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REVIEW ARTICLE

The Role of Oxidative Stress in Diabetic Complications

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Abstract

The morbidity and mortality associated with diabetes is the result of the myriad complications related to the disease. One of the most explored hypotheses to explain the onset of complications is a hyperglycemia-induced increase in oxidative stress. Reactive oxygen species (ROS) are produced by oxidative phosphorylation, nicotinamide adenine dinucleotide phosphate oxidase (NADPH), xanthine oxidase, the uncoupling of lipoxygenases, cytochrome P450 monooxygenases, and glucose autoxidation. Once formed, ROS deplete antioxidant defenses, rendering the affected cells and tissues more susceptible to oxidative damage. Lipid, DNA, and protein are the cellular targets for oxidation, leading to changes in cellular structure and function. Recent evidence suggests ROS are also important as second messengers in the regulation of intracellular signaling pathways and, ultimately, gene expression. This review explores the production of ROS and the propagation and consequences of oxidative stress in diabetes.

Index Entries: Antioxidants, diabetes mellitus; free radicals; glucose autoxidation; lipid asymmetry; lipid oxidation; NADPH oxidase; oxidative phosphorylation; protein kinase C.

INTRODUCTION

Diabetes mellitus is a pervasive disease, affecting nearly 17 million people worldwide *(1)*. The disease is broadly classified by the ability to produce insulin, a key glucose regulatory hormone. A small percentage (5 to 10%) of patients have insulin-dependent (IDDM), or type I, diabetes. IDDM patients are unable to produce insulin because of autoimmune destruction of pancreatic β-cells. The majority of diabetes patients are non-insulin-dependent (NIDDM), however. NIDDM patients are capable, at least initially, of producing insulin, but are deficient in their cellular response. In both cases, decreased uptake of glucose into muscle and adipose tissue leads to chronic extracellular hyperglycemia, which results in tissue damage and pathophysiological complications. These complications, including retinopathy and cataract formation, nephropathy, peripheral nerve damage, heart disease, and atherosclerosis, are responsible for

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the morbidity and mortality associated with the disease *(2–5)*. Because of the enormous societal impact of diabetes, much attention has been focused on the pathology of its complications and the underlying molecular mechanisms.

One of the major hypotheses proposed to explain the hyperglycemia-induced onset of diabetic complications is an increase in oxidative stress *(6–10)*. Similar to their proposed role in the onset of diabetic complications, reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydroxyl radical, and hydrogen peroxide, have been linked to other diseases and injury states, including Alzheimer's disease *(11)*, Parkinson's disease *(12,13)*, chronic obstructive pulmonary disease *(14)*, and ischemia reperfusion injury *(11)*. Evidence suggests that ROS function not only as mediators of destruction, but also as intracellular second messengers that regulate signal transduction cascades and gene expression. This review will examine the source of ROS in diabetes, the mechanisms of propagation of oxidative stress, and the ultimate consequences of increased oxidative stress, including activation of signaling pathways and protein and lipid modification.

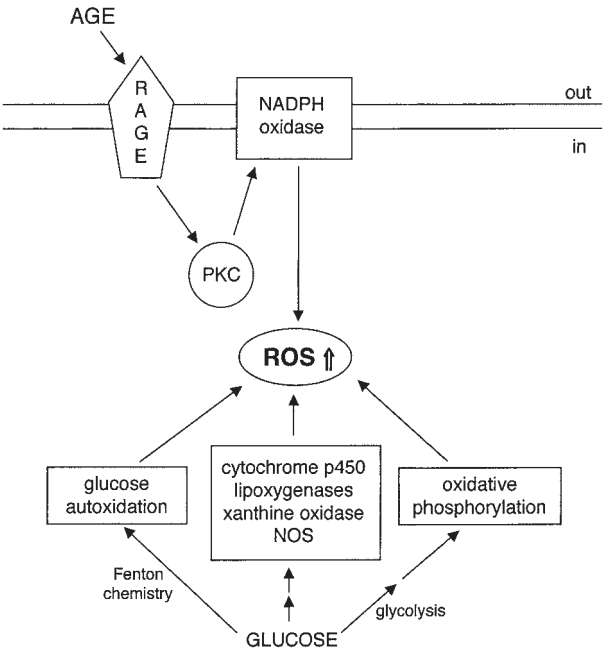

Fig. 1. Reactive oxygen species (ROS), such as superoxide, hydroxyl radical, and hydrogen peroxide, arise from many cellular sources in response to hyperglycemia and diabetes. These sources include oxidative phosphorylation, glucose autoxidation, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and other enzymes, such as xanthine oxidase, lipoxygenase, cytochrome P450 monooxygenases, and nitric oxide syntase. ROS also arise from the production of advanced glycation endproducts (AGE) and activation of AGE receptors (RAGE).

THE SOURCE OF ROS IN DIABETES

Hyperglycemia can stimulate ROS production from a variety of sources. The relative importance of each source varies with cell or tissue type. This review will focus only on the sources for which there is significant evidence to support their role in diabetic complications. A summary of these sources of ROS is provided in Fig. 1.

Oxidative Phosphorylation

The electron transport chain is a major cellular source of ROS, even in nondisease states. Electrons from reduced dinucleotide carriers are transferred between membrane-embedded complexes in the inner mitochondrial membrane with the concomitant generation of a proton gradient that is exploited to synthesize adenosine triphosphate. The electrons are used to reduce molecular oxygen to water at complex IV. However, at times, oxygen is only partially reduced, yielding $O_2^{\bullet-}$, a highly reactive radical. $O_2^{\bullet-}$ generation occurs not only at complex IV, but also anywhere oxygen is accessible to electrons. Under normal conditions, the key sites of $O_2^{\bullet-}$ generation in the mitochondrial membrane are complex I and the ubiquinone-complex III interface, where the presence of long-lived intermediates allows reaction of electrons with molecular oxygen *(15)*. Diabetes alters the primary sites of $O_2^{\bullet-}$ generation, however.

Evidence has linked $O_2^{\bullet-}$ overproduction to hyperglycemic damage. A complex II inhibitor, 2-thenoyltrifluoroacetone, and an uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenyldihydrazone, decrease ROS production in platelets *(16)*, bovine aortic endothelial cells *(17)*, bovine retinal cells *(18)*, and retinal Müller cells *(18)* exposed to high concentrations of glucose, and in retina from streptozotocin (STZ)-treated rats *(18)*. These data indicate that complex II is the primary source of electrons that ultimately contribute to $O_2^{\bullet-}$ formation in diabetes. Treatment of endothelial cells with rotenone, an inhibitor of complex I, has no effect on glucose-induced $O_2^{\bullet-}$ production, indicating this complex is not involved in glucose-mediated ROS production *(17)*. In platelets, complex II inhibitors also reduce platelet aggregation, platelet-derived growth factor release, tyrosine phosphorylation, and protein tyrosine phosphatase activity *(16)*, suggesting the effect of ROS production on platelet function is extensive. Inhibition of oxidative phosphorylation in bovine aortic endothelial cells also reduces protein kinase C (PKC) activity, hexosamine pathway activation, advanced glycation end product (AGE) formation, and sorbitol accumulation through aldose reductase *(17,19)*. In addition, hyperglycemia-induced inhibition of glyceraldehyde-3-phosphate dehydrogenase activity is prevented *(19)*. In cultured retinal cells (endothelial and Müller), hyperglycemia-induced reduction in cell viability and enhanced cell death are suppressed by treatment with superoxide dismutase (SOD) *(18)*. Although these data demonstrate a role for ROS in retinal cell health, it does not definitively establish oxidative phosphorylation as the source of $O_2^{\bullet-}$.

Uncoupling proteins (UCPs) are also proposed to play a role in the regulation of ROS production during oxidative phosphorylation. These proteins facilitate proton leakage across the mitochondrial membrane, dissipating a high membrane protonic potential and thus a limited respiration rate *(20)*. A high protonic potential has been linked to ROS production in mitochondria *(21)* because of a buildup of electron-rich intermediates capable of reducing O_2 to $O_2^{\bullet-}$. Interestingly, UCPs have been shown to be activated by $O_2^{\bullet-}$, demonstrating a feedback response to $O_2^{\bullet-}$ production *(22–24)*. Zucker diabetic fatty rats express reduced levels of UCP2 *(25,26)*, possibly indicating a role for UCP depletion in diabetic oxidative stress. Likewise, UCP3 expression is reduced in dorsal root ganglia from STZ-induced diabetic rats, in dorsal root ganglia cultured in high glucose conditions *(27)* and in skeletal muscle from NIDDM patients *(28)*. UCP2 knockout models exhibit increased atherosclerotic plaque formation *(29)*, a process linked to oxidative stress *(30,31)*. On the other hand, overexpression of UCP1 and UCP3 in dorsal root ganglia prevents ROS production, mitochondrial membrane hyperpolarization, and apoptosis during exposure to high concentrations of glucose *(27)*. Similarly, overexpression of UCP1 in high glucose-treated bovine aortic endothelial cells normalizes ROS production, protein kinase C (PKC) and nuclear factor kappa B (NFκB) activation, hexosamine pathway activation, glyceraldehyde-3-phosphate dehydrogenase inhibition, AGE formation, and sorbitol accumulation *(17,19)*. Although no work has yet established the effect of UCP2 overexpression on models of diabetes or hyperglycemia, UCP2 overexpression does reduce ROS production in other models of neuronal *(32)* and cardiomyocyte *(33)* injury.

Thus it appears that alterations in both electron transport activity and, in some instances, UCP activity/expression contribute to an increase in $O_2^{\bullet-}$ production in diabetes. It is also apparent that mitochondrially derived ROS affect enzyme activity and AGE formation in many models of diabetes. One major drawback to discerning whether mitochondrially derived ROS contribute significantly to the onset of diabetic complications, however, is the toxicity of electron transport complex inhibitors. We must therefore rely on indirect evidence from in vivo models of diabetes.

Nicotinamide Adenine Dinucleotide Phosphate Oxidase

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is best characterized in neutrophils, where its production of $O_2^{\bullet-}$ generates the respiratory burst necessary for bacterial destruction *(34)*. Since the discovery of the neutrophil NADPH oxidase, the enzyme complex, or components thereof has been identified in vascular smooth muscle cells *(35,36)*, vascular endothelial cells *(37,38)*, colonic epithelial cells *(39)*, mesangial cells *(40,41)*, platelets *(42)*, the thyroid gland *(43)*, and polymorphonuclear and mononuclear leukocytes *(44)*. The enzyme complex consists of two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$, which comprise cytochrome b558, the enzymatic center of the complex. After activation, cytosolic components, including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small G coupled proteins Rac and Rap1A, translocate to the membrane to form the active enzyme complex (reviewed in refs. *35,45*). The nonphagocytic NADPH oxidases produce $O_2^{\bullet-}$ at a fraction (1–10%) of the levels produced in neutrophils *(35)* and are thought to function in intracellular signaling.

