

## Forum Review

# Mechanisms for Oxidative Stress in Diabetic Cardiovascular Disease

SUBRAMANIAM PENNATHUR<sup>1</sup> and JAY W. HEINECKE<sup>2</sup>

### ABSTRACT

Obesity, metabolic syndrome, and diabetes are increasingly prevalent in Western society, and they markedly increase the risk for atherosclerotic vascular disease, the major cause of death in diabetics. Although recent evidence suggests a causal role for oxidative stress in insulin resistance, diabetes, and atherosclerosis, there is considerable controversy regarding its nature, magnitude, and underlying mechanisms. Glucose promotes glycooxidation reactions *in vitro*, and products of glycooxidation and lipoxidation are elevated in plasma and tissue from humans suffering from diabetes, but the exact relationships between hyperglycemia and oxidative stress are poorly understood. This review focuses on molecular mechanisms of increased oxidative stress in diabetes, the relationship of oxidant production to hyperglycemia, and the potential interaction of reactive carbonyls and lipids in oxidant generation. Using highly sensitive and specific gas chromatography–mass spectrometry, molecular signatures of specific oxidation pathways were identified in tissues of diabetic humans and animals. These studies support the hypothesis that unique reactive intermediates generated in localized microenvironments of vulnerable tissues promote diabetic damage. Therapies interrupting these reactive pathways in vascular tissue might help prevent cardiovascular disease in this high-risk population. *Antioxid. Redox Signal.* 9, 955–969.

### INTRODUCTION

**D**IABETES MELLITUS is a serious global health problem. A recent study by the World Health Organization estimated that the worldwide prevalence of this disease is expected to grow from 171 million in 2000 to 366 million by 2030 (142). Roughly 21 million people in the United States (7%) are afflicted by this disorder, and 54 million are at risk. Moreover, type 2 diabetes is being diagnosed more frequently in children and adolescents, particularly in American Indians, African Americans, and Hispanic/Latino Americans. Twenty percent of Americans over the age of 60 are diabetic, making the disease one of the most prevalent disorders in the geriatric population (3).

Hyperglycemia is the metabolic hallmark of both type 1 and type 2 diabetes. Type 1, which accounts for 5–10% of all cases in the United States, develops when the immune system

destroys the pancreatic  $\beta$ -cells, drastically decreasing insulin production. Type 2, which accounts for 90% of cases, is a complex, multisystem disease characterized by both insulin resistance in insulin-sensitive tissues (skeletal muscle, liver, and adipose tissue) and progressive pancreatic  $\beta$ -cell dysfunction. These deficits ultimately result in too little insulin to maintain blood glucose levels in the normal range.

Both type 1 and type 2 diabetes markedly increase the risk of microvascular and macrovascular complications. Microvascular complications, which center on dysfunction in the capillary bed of tissues, are wide-ranging, and include the retinopathy, nephropathy, and neuropathy that eventually affect nearly all patients with diabetes. Diabetic retinopathy is the major cause of adult blindness in the United States. Diabetic neuropathy, which affects roughly half of all diabetic patients is the most common cause of nontraumatic amputations (38) and diabetic nephropathy is the major cause of end-stage renal disease (24).

<sup>1</sup>Department of Medicine, University of Michigan, Ann Arbor, Michigan.

<sup>2</sup>Department of Medicine, University of Washington, Seattle, Washington.

Macrovascular complications due to atherosclerosis remain the leading cause of death in diabetic humans. Myocardial infarction, stroke, and peripheral vascular disease are two to four times more prevalent in such patients (24). Moreover, atherosclerosis occurs earlier, and follows a more aggressive course (45). Thus, the cardiovascular event rate in diabetic patients without documented coronary artery disease (CAD) is equivalent to that of nondiabetic patients with CAD (45, 46). Moreover, diabetic patients have higher mortality following myocardial infarction than nondiabetic subjects (45). Women with diabetes lose their premenopausal cardioprotection, and are vulnerable to CAD at the same rate as men (62).

Whereas tissue-specific factors may accentuate diabetic damage, it has become increasingly clear that microvascular and macrovascular diseases share a common pathophysiology. During the past two decades, considerable evidence has implicated oxidative stress in several distinct conditions, including aging, atherosclerosis, neurodegenerative diseases, diabetes, and end-stage renal disease (reviewed in Refs. 4, 5, 15, 56, 72, 123). Oxidative stress occurs when there is an imbalance in the relative rates of oxidant generation and oxidant scavenging, with a subsequent increase in the level of oxidized biomolecules and associated tissue damage.

Accumulating evidence points to hyperglycemia-induced increases in the production of oxidants and other reactive intermediates as one important mechanism for the initiation and progression of endothelial dysfunction in diabetic subjects (16, 61). Recent advances in our understanding of oxidative stress, insulin resistance, and diabetes have focused on mass spectrometric identification of oxidative modifications to tissue proteins and lipids, characterization of redox signaling pathways that are activated by oxidative stress and diabetes, the central role for oxidative stress in all forms of insulin resistance (61), identification of specific cellular organelles (such as mitochondria) and enzymes (such as myeloperoxidase) that generate reactive intermediates, and the discovery that oxidants can activate apoptotic pathways.

In this review, we discuss the proposal that oxidative stress might play a causal role in both atherosclerosis and diabetes, and consider how glucose-induced oxidative stress may lead to microvascular and cardiovascular complications. We will also cover the potential relationship of hyperglycemia and lipids to oxidant-generating pathways and the rationale for therapies aimed at decreasing oxidative stress. One key issue has been to identify specific oxidative pathways that contribute to diabetic complications. Our overall strategy has been to develop sensitive and specific mass spectrometric methods for defining molecular fingerprints of oxidative pathways *in vitro* and, using those tools, to collect evidence that those pathways are pathophysiologically relevant *in vivo*. This is a critical prelude to rationally designing and testing antioxidant therapies.

## PATHOPHYSIOLOGY OF ATHEROSCLEROSIS AND DIABETIC COMPLICATIONS

### *Inflammation, oxidative stress, and atherosclerosis*

Atherosclerosis is a chronic inflammatory disease characterized by infiltration of lipids and inflammatory cells, such

as monocyte-derived macrophages and T-lymphocytes, into the artery wall (117). Although it is well known that elevated levels of low-density lipoprotein (LDL) greatly increase the risk for atherosclerosis (14), *in vitro* studies suggest that LDL by itself is not atherogenic but needs to be modified to initiate atherosclerotic disease (42, 144). This conclusion led to the 'oxidation hypothesis', which proposed that LDL must be oxidatively modified to become atherogenic. Many lines of evidence support this hypothesis. Oxidized LDL is taken up by scavenger receptors of macrophages, which then become lipid-laden foam cells, the pathologic hallmark of early atherosclerotic lesions (129). Oxidized LDL has been isolated from human and animal atherosclerotic tissue, and immunohistochemical studies have detected oxidized lipids in atherosclerotic lesions (44, 146, 147). All major cell types involved in atherosclerosis—smooth muscle cells, endothelial cells, and macrophages—produce reactive oxidants that can oxidize LDL *in vitro* (59, 96, 130). Moreover, oxidized LDL attracts mononuclear cells and stimulates the production of monocyte chemoattractant protein-1 and other inflammatory cytokines, leading to the conversion of fatty streaks to more advanced complex lesions as smooth muscle cells migrate from the media into the subendothelial space. Oxidized LDL may also stimulate smooth cells to synthesize extracellular matrix and activate a signaling cascade by interacting with the lectin-like OxLDL receptor (41, 129). Finally, several structurally unrelated lipid-soluble antioxidants that inhibit LDL oxidation *in vitro* also inhibit atherosclerosis in hypercholesterolemic animals (22, 76, 106).

