Enhanced Diabetes-Induced Cataract in Copper-Zinc Superoxide Dismutase–Null Mice

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PURPOSE. Oxidative stress is thought to contribute to diabetes-induced cataract, and the authors have previously demonstrated that lenses from mice lacking the antioxidant enzyme copper-zinc superoxide dismutase (SOD1) show elevated levels of superoxide radicals and are more prone in vitro to develop glucose-induced cataract than are wild-type lenses. In the present study the effect of streptozotocin-induced diabetes mellitus on cataract formation in SOD1-null and wild-type mice in vivo was examined.

METHODS. Eight weeks after diabetes was established by repeated intraperitoneal streptozotocin injections, the mice were killed and the lenses removed and photographed in retroillumination. The cataract was quantified from the photographs by digital image analysis and the lens contents of glutathione (GSH) as well as the lens protein carbonyl contents suggestive of protein oxidation were analyzed.

RESULTS. The streptozotocin-induced diabetic SOD1-null mice developed more cataract than the diabetic wild-type mice. Also, lens GSH levels were lower in the diabetic SOD1-null mice than in the nondiabetic SOD1-null mice. However, the protein carbonyls were equally raised in the diabetic mice of both genotypes.

CONCLUSIONS. The increased cataract formation and the compromised antioxidant capacity found in the diabetic SOD1-null lenses thus emphasize the involvement of superoxide radicals in diabetes-induced cataract. (Invest Ophthalmol Vis Sci. 2009; 50:2913–2918) DOI:10.1167/iovs.09-3510

Cataract is the leading cause of visual disability across the world and its surgical treatment is expensive and not without risks.1 The incidence and progression of cataract is elevated in patients with diabetes mellitus2–5 who also have higher complication rates from cataract surgery.4 To find a way to delay or inhibit the mechanisms leading to cataract would thus be desirable. Even though the etiology of cataract is still uncertain, researchers have in recent years focused on the impact of oxidative stress on lens transparency and the antioxidant defense against free radicals and reactive oxygen species (ROS).5

The lens is continuously exposed to oxidative stress through ROS generated both endogenously and exogenously in the eye’s surroundings. This chronic exposure may contribute to cataract formation by causing biochemical changes that gradually disarrange the lens fibers resulting in light scattering and loss of transparency.6

In diabetes mellitus, various ROS can be generated in the lens through many pathways. Apart from hyperglycemia, glucose is also elevated in the aqueous humor, causing an increased flux of glucose into the lens. The surplus of glucose in the presence of transition metals7 may facilitate autoxidation reactions between glucose and oxygen, leading to superoxide radical (O2−) production.8 Glucose may also induce glycation of lens proteins that can generate O2− and simultaneously accelerate the formation of advanced glycation end-products (AGEs).9 AGEs may, in turn, further contribute to O2− and hydrogen peroxide (H2O2) generation in the lens epithelium by interaction with specific cell surface receptors (RAGEs).10 Furthermore, O2− generation is also enhanced in the diabetic lens due to the activation of enzymes such as NADPH oxidase and xanthine oxidase.11 In addition to O2−, other ROS may also be elevated by diabetic conditions. Levels of nitric oxide (NO) may, for instance, be increased in the lens,12 as well as in the aqueous humor,13 which may increase peroxynitrite formation. Likewise, H2O2, which is also found to be increased in the aqueous humor,14 enters the lens and can promote generation of hydroxyl radicals (OH•) through Fenton reactions, facilitated by transition metals.7

In addition to the presence of raised levels of ROS, the susceptibility to oxidative insult is enhanced in diabetic lenses through a compromised antioxidant capacity. The flux of glucose through the polyol pathway may contribute to a loss of antioxidants,11 which is further aggravated by the glycation and inactivation of lens antioxidant enzymes such as the superoxide dismutases.12

The dominant superoxide dismutase isoenzyme in the lens is copper-zinc superoxide dismutase (SOD1),15 which catalyzes the degradation of O2− into H2O2 and oxygen.16 The loss of this cytosolic enzyme may thus result in cell damage caused by O2− and secondary oxidants.17 Accordingly, we have shown increased levels of O2− in lenses from mice lacking SOD1, and that these lenses are more prone to photochemical cataract.19 We have previously also studied the effect of in vitro hyperglycemic stress on lenses from SOD1-null mice, finding augmented cataract formation and cell damage,20 possibly through reactions between increased levels of O2− and NO.21 To extend these studies into a more physiological situation we have instead used streptozotocin (STZ)-induced diabetic mice in this study to explore the importance of SOD1 in the protection against cataract development in diabetes mellitus.