In recent years, many researchers have focused on the role of NADPH oxidase in disease states, such as diabetes. Several lines of evidence support NADPH oxidase as a mediator of diabetic complications (reviewed in refs. *46–48*). Superoxide production in the glomerulus of STZ-treated rats and in high glucose-treated aortic endothelial, smooth muscle, and mesangial cells is decreased by treatment with the NADPH oxidase inhibitor diphenylene iodonium (DPI) *(36,38,40,41,49)*. This inhibitor is relatively nonspecific for flavoproteins and may also inhibit other ROS-producing enzymes, such as those involved in oxidative phosphorylation. Some authors *(40,49)* have eliminated the involvement of mitochondrial complex I in $O_2^{\bullet-}$ production by treatment with rotenone, which had no effect on ROS production. However, these studies did not address the involvement of complex II, which has been shown to be the key source of mitochondrial $O_2^{\bullet-}$ production (*see* Oxidative Phosphorylation) *(16,17)*. An increase in NADPH oxidase activity has been demonstrated in the renal cortex of STZ-treated rats *(50)* and in leukocytes from NIDDM patients *(51)*. Expression of several enzyme subunits, including $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$, is increased in aortic and venous endothelial cells and in mesangial cells incubated under high glucose conditions *(37,38, 40,41,52)*. Similarly, expression of $p47^{phox}$ is stimulated in polymorphonuclear and mononuclear leukocytes isolated from individuals challenged with a high glucose intake *(44)*. In addition, the membrane-associated, but not the cytoplasmic fractions, of $p47^{phox}$ and $p67^{phox}$ are increased in the diabetic glomerulus *(40)*. Rac1, one of the associated G proteins, is also increased in the membrane fraction of vascular smooth muscle cells exposed to high concentrations of glucose *(49)*. These data indicate that NADPH oxidases are a major source of glucose-induced ROS production in the vasculature and kidney, but does not support their involvement in other cells.

Interestingly, $p22^{phox}$ expression is upregulated in aortas from hypertensive rats *(53)*, possibly because of an increase in angiotensin II, a strong vasoconstrictor. Deletion of $p47^{phox}$ in hypertensive mice reduces both the hypertensive response and production of ROS observed after angiotensin II infusion *(54)*. Because hypertension is a common complication of diabetes, it is possible that expression of NADPH oxidase is regulated similarly in the two diseases. There is evidence for this hypothesis and of increased angiotensin II labeling in cardiac myocytes and endothelial cells from human diabetic patients *(55)*. DPI treatment blocks an angiotensin II-stimulated increase in ROS production in cardiomyocytes exposed to high concentrations of glucose *(56)*. Conversely, high glucose-induced ROS production and $p47^{phox}$ expression are blocked by cardiomyocyte or kidney homogenate treatment with an angiotensin II type I receptor antagonist *(56–58)*, demonstrating a link between the two pathways of NADPH oxidase activation. In

addition, an angiotensin receptor antagonist, losartan, blocks ROS production in cardiomyocytes from STZ-induced diabetic mice *(59)*.

The NADPH oxidase-dependent production of ROS in diabetes can be decreased by a variety of PKC inhibitors, implicating this family of kinases in the regulation of hyperglycemia-induced NADPH oxidase activity *(36,38,60)*. PKC has also been implicated in the regulation of NADPH oxidase expression. $p47^{phox}$, $p22^{phox}$, and $p67^{phox}$ upregulation in high glucose-treated endothelial cells is normalized by treatment with PKC inhibitors *(37)*. Last, the membrane enrichment of $p47^{phox}$ and $p67^{phox}$ was reduced in the glomerulus of diabetic rats treated with the $PKC\beta_2$ inhibitor ruboxistaurin, indicating a role for this PKC isoform in NADPH oxidase translocation *(40)* (*see* Protein Kinase C).

Glucose Autoxidation

Hyperglycemia-induced oxidative stress also occurs in nonnucleated cells lacking mitochondria and the NADPH oxidase (i.e., erythrocytes) *(61)*. There must, therefore, be another mechanism of ROS formation in these cells. One hypothesis is glucose autoxidation. Glucose, and many of its metabolites, can react with hydrogen peroxide in the presence of transition metals, such as Fe^{2+} and Cu^{2+}, to form hydroxyl radical (•OH), the most reactive ROS *(62,63)*. Most of the data supporting this hypothesis are indirect. For example, many studies demonstrate that metals are required for the induction of glucose-induced ROS production. Protein fragmentation induced by high concentrations of glucose is suppressed by metal chelators, such as diethylenetriamine penta-acetic acid *(64,65)*, whereas Cu^{2+} enhances the effect. Sorbitol, considered an $OH^{\bullet}$ scavenger, greatly reduces protein fragmentation in the presence of both glucose and Cu^{2+}. Collagen fragmentation is induced only in the presence of glucose and metals, particularly Cu^{2+}, and is inhibited by the addition of EDTA *(66)*.

The Fe^{2+} chelator desferrioxamine inhibits glucose and glycosylated collagen-induced lipid hydroperoxide formation in artificial membranes *(67)*. Ou and Wolff *(68)* demonstrated that incubation with high concentrations of glucose increase catalase inactivation in human erythrocytes, a process inhibitable by metal chelation. The extent of inactivation was not substantial, though this could be due to the relatively short incubation time (40 min). Fibroblast cell viability and glutathione levels are reduced in the presence of glucose *(69)*. Fe^{2+} seems to play a role as these effects are abrogated by the addition of 2,2′-dipyridyl, an iron chelator, while a calcium chelator has no effect. However, the most direct evidence in support of this hypothesis comes from the measurement of $O_2^{\bullet-}$ generation in endothelial cells. Both high glucose and 3-*O*-methylglucopyranose (3-OMG), a nonmetabolizable glucose analog, cause an increase in $O_2^{\bullet-}$ production, indicating that metabolism of glucose is not necessary to induce oxidative stress *(70)*. The increase in $O_2^{\bullet-}$ from glucose is inhibitable by iron chelation with desferral, whereas inhibition of enzymatic sources of ROS, such as cyclooxygenases, lipoxygenases, cytochrome P450, and nitric oxide synthase, has no impact. Because the effect of these inhibitors on $O_2^{\bullet-}$ generation induced by 3-OMG was not examined, no conclusion can be made as to the source of ROS during treatment of this analog. These data support a role for metal-catalyzed glucose oxidation in the production of ROS *(71)*, but stop short of a direct demonstration of intermediates in this process, such as formation of the glucose radical or enol tautomer. In addition, all evidence for this hypothesis comes from in vitro models of diabetes. A role for this mechanism in in vivo models of diabetes must still be resolved.

Other Potential Sources of ROS

Nitric Oxide Synthase

Nitric oxide (NO) is an intracellular second messenger that modulates vascular tone and cardiac and neural function. It is produced by constitutive and inducible nitric oxide synthase (NOS); dimeric, zinc-cluster-containing

enzymes that incorporate O_2 into L-arginine and require flavin, NADPH, heme, and tetrahydrobiopterin cofactors *(72,73)*. NOS are expressed in many tissues including cardiac and neuronal tissues and the endothelium. Expression is regulated by cellular factors such as NFκB. NOS has been shown to produce free radicals during the catalytic cycle that may leak from the active site due to enzyme uncoupling *(74)*. NOS can also transfer electrons directly to O_2 to form $O_2^{\bullet-}$ *(75,76)*. In addition, NO can react with $O_2^{\bullet-}$ to form peroxynitrite, a highly reactive oxidant correlated with disease states such as sepsis, inflammation, atherosclerosis, and diabetes *(73)*. Peroxynitrite has been shown to react with the zinc-cluster of NOS, dissociating the monomers and uncoupling enzyme function *(73)*. Therefore, formation of peroxynitrite not only depletes existing NO, but also reduces a tissue's ability to produce more NO, resulting in defective tissue function.

Hyperglycemia contributes to regulation of NOS expression and the production of peroxynitrite (reviewed in ref. *77*). Endothelial NOS (eNOS) mRNA expression is increased in aortas from both STZ-induced diabetic *(60,78)* and fructose-fed hyperinsulinemic *(78)* rats and in human umbilical vein endothelial cells exposed to high concentrations of glucose *(79)*. eNOS protein expression is upregulated in the kidney *(58)*, retina *(80)*, and aorta *(60)* of STZ-induced diabetic rats; in the liver, heart, and kidney of STZ-induced diabetic mice *(73)*; and in human umbilical vein endothelial cells exposed to high concentrations of glucose *(79)*. Inducible NOS protein expression is also increased in the retina of STZ-induced diabetic rats *(81)*. Glucose-induced aortic expression of eNOS can be suppressed by the addition of a PKC inhibitor *(60)*, suggesting PKC activation is a key event in hyperglycemia-induced NOS upregulation, perhaps mediated by PKC-dependent activation of NFκB (*see* Activation of Intracellular Signaling Pathways). Glucose-induced retinal eNOS protein expression can be suppressed by the addition of L-NAME, an NOS inhibitor, or uric acid, a peroxynitrite scavenger *(80)*, suggesting that enzyme activity and peroxynitrite formation also regulate expression.

Despite an increase in mRNA expression, eNOS activity is decreased in the aorta of hyperinsulinemic rats *(78,82)*, indicating direct enzyme inhibition or degradation of RNA. This inhibition of activity can be suppressed by insulin treatment. One hypothesis to explain these data is the depletion of tetrahydrobiopterin. Reduction in the levels of this cofactor leads to dimer uncoupling, preferential reduction of O_2 instead of L-arginine, and a loss of enzyme activity *(75,76,83,84)*. Tetrahydrobiopterin is depleted in fructose-fed, insulin-resistant rats *(78,82)*. Replacement of tetrahydrobiopterin improves blood flow in NIDDM patients *(85)*. In addition, tetrahydrobiopterin treatment improves vascular relaxation, NOS activity, $O_2^{\bullet-}$ formation, and lipid oxidation in the vasculature of fructose-fed hyperinsulinemic rats *(78,82)*.

Another hypothesis to explain these data is diabetes-induced production of peroxynitrite. Treatment of eNOS with peroxynitrite stimulates zinc release, disulfide bond formation, and monomer dissociation, leading to release of $O_2^{\bullet-}$ from the enzyme active site *(73)*. Glucose and diabetes have similar effects on the enzyme. Nitrotyrosine formation, a product of peroxynitrite modification, is increased in retina of STZ-induced diabetic rats (*80,81*), in the pancreas of STZ-induced diabetic mice *(86)*, in the mitochondria of alloxan-induced diabetic mouse hearts *(87)*, and in the plasma *(88,89)* and placenta *(90)* of IDDM patients. NO is concomitantly depleted in the plasma of IDDM patients, indicating either enzyme inhibition, decreased protein expression, or increased NO removal *(88,89)*.

Addition of uric acid, the peroxynitrite decomposition catalyst FP15, or the NOS uncoupler L-NAME reduces diabetes-induced cell dysfunction. FP15 reduces nitrotyrosine formation, improves endothelial relaxation, and suppresses cardiac dysfunction in STZ-induced diabetic mice *(86)*. Uric acid and L-NAME reduce nitrotyrosine formation, lipid oxidation, and retinal permeability (an indica-

tor of retinopathy) in STZ-induced diabetic rats *(80)*. Thus NOS inhibition and peroxynitrite formation contribute appreciably to diabetic vascular complications.

