Oxidative Stress in the Pathogenesis of Diabetic Neuropathy

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Oxidative stress results from a cell or tissue failing to detoxify the free radicals that are produced during metabolic activity. Diabetes is characterized by chronic hyperglycemia that produces dysregulation of cellular metabolism. This review explores the concept that diabetes overloads glucose metabolic pathways, resulting in excess free radical production and oxidative stress. Evidence is presented to support the idea that both chronic and acute hyperglycemia cause oxidative stress in the peripheral nervous system that can promote the development of diabetic neuropathy. Proteins that are damaged by oxidative stress have decreased biological activity leading to loss of energy metabolism, cell signaling, transport, and, ultimately, to cell death. Examination of the data from animal and cell culture models of diabetes, as well as clinical trials of antioxidants, strongly implicates hyperglycemia-induced oxidative stress in diabetic neuropathy. We conclude that striving for superior antioxidative therapies remains essential for the prevention of neuropathy in diabetic patients. (Endocrine Reviews 25: 612–628, 2004)
type 2, have neuropathy (6, 7). Around 10% of these cases of neuropathy are associated with abnormal sensations and pain (8). The incidence of neuropathy increases with duration of diabetes and is accelerated by poor control (9).

The majority of work to date has focused upon the peripheral nervous system, and so, unless stated otherwise, comments in this review refer to the peripheral nervous system. One should note, however, that deficits in the central nervous system are recognized as a feature of diabetes. Generally, spinal cord lesions are considered a rare event in diabetes, although a recent study demonstrated overall slowing of spinal cord potentials in a population of patients with type 2 diabetes at 5–10 yr after disease onset (10). Studies in diabetic rats suggest that the same signal transduction pathways that are implicated in peripheral neuropathy in the dorsal root ganglia are also affected in the brain (11). Again, attempts to unify the mechanisms that ultimately produce neuronal degeneration point to at least a component of oxidative stress. Mild cognitive dysfunction is not uncommon in adults with type 1 diabetes through a mechanism that appears to be linked to the development of vascular complications (12). Similarly, in patients that have Alzheimer’s disease, development of type 2 diabetes with vascular complications accelerates the brain deposition of amyloid protein and neurofibrillar tangles (13).

The mechanisms underlying oxidative stress in chronic hyperglycemia and the development of neuropathy have been examined in animal models (14). This oxidative stress is associated with the development of apoptosis in neurons and supporting glial cells and so could be the unifying mechanism that leads to nervous system damage in diabetes (15, 16). This review explores the evidence for oxidative stress as a significant mediator in the development of diabetic neuropathy as well as the potential for prevention of complications through rigorous antioxidant therapy.

Although this review is mainly focused upon the loss of neuronal function and survival as a cause of diabetic neuropathy, it is important to consider other mechanisms that contribute to the disorder. Neurons not only are lost in diabetes, but their ability to regenerate is also impaired, particularly the small-caliber nerve fibers (17). In patients with diabetic neuropathy, both degeneration and regeneration are present simultaneously, suggesting that the disorder is highly dynamic (7). Over time, the balance between degeneration and regeneration shifts toward more degeneration, and the aim of therapeutic regimens should be to restore the balance on the side of regeneration. The inability to regenerate nerve fibers is related to the degree of neuropathy, suggesting that therapeutic interventions to improve regeneration will be more effective at early stages of disease (17). The mechanisms leading to loss of regeneration may include impaired insulin action (18), loss of growth factor systems (19), and decrease in specific isoforms of protein kinase C (20). Schwann cells are important in the regenerative process, and these also can be impaired in diabetes through hyperglycemia, hypoxia, and oxidative stress (reviewed in Ref. 21). Understanding and the ability to intervene in oxidative stress, therefore, may both prevent neuron degeneration and promote regeneration (7).

II. The Chemistry of Oxidative Stress

Several free radical species are normally produced in the body to perform specific functions. Superoxide ($O_2^{-}$), hydrogen peroxide ($H_2O_2$), and nitric oxide (NO) are three free radical reactive oxygen species (ROS) that are essential for normal physiology, but are also believed to accelerate the process of aging and to mediate cellular degeneration in disease states. These agents together produce highly active singlet oxygen, hydroxyl radicals, and peroxynitrite that can attack proteins, lipids, and DNA. Figure 1 illustrates the different forms of ROS as well as showing examples of their formation and removal within cells. These reactions are described in more detail below.

![Fig. 1. The charged states of oxygen and the formation and detoxification of oxygen radicals in cells. On the left, the various oxidative states of the molecule are illustrated to assist the reader in understanding the terminology of free radicals. As molecular oxygen participates in biochemical reactions in the cell, electrons are shuttled between molecules, and highly reactive intermediates are produced and then removed through the activities of specific enzymes. These reactions are summarized in the schematic on the right.](https://academic.oup.com/edrv/article-abstract/25/4/612/2355264)
A. O$_2^{\cdot-}$

O$_2^{\cdot-}$ is generated by the mitochondrial electron transfer chain during the oxidation of reduced nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide (NAD$^+$) and also as a by-product of many enzymes that act as oxidases. Approximately 4% of electrons that enter the respiratory chain lead to the formation of O$_2^{\cdot-}$ (22). The beneficial effects of O$_2^{\cdot-}$ include regulation of vascular function, cell division (23, 24), inflammation (25), apoptosis (26), and bactericidal activity of neutrophils (27). Decreased levels of O$_2^{\cdot-}$ can lead to an increased susceptibility to bacterial infections, as illustrated in Down's syndrome patients with elevated cytoplasmic superoxide dismutase (SOD)1 (28). Thus, cellular levels of O$_2^{\cdot-}$ are under tight regulation. Excess O$_2^{\cdot-}$ is removed through the activity of a family of SOD enzymes that convert O$_2^{\cdot-}$ to H$_2$O$_2$ and oxygen.

