Paradoxical Effects of Green Tea (Camellia Sinensis) and Antioxidant Vitamins in Diabetic Rats

Improved Retinopathy and Renal Mitochondrial Defects but Deterioration of Collagen Matrix Glycoxidation and Cross-Linking

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We tested the hypothesis that green tea prevents diabetes-related tissue dysfunctions attributable to oxidation. Diabetic rats were treated daily with tap water, vitamins C and E, or fresh Japanese green tea extract. After 12 months, body weights were decreased, whereas glycated lysine in aorta, tendon, and plasma were increased by diabetes ($P < 0.001$) but unaffected by treatment. Erythrocyte glutathione and plasma hydroperoxides were improved by the vitamins ($P < 0.05$) and green tea ($P < 0.001$). Retinal superoxide production, acellular capillaries, and pericyte ghosts were increased by diabetes ($P < 0.001$) and improved by green tea and the vitamins (P variable). Lens crystallin fluorescence at 370/440 nm was ameliorated by green tea ($P < 0.05$) but not the vitamins. Marginal effects on nephropathy parameters were noted. However, suppressed renal mitochondrial NADH-linked ADP-dependent and dinitrophenol-dependent respiration and complex III activity were improved by green tea (P variable). Green tea also suppressed the methylglyoxal hydroimidazolone immunostaining of a 28-kDa mitochondrial protein. Surprising, glycoxidation in tendon, aorta, and plasma was either worsened or not significantly improved by the vitamins and green tea. Glucosepane cross-links were increased by diabetes ($P < 0.001$), and green tea worsened total cross-linking. In conclusion, green tea and antioxidant vitamins improved several diabetes-related cellular dysfunctions but worsened matrix glycoxidation in selected tissues, suggesting that antioxidant treatment tilts the balance from oxidative to carbonyl stress in the extracellular compartment. *Diabetes* 54:517–526, 2005

Green tea is being widely studied for its alleged beneficial properties in the treatment or prevention of human diseases. To date, >1,500 articles referencing "green tea" are listed in Medline. Green tea is reported to delay or prevent certain forms of cancer, arthritis, and cardiovascular and other disorders (rev. in 1). To our knowledge, no systematic study of its efficacy in the prevention of the long-term complications of diabetes exists.

This study was motivated by three considerations. First, diabetes complications have been linked to oxidant stress, in particular the formation of superoxide (2). Second, the major biological mechanisms of action of green tea are being attributed to its antioxidant properties. Third, in a senior high school science research project carried out in part in our laboratory, a commercial green tea extract that was fed to aging C57BL/6 mice delayed collagen cross-linking and fluorescent advanced glycation end product (AGE) accumulation by a mechanism that was duplicated by the combination of vitamins C and E (3). Therefore, this project was designed to examine the ability of green tea to prevent some of the complications and biochemical dysfunctions of diabetes in the rat.

Green tea is rich in catechins, i.e., polyphenolic compounds whose antioxidant oxidant activity is severalfold higher than that of vitamins C and E. According to one study, the total equivalent antioxidant capacity of catechins increases from 0.99 mmol/l for vitamins C and E to 2.40, 2.50, 3.01, 3.82, 4.75, and 4.93 mmol/l for catechin, epicatechin, gallic acid, epigallocatechin, epigallocatechin gallate, and epicatechin gallate, respectively (4). Overall, the catechins represent up to one-third of green tea dry weight (5). Evidence suggests that catechins can prevent lipid hydroperoxide formation and toxicity (6) and scavenge superoxide and other free radicals (7) and peroxyni-

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AGE, advanced glycation end product; CML, carboxymethyl-lysine; DNP, dinitrophenol; ERC, electron respiratory chain; HPLC, high-performance liquid chromatography; TCC, tricarboxylic acid cycle.

trite (8), all of which have been implicated in some aspect of diabetes complications. Catechins were also shown to alter the catalytic activity of oxidative enzymes (7) and to chelate iron and copper, thus preventing metal-catalyzed free-radical formation (9). The latter has been associated with neuropathy in diabetic rats (10).

We tested the hypothesis that green tea displays antidiabetic and antiglycoxidation properties. To better interpret any biological effect, we included a control group that consisted of the classical combination of vitamins C and E. Impact of these treatments on food intake; body weight; indexes of glycemia; lipemia; plasma hydroperoxides; erythrocyte glutathione; retinopathy; nephropathy; renal mitochondrial functions; lens AGE fluorescence; and several markers of glycoxidation, advanced glycation, and cross-linking have been determined in plasma, skin, tendon, and aorta.

RESEARCH DESIGN AND METHODS

Most chemicals were from Sigma-Aldrich (St. Louis, MO). D_8-lysine and D_4-carboxymethyl-lysine were a gift of Dr. Susan Thorpe. Upper-grade (Sencha, Kawanecha) Japanese green tea (East/West Cultural, Kingston, NY) was prepared daily as described by the manufacturer: 10 g of dry tea was added to 750 ml of deionized boiled water cooled to 90°C, brewed for 3 min, decanted, vacuum filtered, placed on ice, and protected from light with aluminum foil. Diabetic rats drank ~100 ml/day, i.e., ~10 times more than expected daily human consumption. Vitamin C dose was 1 g/l drinking water, and vitamin E 10 IU/kg body wt solubilized in Tween 80 (1:3 parts) was administered daily by gavage.

Thirty-eight male Lewis rats (~150 g body wt; Charles River, Wilmington, MA) were divided into four groups: control ($n = 6$), diabetic ($n = 10$), diabetic with vitamins C and E ($n = 10$), and diabetic with green tea ($n = 10$) and housed under specific pathogen-free conditions. Diabetes was induced intravenously with 45 mg/kg streptozotocin (Sigma) in 0.1 mol/l citrate buffer. After 12 months, there were six, nine, eight, and nine survivors in each group, respectively. Animals received 2 IU of ultralente insulin (Humulin; Eli Lilly, Indianapolis, IN) three times per week.

Glycemia was monitored monthly, and glycated hemoglobin was monitored at 3, 6, 9, and 12 months. Glycation of plasma, skin, tendon, and aorta proteins was determined at 12 months by furosine method (see below), glycohemoglobin by affinity chromatography (Sigma kit #442-B, now discontinued), plasma triglycerides with Sigma kit #37 (GPO Trinder), plasma hydroperoxides using the FOX assay (Pierce kit #23285), and erythrocyte glutathione using a Calbiochem kit #354102.

Rats were anesthetized with pentobarbital, and blood was drawn by heart puncture. Eyes, aorta, and kidneys were removed. Kidneys were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid). Aorta, shaved dorsal skin (1–2 cm^2), plasma, and erythrocytes (red blood cells) were stored at −80°C. One eye was placed in buffered formalin for isolation of the retinal vasculature by the trypsin digest technique (11). The other retina was frozen for biochemical analysis. Lenses were removed and stored at −80°C.

Lenses were prepared as water-soluble and -insoluble fractions as previously described (12). The water-insoluble pellet was solubilized with twice 2% (wt/wt) pronase E in phosphate buffer that contained 0.05% sodium azide at 37°C in 30 h. Digested fractions were centrifuged and filtered sterile. Protein concentration in water-soluble fractions was estimated by BCA Protein Assay (Pierce) and in water-insoluble enzyme digest by the ninhydrin method (13). AGE-like fluorescence was determined at 370/440 nm.

Histopathology was performed in retinal vasculature isolated by the trypsin digest technique as previously described (11,14). Acellular capillaries and pericyte ghosts were quantified in a masked manner.

