MECHANISMS OF OXIDATIVE STRESS IN DIABETES: IMPLICATIONS FOR THE PATHOGENESIS OF VASCULAR DISEASE AND ANTIOXIDANT THERAPY

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1. ABSTRACT

Diabetes markedly raises the risk of microvascular and macrovascular disease, the major contributors to higher morbidity and mortality in this increasingly prevalent disorder. Oxidative stress has been postulated as one major contributor to long-term diabetic complications. However, there is considerable controversy regarding the nature, magnitude, and mechanisms of oxidative stress in the diabetic state. Although products of glycoxidation and lipoxidation are elevated in plasma and tissue from humans suffering from diabetes, the exact relationships among hyperglycemia, the diabetic state, and oxidative stress are undetermined. This review focuses on proposed mechanisms for increasing oxidative stress in diabetes, the relationship of oxidant production to hyperglycemia, the contribution of reactive carbonyl compounds that accumulate in the diabetic state to tissue injury, and the potential role of lipids in producing oxidants. Current evidence argues against a generalized increase in oxidative stress in human diabetes, at least in the extracellular milieu. Instead, reactive intermediates generated in certain microenvironments might promote oxidative stress by unique pathways. Thus, many issues need to be addressed, including the suitability of antioxidants for preventing the clinical sequelae of diabetes.

2. INTRODUCTION

Diabetes has reached epidemic proportions in industrialized countries. Roughly 17 million people in the United States—more than 6 percent of the population—have the disorder. Because diabetes damages arterial blood vessels, the leading causes of death are myocardial infarction, stroke, and peripheral vascular disease, which are 2- to 4-times more prevalent in diabetic patients. Moreover, microvascular complications, including retinopathy, nephropathy, and neuropathy, eventually affect nearly all patients with diabetes. In fact, diabetic retinopathy is the major cause of adult blindness in the United States, and diabetic nephropathy is the major cause of end-stage renal disease (1). Diabetic neuropathy, which affects roughly half of all diabetics, is the most common cause of nontraumatic amputations (2).

It has become increasingly apparent that microvascular and macrovascular diseases share a common pathophysiology. During the past two decades, considerable evidence has implicated oxidative stress in diabetes and other diseases, including atherosclerosis, neurodegenerative diseases, and end-stage renal disease (reviewed in ref (3-5)), as well as in aging. Recent advances in our understanding of oxidative stress have included: identification by mass spectrometry of the chemical nature of oxidative modifications in tissue proteins and lipids; characterization of redox signaling pathways and of transcription factors, such as NF-kB, that respond to oxidative stress; establishing of the role of cellular organelles, such as mitochondria, in generating
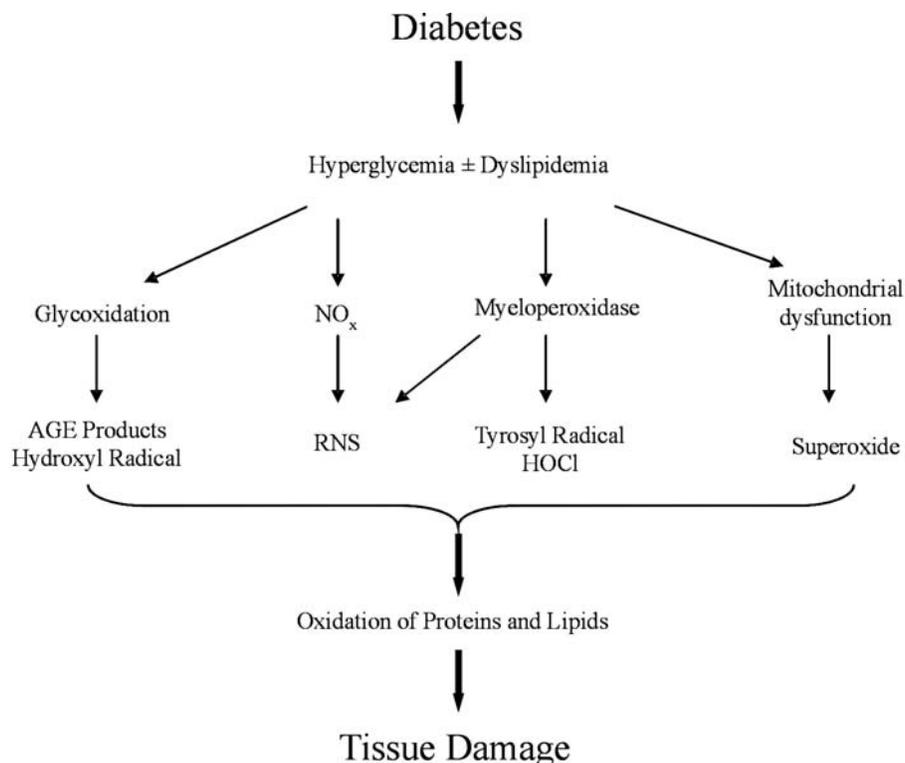
Figure 1. Proposed oxidant-generating pathways in diabetes. AGE: Advanced glycosylation end products; NOx: Nitric oxide derived oxidants; RNS: Reactive nitrogen species; IIOC1: Hypochlorous acid.