Xanthine Oxidase

Xanthine dehydrogenase and xanthine oxidase are two molybdenum-containing flavoenzymes formed from the same gene product, the activity of which is collectively termed *xanthine oxidoreductase*. Xanthine dehydrogenase is an enzyme that catalyzes the oxidation of hypoxanthine to uric acid, using nicotinamide adenine dinucleotide (NAD^+) as a cofactor. After proteolytic cleavage and sulfhydryl oxidation of this enzyme, xanthine oxidase is formed. Xanthine oxidase catalyzes a similar reaction, substituting molecular oxygen as the electron acceptor. Superoxide, hydrogen peroxide, and hydroxyl radical are produced as byproducts of the xanthine oxidase reaction (reviewed in refs. *91–93*). Although xanthine oxidase activity is undetectable in normal endothelial cells *(94)*, it has been proposed to be a major source of ROS in atherosclerosis *(30)*, ischemia-reperfusion injury *(95,96)*, and diabetes mellitus *(97,98)*. Treatment of NIDDM patients with the xanthine oxidase inhibitor, allopurinol, reduces levels of lipid oxidation in plasma and improves blood flow and endothelial cell function *(98)*. Increased xanthine oxidase activity has been demonstrated in the liver of STZ-treated rats *(97)*. Allopurinol treatment of these rats inhibits aortic ring $O_2^{\bullet-}$ production and glutathione and lipid oxidation in the blood, liver, and heart. In addition, hypercholesterolemic rabbits, used as models of atherosclerosis, exhibit $O_2^{\bullet-}$ production in the aortic rings that is inhibitable by allopurinol *(99)*. Atherosclerosis is a major complication of diabetes mellitus and may develop through similar pathways in nondiabetic animals. These data support the hypothesis that the role for xanthine oxidase is tissue-specific. Some conflicting data also exist, however. Aliciguzel *(100)* showed no increase in xanthine oxidase activity in the heart, liver, brain, or kidney of alloxan-treated diabetic rats.

Cytochrome P450 Monooxygenases

Cytochrome P450 monooxygenases are a large class of enzymes involved in the detoxification of endogenous and exogenous molecules (reviewed in refs. *101–103*). Some cytochrome P450 isoforms are also involved in the bioactivation and induction of toxicity of certain molecules, such as acetaminophen. These enzymes form dioxygen intermediates that decompose to release hydrogen peroxide and $O_2^{\bullet-}$ *(103, 104)*. The effect of diabetes on cytochrome P450s varies with respect to the isoform.

Two isoforms, CYP2E1 and CYP4A, are associated with nonalcoholic steatohepatitis, an inflammatory liver injury associated with obesity and NIDDM *(105)*. Only a few isoforms increase in activity or expression in the diabetic state. CYP2E1 metabolizes small molecules such as ethanol and acetone. NIDDM patients exhibit an increase in liver CYP2E1 activity, assessed by chlorzoxazone clearance from the bloodstream *(106)*. There is increased mRNA expression of CYP2E1 in leukocytes of NIDDM *(106,107)* and IDDM *(108)* patients, as well as the livers of *ob/ob* mice *(109)* and STZ-induced diabetic rats *(110)*. Paradoxically, although CYP2E1 mRNA is increased in *ob/ob* mice, protein amounts and activity are unchanged *(109)*, possibly because of a decrease during translation. CYP2E1 protein expression is increased in the livers of STZ-treated Syrian hamsters *(111)*. Hepatic CYP4A10 and CYP4A14 mRNA are also increased in *ob/ob* mice *(112)*. Because the CYP4A isoforms are involved in microsomal fatty acid metabolism, they may be upregulated to alleviate diabetes-associated hyperlipidemia.

It has been reported that the cytochrome P450 family of enzymes is regulated, not by hyperglycemia itself, but by other molecules associated with diabetes. Many isoforms have been shown to be upregulated by treatment with fatty acids and ketone bodies *(113)*, both of which are increased in the diabetic state. There is also evidence that intact leptin signaling is required for constitutive expression of CYP2E1 *(109)*, though expression is still inducible by ketones and fatty acids in its

absence. The latter result may affect the measurement of basal expression levels in animal models deficient in the leptin signaling pathway, such as *db/db* and *ob/ob* mice and *fa/fa* rats.

Unlike CYP2E1 and the CYP4As, many cytochrome P450 isoforms have been shown to decrease in expression in diabetes. These isoforms include CYP1A2, CYP2C22, CYP2C40, and CYP2C11 *(114,115)*. Although this finding should not affect the production of ROS, it may effect the detoxification of other harmful molecules.

Although an increase in mRNA expression of several CYP isoforms is suggestive, it does not directly demonstrate a role for these enzymes in diabetes-induced ROS production. In addition, there are no clinical data demonstrating the effects of cytochrome P450 inhibition on diabetic complications.

Lipoxygenases

Lipoxygenases catalyze the conversion of arachidonic acid into a broad class of signaling molecules, such as leukotrienes, hydroxyeicosatetraenoic acid, and lipoxins (reviewed in refs. *116–122*). Radicals are produced during the enzymatic reactions and normally remain enzyme-bound. These radicals can, under certain conditions, escape the active site and attack surrounding molecules. The 12/15-lipoxygenases are of particular interest for their role in low-density lipoprotein oxidation and atherosclerosis *(123)*. There is also evidence that lipoxygenases play a similar role in atherogenesis in diabetes *(124,125)*.

Leptin receptor deficient *db/db* mice have increased 12/15-lipoxygenase protein expression in aortic endothelial cells and increased eicosanoid formation *(126)*, although it is unclear whether the eicosanoids are formed enzymatically or nonenzymatically (*see* Lipid Hydroperoxides/Eicosanoids). Inhibition of 12/15-lipoxygenase expression in diabetic or high-glucose-treated endothelial cells decreases monocyte adhesion, a key event in atherogenesis *(126,127)*. In addition, 12/15-lipoxygenase knockout mice exhibit resistance to pancreatic β-cell destruction induced by STZ *(128)*, indicating a potential involvement of 12/15-lipoxygenase in the onset of IDDM. It is unclear whether generation of radicals or products by these enzymes is the pertinent function, because, in most cases, researchers have not discerned between enzymatically and nonenzymatically formed lipid hydroperoxides. However, diabetes is associated with an increase in lipoxygenase expression, indicating eicosanoid formation is likely the result of activity of these enzymes. If lipoxygenases have a significant role in the onset of diabetic complications, it is likely to be restricted to atherosclerosis.

PROPAGATION OF OXIDATIVE STRESS

Diabetes mellitus not only stimulates the generation of ROS, but also impairs the ability of a cell or tissue to cope with the increased oxidative burden. After all, an increase in free radicals could do no damage if they were immediately neutralized. Although many enzymes and antioxidants are affected by diabetes, the scope of this review is limited to the most extensively investigated enzymes and antioxidants (reviewed in ref. *129*). A summary of these results is presented in Fig. 2 and Table 1.

Depletion of Antioxidants

Vitamin E

Vitamin E is a major lipophilic antioxidant in plasma and red blood cells that serves to impede the propagation of lipid peroxidation. Vitamin E levels are depleted in the plasma of NIDDM *(130,131)* and IDDM *(132,133)* patients, in normal patients challenged with high concentrations of glucose *(44)* and red blood cells treated with high concentrations of glucose *(134,135)*. The amount of plasma vitamin E is inversely correlated with the existence of diabetic complications in IDDM *(132)* and with the duration of NIDDM *(130)*. Supplementation of vitamin E prevents glucose-induced lipid peroxidation in rat mesangial cells *(136)*, porcine vascular smooth muscle cells *(137)*, the

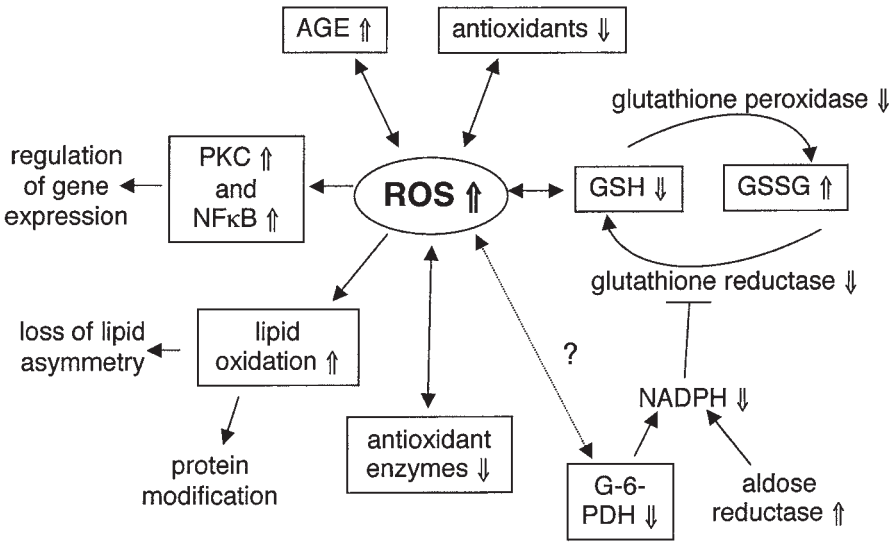

Fig. 2. Production of reactive oxygen species (ROS) depletes antioxidants and antioxidant enzymes, leading to additional ROS accumulation (denoted by double-headed arrows). This ROS accumulation subsequently causes cellular damage, such as lipid and protein oxidation and protein modification, and activates signaling pathways through protein kinase C and NFκB.

renal cortex of STZ-induced diabetic rats *(138)*, and in plasma of IDDM patients (*139,140*). The fact that vitamin E is depleted in diabetes and that supplementation has a protective effect implicates a role for this antioxidant in the prevention of diabetic complications, likely through a decrease in lipid peroxidation.

Vitamin C

Vitamin C is a major hydrophilic antioxidant in both plasma and the cytosol of many cells. Vitamin C contributes to the neutralization of many water-soluble oxidants and acts synergistically with vitamin E to terminate radical-induced lipid oxidation *(141)*. Vitamin C levels are reduced in plasma from patients with NIDDM *(130)* and metabolic syndrome *(142)*, in mononuclear leukocytes from IDDM patients *(143)*, and in the plasma, liver, and kidneys of STZ-induced diabetic rats *(144,145)*. The amount of vitamin C present in the plasma is inversely related to the duration of NIDDM *(130)*. Treatment of diabetic rats with the antioxidant α-lipoic acid *(144)* or insulin *(145)* partially suppresses the loss of vitamin C in the plasma, liver, and kidney, indicating the importance of overall antioxidant status and glycemic control in the maintenance of vitamin C levels. Whether reduction of vitamin C levels in diabetes contributes significantly to diabetic complications is still unclear, however.

Glutathione

Glutathione (GSH) is the primary intracellular free radical scavenger, though it also plays a role in the maintenance of plasma antioxidant status and is the cofactor for several enzymes. GSH contains a free thiol group capable of undergoing disulfide bond formation with another molecule of GSH or another thiol-containing molecule. The relative amount of intracellular reduced and oxidized GSH is a measure of the cellular redox status. GSH is synthesized in two steps, using the amino acids glycine, cysteine, and glutamate, and is the cofactor for many antioxidant enzymes, such as GSH peroxidase, GSH reductase, and GSH-*S*-transferase. The role of these enzymes in diabetic complications will be discussed later in this article.