Detection of superoxide ion in retina. Superoxide anion production was determined by the lucigenin method (15). Freshly isolated retina was equilibrated in Krebs-HEPES buffer in the dark at 37°C in 95% O_2/5% CO_2 for 30 min. After equilibration, 0.5 mmol/l lucigenin was added, and photon emission was measured over 10 s. Repeated measurements were made over a 10-min period in a luminometer (Analytical Luminescence Laboratory, San Diego, CA). Blanks that contained all components except retinas were counted and subtracted from all other readings.

Assessment of nephropathy. Urinary albumin and creatinine were determined by automated instrumentation at University Hospitals of Cleveland. Collagen type IV immunostaining was determined in methacarn fixed renal tissue and sections (5 μm) stained with rabbit antimouse collagen α1 antibodies (Chemicon, Temecula, CA) or nonimmune rabbit IgG for assessment of nonspecific staining. Staining was quantitatively assessed with a Zeiss Image Analysis System (Carl Zeiss Optical, Chester, VA). Twenty-five to 30 glomeruli per area were assessed in blinded manner.

Histochemical detection of redox active iron in kidney sections was performed according to Smith et al. (16) using deparaffinized kidney sections that were fixed in methacarn. The extent of iron deposition was analyzed in a semiquantitative manner by a blinded investigator.

Mitochondrial functions. Mitochondria were isolated from the renal cortex of 12-month Lewis rats according to a previously published standard technique (17). Oxidative phosphorylation was determined using a polarographic Clark electrode (Instech Laboratories, Philadelphia, PA) (18). Coupled respiration was measured by adding 300 nmol of ADP. Uncoupled mitochondrial respiration was measured in the presence of 60 μmol/l dinitrophenol (DNP). DNP increases O_2 consumption independent of ADP transport or ATP synthesis, but its effect is dependent on the supply of reducing equivalents and electron respiratory chain components.

Electron respiratory chain complex activity. For analysis of complex I, 50 μg/ml of mitochondrial proteins were suspended in a hypotonic buffer (25 mmol/l KH_2PO_4, 0.5 mmol/l EDTA). On addition of 5 μmol/l antimycin A, 2 mmol/l potassium cyanide, 60 μmol/l ubiquinone-1, and 100 μmol/l NADH, consumption of NADH was monitored spectrophotometrically at 340 nm (molar absorptivity $\varepsilon = 6{,}200\ mol \cdot l^{-1} \cdot cm^{-1}$). For complex III activity, 2.5 μg/ml proteins in hypotonic buffer were used. Upon addition of 40 μmol/l reduced decylubiquinone, 50 μmol/l cytochrome c, and 2 mmol/l potassium cyanide, the reduction of cytochrome c was measured at 550 nm ($\varepsilon = 18{,}500\ mol \cdot l^{-1} \cdot cm^{-1}$). The coenzyme Q analog decylubiquinone was reduced with $NaBH_4$ to decylubiquinol and used freshly from a stock concentration of 5 mmol/l (pH 7.0). Complex IV activity was assayed polarographically after addition of 5 mmol/l ascorbate, 250 μmol/l TMPD (N,N,N′, N′-tetramethyl-P-phenylenediamine), and 10 μmol/l cytochrome c to 100 μg of mitochondrial proteins.

For Western blot detection of methylglyoxal hydroimidazolone–positive bands, mitochondrial proteins (14 μg/lane) were loaded on duplicate 4–12% (7 × 7 cm) Novex NuPAGE gels (Invitrogen, Carlsbad, CA). Monoclonal antibody to methylglyoxal-derived imidazole AGE (1H7G5) was used at 1:6,000 dilution (2). Antimouse IgG conjugated to horseradish peroxidase (1:40,000 dilution) and chemiluminescent substrate (SuperSignal West Femto, Rockford, IL) were from Pierce.

Isolation and solubilization of collagen. Samples of skin, aorta, and tendon were frozen in dry ice with methanol. Fat and hair were removed from skin with a razor blade. Tissues were minced and delipidated. Insoluble collagen was prepared as described (19). Aliquots (5 mg) of insoluble collagen in buffer H (0.02 M HEPES, 0.1 M $CaCl_2$ [pH 7.5], and 1 μl of toluene and chloroform per ml) were solubilized with 140 units of collagenase (type VII; Sigma C-0773) at 37°C for 24 h with continuous shaking. Tubes were centrifuged, and the pellet was washed and redigested as before. The supernatants were combined, dried, and stored at −80°C for AGE determination.

Determination of glucosepane and other glycation-related cross-links. To the solubilized collagen samples was added 15 μl of internal standard solution that contained 155.5 pmol of $^{13}C_6$-glucosepane, 78.2 pmol of $^{13}C_6$-DOGDIC, 99.5 pmol of $^{13}C_6$-MODIC, and 103.0 pmol of $^{13}C_6$-GODIC cross-links (see below). Digestion at 37°C was carried out by sequential addition every 24 h of 0.120 units of peptidase (Peptidase P-7500; Sigma) in 25 μl of HEPES buffer (pH 7.1), followed by 1 unit of pronase from *Streptomyces* griseum (Roche Diagnostics, Indianapolis, IN) in 50 μl of 0.02 M HEPES (pH 7.1) and 0.2 units of aminopeptidase M (Roche). The freeze-dried product was diluted to 1.5 ml with water and subjected to preparative high-performance liquid chromatography (HPLC) as previously described (20), except for a change in the gradient ammonium formate buffer (10 mmol/l [pH 4.0])/MeOH: 0(0)-2.5(3)-35(12)-95(13–15)-0(18–23). The residue of the lyophilized collected fraction was suspended in 40 μl of water and subjected to liquid chromatography (electrospray ionization) mass spectrometry analysis for glucosepane and other AGEs. The HPLC and LC/MS/MS system was as previously described (21). DOGDIC-ox isomers were monitored mass at m/z 445.

Pentosidine, furosine, and carboxymethyl-lysine. Pentosidine was determined by HPLC with fluorescence detector in 5 mg of acid hydrolyzed tissue samples as previously described (22). Carboxymethyl-lysine (CML) and furosine were determined as trifluoroacetic acid methyl ester derivatives in acid hydrolyzed delipidated protein samples by gas chromatography/MS using an isotope dilution method as described by Miyata et al. (23) using a Hewlett-Packard 5890 Series II Gas Chromatograph with a 5971 Series Mass Selective Detector and a 6890 Series Automatic Injector, a 25-m × 0.2-mm × 0.33-μmol/l Ultra2 column, and the temperature program of Dunn et al. (24). Internal

TABLE 1
Body weight, food intake, and glycemia in studied groups of rats

	n	Final weight (g)	Food intake (g/rat/day)	Plasma glucose (mmol/l)
Nondiabetic control	8	637.75 ± 38.39	17.58 ± 3.56	5.33 ± 1.45
Diabetic control	10	347.55 ± 64.74*	32.13 ± 4.28*	18.61 ± 2.31*
EC-treated diabetic rats	10	316.88 ± 41.82*	33.69 ± 5.17*	19.34 ± 2.38*
GT-treated diabetic rats	10	328 ± 80.95*	27.17 ± 4.00*	19.32 ± 3.75*

Data are means ± SD. Measurements are shown for 1 year and are representative for the results obtained throughout the study. EC, vitamin E + vitamin C; GT, green tea. *$P < 0.0001$ vs. nondiabetic control. Food intake was lower in GT vs. diabetic control ($P = 0.07$).

standards were 121.2 nmol of D_8-lysine and 195 pmol of D_4-CML. Lysine, D_8-lysine, CML, D_4-CML, and furosine were monitored at ions m/z = 320, 328, 392, 396, and 110, which eluted at 23 (lysine, D_8-lysine), 28 (CML, D4-CML), and 35 (furosine) min.

