS, tetrahydrobiopterin (BH_4) has been shown to be a critical component of eNOS regulation [2, 23, 64]. In order for eNOS to remain active, it must remain in a dimeric form and BH_4 contributes to the ability of eNOS to remain in this state [16, 32, 168]. There are many studies that have shown that hyperglycemia/diabetes can produce
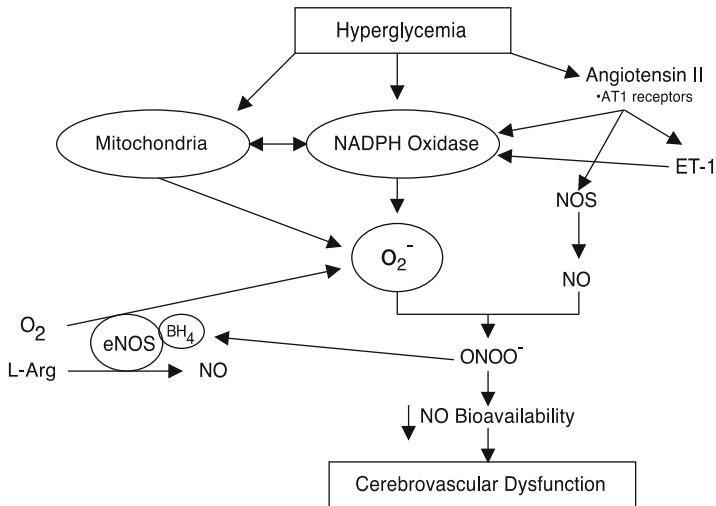
Fig. 2.4 eNOS uncoupling. The formation of ONOO⁻ by an increase in cellular levels of glucose can contribute to cerebrovascular dysfunction by oxidizing tetrahydrobiopterin (BH_4) to dihydro-biopterin (BH_2). This deficiency in the availability of BH_4 would force eNOS from its dimeric form to its monomeric form (an uncoupled state). Once in this uncoupled state, electrons flowing from the eNOS reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine, resulting in the production of superoxide (O_2^-) rather than nitric oxide (NO). Once formed, superoxide can inactivate NO and produce cerebrovascular dysfunction

reductions in the cellular levels of BH_4, leading to an "uncoupling" of eNOS to its monomeric form, thereby increasing the formation of eNOS-derived superoxide [3, 49, 69, 86, 120]. Thus, it is conceivable that eNOS uncoupling is a viable mechanism by which T1D can produce cerebrovascular dysfunction (Fig. 2.4). Support for this concept can be derived from studies that have shown that treatment of type 2 diabetic patients or patients following a glucose challenge with BH_4 can improve eNOS-dependent dilation [71, 76]. In addition, treatment with sepiapterin, a precursor of BH_4, or supplementation with BH_4 produced an improvement in eNOS-dependent responses of peripheral arteries in diabetic rats [11, 120, 121]. Only a limited number of studies have examined the influence of BH_4 on cerebral blood vessels. Early studies have shown that application of BH_4 to cerebral blood vessels could produce dilation or constriction dependent upon the size of the cerebral artery and/or species [82–84, 136]. A more recent study [79] reports that supplementation with sepiapterin in insulin-resistant obese rats improved dilation of cerebral arterioles in response to insulin suggesting eNOS uncoupling in this model. Unfortunately, there are no studies that we are aware of that have examined the influence of chronic treatment with BH_4 or sepiapterin on responses of cerebral arteries or arterioles during T1D. We have, however, shown that supplementation with BH_4 improves impaired responses of cerebral arterioles during other disease states [44, 145, 146], and thus it is conceivable that eNOS uncoupling may play a critical role in impaired vascular function during T1D.
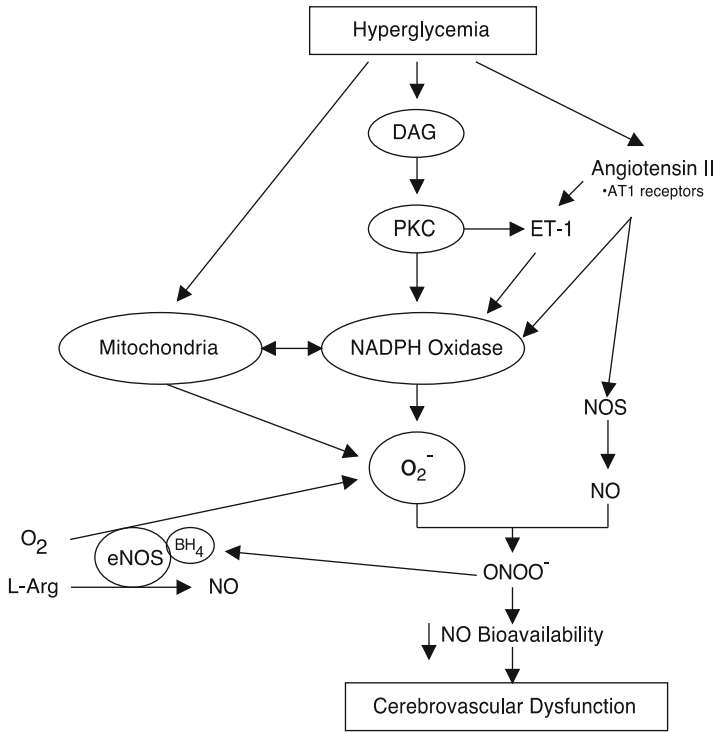
Fig. 2.5 Influence of PKC on cerebrovascular dysfunction. Hyperglycemia can increase the synthesis of diacylglycerol (DAG) which will activate the classical isoforms of protein kinase C (PKC). Once PKC is activated, a variety of events can occur within the cell, including an increase in the expression of endothelin-1 (ET-1) and the activation of NADPH oxidase. These events will lead to cascade of actions to decrease NO bioavailability and cerebrovascular dysfunction

2.2.5 Protein Kinase C

The protein kinase C (PKC) family comprises at least fifteen isoforms. This family of protein kinase enzymes is involved in managing the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these specific proteins. PKC enzymes are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions (Ca^{2+}). Hence, PKC enzymes play important roles in several signal transduction cascades. Increases in cellular levels of glucose can increase the synthesis of DAG which, in turn, will activate the classical isoforms of PKC [60, 72, 77]. Once PKC is activated, a variety of events can occur within the cell, which may result in alterations in vascular permeability and/or vascular function (Fig. 2.5). For example, activation of PKC can lead to a decrease in eNOS, an increase in the expression of endothelin-1, and an increase in oxidative stress via NADPH oxidase [9, 18, 31, 77, 122, 131, 133, 169, 180]. In addition, activation of PKC can induce the activation of

several proinflammatory agents such as tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), and nuclear factor-κB (NF-κB) [47, 50, 101, 142, 158, 166].

Many investigators have reported a role for PKC in impaired endothelial function of peripheral blood vessels during T1D [46, 70, 114, 115, 178]. In addition, a few studies have implicated a role for activation of PKC in impaired responses of cerebral blood vessels during T1D. Studies by Pelligrino et al. [123] have shown that treatment of diabetic rats with staurosporine could restore impaired responses of pial arterioles in diabetic rats. In subsequent studies, Pelligrino and colleagues [170] found that PKCδ activity was increased in the glio-pial tissue of diabetic rats, suggesting that this isoform of PKC may ultimately lead to impaired neurovascular coupling during T1D. Others also have reported impairment in neurovascular coupling in diabetic rats was related to an increase in the activity of PKC [171]. This increase in the activity of PKC appeared to be responsible for a decrease in large conductance (BK) calcium channel and inward rectifier (Kir) calcium channel activity [171]. In addition, we have shown that acute hyperglycemia could impair NOS-dependent responses of pial arterioles in rats and this impairment could be reversed by treatment with a PKC inhibitor [106]. Thus, it appears that activation of PKC, through the stimulation of various downstream events, can influence cerebrovascular function during T1D.

2.2.6 Poly(ADP-Ribose) Polymerase

Poly(ADP-ribose) polymerases (PARPs) are an important set of nuclear enzymes that appear to be involved in the response of the cell to DNA injury/DNA strand breaks [27, 59, 126]. These enzymes, of which PARP-1 is most abundant, normally function in DNA repair, but extensive activation of PARP can promote cellular dysfunction and/or cell death via mechanisms involving depletion of NAD+ and ATP within the cell [27, 59, 126]. Activation of PARP has been implicated in the pathogenesis of several disease states including stroke [29, 43, 109, 126], inflammation [63, 80, 151, 181], myocardial dysfunction [28, 119, 164, 180], autoimmune diseases [125, 126], and cognitive impairment following hypoglycemic cell death [144]. Since oxidative stress can induce the activation of PARP [57, 59] and since oxidative stress is increased in T1D, it is conceivable that PARP activation may contribute to vascular dysfunction during T1D (Fig. 2.6).

Several studies have suggested that PARP activation is increased in T1D and this increase may contribute to cardiovascular and endothelial dysfunction. Pacher et al. [119] have reported an increase in the activation of PARP in the heart of diabetic rats and mice, cardiac dysfunction, and a decrease in NOS-dependent reactivity of the thoracic aorta. In addition, these alterations in cardiac/vascular function observed in diabetic rats and mice could be restored to that observed in nondiabetic animals by treatment with PJ-34 [119]. Studies by others [57, 58] also report that T1D activates PARP and induces endothelial dysfunction of the thoracic aorta. In addition,
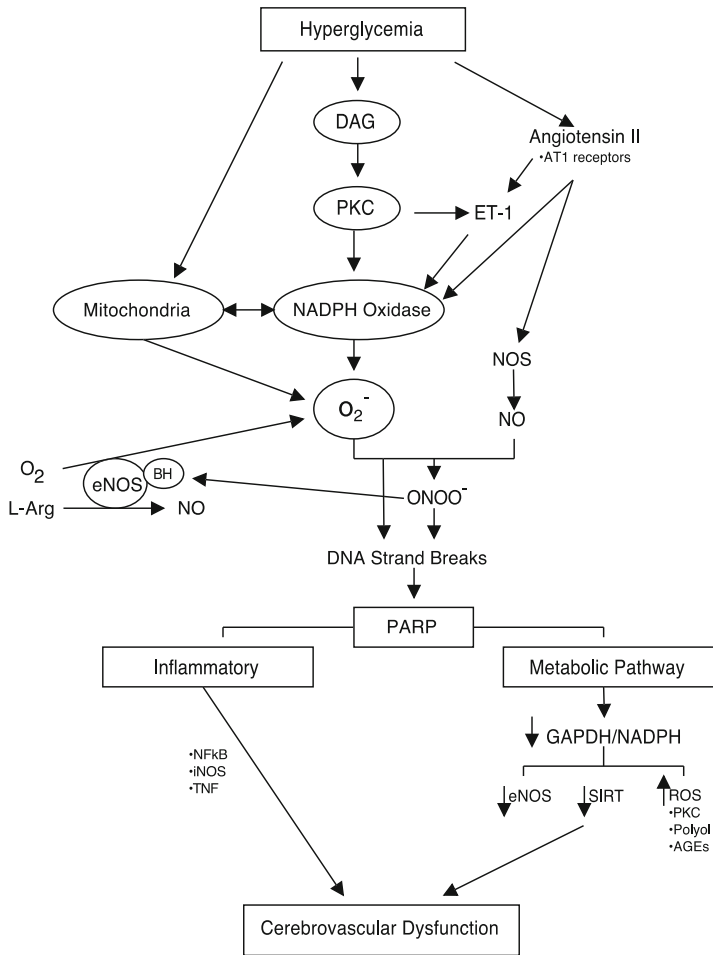
Fig. 2.6 The contribution of PARP. Hyperglycemia during T1D can stimulate increased levels of angiotensin II (AII) and activate PKC. AII and PKC can increase the production of superoxide anion (O_2^-) via activation of NADPH oxidase. Once formed, O_2^- can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), to induce DNA strand breaks thereby activating poly(ADP-ribose) polymerase (PARP). PARP activation can trigger a proinflammatory pathway and the generation of inflammatory mediators (TNFα, iNOS and/or NF-κB) that may lead to cerebrovascular dysfunction. Activation of PARP may, via a metabolic pathway, produce a decrease in NADPH and GAPDH leading to a decrease in cellular energy status (ATP), a decrease in production of eNOS, and a further increase in ROS through activation of several pathways. This decrease in cellular energy status and increase in oxidative stress can lead to cerebrovascular dysfunction

treatment of vascular rings from PARP-deficient mice with glucose (30 mM for 16 h) did not produce endothelial dysfunction, and acute treatment of vascular rings from wild-type mice with PJ-34 prevented endothelial dysfunction induced by an acute episode of hyperglycemia [57]. In studies using T1D rats, we found that acute

treatment of pial arterioles with an inhibitor of PARP (PJ-34) could restore impaired NOS-dependent reactivity [5]. We suggested that the influence of PJ-34 on vascular function was related to its effect on superoxide levels in brain tissue since PJ-34 prevented an increase in superoxide levels found in diabetic rats.

In addition to studies that have examined the role of PARP activation in cerebrovascular dysfunction during T1D, others have suggested that PARP plays an important role in protection of the brain following cerebral ischemia/reperfusion [14, 26, 68, 140]. These investigators have shown that treatment with inhibitors of PARP decreased brain injury and disruption of the blood–brain barrier following ischemia/reperfusion by a mechanism that appeared to be related to preventing an increase in the synthesis/release of inflammatory mediators (TNFα, IL-6, E-selectin, and ICAM-1) [68], thereby preserving endothelial tight junction integrity [96]. Although no studies to our knowledge have examined the influence of PARP inhibition on brain injury following ischemia/reperfusion during T1D, given the results from previous studies, we suggest that future studies should examine the role of this important pathway in the pathogenesis of cerebral ischemia-/reperfusion-induced brain injury and disruption of the blood–brain barrier during T1D. We speculate that the results from these types of studies might have important implications regarding mechanisms for the increased incidence of stroke and cognitive dysfunction observed in diabetic subjects.

Given that PARP activation is a very complex process, it would appear difficult to determine mechanisms that PARP activation produces vascular dysfunction, including cerebrovascular dysfunction, during T1D. Two pathways have been proposed to account for the role of PARP activation in T1D: the proinflammatory and metabolic pathways. The proinflammatory pathway [59, 147, 149] suggests that PARP activates multiple pathways of damage, including NF-κB, PKC, and/or generation of advanced glycosylation end products (AGEs). Activation of these pathways can stimulate the synthesis/release of inflammatory mediators (E-selectin, IL-6, TNFα, ICAM-1, and iNOS) that have been implicated in endothelial dysfunction and brain injury observed in T1D and can generate ROS from additional pathways [68, 94, 103, 117, 128, 134, 181]. Thus, it is possible that PARP activation during T1D can increase the formation of inflammatory mediators that, in turn, produce endothelial dysfunction directly and/or via the production of ROS. The metabolic pathway [57] suggests that hyperglycemia during T1D stimulates the production of oxidants. Although the pathway for the formation of these oxidants in T1D is not entirely clear, it may involve the activation of NADPH oxidase via increased levels of angiotensin II [42, 54, 65, 97, 132, 138]. Evidence suggests that angiotensin II can activate PARP in cultured endothelial cells and can induce DNA strand breaks [150]. In addition, angiotensin II-induced endothelial dysfunction can be prevented by inhibition of NADPH oxidase and PARP [150]. In addition to a possible role for angiotensin II, elevated levels of glucose also have been shown to activate PKC and produce oxidative stress [55, 95, 176]. Further, it has been suggested that oxidative stress may further stimulate the activity of PKC via activation of PARP [41]. It is also conceivable that oxidative stress-induced stimulation of PARP, in turn, activates endothelin-1 to produce vascular dysfunction [110].

Finally, PARP activation can increase ROS formed via an increase in AGEs and the polyol pathway, both of which have been implicated as an important source of ROS during T1D [19, 141, 175]. Regardless of the precise cellular mechanism, once ROS are formed they can induce DNA strand breaks to activate PARP, producing a cellular energy crisis. Without sufficient energy, the endothelium could presumably produce additional levels of ROS and/or have less potential to produce nitric oxide. This metabolic pathway is supported by data obtained from studies that have shown that exposure of endothelium to oxidants can produce depletion of NAD+ in cells that can be prevented by inhibition of PARP, endothelial dysfunction in T1D can be prevented by inhibition of PARP, glucose-induced endothelial dysfunction is prevented in PARP-deficient mice, and altering the energy status within endothelial cells can influence vascular function [22, 58, 59, 119].

2.3 Antioxidant Pathways

Excess production of ROS in the vascular system, the peripheral organ systems, and/or the brain by T1D can be regulated through the expression of a variety of endogenous antioxidant enzymes. These antioxidant enzymes serve to protect the vasculature and/or organ systems, including the brain, by scavenging ROS and interfering with or preventing the activation of downstream signaling events triggered by these ROS. Unfortunately, in T1D where there is a dramatic increase in the levels of ROS, these antioxidant enzyme systems may not be able to adequately regulate these excess levels of ROS and/or may be adversely affected by T1D. This consequence would tip the balance in favor of a prooxidant environment to detrimentally affect vascular function during T1D.

2.3.1 Superoxide Dismutases

The bioactivity of nitric oxide depends, in part, on its ability to interact with ROS, especially superoxide [12]. Early findings suggested that superoxide inactivates nitric oxide [174] and studies since have shown that inactivation of nitric oxide by superoxide contributes to impaired vascular function [36, 37, 107]. While there is considerable attention paid to examining the role of superoxide during disease states, little information is available regarding the functional significance of alterations in the activity/expression of antioxidant pathways during disease states. SODs exist in three isoforms localized within specific cellular compartments. Copper-zinc SOD (SOD-1, CuZnSOD) is located predominately within the cytosol, as well as in the nucleus, and is expressed in all mammalian cells. Manganese SOD (SOD-2, MnSOD) is localized to the mitochondrial matrix, and it is considered to be the primary SOD isoform in relation to oxidative stress in the mitochondria. SOD-2 is needed to protect cellular constituents from superoxide derived

from the electron transport chain. Extracellular SOD (SOD-3, EC-SOD) is also a copper-zinc-containing SOD and is secreted extracellularly. SOD-3 is found bound to heparin sulfate proteoglycans on the surface of cells. It appears that the predominant form of SOD in blood vessels is SOD-1, followed by SOD-2 and the least involving SOD-3 [51–53, 143]. During T1D, superoxide levels are increased in brain tissue [7], but levels of SODs in the brain during T1D are not as clear. Some studies have reported an increase in SOD-2 in brain tissue of diabetic rats [75], but others showing decreases in total SOD activity in the brain [87, 118], and a decrease in SOD-2 and SOD-1 activity and mRNA in the aorta of diabetic rats [78, 85]. In addition, we have reported that SOD-1 and SOD-2 proteins are similar in brain tissue and cerebral microvessels from nondiabetic and diabetic rats, even though levels of superoxide are increased in brain tissue from T1D rats [7].

2.3.2 Glutathione Peroxidases

In addition to SODs, other antioxidant systems tightly regulate cellular redox balance. Cellular protection against ROS and their related by-products involves the activities of endogenous enzymes that belong to the oxidoreductase superfamily [139]. Glutathione peroxidases (Gpx) are a family of antioxidant enzymes that participate in the neutralization of hydrogen peroxide to water utilizing glutathione (GSH) as its substrate. A previous study has shown that Gpx1 plays a functional role in reactivity of cerebral blood vessels in mice [111]. In addition, previous studies have suggested that T1D can reduce Gpx mRNA in patients with T1D [74] and can reduce glutathione levels in the aorta [152] and brain [118] of rats, that the glutathione pathway is susceptible to oxidative stress [39], and that glutathione can protect diabetic rats from neuropathy [165]. However, the role of this endogenous enzyme pathway in protection of cerebral vessels during T1D remains unclear.

Taken together, these findings seem to indicate that antioxidant enzymes (SODs and Gpx) may not be able to compensate for increases in superoxide levels in the brain during T1D and thus may not be able to protect the vasculature from the damaging effects of ROS during T1D.

2.4 A Common Link?

On a cellular/molecular level, there are several major pathways that have been implicated in T1D-induced increases in oxidative stress to account for dysfunction of blood vessels of peripheral organ systems and the brain. Those discussed in this chapter include the cyclooxygenase pathway, NADPH oxidase, eNOS uncoupling, the mitochondria, PKC, and PARP. In addition to these oxidant-producing pathways, it appears that T1D can influence oxidant-protecting pathways (SODs and Gpx) to further alter the balance to favor the damaging effects of ROS. Although not

entirely clear for cerebral blood vessels, based upon findings from previous studies (see [19, 115]), it appears unlikely that oxidant-producing pathways act independently. A unifying hypothesis that has been presented by others [18, 19, 34, 115, 175] suggests that as glucose enters the cell, it stimulates the mitochondria to release superoxide, which in turn activates a number of downstream pathways (PKC, cyclooxygenase, inflammatory cytokines, PARP). These downstream pathways can produce a further increase in the generation of ROS and/or excite other pathways that could contribute to vascular dysfunction. However, this type of unifying hypothesis may not adequately account for the complexity of vascular dysfunction during T1D since inhibition of one of these pathways could not discount the formation of ROS from other distinct pathways, unless there was a linear relationship between the pathways. Faraci [45] has suggested that angiotensin II, acting via AT-1 receptors, can stimulate an increase in the synthesis of ROS from the mitochondria as well as promote inflammation and thus account for cardiovascular-related impairment in vascular function. Others [147, 148] have suggested that increases in cellular levels of glucose can stimulate the production of ROS from a variety of sources, which then activates PARP. Once activated, PARP would stimulate a number of downstream pathways (polyol pathway, PKC, AGEs, inflammatory mediators) that could then lead to the production of more ROS to produce vascular dysfunction. However, one might assume that inhibition of a singular pathway might not restore impaired vascular function given that other oxidant-producing pathways would remain intact. However, studies as outlined in this chapter have shown that inhibition of presumably singular cellular pathways can improve impaired responses of peripheral and cerebral blood vessels during T1D. Thus, although the basic principle that ROS are critical for impaired cerebrovascular function during T1D is certain, what remains uncertain is(are) the cellular pathway(s), indeed networks, that may be activated by ROS during T1D. We suggest that additional studies need to be completed before we can fully address the complexity of the interactions between the various cellular pathways that ultimately contribute to the generation of ROS during T1D.

2.5   Therapeutic Interventions

Based upon the experimental evidence presented in this chapter, one might speculate that inhibition of ROS during T1D would be an attractive therapeutic approach for addressing cerebrovascular dysfunction and its consequences, i.e., cognitive impairment and/or ischemic stroke. The vast majority of studies, several of which are presented in this chapter, have shown that short- and long-term treatment using scavengers of ROS improves vascular function in animal models of T1D. In addition, there are limited data to suggest that treatment of humans with scavengers of ROS improves endothelial function during T1D [159, 160] and brain injury following subarachnoid hemorrhage [10]. However, others have failed to demonstrate a dramatic effect of antioxidant therapy in human subjects with diabetes and/or other cardiovascular-related diseases [99, 113]. There may be several potential key

aspects as to why there are differences with regard to the beneficial effects of inhibition of ROS on vascular function in human subjects. First, the duration of exposure to antioxidant therapy may be important. A recent study reports that reversal of endothelial dysfunction in type 2 diabetic humans was only observed with 5 years of treatment with a combination of agents that lowered blood pressure, blood lipids, and ROS [157]. Second, it is possible that the duration of exposure to ROS during disease states in humans may create a condition whereby the endothelium is less able to respond to antioxidant therapy. Third, it would be rare for a human population not to have multiple risk factors for cardiovascular and cerebrovascular dysfunction. Therefore, the population being studied may not be an appropriate choice due to these multiple risk factors. Fourth, it is difficult to adequately control human subjects during a drug trial and there may be confounding influences in studying this type of population. Fifth, it is certainly possible that mechanisms contributing to vascular dysfunction during disease states, including T1D, are much more complex in humans than in animal models and different modes of therapy need to be examined in more long-term studies before conclusions can be drawn regarding the role of ROS in the pathogenesis of disease states. It may be premature to suggest a single therapeutic approach to limit the production of ROS during cardiovascular-related diseases, including T1D.

2.6   Closing Statement

The production of ROS appears to be the critical component of cerebrovascular dysfunction during T1D. Once ROS are formed, they can damage the endothelium directly and/or activate downstream networks that can lead to the generation of inflammatory mediators and/or produce an additional increase in the levels of ROS. We suggest that these processes not only contribute to impairment of dilator and constrictor responses of cerebral arteries and arterioles but also contribute to impaired neurovascular coupling, leading to an increase in the susceptibility of the brain to injury following ischemia/reperfusion, cognitive dysfunction, and an increase in prospect for ischemic stroke in diabetic humans.

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Chapter 3
Complications in the Coronary Circulation Associated with Diabetes

Christine L. Oltman

3.1 Introduction

The American Heart Association lists the prevalence of diabetes as 27.9 million people 20 years of age or older or greater than 12 % of this population. Another 87.3 million people (about 38 %) have prediabetes (fasting blood glucose of 100 to <126 mg/dL) [1]. In patients with type 2 diabetes, the average life expectancy is reduced by approximately 10 years, and 80 % die from cardiovascular complications. The number of type 2 DM patients is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and lack of physical activity. The total economic cost of diagnosed diabetes in the United States in 2012 was $245 billion [2].

Diabetes mellitus is a chronic disease of glucose metabolic dysfunction. Complications associated with diabetes include retinopathy, nephropathy, neuropathy, and increased risk for developing cardiovascular disease. Diabetic subjects have significantly elevated morbidity and mortality to many cardiovascular-related diseases, including hypertension, stroke, coronary artery disease, myocardial infarction, congestive heart failure, cardiomyopathies, sudden cardiac death, and accelerated atherosclerosis.

The cardiovascular and metabolic risk factors associated with diabetes include insulin resistance, impaired glucose tolerance, hypertension, high cholesterol and triglycerides, hyperglycemia, obesity, decreased coronary blood flow, increased oxidative stress, low-grade inflammation, and altered local vasomotor mechanisms. Dysfunction of the coronary circulation is an important contributor to increased cardiovascular morbidity and mortality in subjects with diabetes.

C.L. Oltman (✉)
Iowa City Veteran Affairs Health Care System and Cardiovascular Diseases Division,
Department of Internal Medicine, University of Iowa, Iowa City, IA 52246, USA

The metabolic syndrome is an emerging epidemic characterized by a cluster of risk factors including insulin resistance, abdominal obesity, atherogenic dyslipidemia, hypertension, and proinflammatory and prothrombotic states and often precedes development of type 2 diabetes. Each individual characteristic is a significant risk factor for development of vascular dysfunction and cardiovascular disease. Vascular dysfunction and progression of coronary artery disease is increased with each additional risk factor.

3.2   The Coronary Circulation

The myocardium has a very limited anaerobic capacity and requires a continuous supply of oxygen from the coronary circulation to meet the metabolic requirement of the heart. If the need for oxygen is not met, there is an immediate and substantial decrease in cardiac function. Decreased coronary blood flow results in under perfusion of the myocardium or ischemia. Several laboratory and clinical studies have shown that cardiac pump function is compromised in diabetes [3–5]. Frequently, many pathologies combine to compromise blood pressure and cardiac conduction resulting in a mismatch between myocardial supply and demand. The cause of the dysfunction is multifaceted, including changes in cardiac myocytes, interstitial fibrosis, and changes in the coronary vasculature. Although some studies have shown coronary blood flow abnormalities are primarily due to accelerated atherosclerosis in the diabetic heart, recent studies suggest coronary vasodilation may be a more pathophysiological response.

Coronary flow reserve is the difference between maximal and baseline coronary blood flow and is a measure of the capacity of the coronary circulation to respond to a vasodilator challenge. The dilator challenge may be induced by an imposed increase in myocardial nutrient demand (i.e., exercise) or by a pharmacological agent that produces dilation (i.e., adenosine). In diabetic patients, coronary flow reserve is reduced [6, 7]. Reduced coronary flow reserve could be related to depressed vasodilator capability, enhanced vasoconstrictor responsiveness, and/or structural remodeling of the coronary microvasculature.

Studies on basal coronary flow in diabetic patients at rest have shown varied results and conclude that baseline coronary flow may or may not be altered [8, 9]. However, when challenged, the ability of coronary arteries to increase blood flow is impaired. Momose et al. [10] used positron emission tomography (PET) to show elevated baseline blood flow, and reduced microvascular resistance is present in asymptomatic, non-insulin-treated type 2 diabetic patients. This suggests a state of activation of endothelial-dependent vasodilation at baseline which limits the flow response to stress conditions (cold pressor test and adenosine-mediated vasodilation), which is present without other symptoms of diabetes. Other studies have shown maximum coronary dilator capacity to pharmacological agents is significantly attenuated in type 2 diabetic compared with nondiabetic patients [6, 7].

These studies also suggest that the primary cause of the altered coronary flow dilator reserve is due to reduction of the dilator reserve of coronary microvessels.

Diabetes impairs the ability of the coronary microcirculation to match myocardial oxygen supply with myocardial oxygen demand. Possible mechanisms include anatomical changes, alterations in endothelial-dependent control of coronary blood flow, altered vasoactive neural–hormonal pathways, and dysfunction of microvascular ion channels.

3.3 Endothelial Dysfunction

Morphologic changes have been reported in the diabetic microcirculation at all levels, including small arteries, arterioles, capillaries, and venules. Some of the pathologies include capillary basal lumina thickening, increased fibrosis and alterations of elastic fibers, perivascular fibrosis, microvascular rarefaction, and reduced coronary capillary density.

Since 1980 when Furchgott and Zawadzki reported that the endothelium is responsible for the vasodilator response to acetylcholine [11], it has been known that the endothelial lining is a physical barrier between blood and the underlying tissue and that this thin layer of cells is an endocrine organ producing and releasing many metabolically active substances. Endothelial cell structure and functional integrity are important in maintenance of the vessel wall and circulatory function.

Endothelial cells produce and secrete numerous compounds that regulate a variety of physiological and pathophysiological processes, including coagulation, inflammation, permeability, cell adhesion, and vasomotor tone. Altered endothelial response is involved in atherosclerosis, hypertension, pulmonary hypertension, sepsis, and inflammatory syndromes.

The endothelium regulates arterial tone and blood flow via production of several vasoactive compounds including dilators nitric oxide (NO), prostacyclin, endothelial-derived hyperpolarizing factor (EDHF), and constrictors endothelin-1 and angiotensin II. There are a variety of endothelial-dependent vasodilators with various signal-transduction mechanisms, including acetylcholine, thrombin, bradykinin, substance P, serotonin, ATP, and ADP.

Endothelial dysfunction is a pathological condition characterized by an imbalance between endothelial-derived relaxing factors and endothelial-derived contracting factors and is due to changes in the synthesis, bioavailability, and/or action of endothelial factors leading to reduction of endothelial-dependent vasodilation and/or increase response to vasoconstrictor agonists. In diabetes, this balance is altered and the increased vasoconstrictor effects can be unopposed leading to increased arterial stiffness and arterial tone and promote vasospasm.

The primary vasodilator released from the endothelium has been identified as NO or a related molecule. Vascular endothelial cells synthesize NO from L-arginine by the action of endothelial nitric oxide synthase (eNOS) as a transduction mechanism for the activation of the soluble guanylate cyclase in vascular smooth muscle.

Increases in 3′5′-cyclic monophosphate cause vascular relaxation. NO is a potent vasodilator and also reduces platelet agreeability, limits vascular smooth muscle cell proliferation, and inhibits leukocyte adhesion. Decreased NO availability appears to play a major role in coronary endothelial dysfunction associated with diabetes. Reduced availability of other vasodilator agents (prostacyclin and EDHF) and simultaneously increased activity of vasoconstrictor substances (including endothelin-1 and angiotensin II) also play a role.

The literature contains inconsistent reports of endothelial dysfunction in diabetes [4, 12–16]. Endothelial-dependent dilation has been shown to be augmented, not altered, and attenuated in coronary arteries from diabetic patients and animals. Discrepancies may be due to species, technique, vessels studied, size of vessels, glycemic status, age, gender, duration of diabetes, or degree of hyperglycemia.

In isolated human arteries, Szerafin et al. have shown bradykinin elicited greater coronary vasodilation in type 1 and type 2 diabetic patients than in controls [17]. Inhibition of cyclooxygenase (COX) by nonspecific inhibitor indomethacin and COX-2 by NS-398, a COX-2 specific inhibitor, did not affect bradykinin-induced dilation in nondiabetic subjects, but significantly reduced bradykinin responses to control level in coronary arterials from patients with diabetes. The authors also show marked COX-2 immunostaining in endothelial and smooth muscle layers in coronary arteries from patients with diabetes, but not in arteries from controls. Thus, increased COX-2 expression contributes to enhanced release of dilator prostaglandins in diabetic humans. This enhanced COX-2 expression may be an adaptive mechanism to compensate for impaired vascular function, aiming to reduce the detrimental effects of diabetes on coronary blood flow.

Many detrimental effects of diabetes are linked to elevations in serum glucose that is accompanied by increased levels of superoxide. Tesfamariam and Cohen [18] incubated rabbit aorta in high glucose (44 mM) for 6 h and showed impairment of endothelial-dependent relaxation to acetylcholine, which was prevented by the presence of superoxide dismutase (a superoxide scavenger), catalase (a hydrogen peroxide scavenger), or allopurinol (an inhibitor of xanthine oxidase and scavenger of free radicals). They conclude that free radicals generated during exposure to elevated glucose are responsible for impaired endothelial cell function and that oxidative stress may be the basis by which hyperglycemia induces vascular complications known to occur in diabetes mellitus. Gutterman and colleagues have shown that 24 h exposure to high glucose (23 mM) increases superoxide production [19] and dilation to isoproterenol, forskolin, and papaverine is impaired in rat coronary arteries [20]. Thus, acute hyperglycemia causes increased free radical formation and produces endothelial dysfunction.

In humans, the course of developing type 2 diabetes occurs over several years and often is preceded by development of the metabolic syndrome. The Zucker obese rat is insulin resistant, hypertensive, and dyslipidemic and is a model of the metabolic syndrome. A model of type 2 diabetes is the Zucker diabetic fatty (ZDF) rat. This rat becomes hyperglycemic by 10 weeks of age when fed a high-fat diet, and glucose remains elevated throughout their life span [21]. At 10 weeks of age, ZDF rats are hyperinsulinemic; however, by 22 weeks of age, serum insulin levels decline
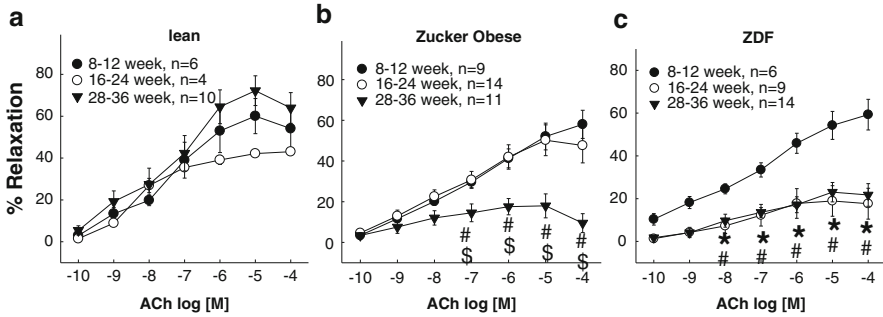
Fig. 3.1 Progression of endothelial dysfunction in coronary arteries from control (lean) insulin-resistant (Zucker) and type 2 diabetic (ZDF) rats. U-46619-induced constriction was similar in all groups. Data are presented as means ± SEM. *N* number of rats. *$P \leq 0.05$, 8–12 vs 16–24 weeks; #$P \leq 0.05$, 8–12 vs 28–40 weeks; $$P \leq 0.05$, 16–24 vs 28–40 weeks

to below levels of insulin in age-matched lean control rats [21]. A similar decrease in insulin levels is observed in human type 2 diabetes, which is thought to be caused by pancreas β-cell exhaustion. Free fatty acids, triglycerides, and cholesterol levels are higher in Zucker obese and ZDF rats compared with lean littermate controls. The Zucker obese and ZDF rat strains have been well characterized as models of the metabolic syndrome and type 2 diabetes [12, 22, 23]. We utilized these rat models to examine the development and progression of coronary vascular dysfunction associated with the metabolic syndrome and type 2 diabetes [24]. In coronary arteries from Zucker obese rats, we showed acetylcholine-mediated dilation was attenuated at 28–36 weeks of age. In coronary vessels from ZDF rats, endothelial dysfunction was observed earlier at 16–24 weeks of age (Fig. 3.1). Responses to sodium nitroprusside were not altered in these coronary arteries. Increases in indices of oxidative stress preceded the development of vascular dysfunction and may serve as a marker of endothelial damage [24]. This study showed the progression and degree of vascular pathology is dependent on the number of risk factors affected.

Another model for type 2 diabetes has recently been established. High-fat-fed rats injected with a low dose of streptozotocin (STZ) have been shown to produce diabetes with similarities to the human type 2 diabetes. Low-dose STZ slightly reduces B cell function, and hyperglycemia is obtained [25, 26]. Treating high-fat-fed rats with a low dose of STZ damages insulin-producing B cells so that hyperglycemia develops even though insulin levels are similar or higher than in chow-fed normoglycemia rats. The diabetes in these rats is analogous to the development of human type 2 diabetes when the decline in hyperinsulinemia is not able to compensate for insulin resistance and hyperglycemia occurs [25]. Using this model, we have shown that high-fat-fed rats gained more weight than the high-fat STZ-treated animals and blood glucose was higher in high-fat STZ-treated animals (20.9 ± 1.2 mM) than control (6.0 ± 0.2 mM) or high-fat-fed rats (6.6 ± 0.3 mM). Serum insulin and leptin levels were increased in high-fat-fed rats, but not in high-fat STZ rats [27]. Endothelial-mediated function was evaluated in isolated coronary

Fig. 3.2 Endothelial
dysfunction in coronary
arteries from high-fat-fed and
high-fat-fed low-dose
streptozotocin rats. U-46619-
induced constriction was
similar in all groups. Data are
presented as means ± SEM
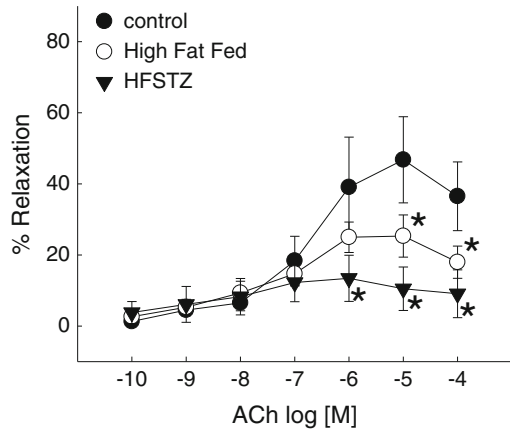
arteries. We found acetylcholine-mediated relaxation was attenuated in coronary
arteries from high-fat-fed rats and further attenuated in high-fat STZ animals
(Fig. 3.2).

Other rat models of type 2 diabetes include the Goto-Kakizaki (GK) rat model,
which is nonobese and spontaneously develops glucose intolerance, moderate
hyperglycemia, hypoinsulinemia, and mild hypertension. Kold-Petersen et al. [13]
have shown coronary arteries from GK rats develop less myogenic tone than control
(Wistar) rats; however, acetylcholine-mediated relaxation was not different. The
authors conclude that the attenuation of myogenic tone is due to a decreased Ca^{2+}
sensitivity and suggest the lack of endothelial dysfunction may be due to modest
hyperglycemia observed in GK rats. The Otsuka Long-Evans Tokushima Fatty
(OLETF) rat develops insulin resistance around 12 weeks of age and shows late
onset hyperglycemia (20 weeks of age) and reduced insulin levels around 60 weeks
of age. Kajikuri et al. [28] found increased vascular superoxide production in
OLETF rats; however, endothelial-mediated dilation in coronary arteries was not
altered at 28 weeks of age. The authors suggest the endothelial function is retained
due to enhanced eNOS protein expression. In contrast, another study evaluated mes-
enteric arteries from aged (36 week old) OLETF rats and showed endothelial dys-
function [14, 29], in this rat model.

We have also studied a model of type 1 diabetes in rats. Rats were made diabetic
with an injection of STZ (55 mg/kg), to destroy pancreatic B cells. Blood glucose
levels were 24 ± 0.08 mM in diabetic animals. In rats with 6-week duration of STZ
diabetes, acetylcholine-mediated (Fig. 3.3) and bradykinin-mediated relaxation was
attenuated in coronary arteries after 14 weeks of diabetes [30]. Sodium nitroprus-
side responses were not altered in coronary arteries from these rats.

EDHF may play an important role in regulating vascular tone and reactivity,
especially in small resistant vessels when NO-mediated control is compromised.
Park et al. have shown that endothelial-mediated vasodilation is NO dependent in
coronary arterioles in control mice; however, that portion of NO-dependent dilation
is reduced in db/db mice, a model of type 2 diabetes [31]. Their study also shows

Fig. 3.3 Endothelial
dysfunction in coronary
arteries from type 1 diabetic
rats (STZ diabetic).
U-46619-induced constriction
was similar in all groups.
Data are presented as
means ± SEM
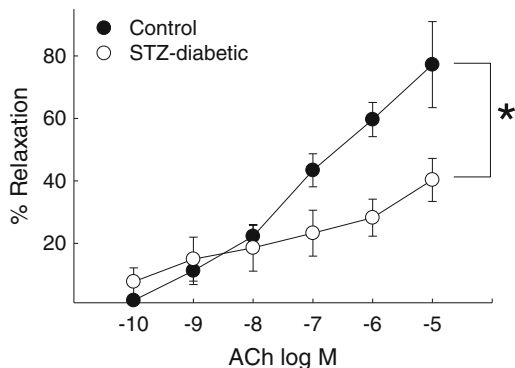
that H_2O_2, K^+, and epoxytrienoic acids, all candidates for EDHF, are involved in ACh relaxation in coronary arterioles from diabetic mice. This data suggest that EDHF may compensate for diminished NO-dependent dilation in diabetes.

3.4 Endothelial-Dependent Vasoconstriction

Endothelin exerts a potent, prolonged vasoconstrictor effect in coronary arteries. Vasoconstriction is produced through the endothelin type A receptor, which is coupled to G-protein signaling, which activates phospholipase and Ca^{2+} channels. In insulin-resistant states and diabetes, plasma endothelin-1 (ET-1) concentration is increased, and this elevated plasma ET-1 concentration is associated with decreased coronary blood flow reserve and is recognized as a mediator of endothelial dysfunction in coronary artery disease [32]. Diabetes is also associated with altered ET-1 signaling, as shown in aorta from obese Zucker rats where ET-1-mediated vasoconstriction is potentiated [33].

There is considerable evidence demonstrating enhanced reactive oxygen species as pathological factor responsible for impaired vasomotor function. Decreased dilation may be mediated by increased production of reactive oxygen species by vascular NADPH oxidase and reduced NO bioavailability. The majority of studies report no change in endothelial-independent vasodilation responses to sodium nitroprusside. This indicates impairment of NO-mediated dilation is not related to alterations in vascular smooth muscle responsiveness to NO.

Increased generation of reactive oxygen species is an underlying cause of vascular dysfunction in diabetes. The source of vascular oxygen free radicals in diabetes is not clear. NAD(P)H oxidase is elevated by hyperglycemia, and increased NAD(P)H oxidase levels have been shown to increase superoxide generation. Gupte et al. have shown that hyperglycemic-induced increases in NAD(P)H oxidase activity did not come from an increase in the expression of the NAD(P)H oxidase subunits, but more likely as a result of chronic activation via intracellular signaling pathways [34]. Huang et al. used db/db mice to characterize complexities of endothelial

dysfunction related to changes in oxidative stress, NO bioavailability, and eNOS signaling during the progression of diabetes [35, 36]. They show vascular superoxide production was progressively increased, and shear stress-induced dilation was reduced in arteries from 3-month mice and further attenuated in arteries from 9-month mice. Elevated levels of free radicals reduce the bioavailability of NO via scavenging or inactivating NO and forming peroxynitrite, which is a highly reactive species, to uncouple eNOS and induce nitrotyrosine formation of signaling molecules. There is also evidence that superoxide and peroxynitrite are mediators of pancreatic cell death and may serve as pathogenic factor precipitating diabetes [37].

An interesting study performed by Belin de Chantemele et al. subjected arteries to normal or high flow by alternatively ligating mesenteric arteries in lean Zucker and Zucker diabetic fatty rats [16]. They found superoxide production (dihydroethidium staining) was higher and ACh-mediated dilation was lower in high-flow arteries when compared to normal-flow arteries. Superoxide overproduction in ZDF rats impaired NO-dependent dilation and high-flow remodeling. The increased ROS production induced by type 2 diabetes altered the ability of arteries to adapt their structure and function in response to a chronic increase in blood flow. The impairment was reversed by an antioxidant treatment.

There may be further defects in agonist-induced signaling in coronary arteries. Possible candidates for defects could include diabetes-induced alterations in protein kinase A and the protein kinase C (PKC) pathways. The link between diabetes and increased activation of the PKC pathway is believed to involve the state of chronic hyperglycemia which leads to an increased level of circulating advanced glycosylated end products (AGEs). The AGEs bind to the endothelial-bound signal-transduction receptor RAGE, which in turn leads to activation of the smooth muscle cell PKC and thus increases oxidative stress.

Ion channels in cells of the vascular wall are important for determining vascular tone. Coronary smooth muscle cells have a relatively steady membrane potential. In coronary smooth muscle cells, membrane potential is maintained by calcium and potassium ions channels. When cells are hyperpolarized, intracellular Ca^{2+} is reduced and promotes vasodilation. A depolarizing stimulus increases intracellular Ca^{2+} to produce vasoconstriction. There are 2 types of Ca^{2+}-mediated channels, the L-type (long lasting) and t-type (transient) channels. There are four classes of potassium (K^+) channels expressed in coronary vascular smooth muscle cells: (1) voltage-dependent (Kv), (2) Ca^{2+}-activated (Kca), (3) ATP-sensitive (Katp), and (4) inward rectifier (Kir) channels. Voltage-gated potassium channels are important for regulating membrane potential and determining coronary vascular resistance and blood flow. Bubolz et al. have shown enhanced peroxynitrite production in diabetic rats contributes to voltage-gated potassium channel dysfunction in the coronary microcirculation [38]. There are three subtypes of calcium-activated K^+ channels: small (SKca), intermediate (IKca), and large or high (BK_{Ca}), named due to their conductance abilities. BK_{Ca} channels may play a compensatory dilator role in disease states such as diabetes due to increased release of EDHF, which activates BK_{Ca} when less NO is available [39]. Katp is the most studied K^+ channel and, under normal metabolic conditions, has a very low open-state probability in vascular smooth muscle cells.

However, during diabetes, aprikalim (Katp opener)-induced dilation has been shown to be enhanced in coronary microvessels of diabetic dogs [40]. It has been shown that glibenclamide, a Katp channel inhibitor, reduces coronary blood flow and coronary venous PO$_2$. This suggests that the role of Katp channels in regulating coronary smooth muscle membrane potential is altered in diabetic states. Kir has the highest expression in resistance vessels, which suggest role in regulation of coronary blood flow; however, few studies have focused on Kir in the diabetic coronary circulation.

Diabetes substantially increases the risk of developing coronary disease. Mechanisms responsible for increased risk of coronary artery disease in patients with diabetes include hyperglycemia, elevated free fatty acids, insulin resistance, reduced NO production, increased NO inactivation, increased inflammatory status, and increased production of advanced glycosylated end products. Each of these factors promotes increased oxidative stress and endothelial dysfunction. In the coronary microcirculation, endothelial dysfunction causes chronic vasodilation that leads to increased capillary pressure and hyperperfusion, which in turn leads to morphologic changes that narrow the lumen and compromise the bioavailability of nitric oxide. These changes limit the ability of the diabetic coronary circulation to increase myocardial perfusion to meet an increase in myocardial nutrient demand.

A better understanding of the underlying microvascular and endothelial pathophysiology associated with diabetes that contributes to cardiovascular disease would help to develop new targets for prevention and treatment of vascular complications associated with diabetes.