O$_2^{\cdot-}$ overproduction occurs in complication-prone tissues when cellular metabolism is perturbed by excess glucose. ATP synthase is inhibited, and electron transfer slows. This can cause overproduction of O$_2^{\cdot-}$ in two ways. First, the half-life of highly reactive quinone intermediates is prolonged, increasing the release of electrons to combine with molecular oxygen and form O$_2^{\cdot-}$. Second, when electron transfer no longer can regenerate NAD$^+$, the enzyme NADH oxidase is activated and generates O$_2^{\cdot-}$ as a byproduct (Fig. 2).

B. Hydrogen peroxide (H$_2$O$_2$)

H$_2$O$_2$ is produced after the spontaneous or SOD-catalyzed dismutation of O$_2^{\cdot-}$ as well as many other enzymatic reactions. Unlike O$_2^{\cdot-}$, which remains at the site of production, H$_2$O$_2$ can diffuse across membranes and through the cytosol (29). This ROS is another component of leukocyte-mediated defense against bacteria. Because H$_2$O$_2$ is a powerful ox-
dizing agent, cells express abundant catalase, glutathione (GSH), and thioredoxin (Trx) that convert H₂O₂ to water. When H₂O₂ reacts with free Fe²⁺, the iron is oxidized and hydroxyl radicals are produced. There are many severe consequences of hydroxyl radical production, including loss of vasodilation that can lead to endothelial injury and tissue hypoxia (30).

C. Nitric oxide (NO)

NO is generated through the activity of a cytosolic enzyme known as NO synthase (NOS). There are both constitutively expressed, calcium-dependent isoforms of NOS and an inducible isoform that is associated with inflammation and cell activation (31, 32). NO plays a major role in regulating vascular tone by activating soluble guanylate cyclases that regulate ion channels. In addition, NO modulates cellular respiration through direct inhibition of cytochrome oxidase by competitively occupying the oxygen-binding site (33). The inducible form of NOS is increased in the arteries of diabetic rats (34). Damaged neurons recover more slowly in the presence of NO, and conversely, NOS inhibitors promote neuronal recovery from injury (35). NO is also believed to act as a neurotransmitter (36). The dual role of NO as both beneficial and detrimental is illustrated in stroke models. Under ischemic insult, endothelial NO produces vasodilation that can improve blood flow, but neuronal NO is produced downstream of calcium dysregulation and can prevent energy generation in the mitochondria (37). More importantly, NO acts as an antioxidant in certain environments and prevents lipid peroxidation (38). However, when O₂⁻ increases, NO reacts with the O₂⁻ to form peroxynitrite and becomes a prooxidant.

III. Cellular Injury through Excess ROS Production

The production of ROS is under tight control in healthy cells, but overproduction during metabolic dysfunction leads to cellular injury. Although both O₂⁻ and NO are relatively inert, when they combine they form the highly reactive peroxynitrite that attacks and inhibits proteins and lipids. In addition, both O₂⁻ and NO can attack iron-sulfur centers of enzymes and other proteins to release iron atoms and consequently inhibit enzyme/protein activities. There are many important proteins that are exquisitely sensitive to this type of inhibition including complexes I–III of the electron transfer chain, aconitase of the trichloroacetic acid cycle, and biotin synthase (39, 40).

The formation of lipid, protein, and nucleic acid adducts involves a complex chain reaction using a range of biological substrates that contain reactive methylene groups. Intermediates in the chain reaction can have extremely high oxidative ability and so cellular damage can be extensive. The chemistry of these reactions has been reviewed previously (41, 42). Lipids present in plasma, mitochondrial, and endoplasmic reticulum membranes are major targets of ROS attack and peroxidation. End products of lipid peroxidation, known as lipid peroxides, can be toxic to a cell and require removal by GSH as described below. Similarly, proteins and nucleic acids can be subject to peroxidation and nitrosylation. Although these end products are not usually directly toxic to the cell, accumulation of inactive proteins can overload the ability of a cell to recycle them, and damage of DNA is known to activate the mechanisms of apoptosis. In addition, accumulation of modified proteins decreases their function, leading to severe loss of normal activity. Axonal transport can be slowed, leading to decreased delivery of growth factors and intermediates from the synapse to the cell body and resulting in induction of apoptosis (43). Oxidative modification of transcription factors not only leads to decreased expression of many proteins such as apoptosis inhibitory factor, complex I, and Bcl-2, but also results in increased expression of stress proteins that may be proapoptotic, including cyclooxygenase 2, poly-ADP ribose polymerase, and Jun kinase (JNK) (44–47).

Production of ROS in all cells not only results in deleterious events but also can play a role in differentiation and development. Redox status can have profound effects on gene expression, so that oxidative stress increases growth factors, stress response elements, and apoptosis pathways (48). In contrast, certain proteins including cytokines, cytochrome c oxidase, and enzymes involved in glucose respiration are repressed by oxidative stress signaling (49). Understanding of gene regulation by reactive oxygen intermediates is rapidly expanding. Once the mechanisms are more fully understood, the ability of a cell to respond to stress by changing gene expression may provide an important therapeutic target.

The most significant consequence of oxidative stress in dividing cells may be DNA modifications that produce genomic instability and mutations (50). Nondividing neurons may suffer less from oxidative damage of DNA. Yet, mitochondrial DNA is particularly sensitive to oxidative damage (51), which would impair energy regulation and thus would be critically important in high energy-requiring neurons. Oxidative stress-mediated neuronal degeneration is implicated in several types of neurodegenerative disease (52–54). In nondividing cells like neurons, damage to proteins and lipids may be more injurious than DNA damage, because this may render proteins unable to perform axonal transport and signaling (43). For example, synaptosomal membranes as well as cytosolic proteins become oxidized, and these changes can be correlated to alterations in brain function (55). Loss of function in neurons rapidly promotes necrotic or apoptotic mechanisms (53, 56).