Tendon breaking time assay. Tendon collagen cross-linking was assayed by the tendon breaking time method as previously described (3,25).

Statistical analysis. The data are expressed as means ± SD. Univariate ANOVA was performed between diabetes and the individual intervention group. Tukey multicomparison tests were also computed using SSPS software for Windows (SPSS, Chicago, IL). Superscripts that share identical letters indicate no significant difference among groups. Nonidentical superscripts indicate statistical difference among groups at $P < 0.05$ level or better.

RESULTS

Body weights and food intake. The body weight of diabetic animals at the end of the study was lower than that of controls ($P < 0.0001$; Table 1). No significant differences between diabetic groups were observed, i.e., neither vitamins E and C nor green tea ameliorated the weight loss. More food was consumed by diabetic than normal rats ($P < 0.001$). Green tea tended to decrease food intake in diabetic rats ($P = 0.07$).

Green tea and antioxidant vitamins have no effect on mean glycemia. The rats developed mild to moderate diabetes with plasma glucose approximately three times higher than in controls (Table 1, Fig. 1*A*). Neither vitamins E and C nor green tea exerted a significant effect on glycemia. Glycated hemoglobin increased with time, probably as a result of worsening glucose tolerance. Furosine (i.e., acid hydrolyzed glycated lysine residues) was elevated in diabetic rat plasma proteins, aorta, and tendon and unaffected by treatment, except in aorta (Fig. 2), in which green tea decreased it to ~30% of diabetic controls ($P < 0.01$). This tissue-specific decrease in glycation by

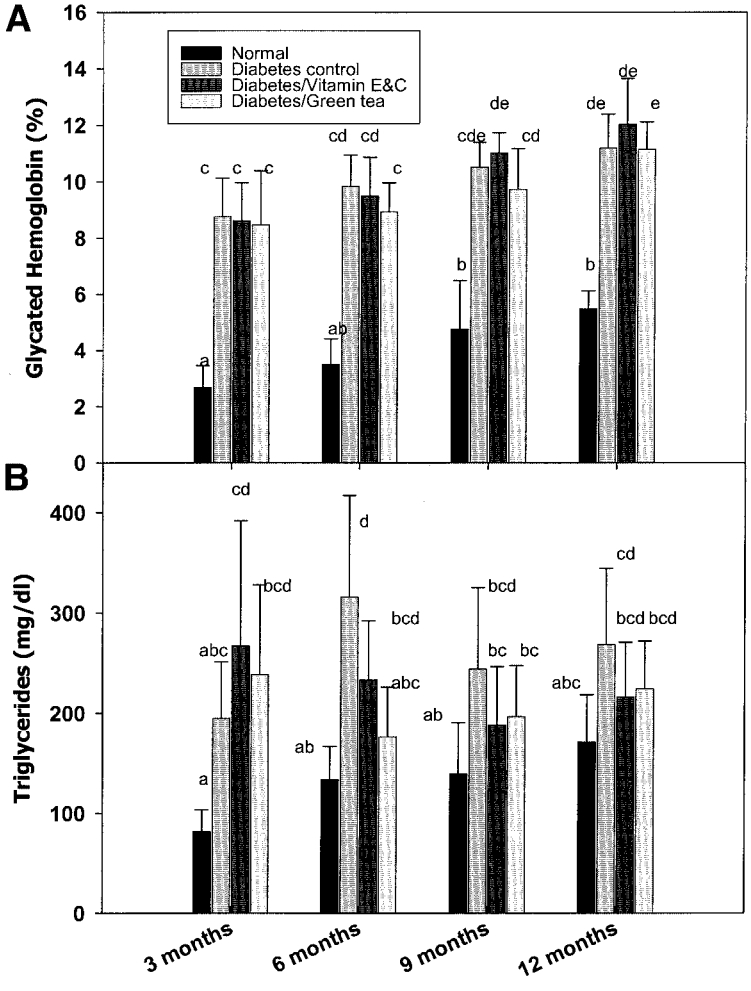


FIG. 1. The effect of diabetes, vitamins E and C, and green tea on glycated hemoglobin (*A*) and plasma triglyceride (*B*) ($P < 0.05$). Bars that do not share identical letter superscripts are different at $P < 0.05$.

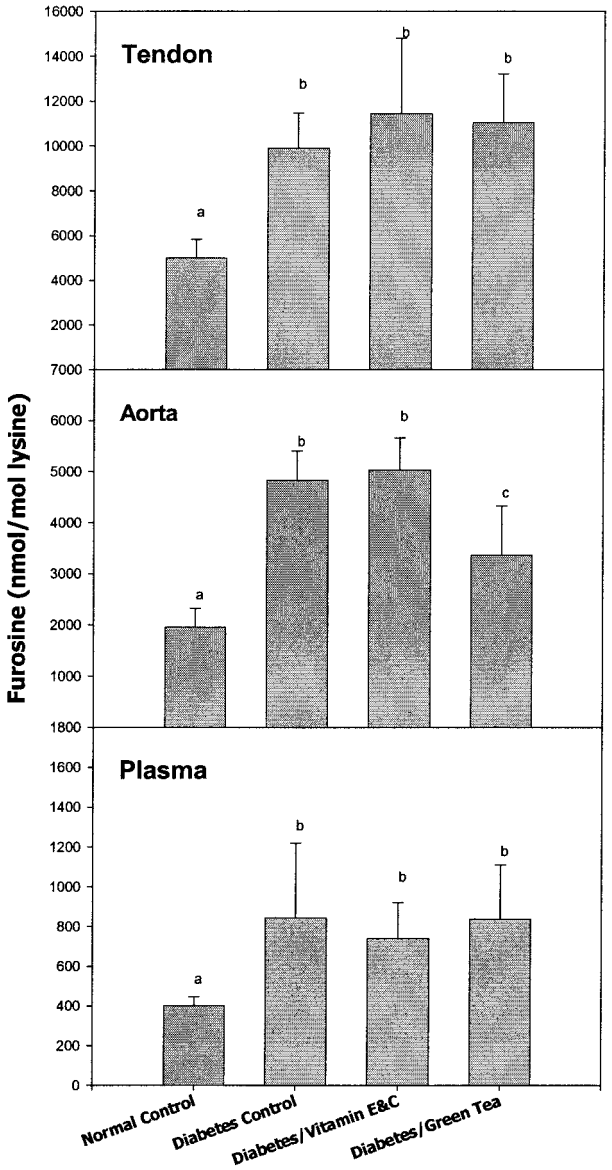


FIG. 2. The effect of diabetes, vitamins E and C, and green tea on furosine accumulation in tail tendon and aorta collagen and plasma proteins. Bars that do not share identical letter superscripts are different at $P < 0.05$.

