Oxidative stress is increased in the retina in diabetes, and it is considered to play an important role in the development of retinopathy. α-Lipoic acid, a thiol antioxidant, has been shown to have beneficial effects on polyneuropathy and on the parameters of oxidative stress in various tissues, including nerve, kidney, and retina. The purpose of this study was to examine the effect of α-lipoic acid on retinal capillary cell apoptosis and the development of pathology in diabetes. Retina was used from streptozotocin-induced diabetic rats receiving diets supplemented with or without α-lipoic acid (400 mg/kg) for 11 months of diabetes. Capillary cell apoptosis (by terminal transferase-mediated dUTP nick-end labeling) and formation of acellular capillaries were investigated in the trypsin-digested retinal microvessels. The effect of α-lipoic acid administration on retinal 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine levels was determined by enzyme-linked immunosorbent assay. α-Lipoic acid administration for the entire duration of diabetes inhibited capillary cell apoptosis and the number of acellular capillaries in the retina, despite similar severity of hyperglycemia in the two diabetic groups (with and without α-lipoic acid). Retinal 8-OHdG and nitrotyrosine levels were increased by over twofold and 70%, respectively, in diabetes, and α-lipoic acid administration inhibited these increases. Our results demonstrate that the long-term administration of α-lipoic acid has beneficial effects on the development of diabetic retinopathy via inhibition of accumulation of oxidatively modified DNA and nitrotyrosine in the retina. α-Lipoic acid supplementation represents an achievable adjunct therapy to help prevent vision loss in diabetic patients. Diabetes 53:3233–3238, 2004

Diabetic retinopathy is the leading cause of acquired blindness among young adults, and studies have shown that hyperglycemia per se initiates its development (1). Diabetes increases oxidative stress, which plays a key regulatory role in the development of its complications (2–4). Reactive oxygen species generated by high glucose are considered as a causal link between elevated glucose and the other metabolic abnormalities important in the development of diabetic complications (5). In diabetes, mitochondria in the retina experience dysfunction (6), and the therapies that inhibit superoxide production in the retina also inhibit the development of diabetic retinopathy (7). However, the mechanism by which oxidative stress can contribute to the development of retinopathy in diabetes remains to be elucidated.

In the pathogenesis of retinopathy in diabetes, retinal microvascular endothelial, Muller, and ganglion cells and pericytes are lost selectively via apoptosis before other histopathology is detectable, or loss of vision is evident (8,9). The detection of terminal transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells in animal models of diabetic retinopathy is shown to serve as a surrogate end point to screen efficacy of interventions to inhibit the development of diabetic retinopathy.

Administration of antioxidants to diabetic rats prevents the development of retinopathy and also retinal metabolic abnormalities postulated to be involved in the development of retinopathy (3,10). Vitamin E supplementation reduces the retinal hemodynamic abnormalities seen in diabetic patients (11), and pyridoxamine inhibits the formation of diabetes-induced retinal acellular strands in rats (12). By contrast, some studies have failed to show any effects of antioxidants on retinal vascular lesions (13), and the differences for such discrepancies are not clear. The antioxidant therapy that inhibits the development of retinopathy in diabetic rats inhibits the activation of nuclear transcriptional factor-κB (NF-κB) and the apoptosis execution enzyme caspase-3 (14,15), suggesting that the beneficial effects of antioxidants on the development of diabetic retinopathy might involve inhibition of activation of NF-κB and caspase-3.

α-Lipoic acid, a disulfide derivative of octanoic acid, can alter the redox status of cells and interact with thiols and other antioxidants (16). Administration of α-lipoic acid to diabetic rats has been shown to inhibit parameters of oxidative stress in various organs of diabetic rats, including kidney, nerve, and retina (10). α-Lipoic acid is also shown to inhibit diabetes-induced activation of a small-molecular weight G-protein in the retina (H-Ras), increased levels of vascular endothelial growth factor, and leukostasis (10,17,18), and these abnormalities have been postulated to play roles in the pathogenesis of retinopathy in diabetes. However, the effects of α-lipoic acid on retinal capillary cell apoptosis and the development of retinopathy are not known.
In the present study, we investigated the effect of long-term administration of α-lipoic acid on retinal capillary cell apoptosis and the development of retinal capillary lesions in the animal model of the early stages of diabetic retinopathy, the streptozotocin-induced diabetic rat. To help interpret the effects of this dietary supplementation on retinal capillary cell apoptosis and histopathology, we have investigated the effect of α-lipoic acid on oxidatively modified DNA and nitrate stress in the retina.