MATERIALS AND METHODS

Animals and Preparation of Lenses

The use of laboratory animals for this study was approved by the regional animal ethics committee. The mice were kept and handled according to the animal welfare legislation of Sweden and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The SOD1-null strain originating in 129/CD122 was backcrossed 10 times into C57BL/6J and thereafter bred and genotyped as detailed.

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previously.  

SOD1-null and wild-type mice originating from the same breeding were given repeated intraperitoneal injections of STZ for five consecutive days.  
The SOD1-null mice had, in preliminary experiments, shown a resistance to STZ (Olofsson EM, unpublished data, 2005), also confirmed by another laboratory, and were consequently given a higher dose of STZ (60 mg/kg) to induce hyperglycemia than were the wild-type mice (52 mg/kg). Mice of both genotypes were also given repeated injections of the STZ vehicle only (100 mg/mL sterile citrate buffer; pH 4.2) in the same manner, to provide nondiabetic control subjects. The mice were housed in a temperature- and light (12 hours)-controlled facility and allowed ad libitum access to water and standard mouse chow. Mean plasma glucose for each mouse was determined from blood samples drawn from the tail and immediately analyzed with a glucometer (GlucoSurePlus; Haemedic AB, Munka Ljungby, Sweden) at 2 and 8 weeks after the initial injection. Mouse weight was recorded before the first injection and after 2 and 8 weeks. Urine samples were collected only at weeks 8 and used for urine (U)-glucose analysis on a six-level scale: 0 mM, 1 to 5.5 mM, 6 to 14 mM, 15 to 28 mM, 29 to 55 mM, and $>$55 mM (Clinitek 500 Urine Chemistry Analyzer; Bayer Health Care AG, Leverkusen, Germany). The mice were nonfasting and not anesthetized at the time of plasma and urine sampling. After 8 weeks (56–58 days) the animals were killed by cervical dislocation. After dissection, the lenses were incubated in tissue culture medium, as described previously.  
The lens leakage of lactate dehydrogenase (LDH) into the culture medium during 2 hours of incubation was used as a marker of dissection damage. Three SOD1-null controls (three males and four females; mean age, 18.4 ± 5.7 weeks) and one wild-type lens showed a leakage of 5% or more analyzed with a colorimetric LDH assay (CytoTox 96 Non-radioactive Cytotoxicity Assay; Promega Corp., Madison, WI) and were thus excluded. Each lens was then weighed, photographed in retroillumination, and crushed and lysed in 500 μL of ice-cold phosphate-buffered saline in a homogenizer (UltraTurrax; IKA, Staufen, Germany) followed by sonication. The resultant homogenate was centrifuged for 10 minutes at 20,000g after which the lens supernatants as well as the lens incubation media were stored at −80°C.

Five SOD1-null mice treated with STZ had to be terminated prematurely due to illness, and one SOD1-null control was taken out of the study due to damaged eyes, leaving 13 SOD1-null mice treated with STZ (six males and seven females; mean age, 15 ± 5 weeks) and 7 SOD1-null controls (three males and four females; mean age, 18 ± 3 weeks). Twenty wild-type mice were injected with STZ (11 males and 9 females; mean age, 17 ± 5 weeks) and 14 with buffer only (7 males and 7 females; mean age, 19 ± 4 weeks). All the wild-type mice survived through the study.

**Analysis of Lens Opacity**

The degree of lens opacity was quantified by digital image analysis of gray scale photographs using ImageJ (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The standard deviations and the mean gray (MG) values obtained by measuring the central 1 mm² of the lenses photographed on top of a steel grid were inserted into the slightly modified formula previously constructed for cataract quantification.

$$\text{Lens opacity in arbitrary units (AU)} = \left(1 - \frac{\text{SD}}{485}\right) \times \left(\frac{255 - \text{MG}}{255}\right) \times 100$$

**Analysis of Glutathione**

The contents of reduced GSH were determined, to assess the redox states of the lenses. After deproteinization of the lens homogenates by 10% metaphosphoric acid and the addition of a freshly prepared 4 M solution of triethanolamine to a concentration of 5%, the samples were analyzed for total GSH content with a colorimetric assay kit (Glutathione assay kit; Cayman Chemical Co., Ann Arbor, MI) based on Ellman's reaction. In the assay, glutathione reductase was used to recycle oxidized glutathione (GSSG) to reduced GSH. The total GSH content in the samples was then calculated from a standard curve at 405 nm and related to the lens wet weight. For GSSG analysis alone, the sulfhydryl groups of GSH were blocked using a 1-M solution of 2-vinylpyridine to a concentration of 1%, before assessment with the assay kit. Finally, GSSG was subtracted from the total GSH to get the reduced GSH content in the lenses.