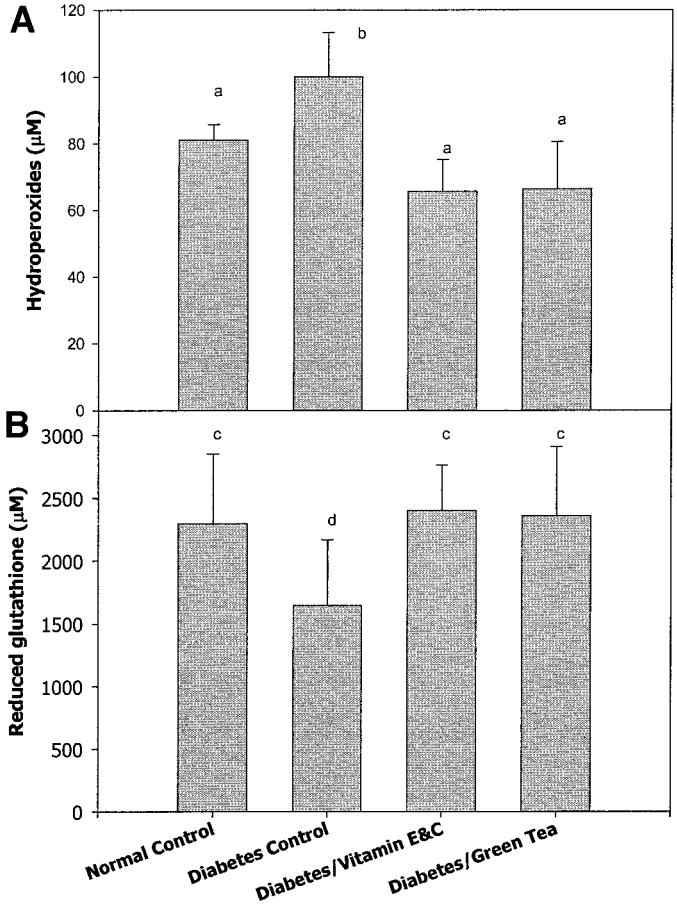


FIG. 3. The effect of diabetes, vitamins E and C, and green tea on plasma hydroperoxide levels (*A*) and erythrocyte glutathione (*B*) at the end of the experiment. Bars that do not share identical letter superscripts are different at $P < 0.05$.

green tea is unexpected and suggests either green tea–induced improvement in collagen turnover or selective blockage of glycation sites by oxidized green tea catechins.

Green tea and antioxidant vitamins tend to suppress hyperlipidemia. Plasma triglycerides were significantly increased in diabetic compared with nondiabetic rats ($P < 0.0001$; Fig. 1*B*). At 6, 9, and 12 months, values were consistently lowered by the vitamins and green tea, but the P value was <0.05 only at 6 months in the green tea group.

Antioxidant vitamins and green tea suppress plasma hydroperoxides and normalizes erythrocyte glutathione. Plasma hydroperoxides were mildly increased in the diabetic rats at 12 months ($P < 0.05$; Fig. 3*A*). Both vitamins E and C and green tea decreased hydroperoxides by ~40% (each, $P < 0.001$). It is interesting that vitamins E and C and green tea decreased the hydroperoxides below control levels by 20% ($P < 0.001$ and $P < 0.05$, respectively), suggesting intense peroxidative activity even in absence of diabetes. The 28% decrease in erythrocyte glutathione ($P < 0.01$) was completely restored by both vitamins E and C and green tea ($P < 0.05$; Fig. 3*B*).

Antioxidant vitamins and green tea improve but do not normalize retinal superoxide formation and morphologic abnormalities. Superoxide production in the retina of diabetic rats was increased by 316% compared with controls ($P < 0.001$; Fig. 4*A*). Vitamins E and C and green tea induced a 49.4% ($P < 0.05$) and 34% ($P < 0.05$) decrease, respectively. As expected, there was a significant loss of pericytes in diabetic retinas (Fig. 4*B*). Thus, 2.9 times more pericyte ghosts were counted in diabetic than control retinas ($P < 0.05$). This was decreased by 14 and 19% (NS) by vitamins E and C and green tea, respectively. Acellular capillaries were almost doubled in the diabetic state ($P < 0.001$; Fig. 4*C*), and green tea decreased them by 23% ($P < 0.05$). The decrease achieved by vitamins E and C was nonsignificant.

Antioxidant vitamins and green tea decrease lens crystallin-bound fluorescence. Previous data revealed that diabetes increased crystallin-bound fluorescence attributable to glycoxidation (12) or dihydropyridinium cross-links (26) and that green tea ameliorated selenite cataract in the rat (27). Here, crystallin-bound fluorescence at 370/440 nm was higher in water-soluble fraction (Fig. 5) in diabetic animals but unchanged in water-

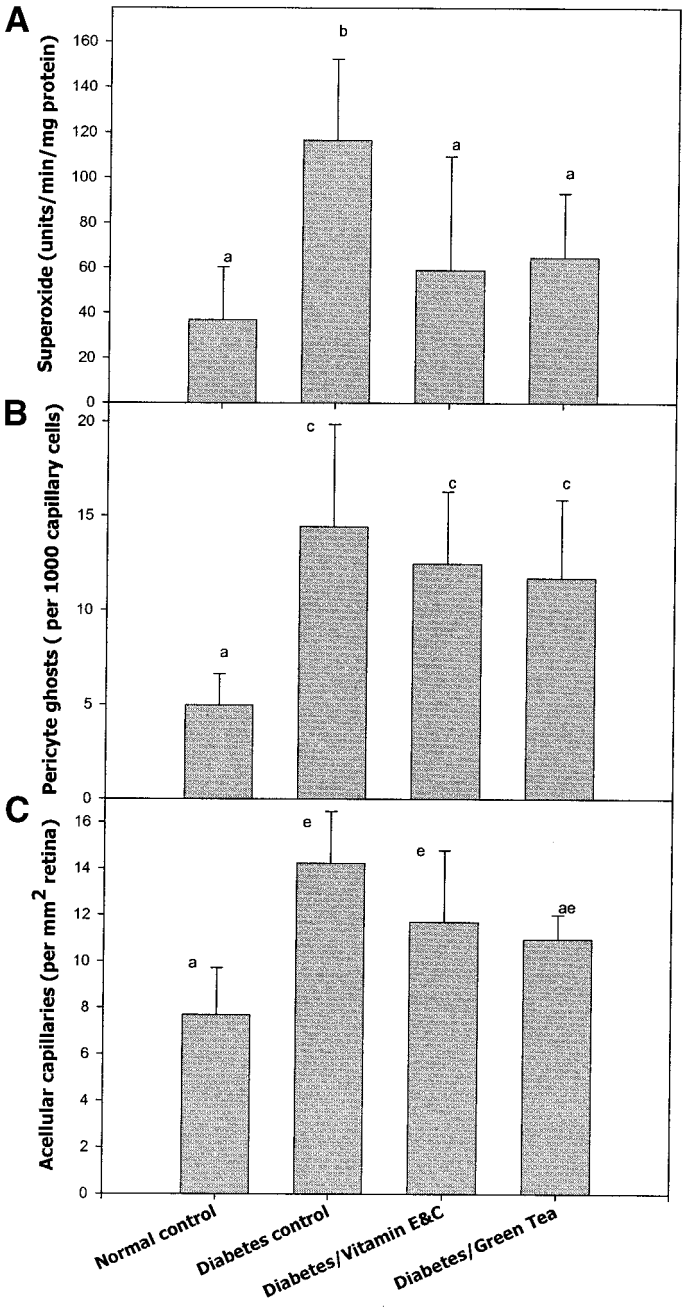


FIG. 4. The effect of diabetes, vitamins E and C, and green tea on superoxide anion production in rat retina (*A*), frequency of pericyte ghosts (*B*), and acellular capillaries (*C*) in retinas of diabetic rats. Bars that do not share identical letter superscripts are different at $P < 0.05$.

insoluble fraction (data not shown). Diabetes induced a 2.9-times increase in relative fluorescence compared with controls ($P < 0.001$). Vitamins E and C and green tea decreased the relative fluorescence by 26 and 32%, respectively. However, only green tea was significant ($P < 0.05$).

Nephropathy. Urinary albumin at 12 months was six times higher in the diabetic compared with control rats ($P < 0.01$; data not shown) but was mild in absolute terms. Neither vitamins E and C nor green tea improved this parameter. Glomerular staining for redox active iron increased 1.7-fold ($P < 0.05$; data not shown) and glomerular collagen IV by 23% ($P < 0.05$; data not shown), but neither the vitamins nor green tea induced a significant decrease. No significant difference in tubular iron staining among the groups was noticed. The failure to develop dramatic alterations in renal parameters may relate to the moderate hyperglycemia of these rats (average HbA_{1c} was 10–11%, whereas it is often 14–15% in other studies [28,29]).

