Abnormalities of Retinal Metabolism in Diabetes and Experimental Galactosemia

VII. Effect of Long-Term Administration of Antioxidants on the Development of Retinopathy

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Antioxidants were administered to diabetic rats and experimentally galactosemic rats to evaluate the ability of these agents to inhibit the development of diabetic retinopathy. Alloxan diabetic rats and nondiabetic rats that were fed 30% galactose randomly received standard diets or the diets supplemented with ascorbic acid and α-tocopherol (vitamins C+E diet) or a more comprehensive mixture of antioxidants (multi-antioxidant diet), including Trolox, α-tocopherol, N-acetyl cysteine, ascorbic acid, β-carotene, and selenium. Diabetes or galactose feeding of at least 12 months resulted in pericyte loss, acellular capillaries, and basement membrane thickening. Compared with diabetic controls, the development of acellular capillaries was inhibited by 50% ($P < 0.05$) in diabetic rats that received supplemental vitamins C+E, and the number of pericyte ghosts tended to be reduced. The vitamins C+E supplement had no beneficial effect in galactosemic rats, but these rats consumed only approximately half as much of the antioxidants as the diabetic rats. The multi-antioxidant diet significantly inhibited (~55–65%) formation of both pericyte ghosts and acellular capillaries in diabetic rats and galactosemic rats ($P < 0.05$ vs. controls), without affecting the severity of hyperglycemia. Parameters of retinal oxidative stress, protein kinase C activity, and nitric oxides remained elevated for at least 1 year of hyperglycemia, and these abnormalities were normalized by multi-antioxidant therapy. Thus, long-term administration of antioxidants can inhibit the development of the early stages of diabetic retinopathy, and the mechanism by which this action occurs warrants further investigation. Supplementation with antioxidants can offer an achievable and inexpensive adjunct therapy to help inhibit the development of retinopathy in diabetes. *Diabetes* 50:1938–1942, 2001

Hyperglycemia induces metabolic disorders that initiate a sequence of events that lead to the development of retinopathy. This is illustrated convincingly by the evidence that nondiabetic animals in which blood hexose concentration is increased with a galactose-rich diet develop retinal capillary lesions that are indistinguishable from those that develop in diabetic humans or animals (1–4). Multiple hypotheses have proposed how hyperglycemia might cause the development of retinopathy (5–11), but it has been difficult to recognize which abnormalities are critical.

Diabetes increases oxidative stress in tissues of both humans and animals, and increased oxidative stress might play a role in the development of diabetic complications (8,12–14). Oxidative stress develops in the retina of diabetic animals and galactose-fed animals (9,13,15), indicating that oxidative stress is at least associated with the development of retinopathy. Consistent with this, Armstrong et al. (8) reported a correlation between increased serum lipid hydroperoxides and the prevalence of retinopathy in diabetic patients. Our previous studies showed that dietary supplementation with antioxidants can inhibit diabetes-induced abnormalities of retinal metabolism (7,9,13,16), but whether antioxidants can inhibit the development of retinopathy has been studied little. A retrospective study in type 2 diabetic patients, based on a single 24-h diet recall, found no evidence that antioxidant supplementation (vitamins C and E and β-carotene) could decrease the severity of retinopathy (17). Hammes et al. (18) observed that nicotinamide, a drug that has cholesterol-lowering and antioxidant properties, partially inhibited pericyte loss in diabetic rats but failed to have any effect on the number of acellular capillaries or microaneurysms. Diabetic rats that were given the water-soluble antioxidant Trolox for 5 months partially normalized a diabetes-induced change in ratio of retinal capillary endothelial cells to pericytes (19), but whether this finding was confounded by differences in severity of hyperglycemia (the initial cause of the retinal lesions) between experimental groups was not considered.

