

# Beneficial Effect of Zeaxanthin on Retinal Metabolic Abnormalities in Diabetic Rats

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**PURPOSE.** Oxidative damage and growth factors are implicated in the pathogenesis of retinopathy in diabetes. Recent studies have shown that two dietary carotenoids, lutein and zeaxanthin (Zx), that are specifically concentrated within ocular tissues, may play important roles in maintaining their integrity. This study is to evaluate the potential protective effects of Zx against retinal oxidative damage and growth factors in diabetes.

**METHODS.** A group of rats received normal powdered diet or powdered diet supplemented with 0.02% or 0.1% Zx soon after induction of diabetes. Age-matched normal rats served as control subjects. At 2 months of diabetes, oxidative stress, vascular endothelial cell growth factor (VEGF), and intercellular adhesion molecule (ICAM)-1 were quantified in the retina.

**RESULTS.** Zx supplementation prevented diabetes-induced increase in retinal damage, and increases in VEGF and ICAM-1. The levels of lipid peroxide, oxidatively modified DNA, electron transport complex III, nitrotyrosine, and mitochondrial superoxide dismutase were similar in the retinas of Zx-treated diabetic rats and normal control rats, and these values were significantly different from those obtained from diabetic rats without any supplementation. In the same rats, Zx also prevented diabetes-induced increases in retinal VEGF and ICAM-1. Both 0.02% and 0.1% Zx had similar effects on diabetes-induced retinal abnormalities, and these effects were achieved without ameliorating the severity of hyperglycemia. However, Zx administration failed to prevent a diabetes-induced decrease in retinal GSH levels.

**CONCLUSIONS.** Zx significantly inhibits diabetes-induced retinal oxidative damage and elevation in VEGF and adhesion molecule, all abnormalities that are associated with the pathogenesis of diabetic retinopathy. The results suggest that Zx supplementation has the potential to inhibit the development of retinopathy in diabetic patients. (*Invest Ophthalmol Vis Sci*. 2008;49:1645-1651) DOI:10.1167/iov.07-0764

Retinopathy, a sight-threatening complication of diabetes, is the major cause of blindness in young adults. Studies have documented that sustained hyperglycemia is the instigating cause of disrupted normal cellular metabolism leading to the development of retinopathy.<sup>1</sup> Diabetes increases oxidative stress, and increased oxidative stress is one of the key regulators in the development of diabetic complications.<sup>2-4</sup> Reactive

oxygen species (ROS) generated by high glucose are considered to act as a causal link between elevated glucose and the other metabolic abnormalities important in the development of diabetic complications.<sup>5</sup> Oxidative stress is increased in the retina in diabetes, the antioxidant defense system is compromised, and superoxide levels are elevated in the mitochondria.<sup>3,6,7</sup> Increased oxidative stress is implicated in the development of retinopathy in diabetes. We have shown that administration of antioxidants prevents diabetes-induced oxidative stress and nitrate stress and the development of retinopathy in diabetic rats.<sup>3,8</sup>

Diabetic retinopathy is considered to be a multifactorial disease with various abnormalities contributing to its development. Vascular endothelial growth factor (VEGF), a major angiogenic factor that is important in vascular permeability, is elevated in the retina and vitreous of diabetic patients and animals, and this increase is associated with the manifestation of diabetic retinopathy.<sup>9</sup> Diabetic retinopathy is also shown to have an inflammatory component, and leukostasis and elevated levels of the adhesion molecule ICAM-1 are observed in the retina in diabetes.<sup>10</sup>

Zeaxanthin (Zx), a natural pigment, is a 40-carbon molecule and has 11 conjugated double bonds (Fig. 1). It is one of the dietary carotenoids that are specifically concentrated in the retina, and a high concentration of carotenoids in the macula is postulated to be crucial in protecting the retina from light-induced damage.<sup>11-13</sup> Zx is considered to act as an antioxidant.<sup>14,15</sup> In diabetes, serum levels of lutein/Zx and lycopene and retinal levels of Zx and lutein are decreased.<sup>16-18</sup>

The purpose of this study is to evaluate the potential protective effects of Zx against diabetes-induced abnormalities in the retina that are implicated in the development of retinopathy. The effect of Zx supplementation was investigated on the parameters of oxidative stress, including oxidatively modified DNA (8-OHdG), complex III (coenzyme Q cytochrome-c reductase), and superoxide dismutase. To determine whether Zx can prevent diabetes-induced increase in retinal growth factors and adhesion molecules, the levels of VEGF and ICAM-1 were also quantified.