IV. Cellular Antioxidant Defense

Antioxidants are defined as any compound that can donate at least one hydrogen atom to a free radical, resulting in the termination of radical chain reactions. An alternative type of antioxidant is defined by its ability to prevent the initiation of a free radical chain reaction rather than to terminate them. This latter type of antioxidant is usually dependent upon the ability to bind metal ions and includes ceruloplasmin, transferrin, and albumin (57). Cells must maintain the levels of antioxidants, often defined as antioxidant potential, through dietary uptake or de novo synthesis. Excess production of free radicals can deplete the intracellular antioxidants, resulting
in oxidative stress. Even brief, acute hyperglycemic episodes such as an oral glucose tolerance test or a meal can decrease the antioxidant capacity of plasma in both normal and diabetic subjects and increase oxidative stress in diabetic patients (58, 59). As a type 2 diabetic patient ages, increased basal levels of free radical production and decreased antioxidants are even further exacerbated by elevated plasma glucose (60). Analysis of individual vitamin and enzyme components of the antioxidant system in man reveals significant changes in diabetes (61). Vitamins A and E and catalase are decreased in both type 1 and 2 patients compared with controls. Whereas GSH-metabolizing enzymes are decreased in type 1 but not type 2 patients, SOD activity is lower in type 2 but not type 1. These changes do not correlate with observed complications (61).

A. Dietary antioxidants

Water-soluble vitamin C and fat-soluble vitamin E together make up an antioxidant system for mammalian cells. Vitamin C, or ascorbic acid, is considered the most important antioxidant in plasma and forms the first line of defense against plasma lipid peroxidation (62). Vitamin E is the generic description for all tocopherol and tocotrienol derivatives that comprise the major lipophilic antioxidant of exogenous origin in tissues (63). Comparison of the isoforms of tocopherol including α-, α-tocopherol, mixed tocopherols (containing R,R,R-α, R,R,R-β, R,R,R-γ, and R,R,R-δ-tocopherol, n-tocopherols, and tocopherol exipients) and Ronoxan MAP demonstrates no significant difference in antioxidant capacity although the antioxidant activity of α-tocopherol acetate is completely lost (63). Different properties of the isoforms have been identified, however. Tocotrienol, but not tocopherol, inhibits angiogenesis of tumors and is recommended as a dietary supplement to decrease tumorogenesis (64). Tocotrienol directly regulates the activity of 12-lipoxygenase that may mediate neuronal excitotoxicity, and so this compound possesses an additional neuroprotective capacity distinct from antioxidant action (65).

Interestingly, for the purposes of considering antioxidant therapy against oxidative stress, antioxidants may act synergistically. In particular, ascorbate regenerates α-tocopherol from the tocopherol radical to reduce the toxicity of tocopherol intermediates (66). Dietary supply of these vitamins leads to a rapid increase in concentration in plasma and cells and a measurable increase in antioxidant potential (67, 68). The use of vitamin supplements for prevention of diabetic neuropathy will be discussed later in Section IX.

B. GSH

GSH is by far the most important antioxidant in most mammalian cells. This ubiquitous tripeptide, γ-Glu-Cys-Gly, performs many cellular functions. In particular, the thiol-containing moiety is a potent reducing agent. GSH is maintained at a concentration of 0.2–10 mM in all mammalian cells (69). Many cells can synthesize GSH de novo by γ-glutamylcysteine synthetase first forming a γ-peptide bond between one cysteine and one glutamate residue. Next, glycine is added by GSH synthetase. Neurons do not contain the γ-glutamylcysteine synthetase enzyme and so require the dipeptide to be secreted from glial cells (70, 71).

The most significant role of GSH is as a water-soluble antioxidant. Toxic lipid peroxides combine with two molecules of GSH under the control of GSH peroxidase to form an inert lipid hydroxyl group, GSH disulfide (GSSG), and water. In addition, GSH is involved in amino acid transport, deoxyribonucleotide synthesis, maintenance of functionally important protein thiol groups in reduced form, and conjugation with toxic compounds such as xenobiotics under the control of glutathione-S-transferase to promote their elimination from the cell (72, 73). After participation in redox reactions, GSH is regenerated from GSSG by the enzyme GSSG reductase using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor.

Depletion of GSH in the cell renders it susceptible to oxidative injury (74). The agent 3-hydroxy-4-pentenoate specifically depletes mitochondrial GSH and enhances cell death induced by prooxidants such as tert-butyl hydroperoxide (75). In contrast, loading the cell, and particularly the mitochondria, with GSH can prevent neuronal apoptosis produced by ischemia (76) and excitotoxicity (77). Overexpression of glutathione-5-transferase in neuroblastoma cells increases their resistance to oxidative stress (78).

C. Trx

Another small protein antioxidant within cells that can maintain redox homeostasis is Trx. Similar to GSH, Trx is regenerated by a NADPH-dependent reductase. In contrast to the critical role GSH plays in chemical detoxification, Trx is essential for maintenance of normal protein structure. Although there is some redundancy between these small molecules, Trx has a more significant role in regulation of catalytic activity, protein-protein interactions, trafficking, activation, degradation, and transcription factors binding to DNA (79). The concentrations of Trx are maintained in the micromolar range in mammalian cells. Mitochondria express distinct isoforms of Trx reductase and Trx synthetase; overexpression of these isoforms confers resistance to prooxidant stress (79).

Additional enzymes that catalyze the reduction of hydrogen peroxide or alkyl peroxide to water, or the corresponding alcohol, are the peroxiredoxins. Detailed analysis of their sequences indicates that these enzymes possess a Trx-like fold and consequently are homologs of both Trx peroxidase and GSH peroxidase (80). There are at least six isoforms of the peroxiredoxin family that are differentially expressed in mammalian tissues (81). These enzymes are rapidly induced after oxidative stress and form part of the early stress response (82).

D. Antioxidant enzymes

In addition to the enzymes that synthesize and maintain antioxidant molecules such as GSH, specific antioxidant enzymes are expressed that detoxify free radical entities in cells, tissues, and extracellular fluids. One of the most ubiquitous of these is SOD. The three major isoforms of SOD are: cy-
Glucose oxidation to ROS in diabetes.