Table 1
Tissue Specificity of the Effects of Hyperglycemia and Diabetes on Antioxidants, Antioxidant Enzymes, and Cellular Damage

	Erythrocytes	Leukocytes	Endothelial cells	Mesangial cells	VSMCs	Kidney	Liver	Aorta	Heart	Retina	Other	Plasma
GSH peroxidase	↓*(132,152)* ↑*(130)*	↓*(150,167)*	↓*(151)*		NC *(137,147)*	NC*(151)*	NC*(151)*					
GSH reductase	↓*(152)*	NC*(155)*	NC*(151)*		NC*(147)*	↑*(144)* NC *(171,151)*	NC*(151)*					
CuZn-SOD	↓*(130)* ↑*(132)*		↓*(151)*	↑*(146)*	↑*(137)*	↑*(144)* NC*(151)*	↓*(151)*					
Mn-SOD			NC*(151)*	↑(146)	↑*(137)*	↓(138) NC*(151)*	↓*(151)*					
Catalase	↓*(130)* ↑*(152)*	↑(167)	↓*(151)*		NC*(147)*	↓*(144)* NC*(151)*	↓*(151)*					
G6PDH	↓*(152,174)*	↓*(175)*	↓*(173)* NC*(177)*			↑*(176)*					↓(174)	
Vitamin E	↓(134,135)											↓(44, 130–133)
Vitamin C		↓*(143)*				↓*(144,145)*	↓*(144,145)*					↓(130,142, 144,145)
Reduced GSH	↓*(130,132, 148,149, 152,153)*	↓*(143,150)*	↓*(151)*	↓*(146)*	↓*(132,147)*	↓*(144,150)* NC*(151)*	↓*(151)*			↓*(81)*	↓*(154)*	↓*(148)*
Oxidized GSH									↑*(156)*			
MDA	↑*(61, 130, 134, 152,209–210)*	↑*(44)*		↑*(146)*	↑*(137,147)*	↑*(138)*		↑*(180)*			↑*(209)*	↑*(130,132, 148,211, 212)*
4-HNE						↑*(150)*	↑*(220)*					
LOOH			↑*(229)*				↑*(150)*			↑*(81)*		↑*(131–133, 143,226,227)*
Isoprostanes						NC*(139)*	↑*(261)*					↑*(247, 271,261)*
AGE	↑*(289–291)*	↑*(289–291)*				↑*(289–291)*	↑*(289–291)*	↑*(289–291)*	↑*(289–291)*	↑*(289–291)*	↑*(289–291)*	↑*(289–291)*
Protein oxidation							↑*(267)*				↑*(266,268)*	↑*(132,211)*

Reference numbers appear in parentheses.

Abbreviations: ↓ = decrease; ↑ = increase; NC = no change; AGE = advanced glycation endproducts; G6PDH = glucose-6-phosphate dehydrogenase; GSH = glutathione; 4-HNE = 4-hydroxynonenal; LOOH = lipid hydroperoxides; MDA = malondialdehyde; SOD = superoxide dismutase; VSMC = vascular smooth muscle cells.

Levels of reduced GSH have been shown to decrease in many models of diabetes/ hyperglycemia and in diabetic patients. Reduced GSH is decreased in cultured mesangial cells *(146)* and vascular smooth muscle cells *(137,147)* exposed to high concentrations of glucose. It is decreased in erythrocytes *(148,149)*, retina *(81)*, and kidneys *(144,150)* of STZ-induced diabetic rats and in the liver and endothelial cells from alloxan-induced diabetic rabbits *(151)*. Reduced glutathione is also depleted in erythrocytes *(130,152,153)*, reticulocytes *(152)*, and platelets *(154)* from NIDDM patients and erythrocytes *(132)*, mononuclear leukocytes *(143)*, and polymorphonuclear leukocytes *(155)* from IDDM patients. There is no change in reduced glutathione amounts in the glomerulus of alloxan-induced diabetic rabbits *(151)*. Conversely, an increase in oxidized glutathione has also been observed in the heart from OVE26 diabetic mice *(156)*.

The erythrocyte-reduced GSH content correlates negatively with diabetic complications *(157)*, including nephropathy, retinopathy, and neuropathy, and with the duration of diabetes *(130)*. The loss of reduced GSH may be due to a drop in activity of glutamyl-cysteine synthetase *(151,153)*, the rate-limiting enzyme in the synthesis of glutathione, and to an increase in oxidation. Lastly, a decrease in oxidized GSH transport has been observed in erythrocytes from NIDDM patients *(153)*, possibly accounting for the accumulation of oxidized GSH. One key piece of data yet to be obtained is the effect of artificial GSH depletion or inhibition of oxidized GSH transport on the manifestation of diabetic complications in diabetic model systems. These results would be more supportive than correlative studies in the establishment of a role for GSH depletion in diabetic complications.

Antioxidant Treatments

Many antioxidants have been studied for their ability to ameliorate the symptoms of diabetic complications. Vitamins E and C are discussed in the preceding sections of this article. Other antioxidants have been tested as well, including carotenoids and N-acetylcysteine. Perhaps the most extensively studied supplement is α-lipoic acid. Although α-lipoic acid functions as a cofactor for some mitochondrial dehydrogenases, it also acts as a metal chelator and antioxidant *(158)*. To our knowledge, no one has yet reported the impact of diabetes on endogenous α-lipoic acid levels. Many studies have reported improvement of several parameters of hyperglycemic damage after treatment with α-lipoic acid, however. STZ-induced diabetic rats treated with α-lipoic acid exhibit decreased levels of lipid oxidation byproducts *(149,159,160)* and $O_2^{\bullet-}$ *(159)*, and an increase in reduced glutathione *(159,160)* and ascorbic acid *(161)* levels, compared with untreated diabetic rats. In addition, this antioxidant restores activity of many antioxidant enzymes, such as superoxide dismutase, catalase, and cytochrome b_5 reductase, to nondiabetic levels *(162)*. α-Lipoic acid has also been shown to increase blood flow and improve nerve conduction *(159,162)* in these animals. Similarly, α-lipoic acid improves vasodilation *(163)* and neuropathic symptoms *(164)* in human diabetic patients. Interestingly, there is some evidence that α-lipoic acid improves insulin resistance and glycemic control in high-glucose fed rats *(165)*, though the mechanism by which this occurs is unknown. These data indicate α-lipoic acid may be useful in both the prevention and treatment of diabetic complications. The question remains whether lipoic acid supplementation acts to replace endogenous levels or acts only as a soluble antioxidant.

Inhibition of Antioxidant Enzymes

Glutathione Peroxidase

Glutathione peroxidases are a class of selenoproteins that use two molecules of GSH to facilitate the reduction of oxidants. There are several isoforms that differ with respect to the oxidants used (reviewed in ref. *166*). GPX1 is the most common cytosolic isoform, whereas GPX2 is found in the gastrointestinal tract and liver. GPX3 is found predominately in plasma and milk. These three enzymes act on hydrogen

peroxide and fatty acid hydroperoxides. GPX4 is found in the testis, possibly in the mitochondria, and can also reduce phospholipid hydroperoxides (i.e., fatty acid hydroperoxides attached to phospholipids). Because of its significance in the interception of ROS, this class of enzymes has been extensively studied for its role in diabetic complications.

The effect of diabetes on GSH peroxidase activity is highly variable with respect to the model of diabetes used and even the tissue type studied. GSH peroxidase activity is increased in the renal cortex of STZ-induced diabetic rats *(138,144)* and in erythrocytes from NIDDM patients *(130)*. Conflicting results have been obtained, however. GSH peroxidase activity was demonstrated to decrease in erythrocytes and reticulocytes from NIDDM *(152)* and IDDM *(132)* patients, though the decrease in IDDM patients was dependent on the presence of complications. The discrepancy in the erythrocyte data may involve the presence of complications in the sample population, though not every study evaluated the impact of complications on GSH peroxidase activity. GSH peroxidase activity is also reduced in polymorphonuclear leukocytes from IDDM patients *(155)* and in aortic endothelial cells from alloxan-induced diabetic rabbits *(151)*. GSH peroxidase mRNA expression is decreased in mononuclear leukocytes from diabetic patients without complications, whereas it is increased in patients with nephropathy *(167)*. No change in GSH peroxidase activity was reported in the liver and glomeruli from alloxan-induced diabetic rabbits *(151)* or in vascular smooth muscle cells treated with high concentrations of glucose *(137,147)*. Therefore, with the exception of erythrocytes and the kidney, GSH peroxidase activity is consistently inhibited in diabetes and is dependent on disease progression. Interestingly, mononuclear cells isolated from normal individuals, diabetics, and diabetics with nephropathy respond differently when placed under high glucose conditions *(167)*. mRNA expression of GSH peroxidase increases in cells isolated from control subjects and diabetics without complications, whereas it decreases in cells isolated from diabetics with nephropathy. Under normal glucose conditions, GSH peroxidase mRNA is decreased in diabetics without complications and slightly increased in patients with nephropathy compared with nondiabetics. Although these data suggest hyperglycemia and diabetic complications affect the regulation of GSH peroxidase expression, the extent to which GSH peroxidase inhibition affects cell health is unclear.

Glutathione Reductase

GSH reductase uses NADPH to reduce oxidized GSH formed by the action of GSH peroxidase. This enzyme is responsible for maintaining a substantial reduced GSH pool (millimolar amounts). Thus a decrease in GSH reductase activity will impair the ability of a cell to cope with ROS. Few data are available on the role of this enzyme in diabetic complications, however. The activity of GSH reductase is decreased in erythrocytes and reticulocytes from NIDDM patients *(152)*, but is increased in kidneys of STZ-induced diabetic rats *(144)*. The increase in the diabetic kidney can be suppressed by the addition of the antioxidant α-lipoic acid, signifying regulation of either activity or expression of GSH reductase by oxidative stress. For the most part, hyperglycemia/diabetes has been determined to have no effect on this enzyme. Hyperglycemic treatment imparted no change in activity on GSH reductase in cultured vascular smooth muscle cells *(147)*. In addition, activity is unchanged in polymorphonuclear leukocytes from IDDM patients *(155)* in the renal cortex of STZ-induced diabetic rats *(138)* and in aortic endothelial cells, liver, and glomeruli from alloxan-induced diabetic rabbits *(151)*. Therefore, it does not appear GSH reductase plays a consistent role in the onset of diabetic complications because of oxidative stress.

Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme in the

pentose phosphate pathway. The pentose phosphate pathway is responsible for ribose synthesis and is the main source of NADPH, for GSH reductase and aldose reductase. G6PDH deficiencies are the most common human enzymopathies and are correlated with a decrease in red cell GSH content *(168)* and an increase in oxidative stress *(169–172)*. Only a few studies have explored the contribution of G6PDH to diabetes-induced oxidative stress, however.

G6PDH activity is decreased in aortic endothelial cells exposed to high concentrations of glucose *(173)*. This decrease is concomitant with an increase in G6PDH phosphorylation and ROS production and a decrease in cellular glutathione. The authors suggest the increase is due to protein kinase A activity, stimulated by a rise in cyclic adenosine monophosphate levels. G6PDH activity is also reduced in reticulocytes *(152)*, erythrocytes *(152,174)*, and lenses *(174)* from NIDDM patients and in lymphocytes from alloxan-induced diabetic rats *(175)*. G6PDH activity is reported to increase in the kidneys of STZ-induced diabetic rats *(176)* and to remain unchanged in venous endothelial cells exposed to high concentrations of glucose *(177)*. These data may indicate that G6PDH regulation varies in different tissues. Clearly, more research is needed to determine the impact of G6PDH inhibition on diabetic complications.