With the increasing prevalence of diabetes, it is worthwhile that significant effort be made to improve our understanding of the etiology underlying cardiovascular complication associated with diabetes. An option for decreasing the late stage complications of diabetes may be intervening earlier in the disease process before vascular dysfunction occurs. Future research should focus on areas to improve strategies to prevent and treat diabetes and its complications at the molecular, cellular, organ, animal, and population levels.

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Chapter 4
Chronic Kidney Disease and Cardiovascular Risk

Jaya P. Buddenini, Kunal Chaudhary, James R. Sowers, and Adam Whaley-Connell

4.1 Introduction

There is a strong relationship between chronic kidney disease (CKD) and cardiovascular disease (CVD) risk [1–3]. The increased risk for CVD in those with CKD is heightened in the presence of traditional Framingham CVD risk factors such as type 2 diabetes, hypertension, and dyslipidemia, and the ensuing CVD contributes to a more rapid progression to end-stage renal disease (ESRD), defined as glomerular filtration rate (GFR) <15 mL/min/1.73 m^2 [4]. It should also be noted that the relationship between CKD and CVD events is a graded one, wherein there exists a strong linear relationship between diminishing GFR and increasing CVD events. In this context, there is an alarming trend wherein younger ESRD patients have an equivalent CVD risk equivalent to those above 65 years of age in the general population. Thereby, there is growing interest in CVD risk reduction strategies in

J.P. Buddenini
Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh, 3459 Fifth Avenue, 628 NW, Pittsburgh, PA 15213, USA

K. Chaudhary
Divisions of Clinical Medicine and Nephrology, Harry S. Truman Memorial Veterans' Hospital, Columbia, MO 65201, USA

J.R. Sowers
Thomas W. and Joan F. Burns Missouri Chair in Diabetes, Director of the MU Diabetes and Cardiovascular Center, Director, Division of Endocrinology, Diabetes Center, University Hospital, Columbia, MO 65212, USA

A. Whaley-Connell (✉)
Harry S. Truman Memorial Veterans Hospital, 800 Hospital Drive, Columbia, MO 65201, USA

Division of Nephrology and Hypertension, University of Missouri-Columbia School of Medicine, 800 Hospital Drive, Columbia, MO 65201, USA
e-mail: whaleyconnela@health.missouri.edu

earlier stages of CKD not only to reduce the CVD burden but also to reduce progression of CKD [5].

The mounting evidence of the kidney cardiovascular relationship prompted the National Kidney Foundation (NKF) task force on CVD in chronic renal disease to consider CKD as a coronary artery disease equivalent for the purposes of risk stratification [6]. Moreover, the work group also recommended considering patients with CKD in the "highest-risk group" for subsequent CVD events [6]. This recommendation was largely based on findings suggesting that, even after adjusting for most traditional Framingham risk factors for CVD, the higher mortality noted in CKD subjects from CVD suggests the possible contribution of uremia-related, nontraditional risk factors [7]. This has led to an understanding of a complex association of both traditional- and nontraditional-related CVD risk factors in CKD patients. To better delineate this dynamic disease process, this chapter will focus on the pathophysiology of CVD in CKD subjects along with early identification of CKD to prevent disease progression.

4.2 Epidemiology

CKD is an ongoing public health dilemma affecting approximately 24–28 million, with an estimated 20 million yet unidentified, with more than one million of them receiving some form of renal replacement therapy [8, 9]. According to the United States Renal Data System 2010 annual report (USRDS), the incidence of Medicare CKD in patients aged 65 or older was 4.3 % in 2008, an increase from 1.2 % seen in 1995. The prevalence of CKD patients among Medicare patients aged 65 and older is noted to be 7.6 %, a 4.6 times increase from the rate of 1.7 % seen in 1995. The rising incident and prevalent rates for CKD are paralleled by an increasing annual cost of Medicare ESRD program in the USA approaching approximately 20.8 billion dollars. The estimated annual Medicare cost to treat patients with CKD is 57.5 billion US dollars, thus contributing to 28 % of the total Medicare expenditure [10].

4.3 Definition and Classification of CKD

The Kidney Disease Outcomes Quality Initiative (NKF KDOQI) established clinical guidelines in 2002 for the definition of CKD for staging purposes that continue to be adapted. The diagnosis of CKD is based on the presence or absence of structural damage to the kidney and the level of kidney function, irrespective of the cause [11]. CKD is defined as either (a) kidney damage ≥3 months, as confirmed by kidney biopsy or markers of kidney damage as noted by the presence of structural or functional abnormalities such as abnormal blood, urine, or imaging studies, with or without decrease in GFR, or (b)

GFR < 60 mL/min/1.73 m^2 for ≥3 months with or without kidney damage [11]. Staging of CKD is based on the level of GFR; stage 1 = 90–120 mL/min/1.73 m^2 and stage 2 = 60–90 mL/min/1.73 m^2 both require the presence of abnormal urine or imaging studies, wherein stages 3–5 do not; stage 3 = 30–60 mL/min/1.73 m^2; stage 4 = 15–30 mL/min/1.73 m^2, and stage 5 < 15 mL/min/1.73 m^2 is roughly equivalent to ESRD.

4.4   Pathophysiology of CKD

There are multiple risk factors that place individuals at risk for the development and more rapid progression of CKD, the most common of which are long-standing diabetes and hypertension. The pathophysiology of CKD involves a sequence of initiating events specific to the underlying etiology, leading to a set of common consequent mechanisms ultimately resulting in the reduction of renal mass and function. In those with diabetes, and to a lesser extent hypertension, there is an initial adaptive hyperfiltration mediated by elevation of glomerular capillary pressure and flow along with functional hypertrophy of the remaining nephrons. These initial adaptive responses eventually become maladaptive over a course of time and predispose to atrophy, fibrosis, and sclerosis of the remaining functional nephrons. There are numerous mechanisms that elicit the initial adaptive hyperfiltration and subsequent maladaptive tissue remodeling of nephrons such as inappropriate activation of the sympathetic nervous system and the renin–angiotensin–aldosterone system (RAAS) [12].

4.5   CKD as Risk Factor for CVD

As compared to age-matched control subjects without kidney disease, patients with CKD have increased CVD mortality even after adjusting for traditional CVD risk factors [10–14]. The strength of this association is driven by CKD patients with GFR < 60 mL/min/1.73 m^2 who are at increased risk for CVD compared to those with an eGFR > 60 [13]. Further, approximately half of the mortality in ESRD patients has been attributed to heart disease [10]. However, it is important to note this is a graded, linear relationship that begins in the earliest stages of CKD with GFR approaching 120 mL/min/1.73 m^2 and with proteinuria. The observation the majority of individuals do not reach the requirement of renal replacement therapy (e.g., dialysis or transplantation) due to the high CVD mortality has led to an increase in scientific exploration in prevention and detection strategies [15, 16]. It is known that individuals with CKD have a high prevalence of other disorders that independently are associated with poor CVD outcomes, such as the presence of diabetes and hypertension; reduced physical activity; and

the presence of high concentration of inflammatory or oxidative biomarkers and deranged lipid parameters.

4.6 Traditional and Nontraditional CVD Risk Factors in CKD

The relationship between CKD and CVD is largely considered to be due to the occurrence of many common traditional Framingham risk factors such as hypertension, diabetes, dyslipidemia, and advancing age [17, 18]. However, there has been little information on successful CVD risk prediction with established equations in subjects with CKD, suggesting the presence of other risk factors that confer additional CVD risk in CKD. Uremia-related risk factors, the term first used by Sahart et al., refers to the risk factors that accumulate in CKD patients as a result of impaired renal clearance [19].

4.6.1 CKD and the Cardiorenal Metabolic Syndrome

Metabolic syndrome (e.g., cardiorenal metabolic syndrome) is a constellation of metabolic abnormalities including the presence of 3 or more clinical abnormalities such as hypertension, diabetes, atherogenic dyslipidemia, abdominal obesity, and albuminuria and/or diminished renal function that are associated with a pro-inflammatory and pro-thrombotic state. This constellation of metabolic and renal disorders is a risk factor for developing both CKD and CVD. Central to the metabolic dysregulation is inappropriate activation of RAAS [20, 21] and insulin resistance with the compensatory hyperinsulinemia that contribute to inflammation and oxidative stress and the development of endothelial dysfunction [17, 22–24]. Multiple cross-sectional [25, 26] and prospective studies [27] support the association between the cardiorenal metabolic syndrome and CKD. Furthermore, the risk for CVD-related outcomes in individuals with CKD increases incrementally with each component of the syndrome (e.g., hypertension, diabetes, obesity, and dyslipidemia) [28].

4.6.2 Role of Common Uremia-Related Comorbidities in the Pathogenesis of CKD-Related CVD

In addition to traditional Framingham CVD risk factors individuals with CKD possess intrinsic uremia-related risk factors such as mineral metabolism disorders, anemia, and increased levels of inflammatory and oxidative markers; abnormal

apolipoprotein levels; elevated plasma homocysteine [6]; and enhanced coagulability that independently contribute to the development of endothelial dysfunction as a precursor to CVD risk [25–31].

4.6.2.1 Mineral Metabolism Disorders: Calcium and Phosphorus Metabolism

During the early stages of CKD, a diminution of 1,25-vitamin D3 formation and gut calcium absorption occurs leading to compensatory increase in parathyroid hormone (PTH). A compensated state of increased phosphorus concentration, normal serum calcium concentration, and low normal vitamin D3, along with mild to moderate increases in PTH, exists until GFR declines to <30 mL/min. As kidney disease progresses to end stage, increases in phosphorus concentration and decrease in vitamin D3 ultimately result in overt secondary hyperparathyroidism (2HPT) in the majority of individuals of CKD. Recent epidemiologic data has shown a strong clinical correlation between hyperphosphatemia and CVD mortality in ESRD patients, manifesting as vascular calcification [32]. A 41 % increase in relative risk of death from coronary artery disease has been noted with serum phosphate concentration greater than 6.5 mg/dL, as has 20 % increase in mortality from sudden death [33]. Further, increased serum phosphate concentration has been noted as an independent predictor for mortality in ESRD patients [34].

The sequential effects of disturbed mineral homeostasis are mediated by promotion of vascular calcification, bone resorption, and direct PTH toxicity [29, 32]. Hyperphosphatemia is considered a potent stimulant of intimal and medial calcification of blood vessels [35]. Intimal calcification involves formation of atherosclerotic plaque, which upon destabilization leads to an adverse cardiovascular event. On the other hand, medial calcification increases arterial stiffness, thus decreasing vascular compliance without compromising arterial lumen [36]. Numerous indices of arterial stiffness such as aortic pulse wave velocity and elastic modulus are noted to be strong independent predictors of CVD in ESRD patients [31]. This association between abnormal bone-mineral metabolism and increased vascular calcification has been suggested as the major uremia-related risk factor contributing to increased risk of CVD in CKD population.

4.6.2.2 Anemia

Anemia is thought to be a contributing risk factor for cardiac remodeling leading to the development of left ventricular hypertrophy (LVH), congestive heart failure (CHF), and CVD mortality [30]. The development of anemia starts early in CKD and is multifactorial. Indeed, decreased levels of erythropoietin, iron depletion, chronic inflammation, bone marrow fibrosis, and impaired erythropoietin response are a few common causes of anemia in individuals with CKD [37]. Two different studies conducted using the National Health and Nutrition Examination Survey

(NHANES) III suggest the prevalence of anemia increases from 1 % at an eGFR of 60 mL/min/1.73 m^2 to 9 % at an eGFR of 30 mL/min/1.73 m^2 and to 33–67 % at an eGFR of 15 mL/min/1.73 m^2 [38–40]. Data from NHANES and the NKF's Kidney Early Evaluation Program (KEEP) support anemia in those 61 years and older with stage 3 or higher CKD [41].

Treatment of anemia with erythropoiesis-stimulating agents (ESAs) has shown to decrease LVH in CKD as well as ESRD patients on dialysis [42]. However, the use of ESAs remains controversial due to two randomized control trials, Correction of Hemoglobin and Outcomes in Renal Disease (CHOIR) [43] and Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) [44], studies which do not support improved outcomes among patients' subgroups randomized to higher hemoglobin level. Further, treatment of anemia in hemodialysis patients with severe cardiac disease was associated with increased risk of death in the Normalization of Hemoglobin trial [45]. However, in another Canadian study, normalization of LV dilatation did not show any increased risk of mortality [46]. Further randomized control studies are needed to delineate whether correction of anemia, and to what level, has with CVD morbidity and mortality.

4.6.2.3 Hyperhomocysteinemia

An elevated plasma homocysteine level is considered an independent CVD risk factor in the general population [6]. There are numerous conflicting studies indicating a potential role for homocysteine in CKD and CVD mortality and morbidity. Homocysteine levels are persistently elevated in ESRD patients and also in patients with cardiac diseases. Another study suggests that the antioxidant drug acetylcysteine reduces plasma homocysteine level to normal range and is associated with improvements in endothelial dysfunction and CVD events when administered long term in patients undergoing hemodialysis [47]. However, it is unclear the role the diminished kidney function and clearance has to elicit this relationship. A recent study would suggest that elevations in plasma homocysteine levels may simply be a function of reductions in GFR [48]. Thereby, further long-term interventional studies are needed to better understand the role of homocysteine for CVD risk in CKD.

4.6.2.4 Inflammatory and Oxidative Stress

C-reactive protein (CRP) has been observed to be elevated in patients with kidney disease [49] and has been shown to be an independent predictor of CVD events in the general population [50]. Recent data would suggest CRP may be a marker for CVD in individuals with CKD undergoing renal replacement therapy with peritoneal dialysis, hemodialysis, or post-kidney transplant [51–54]. In earlier stages, CRP was also noted to be an independent predictor of CVD events in women with creatinine clearance <74 mL/min and with no underlying CVD [54], thereby suggesting a potential role for inflammation in the development of CVD in CKD. To

further substantiate the role of inflammation in CVD, the use of aspirin is associated with CVD risk reduction directly related to CRP levels [50].

Oxidative stress has also been noted as an underlying mechanism for CVD in CKD potentially due to ongoing low-grade inflammation and impaired antioxidant mechanisms [55]. The strength of the association between the cardiorenal metabolic syndrome and CKD underscores the significance of oxidative stress due to metabolic dysregulation in the pathogenesis of CVD in CKD due to excess reactive oxygen species [56].

The evidence derived from numerous cross-sectional, population-based, and prospective studies supports the role of uremia-related risk factors in CVD in CKD. However, a direct relationship between intervention focusing on uremic, nontraditional risk factors and CVD risk reduction has yet to be established, and further large-scale randomized controlled trials are needed to verify these associations.

4.7   Screening and Detection

It is not known whether population-based screening of CKD is cost-effective. In a recent study, population-based screening for CKD with assessment of estimated GFR was found to be not cost-effective in subgroups with hypertension or older people. However, targeted screening of patients with diabetes was associated with cost-effectiveness [57]. Current practice guidelines promote early screening and detection of CKD patients in order to prevent the progression of kidney disease. Several initiatives like NKF-sponsored KEEP, National Institutes of Health (NIH) Healthy People 2010, and the National Kidney Disease Education Program (NKDEP) have emphasized educating patients as well as healthcare professionals about the positive impact of early screening and diagnosis. At this point, it is conventional wisdom that a concerted team effort by primary care physicians and subspecialists is necessary to tackle this public health dilemma [58].

The NKF Kidney Disease Outcomes Quality Initiative (KDOQI) recommends screening at-risk individuals for CKD using blood pressure, GFR estimation, urine albumin to creatinine ratio, urine analysis, and imaging studies of kidneys (in select at-risk individuals) [59]. Those identified as highest risk are individuals with diabetes, hypertension, autoimmune diseases, and patients recovering from an episode of acute renal failure or with family history of kidney diseases.

The most common indices used in clinical practice for evaluation of CKD are serum creatinine (sCr) as a marker for clearance and then estimating GFR as well as determination of proteinuria. Even though sCr is the most commonly used test in clinical practice to assess renal function, sCR may not be the most accurate in early stages of kidney disease when screening and detection are critical. There are multiple reasons including biologic, pharmacologic, and estimation misclassification. In this context, rises in sCr appear only after significant loss of functioning nephrons. Moreover, the generation of sCr is based on muscle mass and diet, and the excretion or secretion of sCr is influenced by drugs such as cephalosporins,

aminoglycosides, cisplatin, cimetidine, and trimethoprim. However, estimating GFR may be the best available index for kidney function. The National Kidney Disease Educational Program (NKDEP) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and American Society of Nephrology (ASN) recommend estimating GFR from serum Cr by using either Modification of Diet in Renal Disease (MDRD) study equation or Cockcroft-Gault equation [60, 61]. Both equations take into account sCr along with age, sex, and weight variables thus minimizing the limitations of using sCr alone. However, there are limitations of the MDRD equation for estimation of GFR due to imprecision and systematic underestimation of GFR at higher levels [62]. Thereby, in 2009 a recent adaption for estimating GFR, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [63], may overcome the limitations of MDRD in earlier stages for screening, detection, and classification of CKD.

Proteinuria is another accepted marker for kidney damage and serves as a guide for screening and detection for CKD especially in earlier stages. However, the presence of increasing levels of proteinuria in CKD is associated with a poor prognosis for both progression of CKD and the development of CVD [64, 65]. Thereby accuracy is important and measurement of albumin to creatinine ratio or total protein to creatinine ratio in untimed spot urine samples is widely accepted for assessment of proteinuria [4]. One of the earliest markers of kidney disease is microalbuminuria, defined as urinary albumin excretion between 30 and 299 mg/24 h. Annual screening allows early identification of CKD in those at highest risk and also serves as a prognostic tool [66].

4.8 Treatment

The treatment options for CKD patients are focused on risk factor reduction and interventions to prevent or slow the progression of CKD and importantly decrease risk for CVD-related outcomes. Treatment guidelines for risk factor reduction focus on blood pressure and glycemic control in CKD. Evidence supports that reduction in systolic blood pressure without decreasing albuminuria is insufficient in preventing the progression of CKD. Thereby, optimization of blood pressure with therapies targeting proteinuria should be a primary goal [67]. The reduction of proteinuria has shown protective effects in CVD risk in those with diabetic kidney disease [67]. Both the Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS) support the decreased risk of development of microalbuminuria and overt nephropathy with intensive glycemic and blood pressure control [68, 69]. However, the UKPDS further supports that blood pressure reduction may take precedence.

There is sufficient evidence to support utilizing interventions that target RAS that have shown to improve CVD-related outcomes and kidney-related outcomes in patients with or without diabetes [70]. The African American Study of Kidney Disease and Hypertension (AASK) addressed the optimal drug regimen

for African Americans with hypertensive renal disease supported by the rationale that ACE inhibitors that improved renal outcomes [71, 72]. Moreover, the Lotrel and Enalapril in African Americans with Diabetes (LEAAD) study, conducted in African American patients with both hypertension and diabetes, concluded that combination therapy with ACE inhibitor/CCB (calcium channel blocker) was much better in achieving RAS blockade and BP reduction compared to monotherapy with ACE inhibitors [73].

4.9 Conclusion

Recent work has clearly established a strong relationship between CKD and an increased CVD risk. In this context, the relationship is a graded linear relationship beginning at the earliest stages of CKD, thereby highlighting the importance for detection of CKD early to improve kidney-related outcomes. Current recommendations by NKF and other societies classify individuals with CKD in the highest-risk group for CVD. Recent studies in CKD population have noted the concurrent presence of uremia-related risk factors along with traditional CVD risk factors leading to the development of CVD. However this association of uremia-related risk factors is yet to be conclusively proven to establish a causal relationship. In the clinical practice, CKD is a compelling indication for aggressive blood pressure control. However, additional risk factor reduction strategies in CKD patients should be pursued by clinicians.

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Chapter 5
Mitochondria and Oxidative Stress in Diabetes

William I. Sivitz

5.1 Introduction

The pathogenesis of type 2 diabetes includes pancreatic β-cell dysfunction and insulin resistance, most importantly in hepatocytes, myocytes, and adipocytes. Type 2 diabetes is also well known to be a progressive disorder [1] characterized by both deteriorating capacity for insulin release and insulin action. Both defects are recognizable early in the course and present even in nondiabetic offspring of patients with type 2 diabetes [2–4]. In contrast, the pathogenesis of type 1 diabetes involves one major organ and cell type, in other words, autoimmune destruction of pancreatic β-cells. At the cellular and molecular levels, the pathogenesis of both type 1 and type 2 diabetes is far more complex. Here the focus will be on the role of mitochondria and mitochondrial reactive oxygen species (ROS).

Type 2 and autoimmune type 1diabetes are clearly *associated* with altered mitochondrial function, including ROS production, although cause and effect relationships remain in dispute. Several studies document the existence of oxidative damage in diabetes. For example, plasma markers of lipid peroxides such as 8-iso-prostaglandin F2α [5], conjugated dienes, and lipid hydroperoxides [6] are elevated early in the course of type 1 diabetes, while antioxidant capacity assayed as total plasma antioxidant capacity (TRAP) is reduced [6]. Moreover, DNA damage is detectable in circulating lymphocytes of subjects with insulin-dependent diabetes and correlates to the extent of glucose elevation [7]. There is also strong evidence for oxidative damage in cells and tissue of persons with type 2 diabetes including blood hydroperoxides [8] and PGF2 alpha [9] and evidence of oxidative damage to DNA [10–12].
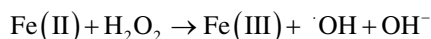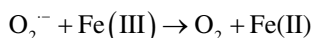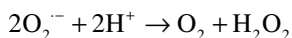
W.I. Sivitz (✉)
Department of Internal Medicine, University of Iowa,
East 422 GH, Iowa City, IA 52242, USA
e-mail: William-sivitz@uiowa.edu

Although ROS clearly originate from multiple cell components, considerable evidence points to mitochondrial ROS as particularly important in disease states including diabetes and its long-term complications of diabetes. It has been suggested that elevated glucose and/or free fatty acids drive the formation of ROS [13–15] impairing both β-cell insulin release and insulin sensitivity and contributing to the complications of diabetes [13, 16, 17]. A common supposition, although probably oversimplified, is that metabolism of these nutrients generates high levels of substrate flux to mitochondria resulting in high mitochondrial NADH/NAD and FADH2/FAD ratios and high potential at low respiration rates (closer to state 4 conditions) and, thereby, more electron leak [17, 18]. In particular, this would apply to cells which take up glucose by facilitated diffusion independent of circulating insulin, characteristic of the classic sites of diabetic complications including the retina, kidney, neurons, and vascular endothelium [19]. On the other hand, insulin-sensitive cells including the muscle, heart, and liver depend on insulin for glucose uptake and/or metabolism. Hence, other factors are likely important in generating the diabetes-related oxidative damage observed in these cells.

The following text will first address mechanisms whereby mitochondria generate ROS. Subsequently, we discuss the detection and quantification of ROS production. This is followed by a review of evidence for oxidative damage to the cell types most relevant to diabetes, including myocytes, hepatocytes, adipocytes, and islet β-cells as well as non-insulin-sensitive cells representing targets for complications.

5.2 Mitochondrial ROS Production

The mitochondrial electron transport system generates superoxide derived from electron leaks as substrates are metabolized [20]. Biologically important ROS include the superoxide radical, $O_2^{\cdot-}$; the hydrogen peroxide, H_2O_2; and the hydroxyl radical, $OH\cdot$. At physiologic pH, superoxide-induced damage is limited in that the species self-reacts (dismutates) or, more efficiently, is catalyzed by superoxide dismutase (SOD) to form H_2O_2 [21] which is scavenged by catalase after exit to the cytoplasm. Thus, superoxide and H_2O_2 per se are not thought to be particularly destructive. However, there is still potential for marked damage due to lipid peroxidation, reaction with nitric oxide (NO) to form peroxynitrite, and through generation of the damaging hydroxyl radical through a series of steps dependent on the presence of redox metals such as iron or copper. This occurs as follows:
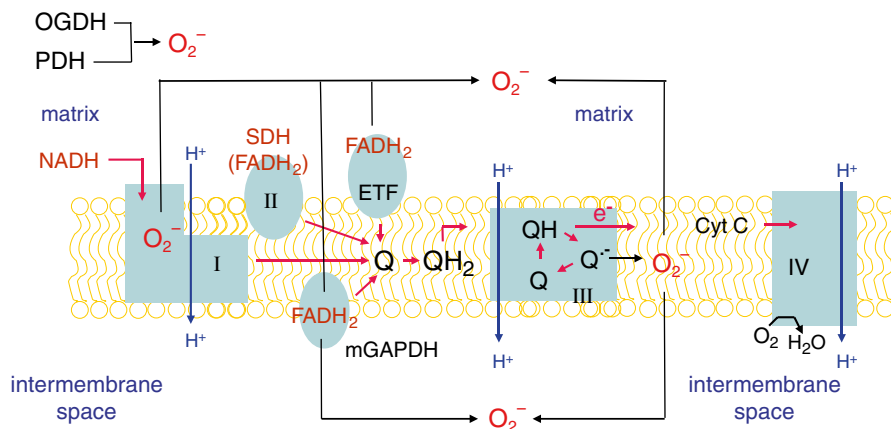
$$2O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2$$

$$O_2^{\cdot-} + Fe(III) \rightarrow O_2 + Fe(II)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\cdot}OH + OH^-$$

Fig. 5.1 Mitochondrial electron transport and ROS production. The schematic diagram depicts the convergent nature of electron donation at one of the four sites, complex I (NADH ubiquinone reductase), complex II (succinate dehydrogenase), the electron transfer flavoprotein (ETF), or a mitochondrial form of GAPDH. Reduced ubiquinone is processed through the Q cycle in complex III where protons are pumped and electrons passed to mobile cytochrome *c* and then cytochrome oxidase. ATP formation through ATP synthase (not shown) is coupled to mitochondrial potential generated by proton pumping at complexes I, III, and IV. Superoxide (O_2^-) is produced at the sites indicated with release to either the matrix, intermembrane space, or both. In addition, superoxide is generated by pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH) although nature of this and localization of the released radical is not clear. *Red arrows* depict electron transport. *Blue arrows* depict H+ movement away from the matrix. *Black arrows* depict electron leaks leading to one electron reduction of oxygen to O_2^-

Mitochondria are considered the major intracellular site of superoxide production [17, 22, 23], albeit exact quantification is difficult. The mitochondrial contribution varies with the respiratory state being greater near state 4 when membrane potential is less mitigated by ATP synthesis [24]. The major sites of superoxide production within mitochondria are somewhat uncertain, but large amounts appear to derive from complexes I and III [23] (Fig. 5.1). Complex I superoxide is released nearly exclusively to the matrix side of the inner membrane, whereas complex III generates superoxide to both the matrix and outward to the intermembrane and extra-mitochondrial space [25, 26]. As recently reviewed [27], there is credible evidence for several sites wherein mitochondria generate superoxide. Prominent among these are two sites in complex I termed site IF (the FMN-containing NADH binding site) and site IQ (an ubiquinone reduction site). Site IF generates superoxide under conditions of forward electron transport during complex I substrate oxidation. Its activity can be increased if downstream transport within complex I is blocked (e.g., by rotenone) in which case upstream redox sites are fully markedly reduced. In contrast, the IQ site becomes highly active when electrons donated at complex II are delivered to complex I through a process termed reverse electron transport [28]. Whether or to what extent reverse transport actually occurs in vivo under physiologic conditions is not clear. Considerable superoxide is also generated

in complex III during redox cycling (cyclic conversion of reduced ubiquinol to oxidized ubiquinone and back) in complex III. This occurs in a site termed IIIQo representing the outer quinone-binding site of the Q cycle, wherein the cycling intermediate semiquinone species leaks electrons to molecular oxygen. The half-life of the semiquinone is highly dependent on mitochondrial membrane potential (or $\Delta\Psi$) and thus can be regulated by uncoupling [29]. Redox cycling of CoQ may also occur within complex I although by a less defined mechanism(s) but also sensitive to $\Delta\Psi$.

In addition to superoxide production in complexes I and III, lesser amounts are derived from other mitochondrial sites. These include pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, the electron transferring flavoprotein (the entry point of electrons from flavin-linked beta-oxidation of fatty acids), and glycerol 3-phosphate dehydrogenase [27]. Some superoxide may also derive from the succinate dehydrogenase complex and cytochrome *c* although this is probably very little [27]. Finally, mitochondrial ROS are generated at the iron-sulfur centers in the aconitase protein where conversion of superoxide to the hydroxyl radical results in inactivation of the enzyme [30, 31].

Although this review is concerned with mitochondrial ROS, it should be noted that considerable ROS is derived from outside this organelle including oxygen radicals from peroxisomal β-oxidation of fatty acids [32], NAD(P)H oxidase [33], xanthine oxidase, arachidonic acid metabolism, microsomal P450 enzymes [34], and the prooxidant heme molecule [35].

5.3   Assessment of Mitochondrial ROS and Oxidative Damage

In general oxidative stress (mitochondrial or other) is evident in two ways, first as ROS production in real time (e.g., superoxide production rates) and second as existent oxidative damage (e.g., lipid peroxides).

5.3.1   ROS Production

It is relatively easy to assess ROS production in isolated mitochondria but more difficult in intact cells or tissues, especially when we are interested in organelle-specific (mitochondrial) ROS. For isolated mitochondria, we and others often use the fluorescent probe, 10-acetyl-3,7-dihydroxyphenoxazine (DHPA or Amplex Red, Invitrogen), considered by some as optimal for ROS production by the isolated organelles [27]. The data can be easily quantified as H_2O_2 production per unit time per unit mitochondrial mass by including a standard curve generated by exogenous H_2O_2. However, it is important to remember that although DHPA is generally

considered a measure of superoxide production, it measures this radical indirectly as H_2O_2 generated by conversion of superoxide by mitochondrial SOD. Other probes including 2′,7′-dichlorodihydrofluorescein diacetate (DCF or H2DCF-DA) [36] and luciferin [37] have also been used to assess mitochondrial superoxide, although we believe with less specificity. Since specificity is concerning for any fluorescent probe, steps should be taken to further document the radical species being observed. For example, catalase or SOD (or analogs) can be used to metabolize H_2O_2 or superoxide, thereby supporting specificity by reducing or completely blocking the observed fluorescent signal.

In contrast to fluorescent probes, a highly specific, albeit far more cumbersome, means to assess mitochondrial superoxide is by electron paramagnetic resonance (EPR) spectroscopy. EPR can be carried out by adding spin trap to mitochondria incubated under desired conditions (substrate, inhibitors, etc.). We and others have used the spin trap, 5,5-dimethyl-l-pyrroline-N-oxide (DMPO) generating a specific signal representing either superoxide or the hydroxyl radical. Moreover, these two possibilities can be separated by adding SOD, which should abolish the signal generated by superoxide but not the hydroxyl radical. In our experience with skeletal muscle and endothelial cell mitochondria, essentially all the signal derives from superoxide.

We suggested a way to measure superoxide from isolated mitochondria in a manner that imparts a degree of specificity for matrix ROS compared to superoxide released external to the organelles [36]. Fluorescent H_2O_2 probes such as DHPA and EPR spectroscopy measure mitochondrial superoxide in different fashion. When DHPA is added to isolated mitochondria, the probe detects H_2O_2 generated from superoxide by matrix MnSOD. H_2O_2 so generated diffuses outward from mitochondria and reacts with horseradish peroxidase in the incubation medium to trigger fluorescence. H_2O_2 produced in this way derives largely from superoxide generated at complex I and released to the matrix [26]. In contrast, the EPR spin trap, DMPO, detects superoxide directly after efflux outward from mitochondria. Superoxide produced in this way derives largely from the Q cycle at complex III [26]. Some complex III superoxide is also released to the matrix. However, DMPO will not easily penetrate mitochondria and matrix superoxide is rapidly converted to H_2O_2, so the spin trap should detect very little matrix superoxide.

Theoretically, it is possible to assess complex III superoxide released to the cytoplasmic side of isolated mitochondria simply by measuring H_2O_2 production, for example, as DHPA fluorescence in the presence and absence of added SOD. Exogenous SOD should increase fluorescence to the extent that it was generated by conversion of externally released superoxide to H_2O_2. Superoxide production has been effectively assessed in this way in studies of the topology of the muscle, heart, and liver mitochondria [26], although that required mathematical correction for fluorescent interference.

5.3.2 Mitochondrial ROS Production in Intact Cells

Several studies measured intact cell total ROS production as H_2O_2 using fluorescent probes such as carboxy dichlorodihydrofluorescein (with more or less attention to radical specificity). However, most intact cell studies do not separate mitochondrial from cytoplasmic ROS. A degree of specificity for intact cell mitochondrial superoxide, as opposed to cytoplasmic, can be detected using mitochondrial-targeted dihydroethidine (DHE) or "MitoSOX." MitoSOX is a DHE derivative conjugated to the cation triphenylphosphonium resulting in potential-dependent accumulation of the probe in the mitochondrial matrix. The accumulation in the matrix should be very large as cationic triphenylphosphonium conjugated molecules accumulate many fold [38]. The difference in fluorescence between untargeted DHE and MitoSOX may provide a semiquantitative index of relative cytoplasmic and mitochondrial superoxide. A concern, however, is the degree to which MitoSOX could undergo oxidation in the cytoplasm, which is difficult to ascertain. Since DHE and MitoSOX do not measure H_2O_2, treatment with a SOD mimetic should decrease fluorescence and serve as a means of validation that superoxide is being measured. It is also important to consider that mitochondrial-targeted DHE is dependent on membrane potential to enter the organelles. Resolution of this requires that potential be monitored and an appropriate correction be applied. Although difficult, this has been accomplished using tetramethylrhodamine methyl ester (TMRM) to measure fluorescence in cerebellar granule neurons [39]. DHE (mitochondrial targeted or not) has been criticized as nonspecific and some advocate analysis of the oxidation products by high-pressure liquid chromatography (HPLC) to document specificity for superoxide as opposed to H_2O_2 [40].

Mitochondrial ROS have also been assessed using the dye, JC-1, whose fluorescence changes from green to red fluorescence dependent on the mitochondrial membrane potential. Difficulties include specificity and difficulty quantifying the signal. Approaches have also been used to assess ROS in intact tissues or even in vivo using DHE or certain dyes whose properties depend on oxidation states within cells [41, 42], although mitochondrial specificity is even more challenging. A novel EPR approach to this issue has recently been described. Differentially targeted EPR spin traps were used to assess mitochondrial ROS in intact lymphocytes in a study describing an interactive effect of mitochondrial ROS with phagocytin NADPH oxidase [43].

5.3.3 Oxidative Damage

As opposed to ROS production or production rates, existent oxidative damage can be assessed by several markers within cells, tissues, blood, and urine. Since these are not specific to mitochondrial ROS, isolation of the organelles or careful

visualization of fluorescent probes is necessary. Commonly used markers are available for DNA damage, lipid peroxidation, and protein oxidation. Examples include 8-hypdoxyguanosine for RNA or DNA damage, 4-HNE (4-hydroxynonenal) or TBARS (thiobarbituric acid reactive substances) for lipid peroxidation, and nitrotyrosine or oxidized glycation products (glycoxidation) for protein damage. A decrease in aconitase activity in isolated tissue or mitochondrial samples can also be used as a marker of mitochondrial oxidative damage [30, 31]. In work by this author and colleagues, we assessed markers of protein glycation and glycoxidation in skin biopsy samples from a large population of well-characterized subjects with type 1 diabetes. Carboxymethyllysine, an advanced glycation end product reflecting glycoxidation, and the glycation marker, furosine, predicted the progression of microvascular complications of diabetes even after adjustment for hemoglobin A_1c levels [44]. The extent of superoxide release (after the fact as opposed to real time) has been measured in situ in whole vessel aortic tissue using DHE or even mitochondrial-targeted DHE [42, 45]. Finally, antioxidant enzyme content and activity can also be assessed as reflecting oxidative stress. However, it is difficult to know whether changes in these parameters reflect actual damage versus adaptive ongoing protection.

5.4 Diabetes-Related Oxidative Stress in Mitochondria in Specific Cell or Tissue Type

Here we will consider mitochondrial ROS and oxidative damage within the cell types most relevant to diabetes including myocytes, hepatocytes, adipocytes, and islet β-cells as well as non-insulin-sensitive cells representing targets for complications. We will attempt to integrate defects in a way consistent with the pathophysiology of diabetes and its complications.

5.4.1 Oxidative Damage and Pancreatic Islet β-Cells

Most cases of type 1 diabetes result from autoimmune destruction of islet β-cells, and ROS may account for a significant part of this damage. Of note is that levels of protective antioxidant enzymes including SOD, catalase, and GPX are relatively low in islets compared to the liver, kidney, brain, lung, skeletal muscle, heart, adrenal gland, and pituitary gland [46]. So, this may account for a particular sensitivity of pancreatic β-cells toward cytotoxic damage, as evidenced by sensitivity to certain toxins, for example, alloxan or streptozotocin, which are agents known to cause free radical damage to islets [47, 48]. Overexpression of SOD [49] or glutathione peroxidase [50] mitigates radical-induced islet damage due to these compounds.

Moreover, antioxidant treatment reportedly improves the function of murine islets after transplantation in mice [51]. There is also evidence that high circulating glucose, once established, increases islet cell H_2O_2 content with subsequent toxicity including reduction of the transcriptional factor PDX-1 which is critical for activation of the insulin gene promoter [52].

Interestingly, prooxidant heme compounds may have a role in the islet pathology of diabetes. Induction of heme oxygenase-1 (HO-1) with cobalt protoporphyrin (CoPP) in nonobese diabetic (NOD) mice increased anti-apoptotic proteins in the pancreas [53]. Heme oxygenase catalyzes the rate limiting step in heme degradation converting heme to biliverdin while consuming oxygen and generating Fe^{2+} and carbon monoxide [54]. This may affect mitochondrial function, at least based on studies in renal mitochondria of diabetic rats. These studies showed that CoPP increased the expression of the carnitine, citrate, deoxynucleotide, dicarboxylate, and ADP/ATP carriers associated with an increase in cytochrome *c* oxidase activity and phosphorylation of the anti-apoptotic proteins AKT and BcL-XL [55].

Oxidative damage to islet β-cells has also been observed in human type 2 diabetes by nitrotyrosine staining of islets obtained at autopsy [56]. Moreover, islets from rats exposed to high fat in the form of oleate infused in vivo demonstrated impaired glucose-stimulated insulin release. This could be inhibited by the antioxidants, taurine or *N*-acetylcysteine which increase glutathione [57]. When incubated ex vivo, the islets which had been exposed to oleate demonstrated increase H_2O_2 production again preventable by the antioxidant compounds or by the SOD mimetic Tempol.

Mitochondrial uncoupling appears important in ROS mediated islet toxicity. This might be expected based on logic since it is well known that high mitochondrial membrane potential increases superoxide generation by the electron transport chain [28, 29]. Of note is that superoxide is itself a signal activating uncoupling protein-2 (UCP2) [58]. This could be construed as an adaptive means to reduce potential and protect against superoxide. Emre et al. [59] found that mice deficient in UCP2 were more sensitive to diabetes induced by multiple low doses of streptozotocin compared to wild-type mice. This was accompanied by evidence for increased damage due to ROS and nitric oxide radicals along with greater intra-islet lymphocytic infiltration.

Based on the above, one could speculate that control of mitochondrial membrane potential through an agent capable of mild uncoupling might be a useful therapeutic tool. However, even if this were feasible, it is important to remember that uncoupling reduces ATP production, a process dependent on mitochondrial inner membrane potential. Further, ATP is critical to insulin release through the well-established mechanism of triggering closure of K_{ATP} channels resulting in calcium entry, depolarization, and insulin release. In fact, UCP2-deficient mice have higher islet ATP levels and increased glucose-stimulated insulin secretion [60]. These concepts are depicted in (Fig. 5.2).
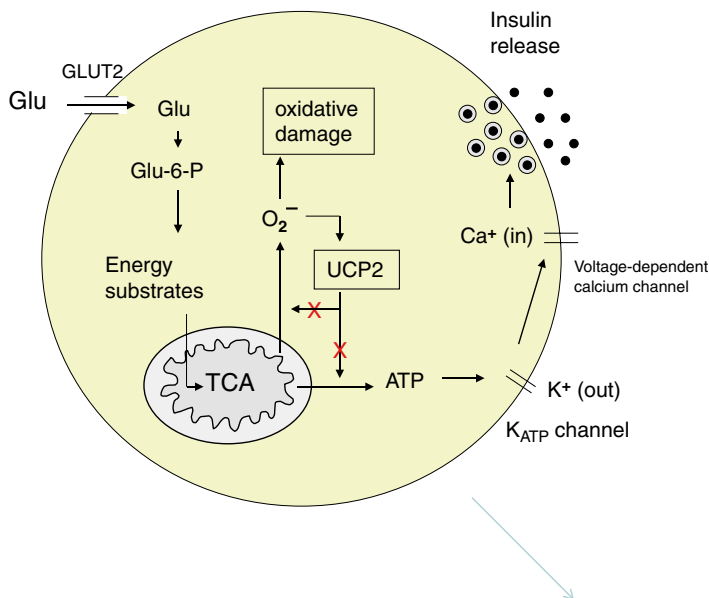
Fig. 5.2 Mitochondrial superoxide and insulin secretion. As shown, glucose sensing and glucose-induced insulin release are dependent on mitochondrial ATP generation and affected by both mitochondrial ROS and uncoupling protein-2 (UCP2). ATP is essential for opening of potassium ATP channels and, therefore, for entry of calcium and insulin release from storage granules. Under conditions of high nutrient flux, it is possible that excess superoxide leads to oxidative damage which, over time, leads to worsening diabetes. As a possible means of compensation, superoxide is shown to activate UCP2 decreasing membrane potential ($\Delta\Psi$) and radical formation but also reducing ATP production. *Red X* indicates negative effect

5.4.2   ROS and Oxidative Damage in Insulin-Sensitive Target Tissues

5.4.2.1   Skeletal and Cardiac Muscle

Skeletal muscle and heart depend strongly on insulin for glucose uptake and metabolism while liver depends on insulin for glucose metabolism. So, any factor that impairs these processes, ROS or other, will lead to insulin resistance. In fact, a common explanation for the duality of insulin resistance and impaired insulin secretion that characterizes type 2 diabetes is ongoing damage to mitochondria of insulin-sensitive peripheral cells [61] along with progressive impairment in mitochondria of islet β-cells [17].

Muscle represents the major peripheral tissue which transports and utilizes glucose in response to insulin. However, because of the dependency of glucose transport on insulin, muscle mitochondria are not subject to glycemia-driven ROS in the same way as non-insulin-sensitive cells. On the other hand, this is not the case for fatty acids, which circulate in higher concentrations in both type 1 and type 2
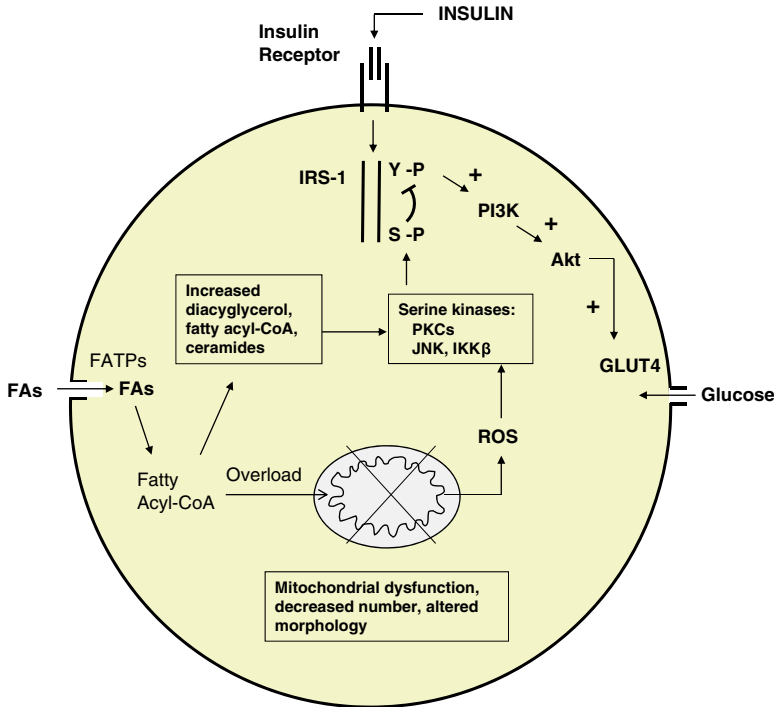
Fig. 5.3 Consequences of excess fatty acyl-coenzyme A (acyl-CoA) and reactive oxygen species (ROS) production on the insulin signaling pathway leading to the insulin-responsive glucose transporter, GLUT4. In response to insulin, tyrosine residues undergo autophosphorylation, and the IR acquires tyrosine kinase activity leading to phosphorylation of the insulin receptor substrate-1 (IRS-1). This initiates a signaling cascade activating serine/threonine kinase-protein kinase B (Akt) and translocation of the GLUT4 to the cell membrane. GLUT4 then fuses with the plasma membrane resulting in glucose uptake by facilitated diffusion. Mitochondrial dysfunction opposes insulin signaling by (1) interfering with oxidation of fatty acyl-CoA and consequent accumulation of intracellular lipid and diacylglycerol and (2) by generation of ROS. Both processes activate serine kinase reactions leading to serine phosphorylation of IRS-1 and interference with insulin signal transduction. *FA* fatty acid, *FATPs* refers to various transport proteins active in fatty acid uptake

diabetes in the untreated state. Intramyocellular lipid content is elevated in humans with obesity, diabetes, and insulin resistance, and much of this lipid is actually localized near mitochondria [62] and potentially sensitive to ROS-induced peroxidation. Indeed, lipid peroxides are elevated in muscle of subjects with obesity and insulin resistance [63]. Figure 5.3 depicts processes triggered by fatty acid exposure leading to insulin resistance.

Fatty acids and lipid peroxides induce uncoupling protein-3 (UCP3)-mediated uncoupling which in theory may be beneficial by reducing ROS and enhancing export of toxic fatty acids [64]. But, as is the case for islet cells, uncoupling may be

a beneficial compensatory response but one that might have a cost in terms of an uncoupling-induced decrease in ATP production.

Boudina et al. [65] reported an increase in ROS production, a decrease in ATP production, and an increase in a marker of oxidative damage (4-HNE) in heart mitochondria of insulin-resistant, obese, and leptin receptor-deficient db/db mice, a model of extreme obesity associated with diabetes. But interestingly, this group noted a decrease in ROS production from heart mitochondria of an insulin-deficient model, the Akita mouse, which more closely resembles human type 1 diabetes [66]. Hence, these findings suggest fundamental differences in the mechanisms underlying ROS production and ROS protection between heart mitochondria of insulin-deficient mice compared to mitochondria isolated from an obese, insulin-resistant strain. We also noted no increase (or an actual decrease) in ROS production measured both as fluorescent H_2O_2 release and as superoxide by EPR from mitochondria of the heart, gastrocnemius muscle, and liver of insulin-deficient rats made diabetic with streptozotocin (STZ) [67]. These findings were associated with an upregulation of MnSOD and UCP3 as well as cytoplasmic catalase in the heart and muscle and an increase in glutathione peroxidase in the liver mitochondria. Hence, the upregulation of antioxidant protection does suggest that islets isolated from insulin-deficient mice had been exposed to antecedent in vivo oxidative stress.

In this regard, we point out an important caveat applicable to the above studies of ROS in insulin-deficient diabetes and to many other studies. This has to do with the interpretation of mitochondrial ROS data. In our studies of STZ diabetes, respiration was also reduced. In isolated mitochondria, respiration is proportional to electron transport culminating in electron transfer to molecular oxygen. Importantly, when superoxide production was normalized to respiration, superoxide was actually significantly increased (not decreased) in muscle mitochondria of STZ-diabetic rats, i.e., ROS per unit electron transport was increased. A simple analogy underscores the importance of the metric, ROS per unit electron transport. ROS production viewed independent of e− transport is analogous to motor vehicle heat generation viewed independent of the speed of the vehicle. In a recent manuscript, we also reported an increase in superoxide generation per unit ATP produced in STZ-diabetic muscle mitochondria [68].

We also examined superoxide production both as H_2O_2 fluorescence and by EPR spectroscopy in mitochondria isolated from the muscle, heart, or liver of rats subject to high-fat feeding along with a low dose of streptozotocin [69]. These treatments led to a state resembling very mild human type 2 diabetes or "prediabetes" defined as an increase in the fasting blood glucose to over 100 mg/dL but not over 125 mg/dL [70]. Our results did not show excess superoxide production (or an alteration in respiration) indicating that the mitochondria, incubated in vitro, were not intrinsically altered to generate excess ROS.