**RESEARCH DESIGN AND METHODS**

Wistar rats (200–220 g, male) were made diabetic by intraperitoneal injection of streptozotocin (55 mg/kg body wt). Insulin was administered to diabetic rats to allow slow weight gain while maintaining hyperglycemia (blood glucose levels of 20–25 mmol/l). Age-matched normal rats served as control. Diabetic rats were divided into two groups: the rats in group 1 received powder diet (Purina 5001) supplemented with α-lipoic acid (400 mg/kg), and in group 2 the diet was without any supplementation; these diets were initiated soon after establishment of diabetes (3–4 days after administration of streptozotocin). Each group had 12–15 rats, and the entire colony of rats was run by exposing the retinal vessels to DNase (2,000 units/ml in 20 mmol/l Tris-HCl, pH 7.5) for 10 min at room temperature before initiation of the TUNEL reaction. TUNEL-positive cells were identified in a masked manner.

**Oxidative stress.** Oxidative stress was measured in the retina by quantifying the levels of oxidatively modified DNA (8-hydroxy-2′-deoxyguanosine [8-OHdG]) and the levels of the intracellular antioxidant reduced glutathione. 8-OHdG levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit from Oxis Research (Portland, OR), as described by us previously (19). To improve the accuracy and reproducibility of 8-OHdG measurement, DNA purified from the retina was digested with DNase (20). The 8-OHdG standard (0.5–40 ng/ml) or 15–20 μg DNA was incubated for 1 h with monoclonal antibody against 8-OHdG in a microtitre plate precoated with 8-OHdG. The final color was developed by the addition of 3,3′,5′-tetramethylbenzidine, and absorbance was measured at 450 nm (19).

Glutathione was measured using a glutathione assay kit from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer’s instructions (19). The retinal sample (50–75 μg) was deproteinized using phosphoric acid, and the amount of 5-thio-2-nitrobenzoic acid produced was measured in the supernatant.

**Nitrotyrosine.** Nitrotyrosine levels were quantified by enzyme immunoassay using a nitrotyrosine enzyme immunosorbent assay kit from Oxis Research according to the manufacturer’s instructions. Nitrotyrosine standard or retinal homogenates were incubated with nitrotyrosine antibody in the microplate for 1 h; this was followed by incubation with streptavidin peroxidase for 1 h. The samples were incubated with tetramethylbenzidine substrate for 30 min, and the reaction was stopped by 2.0 mol/l citric acid. The formation of yellow product was measured at 450 nm. The assay was sensitive as low as 0.05 pmol of nitrotyrosine.

**NF-κB.** Activation of NF-κB was determined in the retina by ELISA, using an NF-κB kit from Active Motif (Carlsbad, CA) and following the manufacturer’s instructions. The assay was based on the principle that only the active form of NF-κB in the sample binds to oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) that is immobilized on the microplate. The primary antibody against the p65 subunit of NF-κB used in the assay system is accessible only when NF-κB is activated and bound to its target DNA. For a sensitive colorimetric readout, the secondary antibody used is conjugated to horseradish peroxidase. Retina was homogenized in the lysis buffer (as provided by the manufacturer) containing diithiothreitol and protease inhibitor, and after removing the cell debris, 8–10 μg protein was used for the ELISA.

Experimental groups were compared statistically using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test for multiple group comparisons. ANOVA with Fisher or Tukey group comparisons gave similar results.