SOD

SOD converts $O_2^{\bullet-}$ to hydrogen peroxide, a less reactive ROS. There are two types of SOD in the cell: the Cu,Zn-SOD found in the cytosol, nuclear compartments, and lysosomes, and the Mn-SOD found in mitochondria. There is also an extracellular SOD found in plasma, lymph, ascites, and cerebrospinal fluid (reviewed in ref. *178*). Mn-SOD works to dismutate $O_2^{\bullet-}$ produced by oxidative phosphorylation, whereas Cu,Zn-SOD neutralizes $O_2^{\bullet-}$ from other cellular sources and that which leaks from the mitochondria. SOD is, perhaps, the most extensively studied antioxidant enzyme in the context of diabetic complications. As such, there is evidence to both support and refute its contribution.

Mn-SOD activity is inhibited in the renal cortex of STZ-induced diabetic rats *(138)* and in the liver of alloxan-induced diabetic rabbits *(151)*. mRNA expression of Mn-SOD is increased in cultured mesangial cells *(146)* and vascular smooth muscle cells *(137)* exposed to high concentrations of glucose. There is no change in Mn-SOD activity in aortic endothelial cells and glomeruli from diabetic rabbits *(151)*.

Cu,Zn-SOD activity is diminished in the liver and aortic endothelial cells of alloxan-induced diabetic rabbits *(151)* and in erythrocytes from NIDDM patients *(130)*. On the other hand, erythrocytes from IDDM patients were found to have increased Cu,Zn-SOD activity *(132)*. There is no obvious reason for this discrepancy, because the common symptom of NIDDM and IDDM, hyperglycemia is likely to be the contributing factor in diabetes-induced Cu,Zn-SOD activity regulation. Cu,Zn-SOD activity is increased in kidneys from STZ-induced diabetic rats *(144)*. mRNA expression is increased in cultured mesangial *(146)* and vascular smooth muscle *(137)* cells exposed to high concentrations of glucose. There is no change in activity in glomeruli from alloxan-induced diabetic rabbits *(151)*. Overexpression of the Cu,Zn-SOD in *db/db* mice exerts a protective effect on the diabetes-induced loss of glutathione, increase in lipid oxidation, and renal injury *(179)*.

Total SOD activity was shown to decrease in the aorta of STZ-treated rats *(180)*, but remain unchanged when cultured vascular smooth muscle cells were exposed to high concentrations of glucose *(147)*. These data may reflect a tissue-specific difference in the regulation of SOD activity within the aorta. It may also reflect differential regulation of the Cu,Zn and Mn forms of SOD.

These data support a role for SOD inhibition in diabetes-induced nephropathy, but stop short of demonstrating its role in other diabetic complications.

Catalase

Catalase mediates the reduction of hydrogen peroxide to water, assisting SOD in the complete neutralization of ROS. Although hydrogen peroxide is not very reactive with most proteins and lipids, it can react with transition metals to form hydroxyl radical, the most reactive of the ROS. Because this reaction is an important source of ROS in hyperglycemia/diabetes (*see* Glucose Autoxidation), catalase performs a vital task. In addition to its enzymatic reactions, catalase binds four molecules of NADPH, which serve to prevent and reverse enzyme inactivation *(181)*. If NADPH levels are depleted, catalase activity may decrease. Many NADPH-dependent enzymes, such as NADPH-oxidase and aldose reductase, are more active in the diabetic state. In addition, the main cellular producer of NADPH, G6PDH, is inhibited in diabetes (*see* Glucose-6-Phosphate Dehydrogenase). Taken together, these data could imply a drop in cellular NADPH levels and a lowered oxidant defense. Indeed, individuals with reduced catalase activity suffer a heightened risk of developing diabetes *(182,183)*.

Catalase activity is decreased in aortic endothelial cells and livers from alloxan-induced diabetic rabbits *(151)*, in kidneys of STZ-induced diabetic rats *(144)*, and in erythrocyte membranes from NIDDM patients *(130)*. On the other hand, Sailaja and coworkers *(152)* determined that activity was increased in erythrocytes and reticulocytes from NIDDM patients. The discrepancy between these erythrocyte results could be because one set was measured in the membrane fraction only *(130)*, whereas the other was measured in whole-cell lysates *(152)*. In addition, the hemolysate catalase activity was measured in the presence of ethanol to stabilize the enzyme, whereas the membrane-associated activity was not. Catalase mRNA expression is increased in mononuclear leukocytes exposed to high concentrations of glucose *(167)*. Depending on the source, these same cells respond differently to high-glucose conditions. Expression is upregulated in cells from control and diabetic subjects without complications which were treated with high concentrations of glucose, whereas expression is downregulated in cells from diabetics with nephropathy. Lastly, activity is unchanged in glomeruli from alloxan-induced diabetic rabbits *(151)* and in cultured vascular smooth muscle cells exposed to high glucose *(147)*. Thus catalase inhibition may only be significant in the onset of complications in certain tissues, such as the kidney and liver.

Stimulation of Aldose Reductase Activity

Aldose reductase is the first enzyme in the polyol pathway, catalyzing the conversion of aldose sugars to their respective alcohols at the expense of NADPH. There is also evidence that aldose reductase reduces certain lipid oxidation byproducts in erythrocytes *(184)*. Therefore, an increase in activity could harm cells by NADPH depletion and alcohol accumulation, while a decrease could lead to a buildup of toxic oxidation products.

Increased aldose reductase activity has been observed in erythrocytes *(185)*, retina *(50)*, lens *(186)*, and kidneys *(150)* from STZ-induced diabetic rats and in erythrocytes from NIDDM *(187)* and IDDM *(188)* patients. There are no known reports of decreased aldose reductase activity in models of diabetes mellitus. Increased aldose reductase activity has been correlated with cataract formation and retinopathy *(50,186,187,189)*, neuropathy *(190,191)*, and nephropathy *(192)*, underscoring its significance in many cell types. Erythrocyte aldose reductase activity has been correlated to that found in other tissues which are difficult to measure *(187,189–191,193–195)*. It has also been demonstrated that individual variation in aldose reductase content significantly influences the erythrocyte redox state and susceptibility to complications *(196)*.

The importance of aldose reductase in the manifestation of many diabetic complications is reflected in the clinical use of aldose reductase inhibitors in the treatment of diabetic complications *(195)*. Fidarestat reduces ROS production in retinal endothelial cells *(50)*. Tolrestat normalizes GSH and NADPH in diabetic erythrocytes *(197)*. Epalrestat reduces advanced glycation end product formation and

lipid oxidation in diabetic erythrocytes *(198,199)*. Lastly, vitamin C has been shown to inhibit aldose reductase activity stimulated by high glucose *(200)* and diabetes *(201)*, indicating its susceptibility to oxidation. Indeed, there is a reactive cysteine residue in the active site of the enzyme that decreases substrate and inhibitor binding after oxidation *(185)*. These data support a significant role for aldose reductase activation in diabetic complications. It is unclear, however, whether accumulation of product or depletion of NADPH is the contributing factor.

CONSEQUENCES OF OXIDATIVE STRESS

If ROS production overwhelms the antioxidant defenses of the cell, either because of copious production of oxidants or depletion of antioxidant defenses, many cellular components can be damaged. The key cellular targets are lipids, protein, and DNA. In addition, gene expression and protein activities can be regulated by oxidants. This is especially true for proteins involved in cell detoxification and signal transduction. This section will focus on lipid and protein damage, as well as activation of signaling pathways. A summary of these data is shown in Fig. 2 and Table 1.

Lipid Oxidation

MALONDIALDEHYDE

Malondialdehyde (MDA) is a late-stage lipid oxidation byproduct that can be formed nonenzymatically or as a byproduct of cyclooxygenase activity (Fig. 3) *(202)*. MDA is a volatile molecule that reacts, via Schiff base formation, with free amine groups of protein, lipid, and DNA. It is estimated that up to 80% of MDA is protein bound *(202)*. In addition, accumulation of MDA affects membrane organization by increasing phosphatidylserine (PS) externalization (*see* Loss of Membrane Asymmetry) *(203)*. Accumulation of MDA and MDA adducts is correlated with many disease states, such as hepatitis C *(204)*, Down syndrome *(205)*, cancer *(206)*, liver injury *(207)*, neurodegenerative diseases *(208)*, and diabetes mellitus *(202)*.

MDA levels are increased in mesangial cells *(146)*, proximal tubule cells *(209)*, vascular smooth muscle cells *(137,147)*, erythrocytes *(61,209,210)*, and mononuclear leukocytes treated with high concentrations of glucose *(44)*. MDA is also increased in plasma *(148)*, the renal cortex *(138)*, and the aorta *(180)* of STZ-induced diabetic rats. Finally, MDA formation is stimulated in the plasma of IDDM patients *(132,211)*, in reticulocytes *(152)*, erythrocytes *(130, 152)*, and plasma *(130)* from NIDDM patients, and in the plasma of obese patients *(212)*. In addition, there is a positive correlation between plasma and erythrocyte MDA accumulation and the duration of diabetes in IDDM patients *(130)*. Underscoring its nonenzymatic production by oxidants, increased MDA levels can be suppressed by antioxidant treatment of diabetic animals, humans, and in glucose-treated cells *(135,137–140,149,213)*.

Interestingly, MDA accumulation has been linked to hyperketonemia in IDDM patients, as well as to elevated glucose levels *(213,214)*. These data are supported by the fact that MDA also accumulates in endothelial cells exposed to acetoacetate *(215)*.

Although the correlative studies are suggestive, more direct evidence on an active role for MDA in the onset of diabetic complications is needed.

