Reactive Carbonyls and Polyunsaturated Fatty Acids Produce a Hydroxyl Radical-like Species: A Potential Pathway for Oxidative Damage of Retinal Proteins in Diabetes

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Running title: A pathway for oxidative damage by glucose and PUFAs
Abbreviations: AGEs, advanced glycation end-products; BHT, butylated hydroxytoluene; CML, N\textsuperscript{ε}(carboxymethyl)lysine; DTPA, diethylenetriaminepentaacetic acid; GC/MS, gas chromatography-mass spectrometry; HODE, hydroxyoctadecadienoic acid; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acid; RNase A, ribonuclease A; STZ, streptozotocin.
Abstract

The pattern of oxidized amino acids in aortic proteins of nonhuman primates suggests that a species resembling hydroxyl radical damages proteins when blood glucose levels are high. However, recent studies argue strongly against a generalized increase in diabetic oxidative stress, which might instead be confined to the vascular wall. Here, we describe a pathway for glucose-stimulated protein oxidation and provide evidence of its complicity in diabetic microvascular disease. Low density lipoprotein incubated with pathophysiological concentrations of glucose became selectively enriched in ortho-tyrosine and meta-tyrosine, implicating a hydroxyl radical-like species in protein damage. Model system studies demonstrated that the reaction pathway requires both a reactive carbonyl group and a polyunsaturated fatty acid, involves lipid peroxidation, and is blocked by the carbonyl scavenger aminoguanidine. To explore the physiological relevance of the pathway, we used mass spectrometry and HPLC to quantify oxidation products in control and hyperglycemic rats. Hyperglycemia raised levels of ortho-tyrosine, meta-tyrosine, and oxygenated lipids in the retina, a tissue rich in polyunsaturated fatty acids. Rats that received aminoguanidine also did not show this increase in protein and lipid oxidation. In contrast, rats with diet-induced hyperlipidemia in the absence of hyperglycemia failed to exhibit increased protein and lipid oxidation products in the retina. Our observations suggest that generation of a hydroxyl radical-like species by a carbonyl/polyunsaturated fatty acid pathway might promote localized oxidative stress in tissues vulnerable to diabetic damage. This raises the possibility that antioxidant therapies that specifically inhibit the pathway might delay the vascular complications of diabetes.
Introduction

Diabetes mellitus markedly increases the risk of microvascular and macrovascular disease (1). An elevated level of glucose is the metabolic hallmark of diabetes, and the degree of glycemic control has long been known to be a major risk factor for diabetic complications (1,2). These observations have given rise to the glucose hypothesis, which proposes that toxic effects of glucose mediate many of the disorder’s deleterious effects (1,2). This hypothesis was strongly supported by the Diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study, which demonstrated that intensive glucose-lowering therapy dramatically lowered the incidence of retinopathy and nephropathy (3,4).

Several mechanisms might explain the link between glucose and vascular disease, including mitochondrial dysfunction (5), formation of advanced glycation end products (AGEs; ref: (6-8)), pseudohypoxia (9,10), altered growth factor activity (11,12), dyslipoproteinemia (13), and increased protein kinase C activity (14). Another important factor might be oxidative stress because in vitro oxidation of glucose produces reactive intermediates, carbonyl compounds, and AGES, all of which have been linked to artery wall damage (5-8,15-18). For example, collagen exposed to glycoxidation reactions in vitro (6) accumulates the oxidized amino acids ortho-tirosine and methionine sulfoxide as well as two well-characterized AGES, pentosidine and Nε-carboxymethyllysine (CML). Levels of CML and pentosidine correlate with the severity of diabetic complications (19). Moreover, decreased levels of antioxidants and increased levels of markers of lipid oxidation products associate with diabetes (6,20), suggesting that oxidative stress might play a causal role in diabetic vascular disease.

Recent observations challenge the hypothesis that hyperglycemia increases oxidative stress, however. Wells-Knecht et al used sensitive and specific methods to demonstrate that age-
adjusted levels of ortho-tyrosine and methionine sulfoxide were similar in skin collagen of diabetic and euglycemic subjects (21). Other studies have failed to find higher levels of glycoxidation products in urine and blood of diabetics than in euglycemic humans (6,22). Collectively, these observations argue strongly against an increase in generalized oxidative stress in diabetes, at least in the extracellular compartment.

None of these studies excludes the possibility of a pathway that increases oxidative stress only in tissues such as the retina and artery wall that are prone to diabetic damage. One potentially destructive pathway involves reactive oxidant species generated by mitochondria (5). Glucose promotes superoxide production in cultured endothelial cells (5) and endothelium of isolated aortic rings (23) and inhibitors of mitochondrial electron transport block this process. In turn, superoxide or its products regulate gene expression and promote AGE formation (5). Increasing evidence implicates cytosolic NAD(P)H oxidase as another potential source of reactive intermediates in the retina and aorta of diabetic rats (24,25). Another important mechanism might involve glucose’s ability to activate protein kinase C (14,26), which could prompt cells to generate oxidants by a variety of mechanisms (13). Localized production of reactive intermediates might therefore contribute to diabetic retinopathy and atherosclerosis.

We recently detected elevated levels of protein-bound ortho-tyrosine and meta-tyrosine in aortic proteins from cynomolgus monkeys subjected to short-term hyperglycemia (27). Levels of these abnormal amino acids correlated strikingly with those of glycated hemoglobin in blood, suggesting that glucose or its metabolites had promoted protein oxidation. In vitro investigations into the stable end products of various oxidation pathways showed that hydroxyl radical generates both ortho-tyrosine and meta-tyrosine when it oxidizes proteins. These observations raise the possibility that the microenvironment of the diabetic artery wall promotes localized
oxidized stress, which could be important in the pathogenesis of early diabetic macrovascular
disease.