### *Hyperglycemia and diabetic complications*

Both the degree of glycemic control and the duration of diabetes predict the risk of diabetic complications (79, 98). These observations have given rise to the "glucose hypothesis," which suggests that glucose mediates many of the deleterious effects of the disease. Both the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) found that strict glycemic control dramatically lowered the incidence of retinopathy, nephropathy, and neuropathy (29, 98, 133, 134). The majority of patients involved in the DCCT trial were subsequently followed up in the Epidemiology of Diabetes Interventions and Complications (EDIC) study. Glycemic control in the intensive treatment group and the conventional therapy group were similar during the EDIC follow-up. Interestingly, the DCCT/EDIC study demonstrated that a period of intensive diabetes therapy during the DCCT had a long-term, beneficial effect on the subsequent risk of microvascular complications (30). The pathophysiological mechanisms responsible for the improvement are unclear and are referred to as "metabolic memory."

Diabetes also strongly increases the risk for atherosclerotic macrovascular disease, which is two to four times more common in diabetic men than in the general population and is prevalent even in premenopausal diabetic women. Recent data from the DCCT/EDIC cohort showed that, during the mean 17 years of follow-up, only 46 cardiovascular events occurred in 31 patients who had received intensive treatment in the DCCT, compared with 98 events in 52 patients who had received conventional treatment. Intensive treatment reduced

the risk of any CAD event by 42% and the risk of nonfatal myocardial infarction, stroke, or death from CAD by 57%, strongly suggesting that glycemic control lowers macrovascular disease end points as well (99). Additionally, intensive therapy during the DCCT associated with decreased progression of intima—medial thickness, a surrogate marker for atherosclerosis, 6 years after the end of the trial (100). The UKPDS suggested a trend toward less macrovascular disease with glucose-lowering therapy, but the difference did not reach statistical significance.

These findings suggest that hyperglycemia promotes atherosclerosis and its associated complications. Therefore, glucose itself may be toxic to the artery wall. However, strict glycemic control alone does not prevent diabetic complications, suggesting the involvement of additional factors. Proposed factors include abnormalities in lipoproteins, metabolic derangements (insulin resistance, elevated free fatty acid levels, etc.), and variations in genes controlling lipid metabolism, all of which might be important in macrovascular as well as microvascular disease (122). Moreover, the presence of nephropathy, as measured by microalbuminuria and albuminuria, associated with a significant increase in the risk of CAD in the DCCT/EDIC cohort, suggesting common pathophysiological mechanisms in microvascular and macrovascular disease (99).

#### *How does hyperglycemia lead to diabetic complications?*

Several mechanisms have been proposed to explain how hyperglycemia mediates diabetic damage. They include formation of advanced glycosylation end products (AGEs) (5, 6, 15, 16, 20, 120, 131), oxidative stress (5, 6, 95), carbonyl stress (5, 6, 95), increased protein kinase C (PKC) activity (70), altered growth factor or cytokine activities (112, 124), reductive stress or pseudohypoxia (67, 143), and mitochondrial dysfunction (16, 101). Some of these hypotheses overlap. For example, AGEs might promote growth factor expression and oxidative stress, and oxidative stress might promote AGE formation (5, 6, 95). Mitochondrial superoxide can result in oxidative stress, form AGE products, and increase PKC activity (16). It is likely that different tissues are differentially vulnerable to hyperglycemia, depending on which biochemical pathways are affected.

#### *Insulin resistance, visceral adiposity, and CAD*

Obesity and visceral adiposity frequently associate with diabetes and insulin resistance. Also, insulin resistance clusters with the metabolic syndrome, a constellation of classic CAD risk factors such as lipid abnormalities, hypertension, and impaired glucose tolerance. Emerging data support the hypothesis that oxidative stress plays a causal role in insulin resistance (61) and might be linked with visceral adiposity. Mature adipocytes function as an endocrine/paracrine organ that secretes numerous adipokines, cytokines, and growth factors, particularly in the setting of insulin resistance. A significant number of these proatherogenic factors can be released from components of visceral fat, such as infiltrating macrophages, that are not adipocytes (139, 140). Several possible mechanisms link obesity to CAD, such as the increased

levels of nonesterified fatty acids (NEFA) that result from increased lipolysis and disturbances in adipokine and cytokine secretion. These processes in turn are linked with insulin resistance (135). NEFAs are independently associated with CAD mortality in older men (113). Several adipokines and cytokines, such as adiponectin, interleukin-6 (IL-6), retinol-binding protein-4 (RBP-4), resistin, and tumor necrosis factor- $\alpha$ , are associated with insulin resistance. RBP-4 is an adipocyte-derived molecule that is elevated prior to the onset of diabetes (43), and it appears to impair insulin signaling in muscle and promote insulin resistance (1, 145). Visceral fat releases IL-6, which can contribute to local and systemic inflammation and elevation of C-reactive protein levels (135). Thus, insulin resistance is intricately linked with visceral adiposity and oxidative stress, and it may promote endothelial dysfunction and CAD.

### **OXIDATIVE STRESS IN DIABETES AND ATHEROSCLEROSIS**

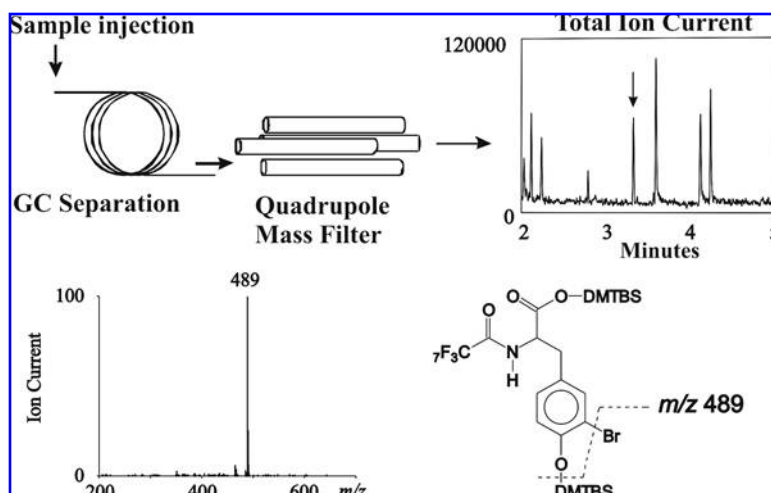
#### *Oxidative damage in diabetes and atherosclerosis*

Although oxidative stress has a well-established role in diabetic complications and atherosclerosis, its origins, magnitude, and localization are controversial. Moreover, it is not known whether oxidative stress is a primary event that occurs early in the disease or whether it represents a secondary phenomenon that merely reflects end-stage tissue damage (6). This distinction has important clinical relevance. If oxidative stress simply reflects tissue damage, interventions that reduce it may fail to affect the disease process. If oxidative stress promotes tissue injury, therapies that interrupt oxidative pathways early in the disease may prevent complications, and those that act later may slow disease progression.