Protective effect of green tea on renal mitochondrial respiration. The growing interest in the role of mitochondrial function in diabetes complications led us to examine this relationship. Renal cortical mitochondria from 12-month normal rats had a NADH-linked state 3 (ADP dependent) and state 4 respiratory rate of 201.2 ± 16.4 and 48.4 ± 4.8 nmol $O_2 \cdot$ mg mitochondrial protein$^{-1} \cdot$ min^{-1}, respectively (Fig. 6*A*). Diabetes induced an 18% decline in state 3 respiration ($P < 0.002$) that was significantly improved by green tea ($P < 0.05$). State 4 respiration was unchanged under these experimental conditions.

Uncoupling agents collapse the proton gradient, thereby promoting maximum rates of mitochondrial respiration dependent on tricarboxylic acid cycle (TCC) and electron respiratory chain (ERC) activity. The renal mitochondria demonstrated decrease of uncoupled respiration (Fig. 6*B*), supporting the idea that diabetes exerts its effect on either TCC or ERC. Green tea induced a significant improvement of uncoupled respiration, suggesting a protective effect on TCC and/or ERC.

For further defining the activity of NADH-linked ERC complexes, the activities of complexes I and IV of the ERC were determined and found not to be affected by chronic diabetes. However, complex III activity was decreased by 20% ($P < 0.05$), and both green tea and the vitamins improved this parameter but significance was reached only in the vitamin group ($P < 0.05$; Fig. 6*C*).

Green tea was found to protect against carbonyl-induced modifications of mitochondrial proteins. Western blot analysis of renal mitochondrial proteins revealed the presence of three methylglyoxal hydroimidzolone–containing proteins between 28–38 kDa and ~14 kDa (Fig. 6*D*, inset). The signal intensity of the ~28-kDa band was increased in mitochondria from all diabetic compared with control animals ($P < 0.01$), and green tea but not vitamins E and C suppressed the immunoreactivity ($P < 0.05$).

Antioxidant vitamins worsen collagen glycoxidation in selected tissues. Pentosidine and CML were quantified in plasma and in insoluble collagen from tendon, skin, and aorta. Diabetes increased pentosidine (Fig. 7*A*) in all tissues and, surprisingly, the vitamins markedly worsened its levels in tendon, aorta, and skin, whereas green tea worsened levels in tendon and aorta. Thus, the 41% diabetes-related increase in tail tendons ($P < 0.0001$) was further increased by 55% in the vitamins E and C group ($P < 0.0001$). The 66% increase in skin ($P < 0.0001$) was further elevated by 60% ($P < 0.0001$) in the vitamin group. Similar results were obtained in aorta with an additional increase of 63% ($P < 0.05$) over diabetes control. The effect of green tea was approaching significance ($P = 0.06$). Green tea had the most enhancing effect in tendons (218% over diabetes control; $P < 0.001$) and a 36% additional increase approaching significance in aorta ($P = 0.06$).

The vitamins and green tea worsened CML in tendon ($P < 0.05$) and plasma (NS; Fig. 7*B*). Similar to glycated lysine (Fig. 2), there was a tendency for green tea to decrease CML in aorta, although not significantly.

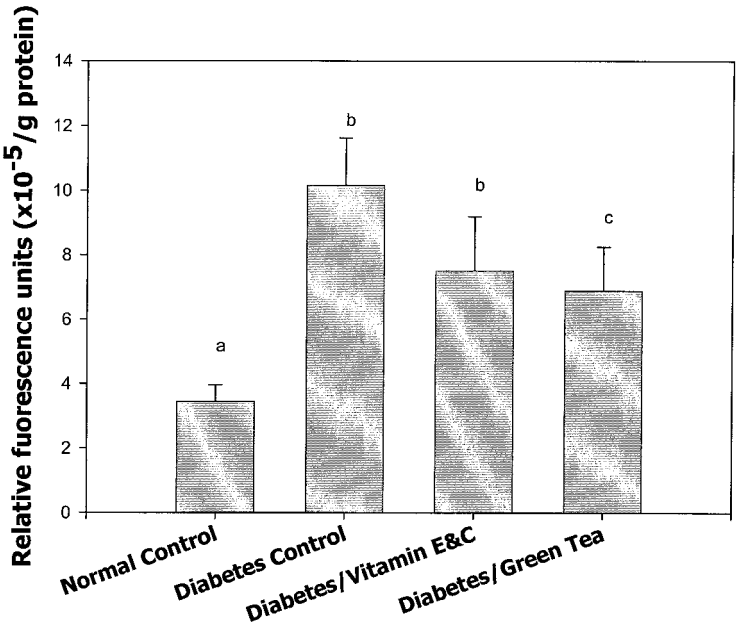


FIG. 5. The effect of diabetes, vitamins E and C, and green tea on relative fluorescence in water-soluble lens fraction. Bars that do not share identical letter superscripts are different at $P < 0.05$.

Effect of antioxidant treatment on glucosepane and other cross-links of the Maillard reaction. The recently described AGEs and lysine-arginine cross-links glucosepane and imidazoline cross-links MODIC, GODIC, and DOGDIC were quantified by LC/MS in enzymatic tendon digests. Glucosepane is derived from Amadori products, whereas the imidazolins are derived from methylglyoxal, glyoxal, and 3-deoxyglucosone, respectively (20). DOGDIC-ox, an oxidized form of DOGDIC (30), was also determined.

Glucosepane was 195% increased compared with controls ($P < 0.0001$; Fig. 8). Vitamins E and C slightly decreased glucosepane formation by 17.6% ($P < 0.05$), whereas the effect of green tea was not significant. Among other cross-links, only DOGDIC was increased by diabetes (NS; Fig. 8*D*). In contrast, MODIC and GODIC behaved like