## METHODS

Diabetes was induced in Lewis rats (200-220 g, male) by administration of streptozotocin (55 mg/kg body weight). Three days after induction of diabetes, the rats with blood glucose levels above 300 mg/dL were divided into three groups: The rats in group 1 received powdered diet (Purina 5001; Ralston Purina, Richmond, IN) without any supplementation, and the rats in groups 2 and 3 received diets supplemented with Zx (0.02% and 0.1%, wt/wt, respectively). Zx was obtained from Zeavision, LLC (Chesterfield, MO). Low-dose insulin (1-2 IU) was administered three to five times a week to the diabetic rats, to avoid ketonuria and to allow slow weight gain while maintaining hyperglycemia (blood glucose levels above 300 mg/dL). Age-matched normal rats served as the control. Each group had 10 or more rats, and the entire rat colony received their respective diets fresh once every 2 weeks. The rats were weighed two times, and their food consumption was measured once every week. Glycated hemoglobin (GHb) was measured by affinity columns (as routinely used in our laboratory<sup>7,8</sup>) 2 days before termination of the experiment. After 8

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Supported in part by grants from the National Institutes of Health, the Juvenile Diabetes Research Foundation, Research to Prevent Blindness, and a charitable gift from the Dennis L. Gierhart Foundation.

Submitted for publication June 20, 2007; revised September 7 and October 8, 2007; accepted February 26, 2008.

Disclosure: **R.A. Kowluru**, None; **B. Menon**, None; **D.L. Gierhart**, Zeavision, LLC (F,I,C,E)

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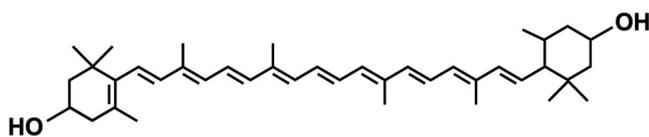


FIGURE 1. Structure of Zx:  $\beta$ -carotene-3,3'-diol C<sub>40</sub>H<sub>56</sub>O<sub>2</sub>.

weeks of diabetes, the rats were euthanized by an overdose of pentobarbital, the eyes were removed, and the retina was isolated and frozen immediately in liquid nitrogen for biochemical measurements. Treatment of the animals conformed to the National Institute of Health Principles of Laboratory Animal Care, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and local institutional guidelines.

Zx levels in the rat retinas were analyzed at Craft Technologies, Inc. (Wilson, NC), by using HPLC analysis, as reported by the company.<sup>19</sup> Retinal homogenate prepared in phosphate buffer with 2 mM EDTA was precipitated with ethanol containing butylated hydroxytoluene. The esterified carotenoids were released by ultrasonic agitation and extracted twice with 90% hexane-10% ethyl acetate. After the organic phase was evaporated, the residue was dissolved in ethyl acetate, sonicated, and analyzed by HPLC (Spherisorb ODS2 column; Waters Corp., Milford, MA).

Lipid peroxide (LPO) levels were quantified in the retina by using an assay kit from Cayman Chemical (Ann Arbor, MI). Hydroperoxide levels were measured directly by the redox reactions with ferrous ions and the resulting ferric ions were detected by using thiocyanate ion as the chromogen.<sup>8,20</sup>

To quantify the concentration of intracellular antioxidant GSH, retinal protein (10–15  $\mu$ g) was deproteinized with phosphoric acid, and GSH was measured in the supernatant by quantifying the amount of 5-thio-2-nitrobenzoic acid produced.<sup>8</sup>

8-OHdG levels were measured by an enzyme-linked immunoassay as routinely used in our laboratory. DNA extracted from the retina was digested with DNase before being used for the assay.<sup>8</sup>

Nitrotyrosine, a measure of peroxynitrite that is formed by the reaction between superoxide and nitric oxide, was quantified in the retina by enzyme immunoassay, as described previously. The assay is sensitive to 0.05 picomoles of nitrotyrosine.<sup>21,22</sup>

The enzyme activity of MnSOD (manganese-superoxide dismutase) was measured in 5 to 10  $\mu$ g retinal protein by a method in which tetrazolium salt is used to quantify superoxide radicals generated by xanthine oxidase and hypoxanthine. MnSOD activity was calculated by performing the assay in the presence of potassium cyanide, to inhibit Cu-Zn SOD and thus measure the residual MnSOD activity.<sup>7</sup>

The amount of VEGF was quantified by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), as routinely performed in our laboratory.<sup>22</sup> Serial dilutions of recombinant human VEGF served as the standard.