#### *Mass spectrometry is a powerful approach for detecting biomarkers of oxidative stress in vivo*

Immunohistochemistry and dihydroethidium fluorescence have been extensively used to study oxidation-specific epitopes and oxidant production in targets of diabetic damage and atherosclerosis. These techniques are highly sensitive, and their ability to provide structural data can localize oxidative events. However, they are nonspecific and, at best, only semi-quantitative. In contrast, mass spectrometry (MS) offers a powerful set of analytical tools for quantifying and identifying biomolecules. Isotope dilution gas chromatography (GC)/MS is a highly sensitive and specific method that we have used to quantify oxidation of specific amino acid markers.

The overall strategy for identifying target analytes by GC/MS is outlined in Fig. 1 (55). Biomolecules such as oxidized amino acids (Fig. 2) derived from plasma or tissue are separated by GC, derivatized and ionized. The mass-to-charge ratios of ions derived by fragmenting the ionized, derivatized parent compound are determined by MS. A full scan mass spectrum obtained for a target analyte, 3-chlorotyrosine (derived by acid hydrolysis from plasma proteins), is shown in Fig. 1. Such a spectrum can unequivocally identify a target biomolecule because each compound has a unique fragmentation pattern. The analyte is quantified by adding a stable,



**FIG. 1. Schematic representation of gas chromatography–mass spectrometry (GC/MS) for identifying oxidized amino acids.** This mass spectrum derived from the dimethylsilyl derivative of 3-chlorotyrosine in protein digests of human plasma is a representative example. Plasma proteins were delipidated and hydrolyzed. Isolated amino acids were derivatized and analyzed by GC/MS.

isotopically labeled internal standard, which is identical to the target analyte except for the heavy isotope. With certain ionization processes, such as electron capture negative-ion chemical ionization, it is possible to detect and quantify sub-femtomole levels of biomolecules.

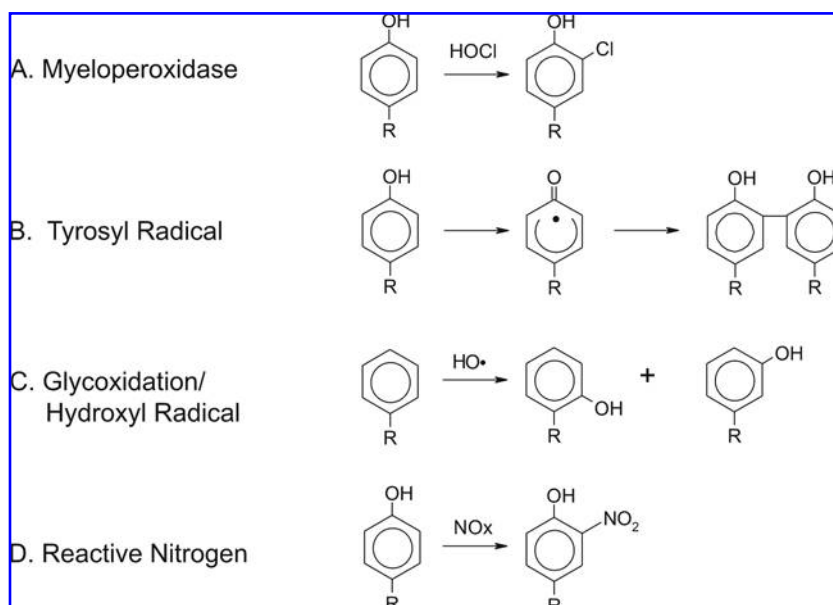
#### Pathways for generating oxidants

Many pathways oxidize proteins *in vitro*. However, the specific pathways that promote oxidative stress in diabetes have not been conclusively identified. One reason is that oxidizing intermediates are difficult to detect *in vivo* because they are short-lived and generated at low levels. Proposed pathways for increased oxidant generation and oxidative stress in diabetes are outlined in Fig. 3.

**The glycoxidation pathway.** Collectively, glucose-mediated oxidative reactions are called glycoxidation pathways.

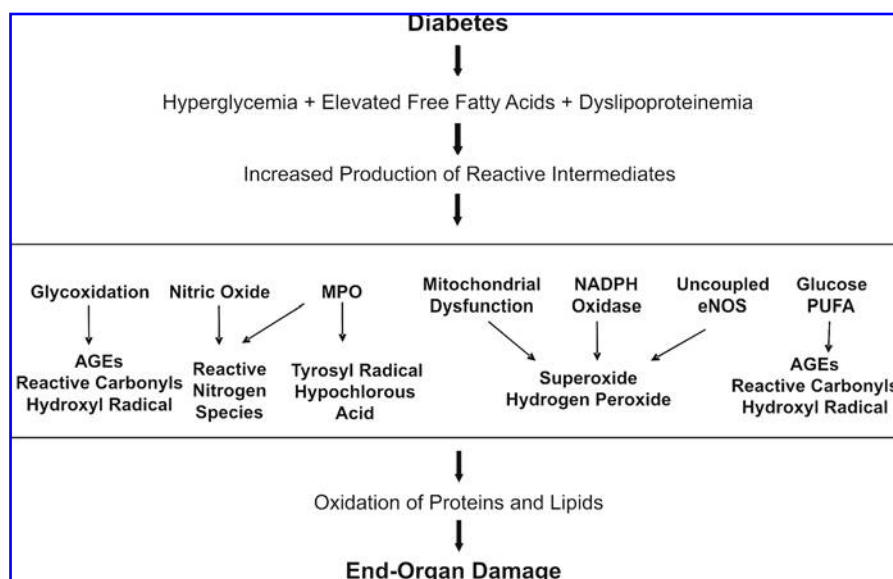
In its open-chain form, glucose has a carbonyl group that can be involved in oxidative chemistry. In the presence of oxygen, glucose can auto-oxidize to reactive oxygen species, such as hydroxyl radical, which cross-links proteins (65). Glucose also reacts nonenzymatically with proteins to form the reversible Schiff base adduct, which subsequently can rearrange itself into the stable Amadori product and AGE products.

*In vitro*, free metal ions catalyze steps in a nonenzymatic glycoxidation pathway that generates AGE products. One important intermediate is hydroxyl radical, which can peroxidize lipids and convert phenylalanine residues of proteins into two unnatural isomers of tyrosine, *ortho*-tyrosine and *meta*-tyrosine (63, 64, 73). Reduced, redox-active metal ions ( $M^{n+}$ ) such as  $Fe^{2+}$  and  $Cu^{1+}$  generate hydroxyl radical ( $HO^{\bullet}$ ) when they react with hydrogen peroxide ( $H_2O_2$ ; Equation 1).



**FIG. 2. Proposed oxidation products of protein-bound aromatic amino acids by myeloperoxidase, tyrosyl radical, glycoxidation/hydroxyl radical, and reactive nitrogen species (RNS).** Myeloperoxidase converts tyrosine to 3-chlorotyrosine; tyrosyl radical cross-links tyrosine to form *o,o'*-dityrosine; RNS convert tyrosine to 3-nitrotyrosine; hydroxyl radical produces *ortho*-tyrosine and *meta*-tyrosine from phenylalanine.

**FIG. 3. Potential pathways for increased oxidant generation in diabetes and atherosclerosis.** AGE, advanced glycosylation end-products; eNOS, endothelial nitric oxide synthase; MPO, myeloperoxidase.