A. Advanced glycosylation end product (AGE)-mediated ROS formation

Glucose at elevated concentrations undergoes nonenzymatic reactions with primary amino groups of proteins to form glycated residues known as Amadori products. After a series of dehydration and fragmentation reactions, the Amadori products are converted to stable covalent adducts known as AGEs (95). These reactions are catalyzed by transition metal ions. Diminished ability in diabetes to bind and sequester transition metals so they are not free to act as catalysts may exacerbate AGE formation. Treatment of diabetic rats with a transition metal chelator can prevent diabetes-induced nerve conduction deficits (96). Glycation of proteins is directly related to the concentration of glucose and therefore is produced through poor glycemic control. A number of common foods contain AGEs that can increase the AGE-induced stress in diabetic patients and promote nephropathy (97).

AGEs bind to a cell surface receptor known as receptor for AGE (RAGE), a multiligand member of the Ig superfamily. This binding initiates a cascade of signal transduction events involving p44/p42 MAPKs, nuclear factor-kB, p21Ras, and other intermediates (98, 99). Interaction of AGEs with RAGE induces the production of ROS through a mechanism that involves localization of prooxidant molecules at the cell surface (100) and a key role for activated NADPH oxidase (101). In neuronal cell lines, application of AGEs depletes GSH, but this is prevented in the presence of antioxidants (102). Antioxidants or antibodies against RAGE prevent both oxidative stress and the downstream signaling pathways that can be activated by ligation of RAGE. AGE-mediated ROS production is particularly implicated in blood vessel endothelial activation and diabetic vascular complications (103, 104).

B. The polyol pathway

The enzyme aldose reductase converts toxic aldehydes to inactive alcohols (2). Glucose is a poor substrate for aldose reductase, but at high concentrations this enzyme converts glucose to sorbitol, initiating the polyol pathway of glucose conversion to fructose. Similar to GSH reductase, the enzyme aldose reductase is dependent upon NADPH as a cofactor. Therefore, excessive activation of the polyol pathway depletes cytosolic NADPH and subsequently depletes GSH, leaving the cell vulnerable to free radicals produced during normal cellular functions such as electron transfer. In addition, accumulation of sorbitol produces a cellular osmotic stress that also generates oxidative stress (105). This pathway has been a target for therapies against diabetes complications including nephropathy (106). Recent human genetic and biochemical data link polymorphisms of the aldose reductase gene to increased risk of diabetic complications, with the principal allele associated with increased disease risk causing a 2- to 3-fold increase in aldose reductase gene expression (106).

C. Protein kinase C (PKC) activation

The activation of the PKC pathway in hyperglycemia is included here for completeness, although the contribution to diabetic neuropathy is likely to occur through its effects in

V. Production of ROS in Diabetes

One unifying mechanism of nervous system injury in diabetes lies in the ability of both metabolic and vascular insults to increase cellular oxidative stress and impair the function of mitochondria (16, 86). Recent studies have supported this hypothesis, including in vivo and in vitro measurement of oxidative stress in sensory neurons as well as neuronal protection by antioxidants. In vitro, application of 10–20 mM glucose to dorsal root ganglia neurons leads to production of O$_2^{-}$ and H$_2$O$_2$ that leads to lipid oxidation and neuronal death. This glucose-induced death is prevented by IGF-I, in part through decreased ROS production (15). Further evidence comes from feeding mice with a high-glucose diet. In this case, the mice experience hyperglycemia that leads to free radical production and oxidative stress (87).

There is a close correlation between oxidative stress in diabetes and the development of complications. In type 1 diabetic patients, oxidative stress is evident within a few years of diagnosis before the onset of complications. As the disease progresses, antioxidant potential decreases, and plasma lipid peroxidation products increase depending upon the level of glycemic control (88). Type 2 diabetic patients have increased lipid peroxidation compared with age-matched control subjects, as well as decreased plasma GSH and GSH-metabolizing enzymes and antioxidant potential, all of which relate directly to the rate of development of complications (89–91). Similarly, oxidative stress is linked to preclinical features of disease, such as vascular endothelial activation that can lead to atherosclerosis (92). The early increase of oxidative stress in diabetes is more pronounced in women and may account for increased cardiovascular disease in female patients (93).

Figure 2 outlines the potential mechanisms underlying the production of excess ROS in the nervous system by high glucose. Each of the potential pathways is discussed below.

A recent review on vascular cell biochemistry outlines similar pathways as instrumental in mediating glucose-mediated endothelial damage (94).
vascular blood flow and microvascular disease rather than directly in neuronal cells. PKC has several unique structural features that facilitate its regulation according to redox status. Prooxidants react with the regulatory domain to stimulate PKC activity, but antioxidants react with the catalytic domain of PKC and inhibit its activity (107). Activity of PKC is increased in the retina, kidney, and microvasculature of diabetic rats, but there is no evidence for altered activity of any of the PKC isoforms in the peripheral neurons (108, 109). This suggests that the lipolytic pathway and production of diacylglycerol are the main causes of PKC activation in non-neuronal cell types (110). Once activated, PKC activates the MAPKs that phosphorylate transcription factors and thus alter the balance of gene expression (111). Specifically, it is the stress genes such as heat shock proteins and c-Jun kinases that increase after PKC activation and can lead to apoptosis or vascular atherosclerosis. A role for PKC in inducing neuronal degeneration possibly at the level of the endothelial cell is implicated by three studies. Inhibition of PKCβ reduces oxidative stress and normalizes blood flow and nerve conduction deficits in diabetic rats (110, 112). High glucose causes nuclear factor-κB activation in endothelial cells, leading to ROS formation, and cellular activation, an effect that is prevented in the presence of a PKC inhibitor (113).

D. MAPK activities

All three classes of the MAPK, ERK1/2, JNK, and p38, are activated in the dorsal root ganglia of diabetic rats. The significance of these signaling pathways in the development of diabetic neuropathy is not clear. Treatment with antioxidants decreases the activation of ERKs, but increases JNK, which may suggest that the ERKs are injurious and JNK is protective (111). Yet, persistent activation of JNK is normally associated with injury (114). Peroxynitrite-induced oxidative stress activates p38 in neuroblastoma cells, and this leads to growth arrest and apoptosis (115). At present, the studies of MAPK involvement in neuropathy are mainly descriptive, and mechanistic studies are required to clarify the role of these signaling pathways.