In the current study, we investigated mechanisms whereby glucose and metabolites of
glucose, such as glyoxal and sorbitol, that accumulate in diabetics (6,15-17,28) can generate
hydroxyl radical or a related species in vitro. Our observations suggest a novel pathway for
glucose-mediated protein damage. The pathway requires both polyunsaturated fatty acids and a
reactive carbonyl, and it involves a species resembling hydroxyl radical. Localized production of
reactive intermediates by this pathway might contribute to glucose-induced retinopathy and other
forms of microvascular and macrovascular disease in diabetes.
Experimental Procedures

Materials. Cambridge Isotope Laboratories (Andover, MA) supplied $^{13}$C-labeled amino acids. Isotopically labeled oxidized amino acids were prepared as described (29). Unless otherwise indicated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemicals (Milwaukee, WI).

Glycoxidation reactions. Buffers were passed over a Chelex-100 resin column (BioRad, Hercules, CA) to remove redox-active transition metal ions (30); then they were filter-sterilized (0.22 μm, Corning, NY). Low density lipoprotein (LDL) was isolated rapidly from plasma obtained from normolipidemic healthy subjects, using ultracentrifugation on a density gradient (31). Reactions were carried out at 37°C under argon in buffer A (100 mM sodium phosphate, 100 μM diethylenetriaminepentaacetic acid (DTPA), pH 7.4) supplemented with 1 mg protein/mL of either LDL or RNase A (fatty acid-free; Sigma). For incubations of >1 week, 1 mM sodium azide was added to the reaction mixture to prevent bacterial growth. Reactions were terminated by adding 300 nM catalase, 100 μM butylated hydroxytoluene (BHT, a lipid soluble antioxidant (30); prepared as a 10 mM stock solution in 95% ethanol), and 200 μM DTPA followed by acid precipitation (27). For reactions involving free fatty acids, the fatty acid was dissolved in chloroform and placed in a glass scintillation vial and the solvent was removed with nitrogen, then the indicated reaction mixture was added, and the vial was sealed under argon and incubated at 37°C with shaking.

Model system studies. Proteins were exposed to hydroxyl radical, tyrosyl radical, and peroxynitrite as described (27). All reactions were carried out at 37°C in buffer A supplemented with 1 mg/mL of protein. Soluble retinal proteins were isolated by centrifuging homogenized rat retina at 100,000 x g for 10 min at 4°C. Bovine serum albumin and soluble retinal proteins were
glycated by incubation with 1 M D-glucose for 6 weeks at 37°C.

**Animals.** The Animal Studies Committee at Washington University and the University of Texas Health Sciences Center at San Antonio approved all studies. To study the effects of hyperglycemia, adult male Sprague-Dawley rats (270-300 g; Harlan, Indianapolis, IN) were housed individually on a 12 h light-dark cycle and allowed *ad libitum* access to water and rat chow (Ralston Purina, Richmond, IN). Diabetes was induced by a single intravenous injection of 50 mg of streptozotocin (STZ) (32). Aminoguanidine (50 mg/kg) was injected subcutaneously once daily. Diabetic animals were not treated with insulin.

To study the effects of hyperlipidemia, adult male Sprague-Dawley rats (210-230 g) were housed individually on a 14-10 h light-dark cycle and allowed *ad libitum* access to water and a low-fat diet (rat chow, 4.4% fat, Harlan Teklad, Madison, Wisconsin). Hyperlipidemia was induced by consumption of a high-fat diet (60% calories from fat (70% animal fat); Harlan Teklad, Madison, Wisconsin, TD97201) for 39 weeks. Levels of hemoglobin A1C were not different between control and hyperlipidemic rats.

At the end of the study, the animals were anesthetized with ketamine hydrochloride (32) and immediately perfused via the left ventricle with ice-cold antioxidant buffer (100 μM DTPA, 50 μM BHT, 0.1% ethanol (v/v), 10 mM 3-amino-1,2,4-triazole, 140 mM NaCl, 10 mM sodium phosphate, pH 7.4) to prevent *ex vivo* oxidation. Tissue was harvested immediately, submerged in ice-cold antioxidant buffer, rapidly frozen by immersion in liquid nitrogen, and stored at –70°C until analysis. Prior to acid hydrolysis, tissue was homogenized at 4°C in buffer B (100 μM DTPA, 50 μM BHT, 1% (v/v) ethanol, 10 mM 3-amino-1,2,4-triazole, 50 mM sodium phosphate buffer, pH 7.4), frozen, and then thawed.

**Protein hydrolysis.** Protein was precipitated with ice-cold trichloroacetic acid (10% v/v),
collected by centrifugation, washed with 10% trichloroacetic acid, and delipidated twice with water/methanol/water-washed diethyl ether (1:3:7 v/v) (33). Isotopically labeled internal standards were added, and samples were hydrolyzed at 110°C for 24 h under argon (33).

**Mass spectrometric analysis.** Amino acids were isolated from the acid hydrolysate using a solid-phase C-18 column (Supelclean SPE, Supelco Inc. Bellefonte, PA). The n-propyl, heptafluorobutyric anhydride derivatives of amino acids were quantified by selected ion monitoring, using isotope dilution negative-ion chemical ionization GC/MS (27) with a Hewlett Packard 5890 Gas Chromatograph equipped with a 12 m DB-1 capillary column and interfaced with a Hewlett Packard 5988A mass spectrometer with extended mass range. Under these chromatography conditions, authentic compounds and isotopically labeled standards were baseline-separated and exhibited retention times identical to those of analytes derived from tissue samples. The limit of detection (signal/noise > 10) was < 10 femtomol for all the amino acids.