reactive oxygen species; and the discovery that oxidants can serve as activators of apoptotic pathways.

To confirm that oxidative stress contributes to diabetic complications, we must identify biologically relevant sources of oxidants that can explain diabetic complications and uncover mechanisms that could cause such damage. This information should make it possible to rationally design and test antioxidant therapies.

In this review, we discuss the proposed mechanisms of oxidative stress in diabetes and the potential relationship of hyperglycemia and lipids to oxidant-generating pathways (Figure 1).

3.   PATHOPHYSIOLOGY   OF   DIABETIC COMPLICATIONS

3.1. Hyperglycemia and diabetic complications
        Two factors that strongly affect the risk of diabetic complications are disease duration and degree of glycemic control (6-8). These observations have given rise to the "glucose hypothesis," which suggests that glucose mediates many of the deleterious effects of diabetes. Although this appears to be an oversimplification of a complex process, it has gained strong support from recent clinical trials in type 1 and type 2 diabetics. Both the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study found that strict glycemic control dramatically lowered the incidence of retinopathy, nephropathy, and neuropathy (7, 9-11). This

salutary finding suggests that hyperglycemia promotes or even initiates these complications. Therefore, glucose itself may be toxic to the microvasculature. However, strict glycemic control alone does not prevent diabetic complications, suggesting the involvement of additional factors.

Diabetes also associates with macrovascular disease, which is 2- to 4-times more common in diabetic men than in the general population and is prevalent even in premenopausal diabetic women. However, the link between hyperglycemia and vascular disease is unclear. Both the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study uncovered a trend toward less macrovascular disease with glucose-lowering therapy, but the difference did not reach statistical significance. Thus, factors other than glucose, such as abnormalities in lipoproteins, metabolic derangements (insulin resistance, elevated free fatty acid levels, etc.), and variations in genes controlling lipid metabolism might be important in macrovascular as well as in microvascular disease (6).

3.2. Mechanisms of tissue damage mediated by hyperglycemia
        Proposed mechanisms for the pathogenesis of diabetic complications include formation of advanced glycosylation end products (AGEs) (4, 12-17), oxidative stress (12, 13, 18), carbonyl stress (12, 13, 18), increased protein kinase C activity (19), altered growth factor or cytokine activities (20, 21), reductive stress or pseudo-

hypoxia (22, 23), and mitochondrial dysfunction (14, 24). Some of these hypotheses overlap. For example, AGEs might promote growth factor expression and oxidative stress, and oxidative stress might promote AGE formation (12, 13, 18). All the hypotheses are supported by extensive data, but a unifying hypothesis remains elusive. The existence of several credible hypotheses might mean that different tissues are differentially vulnerable to various oxidative pathways.