As indicated above, there is evidence for oxidative DNA damage in type 2 diabetes [10–12]. This is supported by cell culture studies wherein L6 myotubes exposed to high fat manifest mitochondrial DNA damage that, interestingly, improved with a targeted DNA repair enzyme [71]. In addition FTO, a gene expressed at higher

levels in muscle from humans with type 2 diabetes associated with obesity, increased oxidative damage and mitochondrial dysfunction when expressed in myotubes [72].

5.4.2.2   Liver

Several reports implicate diabetes-related oxidative stress in the liver. We and others have observed [67, 73] that GSH content is reduced in liver mitochondria of insulin-deficient STZ-diabetic rats. In our work, the reduction in glutathione (GSH) was associated with an increase in GPx expression apparently, in compensation for oxidative stress. Interestingly, mitochondria isolated from fatty liver of obese mice demonstrated opposite alterations, showing increased GSH and a reduction in GPx enzyme activity [74]. On the other hand, proteomic analyses of liver tissue from obese humans with type 2 diabetes revealed decreased levels of GSH and higher levels of protein and lipid oxidative damage [75]. Livers of Goto-Kakizaki (GK) rats, a model of type 2 diabetes, manifest altered mitochondrial complex activities (decreases in I, III, and IV and increases in II and V) as well as oxidative damage in the form of protein oxidation, decreased SOD and glutathione S-transferase, and decreased total antioxidant capacity [76]. However, GSH was increased. So, overall, the above studies implicate oxidative stress to the liver in diabetic models, although GSH levels vary between these reports. Possibly, this is due to differences in the extent of compensation (or lack of) dependent on the model examined.

Impaired aldehyde dehydrogenase (ALDH) has been implicated in diabetic complications. As opposed to excess generation of ROS and products of oxidative damage, ALDH is important in detoxification. Impaired ALDH will increase levels of lipid peroxidation products such as 4-HNE, a reactive aldehyde that modifies proteins. There is evidence that glycoxidation and/or hyperglycemic pseudohypoxia impairs ALDH and leads to accumulation of lipid peroxides in the liver of insulin-deficient diabetic rats [77]. Hyperglycemic pseudohypoxia refers to the increased NADH to NAD^+ ratio observed in insulin-deficient diabetes without a decrease in tissue pO_2 [78].

It is also of interest that cytochromes P450, important in biotransformation and metabolism, are expressed in liver mitochondria where they represent a source of ROS. Further, there is evidence that hepatic CYP2E1 mRNA and/or protein expression is increased in certain conditions including obesity and type 2 diabetes [79].

5.4.2.3   Adipose Tissue

Experimentally induced mitochondrial dysfunction in adipocytes results in increased ROS and impaired insulin signaling [80]. In another study, ROS production altered gene expression in cultured adipocytes, a process that could be alleviated by over-expression of mitochondrial uncoupling protein [81]. It has also been found that high-fat feeding to mice leads to endoplasmic reticulum (ER) stress in adipose tissue, while fatty acid treatment of cultured adipocytes induced ER stress [82].

Interestingly, a recent report showed that adipose oxidative damage observed in obese insulin-resistant animal models could be mitigated by green tea catechins, although how this relates to mitochondria is not clear [83].

On the other hand, it has been reported that ROS may actually have a positive role in insulin signaling since pharmacologic depletion of GSH in mice actually increased energy expenditure and reduced diet-induced obesity [84], so the overall effects of ROS on adipose tissue health are open to some question.

5.4.3   Oxidative Damage in Non-Insulin-Sensitive Cells Relevant to the Long-Term Complications of Diabetes

The major long-term complications of diabetes involve cells that do not depend on insulin for glucose uptake. These cells take up glucose by facilitated diffusion independent of insulin [19] and include the classic sites of diabetic complications including the retina, kidney, neurons, and vascular endothelial cells. It has been suggested that an excess glucose load results in increased substrate flow to mitochondria and consequent enhanced ROS production [85]. In fact, glycemic effects of this nature have been reported for mitochondria of diverse cell types including bovine endothelial cells [14, 86], retinal endothelial cells [13], renal mesangial cells [87], cardiomyocytes [88], and epineural blood vessels [89]. Moreover, diabetes is associated with increased fatty acid oxidation and increased intracellular fat accumulation both of which have been implemented in mitochondrial ROS generation [15, 61].

On the other hand, not all reports show that exposing cultured cells to glucose increases ROS [90, 91]. Moreover, there are reports of increased ROS production on exposure to low glucose [92, 93]. In fact, recent studies in our laboratory using a recently available extracellular oxygen and acidification sensor (Seahorse, Inc) showed that cultured bovine aortic endothelial (BAE) cells exposed to high glucose manifest no greater ROS production and no greater basal or maximal mitochondrial oxygen consumption compared to cells exposed to physiologic glucose [94]. Hence, we believe that effects of glucose on ROS may depend on exact medium and culture conditions that are still unclear.

There are multiple mechanisms whereby ROS could lead to the complications of diabetes. Superoxide reacts with nitric oxide to form peroxynitrite. This will induce lipid peroxidation and consume nitric oxide which can impair endothelial-mediated vasodilation. Superoxide can also damage iron-sulfur centers reducing catalysis by enzymes such as aconitase [31]. Moreover, hydrogen peroxide, produced from superoxide by MnSOD, can react with iron to form the very reactive hydroxyl molecule. Thus, mitochondrial superoxide generates other radicals, thereby imparting diffuse damage to protein, DNA, RNA, and lipids. Moreover, mitochondrial damage and consequent dysfunction will disrupt calcium transits and can induce the mitochondrial permeability transition pore leading to apoptosis [95].

Based on studies in BAE cells, it has been posited that hyperglycemia-induced mitochondrial ROS leads to diabetic complications through pathways including generation of advanced glycosylation end products (AGEs), protein kinase C (PKC) activation, and polyol formation [14]. In the first case, glucose-induced ROS increase levels of methylglyoxal, which is known to induce the formation of AGEs. In addition, ROS-activated PKC can lead to diabetic complications by triggering the production of several proteins. Examples are renal mesangial matrix proteins leading to glomerular damage [96] or platelet-derived growth factor and the vasoconstrictive endothelelin-1 which are associated with diabetic retinopathy [97]. Moreover, antioxidant administration decreases sorbitol accumulation in BAE cells exposed to high glucose. This implies that ROS increase glucose-driven polyol formation through the aldose reductase pathway, a mechanism linked to diabetic complications [14].

Below, we review evidence for mitochondrial-related, diabetes-induced oxidative damage in specific target cells.

5.4.3.1 Retina

Studies using transformed retinal cells (rMC-1) and bovine retinal endothelial cells (BREC) revealed increased superoxide production upon exposure to 25 mM, as opposed to 5 mM, glucose [13]. This was thought to be primarily from mitochondria, since inhibition of the mitochondrial electron transport chain complex II normalized superoxide production whereas inhibition of NADPH oxidase or nitric oxide synthase had little or no effect. On the other hand, Busik et al. [90] showed that 25 mM glucose did not increase ROS production in human retinal endothelial cells. This finding was explained since the increased glucose concentration did not actually increase glucose utilization in these cells. In contrast to the lack of effect of glucose, these authors [90] found that stimulation by interleukin-1β or tumor necrosis factor-α did induce ROS production in human retinal endothelial cells suggesting that diabetes-related endothelial injury may be related more to cytokine production than to excess glucose. This study utilized specific spin traps to verify intracellular production of superoxide by EPR spectroscopy.

Kanwar et al. [98] reported that superoxide production, measured as lucigenin fluorescence, was increased in retinal tissue isolated from streptozotocin-diabetic mice with blood glucose concentrations approximately 400 mg/dL. This was prevented by overexpression of MnSOD in the diabetic mice before isolation of the retinal tissue. These authors also reported that diabetes decreased mitochondrial content of reduced glutathione. Cui et al. [99] used a confocal microscopy approach and reported that high glucose in culture medium increased ROS production in bovine retinal capillary endothelial cells and pericytes associated with apoptosis. These authors also noted increased uncoupling protein expression and MnSOD suggesting mitochondrial compensation for ROS. Oddly the induced UCPs included uncoupling protein-1 (UCP1) generally expressed only in brown fat. But in this respect, a more recent report did describe a -3826A/G polymorphism in the UCP1

gene associated with diabetic retinopathy in type 1 diabetic patients [100]. Consistent with the above studies, Koluru et al. [101] showed that retinal mitochondria from rats after 8 months (but not 2 months) of STZ-induced diabetes are characterized by leakage of markers of apoptosis (cytochrome *c* and the BAX protein). In another report, this group showed that MnSOD overexpression in transgenic mice inhibited oxidative damage to the retina manifest as 8-hydroxy deoxyguanosine (8-OHdG) and nitrotyrosine [102].

5.4.3.2   Renal

Friederich et al. [103] showed that diabetic rats express increased mitochondrial UCP2 in proximal tubular cells associated with increased oxygen use and suggested that the increase in UCP2 was protective against oxidative stress. In another report UCP2 was negatively associated with H_2O_2 production in kidney mitochondria of diabetic rats [104]. Manabe et al. [105] reported that high glucose increased ROS fluorescence in human mesangial cells associated with potentially harmful cytokine expression, an effect that was blocked by astaxanthin, a carotenoid that accumulated in mitochondria. High glucose also reportedly increased H_2O_2 production by dichlorodihydrofluorescein fluorescence in human mesangial cells [87]. This was suppressed by reduction in membrane potential by chemical inhibition or by UCP1 overexpression, but, curiously, also suppressed by MnSOD which should actually increase H_2O_2 production from superoxide.

   Coughlan et al. [106] demonstrated renal mitochondrial oxidative damage in 32-week streptozotocin-diabetic rats manifest as lucigenin luminescence in kidney slices, an effect that was reduced by alagebrium, a cross-link inhibitor of AGE accumulation. Interestingly, renal carboxymethyllysine, an AGE marker of glycoxidation and lipid peroxidation, was also inhibited linking oxidative damage to protein glycosylation. In another report, methylglyoxal formation (a precursor to AGEs) accompanied an increase in superoxide production by renal cortical mitochondria of 12-month STZ-diabetic rats [107]. Mitochondrial ROS were implicated in renal pathology in the Goto-Kakizaki rat, a rodent model of type 2 diabetes [108]. This study showed a reduction in tissue aconitase activity, a mitochondrial enzyme susceptible to inactivation by reactive oxygen, along with an increase in lipid peroxides.

5.4.3.3   Neural Cells

Moreira et al. [109] reported no increase in H_2O_2 production by brain mitochondria isolated from 12-week streptozotocin-diabetic rats. However, that study did show increased H_2O_2 production accompanied by upregulation of glutathione peroxidase in kidney mitochondria of the diabetic rats.

   There is evidence that hyperglycemia-induced oxidative damage induced by insulin-deficient diabetes results in programmed cell death in dorsal root ganglia

and Schwann cells [110]. Moreover, involvement of mitochondrial ROS in this process is evident since the apoptotic changes can be prevented by reduction of membrane hyperpolarization by overexpression of uncoupling proteins [111].

There is also evidence for neurovascular dysfunction in diabetes related to mitochondrial oxidative stress. This is discussed in the next section.

5.4.3.4   ROS and Vascular Cells

Diabetes increases the risk of cardiovascular events two- to fourfold. In part, this could be due to impaired vascular function since both endothelial and smooth muscle cell-mediated vascular reactivities are impaired by diabetes [112, 113]. Therefore, mitochondrial function as affected by diabetes is particularly important with respect to vascular cells.

Interaction of superoxide with nitric oxide will result in lipid peroxidation products [114] suggesting that the oxygen radical would impair vascular function. Impaired endothelium-dependent vasodilation has been demonstrated in various vascular beds of animal models of diabetes and humans with type 1 and type 2 diabetes [115]. Thus, hyperglycemia-induced production of superoxide by mitochondria of endothelial cells has been suggested as a common explanation for diabetes-induced vascular dysfunction [14]. Studies of epineurial arterioles of the sciatic nerve derived from diabetic rats have provided evidence that the generation of oxidative stress through the production of superoxide and peroxynitrite impairs vascular function and endothelium-dependent vascular relaxation [116–119]. It was suggested that complex I of the mitochondrial electron transport chain was responsible for the increase in superoxide formation since pretreating epineurial arterioles from diabetic rats with rotenone reduced formation of this radical [89]. Also, treating diabetic rats with three different types of antioxidants prevented diabetes-induced superoxide production and peroxynitrite formation in the aorta and epineurial arterioles further suggesting that increased oxidative stress contributes to diabetes-induced vascular and neural disease [116–118].

Other studies provide further evidence that antioxidants prevent vascular complications in diabetes. Treating diabetic rats with Tempol, a stable SOD mimetic, abolished the diabetes-induced increase in vascular superoxide, malondialdehyde, and 8-epi-prostaglandin $F(2\alpha)$ and also prevented the impairment in relaxation of aortic rings to acetylcholine [120]. In addition, Keegan et al. demonstrated that treating diabetic rats with α-lipoic acid improved endothelium-dependent vascular relaxation of corpus cavernosum smooth muscle [121]. Cameron and colleagues demonstrated that treating diabetic rats with α-lipoic acid or the metal chelators, hydroxyethyl starch deferoxamine or trientine, prevented impairment of vascular relaxation associated with hyperalgesia and neurovascular deficits [122–126].

Finally, heme oxygenase reportedly protects the vasculature in diabetes. Biliverdin, a product of HO-1 catalysis, has antioxidant properties, while another product, carbon monoxide, has vasodilatory, anti-inflammatory, and antiproliferative effects [35]. The inducible subtype HO-1 is present in many tissues and

upregulated by several stimuli including growth factors, inflammatory cytokines, hypoxia, peroxynitrite, and nitric oxide. HO-1 improves endothelial dysfunction in diabetes [127] and has angiogenic properties [128]. Further, treatment of genetically obese mice by induction of HO-1 with cobalt protoporphyrin ameliorated visceral and subcutaneous fat accumulation, increased adiponectin, and improved insulin sensitivity [129].

5.5 Overall Multicellular Effects of ROS and Type 2 Diabetes

Given the above considerations, we can ask how ROS-induced mitochondrial dysfunction within different cell and tissue types might lead to type 2 diabetes or, if not directly causative, how mitochondrial dysfunction could contribute to the progressive nature of diabetes and its complications. Figure 5.4 depicts a simplistic overview of this process. Obviously, there is considerable detail yet to be resolved. Hopefully, further understanding will lead to approaches that effectively target mitochondria within multiple tissues in a way that mitigates the onset and progression of type 2 diabetes.
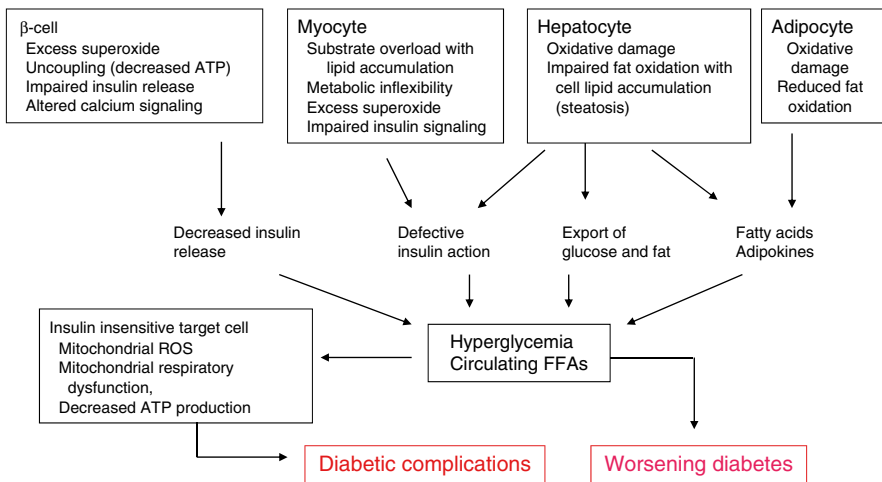

Fig. 5.4 Schematic depicting how defects in different cell types collectively lead to hyperglycemia and elevated free fatty acids (FFAs), worsening diabetic complications, and the progressive worsening of the diabetic state

5.6 Therapeutic Implications

Both lifestyle and pharmacologic interventions have been suggested with variably reported success. These are addressed below.

5.6.1 Lifestyle Modification

Lifestyle modification including exercise and diet decreases the risk for developing type 2 diabetes [130], while physical activity improves glucose tolerance [131]. Moreover, mitochondrial dysfunction may underlie the factors associated with diabetes and the metabolic syndrome including obesity, hyperlipidemia, hypertension, and vascular disease. In this regard, exercise offers several benefits including increased electron transport activity in muscle, stimulation of mitochondrial biogenesis through effects on PGC-1α, and improved sensitivity to insulin [132, 133]. Exercise also activates AMPK which improves both glucose and fat oxidation [132]. Calorie restriction is known to prolong the lifetime of rodents, nematodes, and maybe humans [134]. In this regard, there is evidence that calorie restriction favors mitochondrial biogenesis, oxygen use, ATP formation, and expression of SIRT1 which activates PGC-1α [135, 136], a factor important for mitochondrial biogenesis. Several specific food types have alleged antioxidant properties (not reviewed here), for example, beans blueberries, pecans, cinnamon, etc. However, it is difficult to claim that any specific food or lifestyle intervention targets mitochondrial-specific oxygen radicals. Pharmacologic approaches to this have been suggested as described in the next section.

5.6.2 Pharmacological Intervention

Metformin is most often the initial pharmacologic agent used in type 2 diabetes. Metformin has mitigating effects on ROS production, activates AMPK, and favors mitochondrial proliferation [137, 138]. In clinical use, metformin, unlike insulin or insulin secretagogues, is not associated with weight gain. Another group of drugs that improve insulin sensitivity and enhance mitochondrial biogenesis are the angiotensin receptor blockers or inhibitors of angiotensin-converting enzyme. These agents also reduce oxidative stress, although the mechanisms still need clarification [139].

Newer pharmacologic approaches to improving mitochondrial function may be on the horizon. Resveratrol, an ingredient in red wines, is a polyphenolic SIRT1 activator which, like calorie restriction, has antiaging effects in lower organisms [140–142], reduces signs of aging in mice [143], and extends survival [140]. Other related small molecules have been described which are more potent than resveratrol

to enhance the action of SIRT1 on substrates for deacetylation [144]. Resveratrol is believed to have antioxidant properties [145, 146] although these are not known to be targeted to mitochondria.

As mentioned above (Sect. 5.4.2.1), high fatty acyl-CoA flux may result in mitochondrial overload with adverse consequences toward ROS production and carbohydrate metabolism. Therefore, it may be possible to improve glucose utilization through measures that inhibit mitochondrial uptake of long chain acyl-CoA molecules. For example, lipid suppression of glucose utilization is mitigated by etomoxir, an inhibitor of carnitine palmitoyltransferase 1, or by knockdown of malonyl-CoA decarboxylase, an enzyme that promotes mitochondrial β-oxidation by preventing malonyl-CoA-induced inhibition of CPT-I [147, 148]. Other targets potentially amenable to pharmacologic manipulation include AMPK, which enhances both glucose and fat oxidation [149, 150], pyruvate dehydrogenase [151], or the various shuttle mechanisms regulating uptake of TCA intermediates [152].

Various other compounds or vitamins with antioxidant properties and effects on mitochondria have been used in attempts to prevent, control, or reduce the complications of diabetes. These include coenzyme Q, vitamin E, α-lipoic acid, N-acetylcysteine (NAC), vitamin C, and inducers of heme oxygenase.

As the major mobile mitochondrial electron carrier, coenzyme Q has long been of interest as a therapy for obesity and to improve diabetic states. However, the therapeutic use of CoQ10 and other antioxidants in vivo, particularly in human studies directed at vascular events, has been disappointing [153, 154]. This may be due to concerns about toxicity and, therefore, inadequate dosing or inability to deliver agents to target sites of ROS production. Ubiquinol, the reduced from of CoQ, acts as an antioxidant in mitochondria both by regeneration of vitamin E and by directly reacting with peroxyl radicals. Thus, CoQ acts in mitochondria both as an antioxidant and as a mobile electron carrier [155, 156]. However, in our experience, CoQ10, in either the ubiquinol or ubiquinone redox state, does not appear to have direct effects on mitochondrial ROS [36, 157] and may not easily enter mitochondria.

The antioxidant properties of vitamin E are felt to be based on its oxidation to the tocopheroxyl radical enabling this lipophilic molecule to inhibit lipid peroxidation [156]. However, vitamin E did not improve cardiovascular outcomes in a large multicentered trial and actually increased congestive heart failure [158]. Vitamin E also did not prevent the progression of carotid intima-media thickness in high-risk patients with diabetes [159]. Water-soluble ascorbic acid (vitamin C) is widely marketed for its antioxidant properties [160, 161] and, as stated above, appears to regenerate reduced vitamin E. However, there is no evidence to support a role in the management of diabetes [162].

Other antioxidant molecules of with possible therapeutic action include α-lipoic acid and NAC. In vivo, α-lipoic acid is reduced to dihydrolipoic acid and, as such, is an effective scavenger of superoxide [163]. In this form, the compound regenerates other antioxidants including glutathione, vitamin C, and vitamin E. In retina of STZ-diabetic rats, α-lipoic acid mitigated the diabetes-induced decrease in mitochondrial and cytosolic NAD$^+$/NADH ratios [164]. This compound also prevented

lipid peroxidation when administered to rats [165] and improved β-cell function in apolipoprotein E-deficient mice given STZ [166]. α-Lipoic acid also protected the retinal microvasculature in diabetic rats by reducing nitrotyrosine and oxidized DNA [167]. In human studies, α-lipoic acid has been administered intravenously and improved diabetic peripheral neuropathy [168]. Oral α-lipoic acid also improved peripheral neuropathy but caused nausea, vomiting, and vertigo [169].

Inducers of heme oxygenase mitigated islet damage and improved glycemia in diabetic mice [53] and improved obesity and insulin sensitivity in genetically obese mice [129]. These findings have not, as yet, been translated to human studies.

5.6.3   Mitochondrial-Targeted Antioxidants

The likely role of mitochondrial ROS in human disease has led to efforts to develop effective antioxidant compounds targeted to mitochondria. One approach involves the synthesis of compounds linking agents such as redox forms of quinone (ubiquinol and ubiquinone) or vitamin E to alkylated triphenylphosphonium compounds. These lipophilic cations are avidly taken up into the relatively negative mitochondrial matrix [170]. Two such compounds (alkyltriphenylphosphonium cations) incorporating ubiquinone or vitamin E, termed mitoQ and mitoVit E, respectively, have been synthesized [170]. By virtue of their delocalized positive charge, these agents accumulate several hundredfold in mitochondria [38]. A major mechanism may be to decrease lipid peroxidation by virtue of the quinol moiety acting as a chain-breaking antioxidant [171]. A problem, however, is that these agents, under certain conditions, can also have prooxidant effects [36]. Moreover, they have metabolic effects and above certain concentrations will inhibit ATP production [172].

In addition to the above triphenylphosphonium cationic molecules, other approaches to mitochondrial antioxidant therapy are under investigation. One involves synthetic peptides with antioxidant properties designed to target mitochondria. These penetrate mitochondria targeting the inner membrane by a poorly understood mechanism [173]. Peptides containing tyrosine residues effectively scavenge oxygen radicals and peroxynitrite and inhibit lipid peroxidation [173, 174]. Such peptides were reported to preserve insulin sensitivity in rats fed a high-fat diet [175]. A limitation is that these peptides also possess opioid receptor affinity and activity [176–178].

5.7   Summary

Although we do not suggest that ROS provide a unifying explanation for diabetes, it does seem clear that ROS contribute to defects in both insulin secretion and insulin action seen in type 2 diabetes. Also, the inflammatory damage which characterizes type 1 diabetes is mediated, at least in part, through islet ROS. In persons with

type 2 diabetes, the high nutrient flux and consequent ROS production appear to mediate loss of β-cell function. In insulin-sensitive tissues including the liver, muscle, heart, and adipose, high fatty acid flux leads to oxidative damage. At the same time, non-insulin-sensitive tissues including the eye, kidney, nervous system, and vasculature are exposed to both high circulating glucose and fatty acids and, consequently, ROS-induced diabetic complications.

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Chapter 6
Optimal Measures of Small Fiber Neuropathy in Diabetic Polyneuropathy

M. Tavakoli, H. Fadavi, and R.A. Malik

6.1 Introduction and Objectives

Recently it has been proposed that "If nerve conduction (NC) is normal, a validated measure (with class 1 evidence) of small fiber neuropathy (SFN) may be used" to define and quantify the severity of diabetic sensory-motor polyneuropathy (DSPN) [1]. NC assesses large myelinated nerve fiber function and has been used as an end point in clinical trials of human diabetic neuropathy, based on relative ease of quantification, reproducibility, and reasonable sensitivity and specificity [2]. However, recent data have demonstrated minimal worsening [3] and improvements [4] in electrophysiology in placebo and epidemiological cohorts with little relation to other measures of small fiber and autonomic function in diabetic patients [5].

Small fibers constitute 79.6 % [6] to 91.4 % [7] of peripheral nerve fibers. Damage to this class of fibers underlies the symptoms of painful diabetic neuropathy, which are typically distal, symmetrical, and associated with nocturnal exacerbation. The descriptors used by patients to describe the symptoms can be variable but often include the following: prickling, aching, and burning pain with intermittent sharp stabbing electric-shock-like pains and on examination one can elicit dysesthesia and allodynia. In addition to these troublesome symptoms, dysfunction and damage to this class of fibers are also key to the genesis of foot ulceration through the effect on sudomotor function [8], pressure-induced vasodilation [9, 10], and of course heat and pain perception [11]. Moreover, an increasing body of data shows that small fiber damage may precede large fiber damage in diabetic neuropathy [12–14].

Therefore it appears pertinent to address whether any definition of DSPN should include a measure of small fiber neuropathy. Issues that arise before we can adopt

M. Tavakoli • H. Fadavi • R.A. Malik (✉)

Division of Cardiovascular Medicine, Central Manchester Foundation
Trust & University of Manchester, Manchester M13 9WL, UK
e-mail: rayaz.a.malik@man.ac.uk

the assessment of SFN to diagnose DSPN include establishing the reproducibility, sensitivity, specificity, and accuracy but also the practical viability of any proposed test. For the purposes of this review, we will consider the available evidence for established and emerging measures of "small fiber damage" to diagnose and stratify the severity of DSPN.

6.2 Quantitative Sensory Testing

6.2.1 Thermal Thresholds

Abnormalities in heat-pain thresholds reflect small fiber dysfunction, and a number of instruments including CASE IV, thermoesthesiometer, and Medoc instruments have been used to quantify this parameter. In 498 type 2 diabetic patients and 434 control subjects, an elevated warm threshold was the most frequent abnormality (60.2 %) compared to an abnormal cold threshold (39.6 %) and abnormal sural nerve conduction velocity (12.9 %), and it was related to both symptoms and glycemic control [15]. However, a careful study of 59 diabetic patients with and without symptomatic neuropathy showed that unlike cold perception thresholds and IENFD, warm perception thresholds did not differentiate diabetic patients with and without symptoms [14]. Similarly, in a study of 191 diabetic patients, there was no difference in heat-pain thresholds between those with and without painful neuropathy [16].

6.2.2 Pain-Related Evoked Potentials

In a study of 57 diabetic patients with entirely normal electrophysiology, the latency was increased and amplitude was reduced for pain-related evoked potentials (PREPs), elicited by nociceptive electrical stimulation of the skin [17].

6.2.3 Nerve Axon Reflex/Flare Response

Stimulation of the nociceptive C fiber results in both orthodromic conduction to the spinal cord and antidromic conduction to other axon branches, i.e., the axon reflex (Fig. 6.1) which can stimulate the release of peptides, such as substance P and calcitonin gene-related peptide, resulting in vasodilation and increased permeability. Studies have shown that this neurovascular response mediated by the nerve axon reflex is reduced in diabetic neuropathic patients, correlates with other nerve function measurements, and has reasonable sensitivity and specificity in identifying
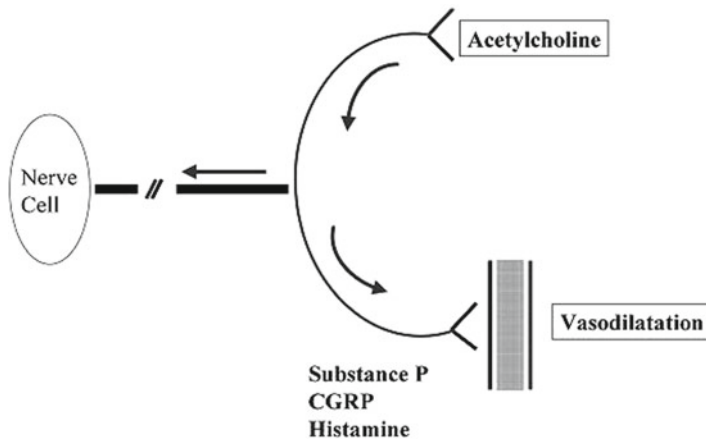
Fig. 6.1 Nerve axon reflex: stimulation of the C nociceptive nerve fibers leads to antidromic stimulation of the adjacent C fibers, which secrete various vasomodulators such as substance P, calcitonin gene-related peptide (CGRP), and histamine that cause vasodilation and increased blood flow

patients with diabetic neuropathy [18, 19]. The LDI flare test evaluates 44 °C heat-induced vasodilation [20] and is reduced in subjects with impaired glucose tolerance (IGT) [21] and type 2 diabetic patients with and without neuropathy [22, 23] but interestingly is normal in patients with type 1 diabetes of long duration [21].

More longitudinal data and perhaps assessment after interventions when compared with established tests are necessary before these techniques can be recommended for clinical use.

6.3   Skin Biopsy

Skin biopsy, a minimally invasive procedure, allows morphometric quantification of intraepidermal nerve fibers (IENF) most commonly expressed as the number of IENF per length of section (IENF/mm) [24, 25] (Fig. 6.2). Intra- and interobserver variability for the assessment of IENF density demonstrates good agreement [25, 26], declines with age, and does not appear to be influenced by weight or height [27]. An international consortium of investigators has recently compiled a normative database for IENFD in 550 participants and shown an effect of age, but no influence of height, weight, or BMI [28]. The blister technique is an alternative less invasive procedure which assesses innervation of the epidermis alone and shows good agreement with punch biopsy [29].
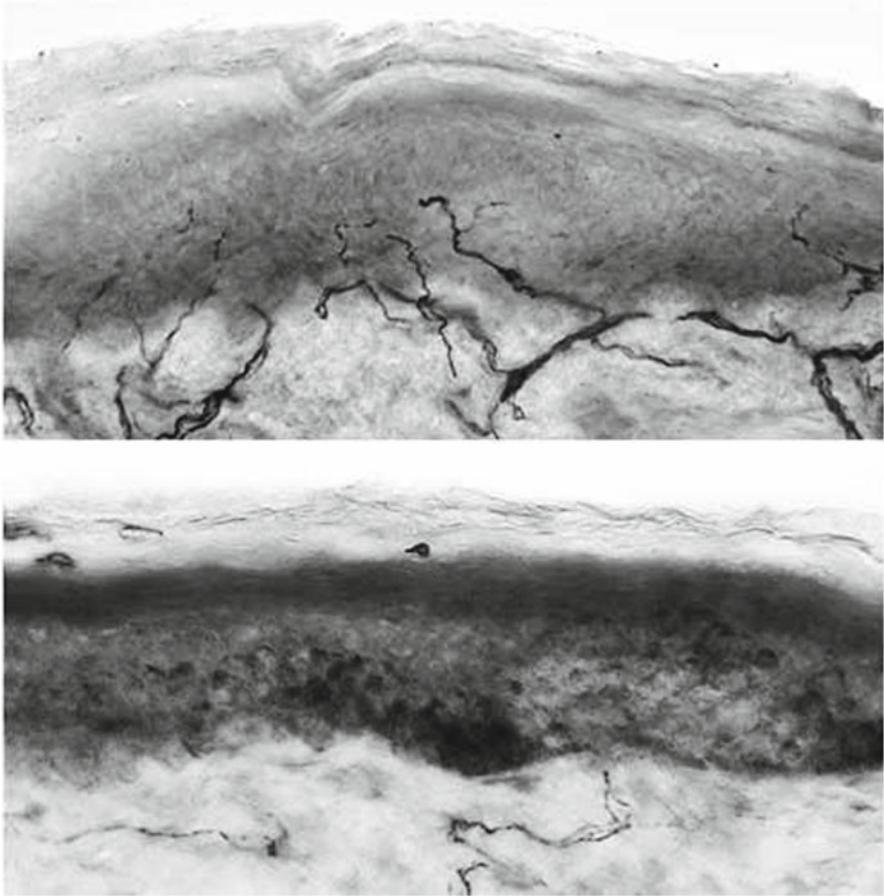
Fig. 6.2 Skin biopsy with PGP 9.5 immunostaining for IENF showing normal IENF (→) in a control subject (*top*) and absence of IENF with only dermal nerve fibers (→) in a diabetic patient with severe neuropathy (*bottom*)

6.3.1 *Diagnostic Yield of IENF Quantification*

No study assessing the sensitivity and specificity of IENF in DSPN is available. However, several studies in SFN have included patients with DSPN. In 58 patients with pure SFN, a cutoff IENF density of ≤8.8 per mm at the ankle was associated with a sensitivity of 77.2 % and a specificity of 79.6 % [30]. Similarly, in 67 patients with pure SFN, a sensitivity of 88 % and a specificity of 88.8 % have been reported [31]. In a study of 210 patients with SFN, which included 65 diabetic patients, the Z-scores and 5th percentile provided the highest specificity (98 and 95 %, respectively) but a very low sensitivity (31 and 35 %, respectively) compared to the ROC analysis (specificity 64 %, sensitivity 78 %) [32]. These findings suggest that the

diagnostic yield of skin biopsy may depend on the reference and cutoff values selected and the definition of SFN adopted. IENF density correlates inversely with thermal thresholds. While some have reported a closer correlation with warm and heat-pain thresholds [30, 33–35] compared to cooling thresholds [36, 37], others have reported the opposite, with a closer correlation with cold rather than heat detection thresholds [16, 38]. A recent study has demonstrated no correlation between IENFD and the neuropathy symptom score, but interestingly an inverse correlation was demonstrated with the severity of pain assessed using the VASmax [39]. The correlation between quantitative sensory testing (QST) and IENF density therefore remains controversial.

The American Academy of Neurology, American Association of Neuromuscular and Electrodiagnostic Medicine, and American Academy of Physical Medicine and Rehabilitation have concluded however that skin biopsy may be considered for the diagnosis of DSPN, particularly SFN, with a level C recommendation [40]. More recently, under the auspices of the European Federation of the Neurological Societies and the Peripheral Nerve Society, revised guidelines on the use of skin biopsy concluded that IENF density is a reliable and efficient technique to confirm the clinical diagnosis of SFN with level A recommendation [41].

Additional morphological features of IENFs include the branch density, length, and mean dendritic length; all show an early reduction which progresses with neuropathic severity [13, 42]. Several studies with serial skin biopsies in patients with SFN have shown that axonal swellings predict a decline in IENF density [43–45]. However, they occur not only in patients with SFN [46] but also in normal individuals [47], and isolated swellings with normal IENF densities have been observed in a variety of other neuropathies [47–50].

6.4 Diabetic Neuropathy

In patients with diabetic neuropathy, the prevalence of abnormal NC, QST, and IENF was comparable [39]. However, IENF density was significantly reduced in patients with normal NC, suggesting early damage to small nerve fibers [12, 14]. Although, a recent study has shown comparable abnormalities in electrophysiology, thermal thresholds and loss of IENF in diabetic patients with mild neuropathy [39]. There is an inverse correlation between IENF density and the severity of DSPN, defined by the neurological disability score [13, 34, 51] and the neuropathy impairment score [14]. Additionally, IENF density appears to be lower in diabetic patients with painful compared to painless neuropathy [13, 34, 52]. A 1-year diet and exercise intervention program in patients with SFN and IGT led to increased IENF density [53]. However, no change was observed in 18 diabetic patients after simultaneous pancreas/kidney (SPK) transplantation [54]. This may reflect the marked IENF loss at baseline [55], particularly in diabetic patients undergoing SPK and the slower regeneration rate of IENF in diabetic patients [56]. These data suggest that

IENF loss is an early feature of diabetes, progresses with increasing neuropathic severity, and may improve with appropriate intervention.

A considerable body of experimental data has been generated recently to show that IENF loss may be an early morphological marker of small fiber damage in animal models of diabetes. A loss of epidermal innervation similar to that observed in diabetic patients has been observed in rodent models of both type 1 and type 2 diabetes, and several therapeutics have been reported to prevent reductions in intraepidermal nerve fiber density in these models [57]. Several studies have assessed cutaneous innervation in mouse footpad [58–60] and showed a reduction in intraepidermal innervation of both flank and footpad skin [61]. There is high interobserver agreement when two experts use the protocol used in humans to quantify the density of IENFs [62]. In a study in nonhuman primates with naturally occurring obesity and type 2 diabetes, hypertrophic epidermal nerve fibers were found in monkeys with short-time hyperglycemia; however, a severe reduction of nerve fibers was demonstrated in those with a duration of diabetes exceeding 8 years [63]. In diabetic mice, although the total epidermal innervation appears unchanged in early diabetes, staining for peptidergic fibers is significantly reduced [64]. These early changes may have a functional relevance, as previous studies in rodents demonstrate behavioral deficits prior to quantifiable intraepidermal nerve fiber loss [65]. Thus IENF density can be reliably quantified in the footpad of healthy and neuropathic rats and interestingly correlates significantly with tail nerve conduction velocity [62]. These findings support the use of IENF quantification as an outcome measurement in experimental neuropathies.

6.5   Nerve Biopsy

Nerve biopsy has traditionally been used to quantify myelinated nerve fiber density which is reduced and correlates with abnormalities in neurophysiology [66, 67] but may also predict development of future neurophysiological deficits [68]. Few studies have quantified unmyelinated nerve fiber damage, but some have shown that it precedes myelinated nerve fiber damage in sural nerve biopsies and therefore it may be used to detect early DSPN [7]. However, nerve biopsy is an invasive and highly specialized procedure which requires neurosurgical expertise to identify and perform, especially when a fascicular biopsy is required. Furthermore, electron microscopy demands considerable expertise and there are very few centers which can perform quantification. It therefore cannot be advocated for use to diagnose DSPN [69].

6.6 Corneal Confocal Microscopy

Corneal confocal microscopy (CCM) is a noninvasive ophthalmic technique that has been shown to detect small sensory corneal nerve fiber loss in diabetic neuropathy (Fig. 6.3) [70], idiopathic small fiber neuropathy and IGT patients [71], and Fabry disease, a condition which is characterized by painful neuropathy [72], by visualizing the subbasal nerve plexus in Bowman's layer of the cornea. Corneal nerve fiber damage correlates with IENF loss and severity of neuropathy in diabetic patients [13, 73] and is more marked in patients with painful diabetic neuropathy [13]. A correlation between loss of corneal nerve fibers and the stage of diabetic retinopathy has also been demonstrated [74]. CCM may also be more sensitive than IENFD in detecting early damage [13] and repair after SPK transplantation [55, 75]. Thus corneal nerve fiber density improves 6 months after combined pancreas/kidney transplantation [75]. CCM has been shown to have high reproducibility [76], with reasonable sensitivity and specificity [77]. To enhance the practical application of this technique, an automated image analysis system has also been developed recently to rapidly quantify corneal nerve pathology [78]. A progressive loss of corneal sensation with increasing severity of neuropathy provides a functional correlate of corneal nerve fiber loss in diabetic patients [79–81].

Therefore as CCM is noninvasive, it may be an ideal technique to assess alterations in small nerve fiber pathology in relation to PDN and progression or regression of neuropathic deficits.
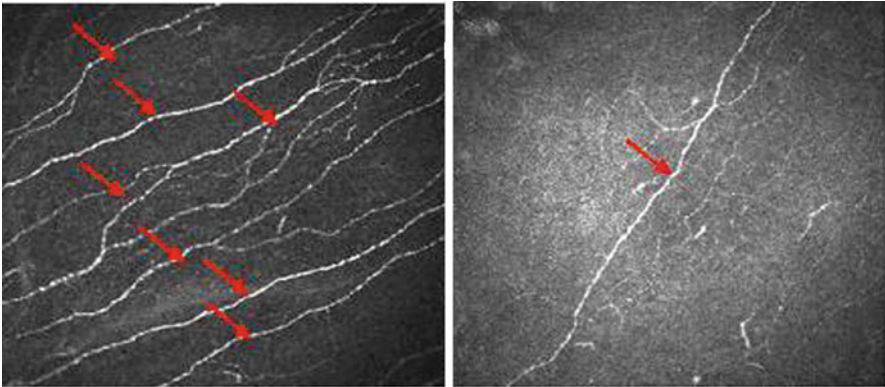

Fig. 6.3 Corneal confocal microscopy image of a control subject (*right panel*) with normal corneal nerve (→) density compared to an image from a diabetic patient with severe neuropathy and marked loss of corneal nerve fibers (*left panel*)

6.7 Sudomotor Dysfunction

6.7.1 Sympathetic Skin Response

Sympathetic skin response (SSR) assesses sudomotor and hence small fiber dysfunction. In an early study it failed to differentiate the presence or absence of neuropathy in a series of 337 diabetic patients [82]. However, it has recently been shown to predict the risk of foot ulceration comparable with abnormalities in NDS and elevated vibration perception [83]. It has also been shown to have a sensitivity of 87.5 % and a specificity of 88.2 % for detecting diabetic autonomic neuropathy [84].

6.7.2 Quantitative Sudomotor Axon Reflex Testing

Quantitative sudomotor axon reflex testing (QSART) evaluates sudomotor function by assessing the local sweat response to iontophoresis of acetylcholine [85] and has been shown to be highly sensitive in the detection of distal SFN [86]. QSART evaluates postganglionic axon function as opposed to the polysynaptic pathways assessed using SSR. In a series of 31 diabetic patients with early neuropathy, it appeared to be better at detecting early neuropathy than SSR [87].

6.7.3 Neuropad

The neuropad test is a simple visual indicator test which uses a color change to define the integrity of skin sympathetic cholinergic innervation. Neuropad responses have been shown to correlate with the modified NDS, QST, CAN, and IENF loss with relatively high sensitivity but lower specificity for detecting DSPN [88, 89]. A recent study has shown that an abnormal neuropad test in those with a normal NDS may predict the development of diabetic neuropathy after 5 years [90]. This appears to reflect early small fiber involvement which is missed using NDS as a measure of neuropathy.

6.7.4 Sudomotor Innervation

Recently, a novel stereologic technique has been applied in skin biopsies and showed a correlation between sweat gland nerve fiber density, neuropathic symptoms, neurological deficits, and sweat production [91]. However, morphometric data in patients with diabetic SFN are limited and further studies are warranted.

6.8 Definition of SFN

Given the overwhelming evidence for the involvement of small fibers in the early and late phases of peripheral nerve damage in diabetic patients, we propose to grade SFN as follows: (1) *possible*, presence of distal symmetrical symptoms and/or clinical signs of small fiber damage; (2) *probable*, presence of distal symmetrical symptoms, clinical signs of small fiber damage, and normal or abnormal sural NC study; and (3) *definite*, presence of length-dependent symptoms, clinical signs of small fiber damage, normal or abnormal sural NC study, and/or abnormal QST thermal thresholds at the foot and reduced IENF density at the ankle.

At present it is not possible to suggest criteria to define the severity of SFN in DPN. However, as normative ranges are established for the different tests of small fiber dysfunction and damage, it may be possible to devise a measure of severity using different percentiles or quartiles as cutoffs.

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Chapter 7
Oxidative Stress and Complications of the Diabetic Foot

Martin J. Stevens and Jayadave Shakher

7.1 Introduction

Lower limb amputations remain common and the principal cause is diabetes despite improvements in clinical care [1–5]. The underlying factor contributing to amputation in diabetes is usually diabetic foot disease and ulceration [4], and the lifetime risk of developing a foot ulcer has been estimated to be as high as 15 % [5, 6]. The annual population-based incidence of foot ulceration has been reported to range from 1.0 to 4.1 % and the prevalence ranges from 4 to 10 % [7]. Despite improvements in the care of diabetes and many of its complications, the burden of diabetic foot disease and ulceration is likely to continue to increase with lower extremity amputations affecting 30 % of subjects with diabetes 40 years and older [8].

Foot ulcers have many effects beyond their immediate physical consequences. For example, ulceration can cause substantial emotional and financial losses [2, 9], and a diabetes-related amputation markedly worsens quality of life and increases the risk of further amputations [10]. Ominously, the presence of foot ulceration and subsequent amputation can predict very poor clinical outcomes with mortality rates after amputation reported to be 40 % at 1 year and 80 % at 5 years which is in fact worse than for many malignancies [11]. The optimal approach to the management of complications of the lower limb in diabetes lies in prevention through the implementation of screening programs aimed at the early detection of neuropathy,

M.J. Stevens (✉)
Centre of Endocrinology Diabetes and Metabolism (CEDAM), School of Clinical and Experimental Medicine, University of Birmingham, Birmingham B15 2TT, UK

Department of Diabetes and Endocrinology, Heart of England NHS Foundation Trust, Birmingham, UK
e-mail: m.j.stevens@bham.ac.uk

J. Shakher
Department of Diabetes and Endocrinology, Heart of England National Health Service Foundation Trust, Birmingham, UK

ischemia, deformity, and edema. These programs have been demonstrated to prospectively reduce the need for subsequent amputations [12, 13]. Still, even with aggressive screening, chronic ulceration in the lower limbs remains one of the most common and most serious consequences of diabetes.

7.2 Diabetic Foot Ulcers: Causes and Complications

Understanding the causes of diabetic foot ulceration is key to developing better preventative and treatment approaches. Contributing factors which predispose to foot ulceration include sensory loss, ischemia, and infection [1, 2, 4]. Many subjects with foot ulceration have some degree of insensitivity reflecting the presence of peripheral somatic neuropathy which can be identified in over 80 % of subjects with diabetic foot ulcers [2]. The presence of autonomic dysfunction is also thought to be important by impairing skin lubrication, altering callus formation, and altering blood flow regulation. Structural deformities in the insensate foot which contribute to abnormally increased pressure [14, 15] are a fundamental factor contributing to foot ulceration. Infection is often present and polymicrobial, but systemic manifestations may be absent despite extensive, limb-threatening sepsis [16]. Subjects with diabetes are prone to develop peripheral vascular disease and calcification which is characteristically worse below the knee and a contributing factor in approximately 60 % of diabetic subjects with non-healing foot ulcers. Impaired lower limb circulation is a factor in up to 46 % of subjects who have a major amputation [17]. A widespread microangiopathy complicates diabetes, and skin blood flow regulation has been reported to be abnormal in many subjects at risk of the development of foot complications and may contribute to the chronicity of the diabetic foot ulcer [17]. Abnormalities of skin blood flow regulation have been implicated in the pathogenesis of diabetic foot lesions by some authors [18–22]. Chronic ischemia in the poorly perfused tissue leads to secondary changes that are the proximal cause of wound-healing failure. One such change is dermal atrophy.

7.2.1 Dermal Atrophy: Occurrence in Diabetic Skin and a Common Intermediate in Chronic Wound Formation

In diabetes, skin structural and functional deficits may contribute to the risk of developing foot ulceration. For example, atrophy of dermal connective tissue (which resembles an accelerated aging process) has been proposed to be important as an early event in the development of a foot ulcer and contribute to impaired healing once ulceration has occurred [23, 24]. In diabetes, proliferation of skin fibroblasts is reduced [25, 26] which in concert with reduced procollagen synthesis and increased levels of connective tissue-degrading matrix metalloproteinases (MMPs) may
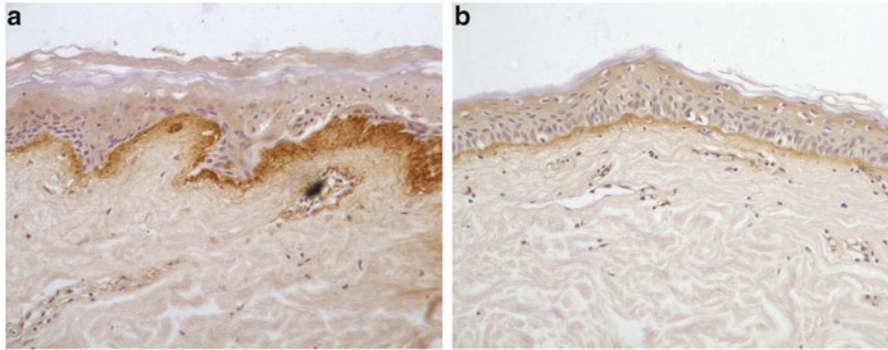
Fig. 7.1 Procollagen 1 immunohistochemistry from a nondiabetic subject and a diabetic subject with foot ulceration showing a reduction in procollagen 1 staining in the diabetes

contribute to ulceration and impaired healing. Levels of procollagen 1 are greatly reduced in the skin of diabetic patients with foot ulcers, which contributes to increased skin fragility (Fig. 7.1).