**Lipid peroxidation.** Lipids were extracted from LDL or tissue using a modified Dole procedure (34,35). Hydroxyoctadecadienoic acids (HODEs) were quantified by reverse-phase HPLC analysis of triphenylphosphine-reduced lipid extracts after base hydrolysis (35). The protein content of tissue pellets was determined by a modified Lowry protein assay, using BSA as standard (36).

**Statistical analysis.** Results represent means ± SE. Differences between two groups were compared using an unpaired Student’s t-test. Multiple comparisons were performed using two-way analysis of variance (ANOVA). Correlations were determined using linear regression analysis and the Spearman rank correlation method for nonparametric data (Sigma Stat, SPSS). A $P$ value < 0.05 was considered significant.
Results

Glucose promotes in vitro oxidation of LDL but not RNase A. To explore potential mechanisms for protein oxidation by D-glucose, we incubated a model protein, RNase A, in Chelex-treated buffer A (100 mM sodium phosphate, 100 μM DTPA, pH 7.4) supplemented with varying concentrations of the sugar. DTPA was included in the reaction mixture to inhibit metal-catalyzed oxidation reactions. We carried out parallel experiments using LDL, a non-covalent complex of protein, cholesterol, cholesteryl ester, triglycerides and phospholipids, to investigate the potential role of lipids in protein oxidation (37). At the end of the incubation, proteins were isolated, delipidated, and hydrolyzed. Free amino acids isolated from the hydrolysate were subjected to isotope dilution GC/MS analysis to measure their content of ortho-tyrosine, meta-tyrosine, o,o'-dityrosine, and 3-nitrotyrosine. After exposure to 25 mM D-glucose for 15 days, LDL contained ~ 40% more ortho-tyrosine and meta-tyrosine than LDL incubated in buffer alone (Fig. 1). However, the D-glucose treatment induced little change in levels of either o,o'-dityrosine or 3-nitrotyrosine. D-Glucose also failed to promote oxidation of RNase A (Fig. 1). Previous in vitro studies have demonstrated that this pattern of protein oxidation products is produced by hydroxyl radical but not by tyrosyl radical or reactive nitrogen species (27,38).

The amount of ortho-tyrosine (Fig. 2A) and meta-tyrosine (data not shown) in LDL increased with length of exposure to D-glucose, and LDL oxidation increased maximally over a range of D-glucose concentrations (5-100 mM; Fig. 2B). Previous studies have suggested that glucose could potentially contribute to LDL oxidation by a metal-ion dependent pathway (39). When DTPA was omitted from the reaction mixture, oxidation of LDL protein increased modestly (Fig. 2A) and RNase A oxidation still failed to occur, suggesting that metal ions could
potentially accelerate the LDL oxidation pathway. The D-glucose-dependent increase in LDL protein oxidation, monitored as an increase in ortho-tyrosine content, also exhibited little dependence on phosphate (Fig. 2C), in contrast to protein glycoxidation catalyzed by redox-active metal ions (40). These results suggest that D-glucose can oxidize LDL by a reaction that yields ortho-tyrosine and meta-tyrosine even in the apparent absence of metal ions. The observation that LDL but not RNase A is susceptible to oxidation suggests that lipids are essential to the pathway.

Glycation fails to generate ortho-tyrosine and meta-tyrosine in model proteins. The Schiff base adduct that D-glucose forms with proteins promotes in vitro glycoxidation reactions much more effectively than D-glucose itself (6,28,41). Certain AGEs bind metal ions, which might promote additional D-glucose oxidation and tissue damage (42). To determine whether interaction of D-glucose with protein might account for the results observed in the previous experiments, extensively glycated albumin and soluble rat retinal proteins (produced by a prolonged incubation with a high concentration of D-glucose as described in Methods) were incubated for up to 15 days in buffer A. However, we failed to see an increase in any of the protein oxidation products we assayed (data not shown). We also oxidized glycated albumin and glycated rat retinal proteins with hydroxyl radical, tyrosyl radical, or peroxynitrite in vitro, but we observed the same patterns of products that appear when nonglycated proteins are exposed to these systems (data not shown). These results suggest that extensively glycated proteins alone cannot promote protein oxidation by a pathway that generates ortho-tyrosine and meta-tyrosine.

Reactive carbonyls promote LDL protein oxidation in vitro. The carbonyl group of glucose accounts for its chemical reactivity (6-8), and levels of a variety of carbonyls derived from glucose are elevated in diabetic humans. We therefore determined whether derivatives of
glucose that accumulate in diabetics, such as glyoxal and sorbitol, might facilitate protein oxidation. LDL and RNase were incubated with 25 mM of D-glucose, fructose, fructose-6-phosphate, ribose, glyoxal, glyceraldehyde, glyceraldehyde-3-phosphate, glycolaldehyde, or sorbitol for 15 days. Reisolated proteins were hydrolyzed and analyzed by GC/MS. More reactive carbonyls caused greater increases in levels of protein-bound ortho-tyrosine (Fig. 3) and meta-tyrosine (data not shown) in LDL. Sorbitol (which lacks a carbonyl group and contains an alcohol group but is otherwise identical to D-glucose) did not raise ortho-tyrosine or meta-tyrosine levels. None of the compounds tested oxidized RNase A (data not shown). Collectively, these observations strongly implicate a carbonyl group in oxidation of LDL protein.