E. ROS formation at the mitochondria

As mentioned earlier, O$_2^-$ is a normal by-product of metabolic processes; therefore, when glycolysis, electron transfer, and oxidative phosphorylation are chronically or acutely overloaded, excess O$_2^-$ produces oxidative stress. The importance of these mechanisms in producing hyperglycemic neuronal degeneration is highlighted in recent studies of uncoupling proteins. 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, these protons that were pumped into the intermembrane space through electron transfer bypass oxidative phosphorylation, and these two processes are said to be uncoupled. Activity of uncoupling proteins, therefore, decreases the inner mitochondrial membrane potential and can relieve the stress of excess NADH entering the electron transfer chain (116). Overexpression of uncoupling proteins in cultured dorsal root ganglia neurons significantly decreases both basal and hyperglycemia-induced ROS formation and prevents glucose-induced neuronal death (117). Interestingly, O$_2^-$ can mediate the activation of mitochondrial uncoupling proteins in skeletal muscle cells, demonstrating that this may be an innate mechanism for protection against excess activity-induced O$_2^-$ in muscle cell mitochondria (118).

Oxidative stress in the mitochondria critically alters energy regulation and survival through at least three mechanisms. First, physiological levels of NO reversibly compete with molecular oxygen for binding to cytochrome c oxidase, producing reversible inhibition and acting as a regulatory switch for electron transfer. In contrast, in the presence of excess O$_2^-$, NO is converted to ONOO$^-$, which competes with molecular oxygen for irreversible binding to cytochrome c oxidase. Thus, ONOO$^-$ profoundly affects mitochondrial function and inhibits ATP synthesis (33, 119). Second, mitochondrial oxidative stress through excess O$_2^-$ and ONOO$^-$ production inhibits the import of essential proteins to the mitochondria that are in turn degraded in the cytosol (120). Finally, oxidative damage of existing inner membrane proteins induces membrane permeability transition, a permeabilization of the mitochondrial inner membrane that precedes cytochrome c release and apoptosis (121).

The mitochondrial mechanisms of ROS production and neuronal injury are activated within 1-2 h of hyperglycemic insult and so may be the greatest contributor to diabetic neuropathy (A. M. Vincent, L. L. McLean, C. Backus, and E. L. Feldman, unpublished data). Many diabetic patients with good overall glucose control still experience neuropathy, so brief postprandial periods of hyperglycemia that produce ROS but no significant AGE formation or polyol pathway activation may be sufficient to injure neurons. Supporting evidence for this conclusion is obtained in patients with impaired glucose tolerance. Many patients with impaired glucose tolerance have significant peripheral neuropathy, and in some cases painful neuropathy is the presenting symptom (17). We would infer that neuropathy in these cases is most likely attributable to brief postprandial hyperglycemic episodes. This suggests that there is a certain higher glucose level that could be at least partially, block the development of diabetic neuropathy.

VI. Neuronal Response to Oxidative Stress

The antioxidant defense system of a cell is clearly not static but can respond to environmental changes. In culture, vascular endothelial cells up-regulate SOD, GSH peroxidase, and catalase through increased gene expression over a period of 3–10 d (122). Because glucose enters neurons via facilitated concentration-dependent transport, neurons are likely more susceptible to glucose flux and subsequent increased oxidative stress. However, a study in 3- and 12-month streptozotocin-treated rats with nerve conduction deficits did not show changes in antioxidant enzymes except for increased catalase at 12 months (123). The changes in antioxidant enzymes and antioxidant reserves in diabetes are discussed under individual sections in Section VII.
B. Antioxidant enzymes

that short episodes of hyperglycemia are more closely linked

cessarily changing overall antioxidant potential, suggesting
type 2 diabetes increases plasma 8-isoprostanes without nec-

measures in diabetic patients (133). Acute hyperglycemia in

A. Antioxidant reserves

Several assays are available for the measurement of total
antioxidant potential in clinical samples, including tissue,
plasma, and urine. The relative merits of these assay tech-
niques are reviewed elsewhere (126–128). The total radical
antioxidant potential assay clearly demonstrates that dia-
betic patients have lower antioxidant defenses and that total
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The presence of oxidative stress in biological fluids can
be simply assessed by examination of spontaneous visible
luminescence. This phenomenon is the result of oxidized
biomolecules with long half-life luminescent intermediates
(124). Measures of spontaneous luminescence were increased
in the urine of patients with known oxidative stress such as
hyperthyroid and muscular dystrophy patients or smokers
compared with healthy controls (125). At present, this
method is not routinely used in diabetes studies, because
more specific end points are selected.

C. Free radical generation

Measurement of free radicals is difficult in animal models
or clinical samples because of their transient nature. Hyper-
glycemia is closely associated with production of $O_2^-$
and peroxides in cell culture models (104). In these models, the
use of fluorescent probes can lead to reliable and reproduc-
ible measures of oxidative stress in real time. These cell-
permeable probes, which are retained in the cell following
the cleavage of an ester conjugate, increase fluorescence at a
specific wavelength after oxidation by free radical attack
(138, 139).

D. Protein, lipid, and DNA adducts

As already stated, the end products of free radical attack
are reliable and relatively straightforward indicators of ox-
idative stress. These modified cell components may be mea-
sured by several different techniques, including HPLC, gas
chromatography-mass spectroscopy, Western blotting, and
ELISA. Biopsy can be used for analysis of oxidized biomol-
ecules in tissues that are particularly at risk from diabetic
complications. These analyses can be performed not only on
tissue but also on plasma and urine. Urine analysis can reveal
nitrosylated proteins (140), lipid oxidation products such as
8-isoprostan es (141), and the DNA adduct 8-hydroxy-2-

VII. Biomarkers of Oxidative Stress

Measuring biomarkers of oxidative stress is an essential
step toward better understanding the pathogenesis and de-
v eloping treatments for diabetic neuropathy. There are sev-
eral approaches that may be adopted, including measure-
ments of the depletion of antioxidant reserves, changes in the
activities of antioxidant enzymes, free radical production,
and presence of protein, lipid, and DNA free radical adducts.