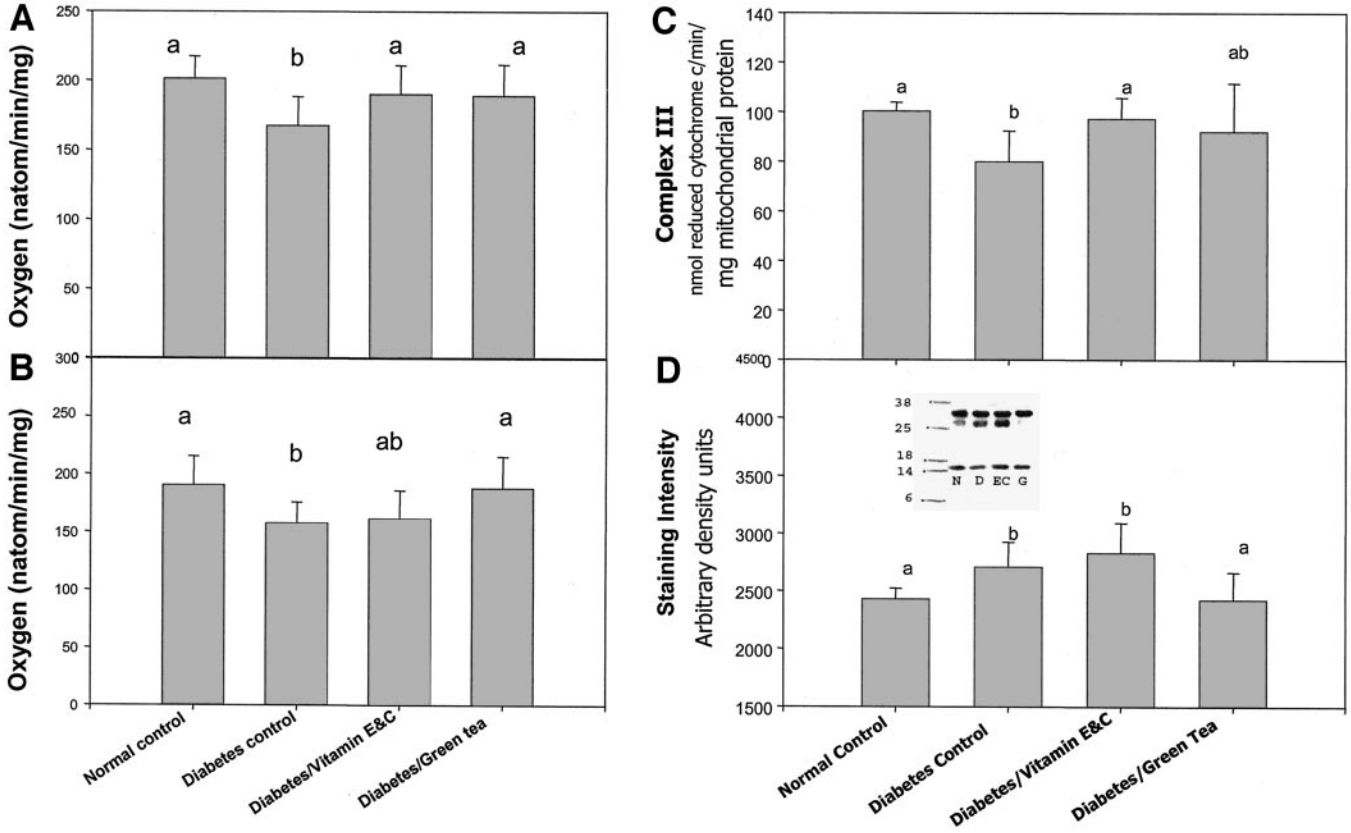
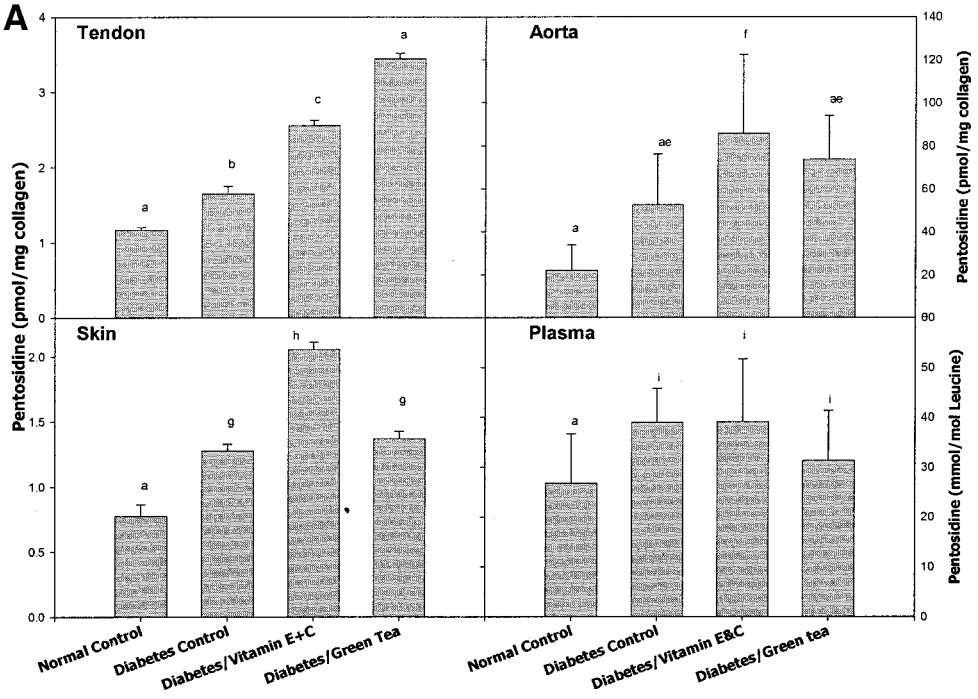


FIG. 6. The effect of diabetes, vitamins E and C, and green tea on NADH-dependent (*A*) and uncoupled (*B*) mitochondrial respiration, mitochondrial complex III activity (*C*), and methylglyoxal modification of a 28-kDa protein probed with an anti-imidazolone antibody (*D*). Bars that do not share identical letter superscripts are different at $P < 0.05$.



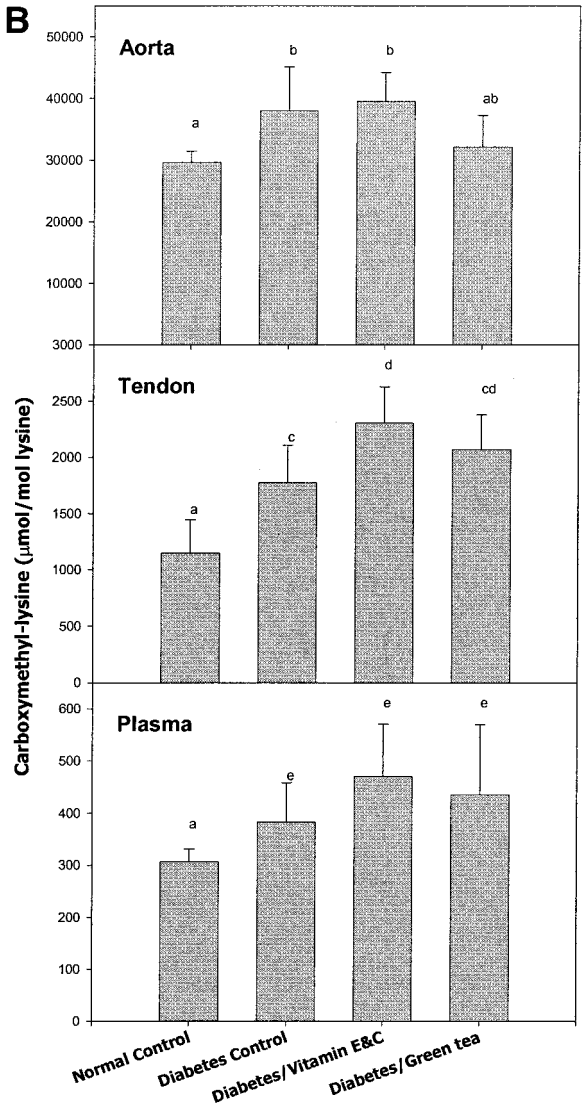


FIG. 7. The effect of diabetes, vitamins E and C, and green tea on pentosidine in tendon, skin, aorta, and plasma (*A*) and on CML in aorta and tendon collagen and plasma proteins (*B*). Bars that do not share identical letter superscripts are different at $P < 0.05$.

a mirror image of glucosepane, i.e., they were depressed (NS) rather than increased in all diabetic animals regardless of the treatment modality.

Effect of antioxidant vitamins and green tea on total tendon cross-linking. Tendon breaking time in urea, a parameter of total cross-linking, increased in diabetic animals from 45 ± 12 min in controls to 1,010 ± 680 min and 1,002 ± 352 min in the diabetes control and vitamins E and C groups, respectively (each $P < 0.001$). Levels further increased to 1,640 ± 450 min in the green tea group ($P < 0.001$). Large SDs were observed because the extent of cross-linking far exceeded the linearity of the method.