The retinal mRNA expressions of MnSOD, Complex III, iNOS, and ICAM-1 were determined by quantitative real-time polymerase chain reaction (Q-RT-PCR; *TaqMan Assays on Demand* for the rat; Applied Biosystems, Inc., [ABI] Foster City, CA), as described by us recently.<sup>23</sup> Briefly, RNA was isolated from the retina (TRIzol reagent; Invitrogen) and converted to cDNA by using a high-capacity cDNA reverse transcription kit with RNase inhibitor. Q-RT-PCR reactions were performed with 50 to 300 ng cDNA templates in 96-well plates on a sequence detection system (model 7500; ABI). Each sample was analyzed in triplicate. Data were normalized to  $\beta$ -2-microglobulin (B2M), and the change ( $x$ -fold) in gene expression relative to normal was calculated by the  $2^{-\Delta\Delta CT}$  method. The GenBank accession numbers and the amplicon length for the target and housekeeping genes used are provided in Table 1 (<http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).

Data are reported as the mean  $\pm$  SD. The results were analyzed by using one-way ANOVA followed by the Fisher test. Similar conclusions

were reached by the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test for multiple group comparisons.

## RESULTS

The severity of hyperglycemia, as measured by body weight, blood glucose, and 24-hour urine volumes, was strikingly increased in rats in the diabetes group compared with the normal control group and was similar in the diabetes and diabetes+Zx groups. Body weight, blood glucose, and urine volumes were comparable in the three diabetic groups (diabetes, diabetes+0.02% Zx, and diabetes+0.1% Zx) and were significantly different ( $P < 0.001$ ) from those in the normal control group (Table 2). The average Zx consumption, calculated from the daily food intake, was approximately fivefold higher in the diabetic rats receiving 0.1% Zx than in the diabetic rats receiving 0.02% Zx (44 and 8 mg/d, respectively).

The levels of Zx in the retinas of the diabetic rats tended to be marginally lower than in the retinas of normal rats; however, the difference did not achieve statistical significance. The Zx levels in the retinas of normal and diabetic rats were in the range of 130 to 180 pg/mg protein and were elevated to approximately 300 pg/mg protein in diabetic rats receiving 0.02% Zx and to approximately 1500 pg/mg protein in the diabetic rats with 0.1% Zx. We acknowledge that although levels of Zx showed a dose response, we cannot exclude the possibility of choroidal blood contamination.

As expected, the levels of LPO and oxidatively damaged DNA, 8-OHdG, were elevated by more than 50% (Fig. 2) in the retina of diabetic rats compared with the age-matched normal rats. Administration of Zx for 2 months prevented a diabetes-induced increase in retinal LPO and 8-OHdG levels. The beneficial effects achieved by 0.02% Zx were not different from those achieved when the concentration of Zx supplementation was increased to 0.1%.

Nitrotyrosine, another parameter of oxidative stress, was elevated twofold in the retinas of diabetic rats compared with that in normal rat retinas, and these levels were similar to those in our earlier reports.<sup>8,22</sup> A diabetes-induced increase in retinal nitrate stress was prevented when the rats were administered Zx soon after induction of diabetes (Fig. 3a). As with the other parameters, increasing the amount of Zx administration from 0.02% to 0.1% did not produce additional beneficial effects. The gene expression of the enzyme responsible for the diabetes-induced increase in NO in the retina, iNOS, was increased by approximately fivefold in diabetes, and supplementation with Zx inhibited this diabetes-induced increase in iNOS mRNA in the retina (Fig. 3b).

Inhibition of the mitochondrial complex III in diabetes is considered to be one of the sources of increased superoxide in the retina in diabetes.<sup>7</sup> The gene expression of complex III in the retina was decreased by 35% in the diabetic rats, and Zx supplementation (0.02%) prevented such reduction in complex III mRNA content (Fig. 4a).