7.2.2 *Oxidative Stress and Ulceration*

Oxidative stress is implicated in the development of diabetic complications [27] including neuropathy [28, 29] and foot ulceration [16, 30]. In addition, chronic wounds of multiple etiologies are characterized by the presence of increased oxidative stress [31–38] which may play a key role in the failure of cellular elements to promote wound healing. In concert with the antioxidant response in other cellular compartments and tissues, inappropriate downregulation of dermal antioxidant defense pathways can be the result of overproduction of oxidants in chronic wounds [38–40]. In turn increased oxidative stress can damage DNA, erode telomeres, and ultimately contribute to cellular senescence [41]. The level of reactive oxygen species (ROS) production can dictate the physiological response with high levels promoting telomere-independent premature senescence, whereas lesser degrees of ROS can accelerate telomere shortening [42]. The complications of diabetes are often viewed as reflecting accelerated aging, and this may well be relevant for the diabetic ulcer since aged tissue is more susceptible to senescence [43, 44].

Exposure to ROS can also result in apoptosis in many cell types. ROS can induce apoptosis via H_2O_2 via activation of c-Jun N-terminal kinase (JNK) pathway [45]. Activated JNK translocates to the mitochondria and inhibits by phosphorylation the anti-apoptotic factor Bcl-2 and phosphorylates and thus activates proapoptotic Bax, Bim, and Bmf [44]. Effectors of apoptosis are ultimately activated by cytochrome *c* release. In the chronic wound, ROS can stimulate the degradation of hypoxia-inducible factor 1 (HIF-1) [46] which is detrimental to wound healing, since induction of HIF-1α-dependent genes such as vascular endothelium-derived growth
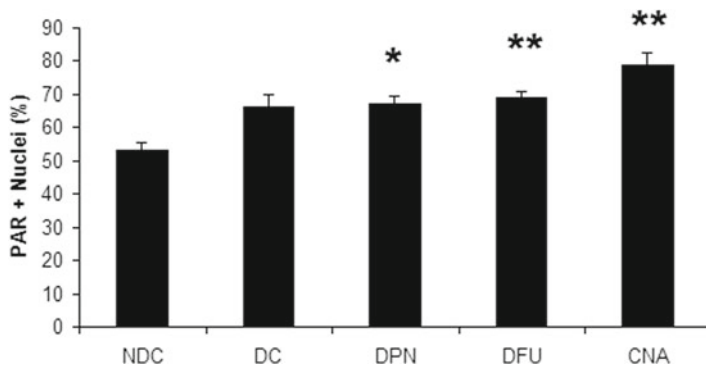
Fig. 7.2 Comparison of percentage of PAR positive nuclei in nondiabetic and diabetic subjects with and without foot complications. $*p<0.05$ vs NDC, $**p<0.01$ VS NDC. *NDC* nondiabetic control, *DC* diabetic control, *DPN* diabetic peripheral neuropathy, *DFU* diabetic foot ulceration, *CNA* Charcot neuroarthropathy

factor, hemoxygenase-1 (HO-1), and endothelial and inducible nitric oxide synthase [47] promotes wound healing via improving perfusion.

Increased ROS can also increase poly(ADP-ribosyl)ation. Poly(ADP-ribosyl) ation is the process by which polymers of ADP-ribose (PAR) are attached via an ester bond to glutamic acid, aspartic acid, or lysine residues, mediated by the enzyme PAR polymerase (PARP) [48]. There are currently 18 known members of the PARP family, two of which, PARP1 and 2, are known to play a role in DNA repair [48]. PARP1 binds as a homodimer to single-strand DNA breaks where it is activated and catalyzes the cleavage of NAD+ forming nicotinamide and ADP-ribose, the polymers of which are added to nuclear proteins [49, 50]. Increased oxidative/nitrosative stress seen in diabetes can result in DNA damage and PARP1 activation [51–53]. Although PARP1 plays a beneficial role in DNA repair, it is possible that hyperactivation in diabetes leads to detrimental effects [50, 53]. Excess cleavage of NAD+ by PARP would exacerbate the effect of increased flux through sorbitol dehydrogenase which results in further depletion of NAD+ aggravating oxidative stress [54]. In addition NAD+ is required as a cofactor for the conversion of GAPDH. GAPDH is modified with PAR in response to diabetes-induced superoxide, reducing GAPDH activity. Hyperglycemia-induced ROS inhibits GAPDH activity in vivo by modifying the enzyme with PAR [54–56]. Hyperglycemia-induced GAPDH suppression by PAR can be prevented by PARP inhibitors [54].

Thus ROS-induced activation of PARP in cells such as keratinocytes, fibroblasts, Schwann cells, and tissues such as the vasculature could have an important effect on the structural and functional integrity of the skin. The effects of diabetes on PARP activation in the skin in subjects at high risk of foot ulceration are shown in Fig. 7.2. Skin punch biopsies were performed on the lower leg skin of healthy nondiabetic subjects and subjects with diabetes complicated by neuropathy for measurements of PARP activation. Diabetic subjects with neuropathy demonstrated an ~40 % increase in PAR-stained nuclei compared to normal controls. Thus oxidative/nitrosative stress in the skin may contribute to foot ulceration by promoting damage to small

Fig. 7.3 Effect of diabetes on skin matrix metalloproteinases in nondiabetic subjects and subjects with diabetes
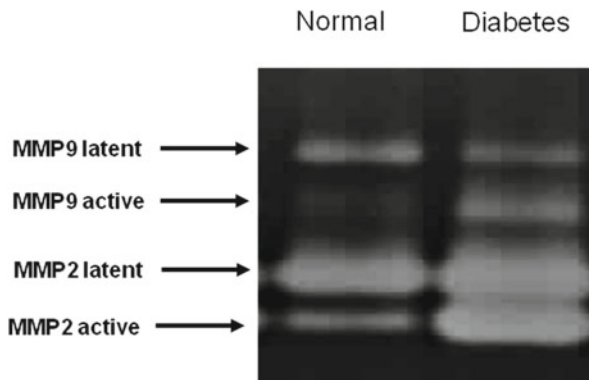
nerve fibers in the skin, by damage to local cellular elements via PARP activation, and by impairing the wound-healing response.

7.2.3 Structural and Functional Skin Deficits in Diabetes

In diabetes, dermal atrophy of the lower limb skin is associated with increased elaboration and activation of MMP, including MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), and MMP-9 (gelatinase B) (Fig. 7.3) [24]. Increased MMP elaboration is thought to be an "early event" in skin degeneration since it precedes overt changes in skin structure. Subsequently, sustained reduction in collagen synthesis occurs in concert with widespread collagen destruction [57–59]. Gene expression of MMP1, MMP-9, and tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 has been reported to be similar in skin samples from nondiabetic and diabetic patents. However levels of MMP-2, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β mRNA were reported to be elevated in subjects with [60]. Levels of oxidative/nitrosative stress as well as TNF-α and IL-1β are increased in chronic non-healing wounds [60–63] which is thought to stimulate secretion of MMPs but inhibit TIMPs [64, 65]. The abundance and activity of some MMPs have been reported to decrease as wounds heal, although this response is complex and poorly understood [66]. Collagenase (MMP-1 and MMP-8) [67, 68] and gelatinase (MMP-2 and MMP-9) [64] activity is increased in chronic wounds and TIMP-1 is decreased [69]. In normal healing, the production and activation of MMPs and degradation of the extracellular matrix and cell migration during the inflammatory phase promote the formation of the new basement membrane [70]. In turn, proteinases activate growth factors which promote granulation tissue and matrix formation and collagen synthesis by fibroblasts, which initially comprises procollagen III which is replaced by procollagen I. The inflammatory phase of healing is thought to be augmented and prolonged in chronic wounds [71, 72] which would serve to

increase the release of local proteases such as the MMPs and inflammatory cytokines [73, 74]. Chronic wounds are also characterized by impaired formation of granulation tissue. In subjects with diabetic foot ulcers, levels of TIMP-1 have been reported to be reduced, whereas MMP-1, MMP-8, and MMP-9 are increased and MMP-2 is activated [69]. Activation of MMP-9 has been associated with impaired healing in pressure ulcers [72].

7.2.4 Oxidative Stress and the Fibroblast

In diabetes impaired fibroblast proliferation and function may play an important role in contributing to damage of the skin [25, 26]. In many patients with diabetes, ulceration occurs in the presence of both sensory loss and ischemia [1–4]. Therefore given that fibroblasts with senescent characteristics can be identified in the lower limbs of patients with vascular insufficiency which precedes wound formation, abnormal fibroblast function may predispose to ulceration rather than being a consequence of the wound environment.

As discussed above, in chronic wounds, oxidative stress [37] and downregulation of type 1 collagen [75, 76] are associated with fibroblast senescence [76]. Conversely, senescent fibroblasts and fibroblasts from chronic wounds [40] have been reported to generate increased oxidative stress [77, 78]. Pro-MMP-2 and pro-MMP-3 have been reported to be increased in fibroblasts from diabetic patients [79]. Mitogen-activated protein kinases have been identified as transducers linking high glucose to biochemical deficits in diabetes [80, 81]. Activation of stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK-2) via ROS and increased lipid peroxidation can lead to upregulation of MMP expression [82] via increased AP-1 [83]. Oxidative stress is increased in fibroblasts cultured in fragmented collagen, and the antioxidant MitoQ10 has been shown to reduce expression of MMP-1 [84]. MMP-1 levels are increased with skin aging [84] which may be related to the increased fragmentation and disorganization of collagen fibrils observed in the dermis [85]. In contrast however, mmp-2 gene expression is not regulated by AP-1 which may explain the attenuated response in high glucose or diabetes [24]. Collagen synthesis is regulated at a transcriptional and posttranslational level [18]. Increased ROS has been reported to increase gene expression and the activity of MMP-1 in concert with a reduction in the expression of pro-α1(I) collagen and pro-α1(III) collagen [86] and reduce collagen production in human dermal fibroblasts (HDF). In HDF, UVB-induced activation of ERK and p38MAPK mediates downregulation of type 1 procollagen [87]. Finally, TNF-α which is increased in subjects with diabetes [88] can also lead to upregulation of MMPs in fibroblasts [89] and suppress pro-α1(I) collagen transcription [90].

7.2.5 Keratinocyte Function Can Be Disrupted by Oxidative Stress

The epidermis plays an important role in wound healing, and so deficits in epidermal function impact the healing of wounds [91]. Wounding results in proliferation of epidermal keratinocytes and upregulation of MMPs including MMP-1 and MMP-9. Keratinocytes migrate over the provisional matrix and close the wound [91]. In aged skin, reduced growth potential [92] and motility [93] of keratinocytes may contribute to the development of superficial wounds which have the potential to develop into deeper chronic ulceration. Less well understood is the role of epidermal changes in diabetes as contributors to the formation of non-healing wounds. Reduced keratinocyte proliferation may contribute directly to the atrophic changes occurring in the epidermis of diabetic subjects [25, 26]. Keratinocyte motility is also impaired in diabetes [31]. Since epidermal motility and proliferation contribute to wound closure [28], it is easy to envision how alterations in these responses directly contribute to slowed repair of wounds in diabetic skin.

7.2.6 Oxidative Stress and Nitric Oxide

Oxidative stress is thought to be critical in the development of the complications of diabetes [28, 29] including foot ulceration [31, 32]. Increased production of vascular superoxide (O^{2-}) in diabetes may inactivate nitric oxide (NO) and contribute to vascular dysfunction [94]. Nitric oxide is almost important in wound repair [95] through a number of mechanisms including angiogenesis [96] and by migration and proliferation of fibroblasts [97], epithelial [98] and endothelial cells [96], and keratinocytes [95]. Decreased wound NO synthase expression and NO levels are associated with impaired wound healing in diabetic mice, and L-arginine improves wound healing [99]. Decreased endothelial NO synthase expression is evident in the skin taken from the dorsum of the foot in diabetic subjects [97]. However the precise mechanisms whereby NO deficiency impairs wound healing remain unclear.

7.2.7 The Role of Advanced Glycosylation End Products

Impaired wound healing in diabetes may also reflect accumulation of advanced glycosylation end products (AGEs) [100, 101]. Indeed, increased skin AGEs has been observed in diabetic subjects with neuropathic foot ulceration [102]. Glycosylation of growth factor receptors may impair cell proliferation. Accumulation of AGEs in diabetic wounds and interaction with the receptor for AGEs (RAGE) can upregulate expression of proinflammatory molecules including endothelin-1, TNF-α, and MMPs [101–103]. The formation and tensile strength of granulation tissue can be

reduced by TNF-α, an effect which may be mediated by increased generation of activated MMPs and an effect which is mediated by IL-1. Upregulation of RAGE in cells important in the inflammatory response, including vascular endothelial cells, mononuclear phagocytes, and fibroblasts [104], can result in decreased collagen deposition, reduced angiogenesis, and a reduction in the quality and quantity of granulation tissue. These changes will ultimately result in poor wound healing and decreased neovascularization of diabetic wounds [101, 104, 105]. Wound healing in rats is impaired by the AGE precursor methylglyoxal which reduced the granulative tissue response [106]. Aberrant cross-linking of matrix proteins promoted by AGEs can also disrupt the deposition of extracellular matrix. Diets rich in AGE delay wound healing in experimental models [105]. Levels of inflammatory cytokines TNF-α, IL-6, and MMPs can be reduced by RAGE blockade which promotes wound healing [101].

7.2.8   Oxidative Stress and Skin Perfusion

Oxidative stress in the diabetic vasculature [107] may impair skin perfusion by a mechanism involving increased diacylglycerol and protein kinase C (PKC) which contributes to vascular dysfunction and skin small vessel disease. Increased lipid hydroperoxides may result in increased cyclooxygenase activity as well as thromboxane synthesis [108, 109] but reduced prostacyclin synthase activity [110] which can result in vasoconstriction [19, 111]. Vasodilatation of the skin in diabetes is reduced in response to occlusive ischemia [111], local [19, 20]] and indirect heating [112], as well as trauma [113]. Damage to unmyelinated primary afferent fibers in diabetes impairs vasodilatation mediated by unmyelinated C fibers [114–116]. The relationship between cutaneous mechano-sensitivity and vasodilation is known as pressure-induced vasodilation (PIV) [117]. PIV permits augmentation of skin blood flow and delays the development of pressure-induced ischemia. This response is NO-mediated and involves capsaicin-sensitive afferent nerve fibers which release calcitonin gene-related peptide in the endothelium [118]. PIV is absent in subjects with diabetes [117] and in diabetic animal models [119]. In diabetes, therefore, foot deformity and increased plantar pressures may contribute to a greater degree of perfusion impairment. Impaired skin circulation may increase oxidative stress and thereby decrease glutathione reductase activity leading to GSH depletion. This, in turn, may contribute to impaired cellular proliferation, decreased collagen and proteoglycans synthesis, and enhanced protease activity [120].

7.2.9   Oxidative Stress and Charcot Neuroarthropathy

Charcot neuroarthropathy is an underdiagnosed complication of the diabetic foot [121–123] which can result in progressive bone and joint destruction, skin

Fig. 7.4 Ulceration of the chronic Charcot foot

breakdown, and ultimately amputation (Fig. 7.4). We have reported that compared to subjects with diabetic peripheral neuropathy alone, patients with Charcot neuro-arthropathy may have distinctive small nerve fiber neurological deficits and skin vascular responsiveness which may predispose to ulceration [122]. The etiology of Charcot neuroarthropathy remains unclear, but increased oxidative/nitrosative stress may play a role. For example, RAGE defense mechanisms have been reported to be impaired in patients with Charcot neuroarthropathy [124], a finding which may contribute to skin blood flow deficits and bony fractures [124, 125]. We recently sought to determine whether activation of PARP could be involved in the pathogenesis of the Charcot foot [126]. Skin punch biopsies were performed in the skin of the upper leg in patients with and without diabetes, neuropathy, and/or a Charcot foot. The percentage of PAR-stained nuclei in the skin was increased by 32 % in subjects with diabetes alone, but the highest levels were measured in subjects with Charcot neuroarthropathy (Fig. 7.2). This increase of PARP suggests that multiple downstream targets of oxidative stress are activated in these subjects which may be involved in the pathogenesis of this disabling complication and also offer a potential therapeutic target.

7.3   Possible Future Therapeutic Options to Prevent Foot Ulceration and Accelerate Wound Healing

7.3.1   Treatment with Topical Retinoic Acid

In diabetes, topical retinoid treatment improves histological structure and biochemical function of the damaged skin [24, 92, 127]. In vitro studies have shown that treatment of skin from subjects with diabetes with retinoic acid or a synthetic retinoid in organ culture can reduce active MMP-1 and MMP-9 by 75 and by 81 %, respectively [24, 128]. Type I procollagen is reduced in diabetic patients with foot ulceration [126], and production is significantly increased in retinoic acid-treated skin in concert with inhibition of MMP elaboration production [24, 128]. Thus retinoic acid can improve the overall structure and function which should make it more resistant to ulcer formation and improve healing should ulceration occur.

The antioxidant effects of retinol and retinoids are well described [129, 130] and have been explored in a number of different cell lines. For example, retinoic acid has been reported to reduce susceptibility to oxidative stress in PC12 cells [131] in neurons [132, 133] and in mesangial cells by a mechanism involving AP-1 [134]. A limited number of studies have assessed whether retinoids can attenuate glucose-induced oxidative stress. In high-glucose-exposed human endothelial cells, for example, 9-*cis* retinoic acid decreases oxidative stress by inhibition of PKC activation [135]. In cortical neurons, retinoic acid prevents the high-glucose-mediated reduction of superoxide dismutase (SOD) activity, reduced glutathione depletion, increased lipoxygenase, and total thiol abundance [136]. The mechanism of the antioxidant actions is unclear but may involve upregulation of antioxidant gene expression [137].

Conversely at supraphysiological concentrations both retinol and retinal can cause DNA breakage via increased superoxide production [138, 139]. Retinol has been reported to increase oxidative stress in rat Sertoli cell which was associated with increased activities of SOD, catalase, and glutathione peroxidase [138]. In HDF, high concentrations (20 μM) of retinol and retinal can increase oxidative stress and apoptosis [140].

7.3.2   Treatment with Alpha Lipoic Acid

Lipoic acid (1,2-dithiolane-3-pentanoic acid) is a potent scavenger of several oxygen radical species including hydroxyl radical, superoxide, singlet oxygen, peroxyl radicals, hypochlorous acid, and nitric oxide [141]. The effects of alpha lipoic acid (ALA) have been extensively evaluated in diabetic rodent models of chronic complications. For example, ALA significantly improves or normalizes deficits in digital sensory nerve conduction velocity, endoneurial nutritive nerve blood flow,

mitochondrial and cytoplasmic NAD+/NADH ratios, GSH and GSH+GSSG content, and the activities of SOD, catalase, and cytochrome b5 reductase and corrects the increased GSSG/GSH ratio [142]. The effects of ALA on nerve function are in part, mediated through a mechanism involving NO. ALA has also been extensively evaluated in man, including seven phase I clinical studies (which included type 1 diabetic patients), three phase II clinical studies in type 2 diabetic subjects, and five phase III clinical studies. ALA has been shown to ameliorate some neuropathic symptoms and deficits in diabetic subjects with DN [143–145]. In vitro studies have demonstrated the ability of lipoic acid to prevent injury of vascular endothelial cells [146, 147] by agents including AGEs. Protection of endothelial cells against injury is thought to reflect the downregulation of several pro-injury events that depend on oxygen radicals. We have previously explored the ability of ALA to promote wound healing in skin abrasion wounds of STZ-D rats [152]. Our results in vivo demonstrated that ALA could restore wound healing to rates observed in healthy nondiabetic animals. At the histological level in STZ-D rats, ALA was found to induce a much denser provisional matrix, a more luxuriant vasculature (evidenced by the presence of large numbers of red blood cells in the provisional matrix), and fewer inflammatory cells in the matrix. These findings are consistent with those of Demiot et al. [148] who demonstrated that PIV in diabetic rodents could be prevented by ALA. In subjects with diabetes, with or without neuropathy, the effect of ALA has been explored using nail-fold video-capillaroscopy on skin capillary blood cell velocity at rest and during postreactive hyperemia (occlusion of the wrist for 2 min, 200 mmHg). ALA was found to precipitate a significant decrease in the time to peak capillary blood cell velocity [149] during postocclusive hyperemia, consistent with an effect on the microcirculation. In contrast, in vitro, ALA treatment of keratinocytes has little effect on proliferation and does not lead to a measurable hyperplasia in the skin of treated rats. Likewise, ALA has no substantial effect on fibroblast proliferation or on elaboration of type I procollagen by these cells. In these regards, the effects of ALA appear to be substantially different but complementary to those observed in the presence of retinoic acid. Finally, the oxidative formation of CML from glycated proteins is reduced by lipoic acid [150].

7.3.3 The Role of Taurine Depletion and Potential Replacement Strategies

In diabetic animal models, antioxidants have been shown to correct experimental diabetic neuropathy [142, 151], improve skin blood flow responses [148], and promote wound healing [152–155]. Taurine is a sulfur-containing free amino acid which can function as an osmolyte calcium modulator and neurotransmitter [151, 156–160]. Taurine also exhibits antioxidant properties in some tissues [159], but the precise mode of its antioxidant actions remains unclear. Intracellular taurine depletion may result in wide-ranging metabolic perturbations including impaired

cellular response to oxidative/nitrosative stress with resultant cytotoxicity [151, 156, 159]. Indeed hyperglycemia-induced taurine depletion has been demonstrated in the nerve [151, 157], lens [161], and mesangial cells [162] of diabetic rodents. Taurine replacement has been shown to attenuate oxidative stress in these tissues [159, 161, 162].

In the skin, taurine has been proposed to play a role in keratinocyte hydration [163] and is highly concentrated in the skin epidermis [164] and can increase wound tensile strength [165]. Taurine is actively transported by its Na^+- and Cl^--dependent, high-affinity transporter [166, 167]. In primary cultures of human keratinocytes, taurine and NAC have been shown to attenuate the TNF-α-induced production of inflammatory cytokines [168]. In high-fructose-fed rats, taurine prevented increases in skin collagen glycation and peroxidation [169]. The application of a taurine-containing gel to full thickness skin wounds of mice was found to increase wound tensile strength by decreasing malondialdehyde and increasing hydroxyproline levels [165]. These data indicate that taurine therapy may be helpful in reversing skin structural deficits complicating diabetes. In cultured HDF exposure to high glucose increases oxidative stress and reduces the expression of types I and III procollagen (α1), an effect which can be reversed by the addition of taurine (MJS unpublished observations). However the lack of beneficial effect of antioxidant therapy on MMP activation or skin structural deficits in organ-cultured skin from diabetic patients at risk for foot ulceration suggests that systemic rather than topical therapy may be required to achieve in vivo.

The formation of non-healing wounds in the skin of diabetic patients—especially in the lower legs and feet—remains a major clinical problem. Although numerous factors contribute to the formation of foot ulcers in diabetic patients, a critical intermediary event is the progressive atrophy of dermal connective tissue in the at-risk skin and the impaired healing response. Although the mechanisms that contribute to these deficits are no doubt multifactorial, increased oxidative/nitrosative stress most likely plays an important role at many levels ranging from fibroblast function to skin perfusion. Better understanding of these deficits may offer the opportunity to develop new therapeutic approaches utilizing agents which can effectively combat oxidative/nitrosative stress in the skin. Of interest, the targets of retinoids and α-lipoic acid action, for example, as well as the mechanisms by which these agents act, appear to be complementary. Therefore, ultimately the combination of retinoic acid with an antioxidant may ultimately prove to be the optimum therapeutic approach to improved overall skin quality and function. Clinical trials are needed to test the efficacy of these treatment approaches.

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Chapter 8
Encephalopathies Accompanying Type 1 and Type 2 Diabetes

Anders A.F. Sima

8.1   Introduction

Diabetic encephalopathies are being increasingly recognized as complications accompanying both type 1 (juvenile) (T1DM) and type 2 diabetes (T2DM). Like other so-called microvascular complication, diabetic encephalopathy appears to differ in the two types of diabetes, suggesting differences in underlying pathobiological mechanisms [1, 2]. Neurobehavioral studies in children with T1DM have shown deficits in attention, processing speech, executive function, and memory [3–5], and imaging studies have demonstrated structural deficits of both gray and white matter structures, particularly in limbic areas [5, 6]. Experimental studies in type 1 diabetic models have suggested that insulin and C-peptide deficiencies as well as hyperglycemia contribute to cognitive deficits [7, 8]. In contrast to earlier belief, recurrent episodes of hypoglycemia do not appear to play a major role on cognition. The incidence of type 1 diabetic encephalopathy is likely to increase due to the global increase in the incidence of T1DM and its occurrence in increasingly younger age groups [9–11] at a time when the brain is still developing and hence particularly susceptible to metabolic insults. In this chapter available clinical data will be reviewed, and underlying mechanisms from data obtained mainly from experimental studies will be discussed.

In the last one and a half decades, several epidemiological studies have described an association between T2DM and dementia and Alzheimer's disease. Studies including several ethnic and racial groups have shown a multifold increased incidence of dementia in T2DM patients [12–14]. Apart from hyperglycemia and insulin resistance in the central nervous system, common comorbidities such as hypercholesterolemia, hypertension, and obesity appear to exacerbate the linkage

A.A.F. Sima (✉)

Departments of Pathology and Neurology, Wayne State University School of Medicine and Detroit Medical Center, 540 E. Canfield Ave., Detroit, MI 48201, USA
e-mail: asima@med.wayne.edu

between T2DM and dementia, besides age alone. Additionally, T2DM appears to accelerate dementia and Alzheimer's disease as compared to patients without T2DM.

As with T1DM, the incidence of T2DM is increasing globally, more so in developing and heavily populated nations like China and India [15]. Interestingly, the projected increases in disease incidences of T2DM and Alzheimer's disease between years 2000 and 2040 show similar trends with a frightening threefold increase over this time period [2, 15, 16].

In this review the epidemiological linkages between T2DM and dementia and Alzheimer's disease will be reviewed. Again, based mainly on fairly limited experimental data, likely underlying pathogenetic mechanisms will be discussed.

Hence, the projected future with respect to diabetic cognitive impairments appears grim. However, recent approaches as how to prevent and modify emerging encephalopathies provide some glimmer of hope and will be discussed at the end of each section.

8.2   Type 1 Diabetic Encephalopathy

8.2.1   Clinical Studies

In the last number of decades, it has become evident that T1DM may have adverse effects on CNS function and cognition and that these effects are accentuated in children with early onset of diabetes [3, 17, 18]. These findings are alarming, since at the present time the greatest incidence of T1DM, globally, is in children under 5 years of age.

Diabetic children are likely to perform more poorly at school than their nondiabetic peers, with lower scores on academic achievement tests and verbal intelligence [19, 20]. Neuropsychological tests have revealed deficits in a variety of cognitive spheres such as sustained attention, psychomotor speed and visuoperceptual function, learning, and memory skills [5, 21–23]. Verbal IQ scores appear to decline with duration of diabetes and tend to be more severe with earlier onset of disease. Apart from age of onset, gender seems to have an effect in that boys perform worse than girls [3, 24]. In contrast to earlier beliefs, recent studies have not associated cognitive deficits with repeated episodes of hypoglycemia [25–27].

8.2.2   Imaging of T1DM Patients

A number of studies have examined brain volumes in patients with T1DM. A high incidence of mesio-temporal lobe sclerosis was demonstrated as being evident already after 7 years of diabetes. This abnormality was unrelated to previous

episodes of severe hypoglycemia [28]. Volumetric studies of patients 12 years after onset of diabetes showed significant white matter atrophy in parahippocampal, temporal, and frontal white matter. These abnormalities were associated with decreased volumes of insular cortex, hippocampus, and thalamic structures [18]. Morphometry of voxel-based analyses has demonstrated decreased densities in thalami, superior, and middle temporal gyri and frontal cortex in patients with duration of T1DM for 15–25 years [6, 29]. These findings are consistent with recent pathological examinations of two patients with early onset of diabetes and who succumbed to ketoacidosis. The postmortem exams showed severe neuronal loss in hippocampus and frontal cortex accompanied by white matter atrophy of the frontal and temporal lobes. The findings were associated with marked down regulation of IGF-1 and insulin receptors and activation of neuroinflammatory factors [30, 31]. They correlated with MRS studies of neurometabolites indicative of neuronal viability [31, 32] and suggest that limbic temporal and frontal structures are particularly vulnerable and probably underlie compromised cognition such as attention, information processing, executive function, and memory as indicated by impaired functional connectivity [33].

The recent increase in the incidence of T1DM and its onset at increasingly younger ages are being observed not only in developed countries but also in heavily populated developing countries [9–11]. The reason for this very concerning trend is not known. Various factors have been put forward, such as formula rather than breast milk feeding during early infancy, increasing incidence of childhood obesity triggering inflammation potentially triggering autoimmunity targeting pancreatic β-cells, and increased incidence of Cesarean sections [34–36].

8.2.3 Factors Underlying Type 1 Diabetic Encephalopathy

Based on the clinical longitudinal data described above, it is evident that age of onset of T1DM is important with a greater impact on the brain when undergoing development. It has been suggested that impaired cerebral blood flow may influence performances on neuropsychological tests. Other likely underlying influences include hyperglycemia with activation of the polyol pathway, compromised neurotrophic support, and cerebral blood flow [3, 18, 31, 37, 38].

Only recently have systematic studies in animal models of type 1 diabetes started to emerge. Neurobehavioral deficits, using the Morris water maze, have been demonstrated in streptozotocin-induced (STZ) diabetic rats. Such deficits were associated with deficits in hippocampal long-term potentiation of the CA1 field, reflective of synaptic plasticity in hippocampus, and information storage in the brain [39]. Interestingly, normalization of hyperglycemia by insulin treatment from onset of diabetes prevented the impairments in long-term potentiation and Morris water maze performances, whereas interventional insulin treatment resulted in only partial effects [39]. In contrast to long-term potentiation, long-term depression was

enhanced in the CA1 field of hippocampus [40]. These data tend to suggest that the underlying pathogenetic mechanisms are operable shortly after onset of diabetes.

Other cerebral abnormalities in somatosensory, visual, and auditory evoked potentials occur in the STZ-diabetic and the spontaneously diabetic Bio-breeding/ Worcester (BB/Wor-rat). Such abnormalities are followed by degenerative axonal changes in the optic nerve and dorsal columns of the spinal cord and are modified by insulin treatment [1, 41–43].

The type 1 diabetic BB/Wor-rat model shows spontaneous onset of diabetes due to an immune-mediated β-cell destruction. It shows complete insulin and C-peptide deficiencies, requiring small sustenance doses of insulin. Compared to the STZ-induced model, it is less affected systemically by emaciation. Longitudinal studies in this type 1 diabetic model have revealed a sequence of metabolic, functional, and structural defects in white and gray matter.

One of the earliest metabolic changes seen in this model is significantly suppressed expression of hippocampal insulin and IGF-1 receptors [44]. These deficits were accompanied by significant suppression of hippocampal IGF-1, IGF-2, and NGF and its receptor NFF-TrkA [2, 44]. Such abnormalities in the hippocampal neurotrophic network were substantially prevented by full replacement of C-peptide [44]. Simultaneous neurobehavioral testing using the radial arm maze [45] showed no delays in latencies in completing the learned tasks. However, the numbers of type 1 and type 2 errors reflecting impaired reference and working memories were significantly increased. Interestingly, these early functional deficits were prevented by C-peptide replacement from onset of diabetes. C-peptide replacement has no effect on systemic hyperglycemia [44]. Not surprisingly, micro-PET examination of 3 months diabetic animals showed a threefold increase in glucose uptake in hippocampus and cerebral cortex, despite an approximately 30 % deficit in the uptake rate constant of the 18_F-fluorodeoxyglucose (FDA) tracer [44].

These early abnormalities may have far-reaching consequences as to impaired cognition. Insulin, IGF-1, and NGF provide important functions in hippocampus with respect to acetylcholine and glutamate synthesis and protection of cholinergic neuronal populations [46–48]. So, for instance, insulin itself promotes choline acetyltransferase (ChAT) and inhibits acetylcholinesterase (AChE), thereby intimately involved in acetylcholine synthesis. Intact insulin signaling and that of other neurotrophic factors are pivotal to normal synthesis of neuroskeletal proteins, their phosphorylation and normal assembly [49]. The early perturbations of trophic factor activities in the BB/Wor-rat were followed by significant degeneration of presynaptic connections in hippocampus coupled with a markedly decreased expression of synaptophysin in 4-month diabetic rats. Again these early degenerative changes were fully prevented by C-peptide replacement [44]. As mentioned above, the regional metabolic rates of glucose in various brain regions showed approximately a threefold increase, which is consistent with data reported in humans [50]. Increased cerebral glucose was associated with an increased expression of the receptor for advanced glycosylation end products (RAGE) mainly colocalized with glial fibrillary acid protein (GFAP)-positive proliferating astrocytes and to a lesser extent with hippocampal pyramidal cells [51]. The upregulation of RAGE was accompanied by

upregulation of tumor necrosis factor alpha (TNF-α) and interleukins (IL) IL-1β, IL-2, and IL-6, whereas the anti-inflammatory IL-10 was significantly downregulated [51]. This upregulation of RAGE is not likely to be solely due to increased exposure to glucose and activation of the polyol pathway, since the nuclear factor kappa light-chain enhancer of activated B-cells (NF-κB), already increased by impaired insulin signaling, is known to be a potent regulator of RAGE expression [2, 51, 52]. Furthermore, TNF-α has an inhibitory effect on insulin signaling, hence providing a self-perpetuating activation of innate inflammatory responses [2, 51]. These perturbations of RAGE and inflammatory interleukins were prevented by C-peptide [69], suggesting that the activation of innate inflammation is mainly mediated via impaired insulin-like signaling by C-peptide.

As mentioned above, deficits in reference and working memory occur early in the BB/Wor-rat. Longitudinal testing using the Morris water maze paradigm revealed normal performances in 4-month diabetic rats. Significant deficits, signifying multiple cognitive spheres such as problem solving, formation of internal representation of the environment, storage, and retrieval of memory, were only evident in 6-month diabetic rats [7, 53, 54]. C-peptide replacement from onset of diabetes showed at 8 months significant but not full prevention of the Morris water maze abnormalities [53]. These data suggest that progressive learning and memory deficits occur in a duration-related fashion. Only at 7 months did diabetic animals show increased expression of the postsynaptic glutamate synaptic subunits involved in Ca^{2+} permeability of AMPA receptor channels playing crucial roles in long-term synaptic plasticity, long-term suppression, and memory formation [55].

Both insulin and C-peptide demonstrate strong antiapoptotic effects. We have previously shown that insulin- and C-peptide-deficient diabetes in the BB/Wor-rat are accompanied by a number of proapoptotic factors, like NGFR-p75, Fas, Bax, PARP, 8-OHdG, and caspase 3 and 12 [7, 53, 56]. Activation of such factors in the hippocampus was associated with increased TUNEL staining of hippocampal pyramidal cell neurons, increased DNA laddering, and decreased density of pyramidal cell neurons, particularly in the CA1 region [7].

In the BB/Wor-rat, white matter changes occur early and precede those of gray matter structures. They are characterized by apoptotic loss of myelinating oligodendroglia cells and a compensatory proliferation of astrocytes in temporal and frontal white matter [2, 44, 57]. Apart from indices of apoptotic and oxidative stress, these changes are also associated with an upregulation of RAGE, TNF-α, and proinflammatory interleukins, similar to what is seen in humans [30]. Similar changes were reported in the STZ-induced diabetic Swiss Webster mouse [8]. In both situations, the abnormalities were preceded by defects in insulin and IGF-1 signaling and prevented by systemic C-peptide replacement and intranasal insulin administration, respectively [8, 44].

These findings underline the central role of impaired insulin-signaling activities in the development of type 1 diabetic encephalopathy. This notion is supported by the effects of C-peptide replacement with a known effect on insulin-signaling elements [58], although it does not bind to the insulin receptor itself as it has sometimes been incorrectly quoted.

The above findings are consistent with those using an intranasal delivery system of insulin itself to the brain [8]. In this system, as with C-peptide replacement, systemic blood glucose levels are not affected. Intranasal insulin delivery to STZ-induced diabetes in Swiss Webster mice over an 8-month period showed prevention of cognitive decline, white matter atrophy, and atrophy of sensory-motor cortices, striatum, and hippocampus. These changes were associated with correction of mRNA of intermediaries of the P13K/Akt pathway as well as prevention of hippocampal and cortical synaptophysin and ChAT levels [8]. Hence, these extensive data demonstrate a preventional effect of direct nasal insulin delivery similar to the effects achieved by systemic C-peptide replacement.

8.2.4 Summary of T1DM Encephalopathy

It is clear that the factors underlying T1DM encephalopathy are complex, interactive, and not fully understood [2]. However, from epidemiological and experimental studies, certain commonalties are starting to emerge. Age of onset of diabetes appears to be of significance, suggesting that metabolic perturbations by T1DM during a stage when the brain is still developing are significant resulting in more severe consequences. The brain development encompasses two hypertrophic growth spurts whereby that of gray matter structures precede those of white matter and peaking around birth in both humans and the rat [59, 60]. The growth spurt of the white matter occurs postnatally in both species. Both spurts last well into early adulthood. This may explain the earlier white matter changes compared to those of the gray matter in rat models, who at the time of diabetes onset still experience the later occurring white matter growth spurt.

As shown particularly in the longitudinal animal studies, insulin deficiency within the brain and its consequences as to the expression of other neurotrophic factors probably play a central and possibly initiating role. Immediate downstream consequences involve synthesis of neurotransmitters, neuroskeletal component with subsequent effects on neurite integrity and connectivity. Additional effects include oxidative stress as well as apoptotic stress with loss of myelinating glia in the white matter and neuronal populations in gray matter structures.

Obviously along this simplified sequence of pathobiological events, other factors certainly play interactive roles. Hyperglycemia most likely plays a contributing role as indicated by clinical studies [3]. It probably contributes to oxidative stress and to activation of the innate immune responses [2]. Other contributing factors not touched upon here that are likely contributors are impaired cerebral blood flow and relative hypoxemia.

8.2.5 What Do We Do About It?

As alluded to above, T1DM encephalopathy is most likely going to become a major medical and social problem over the next decades, although the detailed mechanisms underlying this potential epidemic are not known. However, as indicated by some clinical studies and experimental data, normalization of central insulin-signaling mechanisms should be a prominent goal. We have available to us today relatively simple tools that are highly likely to be of significant benefit in preventing and modifying the development of T1DM encephalopathy. Direct insulin delivery systems to the CNS have repeatedly been shown experimentally to be of tremendous benefits [8, 61]. Likewise systemic C-peptide replacement to sustain insulin-related signaling mechanisms has repeatedly been shown, again in animals, to have significant beneficial effects. Not only would the simple replacement of C-peptide benefit encephalopathy associated with T1DM, but also other so-called chronic T1DM complications both in humans and animal models (see recent review [62]).

This is not to say that these simple and relatively inexpensive measures are going to cure the complications including encephalopathy of T1DM, but from a logical point of view, it would be the best approach easily available to us today to prevent them and modify their clinical expressions.

8.3 Type 2 Diabetic Encephalopathy

8.3.1 Epidemiology and Clinical Studies

The relationship between diabetes and cognitive defects was suggested already in 1922 [63]. A number of studies in different ethnic groups have demonstrated a linkage between T2DM and mild cognitive impairment (MCI) and AD. The projected increases in the prevalence of diabetes and dementia show similar and parallel trends in the various ethnic groups [2, 15, 16] being greatest in heavily populated regions such as China, India, and South America [2, 15, 16]. The coexistence of cerebrovascular disease and T2DM enhances the correlation with MCI and the development of dementia [14, 64, 65], underlining the common coexistence of cerebrovascular disease with T2DM.

Several studies have demonstrated an increase of AD in T2DM patients as compared to nondiabetic individuals. The Rotterdam Study [12] examined some 6,000 patients 55 years and older over a 2-year period using the Mini Mental State Examination (MMSE) and Geriatric Mental State Schedule scores. In this study, T2DM patients showed a twofold increased risk for developing dementia. Patients treated with insulin were at an even higher relative risk being 4.3-fold. Arvanitakis et al. [66] examined over 800 nuns and priests longitudinally over 9 years. Fifteen percent of the cohort had or developed T2DM and showed a 65 % increased risk for

developing AD. The Honolulu-Asia Aging Study [14, 67] examining more than 2,500 Japanese Americans showed a 1.8-fold increased risk for developing AD and 2.3-fold increased risk for vascular dementia. The risk for developing AD increased significantly to 5.5-fold in those T2DM patients who also had the APOE 4ε allele. It should be noted though that the Framingham study found an increased risk for developing AD in patients who were negative for the APOE 4ε genotype [68].

In a follow-up study of the Honolulu-Asia Aging Study [67], in which the authors examined the association between fasting insulin levels and dementia, they found increased risk for dementia in patients with the lowest and highest 15 % percentiles of fasting insulin levels. A recent study of patients older than 75 years of age showed that uncontrolled and/or undiagnosed diabetes increased the risk for AD more than twofold [69]. However, negative studies have also been reported showing nonsignificant relationships between T2DM and AD but with a higher relationship between T2DM and vascular dementia [70].

A number of studies have addressed the different attributes of the metabolic syndrome and cognitive decline. With respect to hypertension, there are generally decreased cognitive performances in hypertensive as compared to normotensive individuals [71]. Follow-up studies showed that hypertension during midlife was associated with an increased risk of cognitive deficits and dementia at a later age [72]. Hypertension causes changes of large cerebral vessels and may severely compromise cerebral perfusion by luminal narrowing of small arterioles resulting in hypoxemia with infarctions and white matter changes so-called leukoaraiosis [73, 74]. Controlled trials employing antihypertensive compounds have provided mixed results. Few studies have reported beneficial effects on dementia [75, 76]. Therefore, hypertensive cerebral vasculopathy may further enhance the effects of diabetic microangiopathy with adverse effects on the cerebral microcirculation. Obesity is associated with poorer cognitive scores, and as with hypertension, obesity in midlife leads to worse cognitive performances in late life [72]. Obesity is associated with leptin metabolism. Impaired leptin homeostasis increases the amount of extracellular amyloid-β and tau phosphorylation in animal models. Administration of leptin results in improvement of cognitive performance, reduction of extracellular amyloid-β, and reduction of tau phosphorylation [77]. In AD reduced circulating levels of leptin are inversely correlated with the severity of cognitive deficits. Hyperlipidemia has in some studies been reported to be associated with increased risk of cognitive deficits [78], whereas others show reversed associations [79]. Pathological and experimental data suggest a pathogenetic role for elevated cholesterol levels in cognitive impairment and dementia (see Sect. 8.3.3).

8.3.2   Imaging Studies in Type 2 Diabetes

It is well known that brain volume decreases with age, being more prominent in the frontal lobe than in other brain regions and that this decline in volume is greater in males than in females [80, 81]. Normal aging is also associated with an increased

incidence of both symptomatic and silent cerebral infarcts [82] and with an increased prevalence of white matter lesions approaching 100 % at age 85 [83].

The incidence of lacunar and silent infarcts is increased up to twofold in T2DM patients as compared to matched nondiabetic individuals [82, 84]. Recent population-based studies demonstrate an increased incidence of white matter lesions in patients with type 2 diabetes [84–86]. T2DM patients show reduced volumes of hippocampus and amygdala [87, 88] and a threefold increased risk for medial temporal lobe atrophy [89] compared to nondiabetic individuals. A relationship between white matter lesions, brain atrophy, and cognitive function has been described in some studies [90, 91]. There is evidence to suggest that these progressive deficits in brain structure may develop already in patients with prediabetes [92]. Single components that comprise the metabolic syndrome also impact on brain pathology. Hypertension without diabetes is a known major risk factor for stroke and white matter atrophy [82, 92], and hyperlipidemia per se is associated with increased risk of stroke [93].

From longitudinal clinical studies, it is therefore clear that the linkages between T2DM, dementia, and Alzheimer's disease are multiple. Age alone is an important factor, which enhances the vulnerability of the brain to other insults. Of the attributes of diabetes alone, hyperglycemia per se is of pathogenetic impact in part responsible for nonenzymatic glycation and oxidative stress (see below). Another not always considered factor is the early perturbations of insulin resistance, leading to impaired insulin signaling and hyperinsulinemia with downstream effects on various nerve growth factors, inflammation, tau, and amyloid handling [94, 95]. Below an attempt will be made to construct a pathogenetic scheme linking type 2 diabetes to Alzheimer's disease.

8.3.3 Mechanisms Underlying Alzheimer's Disease in Type 2 Diabetes

From the epidemiological data referred to above, it is clear that multiple mechanisms contribute to the increased incidence of AD in diabetes and metabolic syndrome. Undoubtedly, advancing age is a major factor. Hyperglycemia is an important factor in reducing cerebral blood flow by decreasing vasoreactivity [96, 97] and contributes to oxidative stress. Vasodilatation is mainly mediated by NO synthesized in endothelial cells by endothelial NO synthase (eNOS). eNOS expression is reduced in a hyperglycemic environment, probably by reduced protein kinase C (PKC) and increased activity of NADPH oxidase [97, 98]. Hence, such effects on vasoreactivity will in addition to pathological changes of the microvasculature referred to above compromise cerebral microcirculation.

8.3.4   Insulin-Related Mechanisms

Most of the mechanistic data linking T2DM with dementia and AD-like pathologies referred to here are obtained from experimental data. Increasing age is accompanied by a decrease in cerebral insulin and IGF levels and a desensitization of their receptors with impaired downstream signaling activities. However, the expression of, for example, the insulin receptor is not necessarily downregulated, whereas that of the IGF-1 receptor usually is [99–101]. Such age-related changes become more pronounced with AD and are accompanied by increased levels of circulating insulin.

Insulin and IGF-1 mediate a myriad of effects in the brain, such as glucose utilization and energy metabolism, oxidative stress, gene regulation of other neurotrophic factors and their receptors, cholinergic gene expression, expression and phosphorylation of neuroskeletal proteins including tau, and regulation of β-amyloid, and they exert anti-inflammatory and antiapoptotic effects [8, 53, 101–103]. Impaired insulin/IGF-1 signaling in insulin-resistant T2DM impairs tyrosine phosphorylation and phosphorylation of IRS molecules with downstream inhibitory effects on the extracellular signal-related kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, as well as the phosphatidylinositol 3-kinase/phosphorylated Akt (P13 kinase/Akt) pathway and glycogen synthase kinase 3β (GSK-3β). Impaired insulin-signaling activity acts unfavorably on the expression and translocation of several transcription factors such as nuclear factor kappa light-chain enhancer of activated β-cells (NFκB) and the cyclic AMP-responsive element-binding protein (CREB) and GSK-3β with effects on proinflammatory factors and apoptosis [8, 53, 103].

Increased expression of NFκB occurs via phosphorylation of I-κB, due to impaired insulin signaling, with disinhibition of NFκB [104, 105]. Activation of NFκB also occurs in the presence of high glucose [106, 107]. NFκB plays a central role in the initiation of the inflammatory cascade with activation of tumor necrosis factor alpha (TNF-α), interleukins, and C-reactive protein [2, 44, 51, 106, 107]. The upregulation of TNF-α has an inhibitory effect on insulin and IGF-1 signaling, thereby providing a self-perpetuating loop [108]. NFκB is also a potent modulator of apoptosis and ROS production.