4-HYDROXY-NONENAL

4-Hydroxy-nonenal (4-HNE) is another lipid oxidation byproduct which can form nonenzymatically. 4-HNE is formed from scission of precursor lipid hydroperoxides and degradation of cyclic intermediates in lipid oxidation *(216,217)*. Compared with MDA, 4-HNE is more reactive with proteins, potentiated by the ability for Michael addition as well as Schiff base formation. 4-HNE will therefore not only react with lysines, but also with cysteines and histidines. In addition, 4-HNE-modified protein is not degraded as efficiently by the multicatalytic

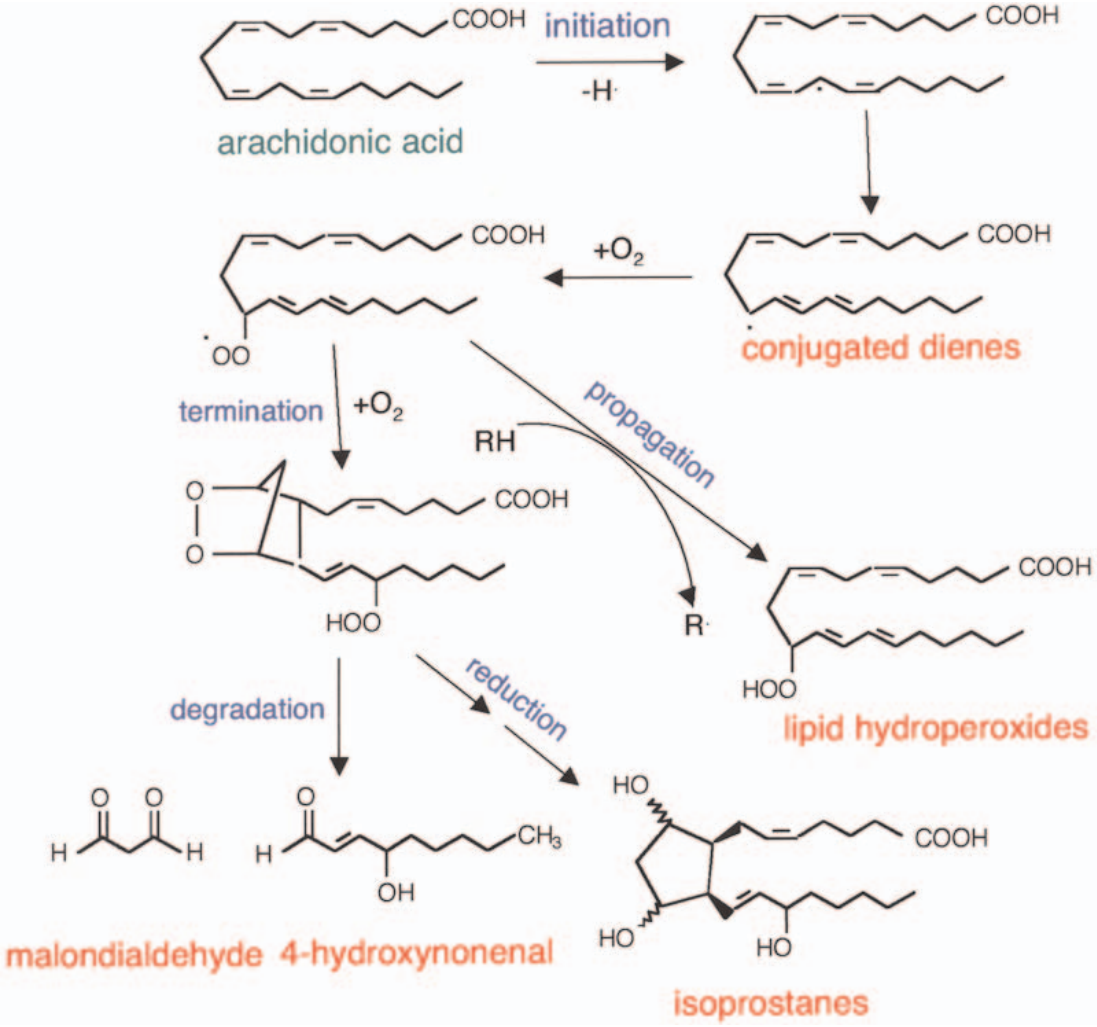

Fig. 3. Polyunsaturated fatty acids can be nonenzymatically oxidized to produce lipid oxidation byproducts. The process is inititated by extraction of a hydrogen radical from afatty acid. Molecular oxygen reacts with the fatty acid radicals to form a variety of products, including lipid hydroperoxides, isoprostanes, and malondialdehyde. These molecules are generally reactive and some, such as isoprostanes, possess biological activity. Many have been measured as markers of oxidative stress in diabetes.

proteasome *(218)*, leading to intracellular accumulation of the modified proteins. 4-HNE induces peroxide formation and activates protein phosphorylation, JNK and p38 activity, and c-Jun and AP1 expression in macrophages, thus stimulating the cellular stress response *(219)*.

Few studies have demonstrated the accumulation of 4-HNE in diabetes mellitus. 4-HNE is increased in microsomes and mitochondria of the IDDM model BB/Wor mice *(220)* and in the kidneys of STZ-induced diabetic rats *(150)*. In addition, disposal of this molecule is impaired in the BB/Wor mice through inhibition of glutathione-*S*-transferase and aldehyde dehydrogenase *(220)*. More attention has been focused on the accumulation of 4-HNE in oxidized low-density lipoprotein, a key process in the onset of atherosclerosis, which is a common diabetic complication. 4-HNE-modified low-density lipoprotein is increased in atherosclerotic lesions

from hyperlipidemic rabbits and humans with atherosclerosis *(221,222)*. To date, no work has demonstrated the role of diabetes-induced 4-HNE production in the activation of signaling pathways, leaving the issue of its importance in the onset of diabetic complications unanswered.

Lipid Hydroperoxides/Eicosanoids

Lipid hydroperoxides are intermediate lipid oxidation byproducts and are also formed enzymatically through the action of lipoxygenases (*see* Lipoxygenases). Both forms are structurally similar and can be further modified to hydroxy fatty acids, leukotrienes, and lipoxins by lipoxygenases and glutathione peroxidase. These molecules are ultimately important in regulation of inflammation and atherosclerosis *(223,224)* and initiate apoptosis in vascular smooth muscle cells *(225)*. There is evidence that both nonenzymatically and enzymatically formed lipid hydroperoxides and their derivatives are elevated in the diabetic state and are associated with a few of the diabetic complications.

Nonenzymatically produced lipid hydroperoxides are increased in the retina of STZ-induced diabetic rats *(81)* and in the plasma of NIDDM *(131,226)* and IDDM *(132,133,143,227)* patients. Because there are few or no data on the formation of nonenzymatically produced lipid hydroperoxides formed in the plasma membrane, the relative amount of these molecules versus other lipid oxidation byproducts is unclear. Elevated lipid hydroperoxide levels can be prevented by good glycemic control through continuous insulin injection *(228)*. However, elevation of these molecules is not reversed upon reinstitution of glycemic control *(81)*.

Lipoxygenase products are elevated in the kidney of STZ-induced diabetic rats *(150)*, in the urine of *db/db* mice *(126)*, and in endothelial cells treated with high concentrations of glucose *(229)*. In addition, 12-lipoxygenase knockout mice exhibit resistance to STZ-induced diabetes *(128)*, reflecting their role in inflammatory processes. Furthermore, lipoxygenase inhibition has been shown to attenuate atherosclerosis in hypercholesterolemic mice *(230, 231)*, whereas overexpression accelerates lesion formation *(232,233)*. On a cellular level, inhibition of lipoxygenases reduces monocyte binding to endothelial cells *(126,234)*, a phenomenon observed under hyperglycemic conditions *(126, 229)*. A role for these molecules in other diabetic complications has yet to be elucidated.

Isoprostanes

Isoprostanes are nonenzymatic products of arachidonic acid oxidation *(235)* that form in situ in the cell membrane and are released through the action of phospholipases. Isoprostanes are chemically similar to enzymatically produced prostaglandins, but possess opposing biological function (reviewed in refs. *12,236–239*). Isoprostanes, especially the F_2-isomers, have been shown to exert such varied action as induction of cardiomyocyte hypertrophy *(240)*, neutrophil adhesion to endothelial cells *(241)*, cell proliferation *(242)*, platelet stimulation *(243–247)*, and vasoconstriction *(235,248,249)*. Isoprostane metabolites, such as epoxyisoprostanes and epoxycyclopentenones, stimulate endothelial cell protein expression and synthesis *(250)*. Others, such as the isolevuglandins, form protein adducts through reaction with lysine side chains *(251–254)*. In addition, isoprostanes have been used extensively as indicators of oxidative stress in cigarette smoking-induced oxidation *(255)*, pesticide exposure *(256)*, chronic obstructive pulmonary disease *(14)*, atherothrombotic disease *(257)*, heart disease *(31,258)*, atherosclerosis *(31)*, and diabetes mellitus *(247,259,260)*. They offer specificity and sensitivity advantages over MDA, the classic oxidant indicator. Recent development of sensitive immunoassays has increased the accessibility of isoprostane measurement, which has traditionally been performed using mass spectrometry.

F_2-isoprostanes are elevated in the plasma and liver of STZ-induced diabetic rats *(261)* and in the plasma and urine of NIDDM patients *(247,260)*. The elevation in NIDDM patients is dependent on the presence of macrovascular complications *(262)*. No increase was observed in the kidney of STZ-induced diabetic rats

(144), indicating a tissue-specific production of these molecules. In addition, F_2-isoprostane elevation is correlated with a risk of IDDM in children *(259)* and with increased plasma homocysteine levels *(263)*. Elevated homocysteine is viewed as a predictor of cardiovascular disease, renal failure, psychiatric disorders, and complications in pregnancy. There is also some evidence that homocysteine may contribute to cardiovascular disease *(264)*. Lastly, STZ-induced diabetic rats exhibit decreased sensitivity to isoprostane-mediated vasoconstriction, possibly implying the presence of adaptive mechanisms toward vasoconstrictors in these animals *(249)*. These data are suggestive, but do not definitively support an active role for isoprostanes in the onset of diabetic complications.

Protein Oxidation

Another potential target of ROS are proteins, whose structure and function can be affected by modification. There are many side chain targets for protein oxidation including cysteine, methionine, and tyrosine. In addition, protein carbonyl content increases during oxidation and is the most commonly measured protein oxidation product *(265)*.

Protein carbonyls are elevated in cultured lens cells exposed to high concentrations of glucose *(266)*, in the liver of STZ-induced diabetic rats *(267)*, in the muscles/skeletal of the NIDDM model Otsuka Long-Evans Tokushima Fatty rat *(268)*, and in plasma from IDDM patients *(132)*. Free sulfhydryl groups are decreased in the plasma of IDDM patients *(211)*, indicating an increase in disulfide bond formation. Furthermore, there is a positive correlation between protein carbonyls and the presence of complications in IDDM patients *(132)*. The accumulation of protein carbonyls may be the result of reduced protein turnover, which is impaired in the liver of diabetic rats *(267)*. Indeed, extensively oxidized protein will inhibit the multicatalytic proteasome, the cellular machinery for protein degradation *(269,270)*. Clearly, further experimentation is necessary to elucidate the role of protein oxidation in diabetic complications.

Advanced Glycation End Products

Glucose can react directly with free amine groups on protein *(271,272)*, DNA *(271,273)*, and lipids *(274)* via Schiff base formation (Fig. 4). The Schiff base is then reduced to an Amadori product and subsequent oxidations and crosslinking occur, yielding a diverse group of modifications referred to as AGE *(275,276)*. AGE are observed primarily in long-lived structural proteins, such as collagen, vitronectin, laminin, and actin, but are also commonly formed on hemoglobin, annexins, catalase, SOD, G6PDH, and membrane proteins *(271,272,277–282)*.

AGE formation is dependent on oxidative processes *(63,283,284)* and can create ROS *(285,286)* through the Maillard reaction. In addition, glycated proteins have an increased affinity for metals *(287)*, increasing the probability of Fenton chemistry and ROS production (*see* Glucose Autoxidation). AGEs can, therefore, propagate oxidative stress in the cells and fluids in which they are produced. Extensively glycated proteins are also less susceptible to degradation by the multicatalytic proteasome *(288)*, leading to their intracellular accumulation.