In the present study, we investigated the effect of long-term administration of two different mixtures of antioxidants on the development of retinal capillary lesions in two animal models of the early stages of diabetic...
retinopathy: alloxan-diabetic rats and galactose-fed rats. To help interpret the effects of the dietary supplements on development of retinal histopathology, we investigated whether hyperglycemia-induced abnormalities of retinal metabolism (oxidative stress, activation of protein kinase C [PKC]), and nitric oxides [NO]) persisted for the entire duration when the retinopathy was developing and could be inhibited by antioxidants over this duration.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats that weighed ~200 g were assigned randomly to be made diabetic, to be made experimentally galactosemic, or to remain as normal controls. Diabetes was induced by an injection of alloxan monohydrate (40 mg/kg i.v.) after a 24-h fast, and insulin was given as needed (0–2 units of NPH insulin subcutaneously, two to five times per week) to achieve slow weight gain without preventing hyperglycemia and glucosuria. Thus, diabetic rats were insulin-deficient but not grossly catabolic. Experimental galactosemia was induced in normal rats by feeding a diet of 30% α-galactose (wt/wt in diet). Representative diabetic rats and galactose-fed rats received diets that were supplemented with one of two antioxidant mixtures. In the first experiment, the “vitamins C+E diet,” both diabetic rats and galactosemic rats were fed ascorbic acid and α-tocopherol (10 and 1 g/kg diet, respectively). In the other antioxidant diet, the “multi-antioxidant diet,” diabetic rats and galactosemic rats were fed a diet that was supplemented with multiple antioxidants consisting of ascorbic acid (1 g/kg), Trolax (500 mg/kg), dl-α-tocopherol acetate (250 mg/kg), N-acetyl cysteine (200 mg/kg), β-carotene (45 mg/kg), and selenium (0.1 mg/kg). The antioxidants were mixed into the powdered diet, antioxidant diets were replaced weekly, and food consumption was measured to calculate the amount of antioxidants consumed. GHb (an estimate of the average level of hyperglycemia over the previous 2–3 months) was measured at least three to four times per year in each animal by affinity chromatography (Glyc-Affin, Pierce, Rockford, IL). Urine volume (24-h duration) was measured over 2–3 consecutive days every 3–4 months. Body weight and average daily food consumption were measured weekly. These experiments conformed to the ARVO Resolution on the Use of Animals in Research.

The rats that received the vitamins C+E diet were killed after 18 months of vitaminc C+E supplementation, and the rats that received the multi-antioxidant diet were killed 12–14 months after initiation of the multi-antioxidant diet. Appropriate normal, diabetic, and galactose-fed controls were killed simultaneously. One eye from each animal was placed in buffered formalin for isolation of the retinal vasculature by the trypsin digest technique (described below). From the other eye, the fresh retinas was collected and used in a microstereology and frozen for biochemical analysis. A small wedge of retina was collected for measurement of capillary basement membrane thickness from representative animals only in the vitamins C+E experiment.

Metabolic abnormalities in the retina.

For estimation of lipid peroxide, levels of thiobarbituric acid substances (TBARS) were quantified in the retina by measuring the absorbance of malonaldehyde-thiobarbituric acid adducts formed by acid hydrolysis at 100°C at 535 nm (20). A standard of 1,1,3,3-tetramethoxypropane, which is sensitive up to 0.3 μmol/l, was used. TBARS in retina were expressed relative to the protein concentration.

Total PKC activity was measured in fresh retina (<0.5 retina/tube) using an in situ assay (7,21). PKC activity was calculated on the basis of the transfer of 32P from [γ-32P]ATP to the octapeptide (KKRRLRL), corresponding to the threonine phosphorylation site of the epidermal growth factor receptor. Residual activity was validated by measuring the enzyme in the presence of phosphol bows esters (100 mmol/l tetradeoxyphosphorol bokeh acid) and staurosporine (50 mmol/l).

NO were determined in retina by measuring the stable metabolites of NO (nitrate+nitrite) using a fluorometric assay kit (Cayman Chemical Company, Ann Arbor, MI). Retinal homogenate was passed through a 10-KDa filter to remove interfering substances in the sample. For complete conversion of nitrate to nitrite, the samples were incubated with nitrate reductase for 2 h. Fluorescence generated by nitrite reaction with 2,3-diaminomethylphthalene was measured at excitation and emission wavelengths of 365 and 450 nm, respectively (22). In each experiment, the retinal tissues obtained from rats in each of the five groups (normal, diabetic, diabetes + antioxidants, galacosemic, and galactose + antioxidants) were run simultaneously. Tissue protein was measured by the Bradford method (23) using bovine serum alburnin as standard.

Evaluation of retinal histopathology.