**RESULTS**

**Effect of α-lipoic acid on capillary cell apoptosis and histopathology.** The number of TUNEL-positive cells in the trypsin-digested retinal vessels (Fig. 1) was significantly higher in diabetic rats than that observed in the vessels from age-matched normal control rats (P < 0.02) (Table 1), thus suggesting increased apoptosis. The total number of nuclei (including pericyte, endothelial cell, and nuclei with undetermined cellular attribution) positive for TUNEL staining was 4.1 ± 2.2 in diabetes compared with 1.6 ± 1.0 in normal control retinal vessels (Table 1). Administration of α-lipoic acid for the entire duration of diabetes prevented an increase in TUNEL-positive nuclei (Table 1).
Microvascular lesions consistent with the early stages of diabetic retinopathy were observed in the trypsin-digested retinal preparation prepared from diabetic rats (Table 1); the number of acellular capillaries was significantly increased in diabetes compared with the age-matched normal control eyes \((P = 0.0002)\). α-Lipoic acid administration also reduced the number of acellular capillaries in diabetic rats; the number of acellular capillaries in the retina was decreased from 3.4 in the diabetic group to 1.2 in the diabetes plus α-lipoic acid group, and the values obtained in the diabetes plus α-lipoic acid group were not statistically different \((P = 0.297)\) from those in the normal control group (Table 1).

**Retinal oxidative stress.** Oxidative stress, as determined by the concentrations of oxidatively modified DNA and intracellular antioxidant, remained elevated in the retina of rats diabetic for 11 months. The levels of 8-OHdG were elevated by over twofold and the concentration of glutathione decreased by ~40% in the retina of rats diabetic for 11 months as compared with those obtained from age-matched normal rat retina (Fig. 2). Long-term administration of α-lipoic acid inhibited the diabetes-induced increase in retinal 8-OHdG levels; 8-OHdG values were not statistically different in the α-lipoic acid group and the normal control group \((P = 0.5101)\). Similarly, the decrease in glutathione levels in the retina seen in diabetes was prevented by administration of α-lipoic acid (Fig. 2).

**Nitrative stress.** In the retina obtained from the rats diabetic for 11 months, nitrotyrosine levels remain elevated by 70% as compared with those obtained from age-matched normal rat retina (Fig. 3). In the same retina, NF-κB was also activated by >70% (Fig. 4). Diabetes-induced changes in retinal nitrative stress were inhibited by administration of α-lipoic acid, and nitrotyrosine levels were similar in the retina obtained from diabetic rats receiving α-lipoic acid and normal control rats. α-Lipoic acid administration also inhibited the activation of NF-κB observed in the retina of diabetic rats (Figs. 3 and 4).

**Severity of hyperglycemia.** The severity of hyperglycemia, as measured by GHb, body weight, and 24-h urine volume, was strikingly increased in the diabetic group compared with the normal control group. Administration of α-lipoic acid did not ameliorate the severity of hyperglycemia in diabetic rats: the values obtained for GHb, body weight, and 24-h urine volumes throughout the duration of the experiment were comparable between the two diabetic groups (diabetes and diabetes plus α-lipoic acid) and were significantly different \((P < 0.001)\) from the normal control group (Table 2).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>TUNEL-positive capillary cells/retina</th>
<th>Acellular capillaries/mm²-retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>13</td>
<td>1.6 ± 1.0</td>
<td>0.69 ± 0.63</td>
</tr>
<tr>
<td>Diabetes</td>
<td>9</td>
<td>4.1 ± 2.2*</td>
<td>3.33 ± 1.41*</td>
</tr>
<tr>
<td>Diabetes plus α-lipoic acid</td>
<td>11</td>
<td>2.1 ± 1.4</td>
<td>1.18 ± 1.08</td>
</tr>
</tbody>
</table>

TUNEL-positive cells were identified in the trypsin-digested retinal microvessel in a masked fashion by scanning the specimen with downward and upward motion beginning at the upper left margin. The same microvessel preparations after TUNEL staining were stained with periodic acid-Schiff and hematoxylin for histologic evaluation. The number of acellular capillaries was counted in multiple mid-retinal fields. \(*P < 0.05\) vs. normal or diabetes plus α-lipoic acid groups.