AGE are found in almost all tissues examined from STZ-induced diabetic rats *(289)* and in human IDDM *(290)* and NIDDM *(291)* patients. Some tissues, such as the liver, kidneys, and erythrocytes, are more susceptible to AGE formation than others *(289,291)*, possibly owing to the longevity of the affected proteins. AGE-modified proteins affect many cellular processes, such as cell adhesion *(271)* and erythrocyte aggregation *(290)*.

Because of the importance of AGE in the development of diabetic complications, AGE inhibitors have been tested clinically *(271, 292–294)*. These molecules, such as aminoguanidine and pyridoxamine, work to trap glycoxidation intermediates and impede crosslink formation *(293,295,296)*. Many AGE inhibitors also possess antioxidant properties, as demonstrated by their ability to prevent lipid oxidation *(274,297–300)*.

AGE formed in plasma and on the surface of cells can bind to receptors known as RAGE

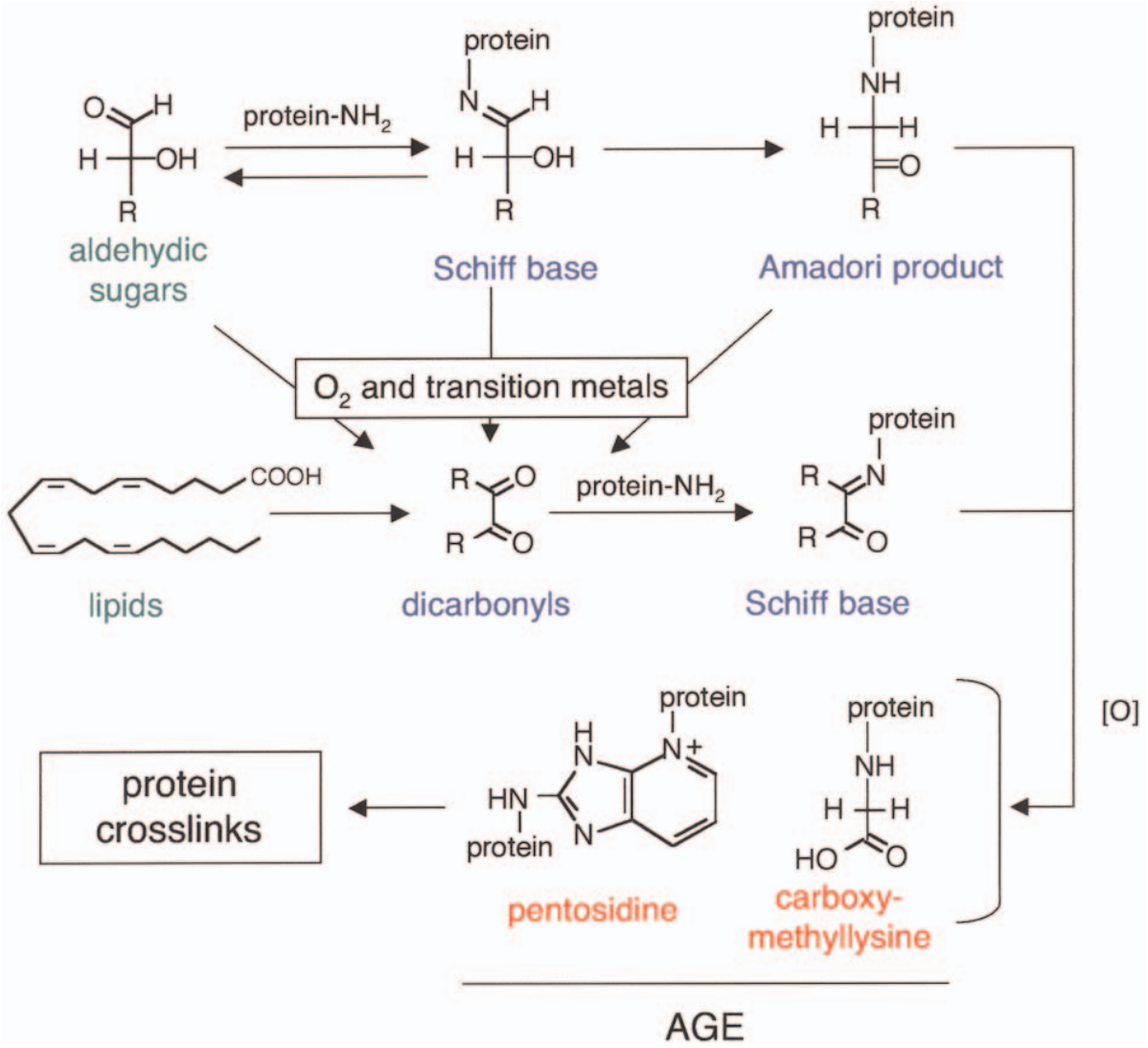

Fig. 4. Aldehydic sugars can react with free amine groups on proteins and lipids to form Schiff bases, which are subsequently reduced to Amadori products. These products react with oxygen, metals, lipids, and additional sugar molecules to create advanced glycation endproducts (AGE), which can create more crosslinks with modified proteins. Two of the most commonly measured AGE, pentosidine and carboxymethyllysine, are shown here.

(receptors for advanced glycation end products) and activate signal transduction pathways (reviewed in refs. *271,301–303*). RAGE are members of the immunoglobulin family of cell surface receptors *(304)* and are pro-inflammatory. They are found in the endothelium, retina, smooth muscle cells, monocytes, and neurons *(305)*. Binding of AGE to RAGE stimulates signaling pathways that activate NADPH oxidase, which produces ROS (*see* NADPH Oxidase) *(306,307)* and NFκB, a nuclear transcription factor (*304,307–309*). Interestingly, divergent pathways of gene expression are activated depending on the ligand bound to RAGE *(310)*. The importance of these receptors in the onset of atherosclerosis has been demonstrated by

increased lesion formation in STZ-induced diabetic mice treated with soluble RAGE *(311)*. Based on these data, AGE production and RAGE activation are likely to be significant contributors to the onset of diabetic complications, particularly atherosclerosis.

Loss of Membrane Asymmetry

Phospholipids are asymmetrically arranged in biological membranes. The choline-containing lipids, phosphatidylcholine and sphingomyelin, are located predominately in the outer leaflet, whereas the amine-containing lipids, PS and phosphatidylethanolamine, are sequestered in the cytoplasmic leaflet. Exposure of PS on the cell surface triggers the blood clotting cascade and thrombin production *(312)*, increases adherence of erythrocytes and platelets to vascular endothelial cells *(313,314)*, and serves as a signal for engulfment by macrophages *(315)*. In addition, a loss of lipid asymmetry in the membranes of intracellular organelles may have profound effects on cellular trafficking and, therefore, cell health.

PS externalization has been demonstrated in erythrocytes from NIDDM patients *(316)* and in erythrocytes exposed to hyperglycemic conditions *(317)*. Diabetic erythrocytes exhibit increased adhesion to vascular endothelial cells *(318)* and thrombin levels are increased in plasma from NIDDM patients *(319)*. In addition, PS externalization has been demonstrated in cells exposed to oxidants *(203,320–322)*, providing a potential mechanism for the loss of lipid asymmetry. The mechanism by which oxidants induce a loss of lipid asymmetry is unknown. One possible mechanism is the oxidant-mediated induction of a phospholipid "scramblase." This protein move lipids bidirectionally in the plasma membrane and has been reported to be activated by oxidant treatment *(323,324)*. To date, there has been no report of diabetes-induced upregulation of this activity. If PS externalization is a significant contributor to diabetic complications, its contribution is likely to be restricted to blood cells and cells undergoing apoptosis (*see* Induction of Apoptosis).

Activation of Intracellular Signaling Pathways

Protein Kinase C

PKC is a peripheral membrane protein through which some lipid-mediated signals are transmitted *(325,326)*. Stimulation of PKC affects vasoconstriction, smooth muscle growth, endothelial cell permeability, and other processes *(327–329)*. There is evidence that PKC is stimulated by ROS, though an increase in diacylglycerol levels may also activate this protein *(330)*. PKC phosphorylates the NADPH oxidase, stimulating translocation to the membrane and $O_2^{\bullet-}$ production *(327,331,332)*, and providing a means for propagation of oxidative stress (*see* NADPH Oxidase).

PKC activity is increased in the glomeruli *(333–335)*, retina *(336,337)*, aorta, and heart *(330, 338)* of STZ-induced diabetic rats and in aortic smooth muscle cells *(339)*, and monocytes *(332)* exposed to high concentrations of glucose. Isoform activation by hyperglycemia is tissue specific. The β_2 isoform is activated in the retina, aorta, and heart *(338)*, whereas the α isoform is activated in monocytes *(332)*. PKC δ is also activated in aortic smooth muscle cells exposed to hyperglycemia or diabetes *(339)*. There is a concomitant increase in diacylglycerol levels in the aorta and heart *(330)*, implying that substrate accumulation may lead to activation of PKC in these tissues. In many diabetic models, the stimulation of PKC activity can also be inhibited by treatment with vitamin E *(332,335–337,340)*, indicating the importance of oxidation in the activation of this enzyme. The reduction in activity on vitamin E treatment is likely due to a decrease in diacylglycerol levels, because this antioxidant has no effect in nondiabetic animals *(335)*.

Inhibition of PKC activity with staurosporine abrogates hyperglycemia–induced-leukocyte adherence to endothelial cells *(341)*, a key event in atherosclerotic plaque formation, and the hyperglycemia-induced increase in retinal endothelial cell permeability *(336, 337,340)*. Because of PKC inhibtion of these events, PKC may be a clinically impor-

tant target for the prevention of diabetic complications *(327)*.

NFκB

NFκB is a transcription factor that regulates expression of genes such as tumor necrosis factor, tissue factor, cell adhesion molecules, and chemotactic proteins *(342)*. It is present in the cytosol and, after activation by phosphorylation of an inhibitor protein or by another means, translocates to the nucleus. NFκB is activated by a diverse set of regulators and disease conditions *(343)*, underscoring its widespread role in the regulation of cellular processes. Several NFκB regulators are important in diabetic complications. These include PKC and oxidants. NFκB is also activated by the stimulation of RAGE by AGE *(307,309)*, an event that activates both PKC and NADPH oxidase, leading to the generation of ROS (*see* NADPH Oxidase and PKC; reviewed in ref. *344)*.

Treatment of endothelial cells with high concentrations of glucose stimulates nuclear translocation *(345)* and DNA binding *(309, 341,346)* of NFκB. Activation of NFκB in these cells induces apoptosis *(346)* and is suppressed by antioxidants *(309)*. Direct inhibition by pyrrolidinedithiocarbamate reduces leukocyte adhesion to endothelial cells, a key event in atherosclerosis *(341)*. Accordingly, activated NFκB is present in atherosclerotic lesions *(347)* and is thought to facilitate the expression of genes involved in plaque formation, such as vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and monocyte chemotactic protein (MCP)-1. NFκB is, therefore, likely a key downstream effector of glucose-mediated damage.