AGEs can damage tissues through a number of mechanisms, including generation of oxidizing intermediates, formation of immune complexes, interaction with a cellular receptor called RAGE (Receptor for AGE), and promotion of cytokine release (5, 131). Although RAGE binds to AGE-modified proteins *in vitro* with high affinity, its ligands *in vivo* are unclear. High levels of AGEs accumulate in renal failure, even in nondiabetic patients, and this process reverses after renal transplantation, implicating the kidneys in AGE production and/or clearance (6, 92–94).

Many studies have shown that age-adjusted levels of pentosidine and N<sup>ε</sup>-carboxymethyllysine (CML), two widely investigated AGE products, correlate with the development of diabetic complications (34, 63, 89, 95, 121). Thus, glycoxidation reactions can be one mechanism for diabetic complications.

**The reactive nitrogen pathway.** Another pathway for generating oxidants involves nitric oxide (NO), which is produced by endothelial cells to regulate vascular tone. NO is also produced during inflammation by macrophages, which are early components of atherosclerotic lesions. NO reacts with superoxide (O<sub>2</sub><sup>•-</sup>) to generate peroxynitrite (ONOO<sup>-</sup>; Equation 2), a potent oxidant that converts tyrosine residues to 3-nitrotyrosine.



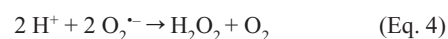
Thus, 3-nitrotyrosine is a marker for the reactive nitrogen pathway. It has been detected in low-density and high-density lipoproteins (LDL and HDL) isolated from human diabetic atherosclerotic lesions (83, 84, 107), and plasma nitrotyrosine levels are elevated in patients with CAD (125, 126). Because acute hyperglycemia promotes vasodilation in humans, glucose might directly or indirectly enhance NO release and oxidant generation (122).

**The myeloperoxidase pathway.** The major pathway through which macrophages and other phagocytic cells of

the innate immune system generate oxidants begins with the cells' membrane-bound NADPH oxidase (NOX), which produces superoxide (Equation 3).



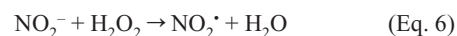
Superoxide dismutates into hydrogen peroxide (Equation 4).



The peroxide can be used by another phagocyte enzyme, myeloperoxidase (77, 78), to convert chloride ion to hypochlorous acid (Equation 5).



Oxidation of NO with oxygen yields nitrite (NO<sub>2</sub><sup>-</sup>), which myeloperoxidase converts to nitrogen dioxide radical (NO<sub>2</sub><sup>•</sup>; Equation 6), a potent nitrating intermediate (37, 40).



Reactive nitrogen species, including peroxynitrite and NO<sub>2</sub><sup>•</sup>, might contribute to the inflammatory process by nitrating lipoproteins and other biomolecules.

Hyperglycemia can activate PKC (70, 71, 75), which leads to phagocyte activation, secretion of myeloperoxidase, and oxidant generation. NEFAs that commonly are overabundant in diabetes can also activate phagocytes *in vitro*. These changes might enhance the production of superoxide and hydrogen peroxide, which myeloperoxidase converts into more potent cytotoxic oxidants, such as hypochlorous acid and nitrogen dioxide radical.

**The mitochondrial pathway.** Mitochondrial electron transport generates superoxide. Glucose promotes mitochondrial electron transport in cells, and a glucose-driven mitochondrial oxidation pathway has been proposed as one

mechanism for damaging cells in diabetes (16). When cultured endothelial cells are exposed to high levels of glucose, their mitochondria increase their superoxide output (33). Endothelial cells transfected with mitochondrial superoxide dismutase, a scavenger of superoxide, do not show this response. Thus, superoxide production by the mitochondrial electron transport chain could be one mechanism for diabetic tissue damage (16, 101). The mitochondrial pathway can be blocked by inhibitors of oxidative phosphorylation (16) or by a superoxide dismutase mimic (118).

Mitochondrial superoxide overproduction mediated by hyperglycemia might also increase polyol pathway activity, PKC activity, and hexosamine flux, resulting in cellular dysfunction and tissue damage (16). Moreover, superoxide inhibits glyceraldehyde phosphate dehydrogenase, a key glycolytic enzyme whose inactivity could make upstream metabolites accumulate. Such inhibition of glycolysis might promote end-organ damage by diverting metabolites into the hexosamine pathway or stimulating the polyol and diacylglycerol-PKC pathways. Benfotiamine, a lipid-soluble thiamine analog that inhibits these pathways by activating transketolase, an enzyme in the pentose pathway shunt, can prevent complications from experimental diabetes in animal models (48).

**Endothelial cell dysfunction and excess superoxide production.** Endothelial dysfunction has been proposed to generate excess superoxide, resulting in oxidative stress. Two enzymes have been implicated: NOX and uncoupled endothelial NO synthase (eNOS). Glucose and NEFAs can directly stimulate NOX because they activate PKC (69). Apocynin, which inhibits both NOX and PKC, reduced vascular superoxide generation in an animal model of type 1 diabetes and two models of type 2 diabetes (obese ob/ob mice and Zucker fatty rats) (23, 128). Additionally, it has been demonstrated that AGEs (138), oxidized LDL (72), and angiotensin II (25) can also increase NOX activity. Angiotensin II may represent a pathophysiologically relevant pathway for stimulating the production of reactive intermediates by artery wall cells because inhibitors of this pathway lower the risk for cardiovascular events in diabetics (60).

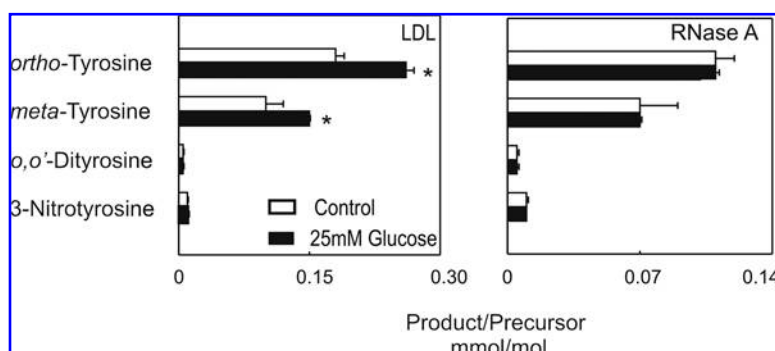
Oxidation of tetrahydrobiopterin, the reduced form of biopterin that is required for arginine reduction and NO production by eNOS, uncouples eNOS from NO synthesis. Under these conditions, eNOS transfers electrons to

molecular oxygen to generate superoxide (136). An alternative mechanism for uncoupling eNOS involves angiotensin II, which can induce dihydrofolate reductase deficiency, perhaps via a hydrogen peroxide-dependent mechanism. Because dihydrofolate reductase maintains tetrahydrobiopterin in its reduced form, dihydrofolate reductase deficiency results in eNOS uncoupling (25). Recently, it has been demonstrated that attenuation of angiotensin II signaling can recouple eNOS and inhibit peroxide production by NOX in diabetic mice (102).