For the purposes of clinical assessment, measurements of end
products of free radical attack may be the most reliable de-
termination of the occurrence of oxidative stress because
enzyme activities and cellular antioxidants are likely to dis-
play transient changes. Yet, the other measures also have
utility depending on the nature of the study.

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B. Antioxidant enzymes

The enzymes responsible for detoxifying free radicals or
regenerating antioxidant molecules can provide an indica-
tion of the level of stress experienced in a cell or tissue. These
enzymes are usually measured by in vitro activity assays,
although changes in transcription can also provide evidence
of cell stress. In long-term diabetes, catalase, GSH reductase,
GSH peroxidase, and SOD decrease in complication-prone
tissue (135). One study reports elevated CuZn-SOD activity
in the blood, although the increased activity did not correct
the deficiency of antioxidant capacity or hyperglycemia-
induced lipid peroxidation (136). The study suggested that
treatment with oral antidiabetic drugs was responsible for
decreases in GSH peroxidase and catalase below control
levels. In a cell culture model of peripheral neuron hyper-
glycemia, there is an initial increase in catalase and SOD as
the neurons attempt to respond to oxidative stress (A. M.
Vincent, L. L. McLean, C. Backus, and E. L. Feldman,
unpublished studies). Initiation of apoptosis, however, occurs
within 3–6 h, after which the antioxidant enzymes rapidly
decrease. Different models of diabetes have produced con-
flicting data regarding increases or decreases in antioxidant
enzymes. In cultured vascular endothelial cells, glucose-in-
duced oxidative stress leads to increased mRNA for antiox-
didant enzymes for a period of 2 wk (122). NADH oxidase
is activated in the brain and kidney of diabetic rats but de-
creased in the liver (137). Because the purpose of this enzyme
is to regenerate NAD$^+$ from NADH to maintain redox status,
this finding strongly suggests that oxidative stress is occur-
r ing in the non-insulin-dependent neurons and kidney cells.

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chromatography-mass spectroscopy, Western blotting, and
ELISA. Biopsy can be used for analysis of oxidized biomol-
ecules in tissues that are particularly at risk from diabetic
complications. These analyses can be performed not only on
tissue but also on plasma and urine. Urine analysis can reveal
nitrosylated proteins (140), lipid oxidation products such as
8-isoprostan es (141), and the DNA adduct 8-hydroxy-2-
deoxyguanosine (8-OH-2dG) (142). These three indicators,
along with other lipid adducts, i.e., malondialdehyde and
4-hydroxyynonenol and carbonyl derivatives of protein side
chains, constitute the most common markers of oxidative
stress in biological systems.

Generally, measures of antioxidants or oxidized end prod-
ucts are more consistently performed in plasma than urine
(143). The excretion of 8-OH-2dG in urine may be mislead-
ning, because this parameter is more strongly influenced by
the degree of oxygen consumption and activity of xenobiotic-
metabolizing enzymes (144). Blood cell 8-OH-2dG is in-
creased in both type 1 and type 2 diabetic patients (145). Nitrotyrosine increases and antioxidant status decreases to similar extents in diabetic patients with or without complications, but oxidized proteins are significantly higher in diabetic patients with complications than in those without any complications (146). This suggests that NO initiation of nitrosylation of proteins may be less significant in producing complications than other free radicals. Nitration of proteins can lead to rapid proteosomal degradation (147); therefore, they can be removed from the cell and resynthesized. Carbonylated proteins and peptides are also inactivated by oxidative stress (148). Measurements of protein carbonyls are highly sensitive, and they can be detected in the plasma of both type 1 and type 2 diabetic patients even without complications (149, 150).

VIII. Antioxidant Therapy for Diabetes Complications

Ten years ago, the Diabetes Control and Complications Trial demonstrated that good glycemic control is the most effective means of decreasing diabetes complications in type 1 patients (2, 151). In another study, uncontrolled diabetes led to pronounced oxidative stress that was reversed when patients attained glycemic control through treatment with glibenclamide or gliclazide (152). Nonetheless, continual tight control is still a challenge in most cases. Therefore, additional therapies that target the pathways leading to hyperglycemia-induced complications are crucial for maintaining long-term quality of life for diabetes patients. Given the hypothesis that oxidative stress may mediate vascular, microvascular, and specific tissue complications in diabetes, antioxidant therapy remains a vital therapy that needs to be exploited. In addition to antioxidants, aldose reductase inhibitors and growth factor therapies also may provide protection through reduction of oxidative stress.

A. Aldose reductase inhibitors

As stated earlier, hyperglycemia-mediated activation of the polyol pathway can produce oxidative stress that may partially underlie diabetes complications. Aldose reductase inhibitors have been tested in experimental diabetic neuropathy, primarily in the streptozotocin rat (153). The aldose reductase inhibitor sorbinil corrects the early deficits in peripheral nerve function with concomitant decreases in parameters of oxidative stress (154). Similarly, the aldose reductase inhibitor WAY-121509 corrects sciatic nerve conduction velocity and endoneurial blood flow and tissue sorbitol accumulation (155). Clinical trials of aldose reductase inhibitors have mostly been disappointing, with a lack of efficacy and unacceptable side effects (6). Zenarestat (Fuji-sawa Pharmaceutical Company, Ltd., Osaka, Japan) is one drug that was effective in type 1 (156) and type 2 (157) diabetic animals. In a phase II clinical trial, zenarestat produced greater than 80% sorbitol suppression and improved nerve conduction velocity slowing and small-nerve fiber density in a 52-wk trial (158). However, a larger phase III trial was suspended because of renal function disorders. The potent aldose reductase inhibitor fidarestat (Sanwa Kagaku KenKyusho, Nagoya, Japan) remains in clinical trial, as current studies are showing therapeutic benefit both in streptozotocin rats (159) and in diabetic patients (160, 161).