**Glucose-mediated LDL oxidation is accompanied by lipid peroxidation and blocked by lipid-soluble antioxidants and aminoguanidine.** Glucose's ability to oxidize LDL but not RNase A suggested that lipid peroxidation might be involved. We therefore incubated LDL for 15 days in the absence or presence of 25 mM D-glucose, extracted the LDL lipids, and analyzed them for HODEs by reverse-phase HPLC. D-Glucose increased the mean concentration of HODEs from 0.93 to 1.30 nmol/mg protein (40% ± 15%, n = 3, P < 0.05). This increase was completely blocked by the lipid-soluble antioxidant BHT (100 μM), the water-soluble antioxidant ascorbate (100 μM), or the carbonyl-reactive compound aminoguanidine (1 mM). In contrast, vitamin E (100 μM), the peroxidase inhibitor azide (1 mM), the peroxide scavenger catalase (170 nM), and the superoxide scavenger superoxide dismutase (250 nM) failed to inhibit LDL oxidation, as did a combination of catalase and superoxide dismutase. These results indicate that D-glucose stimulates peroxidation of LDL lipid *in vitro*. Low molecular weight compounds that react with intermediates in the lipid and aqueous phase block the reaction.
pathway, but peroxidase inhibitors or enzymes that scavenge superoxide or hydrogen peroxide are not inhibitory.

In a parallel set of experiments, we determined levels of ortho-tyrosine in LDL incubated for 15 days in the absence or presence of 25 mM D-glucose and the various inhibitors. The concentration of ortho-tyrosine increased 41% ± 5% (n = 3, P < 0.05) in LDL exposed to D-glucose. As with lipid peroxidation, this enhancement was blocked by BHT, ascorbate, or aminoguanidine, but not by vitamin E, azide, superoxide dismutase, or catalase (data not shown). None of the inhibitors had a significant effect on the extent of lipid or protein oxidation when LDL was incubated in the absence of D-glucose. These observations suggest that D-glucose promotes oxidation of the protein component of LDL by a pathway involving lipid peroxidation and reactive carbonyls.

**D-Glucose can oxidize RNase in vitro if polyunsaturated fatty acids are provided.** To confirm that lipids play a central role when D-glucose oxidizes proteins, we incubated RNase A with D-glucose, various polyunsaturated fatty acids (PUFAs; 25 mM), and DTPA for 15 days (Fig. 4). When the fatty acid was linoleate (C18:2), linolenate (C18:3), or arachidonate (C20:4), levels of ortho-tyrosine in RNase A rose significantly (35%, 40%, and 45%, respectively; P < 0.05). When these fatty acids were provided in the absence of D-glucose, there was a smaller increase in ortho-tyrosine levels. No significant increase occurred in the presence or absence of D-glucose when stearate (C18:0) or oleate (C18:1) was the fatty acid. These results indicate that polyunsaturated, but not saturated or monounsaturated, fatty acids promote protein oxidation and that the carbonyl group of D-glucose accelerates the reaction. The well-known susceptibility of polyunsaturated fatty acids to peroxidation further supports the hypothesis that a carbonyl/PUFA pathway promotes protein oxidation.
**Hyperglycemia increases protein oxidation in the retina of STZ-treated rats.** To explore the physiological relevance of the carbonyl/PUFA pathway, we used isotope dilution GC/MS to quantify levels of oxidized amino acids in retinal tissue from rats rendered hyperglycemic for 6 weeks by STZ. We chose the retina because it contains high levels of esterified polyunsaturated fatty acids and is frequently damaged in diabetes (43,44). Because our *in vitro* experiments showed that aminoguanidine inhibits D-glucose-stimulated LDL oxidation, we also investigated aminoguanidine’s effect on oxidation of retinal proteins.

Male Sprague-Dawley rats were divided into four groups: 8 untreated control rats, 8 diabetic rats, 8 aminoguanidine-treated control rats, and 8 aminoguanidine-treated diabetic rats. D-Glucose levels were monitored at weekly intervals throughout the study. Plasma D-glucose levels in the control and diabetic rats averaged 7.2 mM and 25.4 mM, respectively. Hyperglycemia persisted in the two STZ-treated groups for the entire 6 weeks of the study.

To determine whether proteins from nondiabetic rats contain oxidized amino acids, we assayed freshly isolated retina of control animals. After delipidating the tissue and hydrolyzing it with acid, amino acids were isolated and derivatized with *n*-propanol and heptafluorobutyric anhydride. Analysis of the derivatives by GC/MS in the negative-ion electron capture mode detected compounds that exhibited major ions and retention times identical to those of authentic ortho-tyrosine, meta-tyrosine, 3-nitrotyrosine, and *o,o*'dityrosine. Selected ion monitoring demonstrated that the ions derived from the amino acids coeluted with ions derived from authentic 13C-labeled internal standards (data not shown). These observations indicate that acid hydrolysates of retinal proteins of euglycemic rats contain basal levels of oxidized amino acids.

To determine whether hyperglycemia stimulated protein oxidation in diabetic retina *in vivo*, we used isotope dilution GC/MS to quantify levels of ortho-tyrosine, meta-tyrosine, 3-
nitrotyrosine, and o,o’-dityrosine in acid hydrolysates of retinal tissue from the control and hyperglycemic rats (Fig. 5A,B and Table 1). The samples from the hyperglycemic animals contained 67% more ortho-tyrosine than those from the euglycemic animals (diabetic, 0.35 ± 0.02 mmol/mol phenylalanine (the precursor of ortho-tyrosine); control, 0.21 ± 0.01 mmol/mol phenylalanine; n = 8 per group; P < 0.01). Similarly, levels of meta-tyrosine were 85% greater in the diabetic retinal tissue (diabetic, 0.19 ± 0.02; control, 0.11 ± 0.01 mmol/mol phenylalanine; n = 8 per group; P < 0.05). In contrast, levels of protein-bound 3-nitrotyrosine and o,o’-dityrosine were similar in the control and diabetic groups (Table 1). These results indicate that short-term hyperglycemia can promote protein oxidation in vivo and selectively enrich retinal proteins with ortho-tyrosine and meta-tyrosine.