**FIG. 2.** Effect of administration of α-lipoic acid on diabetes-induced oxidative stress in the retina. Oxidative stress was measured by quantifying the levels of 8-OHdG using the 8-OHdG ELISA kit and 10–15 µg DNA prepared from the retina. Each measurement was performed in duplicate, and the graph represents the means ± SD of the values obtained from five normal, six diabetic, and eight diabetic plus α-lipoic acid rats. Glutathione (GSH) was measured in the deproteinized sample of the retina using colorimetric assay kit from Cayman Chemicals. The results are the means ± SD obtained from 10 rats in the normal control group and 8 rats each in the diabetes and diabetes plus α-lipoic acid groups. \(*P < 0.05\) compared with normal or diabetes plus α-lipoic acid groups. Diab, diabetes; Diab+LA, diabetes plus α-lipoic acid; Norm, normal.

**FIG. 3.** Microvascular lesions consistent with the early stages of diabetic retinopathy were observed in the trypsin-digested retinal preparation prepared from diabetic rats. The same microvessel preparations after TUNEL staining were stained with periodic acid-Schiff and hematoxylin for histologic evaluation. The number of acellular capillaries was counted in multiple mid-retinal fields. \(*P < 0.05\) vs. normal or diabetes plus α-lipoic acid groups.

**FIG. 4.** Nitrotyrosine levels and expression of NF-κB in the retina obtained from the rats diabetic for 11 months. The values obtained for nitrotyrosine levels and NF-κB expression in the retina obtained from diabetic rats receiving α-lipoic acid and normal control rats were similar. **TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>TUNEL-positive capillary cells/retina</th>
<th>Acellular capillaries/mm²-retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norm</td>
<td>5</td>
<td>1.6 ± 0.6</td>
<td>0.69 ± 0.63</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6</td>
<td>4.1 ± 2.2*</td>
<td>3.33 ± 1.41*</td>
</tr>
<tr>
<td>Diabetes plus α-lipoic acid</td>
<td>8</td>
<td>2.1 ± 1.4</td>
<td>1.18 ± 1.08</td>
</tr>
</tbody>
</table>

Diabetes plus α-lipoic acid rats. Glutathione (GSH) was measured in the deproteinized sample of the retina using colorimetric assay kit from Cayman Chemicals. The results are the means ± SD obtained from 10 rats in the normal control group and 8 rats each in the diabetes and diabetes plus α-lipoic acid groups. \(*P < 0.05\) compared with normal or diabetes plus α-lipoic acid groups. Diab, diabetes; Diab+LA, diabetes plus α-lipoic acid; Norm, normal.
Increased oxidative stress in diabetes is considered a contributing factor in the development of diabetic complications, including retinopathy (2-4), and reactive oxygen species generated by high glucose act as a causal link between elevated glucose and the other metabolic abnormalities important in the development of diabetic complications (5). Retinal superoxide levels are elevated (7), mRNA levels of superoxide dismutase are downregulated (21), and glutathione levels are decreased in diabetes (22), suggesting an overwhelming of the endogenous defense system. The therapies that inhibit superoxide dismutase activity and superoxide production and decrease retinal glutathione levels also inhibit the development of diabetic retinopathy (7,23). In addition, we have shown that in diabetes, retinal mitochondria experience dysfunction, and inhibition of superoxide accumulation inhibits the apoptosis of retinal capillary cells (6).