TABLE 1. GenBank Accession Numbers and the Amplicon Length for the Genes Used for qRT-PCR

Gene	GenBank Accession No	Amplicon Length (bp)
<i>MnSOD</i>	NM_017051.2	76
Complex III	NM_001006970.1	98
<i>iNOS</i>	NM_012611.2	77
<i>VEGF</i>	NM_031836.1	75
<i>ICAM-1</i>	NM_012967.1	61
<i>B2M</i>	NM_012512.1	58

TABLE 2. Severity of Hyperglycemia and Level of Zx Supplementation in the Study Groups

Group	Body Wt (g)	Food (g/d)	Zx Consumption (mg/d)	Glucose (mg/dL)	Ghb (%)
Normal	329 ± 26	27 ± 2	NA	80 ± 16	5.5 ± 0.6
Diabetes	250 ± 16*	42 ± 8*	NA	435 ± 97*	15.6 ± 1.5*
Diab+0.02%Zx	245 ± 21*	42 ± 8*	8.4 ± 1.6	454 ± 77*	13.7 ± 1.8*
Diab+0.1% Zx	241 ± 20*	44 ± 8*	44 ± 8	500 ± 72*	15.8 ± 1.7*

Zx consumption was calculated from daily food intake. Ghb was measured by using affinity columns. Data are the mean ± SD of nine rats each in the normal and diabetes+0.1% Zx groups and eight rats each in the diabetes and diabetes+0.02% Zx groups.

\*  $P < 0.02$  compared with normal.

To investigate the effect of Zx on the antioxidant defense system in the retina, the activity and mRNA expression of the superoxide-scavenging enzyme MnSOD and the levels of the intracellular antioxidant GSH were quantified. The enzyme activity and mRNA expression of MnSOD and GSH were decreased significantly in the retinas obtained from diabetic rats compared with levels in retinas of the normal rats ( $P < 0.05$ ; Figs. 4b, 5). Administration of Zx for the entire duration of diabetes prevented the diabetes-induced inhibition of MnSOD (Fig. 5). MnSOD enzyme activities were comparable in the normal, diabetes+0.02% Zx, and diabetes+0.1% Zx groups. In contrast to the beneficial effect of Zx on MnSOD, Zx did not prevent a decrease in retinal GSH levels. The values in the diabetes and diabetes+Zx groups were not significantly different from each other ( $P > 0.05$ ; Fig. 4b).

Two months of diabetes in the rats increased retinal VEGF levels by 55%, but the level was normalized by administration of 0.02% Zx (Fig. 6a). Because diabetic retinopathy is considered to be a low-grade inflammatory disease, and ICAM-1, a representative factor for leukostasis, is increased in the diabetic retina,<sup>10</sup> we investigated the effect of Zx on ICAM-1 expression. Retinal levels of ICAM-1 were elevated by approximately 70% in the diabetic rats compared with that in the age-matched normal rats, and dietary Zx supplementation provided protection from the diabetes-induced increase in ICAM-1 gene expression ( $P < 0.05$  compared with diabetes; Fig. 6b).

## DISCUSSION

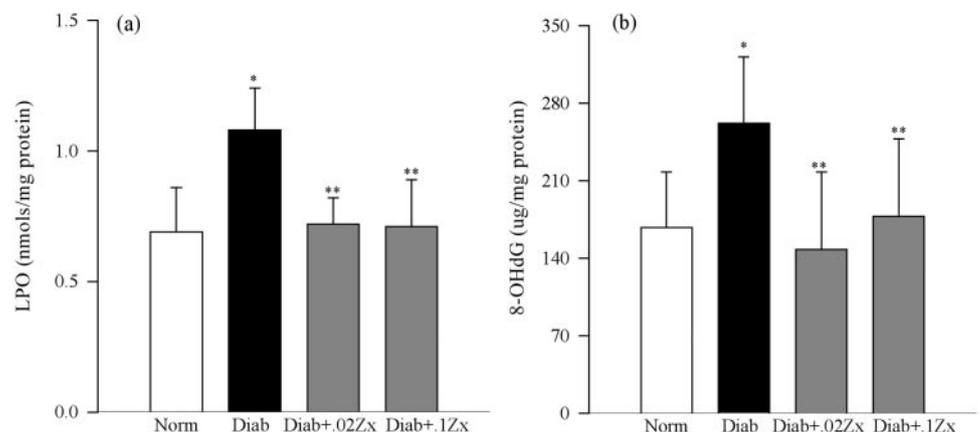
Zx, a member of the carotenoid family, is heavily concentrated in the retina. It protects ocular diseases commonly associated with aging, including cataract and macular degeneration.<sup>14,15,18</sup> Carotenoids are also considered to be beneficial in the prevention of a variety of other major diseases, including cardiovascular disease and cancer.<sup>15</sup> We provide data to show that supplementation of a carotenoid, Zx, inhibits the diabetes-