**Levels of lipid peroxidation markers are elevated in retinal tissue in diabetic rats.** To determine whether lipid peroxidation might enhance D-glucose-stimulated protein oxidation in vivo, we used HPLC to quantify HODE levels in lipid extracts from retinas of control and diabetic animals. Diabetic retinal tissue contained a striking 2-fold higher level of HODEs than tissue isolated from euglycemic controls (Fig. 5C; diabetic, 364 ± 64 pmol/mg protein; control 190 ± 26 pmol/mg protein; n = 8 per group; P < 0.05). These observations indicate that diabetes increases both protein and lipid oxidation in the rat retina.

**Aminoguanidine blocks protein and lipid oxidation in the retina of diabetic rats.** To determine whether aminoguanidine inhibits D-glucose-stimulated protein oxidation in vivo, we quantified levels of oxidized amino acids and HODEs in the tissues of control and diabetic rats treated with the inhibitor (Fig. 5). Retinal tissue isolated from the aminoguanidine-treated diabetic rats failed to accumulate excess ortho-tyrosine, meta-tyrosine or HODEs. In contrast, aminoguanidine treatment did not alter the basal levels of ortho-tyrosine meta-tyrosine or...
HODEs seen in the retinal tissue of control rats. Interestingly, aminoguanidine also failed to alter levels of 3-nitrotyrosine in retina of diabetic rats (Table 1), even though it inhibits nitric oxide synthase \textit{in vitro} and \textit{in vivo} (45).

**Hyperglycemia does not promote protein oxidation and lipid peroxidation in the frontal cortex of STZ-treated rats.** To determine whether hyperglycemia would promote protein oxidation and lipid peroxidation in tissues that are not known to be adversely affected by the diabetic state, we quantified the levels of oxidized amino acids and HODEs in the frontal cortex of diabetic animals. Like retina, this region of the brain contains high levels of esterified PUFAs. Levels of protein-bound \textit{ortho}-tyrosine, \textit{meta}-tyrosine, \textit{o,o'}-dityrosine and 3-nitrotyrosine and HODEs were similar in control and hyperglycemic rats (Table 2). These results indicate that hyperglycemia does not promote protein or lipid peroxidation in the frontal cortex of STZ-treated rats.

**Tissue levels of \textit{ortho}-tyrosine, \textit{meta}-tyrosine and HODEs rise in concert in control and diabetic rats.** To investigate the potential relationship between lipid peroxidation and protein oxidation \textit{in vivo}, we assessed the relationship between HODE levels and oxidized amino acid levels in retinal tissue of control and STZ-treated rats (Fig. 6). Linear regression analysis demonstrated a strong correlation between levels of \textit{ortho}-tyrosine and HODEs ($r^2 = 0.59; P < 0.05$). Levels of \textit{meta}-tyrosine also correlated highly with HODE ($r^2 = 0.50; P < 0.05$). Although there were trends towards correlation between glucose levels and the levels of HODE ($r^2 = 0.12, P = 0.07$), \textit{ortho}-tyrosine ($r^2 = 0.08, P = 0.09$) and \textit{meta}-tyrosine ($r^2 = 0.07, P = 0.09$), respectively, these associations did not reach statistical significance. In contrast, there was no correlation between levels of \textit{o,o'}-dityrosine and 3-nitrotyrosine and HODEs (Fig. 6). These
observations suggest that protein oxidation and lipid oxidation increase in parallel in the retina of diabetic rats and that hyperglycemia may provide the link.

Tissue levels of ortho-tyrosine correlates with meta-tyrosine but not with \( o,o' \)-dityrosine and 3-nitrotyrosine in control and diabetic rats. To determine whether there is a relationship between the levels of the different oxidized amino acids, we compared tissue levels of ortho-tyrosine with meta-tyrosine, \( o,o' \)-dityrosine and 3-nitrotyrosine (Fig. 7). There was a strong correlation between levels of ortho-tyrosine and meta-tyrosine \((r^2 = 0.40; P < 0.05)\). In contrast, there was no correlation between levels of ortho-tyrosine and \( o,o' \)-dityrosine or 3-nitrotyrosine. These results suggest that similar oxidative pathways are likely to produce ortho-tyrosine and meta-tyrosine but that other mechanisms might contribute to formation of \( o,o' \)-dityrosine and 3-nitrotyrosine.

Hyperlipidemia alone fails to increase protein oxidation and lipid peroxidation in the retina of rats. To investigate whether prolonged hyperlipidemia without hyperglycemia would increase protein oxidation and lipid peroxidation in the retina, we used isotope dilution GC/MS and HPLC to quantify levels of oxidized amino acids and HODEs in retinal tissue from control rats and rats with diet-induced hypercholesterolemia. Previous studies have shown that glucose levels are not affected significantly in this animal model of hyperlipidemia (46).

Male Sprague-Dawley rats were divided into two groups: 6 control rats were fed a low-fat (4.4% fat) diet and 7 rats fed a high fat-diet (60% its calories as fat). Animals fed the high-fat diet developed hypercholesterolemia with elevated LDL levels. After 39 weeks on the diet, both the total plasma cholesterol and LDL levels were increased significantly in the animals fed the high-fat diet compared with animals fed the low-fat diet (cholesterol: low-fat, 76 ± 3 mg/dL; high-fat, 196 ± 34 mg/dL; \( P < 0.01 \); LDL levels: low-fat 21 ± 3 mg/dL; high-fat, 114 ± 25
mg/dL; \( P < 0.01 \). In contrast, rats on the two different diets had similar levels of hemoglobin A1C (low-fat, 3.2 ± 0.09; high-fat, 3.1 ± 0.07). Triglyceride levels were similar in both groups of animals, and none of the hyperlipidemic rats developed proteinuria.