4. OXIDATIVE STRESS IN DIABETES

4.1. Protein oxidation in diabetes

Controversy abounds about the nature, magnitude, and localization of oxidant stress in diabetes. Thus, the relationship between hyperglycemia and oxidation is unclear, as is the link between this combination and acceleration of diabetic complications. Moreover, it is not known whether oxidative stress is a primary event that occurs early in the course of the disease or whether it is a secondary phenomenon that merely reflects end-stage tissue damage (13). This distinction has practical clinical relevance because a therapy interrupting early pathogenesis may prevent complications, whereas one that acts later may stop the disease from progressing.

4.2. Pathways for generating oxidants

Many pathways are known to oxidize proteins, at least *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.

4.2.1. The glycoxidation pathway

Because an elevated level of glucose is one of the metabolic hallmarks of diabetes, much attention has focused on the sugar's oxidative chemistry. One widely studied mechanism involves autoxidation of glucose itself, which generates reactive oxygen species such as hydroxyl radical and also cross-links proteins (25, 26). Glucose also reacts nonenzymatically with proteins to form the reversible Schiff base adduct, which subsequently can rearrange to form the stable Amadori product and AGE products. These protein-bound forms of glucose and their oxidized, cleaved, and dehydrated derivatives can produce reactive intermediates.

In vitro, free metal ions catalyze steps in a nonenzymatic glycoxidation pathway that generates AGE products (13). 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 (27-29).

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 (12, 16). Although RAGE binds to AGE-modified proteins *in vitro* with high affinity, its ligands *in vivo* are unclear. High levels of AGEs can accumulate in renal failure and uremia even in nondiabetic patients, and this process ceases after renal transplantation, implicating the kidneys in AGE clearance (13, 30-32).

The glycoxidation hypothesis thus offers one explanation for the proposed link between oxidative stress and the production of AGE products (33). Many studies have shown that age-adjusted levels of pentosidine and carboxymethyllysine (CML), two AGE products, correlate with the development of diabetic complications (18, 34-40).

4.2.2. The reactive nitrogen pathway

Another pathway for generating oxidants involves nitric oxide (NO), which is produced by endothelial cells to regulate vascular tone. NO reacts with superoxide to produce peroxynitrite ($ONOO^-$), a potent oxidant that converts tyrosine residues to 3-nitrotyrosine. Thus, 3-nitrotyrosine is a marker for the reactive nitrogen pathway (42). Its detection in low density lipoprotein isolated from human atherosclerotic lesions suggests that production of reactive nitrogen species increases in diseased vascular tissue (41, 43). Because acute hyperglycemia promotes vasodilation in humans, glucose might directly or indirectly enhance NO release and oxidant generation (6). NO is also be produced during inflammation by macrophages, which are early components of atherosclerotic lesions and persist at all stages of the atherosclerotic process. NO production can also be increased in inflammatory states by induction of inducible nitric oxide synthase (44).

4.2.3. The myeloperoxidase pathway

The major pathway through which macrophages generate oxidants begins with their membrane-bound NADPH oxidase, which produces superoxide and hydrogen peroxide. The peroxide can then be used by another phagocyte enzyme, myeloperoxidase (45, 46). The connection between this pathway and diabetes may be that, at least in the artery wall of diabetic animals, hyperglycemia can activate protein kinase C (19, 47, 48), which leads to phagocyte activation, secretion of myeloperoxidase, and oxidant generation. Free fatty acids that commonly are overabundant in diabetes can also activate phagocytes *in vitro* (6). These changes might enhance the production of superoxide and hydrogen peroxide, which myeloperoxidase converts into more potent cytotoxic oxidants, such as hypochlorous acid.
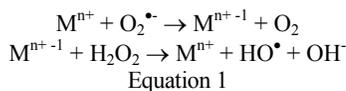
4.2.4. The mitochondrial pathway