DISCUSSION

This study reveals a number of important findings on the relationship between diabetes complications, glycemic and oxidant stress, and the chronic consumption of green tea, i.e., a beverage widely promoted for its beneficial properties that are attributed to its potent antioxidants, the catechins (5). This study is timely in that oxidative stress and the formation of mitochondria-derived superoxide have been recently proposed to be at the center of the pathogenesis of diabetes complications (2).

Overall, the data indicate *1*) that green tea was successful at ameliorating oxidation end points such as plasma hydroperoxides, erythrocyte glutathione, retinal superoxide formation, and renal mitochondrial respiratory chain defects and *2*) that it was in essence acting similarly to the combination of vitamins E and C. The effects on nephropathy were inconclusive, possibly because only mild nephropathy developed. Thus, additional studies will be needed to clarify its effects on this complication, as well as neuropathy and atherosclerosis.

Despite significant effects of the antioxidant vitamins and green tea on the development of biochemical and functional tissue damage, it is surprising that the effects were not more robust in comparison with drugs such as

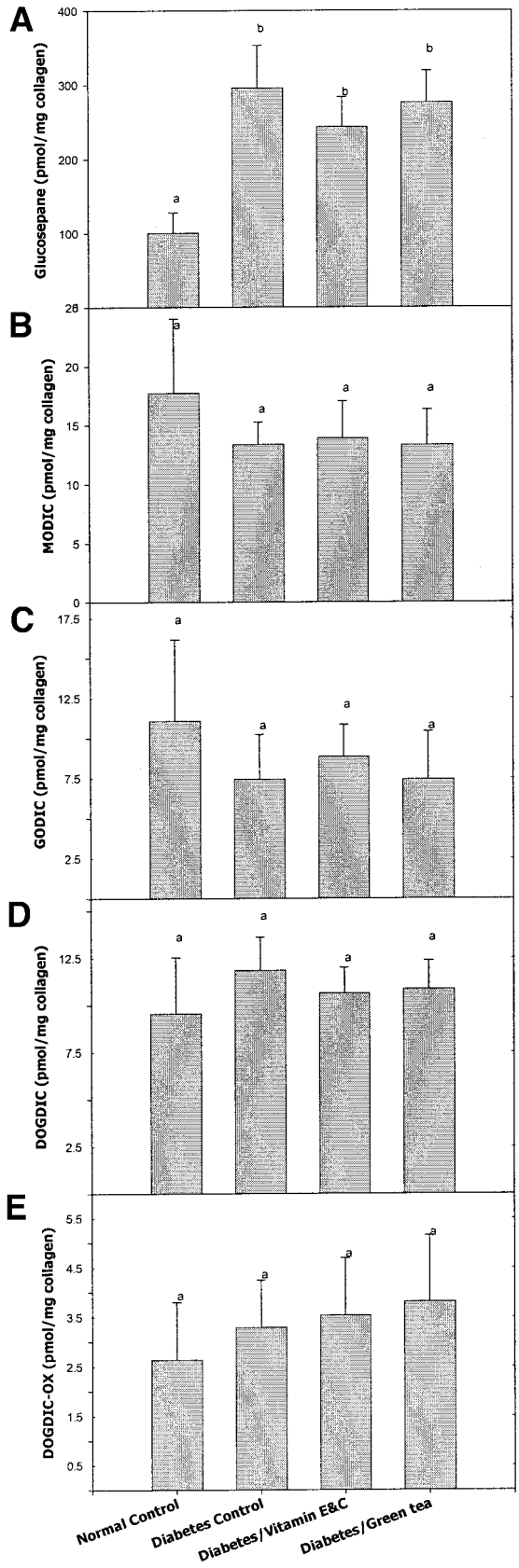


FIG. 8. The effect of diabetes, vitamins E and C, and green tea on glucosepane, MODIC, GODIC, DOGDIC, and DODIC-ox cross-link accumulation in tail tendon collagen. Bars that do not share identical letter superscripts are different at $P < 0.05$.

aminoguanidine (31), pyridoxamine (32), or benfotiamine (33). This suggests that mere suppression of oxidant stress without acting at a "higher" (34) or different level might not be sufficient. This notion is particularly apparent in the pyridoxamine trial with diabetic rats in which pyridoxamine almost completely suppressed albuminuria and retinopathy, whereas α-lipoic acid, enalapril, and vitamin E had milder or no effect (35). One pronounced effect of pyridoxamine was its ability to markedly lower hyperlipidemia to levels not observed in other studies, thereby providing support for the proposed role of lipids in the pathogenesis of diabetic nephropathy (36). In our study, however, only a minor hypolipidemic affect was observed in both treatment groups. Thus, it seems likely that the ability of pyridoxamine to trap both advanced glycation and lipoxidation products may contribute to its powerful activity against diabetes complications.

The marked suppressive effects of diabetes on renal mitochondrial respiratory function and their partial amelioration by the combination of antioxidant vitamins and green tea point to a key role of oxidative stress in mediating damage to the respiratory chain. However, although direct oxidative damage to components of the respiratory chain would be a logical outcome, the finding of a methylglyoxal-modified 28-kDa protein suggests an indirect mechanism involving AGE formation and damage to critical arginine residues. Such a pathway would be fully compatible with a previous demonstration that mitochondrial proteins are subcellular targets of dicarbonyl-induced modifications (17). The capacity of green tea to diminish this modification reinforces the connection between oxidative and carbonyl stress.

The most unexpected and counterintuitive result of this study was the observation that both green tea and antioxidant vitamins worsened a number of glycoxidation parameters in the extracellular matrix. Why would the antioxidant treatment result in increased pentosidine, CML, and cross-linking in selected tissues? A possible explanation was recently offered by Culbertson et al. (36), who reported increased pentosidine, CML, and generic cross-link formation during in vitro glycation reactions in the presence of antioxidants and other inhibitors. In short, both the complexity of the pathways involved in glycoxidation reactions and the multiple pharmacological properties of the various "inhibitors" are such that these may favor alternative nonoxidative pathways of "glycoxidation." Thus, certain inhibitors may actually tilt oxidant stress toward carbonyl stress. In their study, the vitamin E analogue Trolox dramatically increased pentosidine yield during protein ribosylation. Similar effects were noted on CML formation from both ribose and glucose when metals were absent from the incubation buffer.

Except for aorta, CML also increased beyond diabetic control values in tendon and plasma in both the antioxidant vitamin and green tea groups. Because the antioxidant treatment tended to suppress rather than increase lipidemia, it seems less likely that CML in these tissues would originate from lipid peroxidation. The latter mechanism, however, might explain CML formation in aorta, whereby either myeloperoxidase-mediated serine oxidation or peroxynitrite-mediated Amadori product oxidation could be involved (37).

Evidence in support of the importance of anaerobic pathways of AGE formation in diabetes is reflected in the high tendon levels of the collagen cross-link glucosepane (Fig. 8*A*). As expected, levels were not affected by treatment, because no oxidation is required for its formation. The nonsignificant decrease in the vitamin group reflects potential competition for identical reactive sites by ascorbylation products from vitamin C, such as pentosidine and CML. A similar competition for identical modification sites by the minor methylglyoxal and glyoxal-derived cross-links MODIC and GODIC is apparent. In fact, these are present in 10- to 20-fold lower quantities than glucosepane, thereby explaining why levels are depressed and not increased by diabetes.

Finally, the current results differ from our previous study in nondiabetic mice in which decreased cross-linking and AGE formation were observed in several tissues. One possible explanation is that the increased vascular permeability in diabetic tissues led to higher tissue concentrations of oxidized catechins, which are themselves potent tendon cross-linking agents (data not shown). However, the similarity of the green tea data with the vitamin group may also point to the tilting of oxidant to carbonyl stress and anaerobic pathways of cross-link formation. Future studies with various doses of green tea and antioxidant vitamins will be needed to understand precisely the discrepancy between the mice and the diabetic rat study.