Amino acids were isolated from hydrolysates of delipidated retinal proteins of the rats fed the low-fat or high-fat diets, derivatized, and analyzed by isotope dilution GC/MS. Levels of protein-bound ortho-tyrosine and meta-tyrosine and HODEs were similar in control and hyperlipidemic rats (Fig. 8). HPLC analysis of lipid extracts from retinas of control and hyperlipidemic rats similarly revealed no differences in the levels of HODEs between the two groups of animals. Thus, hypercholesterolemia in the absence of hyperglycemia does not promote protein or lipid peroxidation of retinal tissue in this rat model of diet-induced hyperlipidemia.
Discussion

Although hyperglycemia promotes oxidative reactions in vitro (5,6,15,28,39,47,48), there is no consensus about which chemical reactions are relevant in vivo. We therefore began the current study by investigating the oxidation chemistry of the reaction between glucose and model proteins. Pathophysiological concentrations of glucose significantly elevated the level of protein oxidation products in LDL by a pathway that appeared independent of redox-active metal ions. Further studies showed that a variety of carbonyl compounds that are elevated in the diabetic state also promote LDL oxidation.

Through mass spectrometric analysis of LDL that had been exposed to glucose, we detected a selective increase in ortho-tyrosine and meta-tyrosine. This pattern of protein damage is similar to that observed in aortic tissue of diabetic primates (27), and we have shown that it is not generated by glycation. Lipid peroxidation accompanied the oxidation of LDL protein, and production of ortho-tyrosine and meta-tyrosine was blocked by BHT, implicating reactive lipid intermediates in the reaction pathway. Moreover, D-glucose by itself was unable to oxidize a lipid-free protein, RNase A, but a combination of glucose and a PUFA elevated ortho-tyrosine and meta-tyrosine levels. Inhibitors of lipid peroxidation prevented this damage, suggesting an interplay between lipid peroxidation, protein oxidation, and glycoxidation. Oxidation of LDL or RNase A supplemented with PUFA was also blocked by aminoguanidine, a carbonyl scavenger. These in vitro observations indicate that glucose and other reactive carbonyls promote protein oxidation by a pathway that requires PUFA and involves lipid peroxidation.

To explore the pathophysiological relevance of this 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. Pathophysiological abnormalities such as increased
vascular permeability become well established in the retinal tissue as early as 4 weeks after STZ treatment in this model (32,49,50). After the rats experienced 6 weeks of STZ-induced hyperglycemia, their retinal tissue contained markedly elevated levels of ortho-tyrosine and meta-tyrosine. Retinal tissue also contained high levels of HODEs, major products of lipid oxidation. Treatment with aminoguanidine blocked these changes but had little effect on levels of ortho- and meta-tyrosine in control animals. In contrast to diabetic rats, we failed to observe an increase in either oxidized amino acids or HODEs in the retinal tissue of rats with 39 weeks of diet-induced hyperlipidemia. We observed no differences in the levels of D-glucose or hemoglobin A_1C in the rats on the two different diets. These observations suggest that glucose or other reactive carbonyls that abound in the diabetic milieu promote lipid and protein oxidation

_Aminoguanidine’s ability to inhibit both the glucose-promoted oxidation of LDL protein and lipid in vitro and the formation of retinal protein and lipid oxidation products in vivo is consistent with this proposal. Importantly, aminoguanidine inhibits increased retinal blood flow and vascular permeability in diabetic rats (45). Moreover, LDL exposed to glucose is toxic to cultured retinal capillary cells and pericytes, this cytotoxicity associates with a low level of lipid peroxidation, and aminoguanidine blocks both lipid oxidation and cytotoxicity (51). Thus, the carbonyl/PUFA pathway for oxidative damage might play a causal role in this animal model of diabetic retinopathy._

_Model system studies reveal which abnormal amino acids accumulate when proteins are oxidized by different reactive species. Thus, ortho-tyrosine and meta-tyrosine appear when hydroxyl radical oxidizes protein-bound phenylalanine residues (27,33). In contrast, we found that aqueous-phase peroxyl radical produced by the water soluble compound 2,2'-azobis (2-
amidinopropane) dihydrochloride fails to oxidize phenylalanine (data not shown). 3-
Nitrotyrosine accumulates when peroxynitrite oxidizes tyrosine, and o,o'-dityrosine is the major
product when tyrosyl radical reacts with tyrosine (33). A certain pattern of abnormal amino acids
can therefore uncover which reactive species is responsible for protein damage (52). Our
observation that ortho-tyrosine and meta-tyrosine become selectively elevated both when
glucose oxidizes LDL in vitro and in the retina of Sprague-Dawley rats exposed to short-term
hyperglycemia suggests that a hydroxyl radical-like species damages retinal proteins in this
animal model. The requirement for PUFAs in vitro points towards lipid-derived alkoxyl radical
as one potential hydroxyl radical-like oxidizing intermediate in the reaction pathway.

This lipid hydroperoxide-derived species might be involved in macrovascular as well as
in microvascular damage in diabetes because PUFAs are highly enriched in atherosclerotic
lesions as well as in retinal tissue (13,28,37,43,44). In mice deficient in the LDL receptor, both
hyperglycemia and hyperlipidemia are required for macrophages to proliferate in the artery wall
(61), a key step in atherogenesis. Also, glucose-oxidized LDL, but not glucose alone or LDL
alone, promotes the proliferation of cultured peritoneal macrophages. Collectively, these
observations suggest that an oxidative pathway requiring both glucose and lipid, such as the
carbonyl/PUFA pathway, could contribute to both macrovascular and microvascular disease in
diabetes.