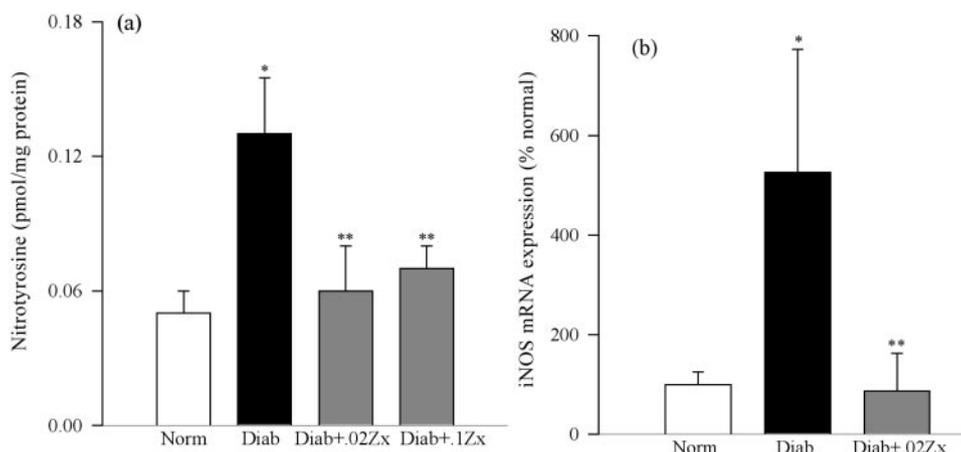
induced increase in oxidative stress and growth factors—retinal abnormalities that are postulated to be involved in the development of diabetic retinopathy. The results could have significant clinical implications, because they imply that Zx supplementation in diabetic patients could help prevent or retard the development of retinopathy.

Carotenoids, including Zx, are powerful antioxidants. Zx is shown to protect against peroxidation of fatty acids in the photoreceptor membrane and blood vessels. The chemical structure of Zx and its position and orientation in the lipid bilayer favors its ability to help reduce lipid peroxidation.<sup>24</sup> Zx protects the eye from ultraviolet and blue-light damage, and helps prevent free radical-induced damage to the retina and lens. Exciting data show that Zx can efficiently inhibit diabetes-induced increased lipid peroxidation in the retina. Our previous studies have shown that the antioxidant therapies that inhibit the diabetes-induced increase in lipid peroxidation in the retina also inhibit the retinal capillary histopathology characteristic of diabetic retinopathy.<sup>3,25,26</sup>

Oxidatively modified DNA, 8-OHdG, is a sensitive marker of increased oxidative stress. Increased 8-OHdG is implicated in the development of diabetic retinopathy. Its levels are increased in the retina in diabetes, and the therapy that inhibits diabetes-induced retinal histopathology also inhibits increased 8-OHdG levels in the retina.<sup>8</sup> Further, the retina of mice overexpressing MnSOD is protected from diabetes-induced capillary histopathology and an increase in 8-OHdG levels.<sup>7,27</sup> The effect of Zx administration on the inhibition of diabetes-induced elevation in retinal 8-OHdG suggests that Zx provides protection, in part, by inhibiting the accumulation of oxidized DNA. In support of this notion, others have shown that Zx reduces DNA damage in UVA radiation-exposed rat epithelial cells.<sup>28</sup> A mixture of antioxidants containing Zx has been shown to protect DNA damage in the photoreceptors of the *rd1* mouse, an animal model of retinitis pigmentosa. The number of TUNEL- and avidin-positive cells was considerably decreased on treatment with the combination of the

FIGURE 2. Effect of Zx on lipid peroxide and oxidatively modified DNA levels. (a) Hydroperoxides were measured in the retina directly by the redox reactions with ferrous ions, and the resulting ferric ions were detected by using thiocyanate as the chromogen. Data represent the mean ± SD of results in seven rats in the diabetes group, eight rats each in the normal and diabetes+0.02% Zx groups, nine rats in the diabetes+0.1% Zx group. (b) 8-OHdG levels were measured by ELISA. Data are expressed as the mean ± SD of results in seven to nine rats in each group. \* $P < 0.05$  compared with the normal group; \*\* $P < 0.05$  compared with the diabetes group.