B. Nerve growth factor (NGF)

The justification for, and outcomes of, clinical trials using NGF have been reviewed elsewhere (162). The discovery of neurotrophic factors such as NGF raised the hope that these agents could be used clinically to combat neurodegenerative disease. Recent in vitro studies demonstrated that NGF can prevent neuronal oxidative stress by increasing intracellular concentrations of GSH (163), suggesting that altering cellular redox potential may be an important function of NGF. NGF also inhibits the up-regulation of NOS in injured neurons (164). Early clinical trials were promising in patients with diabetic neuropathy, but later phase III trials failed, probably because of poor experimental design (162).

IX. Clinical Trials of Antioxidant Therapy in Diabetes Complications

The strongest indicators for the role for oxidative stress in diabetic neuropathy are the trials of antioxidants in both animal models and patients. Animal models of diabetes have limitations including a short life span compared with possibly decades of disease progression in human patients (165). These data require careful analysis, because each therapeutic agent may have effects outside of regulation of antioxidant activity. For example, administration of the antioxidants vitamin C or α-lipoic acid, as well as free amino acids, also improves responses to insulin and thus can provide additional benefit to the proposed reduction of oxidative stress in tissues (166–169). Vitamin E decreases blood glucose in type 1 diabetic rats through an unknown mechanism (170). Following a discussion of more prominent antioxidants, a number of other agents that have been tested in animal models and/or the diabetes clinic are summarized in Table 1.

A. α-Lipoic acid

Probably the most extensively used antioxidant therapy is α-lipoic acid (reviewed in Ref. 171). This agent can be taken up in the diet and can cross the blood-brain barrier. α-Lipoic acid is reduced in cells to the active dihydrolipoate, which potently regenerates other antioxidants such as vitamin C, vitamin E, and GSH through redox cycling. In rats, α-lipoic acid prevents the development of nerve conduction deficits during 6 wk of diabetes after streptozotocin treatment (172). In a similar model, this compound maintains antioxidant and energy status in the lens (173), prevents lipid peroxidation in the retina (135), and maintains peripheral nerve conduction and blood flow (174, 175). Lipoic acid significantly decreases evidence of oxidative stress in multiple tissues (176, 177) and also decreases diabetes-induced caspase 3 activation in brain neurons in rats with experimental diabetes (178). This is one piece of evidence that suggests that central and peripheral nervous system defects can both be mediated, in large part, by oxidative stress. In chronically glucose-
fed rats as a model of type 2 diabetes, lipoic acid prevents hypertension, hyperglycemia, hyperinsulinemia, and increased mitochondrial O$_2^-$ production (179).

α-Lipoic acid is licensed for use in diabetic patients in Germany. Cross-sectional studies continue to demonstrate that supplementation with α-lipoic acid significantly improves antioxidant defense and decreases oxidative stress even in patients with poor glyemic control (180). German trials also suggest that treatment with α-lipoic acid improves the microcirculation, suggesting protective effects other than, or in addition to, decreasing cellular oxidative stress (181). Larger multicenter randomized double-blind placebo trials in Europe and North America have demonstrated limited effects on neuropathic symptoms and electrophysiological testing but suggest that longer-term assessment of neuropathic deficits is merited (182, 183). Slight improvements in cardiac autonomic neuropathy were demonstrated in the DEKAN study (184), and the drug was safe and well tolerated. A recent phase III clinical trial, the SYDNEY trial, demonstrated that IV administration of α-lipoic acid rapidly and significantly improves several neuropathic symptoms and nerve function in patients with stage 2 diabetic sensorimotor polyneuropathy (185). This is strong support for the use of antioxidants in the treatment of diabetic neuropathy, and the use of oral α-lipoic acid is currently in a phase III clinical trial in the United States.

**B. Vitamins E and C**

As a critical antioxidant for the protection of plasma lipids, vitamin C will require supplementation under conditions of prolonged or repeated prooxidant conditions such as hyperglycemia (62). Chronic administration of 1 g/d vitamin C in aged type 2 diabetic patients decreases plasma free radicals and increases cellular GSH levels over a period of 4 months (186). Vitamin C supplementation alone shows limited therapeutic benefit in type 1 diabetes (187) and is more commonly used in combination with vitamin E or other agents. Uses of vitamin C in combination therapies are discussed below.

Vitamin E has been more broadly examined in diabetes models. Interestingly, the incorporation of vitamin E into erythrocyte membranes is impaired in the hyperglycemic state; therefore, decreased antioxidant defense may be further exacerbated in poorly controlled diabetes (188). Rat models of diabetes show some therapeutic benefit in the presence of vitamin E therapy. Diabetes-induced susceptibility to low-density lipoprotein peroxidation is prevented in the presence of vitamin E (189). Dietary vitamin E supplementation also improves fatty acid metabolism and decreases lipid peroxidation in tissues of diabetic rats (190) and improves blood flow and nerve morphometric parameters in the heart (191). In diabetic rats, vitamin E supplementation prevents reactive astrogliosis in the brain that is associated