Other lines of evidence support the idea that interplay among protein oxidation, lipid
peroxidation, and AGE formation is likely to be important in vivo. For example, ortho-tyrosine
and meta-tyrosine (Fig. 4) and CML (53) can form from polyunsaturated fatty acids during lipid
peroxidation, and AGE levels apparently increase in diabetic lipoproteins (6). Because lipids are
oxidized much more readily than glucose and reactive carboxyls, reactions driven by PUFAs
could be an important source of oxidizing intermediates in diabetic tissue. It is noteworthy that levels of reactive carbonyls are also markedly elevated in patients with renal disease and that such patients have a greatly increased risk of atherosclerosis (54). Thus, the carbonyl/PUFA pathway might contribute to localized oxidative stress in renal disease as well as in retinal and artery disease.

We failed to detect elevations of protein or lipid oxidation products in the frontal cortex of brain, a tissue rich in esterified PUFAs that is not known to be affected by diabetic complications. Thus, tissue specific factors might also be critical in determining whether oxidative damage occurs in diabetes. There are several other sources of reactive intermediates that might increase oxidative stress locally in diabetic tissue. For example, glucose increases the production of reactive species by mitochondria inside cultured endothelial cells, and inhibitors of mitochondrial electron transport block this increase (5,55). Cytosolic NADH oxidase could also be a source of reactive oxidants in the retina in the diabetic state (23,25). Retinal tissue exhibits a high rate of oxygen metabolism, suggesting that it could contribute to protein and lipid oxidation in diabetes (49). Also, elevated levels of glucose increase protein kinase C activity in arterial tissue (26), and activation of protein kinase C stimulates the NADPH oxidase of phagocytic cells (13). Protein kinase C also becomes activated in retinal endothelial cells under hyperglycemic conditions and can lead to activation of NADH oxidase (56). In animal models, this pathway increases vascular permeability, neovascularization, and blood flow, which are critically important in diabetic retinopathy.

A pathway involving peroxynitrite is another possible source of oxidative damage. Peroxynitrite forms when nitric oxide reacts with superoxide (57,58), and aminoguanidine inhibits nitric oxide synthase activity (45,59). Moreover, levels of nitric oxide synthase and 3-
nitrotyrosine become elevated in retinal tissue of rats exposed to prolonged hyperglycemia (60). Surprisingly, however, we found that protein extracted from retinal cells of our diabetic animals was not enriched in protein-bound nitrotyrosine. This implies that reactive nitrogen species do not directly promote the oxidation of retinal proteins and lipids during short-term hyperglycemia (though we cannot exclude the possibility of selective nitration of susceptible retinal proteins (59), given that our analytical method quantifies the total amount of protein-bound nitrotyrosine in tissue). Moreover, aminoguanidine did not affect levels of protein-bound nitrotyrosine in either our control or diabetic animals, suggesting that, under our experimental conditions, it affects a pathway not involving nitric oxide synthase.

In summary, our observations suggest a potential pathway for diabetic oxidative stress that requires PUFAs and reactive carbonyls. This pathway selectively enriches proteins in ortho-tyrosine and meta-tyrosine in vitro. The presence of a similar pattern of protein and lipid oxidation products in retinal and aortic tissue of hyperglycemic animals suggests that the carbonyl/PUFA pathway might be relevant to diabetic vascular disease. In future studies, it will be important to determine whether the pathway promotes protein and lipid oxidation in humans. If so, specific therapies that interrupt it might delay the onset of microvascular and macrovascular damage in patients with diabetes.
Acknowledgements

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References


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Figure Legends

Figure 1. Product yields of ortho-tyrosine, meta-tyrosine, o,o′-dityrosine, and 3-nitrotyrosine in LDL and RNase A exposed to D-glucose in vitro. LDL or RNase (1 mg protein/mL) in buffer A (100 mM sodium phosphate, 100 μM DTPA, pH 7.4) was incubated at 37°C for 15 days. The reaction mixtures were supplemented with 0 mM (Control) or 25 mM D-glucose. At the end of the incubation, proteins were delipidated and hydrolyzed. Isolated amino acids were derivatized and analyzed by isotope dilution negative-ion electron capture GC/MS with selected ion monitoring. Values represent the mean ± SEM of triplicate determinations and are normalized to levels of precursor amino acids. Results are representative of those found in 3 independent experiments. *P < 0.05 by Student’s t-test.

Figure 2. Reaction requirements for oxidation of LDL and RNase A by D-glucose. LDL or RNase A was incubated with 25 mM D-glucose in buffer A for 15 days at 37°C (●). Where indicated, the time of the incubation (A), the D-glucose concentration (B), or the phosphate concentration (C) was varied. In parallel incubations, DTPA was omitted from the reaction mixture (○). At the end of incubation, amino acids in the proteins were quantified by isotope dilution GC/MS with selected ion monitoring. Results are representative of those found in 3 independent experiments. Values represent the mean ± SEM of triplicate determinations.

Figure 3. Quantification of ortho-tyrosine in LDL and RNase A incubated with reactive carbonyl compounds and sorbitol. LDL or RNase A was incubated in buffer A 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 isotope dilution GC/MS with selected ion monitoring. Values
represent the mean ± SEM of triplicate determinations. Results are representative of those found in 3 independent experiments.*$P < 0.05$ by Student’s t-test.

**Figure 4. Reaction requirements for oxidation of RNase A by D-glucose.** RNase A (1 mg/mL) was incubated in buffer A at 37 °C for 15 days. Where indicated, 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 was included in the reaction mixture. At the end of incubation, amino acids in the protein were quantified by isotope dilution GC/MS with selected ion monitoring. Values represent the mean ± SEM of triplicate determinations. Results are representative of those found in 3 independent experiments.*$P < 0.05$ by Student’s t-test.