Like macrophages, mitochondria make superoxide, and the glucose-driven mitochondrial oxidation pathway has been proposed as one mechanism for damaging cells in diabetes. When cultured endothelial cells are exposed to high levels of glucose, their mitochondria increase their superoxide output (49); inhibitors of oxidative phosphorylation block this increase. Endothelial cells transfected with mitochondrial superoxide dismutase, a scavenger of superoxide, fail to enhance their production of reactive oxygen species in response to glucose. Thus, superoxide production by the mitochondrial electron

transport chain could be one mechanism for diabetic tissue damage (14, 24, 50). The observation that overexpression of copper-zinc superoxide dismutase protects against early diabetic glomerular injury in transgenic mice is consistent with this hypothesis (51). The mitochondrial pathway can be blocked by inhibitors of oxidative phosphorylation (14) or by a superoxide dismutase mimic (52, 53).

Because superoxide can react with NO to form peroxynitrite (see above) or with hydrogen peroxide to form hydroxyl radical (HO$^{\bullet}$) through the metal catalyzed Haber-Weiss reaction (Equation 1; M, metal ion;), proteins damaged by the mitochondrial pathway might accumulate the stable end products of these pathways, which would serve as markers of damage mediated by glycoxidation, hydroxyl radical, and/or peroxynitrite.

$$M^{n+} + O_2^{\bullet-} \rightarrow M^{n+ -1} + O_2$$
$$M^{n+ -1} + H_2O_2 \rightarrow M^{n+} + HO^{\bullet} + OH^{-}$$
Equation 1

Mitochondrial superoxide overproduction mediated by hyperglycemia might also increase polyol pathway activity, protein kinase C activity, and hexosamine flux, resulting in cellular dysfunction and tissue damage (14). Moreover, superoxide inhibits glyceraldehyde phosphate dehydrogenase, a key glycolytic enzyme whose inactivity could make upstream metabolites accumulate. Inhibition of glycolysis might promote end-organ damage by diverting metabolites into the hexosamine pathway and by stimulating the polyol and diacylglycerol-protein kinase C pathways. Benfotiamine, a lipid-soluble thiamine analog, can prevent complications from experimental diabetes in animal models (54). It inhibits these pathways by activating transketolase, an enzyme in the pentose pathway shunt.

4.3. Lipoxidation in diabetes mellitus

Several recent observations suggest a complex interplay among protein oxidation, lipid peroxidation, and AGE formation. When low density lipoprotein is incubated with glucose or glycated proteins, lipoprotein oxidation increases (55-58). Moreover, CML forms from polyunsaturated fatty acids during lipid peroxidation (59). Thus, CML is a product of both glycoxidation and lipoxidation. CML and related glycoxidation 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 either from carbohydrates or lipids (13). Polyunsaturated fatty acids are oxidized much more readily than glucose, and dyslipidemia is common in diabetics. Thus, elevated levels of plasma lipoproteins might contribute to lipoxidation *in vivo*. It is noteworthy that tissues that are prone to diabetic complications, such as the retina and atherosclerotic intima, are rich in polyunsaturated fatty acids. AGE lipids have also been found in diabetic plasma lipoproteins (55, 56, 60-62), and levels of isoprostanes—specific markers of nonenzymatic lipoxidation—are elevated in diabetic patients (63, 64). CML and other AGE compounds could thus originate *in vivo* when carbohydrates or lipids react with proteins.

Recently, a new class of agents, the Amadorins has been described. These compounds prevent Amadori adducts on proteins from forming AGE products. The Amadorins include aminoguanidine and pyridoxamine. Both inhibit AGE formation from carbohydrates, and pyridoxamine also inhibits advanced lipoxidation reactions (65, 66). Aminoguanidine is effective in preventing diabetic complications in animal models, which may reflect its ability to inhibit AGE formation. However, aminoguanidine also inhibits inducible nitric oxide synthase and this may also contribute to its beneficial effects (67-71). Pyridoxamine is effective in preventing retinopathy and nephropathy in diabetic rats (72, 73). It remains to be seen whether Amadorins can prevent diabetic complications in humans.