### Table 1. Examples of the use of antioxidants against hyperglycemic injury

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Experimental design</th>
<th>Outcome</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>STZ-treated rats, dietary GSH</td>
<td>Suppression of diabetic-induced urinary 8-OH-2dG, albuminuria, and increased tail-flick reaction time.</td>
<td>207</td>
</tr>
<tr>
<td>Coenzyme Q10 (Co Q)</td>
<td>STZ-treated rats ± ischemia, 7-d treatment with Co Q ip</td>
<td>Decreased apoptosis of brain neurons</td>
<td>178</td>
</tr>
<tr>
<td>Aminoguanidine (AG)</td>
<td>Alloxan-treated rabbits, AG in drinking water</td>
<td>Decrease of diabetes-induced retinal lipid peroxidation, also oxidant-induced apoptosis in vitro</td>
<td>208</td>
</tr>
<tr>
<td>Probucol</td>
<td>STZ-treated rats, 1–2 months 1% probucol in diet</td>
<td>Corrected sciatic nerve endoneurial blood flow; improved diabetes-induced motor and sensory nerve conduction deficits</td>
<td>209, 210</td>
</tr>
<tr>
<td>Taurine</td>
<td>STZ-treated rats, 3 wk treated with 5% taurine in the diet</td>
<td>No changes in polyol pathway activation, corrected lens lipid peroxidation, GSSG-GSH, NAD+ -NADH, and ADP-ATP ratios</td>
<td>211</td>
</tr>
<tr>
<td>N-Acetylcysteine</td>
<td>STZ-treated rats, 8 wk 55 mg/d N-acetylcysteine in drinking water</td>
<td>Decreased diabetes-induced motor nerve conduction velocity, corrected erythrocyte GSH, and plasma lipid peroxidation; corrected endothelial dysfunction</td>
<td>212, 213</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>STZ-treated rats, 2 months, 1% dietary supplement</td>
<td>No change in glycemia or polyol pathway, corrected motor and sensory nerve conduction velocity</td>
<td>214</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>11 Type 2 diabetic patients with age-matched controls in parallel study, treated with 300 mg allopurinol/d orally 1 month</td>
<td>Corrected blood flow deficits and decreased plasma lipid peroxidation</td>
<td>215</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>28 Type 1 diabetic patients in a double-blind placebo controlled study of Daflon 500</td>
<td>No change in glycemic control but decreased HbA1c, increased GSH peroxidase activity, increased plasma antioxidant potential</td>
<td>216</td>
</tr>
<tr>
<td>l-Arginine</td>
<td>30 Diabetic patients in a blind placebo controlled study using 2 g/d l-arginine for 3 months</td>
<td>Decreased urinary lipid peroxidation</td>
<td>217</td>
</tr>
<tr>
<td>Zinc</td>
<td>22 Type 1 diabetic patients, oral administration of zinc gluconate 30 mg/d</td>
<td>Untreated patients developed zinc deficiency and reduced activity of GSH peroxidase. Supplementation corrected these deficits and plasma antioxidant potential</td>
<td>218</td>
</tr>
</tbody>
</table>

In addition to the uses of vitamins E and C and α-lipoic acid that are described in more detail in the text, many antioxidants have been examined in hyperglycemia. The upper section of the table contains studies performed in animal models of diabetes and the lower section shows clinical trials in diabetic patients. STZ, Streptozotocin; HbA1c, glycosylated hemoglobin.
with lipid peroxidation (192). Diabetes-induced changes in antioxidant enzymes in different organs are corrected to differing extents by vitamin E, but the combination of vitamin E with another antioxidant, stobadine, provides superior protection against deficits in these enzymes (193).

In healthy human subjects, α-tocopherol decreases evidence of oxidative stress through low-density lipoprotein oxidizability and presence of urinary F2-isoprostane (67). In this regard, α-lipoic acid may be slightly more potent than α-tocopherol in decreasing the same oxidative stress parameters, and there is no added benefit in combining the two agents (67). Small clinical studies demonstrate improvements in a variety of oxidative stress parameters in diabetic patients receiving antioxidant vitamin supplements. Combined oral vitamin C and E therapy reduces oxidative stress in the eye (68) and improves vascular endothelial function in type 1, but not type 2, diabetes (194). Plasma low-density lipoprotein oxidation is decreased after treatment with high doses (1632 mg/d) of vitamin E (195). Topical application of vitamin E improves skin microcirculation and evidence of ROS in type 2 diabetics (196). Finally, urinary 8-isoprostane F2α and 11-dehydro-thromboxane B2 were decreased after treatment with 600 mg/d vitamin E in a population of 85 diabetic patients (197). Direct correlations between improved antioxidant status and the incidence of neuropathy have not yet been made.

Despite many positive clinical trials using vitamin E, some conflicting data exist for diabetes as well as other disorders such as cancer and cardiovascular disease (198, 199). Therefore, broad recommendations for the use of vitamin E and other dietary supplements have not been established. One caution for the preventive intake of α-tocopherol is the evidence that supplementation with α-tocopherol produces deleterious changes in the bioavailability of γ- and δ-tocopherol. The different isomers have different properties, such as in vascular disease and antiproliferative effects, and so additional research into the dietary application of vitamin E is warranted (200, 201).

C. The future of antioxidant therapy in clinical trials

The clinical trials to date have provided strong evidence that oxidative stress is a critical mediator of diabetes complications including neuropathy. To improve future clinical trials, previous studies should be closely examined. High doses of single-antioxidant supplements may perturb the antioxidant-prooxidant balance of cell systems (200, 202). Therefore, mixtures of antioxidant therapies, possibly in combination with trace elements and vitamins that enhance metabolic processes, may provide a better therapeutic option. Monitoring of patients’ antioxidant reserves also may identify development of deficits that could be ameliorated by altering the therapeutic antioxidant regimen. Earlier discussions in this review suggest that GSH may be the most important tissue antioxidant. Therapies aimed at increasing cellular GSH could target the GSH-synthesizing enzymes as well as dietary increases of cysteine or its precursor 2-oxothiazolidine-4-carboxylate, as cysteine is the rate-limiting substrate for GSH synthase (203). GSH is not taken up well by cells, but an esterified form is, and this directly increases the levels of GSH in tissues, plasma, and cerebrospinal fluid (69). Another review of the literature regarding the use of botanicals and dietary supplements in diabetic peripheral neuropathy concludes that evening primrose oil, α-lipoic acid, and capsaicin have been most widely used, but that their efficacy is not yet established (204).

X. Summary

Diabetic neuropathy probably arises from a combination of microvascular and neuronal deficits. Oxidative stress can contribute significantly to these deficits and may be a direct result of hyperglycemia. Brief postprandial peaks in plasma glucose are sufficient to generate hyperglycemic oxidative stress. In contrast, acute glucose deprivation also causes apoptosis of peripheral neurons through a mechanism that at least partially involves oxidative stress (205). Therefore, until we can fully control blood glucose levels, therapies such as antioxidants that are targeted against oxidative stress remain our most promising approach to preventing neuropathy as well as other complications in diabetes.

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