Impaired insulin signaling suppresses early gene responses of c-fos and c-jun with consequences for the expression of IGF-I, IGF-II, NGF, and NT-3 expression and their receptors [56, 109]. Both insulin and NGF provide significant neurotrophic support in hippocampus with respect to cholinergic and glutamergic function [46, 48]. Insulin is closely tied to neurotransmitter synthesis including acetylcholine and glutamate, and NGF exerts a protective effect on cholinergic neurons. Recent advances in our understanding of incretin hormones have led to advances in treating T2DM. Glucagon-like peptide-1 (GLP-1) receptor agonists have shown to be effective in lowering glucose and enhance insulin action [110]. Experimental studies of STZ-diabetic transgenic mice treated with GLP-1 have revealed exciting data showing amelioration of amyloid-β and tau levels [111]. Treatment with PPAR agonists in intracerebrally STZ-treated rats has shown increased insulin receptor expression

and binding [112]. It is therefore almost certain that impaired insulin action plays an important and central role in the increased susceptibility for AD in T2DM. A further evidence for this linkage in patients is the finding that the patients with prediabetes without defined diabetes [113] and with metabolic syndrome alone [114] show poorer cognitive performances with increased rates of decline over time [114].

8.3.5 Amyloid Metabolism

The hallmarks of AD are the deposition of amyloid-beta (Aβ) and the presence of hyperphosphorylated tau isoforms in neurofibrillary tangles. Aβ deposition is associated with impaired insulin signaling, although other mechanisms (see below) are also contributory. Direct effects of insulin on Aβ deposition are twofold. It has been shown both experimentally and in humans that insulin enhances Aβ release from neurons [115]. Furthermore the insulin-degrading enzyme (IDE) degrades both Aβ and insulin [115] Therefore, in a situation of elevated insulin levels, insulin resistance will increase intracellular Aβ and favor extracellular accumulation of Aβ. The net effect of insulin resistance and hyperinsulinemia is therefore increased levels of intracellular and extracellular Aβ levels, respectively. Interestingly, recent data suggest that oligomeric C-peptide may promote amyloid states [116]. Inflammation with activation of microglia promotes Aβ accumulation and amyloid precursor protein (APP) expression and cleavage increase with oxidative stress [117, 118]. In T2DM, C-peptide levels are elevated along with insulin.

8.3.6 Cholesterol and Amyloid Deposition

There is now both overwhelming clinical and experimental data supporting the concept that increased cholesterol levels are involved in amyloidogenesis. The amyloidogenic processing of APP occurs in membrane rafts or so-called caveolae of the cell membrane. These membranous microdomains are enriched in cholesterol, sphingolipids, and saturated phospholipids [119, 120]. Both the insulin and IGF-I receptors are located within these domains. The abnormal processing of APP to Aβ and C-terminal fragment (CTF) of APP occurs in the caveolae and is mediated by β- and γ-secretases. The normal processing of APP to soluble APPα (sAPPα) occurs outside the domains of the caveolae [80]. High cholesterol levels increase the number and the size of caveolae and regulate the levels of caveolin-1, with increased expression of APP, activation of β- and γ-secretases, and hence the formation of Aβ [121–124]. A further factor regulating cholesterol homeostasis is the ε4 allele of Apo E, which is identified as an important risk factor in AD [125, 126]. This is not totally unexpected since Apo 4ε is a lipoprotein that carries and facilitates the transport and incorporation of cholesterol within caveolae. Its expression increases the formation of Aβ fibrils and decreases sAPPα yielding a reciprocal regulation of Aβ

and sAPPα [127, 128]. Indeed in vivo experimental studies show that high-cholesterol diets increase Aβ levels and that cholesterol depletion inhibits Aβ generation [129, 130]. Brain cholesterol is not solely dependent on dietary uptake or hepatic synthesis but is also derived from in situ synthesis [131]. It should be mentioned though that altered signaling of the colocalized insulin receptor also has an impact on APP metabolism [128]. This may not be totally surprising, since there are multiple interactions between the scaffolding of insulin, IGF-1, and caveolin-1 signaling [132, 133]. Evidence suggests that, for instance, statins not only lower cholesterol levels (both systemic and endogenous) but also suppress β-secretase activity in caveolae and promote that of α-secretase, thereby directly attenuating abnormal APP metabolism [120].

The central role of caveolin-1 in the perturbed APP handling and amyloidogenesis has also been shown in vitro. High glucose exposure alone significantly increases caveolin-1 expression, APP, BACE, and Aβ. These increases are significantly greater by addition of cholesterol alone and still further increased by the combination of high glucose (30 mM) and high cholesterol (7 μg/ml). These data suggest a synergistic effect on the perturbed APP metabolism by hyperglycemia and hypercholesterolemia [124]. It is even further enhanced, as would be expected, by incubation of neuroblastoma cells with high cholesterol and Apo 4ε [124].

8.3.7   Abnormal Tau Processing

Tau plays a major role in regulating microtubules, axonal transport, and neuritic outgrowth. Abnormal phosphorylation results in tau dysfunction occurring in multiple neurodegenerative disorders, the so-called tauopathies, and constitutes the major component of paired helical filaments that make up the neurofibrillary tangles in AD.

The linkage between abnormal APP handling and aberrant phosphorylation of tau is not well understood. Activation of several caspases occurs secondary to impaired insulin signaling [7, 94, 134, 135] and to amyloidogenic APP metabolism and is believed to initiate proteolytic cleavage of tau [101, 136]. Once cleaved, tau loses its inhibitory domain of the C-terminal, hence allowing N-terminal fragments to phosphorylate and polymerize. Exposed epitopes are susceptible to phosphorylation by various kinases, some of which emanate from the compromised insulin-signaling cascade such as GSK-3β, PP2A, and Cdk5 [101]. Furthermore, Aβ oligomers induce phosphorylation of tau via inactivation of insulin receptor substrate and upregulation of JNK [137]. Such mechanisms possibly link the amyloidogenic APP handling as well as impaired insulin signaling to abnormal tau disposition in AD.

8.4 Studies in T2DM Animal Models

Numerous studies using transgenic or knockout models with streptozotocin-induced diabetes have linked insulin and IGF-1 signaling to abnormal tau and APP handling. On the other hand, relatively few studies have utilized genetically non-manipulated type 2 diabetic animal models to study the relationship between T2DM and AD.

We reported on the spontaneously type 2 diabetic BBZDR/Wor-rat, which develops obesity, hyperglycemia, and insulin resistance with hyperinsulinemia as well as elevated cholesterol levels, hence closely mimicking the human disorder [101, 138]. Eight months of diabetes, in this model, shows severe neuronal loss in cerebral cortex associated with significant decreases in presynaptic densities and expression of synaptophysin and profound gliosis and a ninefold increase in degenerating neurites as compared to age-matched control rats [101, 138]. The insulin receptor is not downregulated in frontal cortex, whereas insulin-signaling intermediaries such as pAkt and GSK-3β are suppressed signifying insulin resistance. On the other hand, the expression of the IGF-IR localized to caveolae is downregulated in frontal cortex. These abnormalities are accompanied by marked increases in APP, β-secretase, Aβ, and CTF, as well as a 2.5-fold increase in hyperphosphorylated tau. The amyloidogenic APP metabolism was associated with a significant increase in caveolin-1 expression. The latter is linked to insulin resistance and hypercholesterolemia in this model [101]. This was confirmed by in vitro studies and was further accentuated by exposure to Apo4E [124]. Similar but substantially milder changes are observed in the type 1 counterpart model, the BB/Wor-rat [101, 138], which is consistent with recent findings in the T2DM db/db mouse model and in the T1DM STZ mouse model [139]. Therefore, in these models, central insulin resistance and increased exposure to cholesterol can be directly linked to amyloidogenic APP handling and hyperphosphorylation of tau, the very hallmarks of AD.

8.5 Summary T2DM Encephalopathy

Based on the clinical and experimental data described above, there are undoubtedly mechanistic connections between T2DM and AD perpetuating the latter in T2DM patients. It appears that insulin resistance associated with upregulation of caveolin-1 is of central importance with direct effects on amyloid and tau accumulations and indirect and secondary effects via apoptotic and oxidative stressors on neurodegeneration. Furthermore, impaired insulin action affects other neurotrophic factors, neurotransmitters, and structural neuroskeletal proteins contributing to neurite degeneration. Other common clinical abnormalities associated with T2DM, such as hyperlipidemia and obesity, appear to accentuate the abnormalities caused by insulin resistance, such as enhanced amyloidogenic processing of APP and activation of innate inflammatory factors with further reciprocal adverse effects on insulin signaling and oxidative and apoptotic stressors eventually resulting in neuronal loss.

Although many questions remain as to the detailed linkages between the two disorders, certain relationships are starting to become increasingly clear. Therefore, continued investigations are needed in order to start to formulate potential therapeutic interventions in order to curtail the increase of these two major epidemics and their relationship.

8.6 Preventional and Interventional Approaches

As with other chronic complications of diabetes, T2DM-associated AD has been referred to a sT3DM [68], multiple factors are at work. It appears that insulin resistance with downstream effects on amyloidogenesis and tau protein accumulation plays a pivotal role. It therefore seems essential to ameliorate insulin resistance and attending hyperinsulinemia as well as hyper-C-peptidemia. Both insulin and C-peptide form oligomers, so-called amyloid-beta-derived diffusible ligands (ADDLs) [116, 140] which may impact an insulin signaling and Aβ deposition.

Therefore, antidiabetic agents such as insulin sensitizers seem to be a logical starting point. Clinical trials and several experimental studies have demonstrated beneficial effects of PPARγ agonists (see reviews in [141, 142]). Such compounds as rosiglitazone or pioglitazone confer not only an insulin-sensitizing effect but also anti-inflammatory, antioxidative, and anti-amyloidogenic effects. Hence, this group of drugs represents attractive compounds for the treatment of AD in T2DM.

Advances in our understanding of incretin hormones have led to advances in the treatment of T2DM. GLP-1 receptor agonist has demonstrated beneficial effects with respect to enhanced insulin action and glucose lowering [110]. Transgenic mice with STZ-induced diabetes treated with GLP-1 have demonstrated amelioration of Aβ and tau levels [111]. Acetyl-L-carnitine (ALC) is another compound that has undergone clinical trials. It enhances acetylcholine production via improvement in mitochondrial function and enhancement of ATP. It inhibits hippocampal excitotoxicity and promotes responses to NGF. In patients with AD, it improves behavioral deficits like short- and long-term memory, spatial learning task, and those of personal recognition [143, 144]. ALC has also demonstrated beneficial effects on diabetic neuropathy [145]. Apart from these targeted therapy, it is obvious from the above that good glycemic control, control of hypertension, and hyperlipidemia when present should be beneficial.

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Chapter 9
Oxidative Stress, Inflammation and Endothelial Dysfunction: The Link Between Obstructive Sleep Apnoea and Vascular Disease in Type 2 Diabetes

Abd A. Tahrani and Asad Ali

9.1   Introduction

Diabetes mellitus (DM) is a global epidemic that is associated with significant health, social and economic burden [1–4]. Diabetes-related vascular complications, endothelial dysfunction and cardiovascular disease (CVD) remain the main causes of the increased morbidity and mortality of DM. The development of vascular complications in DM is multifactorial. The main putative mechanism includes the activation of poly(ADP-ribose) polymerase (PARP), aldose reductase, protein kinase C (PKC) and the hexosamine pathway and increased production of advanced glycation end products (AGE), increased inflammation and endothelial dysfunction [5, 6]. Hyperglycaemia-induced oxidative stress (OS) and nitrosative stress (NS) seem to play a pivotal role in the activation of these multiple harmful pathways as well as inflammation leading to endothelial dysfunction and vascular complications (Fig. 9.1) [5, 7–9].

Obstructive sleep apnoea (OSA) is a common medical disorder that affects at least 4 % of men and 2 % of women [10] and is very common in patients with type 2 DM (T2DM) [11–20]. OSA was identified recently as an "oxidative stress" disorder, due to the recurrent cycles of deoxygenation followed by re-oxygenation simulating ischaemia–reperfusion injury [21, 22]. In addition, OSA has been associated with increased inflammation and endothelial dysfunction [21–24]. OSA-induced

A.A. Tahrani (✉)
Centre of Endocrinology Diabetes and Metabolism (CEDAM), School of Clinical and Experimental Medicine, University of Birmingham, Birmingham B15 2TT, UK

Department of Diabetes and Endocrinology, Heart of England NHS Foundation Trust, Birmingham, UK
e-mail: a.a.tahrani@bham.ac.uk

A. Ali
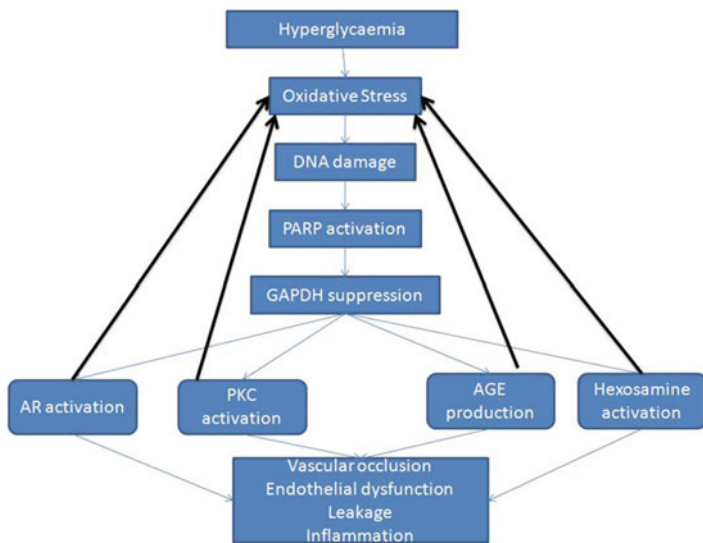Department of Respiratory Medicine, University Hospital of Coventry and Warwickshire, Coventry, UK

Fig. 9.1 Summary of mechanisms that relate hyperglycaemia to microvascular complications in patients with diabetes. *AR* aldose reductase, *PK* protein kinase, *AGE* advanced glycation end products, *PARP* poly(ADP-ribose) polymerase, *GAPDH* glyceraldehydes-3 phosphate dehydrogenase. Adapted from [9] with permission

OS seems to play an important role in the pathogenesis of OSA-related complications and in the associations between OSA and several vascular and metabolic risk factors. Furthermore, as OSA is very common in patients with T2DM, it is plausible that OSA contributes to OS, inflammation and endothelial dysfunction in hyperglycaemic patients and the activation of several pathways resulting in further vascular disease and endothelial dysfunction.

In this chapter, we will review the evidence of the relationship between OS and DM and between OSA and OS, inflammation and endothelial dysfunction, and we will highlight recent advances regarding the impact of OSA in patients with DM.

9.2 Obstructive Sleep Apnoea

9.2.1 Definitions

OSA is characterised by instability of the upper airway during sleep, which results in markedly reduced (hypopnoea) or absent (apnoea) airflow at the nose or mouth [10]. These apnoea/hypopnoea episodes are usually accompanied with cyclical changes in oxygen saturation, blood pressure and heart rate, micro arousals that cause sleep fragmentation, reduction in slow wave and REM and changes in the intrathoracic pressure (as an attempt to overcome the obstruction) (Fig. 9.2) [10].
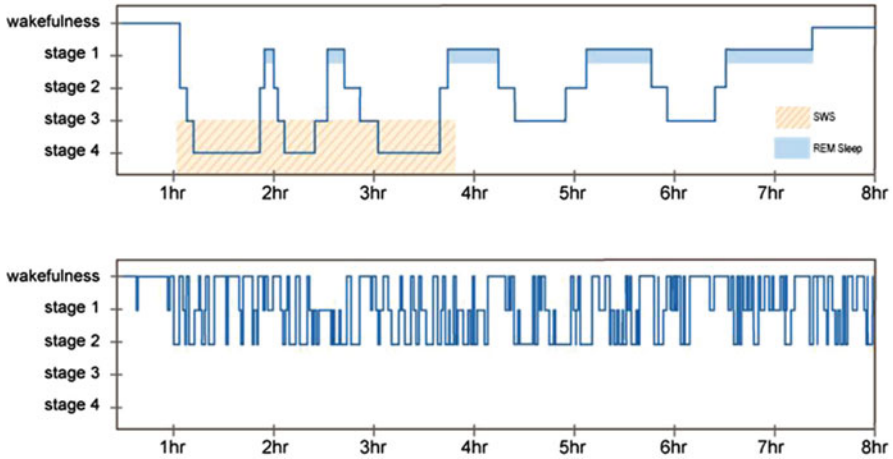
Fig. 9.2 *Top*: normal hypnogram showing sleep stages 1–4 and REM. *Bottom*: hypnogram from a patient with severe OSA showing multiple arousals and lack of SWS and REM sleep. *SWS* slow wave sleep, *REM* rapid eye movement

The American Academy of Sleep Medicine (AASM) guideline has defined apnoea as cessation or ≥90 % reduction in airflow for a period of ≥10 s and hypopnoea as ≥30 % reduction in airflow for ≥10 s associated with ≥4 % drop in oxygen saturation [25]. Apnoeas are classified into obstructive or central based on the presence or absence of respiratory/abdominal efforts. An example of apnoeas and hypopnoeas can be found in Fig. 9.3.

The apnoea–hypopnoea index (AHI) is the average number of apnoea and hypopnoea episodes per hour during sleep and is a marker of the severity of OSA [10]. An AHI ≥5 events/h is consistent with the diagnosis of OSA [26]. OSA can be classified into mild, moderate and severe based on AHI 5 ≤ 15, 15 ≤ 30 and ≥30 events/h. The respiratory disturbance index (RDI) is another OSA measure that includes the AHI in addition to respiratory effort-related arousal, which is defined as a sequence of breaths characterised by increasing respiratory effort leading to an arousal from sleep, but that does not meet criteria for an apnoea or hypopnoea [10].

9.2.2 Epidemiology

OSA prevalence varies considerably between studies (4–26 % in men and 2–28 % in women), mainly due to differences in the population studied, study designs and the method and criteria used to diagnose OSA [10, 27]. In addition, OSA prevalence is affected by many risk factors such as ethnicity (possibly higher risk in Afro-Caribbeans) [27–33], gender (higher risk in men) [27], hormonal status (higher risk in postmenopausal women or men receiving testosterone replacement) [34] and age (increasing risk in older population, but the relationship is not linear as it reaches a
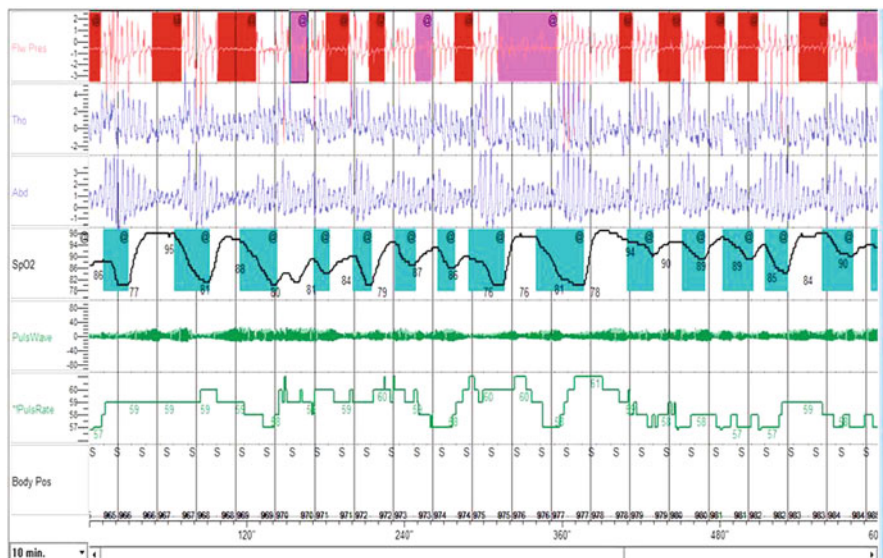
Fig. 9.3 An example of a sleep study from a patient with type 2 diabetes and OSA. The *top row* shows air flow followed by thoracic and abdominal movements followed by oxygen saturation. *Red areas* represent apnoeas, *pink areas* represent hypopnoeas and *green areas* represent oxygen desaturation

plateau around the age of 65–70 years) [27, 28, 34, 35]. Excess body weight, however, is the major risk factor for OSA, although not all OSA patients are obese or overweight [34]. In the Wisconsin Sleep Cohort study, each increase in BMI by one standard deviation resulted in a 4-fold increase in OSA prevalence [36]. In addition, prospective studies showed that weight gain is associated with the development of or worsening of pre-existing OSA [37, 38]. This was further supported by randomised controlled trials showing that weight loss (via lifestyle modifications or surgical intervention) improves/cures OSA [39, 40].

9.2.3   Comorbidities

OSA is associated with several cardiovascular risk factors, but as most OSA patients are obese, it is difficult to state the impacts of OSA from those of obesity.

9.2.3.1   Hypertension

OSA was found to be an independent predictor of the lack of nocturnal dip in blood pressure (BP) in 328 adults enrolled in the Wisconsin Sleep Cohort Study who completed 2- or more 24-h ambulatory BP studies over an average of 7.2 years [adjusted

OR (95 % CI) for baseline AHI 5–14.9 and ≥15 versus AHI<5 were 3.1 (1.3–7.7) and 4.4 (1.2–16.3), respectively] [41]. In another prospective study based on the Wisconsin Sleep Cohort Study, patients with OSA were found to be at increased risk of developing sustained hypertension over a 4-year period (relative to an AHI of 0 at baseline, the adjusted OR for the presence of hypertension at follow-up were 1.42 [95 % CI 1.13–1.78], 2.03 (1.29–3.17) and 2.89 (1.46–5.64) for an AHI of 0.1–4.9, 5.0–14.9 and ≥15.0, respectively; $p=0002$ for the trend] [42]. The associations of hypertension with OSA were seen in men and women, old and young, all ethnic groups and amongst normal-weight and overweight individuals [43].

9.2.3.2 Insulin Resistance, Dysglycaemia and T2DM

OSA is associated with components of the metabolic syndrome and with insulin resistance (IR) independent of obesity [44]. Several studies examined the association between OSA and IR; most showed an association [12, 45–64], but some did not [65–71]. The studies were mostly cross-sectional and the adjustment for confounders varied significantly. Studies that did not show such a relationship included fewer participants and potentially were underpowered.

OSA has also been associated with prediabetes (impaired fasting glucose and impaired glucose tolerance). In a subset of the Sleep Heart Health Study, relative to those with RDI<5, individuals with mild and moderate to severe OSA had adjusted OR of 1.27 (95 % CI 0.98–1.64) and 1.46 (1.09–1.97), respectively, for fasting glucose intolerance [57]. Sleep-related hypoxaemia was also associated with glucose intolerance independently of age, gender, BMI and waist circumference [57]. In another cross-sectional study of 2,588 participants, OSA (RDI≥10) had higher adjusted OR of 1.3 (1.1–1.6) for IFG, 1.2 (1.0–1.4) for IGT, 1.4 (1.1–2.7) for IFG plus IGT and 1.7 (1.1–2.7) for occult diabetes compared to those without OSA [72].

OSA has also been shown to increase the risk of incident T2DM in several prospective studies [73–78]. These studies used a variety of methods to diagnose OSA (from symptoms to polysomnography) and to diagnose T2DM (from self-reported to OGTT); these studies consistently show that OSA is an independent predictor of incident T2DM.

As T2DM and OSA share major risk factors such as obesity and age and OSA is associated with IR, it is not surprising that epidemiological cross-sectional studies showed that OSA is very prevalent in patients with T2DM (23–86 %) [11–20]. The variation in OSA prevalence between studies is due to the differences in population characteristics (primary versus secondary care, long versus short diabetes duration, ethnicity, obesity, etc.) and the differences in the methods and the criteria used to diagnose OSA.

A small number of studies assessed the relationship between OSA and glycaemic control in patients with T2DM, and they showed that OSA and its severity are associated with poorer glycaemic control (both HbA1c and fasting plasma glucose) and glycaemic variability after multivariable adjustments for several confounders such as age, sex, race, BMI, number of diabetes medications, level of exercise, years

of diabetes and total sleep time in some studies [18, 79–82]. These studies were relatively small ($n = 31$–92). The adjusted mean increase in HbA1c between patients with and without OSA varied between 0.7 and 3.69 % depending on the OSA severity.

Despite that some studies showed that CPAP treatment improves insulin sensitivity, the impact of CPAP on glycaemic control in patients with T2DM has been inconsistent. Several studies evaluated the impact of CPAP on glycaemic control in patients with T2DM [11, 83–88], of these, only one is a randomised clinical trial [88], with the rest being uncontrolled pre- to post-assessments. The one randomised controlled study showed no change in HbA1c after CPAP therapy for 3 months. The lack of positive effect could be attributed to the small study sample, the limited duration of follow-up and the suboptimal adherence to CPAP (3.6 h/night). In marked contrast, uncontrolled studies have shown improvements in insulin sensitivity [11, 83], postprandial hyperglycaemia [84], glycaemic variability [87] or HbA1c [84, 85].

9.2.3.3 Cardiovascular Disease and Mortality

Three prospective studies showed that OSA (based on polysomnography) predicts the development of CVD [89–91]. In a study of 182 consecutive middle-aged men free of CVD at baseline who were followed for 7 years, the incidence of CVD was 36.7 % of patients with OSA versus 6.6 % subjects without OSA ($p < 0.001$) with an adjusted OR of 4.9 (1.8–13.6) [89]. CPAP treatment was associated with lower incidence of CVD compared to those non-treated (56.8 versus 6.7 %, $p < 0.001$) [89]. In another prospective study in which men with OSA were followed for a mean of 10.1 years, patients with untreated severe OSA had a higher incidence of fatal and nonfatal CVD than did untreated patients with mild–moderate OSA, simple snorers, patients treated with CPAP and healthy participants [90]. After adjustment for confounders, untreated severe OSA significantly increased the risk of fatal (OR 2.87, 95 % CI 1.17–7.51) and nonfatal (3.17, 1.12–7.51) CVD compared with healthy participants [90]. In another important prospective study, 1,022 patients were followed up for a median of 3.4 years [91]. After adjustment for confounders, OSA was significantly associated with stroke or death (hazard ratio, 1.97; 95 % CI 1.12–3.48; $p = 0.01$) [91]. Furthermore, in patients with stable coronary artery disease, patients with OSA had larger atherosclerotic plaque volume as assessed by intravascular ultrasound, and AHI correlated positively with the plaque volume ($r = 0.6$, $p = 0.01$) [92]. The role of the nocturnal events in OSA to the occurrence of myocardial infarction is further supported by a study that showed patients with OSA were more likely to develop acute myocardial infarction between 12 and 6 am compared to patients matched for comorbidities but do not have OSA (32 versus 7 %, $p = 0.01$) [93].

The impact of OSA on mortality was examined in the Wisconsin Sleep Cohort [94] and the Sleep Heart Health Study [95]. In an 18-year follow-up, there was a stepwise reduction in survival with worsening OSA. The adjusted hazard ratio (HR, 95 % CI) for all-cause mortality with severe versus no OSA was 2.7 (1.3–5.7) [94].

In 6,441 men and women that were followed up for 8.2 years, compared to those without OSA (AHI <5 events/h), the HR (95 % CI) for all-cause mortality in those with mild, moderate and severe OSA were 0.93 (0.80–1.08), 1.17 (0.97–1.42) and 1.46 (1.14–1.86), respectively, after adjustment for age, sex, race, smoking status, BMI and prevalent medical conditions [95]. Measures of intermittent hypoxaemia, but not sleep fragmentation, were independently associated with all-cause mortality [95].

9.3 Oxidative Stress

The term OS refers to the situation of a serious imbalance between the production of free radicals and the antioxidant defence mechanisms, leading to potential tissue damage [96]. Free radical species are a variety of highly reactive molecules that can be divided into different ROS, reactive nitrogen species (RNS) and reactive chlorine species (RCS). A common feature of cells that are damaged by hyperglycaemia is the presence of ROS/RNS causing OS [97, 98]. ROS/RNS can interact with the nitric oxide (NO), which promotes the formation of peroxynitrite while diminishing the bioactivity and bioavailability of NO resulting in endothelial dysfunction, inflammation and atherosclerosis [21, 99]. In addition, OS interacts with lipids, protein, carbohydrates and DNA causing cellular damage and dysfunction [100].

There are four protein complexes (I–IV) in the mitochondrial electron transport chain [5]. Glucose metabolism through the tricarboxylic acid cycle (TCAC) generates electron donors [5]. The main electron donors are NADH, which gives electrons to complex I, and $FADH_2$, which donates electrons to complex II [5]. These electrons are passed to coenzyme Q and then transferred to complex III, cytochrome-C, complex IV and finally molecular oxygen, which they reduce to water [5]. Throughout the electron transport system, ATP levels are precisely regulated [5]. As electrons are transported some of the energy of those electrons is used to pump protons across the membrane at complexes I, III and IV, which generates a voltage across the mitochondrial membrane [5]. The energy from this voltage gradient drives the synthesis of ATP by ATP synthase; alternatively, uncoupling proteins (UCPs) can move down the voltage gradient to generate heat to keep the rate of ATP generation constant [5].

In hyperglycaemia, there is more glucose being oxidised in the TCAC, which pushes more electron donors into the electron transport chain which results in the voltage gradient increase across the mitochondrial membrane [5, 101] until a critical threshold is reached [5]. At this point, electron transfer is blocked resulting in the backup of electrons generating superoxide which is degraded to hydrogen peroxide (which is then converted to H_2O and O_2) by the enzyme superoxide dismutase (SOD) [5]. In experimental studies, abolishing the voltage gradient by using uncoupling protein-1 (UCP-1) results in the lack of ROS production in hyperglycaemia [5, 98]. Similarly, hyperglycaemia does not increase ROS when superoxide is degraded by over-expressing the enzyme manganese SOD (MnSOD) [5]. In endothelial cells
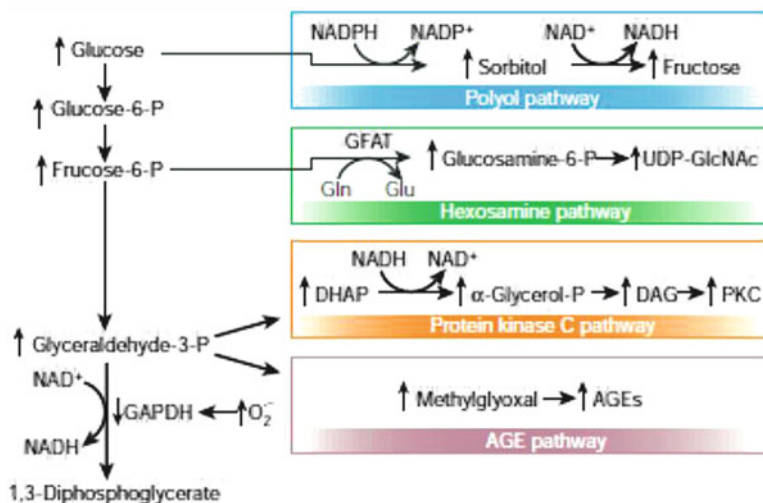
Fig. 9.4 Potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemia damage. Excess superoxide partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into pathways of glucose overutilisation. This results in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDN-*N*-acetylglucosamine increases modification of proteins by O-linked *N*-acetylglucosamine (GlcNAc), and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH. Adapted from [5]

that are deprived of mitochondrial DNA (ρ^0), the impact of hyperglycaemia on ROS production was completely lost [5]. Similarly, in ρ^0 endothelial cells, hyperglycaemia completely fails to activate the polyol, PKC and hexosamine pathways or AGE formation [5]. Inhibiting ROS production and normalising mitochondrial ROS levels prevents the activation of the AGE, PKC and polyol pathways by glucose [98]. This suggests that diabetes-induced ROS and OS are important in stimulating the AGE, PKC and polyol pathways which results in the development of vascular complications, although these same pathways also increase ROS production and OS.

The key glycolytic enzyme GAPDH plays an important role in activating the AGE, PKC, hexosamine and polyol pathways [5]. GAPDH activity is reduced in patients and animals with diabetes, and GAPDH inhibition does not occur when ROS production is prevented by UCP-1 or MnSOD [5, 97]. When GAPDH activity is inhibited, the level of all the glycolytic intermediates that is upstream of GAPDH increases, resulting in the activation of the AGE and PKC pathways because the methylglyoxal (an AGE precursor) and DAG (a PKC activator) are formed from glyceraldehyde-3 phosphate. In addition, the levels of glycolytic metabolite fructose-6 phosphate increase, which activates the hexosamine pathway, and intracellular glucose levels increase, which activates the polyol pathway (Fig. 9.4) [5].

However, experimentally, ROS can inhibit GAPDH activity only at concentrations higher than those found in patients with DM; hence, a different mechanism of

GAPDH inhibition was sought [5]. Poly(ADP-ribosyl)ation is the process by which PAR are attached via an ester bond to glutamic acid, aspartic acid or lysine residues, mediated by the enzyme PARP [102]. PARP 1 and 2 are known to play a role in DNA repair [102]. Increased OS results in DNA damage and PARP1 activation [103–105]. Although PARP1 plays a beneficial role in DNA repair, it is possible that hyper-activation in diabetes leads to detrimental effects [105, 106]. Excess cleavage of NAD+ by PARP would exacerbate the effect of increased flux through SDH which results in depleting NAD+ further, leading to OS [105]. In addition NAD+ is required as a cofactor for the conversion of glyceraldehyde-3-phosphate. Hyperglycaemia-induced ROS inhibits GAPDH activity in vivo by modifying the enzyme with PARP [5, 97, 107]. PARP inhibition reduces OS and inducible NOS (iNOS) expression in high glucose-treated human Schwann cells [108].

In addition to the excess in superoxide production, hyperglycaemia results in reduction in the antioxidant defence system such as GSH, vitamin E, vitamin C, alpha lipoic acid (ALA) and taurine [109]. These antioxidants protect tissues from free radical damage and are recycled or regenerated [109]. GSH is by far the most important antioxidant in most mammalian cells. Hyperglycaemia induces GSH depletion and impairs GSH regeneration; GSH depletion has been linked to the development of diabetes complications [110]. Taurine is a β-amino acid (2-aminoethanesulfonic acid) with antioxidant properties [111, 112]. Taurine depletion is an important mediator of glucotoxicity and OS [111, 112]. Nerve taurine replacement ameliorates deficits in nerve blood flow, NCV and OS in experimental DN and counteracts OS [113, 114]. Furthermore, hyperglycaemia reduces the expression of taurine transporter in Schwann cells which is reversed by the use of antioxidants [115].

9.4   OSA and OS, Inflammation and Endothelial Dysfunction

9.4.1   OSA and Oxidative and Nitrosative Stress

Repetitive episodes of re-oxygenation following hypoxia, as seen in OSA, simulate ischaemia–reperfusion injury which results in the generation of ROS [116]. This hypothesis is supported by several in vivo, in vitro and human studies. It must be noted, however, that studies of intermittent hypoxia in animals may not be transferrable to humans, as the severity of intermittent hypoxia in animal studies is much worse than in patients with OSA and the duration of exposure (days–weeks) is much shorter than in humans, who might have undiagnosed OSA for many years before seeking medical advice. In addition, animal studies that examined the impact of intermittent hypoxia do not take into account that OSA in humans has other aspects than intermittent hypoxia such as sleep architecture disruption and changes in the intrathoracic pressure.

Many markers have been used to demonstrate the relation between OSA and OS including plasma, exhaled breath condensate and urinary 8-isoprostane levels; plasma levels of malondialdehyde (MDA); urinary *o,o'*-dityrosine; plasma levels of TBARS206; urine levels of 8-hydroxy-2'-deoxyguanosine (8-OhdG); and ROS production in monocytes, granulocytes and neutrophils upon in vitro stimulation [22]. Intermittent hypoxia has been associated with mitochondrial dysfunction, OS and increased ROS production [21, 22, 117, 118]. ROS levels have been shown to be 2–3 times higher in patients with OSA compared to healthy controls [21, 119, 120]. Schulz et al. showed increased ROS production in neutrophils from OSA patients, which was reversed by CPAP treatment [120]. Similar results were found in monocytes by other investigators [119]. Multiple studies have shown increased oxidised lipids, DNA and carbohydrates in patients with OSA and animals exposed to intermittent hypoxia [21, 121–127]. In addition, studies showed that patients with OSA have increased levels of lipid peroxidation (interaction between free radicals and lipids) [121, 123], oxidised LDL [125, 128], protein carbonylation (interaction between free radicals and protein) [129] and 8-OhdG (marker of DNA oxidation) [24]. OSA treatment (CPAP and mandibular advancement devices) seems to lower OS levels [120, 121, 123, 130, 131]. In addition, OSA is associated with reduced antioxidant capacity which can be reversed by CPAP treatment [132, 133], adding to the imbalance caused by increased generation of ROS/RNS and resulting in OS and NS.

The evidence for NS is limited in patients with OSA. In one report endothelial expression of nitrotyrosine correlated with AHI despite adjustment for age and adiposity in patients with OSA [134], which is reversible by CPAP treatment [135]. Another study, however, showed no increase in circulating nitrotyrosine levels in patients with OSA [136].

Not all studies showed evidence of increased OS in patients with OSA [137, 138]; these studies were small and had methodological issues regarding the choice of control group and the length of CPAP treatment.

9.4.2   OSA and Inflammation

As OSA is associated with increased OS, then it would be expected that OSA should be associated with increased inflammatory cytokines and adhesion molecules, driven by increased ROS/RNS as well as increased sympathetic activity and obesity that can increase inflammation by increased free fatty acid (FFA) release.

Intermittent hypoxia has been shown to be associated with the activation of the transcription factor nuclear factor κB (NF-κB) in vivo, which was reversible with CPAP treatment [139]. Intermittent hypoxia has also been associated with increased hypoxia-inducible factor-1 (HIF-1) in vivo and in vitro [140–142], which can directly stimulate tens of downstream molecules resulting in IR [142]; increased lipid biosynthesis [143] systemic inflammation [144] and sympathetic activation. HIF-1 activation in OSA can occur either secondary to hypoxia itself

[145], OS [146] or NF-κB activation [147]. Sleep fragmentation/deprivation has also been associated with increased inflammation. Sleep deprivation has been shown to be associated with increased inflammatory markers such as IL-6 and TNF-alpha [148, 149].

OSA has been associated with elevated plasma cytokines such as IL-6, IL-8, TNF-α, CRP, granulocyte chemotactic protein-2 (GCP-2) and monocyte chemotactic protein-1 (MCP-1) independent of obesity [22, 49, 150–157]. CPAP treatment was shown to be effective in reducing these cytokines [158, 159]. Not all studies showed a relationship between OSA and inflammation [160, 161] with obesity being the main confounder.

Adhesion molecules (selectins and integrins) play an important role in inflammation and in the interaction between the endothelium and platelets and white cells. Polymorphonuclear cells, monocytes and T lymphocytes have been shown to have increased adhesion molecules, increased avidity to endothelial cells and increased prolonged lifespan of active polymorphonuclear cells in patients with OSA compared to controls [24, 119, 120, 162–166], which is combined with OS that could result in endothelial damage and vascular disease. In addition, endothelial cells from patients with OSA showed increased expression of adhesion molecules (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM), E-selectin and P-selectin) compared to controls, which might be reversible with CPAP treatment [156, 157, 167–169].

9.4.3 OSA and Endothelial Dysfunction

Endothelial dysfunction is an important step in the development of micro- as well as macrovascular disease. OS, NS and inflammation result in deficits in the production and action of nitric oxide resulting in endothelial dysfunction. In addition, OSA is associated with multiple risk factors of vascular disease such as hypertension, obesity and hyperlipidaemia. Hence OSA would be expected to be associated with endothelial dysfunction.

Several studies have shown reduced circulating as well as endothelial levels of NO in patients with OSA which improves after CPAP treatment [135, 170, 171]. Endothelial-dependent vasodilatation was shown to be impaired in OSA patients independent of hypertension [172] and obesity [173] and cardiovascular risk factors [174]. Brachial artery diameter correlated in one study with hypoxia measures rather than the AHI [174], highlighting the role of hypoxia in the association between OSA and endothelial dysfunction. A study that used laser Doppler flowmetry to examine forearm skin microcirculation found that OSA was associated with lower baseline blood flow compared to subjects without OSA and that the response to acetylcholine and sodium nitroprusside was not impaired by OSA [175]. There is also evidence to suggest that OSA is associated with increased inhibitors of endothelial NO synthase which further contributes to the endothelial dysfunction observed in OSA [176]. Using pulse wave as an indicator of atherosclerosis, a study

showed that OSA patients had significantly higher pulse wave velocity compared to age- and BMI-matched controls [177].

In addition to the impaired vasodilatation, OSA might be associated with increased production of vasoconstrictors. Some studies showed that OSA is associated with increased ET-1 levels [167, 178–180], others did not [181, 182]. Endothelial repair capacity (judged by circulating endothelial progenitor cells) is also impaired in patients with OSA free of overt CVD [135] and endothelial apoptosis increased [183].

CPAP treatment was shown to improve flow-mediated vasodilatation [184], endothelial-dependent vasodilatation [185] and endothelial repair capacity [183] and vasoreactivity [186].

9.5 OSA Molecular Consequence and Vascular Disease in Type 2 Diabetes

In the previous paragraphs we have showed that OSA is associated with increased OS, NS, inflammation and endothelial dysfunction, which contribute to the association observed between OSA and CVD. We have also shown that OSA is very common in patients with T2DM. T2DM of course is a well-recognised cause of OS, NS, inflammation, endothelial dysfunction and vascular disease independent of obesity. Hence, OSA and T2DM share many molecular consequences (Fig. 9.5), whether having OSA and T2DM combined is worse than having T2DM alone is not clear.

This has generated a lot of interest amongst investigators recently, and several studies are ongoing to answer this question. Some of the early results from the
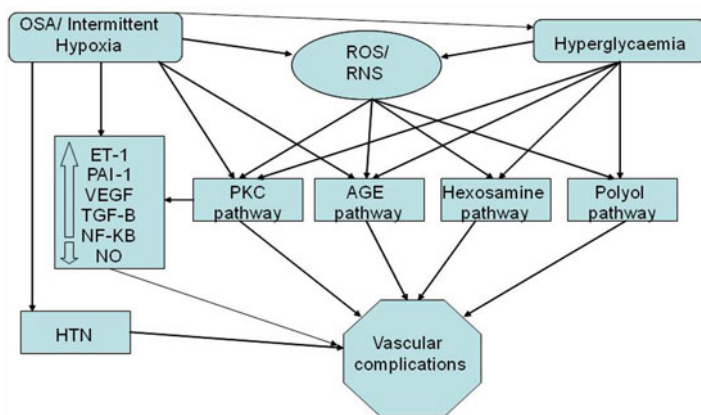

Fig. 9.5 The postulated mechanisms linking OSA to DPN (and microvascular complications). *HTN* hypertension, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *PKC* protein kinase C, *AGE* advance glycation end products. Adapted from [19]

cross-sectional studies suggest that OSA might exacerbate the impact of hyperglycaemia.

In one study of OSA, AHI and nocturnal hypoxaemia were associated with increased NS (as measured by serum nitrotyrosine) and OS (as measured by plasma lipid peroxide) in patients with T2DM [19]. OSA, AHI and nocturnal hypoxia were also associated with PARP activation in patients with T2DM independent of obesity (Tahrani unpublished data). Hence, having OSA in patients with T2DM generates more OS which could drive further endothelial dysfunction and vascular complications. Indeed, using laser speckle contrast imaging, patients with OSA and T2DM were shown to have worse baseline as well as endothelial-dependent and endothelial-independent vasodilatation [19]. AHI and nocturnal hypoxia also correlated with the parameters of endothelial dysfunction [19].

Whether these associations with OS, NS and PARP activation and endothelial dysfunction translate into clinically detectable disease in patients with OSA and T2DM is unknown. However, several studies have shown a cross-sectional association between OSA and diabetes-related microvascular complications such as peripheral neuropathy [19], sight-threatening retinopathy [187], proliferative retinopathy [187, 188] and diabetic nephropathy [189] independent of traditional risk factors including obesity and age. Longitudinal studies have also shown that OSA is associated with the progression of microvascular complications such as the development of pre-proliferative/proliferative diabetic retinopathy and decline in renal function (as measured by estimated glomerular filtration rate) [190, 191].

The relationship between OSA and macrovascular disease was assessed in one observational cross-sectional analysis from the Sleep AHEAD study that found an association between OSA and the prevalence of self-reported stroke but not other CVD [192]. A recent pre- and post-study showed that CPAP reduced systolic blood pressure in patients withT2DM by approximately 9 mm Hg (from a baseline of 149 mm Hg) and lowered pulse rate without an impact of lipids [193].

9.6   Summary and Conclusion

OSA is very common in patients with T2DM and can result in similar molecular consequences similar to those caused by hyperglycaemia including OS, NS, increased inflammation and endothelial dysfunction. Early evidence suggests that patients with OSA and T2DM are at increased risk of OS, NS and endothelial dysfunction compared to those with T2DM alone.

OSA is associated with increased risk of vascular risk factors, CVD and mortality. Whether patients with OSA and T2DM are at increased risk of vascular disease compared to those with T2DM alone requires further investigation, but several cross-sectional studies have shown increased risk of diabetes-related microvascular complications in patients with T2DM and OSA compared to T2DM alone. One observational study also suggested an increased risk of macrovascular disease in patients with OSA and T2DM compared to T2DM only.

CPAP treatment is effective in lowering OS, NS and inflammation and improving endothelial function. CPAP is also effective in lowering BP and has been associated with lower mortality in observational studies. Whether such benefits extend to those who have OSA and T2DM is unknown. One non-randomised trial suggested that CPAP can lower systolic BP in patients with T2DM.

Further observational cohort studies are needed to understand the molecular consequences of OSA in the context of patients with T2DM and to define the role of OSA in the development or progression of diabetes-related micro- as well as macrovascular complications. Interventional studies assessing the role of OSA treatment in managing diabetes-related complications and vascular risk factors are also needed.

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Chapter 10
Taurine Treatment for Complications of Diabetes

Trevor Askwith

10.1 Background

Taurine (2-aminoethanesulphonic acid) (Fig. 10.1) is a free amino acid found as zwitterions in most body fluids. Taurine was first discovered in 1827 as a component of bile where taurine plays an essential role in conjugation to bile acids thus enabling solubility at physiological pH [1]. The major physiological roles of taurine are as an organic osmolyte and antioxidant; however, it has also been reported to act as a scavenger of carbonyl compounds, a modulator of cytosolic calcium and an analgesic and also has neurotrophic properties [2, 3].

10.2 Taurine Biosynthesis

Taurine is synthesised from cysteine by metabolism through the cysteine sulphate pathway. Cysteine is oxidised to cysteine sulphinic acid by cysteine dioxygenase, which is then decarboxylated by cysteine sulphinate decarboxylase (CSD) to form hypotaurine. This latter step is rate limiting; hence the capacity for taurine synthesis is thought to be dependent upon the level of CSD. All cells appear capable of different levels of taurine synthesis, but to a differing extent with CSD activity higher in the liver and brain than in the sciatic nerve [4], and maybe absent from the axon altogether [4–6]. Levels of taurine synthesis vary between mammals; however, all mammals are dependent upon dietary taurine intake [7]. Rodents such as rats and mice have high levels of taurine synthesis, compared to humans [4, 6, 8]. Cats do

T. Askwith (✉)
Translational Drug Discovery Group, School of Life Sciences, University of Sussex, Brighton BN1 9QJ, UK
e-mail: t.askwith@sussex.ac.uk

Fig. 10.1 Chemical
structure of taurine
(2-aminoethanesulphonic acid)
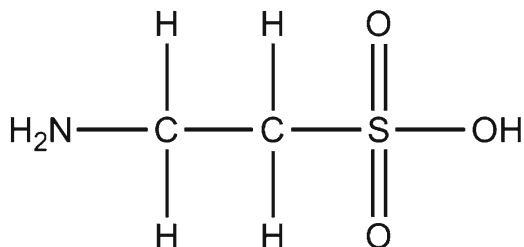
not express CSD and are entirely reliant upon dietary taurine intake; as such cats
with taurine-restricted diets have been used as models of taurine deficiency.