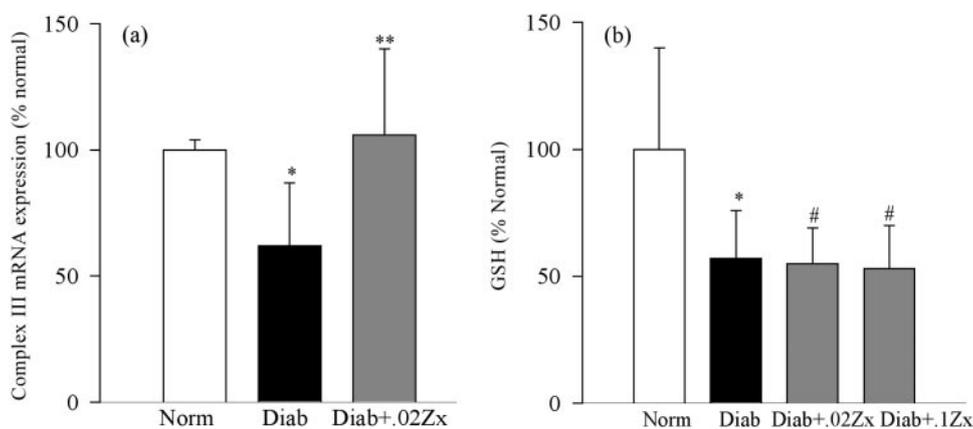


**FIGURE 3.** Effect of Zx on retinal nitrotyrosine and iNOS expression. (a) Nitrotyrosine concentrations in the retina were quantified with a nitrotyrosine EIA kit. Each sample was measured in duplicate. The data are expressed as the mean  $\pm$  SD of results in six rats each in the normal and diabetes + Zx groups and seven rats in diabetes group. (b) mRNA content of iNOS was measured using Q-RT-PCR and gene expression for iNOS was determined in the retina. The levels of iNOS mRNA were normalized to that of the housekeeping gene, *B2M*, in the same sample. The levels measured in normal rat retinas were considered to be 100%. Data are expressed as the mean  $\pm$  SD of results in five or more rats in normal, diabetes, and diabetes + 0.02% Zx. \* $P < 0.05$  compared with the normal group; \*\* $P < 0.05$  compared with the diabetes group.

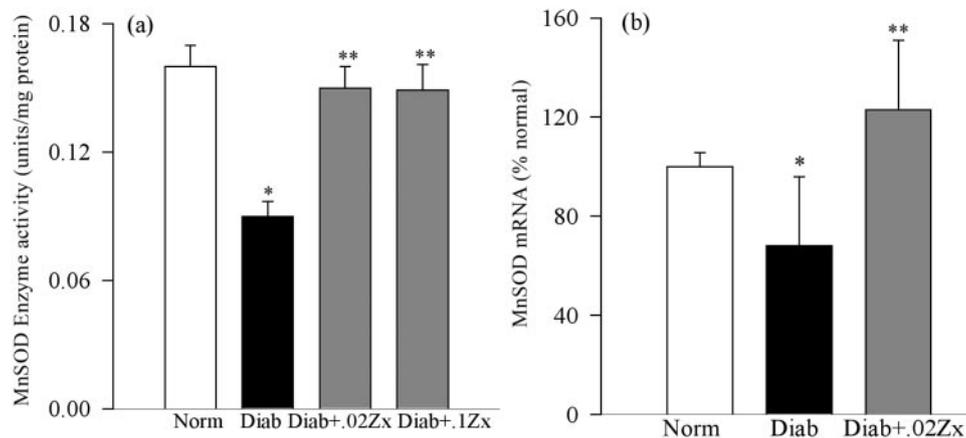
antioxidants.<sup>29</sup> Superoxide levels are elevated in the retina in diabetes, and the activity of the enzyme responsible for scavenging them is decreased.<sup>7,30,31</sup> Our recent studies have shown that the major source of increased retinal superoxide in diabetes is the mitochondria, and the formation of acellular capillaries in the retina that is characteristic of diabetic retinopathy can be prevented in the mice with MnSOD overexpression.<sup>7,27</sup> Mitochondrial superoxide production is considered to be a single unifying mechanism for diabetic complications.<sup>5</sup> The activity of complex III is reduced in the retinal mitochondria of diabetic rodents, leading to a further increase in superoxide accumulation, and administration of antioxidants and overexpression of MnSOD can prevent such decreases.<sup>7,32</sup> The results of the current study showed that diabetes-induced inhibition of complex III and the superoxide scavenging enzyme MnSOD in the retina was prevented by Zx supplementation. Consistent with this, Zx has been shown to scavenge superoxide,<sup>33</sup> and inhibit accumulation of

products of oxidation via both singlet oxygen and free radicals.<sup>34</sup> Further, Zx has been shown to inhibit superoxide generation in UVA-irradiated cells.<sup>28</sup> However, it should be acknowledged that, in the present study, mRNA levels of complex III, but not its activity, were quantified. Our recent study has shown that the antioxidant therapy that inhibits the development of retinopathy in diabetic rats also prevents a decrease in expression of retinal complex III mRNA.<sup>23</sup>