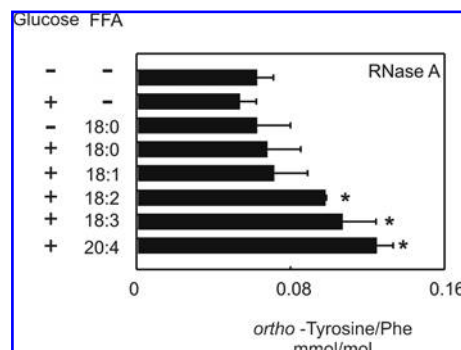
**The glucose-polyunsaturated fatty acid (PUFA) pathway.** To determine whether glucose can generate reactive intermediates by interacting with polyunsaturated fatty acids, we incubated it with LDL or a model protein, RNase (109). The buffer was free of redox-active metal ions because it had been treated with Chelex resin and supplemented with a metal chelator. Under these conditions, pathophysiologically relevant concentrations of glucose modified LDL, as evidenced by the formation of oxidized amino acids, even though metal ions were absent (Fig. 4). In striking contrast, glucose exposure did not increase levels of oxidized amino acids in RNase. These observations suggest that glucose promotes LDL oxidation because the particle contains lipid as well as protein. Our observation that levels of lipid oxidation products rise when LDL is exposed to glucose is consistent with this proposal.

To test the hypothesis that lipid can enhance protein oxidation, we incubated RNase with saturated, monounsaturated, or PUFA (Fig. 5). Glucose stimulated protein oxidation only in the presence of a PUFA. Thus, glucose appears to promote protein oxidation by a pathway involving peroxidation of PUFAs as this reaction is inhibited by lipid-soluble antioxidants (109).

To determine whether glucose's carbonyl group promotes LDL oxidation, we replaced glucose with a variety of short-chain and phosphorylated sugars that have highly reactive carbonyl groups (109). All of the carbonyl compounds promoted oxidation of LDL (but not RNase) protein more effectively than did glucose. In contrast, LDL oxidation was not enhanced by sorbitol, the reduced form of glucose that lacks a carbonyl moiety (Fig. 6). Collectively, these observations indicate that glucose can generate a species resembling the hydroxyl radical by a carbonyl/PUFA pathway. This pathway might therefore promote localized oxidative stress in tissues vulnerable to diabetic damage (109).



**FIG. 4. Product yields of *ortho*-tyrosine, *meta*-tyrosine, *o,o'*-dityrosine, and 3-nitrotyrosine in LDL and RNase exposed to D-glucose *in vitro*.** LDL and RNase (1 mg protein/ml of buffer) were incubated with 0 mM (Control) or 25 mM D-glucose. At the end of the incubation, amino acids in the protein were quantified by GC/MS. \* $p < 0.05$  by Student's *t*-test. Reproduced from (109).



**FIG. 5. Reaction requirements for oxidation of RNase by D-glucose.** RNase was incubated in buffer with and/or without 25 mM of fatty acid (stearate, 18:0; oleate, 18:1; linoleate, 18:2; linolenate, 18:3; arachidonate, 20:4) and/or 25 mM D-glucose. At the end of the incubation, amino acids in the protein were quantified by GC/MS. \* $p < 0.05$  by Student's  $t$ -test. Reproduced from (109).

### OXIDIZED AMINO ACIDS SERVE AS MOLECULAR FINGERPRINTS FOR SPECIFIC OXIDATION PATHWAYS

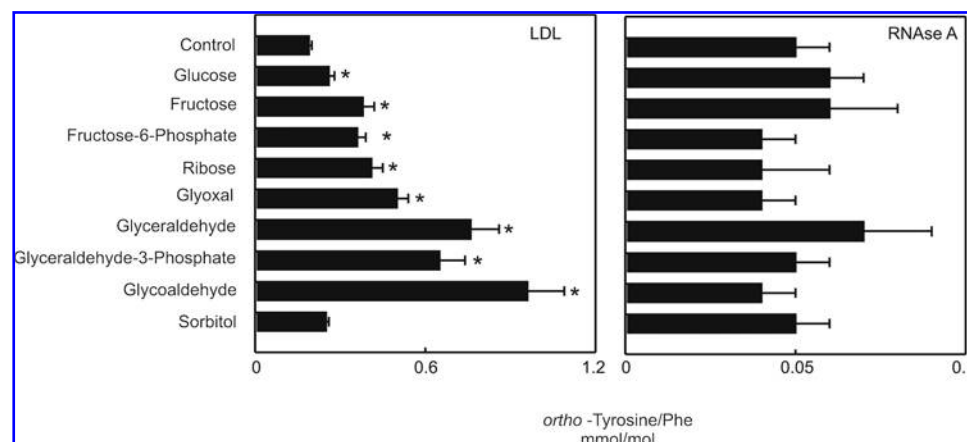
The examples given above illustrate that many different pathways can oxidize lipoproteins *in vitro*. To understand the molecular mechanisms that promote oxidative stress *in vivo*, we first used a chemical approach to define the patterns of oxidation products that are formed by well-characterized oxidant-generating systems *in vitro*. Then we looked for similar patterns in tissue and plasma. We focused on proteins because aromatic amino acids retain the initial footprint of the reactive intermediate that initiates oxidative damage. In contrast, lipid peroxidation products readily undergo subsequent chain-propagating reactions, which mask the products formed early in the pathway. Moreover, many different

oxidizing intermediates give the same spectrum of oxidized lipids, making it difficult to identify specific pathways that trigger lipid oxidation.

Oxidizing intermediates are difficult to detect *in vivo* because they are short-lived and generated at low levels. To sidestep this problem, we and others have identified and are able to monitor acid-stable products of protein oxidation, both *in vitro* and *in vivo* (2, 5, 7–9, 12, 13, 37, 51, 56, 57, 80–84, 107–111). Our overall approach is to use isotope dilution GC/MS to accurately identify oxidized amino acids isolated from tissue proteins. These markers, which include *ortho*-tyrosine, *meta*-tyrosine, dityrosine, 3-nitrotyrosine, and 3-chlorotyrosine (Fig. 2), indicate which biochemical pathway has damaged a protein.

### Evidence for localized oxidative stress in diabetes

Indirect evidence supports the hypothesis that oxidative stress increases in diabetes. For example, numerous investigators have reported elevated levels of products of lipid, protein, and nucleic acid oxidation in the blood of diabetic patients, though most of these products were monitored with nonspecific assays that may not yield valid results with complex biological material (6). Studies using sensitive and specific MS methods to quantify oxidation products have cast doubt on the concept of a generalized increase in oxidative stress in diabetic humans. For example, Wells–Knecht *et al.* (141) performed careful, quantitative studies on collagen, a long-lived protein that is freely exposed to blood glucose and lipids. They concluded that diabetes does not enhance oxidative stress because collagen from diabetic and euglycemic subjects contained similar age-adjusted levels of *ortho*-tyrosine and methionine sulfoxide, two well-characterized markers of protein oxidation *in vitro*. Other mass spectrometric studies have failed to find differing levels of glycooxidation products in urine and blood of diabetic and euglycemic humans (6, 35). These observations argue strongly against a generalized



**FIG. 6. Quantification of *ortho*-tyrosine in LDL and RNase incubated with reactive carbonyl compounds and sorbitol.** LDL or RNase was incubated in buffer supplemented with 25 mM of the indicated sugar for 15 days at 37°C. At the end of the incubation, amino acids in the proteins were quantified by GC/MS. \* $p < 0.05$  by Student's  $t$ -test. Reproduced from (109).

increase in oxidative stress in diabetes, at least in the extracellular compartment. None of the above studies excluded the possibility of localized, tissue-specific increases in oxidative stress in organs vulnerable to diabetic damage: the retina, kidney, vascular wall, and peripheral nerve tissue.