Despite the requirement for dietary taurine intake, taurine deficiency is rare.
Taurine is found ubiquitously in animal products along with nuts, legumes and
sulphur-rich vegetables such as sprouts, cabbage, onions, garlic and turnips.
Additionally there is an inverse regulation of taurine transport in the renal proximal
tubules which controls urinary taurine excretion [9]. Despite this, taurine deficiency
has been observed in both vegans [10] and patients with diabetes.

10.3 Taurine Transport

The plasma taurine concentration is approximately 50 μM [11], whereas the intra-
cellular taurine concentrations range from 5 to 50 mM [2, 12, 13], varying due to the
demand for taurine. For example leukocytes have high taurine concentrations,
20–50 mM, due to the ability of taurine to scavenge hypochlorous acid (HOCl)
generated during an inflammatory response [2]. The high intracellular taurine con-
centration is achieved by active transport across the cell membrane, mediated by a
high-affinity low-capacity transporter known as the taurine transporter (TauT). The
active transport of taurine across the cell membrane is dependent upon the move-
ment of 2 Na^+ ions and 1 Cl^- ions per taurine molecule. The energy required for
taurine transport is derived from the movement of sodium down its electrochemical
energy gradient generated by the Na^+K^+ ATPase; as such taurine uptake is by sec-
ondary active transport [9, 12].

10.3.1 Molecular Identity of the Taurine Transporter

TauT has been cloned from various tissues and animal species such as mouse and
rat brain [14], dog renal MDCK (Madin–Darby canine kidney) cells, human thyroid
[15], placenta [16], retinal pigment epithelial (RPE) cell line, mouse retina and
bovine endothelial cells. These have demonstrated that TauT shares up to 90 %
sequence similarity between mammals, illustrating the importance of TauT in mammals.

These studies also showed that TauT belongs to the superfamily of Na$^+$- and Cl$^-$-dependent transporters, such as those for serotonin, dopamine, noradrenalin, γ-aminobutyric acid (GABA) and creatinine [17]. The TauT gene is located on human chromosome 3p21–25, encoding a protein between 590 and 655 amino acids in length with a molecular weight of approximately 65–70 kDa. Hydropathy plots indicate there are 12 transmembrane regions with the protein having intracellular C and N terminals [17].

10.3.2   Gating of Transport

Several potential phosphorylation sites have been identified on TauT that affect taurine transport. Han et al. [18] identified Ser 322 on S4 loop as a location for protein kinase C (PKC) phosphorylation leading to reduced taurine affinity. Furthermore by substituting charged residues in the S4 loop, other amino acid residues on S4 were found to play an important role in the gating of taurine transport by altering the Km of the transporter, demonstrating direct interaction between this loop and taurine binding [12]. In addition elevations in cyclic adenosine monophosphate and subsequent protein kinase A (PKA) activation increased taurine uptake, an effect blocked by PKA inhibition [9]. As for PKC-mediated phosphorylations, these effects were shown to alter the affinity of TauT for taurine rather than its expression [19, 20].

10.3.3   Regulation of Expression

The promoter region of TauT has been identified in both rat and human leukocytes [21]. In common with the function of taurine as an osmolyte, the promoter region of TauT contains a tonicity response element (TonE), and TauT expression is increased in hypertonic conditions [21]. The other major function of taurine is as an antioxidant, and the TauT promoter also contains an antioxidant response element (ARE), and TauT expression is increased in pro-oxidant conditions. In different studies TauT expression has been seen to be downregulated by glucose, nitric oxide donors, calmodulin as well as taurine itself. Identification of the TauT promoter also identified binding sites for proto-oncogenes such as p53, WT1, ERG the activator protein 1 (AP-1) as well as two oestrogen receptor half sites and the transcription factor Sp1 [12].

10.3.4   Taurine Depletion in Diabetes

The link between taurine and diabetes has been explored since the early 1990s and taurine depletion is measured in many animal and tissue culture models. Table 10.1

Table 10.1 Taurine content in platelets and plasma from patients with diabetes

	Taurine content			
	Plasma (μM)		Platelet (nM/mg protein)	
	Healthy control	Patient	Healthy control	Patient
De Luca et al.[a] [11]	48.6	32.1	183	148
Franconi et al.[b] [22]	93.3	65.6	990	660
Bianchi et al.[a] [23]	46.5	28.7	–	–
Mean	62.8	42.13	586.5	404

[a]Type 1 patients

[b]Type 2 patients

lists the current publications measuring taurine in patients with diabetes. Taurine depletion in patients was initially observed by Franconi et al. [22]. Taurine levels in the plasma and platelets of 39 patients with type 1 diabetes and 34 aged-matched controls with no significant difference in body mass index or rate of albumin excretion were measured. They found patients with diabetes had lower plasma and platelet taurine concentrations. In addition an inverse correlation between taurine concentration and HbA1c was demonstrated. These data have since been repeated in type 2 patients, showing reduced taurine levels in plasma and platelets [23]. They demonstrated reduced uptake of ^{3}H-labelled taurine in platelets of patents as well as increased ^{3}H taurine release, demonstrating a disruption in mechanisms for taurine homeostasis [11]. In the third study, this time in PBMCs isolated from patients with type 2 diabetes, whilst the plasma taurine was again reduced, the mRNA expression of TauT was increased and not associated with HbA1c [23]. This suggests a feedback mechanism to retain PBMC taurine content. Unfortunately neither taurine content within the PBMC nor ^{3}H taurine uptake was measured to ascertain how the mRNA expression paralleled with TauT activity or taurine content. Interestingly when the patients were stratified with complications, TauT mRNA expression was further decreased in patients with retinopathy and a trend towards a decrease in those with macroangiopathy, when compared to patients without complications [23].

In the final study looking specifically at renal taurine clearance, a handful of patients with diabetes and healthy controls (8 of each) were given a taurine load (6×500 mg tablets), and their plasma taurine load as well as urinary excretion was measured. Considering the small number of patients involved in the study, the results were impressive. The peak plasma concentration was significantly smaller in patients than in controls following the taurine load, and the urinary excretion was higher. These suggest that patients with diabetes have reduced lower intestinal taurine absorption, as well as impaired taurine renal tubular reabsorption, potentially explaining why patients with diabetes have lower plasma taurine levels [24].

10.3.5   TauT Expression in Diabetes

Intracellular taurine content is maintained by TauT. The downregulation of TauT by high glucose was first demonstrated by Stevens et al. [25] in RPE cells where glucose decreased taurine transport and TauT expression. This reduction in TauT expression has also been measured in isolated culture of mesangial cells [26] as well as isolated human Schwann cells exposed to chronic high glucose [27]. This reduced taurine uptake due to a reduction in Vmax that corresponded to reduced TauT mRNA and protein expression levels [28]. In animal models TauT expression has been measured in the retina of STZ-D rats by western blot. These showed a gradual decrease in TauT expression, significant after 8 weeks, but not 4 weeks. Interestingly dietary supplement of taurine (5 g/100 g diet) not only increased TauT expression in STZ-D rats but also increased twofold in expression in control animals after only 2 weeks' treatment [29].

In patients with diabetes, TauT expression has been indirectly measured in platelets, where ^{3}H taurine uptake was reduced [11]. The only direct measure of TauT expression in patients is from Bianchi et al. [23] where they measured TauT mRNA expression from isolated PBMCs and showed a fourfold increase in TauT expression from PBMCs. As mentioned in the previous section, this suggests a feedback mechanism to restore intracellular taurine content, since neither taurine content within the PBMC nor ^{3}H taurine uptake was measured to ascertain how the mRNA expression paralleled with a TauT activity or taurine content.

10.3.5.1   Mechanism of Taurine Transport Dysregulation in Diabetes

The major functions of taurine are as an antioxidant and an osmolyte. In concert with this, the promoter region of TauT contains both an ARE and a TonE. In isolated culture models, TauT expression is upregulated by both hypertonic and pro-oxidant conditions [21, 28]. In diabetes these two factors are in conflict. High glucose increases oxidative stress; however, it also results in increased polyol pathway flux increasing intracellular sorbitol content. The compatible osmolyte hypothesis suggests organic osmolytes are coordinately regulated; hence accumulation of one leads to depletion of another. Accumulation of sorbitol leads to depletion of other organic osmolytes such as myoinositol and taurine, which occurs through TauT downregulation. Sorbitol content is regulated by aldose reductase (AR) expression, and overexpression of AR in RPE cells reduces TauT mRNA expression and protein [21].

Inhibiting both of these pathways restores TauT expression and taurine content. Treatment of human Schwann cells exposed to chronic high glucose with either the AR inhibitor sorbinil or the antioxidant α-lipoic acid (ALA) restored TauT expression and taurine uptake [28]. The same is the case in STZ-D rats where inhibition of both restores nerve taurine content [30, 31]. The apparent paradoxical effect of an antioxidant restoring TauT expression appears to be due to the neutralising effect of reactive nitrogen species and nitric oxide. Nitric oxide donors alone reduce TauT
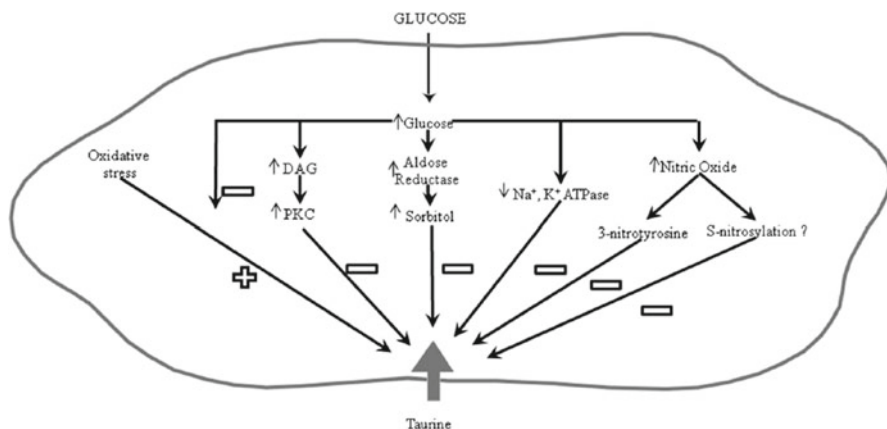
Fig. 10.2 Glucose-induced dysregulation of taurine uptake. Oxidative stress increases taurine uptake, an effect inhibited by glucose. Reduced taurine uptake is influenced by glucose-induced increases in PKC activity, increased aldose reductase flux, reduced Na$^+$K$^+$ ATPase activity and increased NO, possibly due to increased nitrotyrosine of nitrosylation

expression and taurine uptake in human Schwann cells. In human Schwann cells treated with chronic high glucose, inhibition of nitric oxide synthase (NOS) with L-NG-nitroarginine methyl ester (L-NAME) restores TauT expression and taurine uptake [28] (Fig. 10.2). Treatment of rats with L-NAME slows nerve conduction velocity [31], and this could be due to the actions on eNOS, not expressed in isolated human Schwann cells.

10.3.5.2 TauT Activity in Diabetes

As well as a reduction in TauT expression, other factors such as increased PKC activity and reduced Na$^+$K$^+$ ATPase activity may directly reduce the activity of TauT independent of TauT expression, further impairing taurine uptake (Fig. 10.2). The energy required for taurine transport is derived from the movement of sodium down its electrochemical energy gradient generated by the Na$^+$K$^+$ ATPase. As such taurine uptake is reliant upon the Na$^+$K$^+$ gradient, maintained by the Na$^+$K$^+$ ATPase. Impaired Na$^+$K$^+$ ATPase activity is observed in many tissues in animal models of diabetes, such as lens, heat, erythrocytes and sciatic nerve [31], therefore reducing taurine uptake in these tissues.

TauT phosphorylation by PKC has been identified at Ser 322 on the S4 loop leading to reduced taurine affinity [12]. The activation of PKC (primarily β and δ) is increased in various tissues in diabetic animal models, such as the retina, heart, aorta and renal glomerulus. This increased activity reduces not only TauT activity but also expression; in RPE cells exposed to high glucose, inhibition of PKC with bisindolylmaleimide (BIM) overcomes the glucose-induced reduction in taurine uptake [25].

10.3.6 *Mechanism of Action of Taurine Supplementation*

10.3.6.1 Glucose Uptake and Insulin Resistance

As early as the 1930s, studies reported the hypoglycaemic effects of taurine [6]. These results are by no means consistent and there are many studies that demonstrate the beneficial effects of taurine occur with no change in blood glucose or HbA1c [3, 32, 33]. A longer-term study has demonstrated taurine supplementation for 6 months did reduce blood glucose levels in STZ-D rats [34], suggesting longer-term taurine supplementation may be required for the hypoglycaemic effect to be observed.

More recent work has been conducted by one group looking at the effect of taurine on β-islet function and insulin secretion. Carneiro et al. [35] studied the effect of taurine supplementation on β-islet function in nondiabetic mice. Taurine-supplemented mice had higher glucose-induced insulin secretion and greater glucose tolerance. The islets of these mice also had higher insulin content and slower cytosolic Ca^{2+} oscillations in response to glucose stimulation. This group also demonstrated taurine improves insulin sensitivity in mice fed a high-fat diet and have become hyperglycaemic and insulin resistant. In these mice taurine supplementation improved both insulin resistance and glucose tolerance [36]. It could therefore be possible that taurine is aiding insulin sensitivity in type 2 diabetes; however, whether and how taurine may be effecting insulin signalling in type 1 diabetes is unclear.

10.3.6.2 Blood Flow and Platelet Aggregation

In diabetic neuropathy there is a considerable debate about the role of metabolic versus vascular disturbances in the progression of the condition. Sections 10.7.3–10.7.8 discuss the metabolic effects of taurine supplementation; however, taurine also has beneficial effects on the vascular disturbances and blood flow. In diabetic animal models, taurine partially restored nerve blood flow in both STZ-D mice [37] and Zucker diabetic fatty rats [38] as well as induced VEGF expression in the retina of STZ-D rats [39]. The mechanism of these effects is not clear; however, the results are similar to that of antioxidants observed in STZ-D rats [30, 40], demonstrating they could be due to reducing ROS and restoring function in vascular smooth muscle cells [2] or restoring NO signalling.

Taurine is able to increase cholesterol solubility increasing its excretion, and several studies have demonstrated that taurine administration is able to reduce serum cholesterol in both diabetic animals [41] and human subjects. In particular Zhang et al. [42] demonstrated taurine supplementation reduced circulating triglycerides and reduced BMI in obese young nondiabetic adults. Platelet hyper-aggregation is a contributing factor for complications, and there is a close relationship between platelet aggregation in diabetic patients and diabetic complications [6]. Taurine is found in high concentration in platelets (10–50 mM) [6], and clinical

studies have demonstrated this concentration is reduced in diabetic patients. Oral taurine supplementation, however, can reverse this depletion and normalise platelet hyper-aggregation [2]. Interestingly this effect was not found in obese, nondiabetic men [43]. In this study baseline plasma taurine levels were normal, and although this was increased by taurine supplementation, platelet taurine content was not measured. It therefore appears taurine depletion, possibly induced by hyperglycaemia, is required for taurine to be effective at normalising platelet aggregation. Finally in a short 2-week study of patients with type 1 diabetes, supplementation with 1.5 g taurine per day (3×500 mg) restored arterial stiffness and brachial artery reactivity with no effect on other measurements such as HbA1c, blood pressure, cholesterol or heart rate [44]. These results are suggestive of a taurine having a beneficial effect in endothelial cell dysfunction.

Taurine is regularly seen to have anti-hypertensive effects in both nondiabetic animal models and humans [45, 46]. The anti-hypertensive effects of taurine could be due to antioxidant or anti-inflammatory effects; however, taurine also attenuates the actions of angiotensin II on Ca^{2+} signalling and protein synthesis demonstrating other possible mechanisms for the anti-hypertensive action of taurine [2]. Many of the actions of taurine on hypertension are by suppression of the sympathetic nervous system which may also have effects on nerve blood flow, thereby reducing hypertension [46].

10.3.6.3   Antioxidant Actions of Taurine

One of the major actions of taurine is as an antioxidant, and in vitro TauT expression and taurine uptake are increased in response to pro-oxidants [21, 27], suggesting increased taurine uptake as a defensive response. In isolated culture and animal models of diabetes, taurine supplementation reduces markers of oxidative stress in different tissues, i.e. lipid peroxidation in plasma [47], heart, muscle, liver, kidney [48] and sciatic nerve [3]; nitrated proteins, oxidative stress and lipid peroxidation in isolated human Schwann cells [27, 28] and decreased superoxide formation in β-islet cells from rats infused with high glucose [49].

The mechanism(s) by which taurine acts as an antioxidant, however, is(are) unclear. The taurine precursor hypotaurine is able to neutralise classic ROS, but Aruoma et al. [50] established that taurine is incapable of directly scavenging classic ROS, O_{2-}, OH and H_2O_2. It has been speculated that the antioxidant effects of taurine are via indirect mechanism by increasing antioxidant defence enzyme; however the effects seem to be a reversal of toxic effects rather than directly increasing antioxidant defence enzymes [51]. One example of this is the prevention of glutamate-induced neurotoxicity and subsequent ROS increase. Prolonged activation of the N-methyl D-aspartate (NMDA) receptor by glutamate results in overload of intracellular and mitochondrial Ca^{2+}, causing mitochondrial damage and ROS production [52]. Taurine reduces ROS production in glutamate-induced neurotoxicity, but neither by a direct antioxidant mechanism nor by increasing the antioxidant

defence system. Instead taurine has a direct effect on Ca^{2+} uptake via the Na^+/Ca^{2+} exchanger, reducing Ca^{2+} influx and thereby reducing cellular toxicity and ROS [53]. In isolated human Schwann cells, chronic high glucose resulted in increased SOD, CAT activity and GSH abundance; however although taurine supplementation reduced oxidative stress and lipid peroxidation, this was without effect on the anti-oxidant defence system [27]. Hence, it is possible that the effects of taurine on glucose-induced oxidative stress are due to an indirect effect on other mechanisms increasing oxidative stress such as a carbonyl scavenging, rather than directly on ROS, or antioxidant defence [51].

10.3.6.4 Anti-inflammatory Actions

HOCl is a major bactericidal agent generated by polymorphonuclear leukocytes and eosinophils. Although taurine is unable to scavenge classic ROS, it does react with HOCl in a 1:1 ratio neutralising HOCl and forming the less toxic oxidant taurine chloramines [2]. Hence, taurine acts as an anti-inflammatory agent by neutralising HOCl. It has been demonstrated that taurine chloramines act as an inflammatory mediator to reduce iNOS, TNF-α, IL6 and IL8 in polymorphonuclear cells [54]. These pro-inflammatory mediators are increased in patients with diabetes [55] and due to hyperglycaemia [56].

10.3.6.5 Carbonyl Scavenging

Taurine is a free amino acid and therefore it has a free amino group which can react with carbonyl groups forming a Schiff base with the sugar carbonyl, sparing the proteins from glycation [6]. Taurine supplementation reduces AGE and protein glycation in many diabetic models: isolated human erythrocytes, fructose-fed rats and fructose-treated bovine lens. Taurine is able to scavenge both toxic aldehydes and other carbonyl compounds [2, 51].

10.3.6.6 Calcium Signalling

Abnormal $[Ca^{2+}]$ in diabetes is thought to contribute to the development of diabetic neuropathy and the associated pain. Resting $[Ca^{2+}]$ is increased in sensory neurons and DRGs in both STZ-D rats and mice, and the amplitude of multiple voltage-gated calcium channels is also increased in diabetic models. Ca^{2+} overload is reported in diabetic mitochondria, which is important as mitochondrial dysfunction is repeatedly cited as a result of the metabolic dysfunction observed in diabetes [56].

Taurine is known to modulate Ca^{2+} homeostasis [2]. It is able to counter glutamate-induced Ca^{2+} elevations [57], inhibit excessive Ca^{2+} accumulation in

cardiomyocytes and attenuate abnormal Ca^{2+} signalling in sensory neurons of STZ-D rats [58]. How taurine does this is unclear and may differ in different situations. For instance, in glutamate-induced neurotoxicity, taurine is able to lower $[Ca^{2+}]$ by directly affecting the Ca^{+}/Ca^{2+} exchanger. However, other studies have demonstrated interactions between taurine and either phospholipids or phosphoinositol, by inhibiting phosphoinositide turnover [57] or by altering Ca^{2+} binding to membrane phospholipids [58].

10.3.6.7 Na⁺K⁺ ATPase

$Na^{+}K^{+}$ ATPase is an ubiquitously expressed membrane pump that utilises ATP to export three Na^{+} ions and import $2K^{+}$ ions [59]. Many cellular functions are coupled to the movement of sodium ions, for example, in the nerve, the Na^{+} gradient is required for nerve impulses to travel; however, it is also required for the transport of molecules such as myoinositol and taurine. $Na^{+}K^{+}$ ATPase activity is reduced by oxidative and nitrosative stress, and in diabetes $Na^{+}K^{+}$ ATPase activity is disrupted [60]. In the sciatic nerve, lens, heart and erythrocytes, this disruption results in a decrease in $Na^{+}K^{+}$ ATPase activity. Considering the role of the $Na^{+}K^{+}$ ATPase in the nerve, decreased activity impairs nerve impulses and there is a close correlation between decreased $Na^{+}K^{+}$ ATPase activity and diabetic neuropathy [59]. Taurine supplementation restores $Na^{+}K^{+}$ ATPase activity in the nerve of STZ-D rats [37], as well as the retina of STZ-D rats and in peroxynitrite-treated liver samples [61]. Although the mechanism of this action is unclear, since the $Na^{+}K^{+}$ ATPase is downregulated by oxidative and nitrosative stress, it is likely to be an antioxidant effect.

10.3.6.8 Role of Taurine in Mitochondrial Function

The regulation of mitochondrial taurine content appears to be independent of cytosolic taurine content. This can be elucidated from the existence of a mitochondrial TauT which has been identified, but not characterised [62], that mitochondrial taurine content is approximately a third of the cytosolic taurine content [51] and depletion of taurine by β-alanine treatment does not reduce mitochondrial taurine [51].

In the mitochondria, taurine plays a role in protein synthesis where it forms conjugates with uridines of mammalian mitochondrial tRNA [62, 63]. In certain mitochondrial diseases, these taurine modifications are lacking, reducing synthesis of certain mitochondrial proteins. Exposure of taurine to β-alanine depleted the cytosolic (but not mitochondrial) taurine content by 45 %. This led to a reduction in synthesis of respiratory chain subunits ND5 and ND6, which led to an increase in oxidative stress and decline in electron transport chain activity, an effect reversed by taurine supplementation [64].

It has also been reported that taurine has buffering properties in the mitochondrial matrix, disruption of which could lead to insufficient buffering of the matrix [65]. Therefore taurine is potentially able to stabilise the environment in the mitochondria

and prevent leakage of reactive compounds into the mitochondrial environment, indirectly acting as an antioxidant [65].

10.3.7 Taurine Supplementation in Diabetes

The reduction in circulating and intracellular taurine can be replenished by taurine supplementation. This has been demonstrated in rats as well as in diabetic patients. In a short, 90-day trial, patients with diabetes received 500 mg taurine supplementation three times a day for 90 days. This restored both platelet and plasma taurine content. Taurine supplementation also reduced platelet aggregation in patients with diabetes, with no effect on the healthy controls. However, there was no effect on HbA1c or cholesterol [22].

10.3.7.1 Diabetic Neuropathy

In STZ-D rats TauT is expressed in the vascular endothelium, Schwann cells, axons and neurovasculature; therefore, downregulation of TauT could result in taurine depletion in key sites implicated in diabetic neuropathy. Indeed, taurine depletion has been identified in the sciatic nerve of STZ-D rats [30] as well as isolated human Schwann cells exposed to chronic high glucose. This reduced taurine uptake due to a reduction in Vmax that corresponded to reduced TauT mRNA and protein expression levels [27].

The effect of taurine supplementation in neuropathic animal models has been extensively studied. In STZ-D rats taurine supplementation prevents nerve conduction velocity deficits, hyperalgesia as well restoring nerve blood flow [37, 58]. Taurine also reduced nerve oxidative stress, restored normal calcium signalling and reduced nerve growth factor deficits [3]. These studies have been repeated in part in Zucker fatty rats where taurine supplementation restored nerve conduction velocity and nerve blood flow [38].

10.3.7.2 Diabetic Retinopathy

In isolated culture of RPE cells, TauT expression and activity is downregulated by high glucose [25]. Taurine supplementation also protects isolated rat ganglion cells from hypoxia-induced apoptosis by preventing mitochondrial dysfunction [66]. Similarly in vivo taurine supplementation reduces glial cell apoptosis, as well as attenuating the induction of glial fibrillary acid protein (GFAP) expression, a marker of gliosis in STZ-D rats [29, 39]. As with the improvement in nerve blood flow observed in neuropathy, it is also possible that taurine improved retinal vascular function, as taurine supplementation attenuates induced VEGF expression in the retina of STZ-D rats [39]. Finally in STZ-D rats, taurine also reduces retinal glutamate content as well as in cultured Müller cells [29, 39].

10.3.7.3 Diabetic Nephropathy

In isolated culture of renal tubular cells exposed to high glucose, taurine blocked many of the markers of nephropathy, stimulating p42/44 MAPK, JAK2, STAT1, STAT3, fibronectin, type IV collagen synthesis as well as increased concentration of cyclin D/CDK4 and suppressed p21 NafI/Cup1 and p2KIPI [67]. In STZ-D rats co-administration of taurine and streptozotocin reduced the histological appearance of renal injury as well as renal monoaldehyde and oxidised low-density lipoprotein, suggesting the protective effect of taurine against early-stage renal injury [68]. Four months after STZ-D administration, by which time animals demonstrated proteinuria, rats were treated with 1 % taurine added to their drinking water. Taurine treatment prevented a further rise in proteinuria and improved renal histology and reduced TGF-β expression in the glomeruli as well as several markers of oxidative stress, such as pentosidine and nitrotyrosine [69]. These suggest that taurine could prevent progression of nephropathy in patients with established nephropathy.

10.4 Conclusion

In the past 20 years of research, a link between diabetes/taurine depletion and diabetic complications has been established. The mechanisms behind the depletion in taurine appear to surround dysfunction of TauT regulation. In animal models, taurine has the ability to restore many of the molecular biomarkers as well as physiological measurements associated with retinopathy, nephropathy and neuropathy. Whilst the mechanisms behind these effects are unclear and require further study such as the utilisation of TauT knockout animal and in vitro models, the potential benefits of these effects to patients with diabetes demonstrate the requirement for further clinical studies to be performed.

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Chapter 11
Oxidative Stress and Cardiovascular Disease in Diabetes

Rodica Pop-Busui, Mitali Mehta, and Subramaniam Pennathur

11.1 Introduction

The world prevalence of diabetes among adults (aged 20–79 years) has reached epidemic proportions and unfortunately continues to rise. A recent study that included all 216 countries of the United Nations as an update to prior analyses performed by the World Health Organization and International Diabetes Federation supports this notion. It was reported that the worldwide prevalence of diabetes is expected to grow from a rate of 6.4 % in 2010, affecting 285 million adults, to 7.7 % affecting 439 million adults, by 2030 [1]. In the USA, there are currently more than 26 million adults with diagnosed diabetes, with an estimated 36 million adults to be diagnosed by 2030 [1]. Type 2 diabetes accounts for 90 % of cases, as a consequence of increased insulin resistance in skeletal muscle, liver, and adipose tissue, and impaired insulin secretion from the pancreatic β-cell due to islet cell dysfunction. Of particular concern is that type 2 diabetes is also now being diagnosed frequently in young children and adolescents, which may add unforeseeable socioeconomical burdens. The high prevalence of diabetes is explained in part by an increasing incidence of obesity and metabolic syndrome as a consequence of the adaptation to a westernized diet and a sedentary lifestyle. These data, based on a larger number of studies, indicate a growing burden of diabetes, particularly in developing countries.

R. Pop-Busui (✉)
Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine,
University of Michigan, 5329 Brehm Tower, 1000 Wall Street, Ann Arbor, MI 48105, USA
e-mail: rpbusui@umich.edu

M. Mehta
University of Michigan Medical School, Ann Arbor, MI, USA

S. Pennathur
Division of Nephrology, Department of Internal Medicine, University of Michigan,
Ann Arbor, MI, USA

Cardiovascular disease (CVD) mortality remains the main cause of excess mortality among patients with diabetes and also represents a significant cause of morbidity. For instance, myocardial infarction, stroke, and peripheral vascular disease are two to four times more prevalent in diabetic patients [2, 3]. Moreover, vascular disease occurs earlier and follows a more aggressive course [3]. Thus, the cardiovascular event rate in diabetic patients without documented coronary artery disease (CAD) is equivalent to that of nondiabetic patients with CAD [2, 3]. Moreover, diabetic patients have higher mortality following myocardial infarction than nondiabetic subjects [2, 3]. Women with diabetes lose their premenopausal cardioprotection and are vulnerable to CAD at the same rate as men [4].

The development of CVD in diabetes is dependent on underlying genetic predisposition and on coexisting independent accelerating factors such as hypertension and dyslipidemia, which when acted on by various initiating events, result in inflammatory changes. The contribution of hyperglycemia to the pathogenesis of diabetes complications in both type 1 [5, 6] and type 2 diabetes [7] is beyond dispute. Inflammation and hyperglycemia unleash a cascade of events that affects cellular proteins, gene expression, and cell-surface receptor expression in the endothelium, ultimately resulting in progressive pathologic changes and subsequent vascular complications. Several critical mechanisms of hyperglycemia-induced diabetic vascular damage have been described including redox imbalances secondary to enhanced aldose reductase (AR) activity and increased polyol pathway flux, increased advanced glycation end products (AGEs), and increased expression of the receptor for AGEs, activation of protein kinase C (PKC) isoforms, and overactivity of the hexosamine pathway [8]. However, clinical studies designed to block these pathways individually have failed to prevent the development and progression of diabetes vascular complications [8, 9]. In addition, while tissue-specific factors may accentuate diabetic damage, it has become increasingly apparent that all diabetic complications share a common pathophysiology.

During the past two decades, considerable evidence has implicated oxidative stress in several distinct conditions, including aging, atherosclerosis, neurodegenerative diseases, diabetes, and end-stage renal disease (reviewed in [10–17]. These observations led to the seminal theory described by Brownlee in 2000 that all of the different pathogenic mechanisms described above stem from a single hyperglycemia-induced process, namely, overproduction of superoxide by the mitochondrial electron-transport chain [18].

In this chapter, we discuss the link between oxidative stress, endothelial dysfunction, and diabetic vascular disease. We will also discuss the potential relationship of carbonyls and lipids to oxidant-generating pathways and the rationale for therapies aimed at decreasing oxidative stress. Identifying specific pathways of reactive oxidant generation in diabetes will ultimately lead to rational design of drugs to interrupt this process and prevent diabetic complications.

11.2   Oxidative Stress in Diabetes

11.2.1   *Excessive Free Radical Production and Oxidative Stress*

Oxidative stress occurs when there is an imbalance in the relative rates of oxidant generation and oxidant scavenging, with a subsequent increase in the level of oxidized biomolecules and associated tissue damage [19]. The term "oxygen free radicals" summarizes a variety of highly reactive molecules that can be divided into different categories, e.g., reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species (RCS). Superoxide (O_2^-) is the initial oxygen free radical formed by the mitochondria, which is then converted to other more reactive species that can damage cells in numerous ways [20].

The most prominent members of such categories include superoxide (O_2^-), hydroxyl radical (OH^-), peroxyl radical (ROO^-) in the ROS group, and nitric oxide (NO) in the RNS group and are summarized in Table 11.1.

Although free radical reactions are essential for host defense mechanisms utilized by neutrophils, macrophages, and other cells of the immune system, the overproduction of free radicals may cause tissue injury and cell death [21–27]. In vitro and human studies have demonstrated ROS-induced DNA and protein damage

Table 11.1   Reactive species generated from oxygen, nitrogen, and chlorine

Radicals	Non-radicals
Reactive oxygen species (ROS)	
Superoxide, O_2^-	Hydrogen peroxide, H_2O_2
Hydroxyl, OH^-	Hydrochlorous acid, HOCl
Peroxyl, RO_2^-	Hypobromous acid, HOBr
Alkoxyl, RO^-	Ozone, O_3
Hydroperoxyl, HO_2^-	Singlet oxygen $^1\Delta g$
Reactive nitrogen species (RNS)	
Nitric oxide (nitrogen monoxide), NO	Nitrous acid, HNO_2
Nitrogen dioxide, NO_2^-	Nitrosyl cation, NO^+
	Nitrosyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO_2^+ (e.g. as nitryl chloride, NO_2Cl)
	Alkyl peroxynitrites, ROONO
Reactive chlorine species (RCS)	
Atomic chlorine, Cl^-	Hypochlorous acid, HOCl
	Chlorine, Cl_2
	Nitronium (nitryl) chloride, NO_2Cl

[28–32], with subsequent inactivating effects on the function of a large variety of receptors, antioxidant defense and repair enzymes, or transport proteins [33].

The end products of free radical attacks are relatively straightforward indicators of oxidative stress. Nevertheless, some controversy continues to exist about which markers of oxidative stress are most reliable in predicting long-term outcomes and/or are most suitable as future clinical practice indicators. An important reason was related to the sensitivity of the methods used for detecting these biomarkers.

11.2.2   Detection of Oxidative Stress In Vivo by Mass Spectrometry

Antibody-based assays and dihydroethidium fluorescence have been extensively used to study oxidation-specific epitopes and oxidant production in targets of diabetic damage and atherosclerosis. These techniques are highly sensitive, and the ability of immunochemical studies to provide anatomical data can localize oxidative events. However, they are nonspecific and, at best, only semiquantitative. In contrast, mass spectrometry (MS) offers a powerful set of analytical tools for quantifying and identifying biomolecules. Isotope dilution gas-chromatography (GC)/MS is emerging as a highly sensitive and specific method to quantify oxidation of specific amino acid markers. Biomolecules such as oxidized amino acids derived from plasma or tissue are separated by GC, derivatized and ionized (Fig. 11.1).

The mass-to-charge ratios of ions derived by fragmenting the ionized, derivatized parent compound are determined by MS [14]. Such a spectrum can unequivocally identify a target biomolecule because each compound has a unique fragmentation pattern. The analyte is quantified by adding a stable, isotopically labeled internal standard, which is identical to the target analyte except for the heavy isotope. With certain ionization processes, such as electron capture negative-ion chemical ionization, it is possible to detect and quantify sub-femtomole levels of biomolecules.

11.2.3   Proposed Pathways for Generating Oxidative Stress in Diabetes

Many pathways oxidize proteins in vitro. However, the specific pathways that promote oxidative stress in diabetes have not been conclusively identified. One reason is that oxidizing intermediates are difficult to detect in vivo because they are short-lived and generated at low levels. Proposed pathways for increased oxidant generation and oxidative stress in diabetes and prediabetes are outlined in Fig. 11.2 [10, 13] and are discussed below.
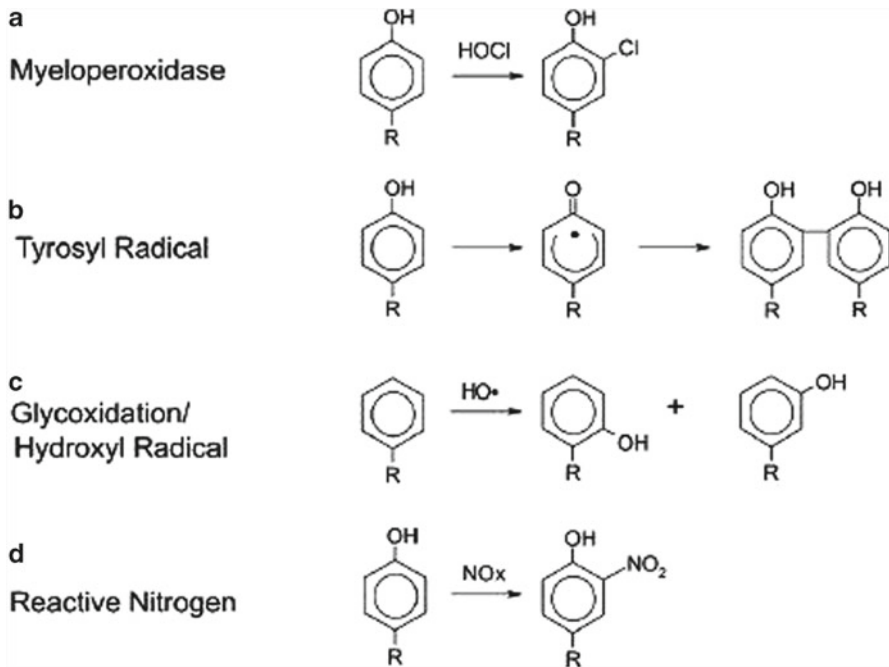
Fig. 11.1 Proposed oxidation products of protein-bound aromatic amino acids by myeloperoxidase, tyrosyl radical, glycoxidation/hydroxyl radical, and reactive nitrogen species (RNS). Myeloperoxidase converts tyrosine to 3-chlorotyrosine; tyrosyl radical cross-links tyrosine to form *o,o'*-dityrosine; RNS convert tyrosine to 3-nitrotyrosine; hydroxyl radical produces ortho-tyrosine and meta-tyrosine from phenylalanine. Reproduced from [14]
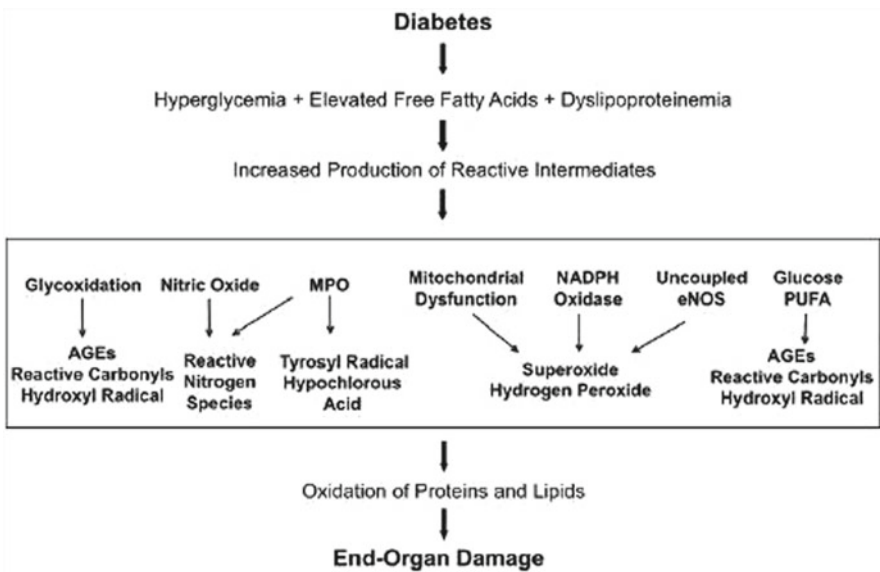

Fig. 11.2 Potential pathways for increased oxidant generation in diabetes and atherosclerosis. *AGE* advanced glycosylation end products, *eNOS* endothelial nitric oxide synthase, *MPO* myeloperoxidase. Reproduced from [14]

11.2.3.1 The Glycoxidation Pathway

Glucose-mediated oxidative reactions are collectively called glycoxidation pathways. In its open-chain form, glucose has a carbonyl group that can be involved in oxidative chemistry. In the presence of oxygen, glucose can auto-oxidize to a hydroxyl radical or other ROS, which cross-links proteins [34]. Glucose also reacts nonenzymatically with proteins to form the reversible Schiff base adduct, which subsequently can rearrange itself into the stable Amadori product and advanced AGEs. In diabetes, AGEs are found in increased amounts in the extracellular matrix. In vitro, free metal ions catalyze steps in a nonenzymatic glycoxidation pathway that generates AGEs. One important intermediate is the hydroxyl radical, which can peroxidize lipids and convert phenylalanine residues of proteins into two unnatural isomers of tyrosine, *ortho*-tyrosine and *meta*-tyrosine [35–37]. Reduced, redox-active metal ions (M^{n+}) such as Fe^{2+} and Cu^{1+} generate hydroxyl radical (HO·) when they react with hydrogen peroxide (H_2O_2). Thus, glycoxidation reactions can be one mechanism for diabetic complications.

11.2.3.2 The Reactive Nitrogen Pathway

Another pathway for generating oxidants involves NO, which is produced by endothelial cells to regulate vascular tone. NO is also produced during inflammation by macrophages, which are early components of atherosclerotic lesions. NO reacts with superoxide (O_2^-) to generate peroxynitrite ($ONOO^-$), a potent oxidant that converts tyrosine residues to 3-nitrotyrosine. Thus, 3-nitrotyrosine is a marker for the reactive nitrogen pathway.

Enzymatic Pathways That Generate Superoxide, Hydrogen Peroxide, and Peroxynitrite

At neutral pH, O_2^- is a reducing agent rather than an oxidant. However, it dismutates enzymatically or nonenzymatically into hydrogen peroxide (H_2O_2), which can oxidize thiol residues and act as an oxidizing substrate for heme proteins such as myeloperoxidase (MPO). O_2^- also reacts at a diffusion-controlled rate with NO to form $ONOO^-$, a powerful RNS that nitrates tyrosine residues and damages a wide range of biomolecules.

11.2.3.3 The Mitochondrial Electron-Transport Pathway

In tissues and organs, mitochondrial electron transport mediated by the cytochrome enzyme complex is an important source of O_2^- and consequently of H_2O_2. Plasma levels of both glucose and free fatty acids (FFAs) are elevated in diabetes, and both

of these substrates promote an increased flux of electron donors (NADH and $FADH_2$) in the mitochondrial electron transport in cells. Substrate-driven mitochondrial oxidation pathway has been proposed as one mechanism for damaging cells in diabetes [17]. As a result, the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside complex III is blocked, causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide. The mitochondrial isoform of the enzyme SOD degrades this oxygen free radical to hydrogen peroxide, which is then converted to H_2O and O_2 by other enzymes. In primary arterial endothelial cells in culture, intracellular hyperglycemia increases the voltage across the mitochondrial membrane above the critical threshold necessary to increase superoxide formation [8] and, subsequently, increases production of ROS. Neither hyperglycemia nor increased fatty acid oxidation in vascular endothelium increases ROS nor activates any of the pathways either when the voltage gradient across the mitochondrial membrane is collapsed by uncoupling protein 1 (UCP-1) or when the superoxide produced is degraded by MnSOD [38].

Mitochondrial O_2^- overproduction mediated by hyperglycemia might also increase polyol pathway activity, PKC activity, and hexosamine flux, resulting in cellular dysfunction and tissue damage [17]. Exposing endothelial cells to exogenous oxidants leads to mitochondrial damage and can augment O_2^- production, a mechanism whereby oxidative stress perpetuates oxidative stress [39]. Moreover, superoxide inhibits glyceraldehyde phosphate dehydrogenase, a key glycolytic enzyme whose inactivity could make upstream metabolites accumulate. Such inhibition of glycolysis might promote end-organ damage by diverting metabolites into the hexosamine pathway or stimulating the polyol and diacylglycerol (DAG)–PKC pathways. Benfotiamine, a lipid-soluble thiamine analog that inhibits these pathways by activating transketolase, an enzyme in the pentose pathway shunt, can prevent complications from diabetes in experimental animal models [40].

11.2.3.4 NADPH Oxidases

A family of NADPH oxidases, also known as the NOX enzymes, are major producers of O_2^- in the vasculature [39]. Several NOX isoforms present in the endothelium and smooth muscle cells are selectively upregulated by pathologic stimuli. Potential factors include angiotensin II, endothelin-1, hypercholesterolemia, shear stress, nonesterified fatty acids (NEFAs), hyperglycemia, and growth factors. Angiotensin II may represent a pathophysiologically relevant pathway for stimulating the production of reactive intermediates by artery wall cells because inhibitors of this pathway lower the risk for cardiovascular events [41]. In humans, NADPH oxidase activity correlates inversely with endothelial function, even after other major risk factors for atherosclerosis, including diabetes and hypercholesterolemia, are taken into account [42, 43].

11.2.3.5   Uncoupled Endothelial Nitric Oxide Synthase

Endothelial nitric oxide synthase (eNOS) synthesizes NO in endothelial cells, and its uncoupling has been described in various conditions, including diabetes, hypertension, and hypercholesterolemia. One proposed mechanism involves oxidation of its cofactor, tetrahydrobiopterin (BH_4) [44]. Under those conditions, eNOS transfers electrons to molecular oxygen, generating O_2^- [45]. An alternative mechanism for uncoupling eNOS involves overproduction of angiotensin II, which can induce dihydrofolate reductase deficiency. Because dihydrofolate reductase maintains BH_4 in its reduced form, its deficiency uncouples eNOS. BH_4 oxidation and NOS uncoupling have been demonstrated in hypertension, diabetes, and hypercholesterolemia. Moreover, administering BH_4 improves endothelium-dependent vasodilation in experimental animals and humans with those conditions [42].

11.2.3.6   Xanthine Oxidase

Another possible source of O_2^- and H_2O_2 in mammalian cells is xanthine oxidase, which converts hypoxanthine and xanthine to uric acid while reducing molecular oxygen. Hydrogen peroxide can increase levels of xanthine oxidase, further accentuating O_2^- production. Inflammatory cytokines, such as tumor necrosis factor-alpha, and oxidation of cysteine residues by oxidants such as peroxynitrite can result in the conversion of xanthine dehydrogenase to xanthine oxidase [42]. Xanthine oxidase is an important source of oxidants in a variety of pathophysiological states, including diabetes, hypertension, atherosclerosis, ischemia–reperfusion, and heart failure [42]. Endothelial levels of xanthine oxidase are elevated in humans with heart failure and subjects with CAD, and they correlate with degree of impairment in endothelium-dependent vasodilation [42].

11.2.3.7   The Myeloperoxidase Pathway

The major pathway through which macrophages and other phagocytic cells of the innate immune system generate oxidants begins with the cells' membrane-bound NADPH oxidase (NOX), which produces superoxide, which can be converted by superoxide dismutases into hydrogen peroxide. The hydrogen peroxide can be used by another phagocyte enzyme, MPO [46, 47], to convert chloride ion to hypochlorous acid.

Oxidation of NO with oxygen yields nitrite (NO_2^-), which MPO converts to nitrogen dioxide radical, a potent nitrating intermediate [48, 49].

RNS, including peroxynitrite and $NO_2\cdot$, might contribute to the inflammatory process by nitrating lipoproteins and other biomolecules. Hyperglycemia can activate PKC [50–52], which leads to phagocyte activation, secretion of MPO, and

oxidant generation. NEFAs that commonly are overabundant in diabetes can also activate phagocytes in vitro. These changes might enhance the production of superoxide and hydrogen peroxide, which MPO converts into more potent cytotoxic oxidants, such as hypochlorous acid and nitrogen dioxide radical.

11.2.3.8 The Glucose–Polyunsaturated Fatty Acid Pathway

Recent studies indicate that high glucose can promote localized oxidative stress in tissues vulnerable to diabetic damage by interacting with polyunsaturated fatty acids (PUFAs) via a carbonyl/PUFA pathway [53]. For instance, it was reported that by incubating glucose with low-density lipoproteins (LDL) or a model protein, ribonuclease (RNAse), pathophysiologically relevant concentrations of glucose modified LDL, as evidenced by the formation of oxidized amino acids, even though metal ions were absent [53]. In striking contrast, glucose exposure did not increase levels of oxidized amino acids in RNAse. These observations suggest that glucose promotes LDL oxidation because the particle contains lipid as well as protein. In subsequent experiments incubating RNAse with saturated, monounsaturated, or PUFA, it was found that glucose stimulated protein oxidation only in the presence of a PUFA. Thus, glucose appears to promote protein oxidation by a pathway involving peroxidation of PUFAs, as this reaction was inhibited by lipid-soluble antioxidants [53]. Additional experiments replacing glucose with a variety of short-chain and phosphorylated sugars that have highly reactive carbonyl group described that all of the carbonyl compounds promoted oxidation of LDL (but not RNAse) protein more effectively than did glucose. In contrast, LDL oxidation was not enhanced by sorbitol, the reduced form of glucose that lacks a carbonyl moiety [53].