In diabetes, retinal microvascular cells undergo accelerated apoptosis before other histopathology is detectable or loss of vision is evident (8,9). The levels of the proapoptotic protein Bax are increased in the retinal capillary cells (6,24), and the activity of apoptosis execution enzyme is increased in the rat retina at a duration of diabetes when capillary cell death and histopathology can be detected (14). Both retinal endothelial cells and pericytes undergo accelerated apoptosis in diabetes, and the loss of both of these microvascular cells could lead to the histopathology characteristic of retinopathy. Accelerated apoptosis of endothelial cells can result in capillary closure because the increased turnover can prematurely exhaust the cell’s replicative capability and because pericytes probably do not replicate in the adults (25), their accelerated death is likely to result in pericyte ghosts. Histopathology of diabetic retinopathy takes over a year to develop in rats (17,26), but apoptosis is a rapidly consummated phenomenon, and the cell contains fragmented DNA for only a few hours (27). Thus, a small number of TUNEL-positive cells observed in diabetic retina (8,9) along with defective endothelial replication may well be sufficient to account for the pericyte loss and formation of acellular capillaries. However, in our studies we have not distinguished TUNEL-positive pericytes from endothelial cells, but it is likely that α-lipoic acid administration could be inhibiting diabetes-induced apoptosis of both endothelial cells and pericytes.

Here, for the first time, data are included to show that long-term administration of α-lipoic acid can prevent accelerated apoptosis and formation of acellular capillaries in the retinal vasculature, suggesting a beneficial effect of α-lipoic acid on the development of retinopathy in diabetes. In agreement, others have shown that α-lipoic acid has beneficial effects on the upregulation of retinal vascular endothelial growth factor and mitochondrial and cytosolic NAD+/NADH ratios (10). Furthermore, α-lipoic acid administration to diabetic rats has been shown to inhibit leukocyte adhesion to the retinal capillaries, but it has failed to show any beneficial effect on the abnormal retinal blood flow (18). Although both of these abnormalities are some of the early abnormalities seen in the retina in diabetes and are postulated to contribute to the pathogenesis of retinopathy (18,28,29), our data provides evidence that α-lipoic acid can effectively inhibit the development of retinopathy in diabetes.

In the present study, the concentrations of α-lipoic acid...
that inhibited microvascular apoptosis and pathology in the retina in diabetes prevented an increase in oxidative stress in the retina. The levels of retinal cytosolic antioxidant glutathione were similar in the diabetes plus α-lipoic acid and age-matched normal control groups. The concentration of retinal glutathione that we have obtained in the present study (including that in normal and diabetes groups) is significantly lower than our previous reports (22,30); however, the results are consistent and show that the amount of glutathione is significantly decreased in diabetes. Although we cannot pinpoint the reasons for such discrepancies in the absolute numbers, here we have used a colorimetric assay based on the enzymatic recycling that uses 5,5′-dithio-bis-2-nitrobenzoic acid, and the previous measurements were performed fluorometrically using o-phthalaldehyde. Because α-lipoic acid is a powerful free radical scavenger that can directly chelate metal ions and regenerate cytosolic antioxidants, the beneficial effects of α-lipoic acid on the apoptosis of retinal capillary cells and histopathology seen in this study could include both scavenging of free radicals and increasing glutathione. In support, others have reported beneficial effects of α-lipoic acid on oxidative stress in diabetes, including a decrease in mitochondrial and cytosolic NAD+/NADH, malondialdehyde plus 4-hydroxyalkenal concentrations in the retina, and glutathione levels in the kidney (10,31,32).

Increased levels of 8-OHdG are reported in the leukocytes of patients with idiopathic retinal inflammatory disease (33), and our data show that diabetic retinopathy, a disease that shares many similarities with a chronic inflammatory disease (34,35), also has increased oxidatively modified DNA in the retina. Higher levels of 8-OHdG are observed in diabetes in cardiomyocytes, kidney, and urine (19,36,37). Although α-lipoic acid is shown to terminate free radicals and chelate transition metal ions (16,38), we believe this is the first report showing that α-lipoic acid can effectively inhibit the accumulation of oxidatively modified DNA; the diabetes-induced increase in retinal 8-OHdG was inhibited by α-lipoic acid administration. This suggests that oxidative modification of DNA might be playing an important role in the pathogenesis of retinopathy in diabetes.