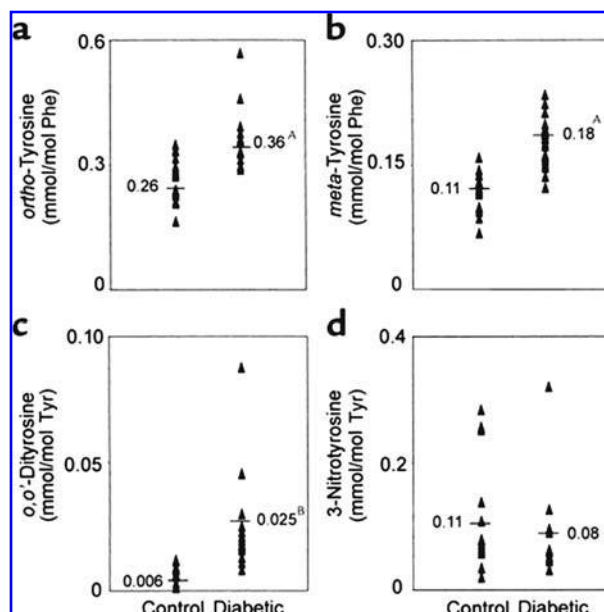
### MS quantitation of oxidized amino acids in aortic proteins of diabetic *Cynomolgus* monkeys reveals localized oxidative damage by hydroxyl radical

To investigate the potential role of localized oxidative stress in diabetic macrovascular disease, we used a primate model of diabetic atherosclerosis. These studies involved *Cynomolgus* monkeys that had been hyperglycemic for 6 months due to streptozotocin-induced diabetes (111). We analyzed samples from 7 controls and 8 diabetic *Cynomolgus* monkeys, sampling three different areas from the thoracic aorta of each animal. Compared with the control samples, the aorta wall proteins from the diabetic animals showed a significant 40% increase in *ortho*-tyrosine (Fig. 7A) and a similar (60%) increase in *meta*-tyrosine (Fig. 7B). *In vitro* studies identify these oxidized amino acids as products of hydroxyl radical, implicating a hydroxyl radical-like species in damage to artery wall proteins in diabetic monkeys.

The concentration of *o,o'*-dityrosine, a marker of tyrosyl radical that is produced in lower yield by hydroxyl radical (Fig. 7C), was fourfold greater in the diabetic samples than in the control samples. Thus, *ortho*-tyrosine, *meta*-tyrosine, and *o,o'*-dityrosine levels were higher in aortic proteins from diabetic monkeys than in those from control animals. However, we found no significant difference in 3-nitrotyrosine levels in proteins isolated from aortic tissue of control and diabetic *Cynomolgus* monkeys (Fig. 7D). Collectively, these results indicate that *ortho*-tyrosine, *meta*-tyrosine, and *o,o'*-dityrosine levels are selectively elevated in aortic proteins of diabetic monkeys, whereas 3-nitrotyrosine levels remain unchanged. This pattern of oxidized amino acids suggests that a hydroxyl-radical like oxidant promotes aortic damage in this animal model of type 1 diabetes.

### Correlations between aortic tissue levels of the oxidized amino acids and serum levels of glycated hemoglobin

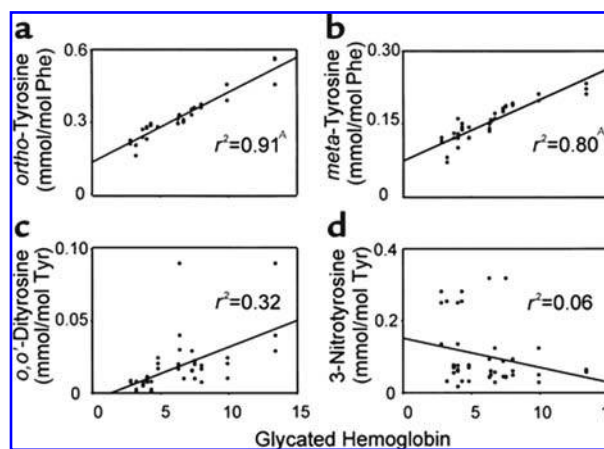
To determine whether glucose promotes protein oxidation *in vivo*, we assessed the relationship between level of glycemic control (measured as serum glycated hemoglobin) and levels of amino acid oxidation products in aortic tissue in control and diabetic *Cynomolgus* monkeys (Fig. 8). Linear regression analysis demonstrated a strong correlation between levels of both *ortho*-tyrosine and *meta*-tyrosine and glycated hemoglobin ( $r^2 = 0.9$  and  $0.8$ , respectively; both  $p < 0.001$ ). Levels of *o,o'*-dityrosine and glycated hemoglobin correlated less strongly ( $r^2 = 0.3$ ;  $p = 0.07$ ). In contrast, there was no correlation between levels of 3-nitrotyrosine and glycated hemoglobin. These observations support the hypothesis that glucose promotes the formation of *ortho*-tyrosine and *meta*-tyrosine in the artery wall and suggest that both glucose and other pathways contribute to the generation of *o,o'*-dityrosine (111).



**FIG. 7.** Quantification of *ortho*-tyrosine (a), *meta*-tyrosine (b), *o,o'*-dityrosine (c), and 3-nitrotyrosine (d) in aortic proteins isolated from control and diabetic *Cynomolgus* monkeys. Aortic tissue was harvested from control and diabetic animals at the end of the 6-month study. Tissue was delipidated, hydrolyzed, and amino acids were quantified by GC/MS. \* $p < 0.01$  by Student's *t*-test. Reproduced from (111).

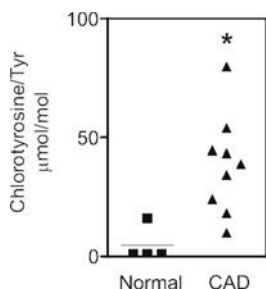
### Plasma and urinary levels of oxidized amino acids are potential markers for assessing oxidative stress *in vivo*

There is increasing evidence that oxidized amino acids in plasma and urine can serve as markers for noninvasive



**FIG. 8.** Concentrations of *ortho*-tyrosine (a), *meta*-tyrosine (b), *o,o'*-dityrosine (c), and 3-nitrotyrosine (d) in aortic proteins of *Cynomolgus* monkeys as a function of glycated hemoglobin. Lines represent the linear least squares fit of the data. Glycated hemoglobin was determined in serum of both control and diabetic animals at the end of the 6-month study. \* $p < 0.001$  by Spearman rank correlation. Reproduced from (111).





**FIG. 9. MS quantification of 3-chlorotyrosine in HDL isolated from plasma.** Plasma was obtained from healthy control subjects and subjects with established CAD. Oxidized amino acids isolated from hydrolyzed HDL proteins were quantified by GC/MS.  $p < 0.01$  by Student's  $t$ -test. Reproduced from (8).

assessment of oxidative stress *in vivo*. Plasma and urinary levels of these markers are likely to be proportional to the rate of generation and thus can serve as indices of chronic oxidative stress *in vivo* (8, 9, 82, 107, 108, 125–127, 150).