4.4. Carbonyl stress in diabetes

In its open chain form, glucose is a reactive carbonyl that can generate even more reactive compounds through both enzymatic and nonenzymatic reactions. The carbonyl stress hypothesis of Baynes and Thorpe cites this property to explain the increased protein and lipid modification that typifies diabetes and uremia. In this model, reactive carbonyl production increases due to increased substrate stress (increased 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 modification of proteins and lipids. Oxidative stress is thus a primary event that increases carbonyl production and also a secondary event due to elevated carbonyl levels (13).

4.5. Evidence for a generalized increase in 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 diabetics, though most of these products were monitored with nonspecific assays that are not valid for complex biological materials. Levels of glutathione in red blood cells are also consistently lower in diabetic subjects than in apparently healthy controls, again suggesting oxidative stress. The difference is small, however, and other factors associated with the diabetic milieu might be responsible for reducing intracellular levels of the thiol-containing peptide (6).

Recent studies using sensitive and specific mass spectrometric 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* (74) performed careful, quantitative studies of collagen, a long-lived protein that is freely exposed to blood glucose and lipids. They concluded that diabetes does not enhance oxidative stress because collagen from diabetic and euglycemic subjects contained similar age-adjusted levels of *ortho*-tyrosine and methionine sulfoxide, two well-characterized markers of protein oxidation *in vitro*. Other mass spectrometric studies have failed to find differing levels of glycoxidation products in urine and blood of diabetic and euglycemic humans (13, 75). These observations argue strongly against a generalized increase in oxidative stress in diabetes, at least in the extracellular compartment. Thus, there is still controversy about the

Table 1. Patterns of tyrosine and phenylalanine oxidation products in aortic proteins of diabetic monkeys exposed to various oxidants *in vitro*

	ortho-Tyrosine	*meta*-Tyrosine	*o,o'*-Dityrosine	3-Nitrotyrosine
Diabetic aortae	↑	↑	↑	–
Hydroxyl radical	↑	↑	↑	–
Peroxynitrite	↑	↑	↑	↑↑
Tyrosyl radical	–	–	↑	–

↑, Increased; ↑↑, markedly increased; –, no change; Reproduced with permission from ref.43.

possible link between diabetic hyperglycemia and oxidative stress and the role of oxidative stress in diabetic microvascular and macrovascular disease.

4.6. Evidence for localized oxidative stress in diabetes

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. Indeed, several lines of evidence suggest that localized oxidative stress might contribute to diabetic complications.

We have proposed that hyperglycemia and other components of the diabetic metabolic state might favor oxidative reactions in the microenvironment of susceptible organs. Potential mechanisms include glucose-stimulated mitochondrial oxidative phosphorylation (24), production of reactive intermediates by phagocytic white blood cells at sites of inflammation (e.g. atherosclerotic tissue), and peroxidation of polyunsaturated fatty acids, all of which have been implicated in the generation of reactive intermediates *in vitro* (13, 59, 60, 62). These observations suggest that oxidative stress might increase locally in certain diabetic tissue microenvironments, perhaps due, in part, to the interplay of oxidative reactions involving lipids, carbohydrates, and cells.

To investigate the potential role of localized oxidative stress in diabetic macrovascular disease, we used a highly sensitive and specific method, isotope dilution gas chromatography-mass spectrometry, to quantify oxidized amino acids in artery wall proteins of nondiabetic and diabetic nonhuman primates. We measured levels of *ortho*-tyrosine, *meta*-tyrosine, *o,o'*-dityrosine, and 3-nitrotyrosine. Our previous studies demonstrated that these amino acids are stable markers of protein oxidation *in vitro* and that different reaction pathways generate distinct patterns of products.