Nitric oxide production is elevated in the retina and the capillary cells in diabetes, and iNOS is considered to be the enzyme that contributes to increased nitric oxide levels.<sup>3,25,35</sup> Superoxide and nitric oxide react to form peroxynitrite, which can interact with lipids, DNA, and proteins and trigger cellular responses including oxidative damage and apoptosis.<sup>36</sup> Nitration of proteins is considered to play a role in apoptosis. It can disrupt protein assembly and functions with possible pathologic consequences and result in oxidation of protein sulfhydryls.<sup>37</sup> Nitritative modifi-



**FIGURE 4.** Effect of Zx on mitochondrial complex III and GSH in the retina. (a) Complex III mRNA content was measured by Q-RT-PCR, with *B2M* used as the housekeeping gene. Complex III mRNA content in the normal rat retinas was considered to be 100%. The data are the mean  $\pm$  SD of results in at least five rats in each of the three groups. (b) GSH was estimated in the supernatant obtained from deproteinization of the retinal homogenate. Each sample was analyzed in duplicate, and the data are expressed as the mean  $\pm$  SD of results in eight or more rats in each group. GSH content of the normal rat retinas was considered to be 100%. \* $P < 0.05$  compared with the normal group, and \*\* $P < 0.05$  and # $P > 0.05$  compared with the diabetes group.



**FIGURE 5.** Effect of Zx on the enzyme activity of MnSOD and its mRNA levels. (a) Activity of SOD was determined by using 5 to 10  $\mu$ g retinal protein. The activity of MnSOD was calculated by subtracting the activity that was inhibited by potassium cyanide from the total SOD activity. The data are the mean  $\pm$  SD of results in seven to eight rats in each group. (b) Gene expression of MnSOD was determined with a sequence detection system. The mRNA levels of MnSOD were normalized to that of *B2M* mRNA in the same sample. The amount of mRNA in the normal rat retina was considered to be 100%. \* $P < 0.05$  and \*\* $P < 0.05$  compared with the normal and diabetes groups, respectively.

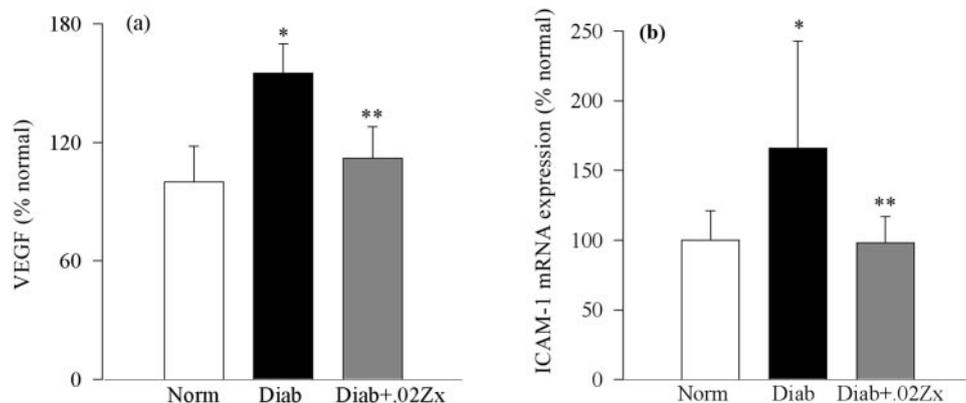
cations of retinal proteins are formed early in the course of development of retinopathy in diabetes, and levels of nitrotyrosine remain elevated when the histopathology is developing.<sup>38</sup> Supplementation with lipoic acid, an antioxidant that inhibits capillary cell apoptosis and pathology in the retina of diabetic rats, also prevents an increase in diabetes-induced oxidative stress in the retina.<sup>8</sup> The effect of Zx on the inhibition of the diabetes-induced increase in retinal iNOS expression and nitrotyrosine levels observed in our study suggests that Zx inhibits nitrotyrosine production by blocking both free radicals and nitric oxide. This finding is supported by results from others showing that Zx has the potential to protect macular tissue<sup>39</sup> and also acts in the initial stages of atherosclerosis<sup>40</sup> by prevention of peroxynitrite accumulation.