To determine whether oxidized amino acids in urine might serve as markers of oxidative stress, we determined the urinary excretion of *o,o'*-dityrosine in a mouse model of peritoneal inflammation (9). To correct for individual differences in glomerular filtration, we normalized *o,o'*-dityrosine levels to those of urinary creatinine. The concentration of *o,o'*-dityrosine was three-fold higher in the urine of mice with active peritoneal inflammation, but it failed to increase in mice lacking phagocytic gp91<sup>phox</sup> subunit of NOX. These results suggest that degradation of oxidatively damaged proteins in phagocytes releases free *o,o'*-dityrosine that enters the blood and is excreted in urine.

These observations may be relevant to human pathophysiology. For example, a case-control study demonstrated that systemic levels of protein-bound nitrotyrosine were significantly higher in patients with CAD than in controls with healthy arteries. Moreover, statin therapy lowered levels of oxidation markers in plasma, raising the possibility that these drugs can potentially be antioxidants (125–127). Therefore, these markers might serve not only to assess oxidative stress but also to monitor the efficacy of therapy.

#### *Oxidized HDL is a plasma marker of oxidative stress in subjects with CAD*

Because lipoprotein oxidation is implicated in atherogenesis, we analyzed plasma lipoproteins for oxidized amino acids. Preliminary studies demonstrated that HDL, but not LDL, isolated from plasma of subjects with established CAD contained high levels of 3-chlorotyrosine, a highly specific marker for the myeloperoxidase pathway. The level of 3-chlorotyrosine was 13-fold higher in HDL isolated from plasma of subjects with established CAD than in HDL from plasma of healthy subjects (Fig. 9); (8). We also found that levels of 3-nitrotyrosine were twice as high in HDL from plasma of patients with established CAD (Fig. 10); (107). These observations raise the exciting possibility that oxidized HDL might be a novel marker for clinically significant CAD. It will clearly be important to determine whether levels of oxidized amino acids are elevated in HDL and LDL of diabetic humans.

#### *Lipoprotein oxidation in diabetes mellitus*

Several observations suggest a complex interplay among protein oxidation, lipid peroxidation, and AGE formation.

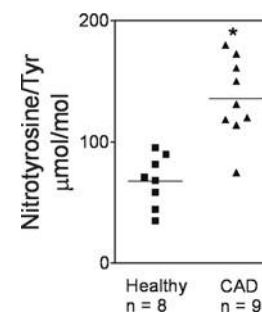
When LDL is incubated with glucose or glycated proteins, lipoprotein oxidation increases (18, 19, 66, 74). Moreover, CML, an extensively studied AGE, forms from PUFAs during lipid peroxidation (39). Thus, CML is a product of both glycooxidation and lipoxidation. CML and related glycooxidation products can form not only from early intermediates in the Maillard reaction but also directly from glyoxal or methylglyoxal, which may in turn be derived from either carbohydrates or lipids (6). PUFAs are oxidized much more readily than is glucose, and dyslipidemia is common in diabetic patients. Thus, elevated levels of plasma lipoproteins might contribute to lipoxidation *in vivo*. AGE lipids have also been found in diabetic plasma lipoproteins (18, 19, 114–116), and levels of isoprostanes—specific markers of nonenzymatic lipoxidation—are elevated in diabetic patients (28, 91). CML and other AGE compounds could thus originate *in vivo* when carbohydrates or lipids react with proteins.

Aminoguanidine and pyridoxamine comprise of a new class of agents termed “amadorins” that prevent the amadori product from forming AGEs. Both inhibit AGE formation from carbohydrates, and pyridoxamine also inhibits advanced lipoxidation reactions (104, 137). Aminoguanidine prevents diabetic complications in animal models, which may reflect its ability to inhibit AGE formation and inducible nitric oxide synthase (17, 36, 47, 49, 68). Clinical trials were halted, however, because aminoguanidine proved too toxic to humans. Pyridoxamine is effective in preventing retinopathy and nephropathy in diabetic rats (31, 132), but its effects on humans remain to be determined.

#### *Carbonyl stress in diabetes*

Glucose is a reactive carbonyl that can generate additional carbonyls and other reactive compounds through both enzymatic and nonenzymatic reactions. Baynes and Thorpe proposed that diabetes is characterized by carbonyl stress that in turn may account for the increased protein and lipid modifications that typify diabetes and uremia (6). In this model, reactive carbonyl production rises due to increased substrate stress (elevated levels of glucose and its derivatives), and is compounded by deficiency or overload of pathways that detoxify carbonyl compounds. The abundance of reactive carbonyls enhances protein and lipid modification. Oxidative stress is thus a primary event that increases carbonyl production, but is also a secondary event due to elevated carbonyl levels (6).

**FIG. 10. MS quantification of 3-nitrotyrosine in HDL isolated from plasma.** Plasma was obtained from healthy control subjects and subjects with established CAD. Oxidized amino acids isolated from hydrolyzed HDL proteins were quantified by GC/MS.  $*p < 0.01$  by Student's  $t$ -test. Reproduced from (107).



### *Glucose promotes lipid and protein oxidation in diabetic retinal tissue by a carbonyl- and PUFA-dependent pathway*

To begin to explore the pathophysiological relevance of the carbonyl-PUFA pathway, we quantified levels of protein and lipid oxidation products in the retina of hyperglycemic Sprague–Dawley rats, a well-characterized model of diabetic retinopathy (109). Abnormalities such as increased vascular permeability became well established in retinal tissue as early as 4 weeks after STZ treatment (85, 149). After 6 weeks, retinal tissue contained markedly elevated levels of *ortho*-tyrosine and *meta*-tyrosine and high levels of hydroxy-octadecadienoic acid, a major product of lipid oxidation. Treatment with the carbonyl scavenger aminoguanidine blocked these changes but failed to affect levels of *ortho*- and *meta*-tyrosine in control animals. We observed no differences in levels of D-glucose or hemoglobin A<sub>1c</sub> in the rats on the two different diets. Thus, glucose or other reactive carbonyls that abound in the diabetic milieu promote lipid and protein oxidation *in vivo* by a pathway requiring PUFAs. These observations suggest that glucose might operate locally by a variety of mechanisms, damaging artery wall proteins even in the absence of generalized oxidative stress. It will be important to determine whether the pattern of oxidation products in diabetic humans resembles the ones we observed in the monkey and rat studies.

### *Hyperlipidemia in concert with hyperglycemia stimulates the proliferation of macrophages in atherosclerotic lesions: potential role of glucose-oxidized LDL*

Macrophage proliferation has been implicated in the progression of atherosclerosis. Recent studies have investigated the effects of hyperglycemia and hyperlipidemia on macrophage proliferation in murine atherosclerotic lesions and isolated primary macrophages (80). Glucose promoted lipid and protein oxidation of LDL *in vitro*. Oxidation of LDL with glucose resulted in a selective increase in protein-bound *ortho*-tyrosine and *meta*-tyrosine. Moreover, glucose-oxidized LDL—but not elevated levels of glucose alone—stimulated proliferation of isolated macrophages. These observations may be pertinent to diabetic vascular disease because macrophage proliferation in atherosclerotic lesions was observed in LDL receptor-deficient mice that were both hypercholesterolemic and hyperglycemic but in not mice that were only hyperglycemic (80).