We studied *Cynomolgus* monkeys that had been exposed for 6 months to streptozotocin-induced hyperglycemia, selecting this model because diabetes accelerates atherosclerosis as assessed biochemically and histologically. In contrast, the disease fails to promote atherosclerosis (and is often protective) in most other animal models. When diabetic monkeys were compared with euglycemic controls, there were only minimal differences in blood cholesterol and triglyceride concentrations, two other important risk factors for vascular disease (76, 77). Thus, the contribution of hyperglycemia to artery wall damage could be investigated without having to account for differential lipid levels. We found that

hyperglycemia elevated levels of *ortho*-tyrosine, *meta*-tyrosine, and *o,o'*-dityrosine in aortic proteins (43). In striking contrast, the two groups had similar 3-nitrotyrosine levels, which suggest that hyperglycemia does not increase the production of reactive nitrogen species in the artery wall of this animal model.

We observed a similar pattern of amino acid oxidation products when we incubated aortic proteins with hydroxyl radical *in vitro* (Table 1; ref. (43)) but not when we incubated them with myeloperoxidase, reactive nitrogen species, or tyrosyl radical. Moreover, there were strong correlations between aortic tissue levels of both *ortho*-tyrosine and *meta*-tyrosine and serum glycated hemoglobin, an index of glycemic control (Figure 2; ref. (43)). These observations strongly suggest that hyperglycemia in this primate model promotes the formation of *ortho*-tyrosine and *meta*-tyrosine in artery wall proteins early in diabetes and that the oxidative pathway must therefore involve hydroxyl radical or a related species. This raises the possibility that localized oxidative stress mediated by a similar pathway might play an important role in human diabetic atherogenesis.

4.7. Potential pathways for generating localized oxidative damage in diabetes

Cellular pathways such as mitochondria and the NADPH oxidase of phagocytes clearly represent one potential mechanism for the local production of oxidants. Other factors might also be important. Glucose promotes *ortho*-tyrosine formation through nonenzymatic glycoxidation reactions that require redox-active transition metal ions, and such ions may be present in advanced human atherosclerotic lesions (78, 79). Thus, catalytic metal ions might help generate a species resembling hydroxyl radical in the microenvironment of the diabetic artery wall. Moreover, AGEs can bind metal ions, making them available for glucose oxidation and tissue damage (80-82).