Histopathology was detected in retinal vasculature isolated by the trypsin digest technique as used previously by us (24,25). The trypsinized retinal vessels were stained with periodic acid-Schiff and hematoxylin for histologic evaluation. Retinal cells, lesions, and capillary basement membrane thickness were quantified in a masked manner. The number of pericyte “ghosts” (indicating a site on a capillary where a pericyte had been lost) were determined in the mid-retina by examining ~1,300 capillary cells per retina. The number of acellular capillaries was counted in multiple mid-retinal fields (one field adjacent to each of the five to seven retinal arterioles radiating from the optic disc) and expressed relative to retinal area examined. Pericyte ghosts were counted only on capillaries that had one or more endothelial cells, because capillaries that lacked pericytes as well as endothelial cells were by definition acellular. The methods of counting pericyte ghosts differed between the two experiments, resulting in different absolute numbers even though the conclusions were the same. In the vitamins E+C experiment, pericyte ghosts were counted while the vascular preparation was viewed at high magnification (~400×) in the microscope; in the multi-antioxidant experiment, areas of the vascular bed were systematically photographed at the same magnification, and ghosts were determined by examination of digital images. Ghosts could be seen clearly in the microscope, but the digital method allowed us to mark positively each ghost and cell observed on the image (and to refer to the microscope for structure where the decision to classify the object as a ghost was more difficult). The difference in the absolute numbers of ghosts detected in the two studies presumably reflects these differences. Capillary basement membrane width was determined in the superior portion of the retina (mid-retina of the right eye) for representative animals using the method of Siperstein et al. (29).

Statistical analysis.

Data are reported as means ± SD and analyzed statistically using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney U test for multiple-group comparisons. Similar conclusions were reached also by using analysis of variance (ANOVA) followed by Tukey’s test.

RESULTS

Average blood hexose concentrations, as measured by GHb, did not differ between the two experiments and are therefore pooled. GHb remained significantly (P < 0.001) greater than normal in all of the diabetic groups and galactose-fed groups throughout the study (averaging 4.0 ± 0.6 for the normal group, 12.5 ± 0.8 for diabetic rats, 13.3 ± 2.2 for diabetic rats treated with antioxidants, 6.3 ± 0.9 for galactosemic controls, and 6.2 ± 0.5 for galactosemic rats treated with antioxidants). Neither antioxidant supplement had a significant effect on GHb, urine volume, or body weight compared with that in control diabetic rats or galactose-fed rats.

Oxidative stress, as estimated by TBARS, remained elevated (~twofold above normal) in the retina of rats that were diabetic or experimentally galactosemic for at least 1 year. Likewise, total PKC activity in the whole retina remained increased above normal (35%), and NO remained elevated >50% above normal in both diabetic and galactosemic animals (Table 1).

Microvascular lesions consistent with the early stages of diabetic retinopathy were present in diabetic control and

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<table>
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<th>Hemoglobin (GHb)</th>
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<th>Diabetes + antioxidant</th>
<th>Galactose</th>
<th>Galactose + antioxidant</th>
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Values are mean ± SD of 6–9 rats in each group. Multi-antioxidant, multi-antioxidant supplements; *P < 0.05 vs. normal rats. †P < 0.05 vs. respective untreated diabetic rats or untreated galactose-fed rats.
galactose control rats after at least 1 year of elevated hexose concentration ($P < 0.01$ vs. normal controls; Figs. 1 and 2). Lesions included pericyte ghosts, acellular capillaries, and thickened retinal capillary basement membrane. Saccular capillary microaneurysms, intraretinal microvascular abnormalities, hemorrhages, and neovascularization were not observed in any group.

**Dietary supplementation with vitamins C+E.** Administration of vitamins C+E for 18 months significantly inhibited the development of acellular capillaries in retinas of diabetic rats ($P < 0.05$; Fig. 1). In the same vitamins C+E-treated diabetic rats, the number of pericyte ghosts likewise tended to be inhibited, but the results did not achieve statistical significance. A diabetes-induced thickening of capillary basement membrane was not affected by this vitamin mixture (capillary basement membrane width for normals = 135 ± 18 nm, diabetes = 215 ± 51 nm, diabetes + vitamins C+E = 192 ± 44 nm). Supplementation with this dose of vitamins C+E had no beneficial effect on the development of any parameter of retinopathy in galactose-fed animals, including acellular capillaries, pericyte ghosts, and basement membrane thickness. This may be because the galactose-fed animals that received this vitamin mixture did not consume as much of the antioxidants as did the diabetic rats (due to hyperphagia in the diabetic rats); average supplemental intake of α-tocopherol and ascorbic acid was 142 ± 24 and 1,422 ± 236 mg·kg$^{-1}$·body wt·day$^{-1}$, respectively, for the vitamins C+E-treated diabetic animals and 69 ± 9 and 685 ± 88 mg·kg$^{-1}$·body wt·day$^{-1}$, respectively, for the vitamins C+E-treated galactose-fed animals.

**Dietary supplementation with the multi-antioxidant mixture.** Because diabetic rats consumed more food than experimentally galactosemic rats (20), the concentration of each antioxidant in the diet that was fed to galactosemic rats was increased by 50% (and adjusted periodically) in this experiment so that antioxidant intake (per kilogram of body weight per day) was comparable between antioxidant-treated diabetic rats and galactosemic rats.