Mitogen-Activated Protein Kinases

Mitogen-activeated protein (MAP) kinases are regulatory proteins, involved in signal transduction, which transmit signals from the membrane to the nucleus through phosphorylation of downstream proteins (reviewed in ref. *348–350*). These proteins respond to extracellular stimuli, such as radiation, osmotic and pH changes, mechanical and oxidative stress, growth factors, and cytokines. They serve to regulate such diverse events as embryogenesis, cell division, cell proliferation, and cell death *(348–350)*. There are several classes of MAP kinases, each responding to different stimuli and regulating expression of a unique set of genes *(348)*. The three classes of MAP kinases proposed to be involved in the onset of diabetes or diabetic complications are p38 MAP kinases, c-jun N-terminal kinases (JNKs), and extracellular signal-related kinases (ERKs).

p38 MAP kinases translate signals from chemokines, neurotransmitters, radiation, physical, and chemical stresses *(348)*. This subfamily regulates inflammation, differentiation, and proliferation, among other processes *(351,352)*. p38 MAP kinase activity is increased in vascular smooth muscle cells *(339)*, retinal pericytes *(339)*, and mesangial cells *(339)* exposed to high concentrations of glucose. MAP kinases are activated via phosphorylation by upstream kinases *(348,351,352)*. Thus p38 phosphorylation, rather than MAP kinase activity, is routinely used as a measure of activation. Indeed, high concentrations of glucose stimulates p38 phosphorylation in renal tubule cells *(353)*. Treatment of mesangial cells with methylglyoxal, an AGE intermediate and byproduct of glycolysis, also increases p38 phosphorylation *(354)*. In addition, phosphorylated p38 is increased in aorta *(339)*, dorsal root ganglia *(355)*, and sciatic nerve *(355)* from STZ-induced diabetic rats; in skeletal muscle *(356)*, adipocytes *(357)*, and platelets *(358)* from NIDDM patients; and in the livers of *ob/ob* mice *(359)*. Diabetes-induced p38 MAP kinase activation and activity can be suppressed by inhibitors of p38 MAP kinase *(339,353–355)*, PKC *(339)*, aldose reductase *(355)*, AGE *(354)*, antioxidants *(354)* and insulin treatment *(355,356)*. In addition, inhibition of MEK, the upstream kinase that activates p38, also reduces p38 phosphorylation *(353)*. Inhibition of p38 MAP kinase reduces apoptosis *(354)* and arachidonic acid release *(339)* and improves sensory nerve conduction *(360)*. In addition, translocation of the insulin-responsive glucose

transporter, GLUT4, is increased over untreated controls *(357)*, indicating a role for p38 in insulin resistance.

Fewer studies have been performed to elucidate the involvement of JNKs and ERKs in diabetic complications. Similar to p38, JNKs and ERKs are activated by phosphorylation *(348)*. JNKs are a family of MAP kinases, also known as stress-activated protein kinases, that respond to radiation, growth factor deprivation, cytokines, and environmental stressors *(348–350)*. Phosphorylated JNK is increased in adipocytes from NIDDM patients *(357)*; in liver, fat, and muscle from obese mice *(361)*; and in vascular smooth muscle cells exposed to high concentrations of glucose *(362)*. Deletion of JNK1 reduces body weight, plasma glucose, and plasma insulin levels and improves insulin sensitivity in *ob/ob* mice, most likely because of a decrease in the inhibitory phosphorylation of the insulin receptor substrate-1 *(361)*. ERKs are activated by growth factors, neurotransmitters, and hormones, thus mediating the cellular response to these molecules *(348)*. ERKs are activated in mesangial cells cultured under hyperglycemic conditions *(353)* and in adipocytes from NIDDM patients *(357)*. In addition, high concentrations of glucose stimulate ERK phosphorylation in vascular smooth muscle cells from aged rats *(362)*. Glucose does not stimulate ERKs in vascular smooth muscle cells from young rats *(362)*, indicating an age-dependent regulation of these proteins. Although these results are suggestive, a direct link between MAP kinases and diabetes-induced oxidative stress has yet to be established.

Induction of Apoptosis

Apoptosis, or programmed cell death, is a process triggered by extensive cellular damage. After it is triggered, a highly regulated order of events occurs in which DNA is cleaved into small fragments, proteins are degraded, PS is exposed on the outer surface of the cell, and cellular structures are disassembled *(363)*. The cell remnants are engulfed by macrophages that recognize the surface-exposed PS by means of a PS receptor *(315)*. The classical apoptotic pathway involves proteolytic caspases, which function in the breakdown of cellular proteins *(363–366)*.

Induction of apoptosis has been implicated in diabetic neuropathy *(367–369)*, cardiomyopathy *(370)*, and in atherosclerotic plaque instability *(225)*. Apoptosis in diabetic models is correlated with disruption of mitochondrial membrane potential *(371)*, cytochrome c leakage to the cytosol *(371)*, and caspase 3 activation *(370,372)*. These data indicate diabetes-induced apoptosis proceeds through the classical pathway of protease activation. Apoptosis is induced in endothelial cells *(373)*, dorsal root ganglia *(374)*, and cardiac myocytes *(370)* exposed to hyperglycemic conditions, in dorsal root ganglia from STZ-induced diabetic mice *(371,372,374,375)* and in myocytes, endothelial cells, and fibroblasts from NIDDM patients *(55)*. Treatment of diabetic rats with insulin *(371)* or overexpression of UCP1 *(367)* inhibits apoptosis in the dorsal root ganglia, indicating that glucose induces apoptosis through mitochondrially derived oxidative stress. These findings are supported by research suggesting vascular endothelial cell apoptosis is inhibited by antioxidant treatment *(376)*. Although the studies to date provide intriguing results, this line of research is still relatively new. More insight is needed to discern the relationship between apoptosis and diabetic complications, particularly cardiomyopathy and atherogenesis.

Induction of Cyclooxygenase Expression

Cyclooxygenases (COXs) mediate a myriad of tissue functions through the production of cyclic arachidonic acid derivatives such as prostaglandins, prostacyclins, and thromboxanes. COX exist in multiple isoforms: the constitutively expressed COX1 and the inducible COX2 are the most extensively studied. These isoforms work together to regulate such varied processes as blood flow, renal function, nerve and brain function, uterine contraction, bone resorption and formation, inflammation, and

pain *(377)*. COX2 expression is induced by proinflammatory cytokines through NADPH oxidase stimulation and ROS production *(378)*

Because of their diverse roles in tissue function, cyclooxygenases, specifically COX2, have been proposed to mediate many of the diabetic complications, including nephropathy, retinopathy, neuropathy, and atherosclerosis. COX2 expression is induced in the sciatic nerve *(379)*, renal cortex *(380)*, and renal medulla *(381)* of STZ-induced diabetic rats. Monocytic cells under hyperglycemic conditions upregulate COX2 expression *(382)*. COX2 expression is also induced in human mesangial cells exposed to high concentrations of glucose *(383)*, in atherosclerotic plaques from NIDDM patients *(384)*, and in retina from IDDM and NIDDM patients *(385)*. In addition, COX1 expression is induced in the renal medulla *(381)* of STZ-induced diabetic rats. Expression of COX enzymes is normalized by glycemic control *(381,384)*, by inhibition of oxidative phosphorylation *(382,383)*, protein kinase C, NFκB, p38 MAP kinase *(382)*, or RAGE *(384)*, or by mutating the NFκB binding elements in the COX2 promoter (*383*). The latter observations indicate the importance of oxidative stress in the induction of COX expression. Inhibition of COX2 normalizes nerve blood flow and conductance *(379)* and kidney perfusion pressure *(380)* in diabetic rats, indicating the potential importance of these enzymes in diabetic neuropathy and nephropathy.

SUMMARY AND CONCLUSIONS

Oxidative stress is an important mediator of diabetic complications. ROS alter membrane and protein structure, protein function, and interfere with signaling pathways. The sources of ROS are several. Arguably the most important hyperglycemia-inducible ROS generator is the mitochondrion, which produces $O_2^{\bullet-}$ from the partial reduction of oxygen. Lipoxygenases, NOS and cytochrome P450 monooxygenases produce ROS through enzyme uncoupling and release of product intermediates. In addition, NOS can substitute molecular oxygen for its substrate, yielding $O_2^{\bullet-}$. Xanthine oxidase produces ROS as a byproduct of its reactions. NADPH oxidase produces $O_2^{\bullet-}$ as its product, a bactericidal function originally detailed in neutrophils. In nonphagocytic cells, this $O_2^{\bullet-}$ production has been co-opted for use in signaling pathways. Another mechanism of ROS production is through the autoxidation of glucose or other aldehydes. Glucose radicals can react with metals and hydrogen peroxide to create hydroxyl radical, the most reactive ROS. This mechanism may be especially important in cells lacking mitochondria, such as erythrocytes.

After ROS are created, they deplete cellular antioxidant defenses, including vitamins E and C and GSH and, in many cases, reduce antioxidant enzyme activity. GSH peroxidase, reductase, catalase, superoxide dismutase, and G6PDH activities have been shown to decrease in various tissues exposed to hyperglycemia or diabetes. Aldose reductase activity is stimulated by hyperglycemia and may be responsible for depletion of NADPH, an important cofactor of GSH reductase. Collectively, these alterations impede the ability of a cell to neutralize additional production of oxidants. In addition, NO, produced by NOS, will react with $O_2^{\bullet-}$, creating peroxynitrite, another potent oxidant, and depleting NO, a vasorelaxant. Reports of the effect of diabetes on these antioxidant defenses are mixed, however, and the effects appear to be tissue-specific.

When oxidants are produced beyond the capability for neutralization, cellular components are damaged. Lipids, especially those containing polyunsaturated fatty acids, are oxidized to form reactive molecules such as MDA and 4-HNE. These aldehydes will react with protein and DNA, altering their function and potentially inducing genetic mutations. Both molecules are produced in diabetes. Isoprostanes, arachidonate-derived lipid oxidation byproducts, are potent vasoconstrictors and stimulate gene expression in endothelial cells and cardiomyocytes. Isoprostanes have become popular markers of lipid oxidation in diabetes, and other diseases, in part because of the

specificity and sensitivity of detection. Lipid oxidation may also induce a loss of membrane asymmetry, leading to activation of blood clotting factors, cell adhesion, and engulfment by macrophages. A loss of lipid asymmetry may also occur in the membranes of internal organelles. This could potentially affect cellular processes, such as membrane trafficking, and protein activity, such as that of PKC. Protein oxidation and AGE formation can alter protein structure and impede activity. Extensively modified proteins are not degraded efficiently by the multicatalytic proteasome, leading to accumulation of damaged protein. In addition, AGE bind to cell surface receptors, activating signal transduction pathways and stimulating gene expression. Signal transduction pathways can also be activated by ROS directly, through proteins such as PKC and NFκB. Depending on the genes expressed, cellular processes and cell–cell interactions may be affected. This may be especially pertinent in the onset of atherosclerosis, which is initiated by monocyte adhesion to endothelial cells.

In conclusion, there is considerable evidence that induction of oxidative stress is a key process in the onset on diabetic complications. The mechanisms and pathways involved are complex. ROS are produced by mitochondria and various enzymes, including NADPH oxidase, xanthine oxidase, and NOS. The role of the individual sources varies significantly with the tissue and the model of diabetes studied. After it is formed, ROS can deplete antioxidant defenses, such as free radical scavengers and ROS detoxifying enzymes. If ROS are not detoxified, cellular components are damaged and signaling pathways are altered. Further elucidation of these pathways may lead to new methods for the prevention of diabetic complications, as well as novel treatment options for existing conditions.

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