### *Antioxidants for preventing diabetic complications*

The proposed role of oxidized LDL in atherogenesis suggests that a high dietary antioxidant intake might prevent premature vascular disease in humans. However, the majority of prospective, double-blind, placebo-controlled trials of one proposed lipid-soluble antioxidant, vitamin E, have failed to demonstrate any reduction of clinical events in patients with established atherosclerosis (54). The disappointing results of these trials have led many to question the role of oxidative damage in the pathogenesis of coronary artery disease in humans.

It might be, however, that vitamin E's ability to serve as an antioxidant *in vivo* should be questioned (54). Thus, despite the impressive ability of other lipid-soluble antioxidants to block atherosclerosis in hypercholesterolemic animals, vitamin E—at doses that fail to lower cholesterol levels—has not exerted a consistent inhibitory effect in such experiments. These observations (53, 54, 87) emphasize the importance of documenting that a proposed antioxidant intervention actually inhibits oxidative reactions *in vivo*.

There is also remarkably little information about the influence of vitamin E supplementation on lipid oxidation in humans. Indeed, a recent study of healthy humans taking dietary supplements as high as 2,000 IU/day for 8 weeks found no change in levels of three lipid oxidation products: 4-hydroxynonenal and two isoprostanes (90). The investigators assessed products of lipid peroxidation using GC/MS, a sensitive and specific method. These results strongly suggest that vitamin E failed to inhibit lipid peroxidation in these individuals.

We have shown that one pathway for LDL oxidation in the human artery wall involves myeloperoxidase (27, 55). It is therefore noteworthy that vitamin E also fails to inhibit many of the reactions executed by that enzyme, that myeloperoxidase and its characteristic products are found at high levels in human atherosclerotic tissue, and that myeloperoxidase oxidizes LDL by reactions that are independent of lipid peroxidation (50, 52, 58, 119). These observations highlight the importance of identifying both the targets of oxidation in the artery wall and the biochemical pathways that promote oxidative reactions *in vivo*, using sensitive and specific analytical techniques such as MS.

Trials of antioxidants and carbonyl trapping agents in humans suffering from diabetes have also yielded discouraging results. Chronic treatment with vitamin E failed to decrease cardiovascular events in a large study that included a high percentage of diabetic patients (148). One possible reason is that antioxidant therapy might benefit only subjects who exhibit increased oxidative stress. Indeed, the renal failure patients who benefited from vitamin E therapy (10) might have been a subset with greatly increased carbonyl and oxidative stress (94).

Certain inhibitors of oxidant generation and proposed antioxidants have shown promise in preliminary studies of diabetic neuropathy. These include the xanthine oxidase inhibitor, allopurinol, and the chain-breaking antioxidant alpha-lipoic acid (ALA). A recent report (21) examined the effects of allopurinol on endothelial function and oxidative stress in type 2 diabetic patients. The investigators showed that allopurinol increased the endothelium-dependent mean blood flow response to acetylcholine by 30% and decreased systemic levels of malondialdehyde. However, the relevance of endothelium-dependent mean blood flow response as a surrogate marker for cardiovascular disease has been questioned because this technique predicted that vitamin E would prevent vascular disease.

ALA is an endogenous free radical scavenger (97, 105) and metal chelator (105). ALA is reduced metabolically to dihydro-lipoate, which combines free radical scavenging and metal chelating properties with the ability to regenerate high concentrations of nonenzymatic and enzymatic antioxidants (86,

103, 105). In the Alpha-Lipoic Acid in Diabetic Neuropathy (ALADIN) Study, neuropathic symptoms lessened in type 2 diabetic subjects treated with intravenous ALA for 3 weeks (152). In the ALADIN III Study, 509 subjects with type 2 diabetes and symptomatic diabetic neuropathy were administered sequential treatment with 600 mg/day ALA intravenously for 3 weeks followed by 600 mg thrice daily orally for 6 months. The total symptom score for neuropathy symptoms was significantly better at early time points in the ALA-treated group and neuropathic deficits were consistently improved throughout the study (151). In the Deutsche Kardiale Autonome Neuropathie (DEKAN) Study, heart rate variability improved after 4 months of oral treatment with ALA (800 mg/day) in type 2 diabetic patients with neuropathy (153). Whether long-term oral therapy with ALA will be effective in diabetic neuropathy remains to be established. The clinical significance of affecting heart rate variability in diabetics is also unclear.

Pharmacologic agents currently in clinical practice with demonstrated benefit in CAD may act in part by serving as antioxidants. Angiotensin converting enzyme (ACE) inhibitors and statins have lowered CAD event rates in randomized controlled trials in diabetic patients (26, 60). As noted above, many lines of evidence suggest that angiotensin II triggers oxidant production by endothelial cells and other cells of the artery wall. Interestingly, the ACE inhibitor ramipril slowed the onset of type 2 diabetes in the Heart Outcomes Prevention Evaluation (HOPE) trial (60), though this effect was not confirmed in a more recent trial (11). Ramipril's well-recognized ability to mitigate the pro-oxidant effect of angiotensin II may in part account for its efficacy. A case-control study demonstrated that systemic levels of protein-bound nitrotyrosine were significantly higher among patients with CAD and that statin therapy lowered levels of oxidized amino acids in plasma, raising the possibility that statins can potentially be antioxidants (125–127). Thiazolidinediones (TZD), which have been proposed to improve endothelial dysfunction in part by decreasing oxidative stress via activation of the peroxisome-proliferator-activated receptor  $\gamma$ , have now been shown to decrease carotid intimal thickness in diabetic subjects independently of their ability to boost glycemic control (88). Additionally, TZD treatment also decreased progression to overt diabetes in subjects with impaired glucose tolerance (32). The oxidative pathways modulated by these agents have not been defined and should be the focus of future investigations.

## CONCLUSIONS AND PERSPECTIVES

The ability to accurately quantify amino acid oxidation markers in tissue samples, plasma, and urine can provide invaluable mechanistic insights into disease pathogenesis. Therefore, further studies of the biochemical basis for oxidant generation might facilitate the development of specific antioxidant therapies designed to retard diabetic complications.

The potential role of antioxidant therapies in preventing microvascular and macrovascular disease in diabetic humans is another important issue. To examine the oxidative stress hypothesis in diabetes, suitable test subjects and optimal an-

tiioxidant regimens need to be identified. Such a trial should involve diabetic patients with evidence of increased oxidative stress (for example, subjects with elevated levels of oxidized amino acids in their plasma or urine). These high-risk individuals are most likely to benefit from antioxidant therapy. The optimal regimen for inhibiting oxidative tissue injury in humans also needs to be determined so that test compounds can be administered in effective antioxidant regimens.

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## ABBREVIATIONS

ACE, angiotensin converting enzyme; AGEs, advanced glycation end products; ALA, alpha-lipoic acid; CML, N<sup>ε</sup>-(carboxymethyl)lysine; eNOS, endothelial nitric oxide synthase; GC/MS, gas chromatography–mass spectrometry; HDL, high density lipoprotein; LDL, low density lipoprotein; NEFA, nonesterified fatty acid; NO, nitric oxide; NOX, NADPH oxidase; PKC, protein kinase C; PUFA, polyunsaturated fatty acid; RBP4, retinol-binding protein 4; TZD, thiazolidinedione.

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Address reprint requests to:  
 Subramaniam Pennathur  
 Division of Nephrology  
 University of Michigan  
 1150 W Medical Center Drive  
 Ann Arbor MI 48109-0676

E-mail: spennath@umich.edu

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