Administration of the multi-antioxidant mixture for the entire duration significantly inhibited the diabetes- and galactosemia-induced increases in retinal oxidative stress and also corrected hyperglycemia-induced increases in retinal PKC and NO ($P < 0.05$; Table 1). Unlike findings in the rats that were fed the vitamins C+E supplement, the multi-antioxidant supplement significantly inhibited the development of acellular capillaries and pericyte ghosts ($P < 0.05$) in both diabetic rats and galactose-fed rats (Fig. 2).

**DISCUSSION**

This study shows that increases in oxidative stress, PKC activation, and NO production remain elevated for at least 12 months in retinas of diabetic or galactosemic animals and can be inhibited significantly by the administration of antioxidants. Moreover, our results indicate that administration of antioxidants can inhibit the development of diabetes-induced retinal histopathology. The results demonstrate that there is at least a strong association between hyperglycemia-induced oxidative stress and retinal histopathology.

Possible sources of oxidative stress in diabetes include...
increased generation of reactive oxygen species by autoxidation of glucose, decreased tissue concentrations of low-molecular-weight antioxidants (13,27–29), and impaired activities of antioxidant defense enzymes (13). We reported previously that retinal glutathione levels are decreased and antioxidant defense enzymes are impaired in diabetes and that these defects are corrected by supplementation with either antioxidant mixture (13,20). Which retinal cell types manifest the oxidative stress in diabetes cannot be identified in the present study because metabolic abnormalities were measured in the whole retina. Reactive oxygen species have been reported to be increased in endothelial cell in the early stages of diabetes in BBZ/W rats, and this is accompanied by endothelial cell dysfunction (30). Li et al. (31) observed alterations in levels of mRNA for antioxidant enzymes in retinal pericytes from diabetic patients.

Both antioxidant supplements inhibited oxidative stress (9,13,20) and vaso-obliteration of retinal capillaries (as reflected by the number of acellular capillaries) in diabetic animals, but only the multi-antioxidant–supplemented diet inhibited retinal histopathology in galactose-fed animals. This observation is consistent with evidence reported by us previously, showing that the multi-antioxidant diet corrected a galactose-induced decrease in retinal catalase activity (20) that was not corrected by supplementation with vitamins C+E (13). It is not clear whether the beneficial effect of antioxidants in galactosemic animals that were fed the multi-antioxidant supplement is due to the greater intake of antioxidants in that experiment compared with that in the vitamins C+E experiment or whether the effect is due to benefits of the additional antioxidants. Increasing the diversity of antioxidants in the diet has been shown to provide significantly more protection against oxidative stress in various organs than any individual antioxidant (32,33). Vitamins C+E had effects similar to those observed with aminoguanidine, an agent that has antioxidant as well as other properties (34–37), in that it inhibited both parameters of oxidative stress and capillary lesions (acellular capillaries and pericyte ghosts) in diabetic rats but inhibited only oxidative stress in galactose-fed rats. It is possible that both vitamins C+E and aminoguanidine have fewer antioxidant properties than the multi-antioxidant mixture, which may not be apparent from the parameters of oxidative stress that were measured. Others have observed beneficial effects in peripheral nerve and heart at relatively high levels of the antioxidants (33,38). Moreover, we cannot rule out the possibility that the antioxidants that were administered to hyperglycemic rats might have been acting via mechanisms in addition to those related to correction of oxidative stress.

Inhibition of PKC might occur by multiple mechanisms, some of which might be independent of oxidative stress. α-Tocopherol has been found to normalize diabetes-induced increases in PKC activity via inhibition of diacylglycerol accumulation (a mechanism that seems not to involve oxidative stress) (39), but because reactive oxygen species directly increase the activity of PKC (40,41), it seems likely that oxidative stress also can contribute to activation of the enzyme. Hyperglycemia-induced abnormalities of retinal PKC and oxidative stress are closely interrelated, as indicated further by evidence that normalization of PKC activity in diabetes with selective inhibitors can inhibit the diabetes-induced increase in free-radical accumulation (42,43). The ability of the antioxidant mixture to prevent NO accumulation in diabetes might be due to the fact that nuclear factor κB–mediated regulation of the inducible form of NO synthase involves reactive oxygen species (44) or because antioxidants can scavenge NO directly and inhibit formation of cGMP (45).

In summary, long-term administration of antioxidants can inhibit the development of the early stages of diabetic retinopathy. The mechanism by which this action occurs warrants further investigation. Supplementation with antioxidants represents an achievable and inexpensive adjunct therapy to help inhibit the development of retinopathy in diabetic patients.

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