VEGF, a hypoxia-induced vascular growth factor, is elevated in the retina in diabetes, and this elevation plays a pivotal role in the increased permeability and angiogenesis in diabetic retinopathy.<sup>9,22</sup> A strong correlation is observed between lipid peroxides and VEGF in the vitreous of patients with proliferative diabetic retinopathy.<sup>41</sup> Antioxidants, including lipoic acid

and curcumin inhibit increased levels of VEGF seen in the retina of diabetic rats.<sup>22,41</sup> In this study, we show that Zx administration prevented the diabetes-induced increase in retinal VEGF levels. Consistent with this, Zx administration has been shown to normalize VEGF expression in RPE from APO-E<sup>-/-</sup> mice.<sup>42</sup> Thus, the beneficial effect of Zx supplementation on increased levels of VEGF are significant and imply that Zx has the potential to prevent the development of diabetic retinopathy by regulating growth factors.

Leukostasis is increased in the retina in diabetes, and increased leukostasis is postulated to contribute to the development of diabetic retinopathy.<sup>10</sup> ICAM-1 is linked to increased leukostasis, and the expression of ICAM-1 is upregulated by VEGF.<sup>43</sup> Amelioration of the diabetes-induced increase in ICAM-1 gene expression by Zx suggests that the protection of diabetic retinopathy by Zx could also include its beneficial effects on leukostasis.

Zx is a fat-soluble carotenoid, and its release from the food matrix and dissolution in the lipid phase appears to be critical



**FIGURE 6.** Effect of Zx on the diabetes-induced increase in retinal growth factors. (a) The amount of VEGF was quantified in the retinas of rats in the normal, diabetes, and diabetes+0.02% Zx groups by ELISA. VEGF content of the retinas of normal rats was considered to be 100%. The data are expressed as the mean  $\pm$  SD of four rats in the normal and five rats each in the diabetes and diabetes+Zx groups. (b) mRNA content of ICAM-1 was measured using Q-RT-PCR. The mRNA levels for the respective target genes were normalized to *B2M* mRNA in the same sample. The levels measured in normal rat retinas were considered to be 100%. \* $P < 0.05$  and \*\* $P < 0.005$  compared with the normal and diabetes groups, respectively.

in its absorption by the digestive tract.<sup>44</sup> We show that Zx levels in the retina were elevated fivefold in the rats receiving 0.1 mg/kg Zx compared with 0.02 mg/kg Zx. This finding strongly suggests that Zx is able to cross the blood-retinal barrier to reach the retina. Consistent with this, others have shown that women with diabetes have significantly lower levels of Zx and lutein in the retina.<sup>16</sup> Thus, the beneficial effects of Zx supplementation observed in our study can be attributed to the increased Zx concentration in the retina and are not due to other confounding effects.

Zx is considered a safe supplement. The daily recommended dose is approximately 6 to 30 mg, which is easily tolerated without any side effects. Although Zx and lutein are available from a wide variety of foods, the daily consumption is routinely lower than the concentration necessary for protection. Zx has been shown to improve the cytotoxic action of anticancer chemotherapy drugs and induce apoptosis of cancer cells in lymphoma and breast cancer.<sup>45,46</sup> A long-term study of healthy women has shown that Zx and lutein can reduce the risk of breast cancer in premenopausal women and help prevent atherosclerotic build-up by inhibiting fatty plaque formation and endothelial cell damage.<sup>47</sup> The Coronary Artery Risk Development in Young Adults/Young Adult Longitudinal Trends in Antioxidants trials have shown that carotenoids, including Zx are inversely associated with the markers of oxidative stress, inflammation, and endothelial dysfunction.<sup>17</sup> Serum carotenoids in patients with type 2 diabetes are shown to be inversely associated with impaired glucose metabolism.<sup>18</sup> We recognize that a long-term diabetic rat study (12–14 months) is necessary to determine the effects of Zx on the histopathologic characteristics of diabetic retinopathy, but our results strongly suggest that Zx has the potential to inhibit the development of diabetic retinopathy via ameliorating oxidative stress, growth factor expression, and inflammation, which raises the possibility that it could be used as an adjunct therapy to help prevent vision loss in patients with diabetes.

### Acknowledgments

The authors thank Mamta Kanwar, Sarah Odenbach, and Divyesh Sarman for technical assistance.

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