11.3 Oxidized Amino Acids as Potential Markers of In Vivo Oxidative Stress

There is increasing evidence that oxidized amino acids in plasma can serve as markers for noninvasively assessing oxidative stress in vivo. In steady state, plasma levels of these markers are proportional to their rate of generation and can serve as indices of chronic oxidative stress in vivo [13, 15, 54–58]. A recent case–control study demonstrated that systemic levels of protein-bound nitrotyrosine were significantly higher among patients with CAD compared with those with healthy arteries and that statin therapy lowered levels of oxidation markers in plasma raising the possibility that statins can potentially be antioxidants [56, 57, 59]. Therefore, these markers can be used to assess degree of oxidative stress and to monitor efficacy of therapy.

11.4 Mechanisms Linking Oxidative Stress with Vascular Damage in Diabetes

11.4.1 Oxidative Stress and Increased AGE in Diabetic Vascular Disease

Critical intermediaries in the formation of AGEs are 3-deoxyglucosone (3DG) from fructoselysine and glyoxal and methylglyoxal from either Amadori compounds, Schiff base intermediaries, or direct oxidation of sugars [60]. With time these products undergo chemical rearrangement, dehydration and fragmentation reactions, and cross-linking to form irreversible AGEs. AGEs can damage tissues through a number of mechanisms, including generation of oxidizing intermediates [61–65], modification of intracellular proteins that promote altered function, formation of immune complexes, and interaction with a cellular receptor called RAGE (*receptor for AGE*). RAGE binding induces the production of ROS, which in turn activates the pleiotropic transcription factor nuclear factor (NF)-B [66], causing multiple pathological changes in gene expression and promotion of cytokine release [10, 67]. Giardino et al. [68] have shown that the intracellular formation of AGE and the lipid peroxidation are closely interdependent processes, in that inhibition of lipid peroxidation prevents the formation AGE products. Although RAGE binds to AGE-modified proteins in vitro with high affinity, its ligands in vivo are unclear. High levels of AGEs accumulate in renal failure, even in nondiabetic patients, and this process reverses after renal transplantation, implicating the kidneys in AGE production and/or clearance [69–72].

Many studies have shown that age-adjusted levels of pentosidine and Nε-carboxymethyllysine (CML), two widely investigated AGE products, correlate with the development of diabetic micro- and macrovascular disease [36, 73–76]. Animal and human studies have also shown that diabetes is associated with poor outcomes following acute vascular occlusive events. For instance, diabetic animals have a decreased vascular density following hind limb ischemia [77] and impaired wound healing [78]. Human angiograms demonstrate fewer collateral vessels in diabetic patients compared with nondiabetic controls [79]. Clinically, this contributes to increased rates of lower limb amputation, heart failure, and increased mortality after ischemic events. These defects that result, in part, from a failure to form adequate compensatory vasculogenesis in response to ischemia appear to be mediated by AGEs. High glucose induces a decrease in transactivation by the transcription factor hypoxia-inducible factor (HIF)-1, which mediates hypoxia-stimulated chemokine and vascular endothelial growth factor (VEGF) production by hypoxic tissue, as well as chemokine receptor and eNOS expression in endothelial precursor cells in the bone marrow. AGE-modified proteins in the circulation can affect a range of cells and tissues. A specific RAGE has been shown to mediate signal transduction via generation of ROS, activation of NF-B, and p21 ras [80, 81]. In endothelial cells, AGE binding to its receptor alters the expression of several genes, including thrombomodulin, tissue factor, and VCAM-1 [82–84]. These effects induce

procoagulatory changes on the endothelial cell surface and increase the adhesion of inflammatory cells to the endothelium. In addition, endothelial AGE receptor binding appears to mediate, in part, the increased vascular permeability induced by diabetes, probably through the induction of VEGF [85, 86]. RAGE deficiency attenuates the development of atherosclerosis in the diabetic apoE$^{-/-}$ model of accelerated atherosclerosis. Diabetic RAGE$^{-/-}$/apoE$^{-/-}$ mice had significantly reduced atherosclerotic plaque area. These beneficial effects on the vasculature were associated with attenuation of leukocyte recruitment; decreased expression of proinflammatory mediators, including the NF-B subunit p65, VCAM-1, and MCP-1; and reduced oxidative stress [87].

11.4.2 Oxidative Stress, Increased Polyol Pathway Flux, and Vascular Damage

Among the proposed mechanisms that could explain how hyperglycemia-induced increases in polyol pathway flux could damage the tissues involved, an increase in redox stress caused by the consumption of NADPH appears to be most widely accepted. NADPH is a cofactor required to regenerate reduced glutathione (GSH), and GSH is an important scavenger of ROS; a decreased NADPH could induce or exacerbate intracellular oxidative stress. Overexpression of human AR increased atherosclerosis in diabetic mice and reduced the expression of genes that regulate regeneration of GSH [88]. In diabetic rats, decreased glutathiolation of cellular proteins is associated with decreased NO availability and restoring the NO levels in diabetic animals was shown to increase glutathiolation of cellular proteins, to inhibit AR activity, and prevents sorbitol accumulation.

11.4.3 Oxidative Stress, PKC Activation, and Vascular Damage

PKC is a serine/threonine kinase involved in signal transduction events in response to specific hormonal, neuronal, and growth factor stimuli [89], a process dependent on Ca^{2+} ions and phosphatidylserine and enhanced by DAG [90]. PKC has several unique structural features that facilitate its regulation according to redox status. Prooxidants react with the regulatory domain to stimulate PKC activity, and antioxidants react with the catalytic domain of PKC and inhibit its activity [91]. At least 11 isoforms have been identified to date, which differ in structure and substrate requirements [89] and have wide differences in tissue localization. For example, PKCβ is present in pancreatic islet cells, monocytes, the brain, and many vascular tissues including the retina, kidney, and heart [92–96].

Hyperglycemia primarily activates the β and δ isoforms of PKC in cultured vascular cells [97], and their excessive activation operates as a third common pathway mediating tissue injury induced by diabetes-induced ROS. Increased ROS inhibit the

activity of the glycolytic enzyme GAPDH, raising intracellular levels of the DAG precursor triose phosphate and subsequent enhanced de novo synthesis of DAG from glucose via triose phosphate [96]. Several signaling cascades induced by glucose-induced activation of PKC and of p38 mitogen-activated protein kinase (MAPK) and by fatty acid oxidation in insulin-resistant arterial endothelial cells and heart may play important role in diabetic atherosclerosis and cardiomyopathy. These include platelet-derived growth factor (PDGF) receptor-β dephosphorylation and pericyte apoptosis [98], decreased NO production in smooth muscle cells [99], inhibition of insulin-stimulated expression of eNOS in cultured endothelial cells [100], increased expression of the permeability-enhancing factor VEGF in vascular smooth muscle cells [101], overexpression of the fibrinolytic inhibitor, plasminogen activator inhibitor (PAI)-1, and the activation of NF-B in cultured endothelial cells and vascular smooth muscle cells [102, 103]. PKC activation has also shown to play a role in mediating increased O_2^- production, activation of cyclooxygenase-2 (COX-2) pathway by glucose, and reduced NO availability, contributing therefore to endothelial dysfunction [104, 105].

11.4.4 Mitochondrial Superoxide Production Links Pathways of Vascular Damage in Diabetes

Experimental evidence from in vitro and in vivo studies shows that hyperglycemia inhibits GAPDH activity. GAPDH is commonly thought to reside exclusively in the cytosol. However, it normally shuttles in and out of the nucleus, where it plays a critical role in DNA repair [106]. A decreased GAPDH activity induces an increase in the levels of all the upstream glycolytic intermediates [8], which in turn activates the polyol, the PKC, the AGE, and hexamine pathways, and increases the expression of the receptor for AGEs and its activating ligands in the vasculature as described above [8].

It was shown that hyperglycemia-induced increased superoxide production inhibits GAPDH activity by modifying the enzyme poly(ADP-ribose) polymerase (PARP) [106]. When increased intracellular glucose generates increased ROS in the mitochondria, free radicals induce DNA strand breaks, thereby activating PARP. PARP is a profuse nuclear enzyme of eukaryotic cells that has been implicated in response to DNA injury. When activated by single-strand DNA (ssDNA) breaks, PARP initiates an energy-consuming cycle by transferring ADP-ribose units from NAD^+ to nuclear proteins, resulting in a rapid depletion of the intracellular NAD^+ and ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction [107–113].

By inhibiting mitochondrial superoxide production with either uncoupling protein-1 (UCP-1) or MnSOD, both modification of GAPDH by poly(ADP-ribose) (PAR) and reduction of its activity by hyperglycemia were prevented [8]. Uncoupling proteins are a family of proton carriers that are expressed at the inner mitochondrial

membrane and are responsible for proton leak across the membrane into the cristae. Thus, protons pumped into the intermembranous space through electron transfer bypass oxidative phosphorylation, and these processes are said to be uncoupled. Activity of uncoupling proteins decreases the inner mitochondrial membrane potential and can relieve the stress of excess NADH entering the electron transfer chain [114]. Therefore, blocking of mitochondrial overproduction of superoxide by either UCP-1 or MnSOD prevents the inhibition of GAPDH activity by hyperglycemia.

11.5 Oxidative Stress in Diabetes and Atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by infiltration of lipids and inflammatory cells, such as monocyte-derived macrophages and T lymphocytes, into the artery wall [115]. It is well known that elevated levels of LDL cholesterol greatly increase the risk for atherosclerosis [116]. However, in vitro studies suggest that LDL by itself is not atherogenic and needs to be modified to initiate atherosclerotic disease [117, 118]. This conclusion led to the "oxidation hypothesis," which proposed that LDL must be oxidatively modified to become atherogenic.

Although oxidative stress has a well-established role in diabetic complications and atherosclerosis, its origins and magnitude remain poorly understood. Moreover, it is not known whether oxidative stress is a primary event that occurs early in the disease or whether it represents a secondary phenomenon that merely reflects end-stage tissue damage [28]. This distinction has important clinical relevance. If oxidative stress simply reflects tissue damage, interventions that reduce it may fail to affect the disease process. If oxidative stress promotes tissue injury, therapies that interrupt oxidative pathways early in the disease may prevent complications, and those that act later may slow disease progression.

There seems to be general agreement that the production of free radicals is increased in diabetic patients. For example, numerous investigators have reported elevated levels of products of lipid, protein, and nucleic acid oxidation such as 8-epi-prostaglandin F_2 (8-epi-PGF_2), 8-hydroxy-2-deoxyguanosine (8-OHdG), and oxidized LDL in subjects with both type 1 and type 2 diabetes when compared to healthy age-matched subjects [119–132]. Davi et al. [132] also reported that urinary levels of 8-epi-PGF2-α are increased in patients with both type 1 and 2 diabetes and decrease significantly with aggressive control of hyperglycemia. Several studies in experimental models of diabetes reported that free radicals contribute to the onset and progression of diabetes complications [133–138]. Others reported that lipid peroxidation correlates closely with all diabetic complications in vivo and contributes to the development of atherosclerosis [139–141]. We have recently demonstrated that systemic oxidative stress is increased in type 1 diabetic patients with early microangiopathic complications and subclinical cardiovascular autonomic neuropathy (CAN) [142]. Compared to healthy control subjects, asymptomatic diabetic subjects with subclinical microangiopathy presented increased levels of urine

8-epi-PGF2-α, and the highest levels of 8-epi-PGF2-α were found in subjects with more advanced CAN [142].

However, Baynes [143] presented evidence that oxidative stress may not occur early in the disease process, but may rather be an underlying pathogenic factor in the progression of the disease.

One of the major problems in assessing when oxidative damage occurs in the disease process or whether there is an accumulation of free radical-derived tissue damage with duration of disease is the stability of the oxidation products [143].

Oxidizing intermediates are difficult to detect in vivo because they are short-lived and generated at low levels. To sidestep this problem, more recently several groups of investigators have identified and are able to monitor acid-stable products of protein oxidation, both in vitro and in vivo [10–15, 48, 53–55, 144–155]. The overall approach is to use isotope dilution GC/MS to accurately identify oxidized amino acids isolated from tissue proteins. These markers, which include *ortho*-tyrosine, *meta*-tyrosine, dityrosine, 3-nitrotyrosine, and 3-chlorotyrosine (Fig. 11.1), are sensitive indicators of the biochemical pathway affected by oxidative stress occurring in the disease process.

Studies using sensitive and specific MS methods to quantify oxidation products have cast doubt on the concept of a generalized increase in oxidative stress in diabetic humans. For example, Wells-Knecht et al. [156] performed careful, quantitative studies on collagen, a long-lived protein that is freely exposed to blood glucose and lipids. They concluded that diabetes does not enhance oxidative stress because collagen from diabetic and euglycemic subjects contained similar age-adjusted levels of *ortho*-tyrosine and methionine sulfoxide, two well-characterized markers of protein oxidation in vitro. Other mass spectrometric studies have failed to find differing levels of glycoxidation products in urine and blood of diabetic and euglycemic humans [69, 157]. These observations argue strongly against a generalized increase in oxidative stress in diabetes, at least in the extracellular compartment. None of the above studies excluded the possibility of localized, tissue-specific increases in oxidative stress in organs vulnerable to diabetic damage: the retina, kidney, vascular wall, and peripheral nerve tissue.

11.5.1 Diabetic Endothelial Dysfunction, Oxidative Stress, and Atherosclerosis

Endothelial dysfunction is a key early feature in atherogenesis [158, 159]. It is characterized by a reduction in the bioavailability of vasodilators such as endothelium-derived NO and a relative or absolute abundance of vasoconstrictors. This imbalance impairs endothelium-dependent vasodilation, the functional hallmark of endothelial function [158, 159]. Endothelial dysfunction is also involved in plaque progression and its complications [158, 159] and may contribute to inflammatory responses.

NO mediates vasodilatation by activating soluble guanylate cyclases that regulate ion channels, macrophage cytotoxicity, and neurotransmission [160]. A decline

in NO bioavailability can result from multiple factors, including accelerated NO degradation, decreased expression of eNOS, lack of substrates for eNOS, and decreased eNOS activation [39, 42].

Oxidative stress-induced disruption of endothelium-dependent vasodilation is involved in the pathogenesis of diabetes complications, atherosclerosis and CVD. Several lines of evidence support a role for ROS in inducing impaired endothelium-dependent vasodilation. For example, in experimental diabetes, the superoxide radical was shown to induce rapid NO inactivation, as NO reacts with the O_2^- to form peroxynitrite and becomes a prooxidant [133, 161–169]. In addition, NO modulates cellular respiration through direct inhibition of cytochrome oxidase by competitively occupying the oxygen-binding site [170]. In vascular smooth muscle cells, oxidants have also been implicated in changes to signaling pathways downstream of cGMP, the second messenger of NO [171].

NO synthase, the enzyme catalyzing the conversion of L-arginine to citrulline and NO, is critically situated in endothelial cells, vascular smooth muscle cells, and sympathetic ganglia. Both constitutively expressed, calcium-dependent isoforms of NOS and an inducible isoform associated with inflammation and cell activation [172, 173] have been described.

Constitutively expressed eNOS is instrumental in the regulation of vascular function and can generate both NO and O_2^-. In the presence of Ca^{2+}/calmodulin, eNOS produces NO from L-arginine by means of electron transfer from NADPH through a flavin-containing reductase domain to oxygen bound at the heme of an oxygenase domain, which also contains binding sites for tetrahydrobiopterin and L-arginine. In the absence of tetrahydrobiopterin, NO synthesis is shifted to the generation of O^{2-} [174]. Very recently it was reported that oxidative stress alters eNOS activity by promoting S-glutathionylation, a reversible protein modification involved in cellular signaling and adaptation, in endothelial cells, and in intact and hypertensive vessels. This in turn reversibly decreases NOS activity with an increase in O_2^- generation resulting in impaired endothelium-dependent vasodilation [174]. This suggests that agents with thiol-reducing properties may be beneficial in reversing endothelial dysfunction and ameliorating CVD.

Inducible forms of NOS are increased in the vascular muscle cells of diabetic rats [175] and other tissues [176] and may promote upregulation of net NO production. High NO, in the presence of excess O_2^-, results in the formation of peroxynitrite, a potent oxidant that promotes nitration of protein tyrosine residues, producing a distinctive molecular fingerprint for nitric oxide-derived oxidants, 3-nitrotyrosine. An alternative mechanism for generating nitric oxide-derived oxidants involves MPO [48], a leukocyte-derived enzyme enriched in atherosclerotic lesions that serves as an independent predictor of cardiovascular risk.

In human studies, nitrotyrosine has been detected in LDL and high-density lipoproteins (HDL) isolated from human diabetic atherosclerotic lesions [15, 144, 145], and plasma nitrotyrosine levels are elevated in patients with CAD [56, 59]. It was also suggested that nitrotyrosine may serve as an inflammatory marker for CAD, as in multivariable analysis systemic levels of protein-bound nitrotyrosine were independently associated with the presence of CAD [56]. In addition, statin therapy was

shown to promote significant reductions in systemic nitrotyrosine, independent of the reduction in lipoprotein levels [56, 59].

Impaired endothelial function has been demonstrated in subjects with both type 1 and type 2 diabetes and in obese, insulin-resistant subjects [171]. Because acute hyperglycemia promotes vasodilation in humans, glucose might directly or indirectly enhance NO release and oxidant generation [177].

In subjects who develop type 2 diabetes, endothelial dysfunction predates hyperglycemia, suggesting that other factors such as insulin resistance and increased concentrations of FFAs initiate endothelial dysfunction in this setting. In established diabetes, hyperglycemia acts in concert with hypercholesterolemia, hypertension, and other factors to induce worsening of endothelial dysfunction. Hyperglycemia and elevated levels of FFAs promote oxidative phosphorylation in mitochondria and also boost the production of reactive intermediates such as superoxide that accelerate NO degradation [17].

In addition, more recent studies have suggested that T-786C single-nucleotide polymorphism (SNP) in the promoter of eNOS is associated with blunted NO bioactivity and is associated with changes in markers of oxidative stress [178].

11.5.2   *Oxidative Stress, PARP Activation, and CVD in Diabetes*

One of the important pathways of peroxynitrite-mediated vascular dysfunction in diabetes involves the activation of PARP [179]. For many decades, PARP was mainly viewed as an enzyme primarily involved in DNA repair and maintenance of genomic stability. Mild activation of PARP regulates multiple cellular reactions such as DNA repair, gene expression, and cell survival [180]. However, overactivation of PARP could initiate a series of cellular processes that culminate instead with cellular damage [106].

Over the last decade, additional roles of PARP have been identified in the sequelae of nitrosative stress, including contributing to the pathogenesis of endothelial dysfunction in diabetes [181]. In experimental diabetes, both pharmacological PARP inhibition and the PARP$^{-/-}$ phenotype prevented the activation of PARP, but had no effect on the DNA single-strand breakage [182]. Subsequent studies demonstrated that the diabetes-associated loss of endothelial function is not only preventable but also rapidly reversible with PARP inhibition [182]. Treatment with the PARP inhibitor ameliorated vascular PAR accumulation in the diabetic blood vessels and restored normal vascular function without altering systemic glucose levels, plasma-glycated hemoglobin levels, or pancreatic insulin content [183]. The potential of PARP inhibition in reversing endothelial dysfunction has also been demonstrated in an autoimmune nonobese diabetic model of diabetes [184] and in leptin-deficient db/db mice.

In humans, PARP activation is present in healthy subjects at risk of developing diabetes as well as in established type 2 diabetic patients, and it is associated with impairments in the vascular reactivity in the skin microcirculation [185]. In diabetes, increased oxidative and nitrosative stress also occurs in cardiomyocytes and endothelial cells and, in concert with PARP activation, may contribute to cardiomyopathy [186]. PARP activation may cause an energy deficit and cell death through depletion of NAD+, an ATP-consuming process. PARP, through inhibition of GAPDH, diverts glucose from glycolytic pathways into alternative fates, including AGE formation, hexosamine, polyol pathway flux, and PKC activation, which mediate hyperglycemia-induced cardiac tissue damage [186, 187].

11.5.3 Inflammation, Oxidative Stress, Oxidized LDL, and Atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by infiltration of lipids and inflammatory cells, such as monocyte-derived macrophages and T lymphocytes, into the artery wall [115]. It was shown that elevations of inflammation-sensitive plasma proteins precede clinical CVD and are intricately linked with the development of cardiovascular events [188–190]. It was also proposed that in diabetes, oxidative stress and chronic inflammation act in concert in the development and progression of atherosclerosis [10, 13].

Although it is well known that elevated levels of LDL greatly increase the risk for atherosclerosis [116], in vitro studies suggest that LDL by itself is not atherogenic but needs to be modified to initiate atherosclerotic disease [117, 118]. This conclusion led to the "oxidation hypothesis," which proposed that LDL must be oxidatively modified to become atherogenic. Several lines of evidence support the hypothesis that LDL must be oxidatively modified to become atherogenic. For instance, oxidized LDL (OxLDL) has been isolated from human and animal atherosclerotic tissue, and immunohistochemical studies have detected oxidized lipids in atherosclerotic lesions [191–193]. All major cell types involved in atherosclerosis—smooth muscle cells, endothelial cells, and macrophages—produce reactive oxidants that can oxidize LDL in vitro [194–196]. Oxidized LDL is taken up by scavenger receptors of macrophages, which then become lipid-laden foam cells, the pathologic hallmark of early atherosclerotic lesions [197]. Moreover, OxLDL attracts mononuclear cells and stimulates the production of monocyte chemoattractant protein-1 and other inflammatory cytokines, leading to the conversion of fatty streaks to more advanced complex lesions as smooth muscle cells migrate from the media into the subendothelial space. Oxidized LDL may also stimulate smooth cells to synthesize extracellular matrix and activate a signaling cascade by interacting with the lectin-like OxLDL receptor [197, 198]. Finally, several structurally unrelated lipid-soluble antioxidants that inhibit LDL oxidation in vitro also inhibit atherosclerosis in hypercholesterolemic animals [199–201].

11.5.4 Glucose-Oxidized LDL and Atherosclerosis in Diabetes

Macrophage proliferation has been implicated in the progression of atherosclerosis. Recent studies have investigated the effects of hyperglycemia and hyperlipidemia on macrophage proliferation in murine atherosclerotic lesions and isolated primary macrophages [152]. Glucose promoted lipid and protein oxidation of LDL in vitro. Oxidation of LDL with glucose resulted in a selective increase in protein-bound *ortho*-tyrosine and *meta*-tyrosine. Moreover, glucose-oxidized LDL—but not elevated levels of glucose alone—stimulated proliferation of isolated macrophages. These observations may be pertinent to diabetic vascular disease because macrophage proliferation in atherosclerotic lesions was observed in LDL receptor-deficient mice that were both hypercholesterolemic and hyperglycemic but in not mice that were only hyperglycemic [152].

11.5.5 Oxidative Stress, Insulin Resistance, Visceral Adiposity, and CVD

Obesity and visceral adiposity frequently associate with diabetes and insulin resistance. Also, insulin resistance clusters with the metabolic syndrome, a constellation of classic CAD risk factors such as lipid abnormalities, visceral adiposity, impaired glucose tolerance, and hypertension, which is considered a prediabetic state. Emerging data support the hypothesis that oxidative stress plays a causal role in insulin resistance [202] and might be linked with visceral adiposity. For instance, increased levels of 8-epi-PGF2-α were associated with body mass index and blood glucose levels in a cohort of the Framingham Heart Study [203] and with visceral adiposity and insulin resistance in men in a smaller study [204]. Although correlation does not prove causation, the results of these studies suggest that obesity is an important factor for enhanced oxidative stress and that this oxidative stress may trigger the development of insulin resistance. Mature adipocytes function as an endocrine/paracrine organ that secretes numerous adipokines, cytokines, and growth factors, particularly in the setting of insulin resistance. Several adipokines and cytokines, such as adiponectin, interleukin-6 (IL-6), retinol-binding protein-4 (RBP-4), resistin, and tumor necrosis factor-α, are associated with insulin resistance. RBP-4 is an adipocyte-derived molecule that is elevated prior to the onset of diabetes [205], and it appears to impair insulin signaling in muscle and promote insulin resistance [206, 207]. Visceral fat releases IL-6, which can contribute to local and systemic inflammation and elevation of C-reactive protein levels [208]. Moreover, tumor necrosis factor-α mediates its effect through hydrogen peroxide generation [209]. It was also reported that hydrogen peroxide impairs insulin signaling [210] and inhibits glucose transport [211], two cardinal features of insulin resistance. Similar results were reported in children. For instance, Molnar et al. [212] demonstrated a reduced antioxidant capacity in obese children with metabolic syndrome in whom plasma alpha-tocopherol and β-carotene levels corrected for plasma lipids (cholesterol + triglyceride)

were significantly lower compared with healthy controls [212]. Some have speculated that oxidative stress is a potential consequence of insulin resistance after it was reported that insulin promotes hydrogen peroxide generation in fat cells [213]. Thus, insulin resistance is intricately linked with visceral adiposity and oxidative stress, and it may promote endothelial dysfunction and CAD.

11.5.6 Cardiovascular Autonomic Neuropathy (CAN) in Diabetes and Oxidative Stress

CAN is an important complication of diabetes [214], associated with a high risk of cardiac arrhythmias and sudden death [215–220] and with high cardiovascular morbidity [214, 221]. In diabetes, the development of CAN is a function of complex interactions among degree of glycemic control, disease duration, age-related neuronal attrition, and systolic and diastolic BP [222, 223]. Hyperglycemia plays the key role in the activation of various biochemical pathways related to the metabolic and/ or redox state of the cell that act in concert to impact autonomic neuronal function in diabetes including the increased oxidative/nitrosative stress [224–227].

Autonomic innervation is the primary extrinsic control mechanism regulating heart rate variability and cardiac performance. It has been shown that chronic hyperglycemia promotes progressive autonomic neural dysfunction in a fashion which parallels the development of peripheral neuropathy, e.g., beginning distally and progressing proximally. The earliest manifestations of CAN in diabetes tend to be associated with various degree of parasympathetic denervation. As such, the initial development of CAN in diabetes is characterized by early augmentation of sympathetic tone [228]. Our data [142] and others [229] confirmed that, early in the progression of CAN complicating type 1 diabetes, there is a compensatory increase in the cardiac sympathetic tone in response to subclinical peripheral denervation. Later, sympathetic denervation follows beginning at the apex of the ventricles and progressing toward the base.

The initial prevalent cardiac sympathetic activity with subsequent abnormal norepinephrine signaling and metabolism, increased mitochondrial oxidative stress [230], and calcium-dependent apoptosis [231] may contribute to myocardial injury [230, 232] and explain the high risk of cardiac events and sudden death in these patients. The sympathetic imbalance associated with CAN may also critically influence myocardial substrate utilization [233] and contribute to mitochondrial uncoupling [234], regional ventricular motion abnormalities, functional deficits, and cardiomyopathy [142].

11.5.7 Oxidative Stress and Diabetic Cardiomyopathy

In type 1 DM (T1DM), left ventricle (LV) dysfunction often precedes or occurs in the absence of significant CAD or hypertension [142, 235–240]. Indeed, alterations of diastolic [142, 237, 241] and systolic [242] function are reported in otherwise

healthy diabetic subjects and often predate the development of other chronic diabetic complications. This suggests that diabetes has direct effects on the heart, which can contribute to the development of cardiomyopathy and LV dysfunction in the absence of overt large vessel disease. Such effects may occur via various other mechanisms including subclinical microvascular disease, presence of CAN, impairment in myocardial metabolism, efficiency and energetics, and activation of oxidative stress. Sympathetic activation associated with CAN generates high myocardial norepinephrine levels with abnormal norepinephrine signaling and metabolism and subsequent catecholamine toxicity [232, 243]. These may contribute to myocardial injury via cytotoxic effects to the heart associated with increased production of mitochondrial ROS [230, 244], and calcium-dependent apoptosis [230–232, 245], and may explain the progression to LV dysfunction and future risk for CVD events. For instance, CAN is accompanied by depressed diastolic filling [246] and reduced LV ejection fraction [247], which correlates with heterogeneous cardiac [^{123}I]*meta*-iodobenzylguanidine (MIBG) and [^{11}C]*meta*-hydroxyephedrine (HED) retention [248, 249]. Our prior studies identified diastolic dysfunction early in the course of T1DM [142].

The heart is unique among organ systems in its continuous need for high-energy phosphates to maintain contractile function. It can switch between different substrates depending on substrate availability, hormonal milieu, oxygen availability, and metabolic demands [250]. Myocardial glucose and FFA metabolism are tightly coupled, with increased FFA metabolism inhibiting myocardial glucose metabolism and vice versa. Sympathetic toxicity induces insulin resistance and may compromise regional glucose utilization [186]. This alteration, together with the increased FFA supply, due to catecholamines' induced fatty acids extraction and oxidation [251, 252], switches cardiac energy generation to utilization of FFA. Therefore, FFA, an inefficient energy source [253], may contribute to more than 90 % of the myocardial oxygen (O_2) consumption in the diabetic heart [254, 255], which may induce mitochondrial uncoupling [234, 256], increased O_2 demand [234, 256], and generation of ROS (Fig. 11.1). Mitochondrial uncoupling when associated with deficits in glucose metabolism may also predispose to programmed cell death and fibrosis [235, 236, 257]. All these changes in the type of substrate in the presence of sympathetic activation may reduce cardiac efficiency—the ratio of cardiac work to myocardial oxygen consumption [246, 258–261].

In addition, increased myocardial catecholamines associated with sympathetic activation increase oxygen consumption, cause energy depletion [246, 253, 262, 263], and promote increased glycolysis and subsequent myocardial acidosis [253, 264]. Reduced cardiac efficiency and increased oxygen demand make the heart especially vulnerable to damage following increased workload or ischemia. Therefore, these evidences suggest that, in T1DM, chronic adrenergic stimulation and LV sympathetic imbalance may lead to cell injury, impaired myocardial efficiency, myocardial remodeling, abnormal myocardial contractile patterns, and subsequent cardiomyopathy.

11.6 Hyperglycemia and Diabetic Cardiovascular Complications: Evidence from Clinical Trials

Diabetes strongly increases the risk for atherosclerotic macrovascular disease. Many epidemiological studies have reported that progressively higher fasting [265, 266] or post-load glucose [267, 268] or HbA$_{1c}$ levels [269, 270] predict a progressively higher incidence of cardiovascular outcomes. It was also reported that the rate of death from CAD is two to four times higher in diabetic men than in the general population [2] and that CAD is more prevalent even in premenopausal diabetic women. The significant gains that have been made in reducing mortality from CVD for the general population have not been as dramatic in the diabetic population, and several groups of diabetic individuals even show an increase in cardiovascular mortality [271]. Both the degree of glycemic control and the duration of diabetes predict the risk of diabetic complications [272]. The importance of hyperglycemia as a risk factor for CAD in the general population and in diabetes is further highlighted by the finding of the INTERHEART study that HbA$_{1c}$ is an independent risk factor for MI in the presence of multiple other independent cardiovascular risk factors and across most geographical regions and ethnicities. The INTERHEART study, a large case–control study of MI conducted in 29,972 people in 52 countries, reported that self-reported diabetes and eight other cardiovascular risk factors confer more than 90 % of the population-attributable risk of MI globally [273]. The same study also found that the degree of glycemic control, as assessed by HbA$_{1c}$, provides more information on MI odds than self-reported diabetes status or many other established risk factors including age, sex, hypertension, dyslipidemia, smoking, obesity, and psychosocial stress, as every 1 % HbA$_{1c}$ increment independently predicts 19 % higher odds of MI [274]. These findings suggest that hyperglycemia may be toxic to the artery wall and increases cardiovascular risk through a mechanism that appears to be independent of these other cardiovascular risk factors and that this mechanism is relevant with and without a history of diabetes and operates on a global level.

In type 1 diabetes, the Diabetes Control and Complications Trial (DCCT) found that strict glycemic control dramatically lowered the incidence of microvascular complications [5, 6, 275]. These observations have given rise to the "glucose hypothesis," which suggests that glucose mediates many of the deleterious effects of the disease. At DCCT closeout, all subjects were encouraged to adopt intensive treatment and most agreed to participate in the observational Epidemiology of Diabetes Interventions and Complications (EDIC) study [276].

Subsequent EDIC evaluations demonstrated long-term benefits of prior intensive glycemic control on microvascular complications [277–279], and CVD [280] in spite of the fact that the HbA$_{1c}$ separation between former DCCT intensive and conventional groups narrowed substantially at EDIC year 1 and was no longer statistically significant by EDIC year 5 [280] (Table 11.2). Intensive treatment reduced the risk of any CAD event by 42 % and the risk of nonfatal myocardial infarction, stroke, or death from CAD by 57 %, strongly suggesting that glycemic control

Table 11.2 Clinical trials exploring the effects of glucose control on cardiovascular disease

Study	Patient population	Subjects enrolled	Target A1c	Duration	Primary end point	Outcome
DCCT [5]	T1DM	1,441	7 % INT vs. 9 % CONV	6.5 years	Microvascular events	Significant prevention in INT for all
DCCT/EDIC [273]	T1DM (former DCCT cohort)	1,300	Standard of care, A1c no longer different as of year 5	10 years follow up post DCCT	Composite CV events	Significant prevention in former INT
UKPDS [283]	T2DM	5,102	7 % INT vs. 7.9 % CONV	6 years	1. Microvascular 2. Outcome Composite CV events	1. Significant prevention in INT 2. Nonsignificant trend with INT, MI prevention in metformin substudy
UKPDS follow up [284]	T2DM (former UKPDS cohort)		Standard of care, A1c no longer different	10 years follow up	Microvascular outcome Composite CV events	Significant prevention in former INT in the entire cohort; metformin benefit preserved
ADVANCE [284]	T2DM	11,140	6.4 % INT vs. 7.4 standard	4.5 years	Composite CV events	Nonsignificant trend
ACCORD [285]	T2DM	10,000	6.4 INT vs. 7.5 % standard	5.6 years	Composite of nonfatal MI nonfatal stroke, or death from CVD causes.	Increased mortality with INT
VADT [286]	T2DM	1,791	6.9 % INT vs 8.4 standard	6.5 years	Composite CV events	Nonsignificant trend
STENO-2	T2DM	160	Targeted A1c <6.5 % (reached 6.8)+intensive control of other risk factors	7.8 years	Composite of death from cardiovascular causes, nonfatal myocardial infarction, nonfatal stroke, revascularization, and amputation	Significantly decreased the composite primary CVD endpoint. However the effects of the individual glucose treatment component cannot be estimated

DCCT Diabetes Control and Complications Trial, *T1DM* Type 1 Diabetes Mellitus, *INT* interventional group, *CONV* conventional control group, *EDIC* Epidemiology of Diabetes Interventions and Complications Study, *CV* cardiovascular, *UKPDS* United Kingdom Prospective Diabetes Study, *MI* myocardial infarction, *T2DM* Type 2 Diabetes Mellitus, *ADVANCE* Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation, *ACCORD* Action to Control Cardiovascular Risk in Diabetes Trial, *VADT* Veteran's Affairs Diabetes Trial

lowers macrovascular disease endpoints as well [280]. Most recently we reported persistent beneficial effects of intensive versus conventional therapy on measures of CAN, a complication associated with high mortality risk, up to 14 years of follow-up in EDIC [281]. Additionally, intensive therapy during the DCCT associated with decreased progression of intima–medial thickness, a surrogate marker for athero-sclerosis, 6 years after the end of the trial. This beneficial effect of prior intensive glucose control is termed "metabolic memory" [279], but the pathophysiological mechanisms responsible for this effect are still unclear.

In type 2 diabetes the United Kingdom Prospective Diabetes Study (UKPDS) also showed microvascular benefits with intensive glucose control and suggested a trend toward less macrovascular disease with intensive glucose-lowering therapy, but the difference did not reach statistical significance [7, 282] (Table 11.2). However, most recently, a significant persistent benefit for myocardial infarction and death from any cause was reported after intensive glucose-lowering and metfor-min therapy in this cohort during 10 years of posttrial follow-up of the UKPDS participants [283] (Table 11.2).

However, in type 2 diabetes, strict glucose control alone does not prevent cardio-vascular events in patients with more advanced disease [284–286] or requires long time of follow-up in patients newly diagnosed [283] (Table 11.2). Also, the increased mortality associated with tight glucose control in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial [285] has challenged the "tight glucose" concept, raising the possibility of detrimental effects associated with a tight glucose control including higher incidence of severe hypoglycemia, important weight gain, and drug interactions. In addition, recent evidence suggests that glycemic variability may also influence the risk of cardiovascular complications, possibly through a mechanism mediated by activation of oxidative stress [287]. Among patients with type 2 diabetes, markers of oxidative stress levels were four times higher in patients with the greatest glycemic variability compared with patients having the lowest glycemic variability, and the acute glucose variability was a strong predictor of total free radical production [287].

11.6.1 Antioxidants in Preventing Cardiovascular Disease

The proposed role of oxidized LDL in atherogenesis suggests that a high dietary antioxidant intake might prevent premature vascular disease in humans. Antioxidants such as vitamin E, vitamin C, α-lipoic acid (thioctic acid), taurine, GSH, flavonoids, uric acid, and various enzymes (catalase, superoxide dismutase, GSH peroxidase) are metabolic intermediaries or substrates, which protect biologi-cal tissues from free radical damage, and are recycled or regenerated by biological reductants [288].

11.6.2   Epidemiological Evidence: Prospective Cohort Studies (Table 11.3)

A wide range of prospective cohort studies confirm the above considerations (Table 11.3). For instance, the lower cardiovascular mortality observed in Mediterranean populations when compared with Northern European countries has been attributed to differences in the intake of antioxidant-rich foods and beverages [289]. In line with this, a meta-analysis of cohort studies including almost 4,00,000 patients [290] reported that high vitamin E and vitamin C intake was associated with a lower rate of coronary heart disease. Higher quintiles of serum vitamin E (within the physiological range) were associated with lower mortality for cancer and CVD after a follow-up of 19 years in 29,092 male smokers enrolled in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study [291]. In the roughly 8,000 subjects of the NHANES-II study, the lowest quartile of serum vitamin C had an increased mortality for cancer and cardiovascular mortality in men but not in women, who had higher ascorbate levels at baseline [292]. Notably, the major limitation of these studies was that higher consumption of antioxidant-rich vegetables and fruits is also associated with generally "healthier" lifestyle, including physical exercise and abstinence from smoking.

11.6.3   Interventional Trials on Antioxidant Vitamins (Table 11.3)

A number of interventional trials were conducted between 1996 and 2002, mainly administering vitamin E, in the synthetic or natural form, β-carotene, and vitamin C, alone or in combination, and at different dosages. Some studies showed a benefit of vitamin E supplementation in the secondary prevention of CVD [293] and of vitamin E plus C supplementation in slowing carotid intima–media thickening in hypercholesterolemic patients [294]. However, a meta-analysis that pooled data from clinical trials using β-carotene and vitamin E in diverse population groups failed to demonstrate a beneficial effect of antioxidant supplements on cardiovascular morbidity and mortality [295]. Similarly, a meta-analysis of randomized, placebo-controlled trials published until January 2010 evaluating the effects of vitamin E supplementation of ischemic and hemorrhagic strokes demonstrated a relatively small risk reduction of ischemic stroke and a generally more severe outcome of hemorrhagic stroke, advocating against the widespread use of vitamin E [296]. A recent Cochrane systematic review of all primary and secondary prevention randomized clinical trials on antioxidant supplements (β-carotene, vitamin A, vitamin C, vitamin E, and selenium) versus placebo or no intervention found no evidence to support antioxidant supplements for primary or secondary prevention and suggested that vitamin A, β-carotene, and vitamin E may increase mortality [297].

Table 11.3 Clinical trials exploring the effects of antioxidants on CVD outcomes

Name	Type of study	Patient population	N	Agent	Duration	Primary end point/outcome
ATBC [290]	Observational prospective cohort	Men smokers	29,092	Vitamin E	19 years	Lower CVD mortality with higher serum vitamin E
NHANES-II [291]	Observational prospective cohort	Men and women	8,000	Vitamin C	12–16 years	Higher CVD mortality with lower serum vitamin C levels in men, not in women
Meta-analysis [289]	Cohort studies	Men and women	400,000	Vitamins C and E		Lower rate of coronary heart disease with higher intake of vitamins C and E
WHO MONICA Project [288]	Population cohort	Men and women aged 25–64 years	15 millions	Daily intake of antioxidant-rich foods and beverages	10 years	Lower cardiovascular mortality in Mediterranean populations vs. Northern European countries due to higher intake of antioxidant-rich foods and beverages in former
Physicians Health Study [294]	Randomized placebo controlled	Diabetes and non-diabetes	14,641	Vitamins C and E	10 years	Composite CV events
CHAOS [292]	Double-blind, placebo-controlled	CAD confirmed by angiography	2,002	Vitamin E	510 days	Vitamin E significantly reduced the risk of the primary endpoint of cardiovascular death and non-fatal MI
ASAP [293]	Randomized, 3-year double-masked treatment period, followed by 3-year open treatment	Men and women, with high cholesterol, 45–69 years	520	Vitamins C and E supplement in physiological ratios	6 years	In both parts of the study, the supplementation with 136 IU of vitamin E plus 250 mg of slow-release vitamin C twice daily slowed down the progression of carotid atherosclerosis in men but not women

(continued)

Table 11.3 (continued)

Name	Type of study	Patient population	N	Agent	Duration	Primary end point/outcome
HOPE [303]	Randomized placebo controlled, 2×2 factorial	High risk CVD or diabetes	9,297	Ramipril/vitamin E	4.5 years	Ramipril significantly reduced the rates of death, myocardial infarction, and stroke, and new diabetes development Vitamin E had no apparent effect on cardiovascular outcomes
Meta-analysis [325]	Interventional studies	Non-diabetics	6,000	Polypheonols		Polyphenols ameliorate endothelial function and significantly reduce blood pressure
Chocolate intake and the incidence of heart failure [328]	Prospective study	Non-diabetic, middle-aged and elderly women	31,823	Chocolate	8 years	Moderate, habitual chocolate intake associated with lower rate of heart failure hospitalization and death
Mediterranean Adequacy Index: correlation with 25-year mortality from coronary heart disease in the Seven Countries Study [315]	Cohort study	Middle-aged men	12,763	Mediterranean diet	25 years	Mediterranean diet protects against coronary heart disease
Nurses Health Study [316]	Cohort study	Women without diabetes and CVD	74,886	Mediterranean diet	20 years	Greater adherence to Mediterranean diet associated with lower risk of CHD and stroke incidence
HALE project [317]	Cohort study	Elderly European men and women	1,507	Mediterranean diet	10 years	Adherence to a Mediterranean diet is associated with a lower mortality
EPIC Study—Greek Arm [318]	Prospective cohort study	Men and women without cancer, diabetes, CHD	23,349	Mediterranean diet	8.5 years	Higher adherence to Mediterranean diet is associated with lower total mortality

Study	Study type	Population	N	Intervention	Duration	Outcome
Lyon Diet Heart Study [322]	Randomized clinical trial	Patients after an MI	605	Mediterranean alpha-linolenic acid-rich diet vs. prudent control diet	5 years	Alpha-linolenic acid-rich Mediterranean diet more efficient in preventing secondary coronary events and death
THIS DIET [323]	Randomized clinical trial	Patients after an MI	202	Mediterranean diet or low-fat diet vs. control diet	46 months	Low-fat or Mediterranean diet benefit overall and cardiovascular-event-free survival after MI
[310]	Randomized clinical trial	Overweight people with T2DM	215	Low-carbohydrate, Mediterranean diet vs. low-fat diet	4 years	Compared to a low-fat diet, a low-carbohydrate, Mediterranean-style diet lead to more favorable changes in glycemic control and coronary risk factors, and delayed the need for antihyperglycemic drug therapy
[311]	Randomized clinical trial	Men and women with metabolic syndrome	180	Mediterranean diet vs. control diet	2.5 years	Mediterranean-style diet may reduce prevalence of metabolic syndrome and its associated CV risk
[330]	Randomized clinical trial	Men and women with Type 1 DM	44	Treatment with triple antioxidant with allopurinol, α-lipoic acid and nicotinamide vs. placebo	2 years	The combination antioxidant treatment regimen did not prevent progression of cardiovascular autonomic neuropathy and had no beneficial effects on myocardial perfusion

ATBC Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, *NHANES-II* National Health and Nutrition Examination Survey II, *MONICA* Multinational Monitoring of Trends and Determinants in Cardiovascular Disease, *CHAOS* Cambridge Heart Antioxidant Study, *ASAP* Antioxidant Supplementation in Atherosclerosis Prevention, *HOPE* Heart Outcomes Prevention Evaluation Study, *CVD* cardiovascular disease, *HALE* Healthy Ageing: A Longitudinal study in Europe, *EPIC* European Prospective Investigation into Cancer and Nutrition, *THIS DIET* The Heart Institute of Spokane Diet Intervention and Evaluation Trial), *T2DM* Type 2 Diabetes Mellitus

Recently, the neutral effect of vitamin C plus E supplementation was confirmed by the results of the Physicians Health Study, which enrolled 14,641 middle-aged male physicians with low prevalent CVD, followed up for about 10 years [295].

Additional attempts made with the supplementation of folic acid, which might compensate for the oxidation of the NOS coenzyme BH$_4$, also did not show a prognostic impact of this type of supplementation. Thus, the 2004 AHA Committee for Nutrition, Physical Activity, and Metabolism discouraged the use of antioxidant supplementation for the prevention of CVD [298]. As a further confounding factor, questions have been raised about the safety of prescribing antioxidant vitamins, with an increased overall mortality associated with β-carotene, vitamin A, and vitamin E supplementation, possibly due to increases in cancer mortality, reported in some of these trials [299].

However, the majority of prospective, double-blind, placebo-controlled trials of one proposed lipid-soluble antioxidant, vitamin E, have failed to demonstrate any reduction of clinical events in patients with established atherosclerosis [300]. The disappointing results of these trials have led some to question the role of oxidative damage in the pathogenesis of CAD in humans. It might be, however, that vitamin E's ability to serve as an antioxidant in vivo should be questioned [300]. Thus, despite the impressive ability of other lipid-soluble antioxidants to block atherosclerosis in hypercholesterolemic animals, vitamin E at doses that fail to lower cholesterol levels has not exerted a consistent inhibitory effect in such experiments. These observations [300–302] emphasize the importance of documenting that a proposed antioxidant intervention actually inhibits oxidative reactions in vivo.

There is also remarkably little information about the influence of vitamin E supplementation on lipid oxidation in humans. Indeed, a recent study of healthy humans taking dietary supplements as high as 2,000 IU/day for 8 weeks found no change in levels of three lipid oxidation products: 4-hydroxynonenal and two isoprostanes [303]. The investigators assessed products of lipid peroxidation using GC/MS, a sensitive and specific method. These results strongly suggest that vitamin E failed to inhibit lipid peroxidation in these individuals.

Trials of antioxidants and carbonyl-trapping agents in humans suffering from diabetes have also yielded discouraging results. Chronic treatment with vitamin E failed to decrease cardiovascular events in a large study that included a high percentage of diabetic patients [304]. One possible reason is that antioxidant therapy might benefit only subjects who exhibit increased oxidative stress. Indeed, the renal failure patients who benefited from vitamin E therapy [305] might have been a subset with greatly increased carbonyl and oxidative stress [70].

The effects of the xanthine oxidase inhibitor, allopurinol, were studied extensively in clinical trials in subjects with or without diabetes. A recent report [306] examined the effects of allopurinol on endothelial function and oxidative stress in type 2 diabetic patients. The investigators showed that allopurinol increased the endothelium-dependent mean blood flow response to acetylcholine by 30 % and decreased systemic levels of malondialdehyde. However, the relevance of endothelium-dependent mean blood flow response as a surrogate marker for CVD has been questioned because this technique predicted that vitamin E would prevent vascular disease.

Pharmacological agents currently in clinical practice with demonstrated benefit in CAD may act in part by serving as antioxidants. Angiotensin-converting enzyme (ACE) inhibitors and statins have lowered CAD event rates in randomized controlled trials in diabetic patients [41, 307]. As noted above, many lines of evidence suggest that angiotensin II triggers oxidant production by endothelial cells and other cells of the artery wall. Interestingly, the ACE inhibitor ramipril slowed the onset of type 2 diabetes in the Heart Outcomes Prevention Evaluation (HOPE) trial [41], though this effect was not confirmed in a more recent trial [308]. Ramipril's well-recognized ability to mitigate the prooxidant effect of angiotensin II may in part account for its efficacy. A case–control study demonstrated that systemic levels of protein-bound nitrotyrosine were significantly higher among patients with CAD and that statin therapy lowered levels of oxidized amino acids in plasma, raising the possibility that statins can potentially be antioxidants [56, 57, 59].