Model system studies demonstrate that Schiff base adducts of methylglyoxal generate radical cations and superoxide (80, 82) without metal ion involvement. Therefore, this pathway might also contribute to localized oxidative stress in diabetes. Moreover, we recently showed that glucose and other carbonyl compounds promote protein and lipid oxidation *in vitro* by a reaction pathway that requires polyunsaturated fatty acids but not free metal ions (Pennathur and Heinecke, unpublished observation). Because atherosclerotic tissue is enriched in polyunsaturated fatty acids, reactions involving lipids and carbonyl compounds might increase oxidative stress in the
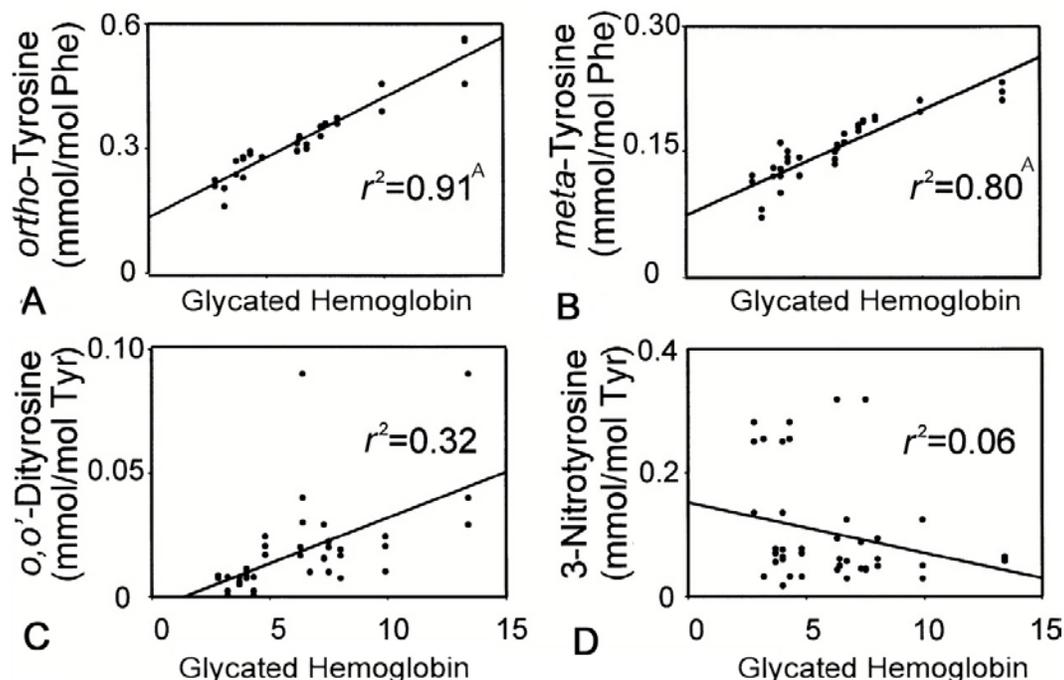
Figure 2. Concentrations of *ortho*-tyrosine (A), *meta*-tyrosine (B), *o,o'*-dityrosine (C), and 3-nitrotyrosine (D) in aortic proteins of *Cynomolgus* monkeys as a function of glycated hemoglobin. Lines represent the linear least squares fit of the data. Glycated hemoglobin was determined in serum of both control and diabetic animals at the end of the 6-month study. A, $p < 0.001$ by Spearman rank correlation. Reproduced with permission from ref. 43.

microenvironment of the diabetic artery wall. This localized process could also occur in other tissues that are rich in polyunsaturated fatty acids, including the retina, kidney and nervous tissue, and it would not necessarily be detected by assays of generalized oxidative stress.

4.8. Antioxidants for preventing diabetic complications

If generalized or localized oxidative stress proved to be a major contributor to diabetic complications, effective antioxidant regimens would be important therapies. Over the past decade, there has been an explosion of interest by scientists and the public in the possibility that dietary and supplemental antioxidant vitamins, such as beta-carotene, vitamin E, and vitamin A, might prevent human disease. However, trials of antioxidants and carbonyl trapping agents in humans suffering from diabetes have yielded mixed results, despite impressive findings from rat studies. Chronic treatment with vitamin E failed to decrease cardiovascular events in a large study that included a high percentage of diabetic patients (83). Also, trials of lipoic acid—a proposed antioxidant—for treating diabetic neuropathy have been equivocal (84, 85). One possible reason for these discouraging results is that antioxidant therapy might benefit only subjects who exhibit increased oxidative stress. Indeed, the renal failure patients who benefited from vitamin E therapy (86) might have been a subset with greatly increased carbonyl and oxidative stress (30). However, there is remarkably little evidence that

compounds such as vitamin E and vitamin C actually inhibit oxidative reactions in humans.

5. CONCLUSION AND PERSPECTIVES

Two critical questions that remain unanswered are the nature of oxidative injury in diabetes and the selective vulnerability of tissues to diabetic damage. A possible answer is that reactive intermediates generated in certain microenvironments might promote protein oxidation by pathways unique to the diabetic state. Local factors, such as a locally high concentration of polyunsaturated fatty acids or differences in gene expression, might influence the susceptibility of particular tissues to oxidant stress. 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 antioxidant regimens need to be identified. Such a trial should involve diabetics with evidence of increased oxidative stress, just as the statin trials studied subjects with high cholesterol levels rather than the general population. 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: AGE, advanced glycosylation end products; CML, carboxymethyllysine; NO, nitric oxide; RNS, Reactive nitrogen species; NO_x, nitric oxide derived oxidants; HOCl, Hypochlorous acid

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