Our studies and those of others have shown that the retina experiences increased nitrative stress in diabetes: the levels of peroxynitrite (formed by the reaction between NO and superoxides) (39) are elevated and NF-κB is activated in diabetes, and they remain elevated when capillary cell apoptosis and pathology can be seen in the retina of diabetic rats (15,24,40). The activation of NF-κB in the present study was demonstrated by using the antibody against its p65 subunit; this is because the expression of p65 subunit is increased in the retinal microvasculature obtained from diabetic patients and in the isolated retinal capillary cells incubated in high glucose medium (15,24). In addition, Podesta et al. (24) have shown that in bovine retinal pericytes, only p65 antibody, but not p50 antibody, inhibits electrophoretic migration of the NF-κB complex, and this activation is considered responsible for the hyperglycemia-induced accelerated loss of pericytes observed in diabetic retinopathy.

Nitrative modifications in retina that are formed early in the course of development of retinopathy in diabetes appear to contribute to the progression of retinopathy after reinstitution of good glycemic control (22). The therapies that inhibit the activation of apoptosis execution enzyme development of retinopathy in diabetic rats inhibit NO and nitrotyrosine levels and NF-κB activation in the retina (3,14,15). Here we provide data showing that the concentration of α-lipoic acid that inhibits capillary cell apoptosis and pathology in the retina of diabetic rats also prevents the increase in diabetes-induced nitrative stress in the retina. Nitration of proteins is considered to play a role in the apoptosis of retinal cells; it can disrupt protein assembly and functions, with possible pathological consequences, and results in oxidation of protein sulfhydryls (41–43). Our results show that α-lipoic acid inhibits the diabetes-induced increase in nitrotyrosine and capillary cell apoptosis in the retina. α-Lipoic acid treatment, by preventing the formation of nitrotyrosine, has been shown to protect the endothelial NO system of the mesenteric vasculature and the nitricergic innervation of corpus cavernosum in diabetic rats (43). In addition, α-lipoic acid reduces the advanced glycation end product–induced activation of NF-κB by the reduction of oxidative stress in the cell (44) via inhibition of the release and translocation of NF-κB from the cytoplasm into the nucleus.

α-Lipoic acid inhibits capillary cell apoptosis and pathology in the retina, despite similar severity of hyperglycemia in diabetic rats receiving diets supplemented with and without α-lipoic acid. Although α-lipoic acid has been shown to stimulate glucose uptake into fat cells by activating the insulin signaling pathway (45), our results show that the beneficial effects of α-lipoic acid seen in the present study are not caused by amelioration of blood glucose levels, because the levels of GHB, a parameter of long-term blood glucose, were not different in diabetes and diabetes plus α-lipoic acid groups.

In summary, long-term administration of α-lipoic acid can inhibit the apoptosis of retinal capillary cells and the development of the early stages of diabetic retinopathy. The mechanism by which α-lipoic acid inhibits retinopathy possibly involves inhibition of both oxidative damage to DNA and nitrative stress in the retina. Thus, supplementation with α-lipoic acid represents an achievable adjunct therapy to help prevent loss of vision in diabetic patients.

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GHb (%)</th>
<th>Body weight (g)</th>
<th>Urine volume (ml/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.9 ± 0.8</td>
<td>507 ± 45</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>Diabetes</td>
<td>12.7 ± 1.2*</td>
<td>296 ± 34*</td>
<td>122 ± 25*</td>
</tr>
<tr>
<td>Diabetes + α-lipoic acid</td>
<td>11.5 ± 2.1†</td>
<td>274 ± 27*†</td>
<td>95 ± 21*†</td>
</tr>
</tbody>
</table>

Data are means ± SD of seven rats each in normal group and eight rats each in diabetes and diabetes plus α-lipoic acid groups. GHb and 24-h urine excretion (measured over 2–3 consecutive days) were quantified at 8 weeks of diabetes, and the process was repeated every 3 months thereafter. *P < 0.02 vs. normal; †P > 0.02 vs. diabetes.
ACKNOWLEDGMENTS

This study was supported in part by grants from the Juvenile Diabetes Research Foundation, the Thomas Foundation, and Research to Prevent Blindness. We thank Saiyeda Noor Abbas for technical assistance.

REFERENCES