Despite a sound biological rationale and a number of preclinical and clinical lines of evidence, studies testing the effects of classical antioxidants such as vitamin C, vitamin E, or folic acid in combination with vitamin E have been disappointing. Rather, substances such as statins, angiotensin-converting enzyme inhibitors, or AT1-receptor blockers, which possess indirect antioxidant properties mediated by the stimulation of NO production and simultaneous inhibition of superoxide production (e.g., from the NADPH oxidase), have been shown to improve vascular function in preclinical and clinical studies and to reduce the incidence of cardiovascular events in patients with CVD.

11.6.4 Diet and Polyphenols

The recommendation of a healthy diet, rich in fruits and vegetables and whole grain foods, is still standing [309]. Adherence to the Mediterranean diet has been suggested to have a beneficial effect on mortality from all causes and on the primary and secondary prevention of CVD [310–313]. It was suggested that the Mediterranean diet may exert positive influences on human health and coronary heart disease in particular, due to its antioxidant and anti-inflammatory effects [314, 315]. Results from the 25-year follow-up of the Seven Countries Study, the Nurses' Health Study, the HALE project, and the Greek arm of the EPIC study [316–319] underline its protective role in regard to coronary heart disease. Adherence to the Mediterranean diet protects against the development of coronary heart disease in patients with hypertension, hypercholesterolemia, and the metabolic syndrome [320–322]. Adherence to the Mediterranean diet was also shown to have beneficial effects on the secondary CVD prevention. For instance, two randomized clinical trials performed in patients surviving a first myocardial infarction, the Lyon Diet Heart Study and THIS DIET [323, 324], adherence to the Mediterranean diet was associated with lower mortality risk and overall and cardiovascular event-free survival, although body weight was not significantly changed . In a randomized trial of 215 overweight people with newly diagnosed type 2 diabetes who were never treated

with antihyperglycemic drugs, a Mediterranean-style diet led to more favorable changes in glycemic control and coronary risk factors and delayed the need for antihyperglycemic drug therapy compared with a low-fat diet and a low-carbohydrate diet [311]. In another randomized trial, a Mediterranean-style diet was also shown to improve endothelial function and vascular inflammatory markers in patients with the metabolic syndrome compared with a prudent diet [312].

In line with this concept, attention has been focused on another family of antioxidant compounds, i.e., polyphenols, a group comprising about 8,000 different molecules, among which flavonoids are the most studied family. Polyphenols are potent antioxidants abundant in vegetables and particularly in derived products such as chocolate, tea, and wine. This more "natural" approach to antioxidant supplementation seems to be promising, since the antioxidant capacity of these compounds is not simply related to direct ROS scavenging but also to inhibition of enzymatic sources of oxidative stress and stimulation of endogenous antioxidant enzymes. Benefits from polyphenol-rich foods and beverages are likely to arise from multiple pathways, and the antioxidant power appears to be only one of these [325]. A meta-analysis of 113 interventional studies for a total of roughly 6,000 subjects who underwent different kinds of food/beverages or extracts supplementation at different doses demonstrated that these compounds ameliorate endothelial function, an intermediate endpoint strongly associated with cardiovascular prognosis, both in healthy subjects and in patients with cardiovascular risk factors, while significantly reducing blood pressure [326].

Two meta-analyses of small, relatively short-duration randomized clinical trials suggested that chocolate reduces both systolic and diastolic blood pressure [327] and increases flow-mediated dilation after acute and chronic intake. Others have shown that cocoa flavonoids are associated with decreased susceptibility to low-density lipoprotein oxidation [328] and improved endothelial function. A prospective study of 31,823 women aged 48–83 years without baseline diabetes or a history of HF or myocardial infarction, followed for a mean of 8 years, found that moderate habitual chocolate intake was associated with a lower rate of HF hospitalization or death [329].

Despite these promising data, further questions remain to be solved. First of all, it remains unclear how potent are the antioxidant properties of polyphenols and which molecules in this class are the most potent ones. Further, it needs to be clarified whether these compounds possess other properties beyond their chemical antioxidant ones. Also, concentrations of active substances present in food and beverages show remarkable variability due to genetic and agronomic factors, postharvest handling, and subsequent processing steps. Such problems could theoretically be overcome by using standardized formulations for supplementation, but this field still awaits exploration. In addition, in recommending an increase in flavonoid-rich substances such as chocolate and wine consumption, physicians must be careful in balancing caloric and alcohol intake, and this therapeutic approach requires patients' compliance with chronic lifestyle changes. At the moment, strong evidence obtained with long-term randomized controlled trials is still lacking, and no conclusion on the efficacy and safety of flavonoid supplementation can be reached.

Although more promising than other direct antioxidants, whose clinical efficacy is limited by the disadvantageous biochemical properties described previously, the available evidence with flavonoids consists mainly of prospective cohort studies and of mechanistic studies, in vitro or in animals; short-term interventional randomized trials only addressed blood pressure reduction or endothelial function as surrogate endpoints of cardiovascular health. In the meta-analysis mentioned earlier, the positive results on endothelial function and blood pressure were obtained only after several weeks' administration of certain flavonoid-rich foods, particularly tea and chocolate [326].

Furthermore, it needs to be mentioned again that an increased antioxidants intake, rather than being the cause of improved outcome, could be simply a marker of a healthier lifestyle. Thus, future research should more clearly address differences between different kinds of polyphenols, in order to identify which type of intervention would constitute the most feasible and effective approach for cardiovascular patients. Additionally, research should aim to clarify whether these encouraging results can be translated into reduction of events in our patients.

11.7   Conclusions

Strong evidence emerged in the last decade shows that oxidative stress is one major factor in the onset and the development of diabetes vascular disease. Until we can fully control blood glucose levels, antioxidants might be helpful for treating diabetic patients and their complications. It is more reasonable to assume that mixtures of antioxidant therapies, possibly in combination with trace elements and vitamins that enhance metabolic processes, may provide a better therapeutic option. Large-scale clinical trials are needed to evaluate the long-term effects of these antioxidants in diabetic patients.

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Chapter 12
Oxidative Stress in Diabetes Mellitus and Possible Interventions

Triantafyllos Didangelos, John Doupis, and Aristidis Veves

12.1 Introduction

Diabetes mellitus is considered one of the most important diseases of our time as its prevalence is globally increased every year. A large amount of evidence has proved that there is a strong association between diabetes, oxidative stress, and endothelial dysfunction. It is also well recognized that endothelial dysfunction, which is present even in people at risk of developing diabetes, is strongly connected with oxidative stress and considered as a preliminary risk factor for the development of atherosclerosis and cardiovascular disease. Thus, a lot of research effort has been focused during the last years toward the direction of reducing diabetes-related oxidative stress, either with the use of different pharmaceutical agents or with life style interventions.

In this chapter we are going first to analyze briefly the basis of oxidative stress in diabetes and then to focus on the different studied interventions for the diabetes-related oxidative stress reduction.

T. Didangelos
Diabetes Center, First Propeudetic Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, American Hellenic Educational Progressive Association Hospital, S. Kyriakidi 1, 54636 Thessaloniki, Greece

J. Doupis
Internal Medicine and Diabetes Department, Salamis Naval Hospital, Salamis, Greece

A. Veves (✉)
Microcirculation Laboratory and Joslin-Beth Israel Deaconess Foot Center, Beth Israel Deaconess Medical Center and Harvard Medical School, West Campus, One Deaconess Road, Boston, MA 02215, USA
e-mail: aveves@caregroup.harvard.edu

12.2 Oxidative Stress in Diabetes Mellitus

Helmut Sies was the first to define oxidative stress in the following way: "Oxidative stress is a change in the pro-oxidant/antioxidant balance in the favor of the former, potentially leading to biological damage" [1]. Diabetes is currently recognized as an oxidative stress disorder [2]. Oxidative stress per se is characterized by high accumulation of reactive oxygen species (highly reactive molecules generated during oxidative metabolism and energy production) that cannot be coerced by the endogenous circulating neutralizing agents and antioxidants [3]. The causative mechanisms of oxidative stress due to hyperglycemia are shown in Fig. 12.1.

12.3 Increased Superoxide Production

Diabetes mellitus is associated with increased production of superoxide (O_2^-), mainly due to hyperglycemia [3]. Hyperglycemia causes an increase in intracellular glucose concentration in insulin-independent cell types, such as endothelium. More particular, increased intracellular glucose concentration results in an increased rate of glycolysis, which in turn increases the flux of pyruvate (the product of glycolysis) through the tricarboxylic acid (TCA) cycle. This increased flux of pyruvate through the TCA cycle appears to be responsible for overproduction of superoxide [3].
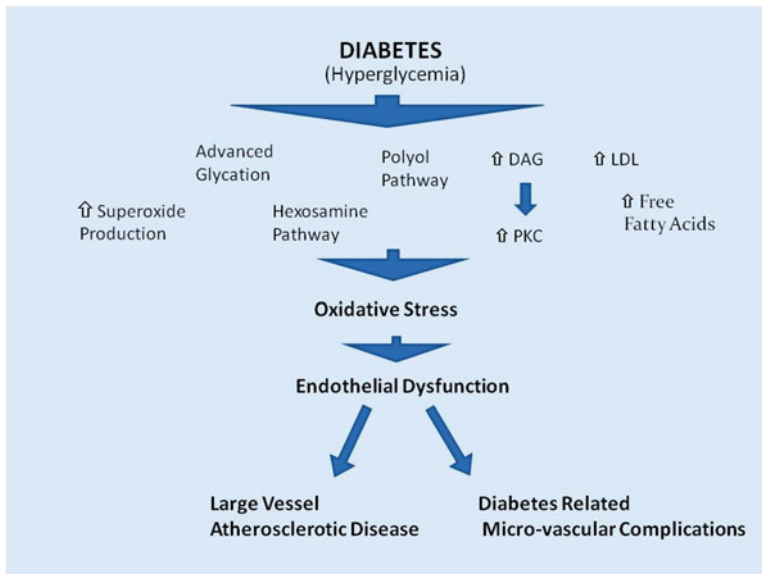
Fig. 12.1 Mechanisms of oxidative stress in hyperglycemia
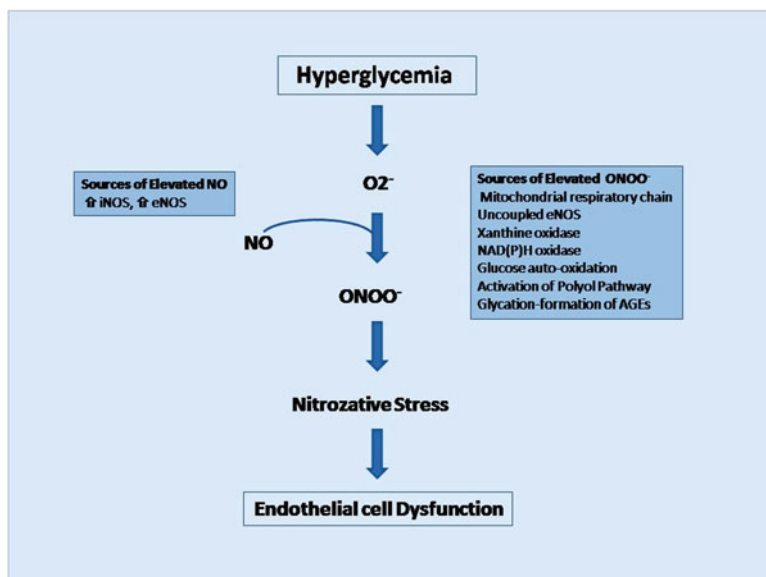
Fig. 12.2 Hyperglycemia-induced endothelial dysfunction. Superoxide produced secondary to hyperglycemia combines with NO to form peroxynitrite. This reduces the bioavailability of NO and induces nitrosative stress by multiple mechanisms including modifications of macromolecules and PARP induction

Hyperglycemia, however, is not the only mechanism by which diabetes causes increased superoxide production. Diabetes is also associated with increased levels of free fatty acids, which contribute to increased superoxide production [4]. Other circulating factors that are elevated in diabetes, such as leptin, also contribute to increased ROS generation [5].

12.3.1 Oxidative Stress and NO

Nitric oxide (NO) plays a key role in vascular health, regulating the endothelial vasodilatation and protecting the vascular wall by inhibiting inflammation, cellular proliferation, and thrombosis [3]. Increased superoxide and reactive oxygen species negatively affect vascular health by downregulating endothelial-derived NO. Decreased NO bioavailability increases the vascular tone, promoting also structural and biological changes that lead to atherosclerosis [3, 4]. NO quenching by peroxynitrite (ONOO⁻) and decreased NO production are the main causes of decreased NO bioavailability [3, 4, 6]. In addition, under certain conditions, the superoxide anion reacts with NO to form peroxynitrite, further reducing the bioavailability of NO in the vasculature leading to impaired protein and lipid function (see Fig. 12.2) [7]. Peroxynitrite, in turn, inactivates the factor (6R)-5,6,7,8-tetrahydro-L-biopterin

(BH4), which plays a significant role in NO production by the endothelial NO synthase (eNOS), leading to further reduction of NO bioavailability. BH4 deficiency uncouples the eNOS complex and promotes production of superoxide by eNOS, thus producing more oxidative stress promoting vascular dysfunction and atherosclerosis [7].

12.3.2   Other Effects of Oxidative and Nitrosative Stress

The degradation of tyrosine nitrated proteins produces free nitrotyrosine. This marker of nitrosative stress has been found in tissues, atherosclerotic lesions, and blood [8–10]. In addition to the modification of biomolecules, peroxynitrite affects important signaling pathways triggering mitochondrial dysfunction and cell death in endothelial cells and cardiomyocytes [11].

12.3.3   PARP Activation

Oxidative and nitrosative stress has been proved to activate poly(ADP-ribose) polymerase (PARP), which is an important mediator of vascular dysfunction in diabetes [7, 11–13] even prior to the onset of microvascular disease [14]. PARP activation initiates a series of cell cycle events (see Fig. 12.2) that deplete intracellular nicotinamide adenine dinucleotide (NAD) and adenosine 5′-triphosphate (ATP) pools, thus limiting glycolysis and mitochondrial respiration, leading to vascular cell dysfunction and death [6].

12.3.4   Protein Kinase C Activation

Hyperglycemia and increased production of free fatty acids increase the activity of protein kinase C (PKC) promoting oxidative stress through activation of mitochondrial NADPH oxidase. Increased PKC activity has also a number of other effects including decreased NO production, increased vascular permeability, increased microvascular protein accumulation, increased plasminogen activator inhibitor-1 (PAI-1) expression and activation of nuclear factor-kappa B (NF-κB) in endothelial cells and vascular smooth muscle, and increased endothelin-1 (ET-1) production. All these actions promote vascular occlusion, stimulate inflammation, and ultimately lead to endothelial dysfunction [2, 15]. PKC may also be activated by increased diacylglycerol (DAG) levels either from de novo synthesis of DAG (from glycolytic intermediates) or from increased activity of the polyol pathway and via ligation of RAGE [16]. Inhibition of PKC with ruboxistaurin (or LY333531) greatly improves microvascular flow to the retina, kidney, endoneural blood supply, and

mesenteric bed in animal models [17–19]. Despite these promising findings, ruboxistaurin has had less robust results in humans [20].

12.3.5 Advanced Glycation End Products

Hyperglycemia may also promote oxidative stress by contributing to the production of advanced glycation end products (AGEs) which are nonenzymatically glycated proteins or lipids susceptible to oxidation after exposure to aldose sugars [21]. AGEs can produce ROS and trigger mechanisms that generate the production of intracellular oxidants. In addition, AGEs have been found to alter extracellular matrix protein function, cause vascular leak, decrease the bioavailability of endothelium-derived nitric oxide (NO), and promote inflammation and endothelial dysfunction [22].

Additionally, AGEs may also induce oxidative stress and endothelial dysfunction by binding and activating RAGE which results in a sustained activation of NF-κB and its target genes increasing also the endothelial cell permeability to macromolecules [23]. Elevated levels of AGEs have been noted in the serum of diabetic patients and correlate with progression of diabetic complications such as nephropathy [24, 25]. Treatment of animals with inhibitors of AGE formation, such as aminoguanide, can prevent diabetic microvascular complications [26].

12.3.6 Polyol Pathway

Hyperglycemia may also promote oxidative stress by increasing polyol pathway flux [27]. The enzyme aldose reductase usually presents low affinity to glucose. However, in a high glucose concentration environment, the increased intracellular glucose results an increased activity of aldose reductase and a consequent increase of the glucose reduction to sorbitol which is further oxidized to fructose. This procedure, which consumes NADPH, decreases the reduced glutathione and increases the PKC activation, subsequently increasing the oxidative stress [3]. Inhibition of aldose reductase has been shown to prevent diabetic nephropathy, retinopathy, and neuropathy in animal models [27]. Larger clinical trials in humans, however, have had mixed results, thus raising questions regarding the importance of this mechanism [28, 29].

12.3.7 Hexosamine Pathway

Hyperglycemia, finally, may also shunt excess glucose through the hexosamine pathway [30]. Excessive intracellular glucose results in conversion of fructose-6-phosphate

to glucosamine-6-phosphate and ultimately to *N*-acetylglucosamine, promoting a series of reactions that increase oxidative stress by NADPH depletion, TGF-beta and plasminogen activator inhibitor-1 (PAI-1) gene expression increase, and endothelium nitric oxide synthase (eNOS) activity inhibition [31].

12.3.8 Diabetes and Cellular Adhesion Molecules (CAMs)

Endothelium can be activated by the effect of various factors including oxidative stress, producing inflammation molecules like iCAM and vCAM MCP and inducing the adhesion and accumulation of monocytes at the arterial wall. This is the first step for the development of endothelial dysfunction and atherosclerosis. This process has been proved to be present not only in diabetes but also in the prediabetic state many years before the diagnosis of diabetes [32].

Diabetes has been found to be closely associated with endothelial dysfunction in both resistance and conduit vessels of the peripheral circulation [33–37] as well as in the coronary circulation [38, 39]. The soluble adhesion molecules, E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1, the presence of which is highly associated with vascular inflammation and oxidative stress, are found to be elevated in subjects with T2DM [40–43]. Similarly, increased levels of von Willebrand factor (vWF), a measure of endothelial cell damage and activation, are found in diabetes [40, 42, 43]. Furthermore, microalbuminuria, which has been proved to be an independent predictor of endothelial dysfunction, may possibly indicate a widespread vascular dysfunction in diabetes [40, 44].

The pathogenetic mechanisms underlying the development of endothelial dysfunction in diabetes have not been fully identified. Oxidative stress and the subsequent reduction on NO bioavailability seem to play the most significant role according to the data so far.

12.4   Methods of Assessing Endothelial Function

Prior to the development of macrovascular and microvascular clinical disease, early changes in endothelial function can be measured. These changes reflect alterations in the regulation of vascular tone or reactivity which is influenced by endothelial NO production (endothelium-dependent vasoreactivity) as well as vascular smooth muscle relaxation in response to NO (endothelium-independent vasoreactivity). In endothelium-dependent vasodilation, acetylcholine, shear stress, or hypoxia can activate endothelial cells to release NO. The stimuli of shear stress and hypoxia are utilized in the flow-mediated dilation (FMD) technique to produce endothelium-dependent vasodilation. In contrast, endothelium-independent vasodilation occurs as a result of smooth muscle cell relaxation in direct response to exogenous NO (from NO donors such as nitroglycerin or nitroprusside). Vascular reactivity refers to both endothelium-dependent and endothelium-independent vasodilation.
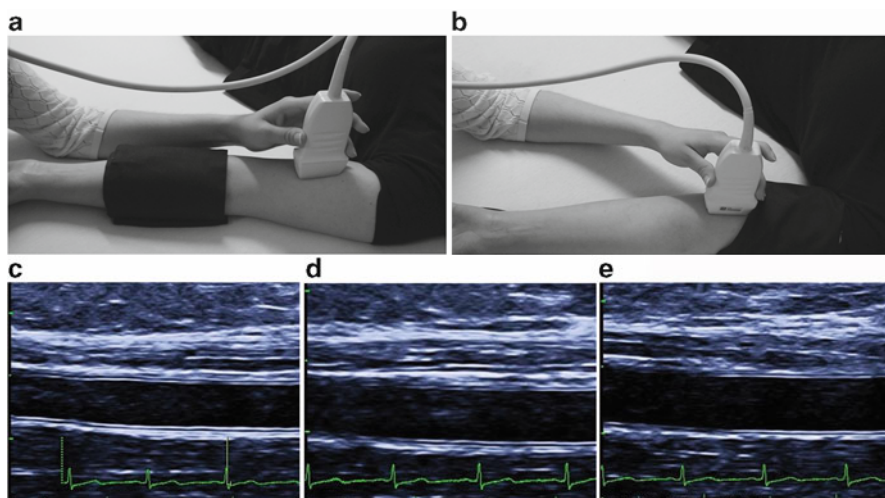
Fig. 12.3 a The assessment of flow-mediated vasodilation in the brachial artery. A 7.0 MHz or greater linear array transducer is used to image the brachial artery above the antecubital fossa in the longitudinal plane. A regular sphygmomanometer is employed to occlude the artery blood flow. The sphygmomanometer can be placed either at the forearm (**a**) or at the upper arm level (**b**). Two-dimensional grayscale scans are taken, one at rest, before the cuff inflation (**c**), and 1 min after the cuff deflation that leads to artery dilation (**d**). The percentage of the post-occlusive artery diameter increase over the baseline represents the FMD

12.4.1   Vascular Reactivity Measurements in the Macrocirculation

Macrovascular disease is most commonly assessed by ultrasound measurements of brachial artery diameter and the common carotid intima–media thickness (IMT). Changes in brachial artery diameter after stimuli measure early functional changes associated with atherosclerosis. Endothelium-dependent vasodilation of the brachial artery can be assessed by intra-arterial infusion of substances that act on the endothelium to release NO, such as acetylcholine, or by FMD. FMD is induced by occluding the brachial artery with a pneumatic tourniquet to the upper limb for a total of 5 min [45]. Tissue hypoxia and pH changes in the area distal to the occlusion, causes reactive vasodilation in the skin and muscle microcirculation immediately after release of the occlusion. This process causes a brief period of high blood flow and increased shear stress in the brachial artery that stimulates the endothelial production of NO and vasodilation that can be measured on high-resolution ultrasound (see Fig. 12.3). Endothelium-independent vasodilatory function of the brachial artery can be assessed by intra-arterial or sublingual administration of NO donors such as nitroglycerin or nitroprusside.

In contrast, common carotid IMT identifies anatomic changes consistent with early atherosclerosis. Carotid artery IMT is an ultrasound measure of the distance between the intima to the outer edge of the media. Increased intima–media thickness occurs early in the process of atherosclerotic plaque formation prior to luminal
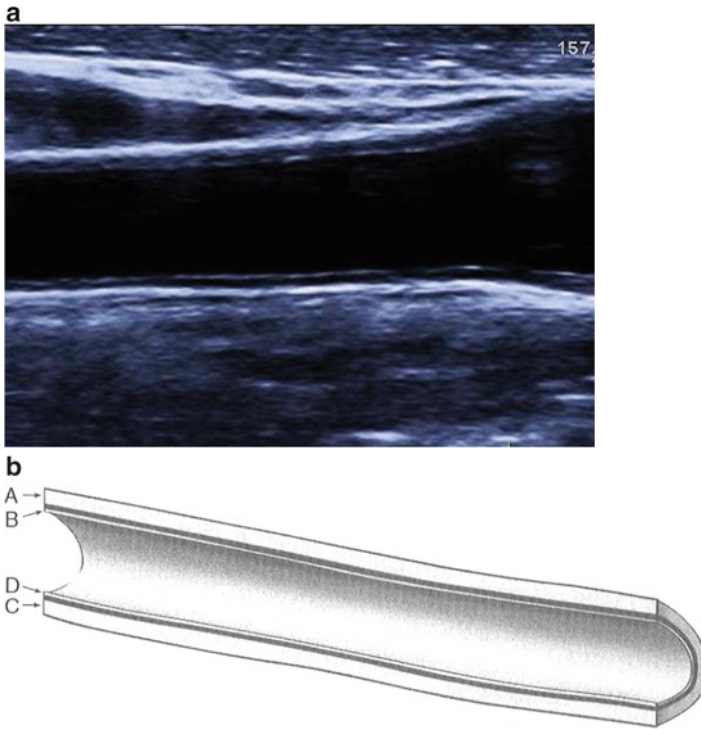
Fig. 12.4 **a** Image of the common carotid artery. A 7.5 MHz linear array transducer and high-resolution ultrasound were used. The carotid bifurcation can be seen on the right of the picture. **b** Simplified diagram of the arterial wall boundaries indicating the adventitia–media (*A*) of the near wall, intima–blood boundaries (*B*) for the near wall, and adventitia–media (*C*) and intima–blood boundaries (*D*) for the far wall

narrowing. IMT is associated with the presence of conventional atherosclerotic risk factors and can predict the development of cardiovascular events [46, 47] (see Fig. 12.4).

12.4.2 Microcirculatory Measurements

Microcirculatory vascular reactivity is most commonly assessed by laser Doppler flowmetry to measure blood flow in the skin. Blood flow is estimated from the combination of number and velocity of moving red cells within arterioles, capillaries, and postcapillary venules. A laser beam is delivered to the skin via a fiber optic light guide, and reflected light is gathered by a second set of photodetectors. Light reflected by moving objects, such as red blood cells, is reflected at a different frequency. The Doppler shifted fraction of the light signal and the mean Doppler frequency shift is calculated to generate a value in mV, which is proportional to the
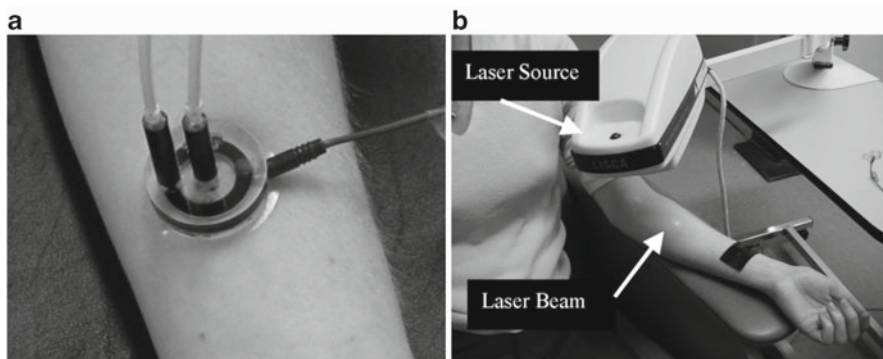
Fig. 12.5   a Measurements of direct and indirect effect of vasoactive substance using single-point laser probes: one probe is used in direct contact with the iontophoresis solution chamber (*colored ring*) and measures the direct response. The center probe measures the indirect response (nerve axon-related effect). A small quantity (<1 mL) of 1 % acetylcholine chloride solution or 1 % sodium nitroprusside solution is placed in the iontophoresis. A constant current of 200 mA is applied for 60 s achieving a dose of 6 mC/cm^{-2} between the iontophoresis chamber and a second non-active electrode placed 10–15 cm proximal to the chamber (black strap around the wrist). This current causes a movement of solution to be iontophoresed toward the skin. **b** Laser Doppler flowmetry: A helium-neon laser beam is emitted from the laser source to sequentially scan the circular hyperemic area (seen surrounding the laser beam) produced by the iontophoresed vasoactive substance to a small area on the volar surface of the forearm

quantity and velocity of red blood cells with the measured superficial skin microcirculation [48].

The microcirculation can be studied without systemic side effects by using iontophoresis and microdialysis techniques that allow for precise, local delivery of vasoactive agents. Iontophoresis uses a small charge to facilitate transcutaneous delivery of charged substances into the skin without trauma or pain (Fig. 12.5). The length of stimulation, strength of current used, and area of delivery determine the number of molecules transported. Endothelium-dependent vasodilation is assessed by delivery of acetylcholine using anodal current given its positive charge, whereas endothelium-independent vasodilation is assessed by the delivery of the anion sodium nitroprusside using cathodal current. Microdialysis can be used to deliver larger, water-soluble vasoactive agents that lack a charge. These techniques allow for noninvasive measurement of abnormal endothelial function prior to the development of overt clinical disease.

12.5   Therapeutic Interventions That Modify Oxidative Stress

Significant amount of evidence has proved that oxidative stress may be very harmful for the vasculature, especially in individuals with diabetes; thus, research has been focused the late years in investigating possible therapeutic ways against

oxidative stress in patients with diabetes including the use of therapeutic agents or lifestyle interventions. Agents, including vitamins E, C, α-lipoic acid, statins, angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin II receptor blockers (ARBs), and thiazolidinediones, as well as lifestyle interventions, have been evaluated in large clinical trials and will be discussed in the following section. Many other agents have been noted to have antioxidant properties, but have not been evaluated in human clinical trials and are beyond the scope of this chapter.

12.5.1   Vitamin E

Vitamin E is a fat-soluble vitamin that has been found to present significant antioxidant properties. Initial studies showed that vitamins E and C supplementation may improve markers of oxidative stress and endothelium-dependent vasodilation in both experimental diabetic models and clinical trials [17, 49–51]. Specifically, vitamin E supplementation has been initially proved to ameliorate endothelial dysfunction in both cholesterol-fed rabbits and streptozotocin-diabetic rats [49, 52]. Furthermore, in human studies, acute administration of vitamin E has generally been shown to improve endothelium-dependent vasodilatation in both type 1 and type 2 diabetes [53]. The Cambridge Heart Antioxidant Study (CHAOS) that employed vitamin E (400–800 IU) reported a significant risk reduction from nonfatal myocardial infarction after an 18-month follow-up period, accompanied, though, by a nonsignificant increase of cardiovascular deaths in the same group [54].

However, the initial enthusiasm regarding the possible vaso-protective role of vitamin E dropped after the results of subsequent animal and human studies. More particular, animal studies reported that the supplementation of vitamin E or/and C may lead to endothelial dysfunction in both diabetic and healthy animals [16, 17] possibly due to pro-oxidant effects of vitamin E on vitamin C in the presence of NO and/or the de novo synthesis of vasoconstrictive prostanoids [16]. In addition, the PPP trial that included diabetic patients revealed no reduction in cardiovascular events or death after vitamin E supplementation. The study showed also an increased risk of adverse events with vitamin E supplementation, raising further concerns about its use [55].

A study from our unit, which included patients with both type 1 and type 2 diabetes treated with high dose of vitamin E (1,800 IU daily) for 12 months, found no improvement in endothelium-dependent or endothelium-independent vasodilation, in both skin microcirculation and brachial artery macrocirculation tests [56]. In addition, vitamin E supplementation had no effect in left ventricular function [56]. Interestingly in the same study, endothelin (a potent vasoconstrictor) was increased in the treatment group after 6 months and normalized by 12 months. In addition, endothelium-independent vasodilation and systolic blood pressure slightly worsened by the end of the 12-month treatment period. Of interest, C-reactive protein (CRP), a marker of inflammation, was decreased in the vitamin E-treated group, concluding that, although vitamin E may present a beneficial anti-inflammatory

effect, reducing CRP does not seem to have a positive effect on cardiovascular function.

The GISSI-Prevenzione trial employed vitamin E (300 mg per day) and *n*-3 polyunsaturated fatty acids (PUFA) or placebo for a median of 3.5 years [57]. Patients treated with vitamin E had no benefit in preventing cardiovascular events. On the contrary, patients with left ventricular dysfunction (ejection fraction < 50 %) presented a 50 % increased risk of developing congestive heart failure [57, 58]. In the Heart Outcomes Prevention Evaluation study (HOPE), conducted in more than 9,500 subjects, it was concluded that vitamin E supplementation had no effect on cardiovascular outcomes in all subgroups including the individuals with diabetes [59].

The HOPE trial was extended to the HOPE—The Ongoing Outcomes (HOPE-TOO) trial reported no difference in cardiovascular outcomes (including myocardial infarction, stroke, and death from cardiovascular causes) between the vitamin E treatment and placebo groups. On the contrary, subjects treated with vitamin E had higher rates of heart failure and heart failure-related hospital admissions. These findings were present in all groups of patients including the patients with diabetes and were persistent through both HOPE and HOPE-TOO [60]. The reason for the association between the increased rate of heart failure and vitamin E supplementation was unclear; however, the authors expressed the hypothesis that a pro-oxidative effect of vitamin E, in certain circumstances, could possibly depress the myocardial function. Finally initial meta-analyses did not show any effect of vitamin E on survival [61, 62].

In a recent meta-analysis of 19 clinical trials, the relationship between vitamin E supplementation and total mortality was examined. The results showed that in 9 of 11 trials testing high-dose vitamin E (≥400 IU/day), the all-cause mortality risk increased, prompting the conclusion that high doses of vitamin E (≥400 IU/day) should be avoided [63]. Finally, both cardiovascular outcomes and atherosclerosis progression by carotid intima–media thickness are not improved by vitamin E in a group of high-risk patients with vascular disease or diabetes in both HOPE study and SECURE trial [60, 64, 65].

Vitamin E has been also tested in the prevention of type 2 diabetes. Two interventional studies that used vitamin E or β-carotene supplementation did not show any positive effect on the delay of the development of type 2 diabetes [66, 67]. In another recent study [63], vitamin C supplementation was added to vitamin E, for testing the hypothesis that vitamin C is necessary for the regeneration of the oxidized vitamin E. However, the analysis of the study revealed neither benefit nor harm, by the supplementation of vitamin C, vitamin E, and β-carotene on the primary prevention of type 2 diabetes.

In conclusion, as the data, so far, indicate, there is currently no compelling evidence to support the use of vitamin E for preventing cardiovascular disease in diabetes. On the contrary, high doses of vitamin E may be associated with serious side effects. Thus, it is reasonable to suggest that such high dose should be avoided.

12.5.2   *Vitamin C*

Vitamin C (or ascorbic acid) is a water-soluble vitamin that, except its numerous biological effects, demonstrates a significant antioxidant role. It prevents oxidation of LDL and, as already mentioned, regenerates oxidized vitamin E. In addition, it stabilizes BH4, an eNOS cofactor, subsequently increasing NO production. Initial studies involving acute increases of the vitamin C plasma levels reported a significant improvement of endothelial function in multiple disease models of oxidative stress. Indeed, in a study by Beckman et al., it was reported that hyperglycemia-induced endothelial dysfunction in healthy volunteers was reversed by vitamin C infusion [68]. In addition, intra-arterial infusion of vitamin C has been reported to improve endothelial function in both type 1 and type 2 diabetic patients [69, 70]. Furthermore, other studies presented an immediate improvement of the endothelial function in subjects with essential hypertension, after vitamin C infusion, whereas other antioxidants such as *N*-acetylcysteine did not have similar effect [71].

In a cohort study of 11,348 adults for 10 years (the first National Health and Nutrition Examination Survey (NHANES I) [72], increased vitamin C intake (approx 300 mg per day) was associated with a 45–25 % risk reduction in all-cause mortality including mortality from cardiovascular events in men and women, respectively. Additionally, in an observational study in 85,118 female nurses followed for 16 years, vitamin C supplementation was associated with a significantly lower risk (28 %) of coronary disease (relative risk of 0.72) after statistical correction for other cardiovascular risk factors [7, 73]. This benefit was noted again by researchers in the EPIC-Norfolk prospective population study [74].

Although initial acute studies have shown significant improvement in endothelial function with vitamin C administration, long-term therapy did not present similar results. In a recent study, the combined therapy with vitamins C and E in types 1 and 2 diabetic patients showed an improvement in endothelial function only in patients with type 1 diabetes [53]. In another study, high oral doses of vitamin C did not improve endothelial function in type 2 diabetic subjects [75].

In summary, according to the current data, there is no compelling evidence to support the use of vitamin C for preventing cardiovascular disease in diabetes. New randomized, placebo-controlled studies addressing the cardiovascular benefits of vitamin C supplementation, independent of other vitamin supplements, need to be conducted to support evidence regarding the possible cardiovascular benefit of vitamin C supplementation in patients with diabetes.

12.5.3   *α-Lipoic Acid*

α-Lipoic acid is a hydrophilic antioxidant allowing it to exert beneficial effects in both aqueous and lipid cellular environments. α-Lipoic acid is reduced to its

conjugate base, dihydrolipoate, which is able to regenerate other antioxidants such as vitamins C and E, as well as reduced glutathione.

A long-term treatment with α-lipoic acid in diabetic animal models demonstrated improvements in metabolic profile including blood glucose, plasma insulin, cholesterol, triglycerides, and lipid peroxidation as well as the microvasculature [76]. In contrast, short-term treatment with α-lipoic acid in rat models of insulin resistance and insulin deficiency did not improve hyperglycemia or fasting triglycerides [77].

In the microcirculation of diabetic rats, α-lipoic acid reduces nitrotyrosine levels and prevents pathologic retinal vessel changes [78]. Additionally, α-lipoic acid has been proved to prevent AGE-dependent depletion of reduced glutathione and ascorbic acid and the subsequent activation of NF-kappa B in endothelial cell culture [79]. Thus, it appears that α-lipoic acid supplementation may reduce oxidative stress improving the metabolic derangements and microvascular function in animal and in vitro models.

Human studies with α-lipoic acid have been mainly focused in the treatment of diabetic polyneuropathy. In initial studies, a 19-day supplementation with α-lipoic acid improved the symptoms of diabetic polyneuropathy [80], while a longer-term therapy (initial IV infusions, then oral treatments for 2 years) objectively improved peripheral nerve function [81].

On the contrary, another trial followed the patients for 7 months, demonstrated no improvements in symptoms in the group with α-lipoic acid [82], while 4 years treatment in the NATHAN 1 trial reported improvements in only some neuropathic deficits and symptoms, but not objective nerve conduction, in patients with mild to moderate distal symmetric neuropathy [83]. In addition, there was a nonsignificant trend of developing serious adverse events in the treatment group indicating that although there may be a possible improvement in neuropathy, the long-term oral therapy may increase the risk of serious adverse events [83].

The effects of α-lipoic acid have been studied also in autonomic diabetic neuropathy and surrogate markers of macrovascular disease in a small number of subjects. A 4-month treatment with α-lipoic acid showed a slight improvement in heart rate variability measurements, without, though, changing the symptoms of autonomic dysfunction [84]. Finally, in a study of 4 weeks of oral α-lipoic acid supplementation, it was reported that there was a significant improvement of the endothelium-dependent vasorelaxation of the brachial artery compared to the placebo group, accompanied by a significant reduction in markers of endothelial activation (interleukin-6 and plasminogen activator-1) [85].

Concluding, the impact of lipoic acid on clinical cardiovascular end points is still unknown. Given also the increased risk of serious adverse events in long-term administration, the use of α-lipoic acid supplements cannot be recommended for patients with diabetes.

12.5.4   Statins

Statins improve the lipid profile by inhibiting the enzyme hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase) reducing the risk of cardiovascular morbidity and mortality [86]. Several studies have proposed that statins may decrease oxidative stress consequently improving the endothelial function.

Indeed, statins decrease NADPH activity, reducing the formation of reactive oxygen species and downregulating the renin–angiotensin system. They also reduce the oxidation of ROS and LDL cholesterol by reducing the activity of the NADPH oxidase in endothelial cells [87–94]. In addition, statins reduce the foam cells formation (responsible for atherosclerotic lesions formation) by decreasing the oxidized LDL uptake by the monocytes [95, 96]. Furthermore, statins downregulate AT1 receptor at the transcriptional level, improving measures of oxidative stress and vascular function [90]. Interestingly, atrovastatin has been proved to demonstrate free radical scavenging abilities through its hydroxymetabolites [97].

By reducing the oxidation of LDL, statins upregulate eNOS expression, consequently improving the vascular function in animal models of type 2 diabetes and hypercholesterolemia [98–100]. Statin-mediated increment in eNOS function was reported to be critical in vascular regeneration and restored myocardial vasorelaxation after experimentally induced myocardial infarction in the mouse model. This benefit was not observed in eNOS−/− mice [101].

It is a common knowledge that treatment with statins reduces the risk of major vascular events [102, 103]. However, its benefit in improving endothelial dysfunction has not been clearly identified so far. Indeed, treatment with statins did not improve vasoreactivity in patients with poorly controlled diabetes [104]. On the other hand, endothelium-dependent vasodilation significantly improved, independently of lipid lowering, in patients with better glycemic and lipid control in both type 1 and type 2 diabetes [105–110].

Statins were also reported to ameliorate postprandial hypertriglyceridemia- and hyperglycemia-induced endothelial dysfunction, reducing also the serum nitrotyrosine levels in type 2 diabetes suggesting that its short-term, lipid-independent vascular benefits are secondary to decreased oxidative and nitrosative stress [111].

In conclusion it seems that statins improve endothelial function prior to reductions in LDL unless there is overwhelming oxidative stress related to type 2 diabetes. The reduced response to statins may also be related to the increased levels of asymmetric dimethylarginine (ADMA), a competitive inhibitor of eNOS. Indeed, a recent study has been shown that a 3-week treatment with statin failed to improve vasoreactivity in patients with increased levels of ADMA [112].

12.5.5   ACE Inhibitors and ARBs

ACE inhibitors and ARBs exert their clinical effects by decreasing the binding of angiotensin II to the AT1 receptor, by decreasing levels of angiotensin II and by inhibiting the interaction of angiotensin II to the AT1 receptor, respectively. ACE inhibitors and ARBs have been proposed to improve endothelium-dependent vaso-relaxation by decreasing superoxide production and increasing NO bioavailability [113–116]. These actions are mainly derived by the inhibition of angiotensin II which opposes many of the actions of NO. In particular angiotensin II causes vaso-constriction, altered vascular smooth muscle function, increased inflammation via NF-κB, and hypercoagulability by increased formation of PAI-1. In addition angiotensin II induces vascular superoxide production by uncoupling eNOS upon loss of dihydrofolate reductase (DHFR), which is a BH4 salvage enzyme [113].

Recent studies have shown that ACE inhibitors and ARBs improve vascular function and cardiovascular outcomes in type 2 diabetes. Both agents unequivocally improve endothelial function in patients with type 2 diabetes [117–120]. Valsartan therapy improved resting forearm skin blood flow and resting brachial artery diameter after a 12-week treatment in patients with type 2 diabetes. However, their impact on endothelial function in patients with type 1 diabetes is less clear [121–124].

HOPE and LIFE studies have shown that ACE inhibitors and ARBs improve cardiovascular as well as all-cause mortality outcomes in patients with diabetes. The benefit seemed to be higher in patients with diabetes than in nondiabetics [125, 126]. The presence of native LDL increases AT1 receptor expression at least two-fold in a sustained manner for 24 h by stabilization of posttranscriptional mRNA [127]. Furthermore, angiotensin II is binding with the AT1 receptor, upregulating the endothelial oxidized LDL receptor (LOX-1) in endothelial cells. This upregulation of LOX-1 receptor is prevented by ARBs and ACE inhibitors, limiting the potential diffusion of oxidized LDL from the blood into the vessel wall, thus reducing the possibility of plaque formation [128]. Given that statins decrease the levels of native LDL which is responsible for the at least twofold increase of theAT1 receptor expression [127], a coadministration of ACE inhibitors/ARBs with a statin may produce a synergic decrease in oxidative stress and vasoconstriction, as well as a decreased uptake of oxidized LDL and improved endothelial function [128].

12.5.6   Thiazolidinediones

Thiazolidinediones is an antidiabetic agent category also known as PPAR-gamma agonists that bind nuclear PPAR-gamma receptors in adipocytes which function as transcription factors for genes important in adipocyte differentiation, lipid metabolism, and insulin sensitivity. PPAR-gamma receptors are also expressed in cells

involved in the process of atherosclerosis including endothelial cells, vascular smooth muscle cells, monocytes/macrophages, and T cells.

Increased amount of evidence supports that apart from enhancing glycemic control, thiazolidinediones improve surrogate measures of vascular disease. Indeed, thiazolidinediones have been proved to improve endothelium-dependent vasodilation as well as measurements of carotid IMT in patients with diabetes [129–133]. In addition, both rosiglitazone and pioglitazone have been reported to increase the regenerative capacity of endothelial progenitor cells in individuals with diabetes [134, 135]. This improvement in vascular function has been found to be associated with reduced NADPH oxidase activity, decreased LDL oxidation, and reduction in vascular inflammation [133, 136].

However, although thiazolidinediones proved to have a significant improvement in oxidative stress and vascular function, there are serious concerns that one of them, rosiglitazone, worsens clinical cardiovascular outcomes. Thus, rosiglitazone has been reported to be associated with increased risk of congestive heart failure, as well as myocardial infarction [137, 138]. Thus, the current consensus is that rosiglitazone may have detrimental effects in patients with previous heart disease and diabetes, and its use cannot be recommended in these patients. Unlike rosiglitazone, larger clinical trials of pioglitazone in high-risk patients with type 2 diabetes and prior MI have demonstrated an improvement in rates of myocardial infarction, but increased edema formation and heart failure remain concerns [139, 140].

12.5.7 Antioxidants and Mediterranean Diet

A study in 34.486 postmenopausal women reported that increased intake of vitamin E through diet was associated with decreased risk of death from coronary artery disease, while vitamin E supplementation did not affect the risk of death from cardiovascular disease [141]. This study exemplifies the paradox noted in several large-scale clinical and epidemiologic studies that diet but not vitamin supplementation seems to improve cardiovascular outcomes.

A great amount of evidence the last few decades has shown that this type of diet has impressive effects in reducing cardiovascular risk [142]. In addition, low adherence to Mediterranean diet has been proven to increase the risk for metabolic syndrome [143]. Olive oil, a main component of the diet, has significant antioxidant properties and is considered one of the primary factors that contribute to these beneficial effects [144].

In a recent study involving subjects with metabolic syndrome, the Mediterranean diet presented anti-inflammatory and antithrombotic properties improving the endothelial function and insulin sensitivity [145]. Therefore, the current consensus is that a diet that encompasses the main components of the Mediterranean diet can greatly reduce cardiovascular risk in diabetic patients.

12.5.8   *Green Tea and Coffee*

Coffee, a common beverage in western countries, has been reported to possibly have antioxidant effects through minerals (such as magnesium), phytochemicals (in caffeine), and antioxidants. Several studies have shown that coffee decreases the risk of type 2 diabetes although there have been reports that caffeine itself may impair glucose metabolism in type 2 diabetics [146, 147]. However, it is not clear how coffee decreases the risk of type 2 diabetes especially since caffeine (and its phytochemicals) does not seem to play a significant role.

Green tea, another widely consumed beverage, also seems to have protective effects as its polyphenols have antioxidant properties. A study that followed Japanese subjects for 11 years reported that the consumption of green tea was associated with a decrease in all-cause mortality as well as mortality from cardiovascular disease [148]. In another Japanese study, consumption of green tea, coffee, and total caffeine was associated with a decreased risk for type 2 diabetes in a 5-year follow-up period [149].

12.5.9   *Exercise*

Exercise or physical activity is recommended for the prevention or the initial therapy of type 2 DM and ischemic heart disease [150]. Many studies have also shown that exercise can reduce blood glucose, apolipoprotein B-rich lipoproteins, oxidative stress, or inflammatory cytokines and elevate HDL cholesterol, insulin sensitivity, antioxidant capacity, or mitochondrial function [151–154].

Other studies indicated also that exercise may inhibit the expression of NOX in human arteries [155], possibly providing a novel mechanism for the beneficial effect of exercise and may help diabetic patients to prevent cardiovascular disease. NOX is a transmembrane enzyme located in intracellular organelles and functions in the generation of superoxide.

12.6   Conclusions

Although there was initially much enthusiasm for the antioxidant therapy in diabetes, especially in the form of supplemental vitamins, clinical trials have not shown evidence of decreased risk of cardiovascular outcomes. Vitamins E and C supplementation, therefore, cannot be currently recommended. On the other hand, diet rich in antioxidants, especially Mediterranean diet, can provide considerable reduction in cardiovascular risk and may be of particular benefit to subjects with diabetes. Finally, statins, ACE inhibitors, and ARBs, alone or in combination, seem to present antioxidative properties. However, its use cannot be recommended, as their

indications so far are limited to hypercholesterolemia and hypertension, respectively. Further research is needed in order to be determined whether they could be possibly used for their antioxidant vascular protective properties.

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