**Figure 5. Quantification of oxidized amino acids and lipids in retinal tissue isolated from control and hyperglycemic Sprague-Dawley rats.** Male Sprague-Dawley rats were divided into four groups: 8 untreated control rats, 8 diabetic rats, 8 aminoguanidine (AG)-treated control rats, and 8 aminoguanidine-treated diabetic rats. Rats were rendered hyperglycemic with STZ. At the end of the 6-week study, retinal tissue was harvested from the animals. Retinal proteins were delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by isotope dilution negative-ion electron capture GC/MS with selected ion monitoring (A, B). Lipids were extracted from retinal tissue, reduced with triphenylphosphine, and hydrolyzed. HODEs were quantified by reverse-phase HPLC (C). Each value represents the mean of triplicate analyses of each tissue sample. *$P < 0.05$ by ANOVA.
Figure 6. Correlation of ortho-tyrosine, meta-tyrosine o,o’-dityrosine, or 3-nitrotyrosine with HODEs in retinal tissue of control and diabetic Sprague-Dawley rats. At the end of the 6 week study, retinal tissue harvested from control animals, control animals treated with aminoguanidine, diabetic animals, and diabetic animals treated with aminoguanidine was analyzed for oxidation products as described in the legend to Fig. 5. Lines represent the linear least-squares fit of the data.

Figure 7. Correlation of meta-tyrosine o,o’-dityrosine, or 3-nitrotyrosine with ortho-tyrosine in retinal tissue of control and diabetic Sprague-Dawley rats. Retinal tissue harvested from control animals, control animals treated with aminoguanidine, diabetic animals, and diabetic animals treated with aminoguanidine was analyzed for oxidation products as described in the legend to Fig. 5. Lines represent the linear least-squares fit of the data.

Figure 8. Quantification of oxidized amino acids and lipids in retinal tissue isolated from control and hyperlipidemic Sprague-Dawley rats. Male Sprague-Dawley rats were divided into two groups: 6 control rats and 7 rats rendered hyperlipidemic by feeding with a high-fat diet (HFD). At the end of the 39 week study, retinal tissue was harvested from the animals. Retinal proteins were delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by isotope dilution negative-ion electron capture GC/MS with selected ion monitoring. Lipids were extracted from retinal tissue, reduced with triphenylphosphine, and hydrolyzed. HODEs were quantified by reverse-phase HPLC.
Table 1: Quantification of \( o,o' \)-dityrosine and 3-nitrotyrosine in retinal proteins isolated from control and diabetic Sprague-Dawley rats by isotope dilution GC/MS.

<table>
<thead>
<tr>
<th>Oxidized Amino Acids (( \mu \text{mol/mol tyrosine} ))</th>
<th>( o,o' )- Dityrosine</th>
<th>3- Nitrotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30 ± 1</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>Control + AG</td>
<td>32 ± 2</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>Diabetic</td>
<td>28 ± 3</td>
<td>79 ± 10</td>
</tr>
<tr>
<td>Diabetic + AG</td>
<td>31 ± 3</td>
<td>78 ± 8</td>
</tr>
</tbody>
</table>

At the end of the 6 week study, retinal tissue was harvested from 8 control animals, 8 control animals treated with aminoguanidine (AG), 8 diabetic animals, and 8 diabetic animals treated with aminoguanidine. Retinal proteins were delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by negative-ion electron capture GC/MS with selected ion monitoring. Each value represents the mean of triplicate analyses of each tissue sample. Values are normalized to the precursor amino acid tyrosine and are expressed as mean ± SEM.
Table 2: Quantification of oxidized amino acids and HODEs in frontal cortex of control and diabetic Sprague-Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>Oxidized Amino Acids (μmol/mol precursor)</th>
<th>HODE (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ortho-tyrosine</td>
<td>meta-tyrosine</td>
</tr>
<tr>
<td>Control</td>
<td>440 ± 130</td>
<td>94 ± 16</td>
</tr>
<tr>
<td>Control + AG</td>
<td>409 ± 120</td>
<td>106 ± 18</td>
</tr>
<tr>
<td>Diabetic</td>
<td>471 ± 110</td>
<td>104 ± 14</td>
</tr>
<tr>
<td>Diabetic + AG</td>
<td>430 ± 120</td>
<td>98 ± 18</td>
</tr>
</tbody>
</table>

At the end of the 6 week study, frontal cortex was harvested from the brains of 8 control animals, 8 control animals treated with aminoguanidine (AG), 8 diabetic animals, and 8 diabetic animals treated with aminoguanidine. Proteins were delipidated, hydrolyzed, and subjected to solid-phase extraction. The isolated amino acids were derivatized and analyzed by negative-ion electron capture GC/MS with selected ion monitoring. Lipids were extracted from retinal tissue, reduced with triphenylphosphine, and hydrolyzed. HODEs were quantified by reverse-phase HPLC. Each value represents the mean of triplicate analyses of each tissue sample. Values are normalized to the precursor amino acids tyrosine and phenylalanine for the oxidized amino acids and as pmol/mg of protein for HODEs. Data is expressed as mean ± SEM.
Figure 1

- **ortho-Tyrosine**
- **meta-Tyrosine**
- **o,o’-Dityrosine**
- **3-Nitrotyrosine**

**Product/Precursor mmol/mol**

- **Control**
- **25 mM Glucose**

**Legend:**
- LDL
- RNase A
Figure 2
Figure 3
Glucose FFA

RNase A

- -
+ -
- 18:0
+ 18:0
+ 18:1
+ 18:2
+ 18:3
+ 20:4

ortho-Tyrosine/Phe
mmol/mol

Figure 4
Figure 6
Figure 8
Reactive carbonyls and polyunsaturated fatty acids produce a hydroxyl radical-like species: A potential pathway for oxidative damage of retinal proteins in diabetes
Subramaniam Pennathur, Yasuo Ido, Jozsef I. Heller, Jaeman Byun, Ratna Danda, Pablo Pergola, Joseph R. Williamson and Jay W. Heinecke

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