In summary, the data support the notion that oxidative stress plays a role in the pathogenesis of diabetes complications in the rat. However, the study also reveals that interventions based on antioxidants alone, in contrast to carbonyl trapping and other agents such as pyridoxamine, aminoguanidine, and thiamine, only incompletely prevent diabetes-like complications. Finally, the study also provides strong evidence for a dissociation between cellular and extracellular processes, as reflected by a paradoxical increase in tissue glycation, glycoxidation, and cross-linking that might be attributed to the antioxidant treatment itself. Although the primary focus of this study was on green tea, further studies will be needed to clarify which vitamin has the most deleterious effect on collagen glycoxidation and cross-linking.

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REFERENCES

1. Higdon JV, Frei B: Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* 43:89–143, 2003
2. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
3. Rutter K, Sell DR, Fraser N, Obrenovich M, Zito M, Starke-Reed P, Monnier VM: Green tea extract suppresses the age-related increase in collagen crosslinking and fluorescent products in C57BL/6 mice. *Int J Vitam Nutr Res* 73:453–460, 2003
4. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB: The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic Res* 22:375–383, 1995
5. Dufresne CJ, Farnworth ER: A review of latest research findings on the health promotion properties of tea. *J Nutr Biochem* 12:404–421, 2001
6. Kaneko T, Matsuo M, Baba N: Inhibition of linoleic acid hydroperoxide-induced toxicity in cultured human umbilical vein endothelial cells by catechins. *Chem Biol Interact* 114:109–119, 1998
7. de Groot H, Rauen U: Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam Clin Pharmacol* 12:249–255, 1998
8. Fiala ES, Sodum RS, Bhattacharya M, Li H: (-)-Epigallocatechin gallate, a polyphenolic tea antioxidant, inhibits peroxynitrite-mediated formation of 8-oxodeoxyguanosine and 3-nitrotyrosine. *Experientia* 52:922–926, 1996
9. Kashima M: Effects of catechins on superoxide and hydroxyl radical. *Chem Pharm Bull (Tokyo)* 47:279–283, 1999
10. Cameron NE, Cotter MA: Neurovascular dysfunction in diabetic rats. Potential contribution of autoxidation and free radicals examined using transition metal chelating agents. *J Clin Invest* 96:1159–1163, 1995
11. Engerman RL, Kern TS: Retinopathy in animal models of diabetes. *Diabetes Metab Rev* 11:109–120, 1995
12. Nagaraj RH, Monnier VM: Non-tryptophan fluorescence and high molecular weight protein formation in lens crystallins of rats with chronic galactosemia: prevention by the aldose reductase inhibitor sorbinil. *Exp Eye Res* 51:411–418, 1990
13. Moore S, Stein WH: A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J Biol Chem* 211:907–913, 1954
14. Kowluru RA, Tang J, Kern TS: Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. *Diabetes* 50:1938–1942, 2001
15. Du Y, Miller CM, Kern TS: Hyperglycemia increases mitochondrial superoxide in retina and retinal cells. *Free Radic Biol Med* 35:1491–1499, 2003
16. Smith MA, Harris PL, Sayre LM, Perry G: Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci U S A* 94:9866–9868, 1997
17. Rosca MG, Monnier VM, Szweda LI, Weiss MF: Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal. *Am J Physiol Renal Physiol* 283:F52–F59, 2002
18. Humphries KM, Yoo Y, Szweda LI: Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry* 37:552–557, 1998
19. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S: Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA1c as markers of diabetic complications. DCCT Skin Collagen Ancillary Study Group. Diabetes Control and Complications Trial. *Diabetes* 48:870–880, 1999
20. Biemel KM, Friedl DA, Lederer MO: Identification and quantification of major maillard cross-links in human serum albumin and lens protein. Evidence for glucosepane as the dominant compound. *J Biol Chem* 277:24907–24915, 2002
21. Nagai R, Unno Y, Hayashi MC, Masuda S, Hayase F, Kinae N, Horiuchi S: Peroxynitrite induces formation of N(epsilon)-(carboxymethyl) lysine by the cleavage of Amadori product and generation of glucosone and glyoxal from glucose: novel pathways for protein modification by peroxynitrite. *Diabetes* 51:2833–2839, 2002
22. Sell DR, Monnier VM: Structure elucidation of a senescence cross-link from human extracellular matrix: implication of pentoses in the aging process. *J Biol Chem* 264:21597–21602, 1989
23. Miyata T, Fu MX, Kurokawa K, van Ypersele de Strihou C, Thorpe SR, Baynes JW: Autoxidation products of both carbohydrates and lipids are increased in uremic plasma: is there oxidative stress in uremia? *Kidney Int* 54:1290–1295, 1998
24. Dunn JA, McCance DR, Thorpe SR, Lyons TJ, Baynes JW: Age-dependent accumulation of N epsilon-(carboxymethyl)lysine and N epsilon-(carboxymethyl)hydroxylysine in human skin collagen. *Biochemistry* 30:1205–1210, 1991
25. Richard S, Tamas C, Sell DR, Monnier VM: Tissue-specific effects of aldose reductase inhibition on fluorescence and cross-linking of extracellular

matrix in chronic galactosemia: relationship to pentosidine cross-links. *Diabetes* 40:1049–1056, 1991
26. Gupta SK, Halder N, Srivastava S, Trivedi D, Joshi S, Varma SD: Green tea (Camellia sinensis) protects against selenite-induced oxidative stress in experimental cataractogenesis. *Ophthalmic Res* 34:258–263, 2002
27. Degenhardt TP, Alderson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, Thorpe SR, Baynes JW: Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61:939–950, 2002
28. Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G: Upregulation of the vascular endothelial growth factor/vascular endothelial growth factor receptor system in experimental background diabetic retinopathy of the rat. *Diabetes* 47:401–406, 1998
29. Reihl O, Biemel KM, Eipper W, Lederer MO, Schwack W: Spiro cross-links: representatives of a new class of glycoxidation products. *J Agric Food Chem* 51:4810–4818, 2003
30. Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M: Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci U S A* 88:11555–11558, 1991
31. Stitt A, Gardiner TA, Alderson NL, Canning P, Frizzell N, Duffy N, Boyle C, Januszewski AS, Chachich M, Baynes JW, Thorpe SR, Anderson NL: The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. *Diabetes* 51:2826–2832, 2002
32. Hammes HP, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeld R, Giardino I, Brownlee M: Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat Med* 9:294–299, 2003
33. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
34. Alderson NL, Chachich ME, Youssef NN, Beattie RJ, Nachtigal M, Thorpe SR, Baynes JW: The AGE inhibitor pyridoxamine inhibits lipemia and development of renal and vascular disease in Zucker obese rats. *Kidney Int* 63:2123–2133, 2003
35. Inman SR, Stowe NT, Cressman MD, Brouhard BH, Nally JV Jr, Satoh S, Satodate R, Vidt DG: Lovastatin preserves renal function in experimental diabetes. *Am J Med Sci* 317:215–221, 1999
36. Culbertson SM, Vassilenko EI, Morrison LD, Ingold KU: Paradoxical impact of antioxidants on post-Amadori glycoxidation: counterintuitive increase in the yields of pentosidine and Nepsilon-carboxymethyllysine using a novel multifunctional pyridoxamine derivative. *J Biol Chem* 278:38384–38394, 2003
37. Anderson MM, Requena JR, Crowley JR, Thorpe SR, Heinecke JW: The myeloperoxidase system of human phagocytes generates Nepsilon-(carboxymethyl)lysine on proteins: a mechanism for producing advanced glycation end products at sites of inflammation. *J Clin Invest* 104:103–113, 1999