**Analysis of Protein Oxidation**

For further assessment of the redox states of the lenses, their contents of protein carbonyls, suggestive of oxidative stress-modified proteins, were determined with an immunoblot assay (OxyBlot Protein Oxidation Detection Kit; Chemicon International, Temecula, CA). To begin with, the water-soluble proteins in the lens supernatants were denatured by 12% SDS (sodium dodecyl sulfate), derivatized with 2,4-dinitrophenylhydrazine (DNPH), and neutralized and 2-mercaptoethanol after which they were separated by 15% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and electroblotted onto polyvinylidene difluoride membranes (GE Health care, Buckinghamshire, UK). Blots were blocked and probed with provided antibodies. Bands were visualized using a Western blot detection system (ECL Advance; GE Health Care, Buckinghamshire, UK) and the chemiluminescence recorded on an imager (Chemidoc XRS, using Quantity One software; Bio-Rad, Hercules, CA). The protein carbonyl content was calculated by relating the intensities of the bands to a standard curve attained by running a series of dilutions of a protein mixture (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and trypsin inhibitor) with known amounts of carbonylated proteins, on each blot. The resulting amount of carbonyls was finally divided by the wet weight of respective lens.

**Statistical Analyses**

Commercial software was used for data analysis (SPSS, Chicago, IL). For each mouse, the mean value of digital cataract quantification and lens wet weight were related to the mean plasma glucose levels and analyzed statistically with a general linear model with plasma glucose as a continuous factor and the genotype of the mouse as a categorical factor.

For each lens, the cataract quantification was related to GSH and protein carbonyl contents and analyzed by a general linear model with GSH and protein carbonyls as continuous factors and the genotype as a categorical factor. Also, regression analyses were performed for each separate genotype to assess for associations between cataract and GSH and carbonyl contents respectively. $R^2$ was calculated to describe how well the data fitted the regression models.

Student’s $t$-test and one-way analysis of variance followed by Tukey post hoc testing were applied when testing the differences between means of two or more groups. Transformations were performed on data that violated the assumptions of parametric testing. The results are expressed as the mean ± SD and $P \leq 0.05$ is considered significant.

**RESULTS**

The diabetic SOD1-null mice developed more cataract than did the diabetic wild-type mice or the nondiabetic mice (Fig. 1). The cataract was classified as cortical according to an established subjective cataract staging system, also used in our previous work (Fig. 2).  

Levels of GSH were equal in the nondiabetic control lenses of both genotypes, but were reduced by 19% in the STZ-induced diabetic SOD1-null lenses compared with the nondiabetic SOD1-null control animals. There was, however, no difference in GSH content between the STZ-treated and untreated wild-type mice (Table 1). Still, GSH contents declined as cataract developed in both the SOD1-null and the wild-type lenses (Fig. 3). GSSG (data not shown), on the other hand, was
The carbonylated protein bands visualized in the immunoblot assay (OxyBlot Protein Oxidation Detection Kit; Chemicon International) appeared mainly between the bands for masses of 29 and 21 kDa, respectively (Fig. 4B). The amount of protein carbonyls was equal in the lenses from the SOD1-null and wild-type mice not subjected to STZ. However, STZ-induced diabetes increased the carbonyl content equally in the SOD1-null and wild-type lenses by 60% to 70% compared to the nondiabetic controls (Fig. 4B, Table 1). As opposed to GSH, the amount of carboxyls increased as cataract developed in both the SOD1-null and wild-type lenses, also regardless of genotype (Fig. 4A).

The SOD1-null mice became less diabetic on STZ-treatment and also showed less weight loss than did the wild-type mice, despite being given a higher dose of STZ (Table 1). Only 31% (4/13) of the STZ-treated SOD1-null mice compared with 75% (15/20) of the wild-type mice showed a mean plasma (P)-glucose ≥ 15 mM and U-glucose ≥ 14 mM. Of interest, only SOD1-null mice treated with STZ became visibly ill and had to be terminated prematurely (5/18). Of these mice, two were terminated within 2 weeks from the first injection, thus before the first evaluation of P-glucose. At 2 weeks, the other three showed a P-glucose of 14.7 ± 4.8 mM and a weight loss of −3.0 ± 2.1 g, but were later considered to be in such poor condition that they were terminated before the study endpoint. As expected, The SOD1-null mice showed lower body weights at the start of the study compared with the wild-type mice (25.3 ± 4.8 compared with 32.6 ± 6.7 g; P < 0.001) which may explain why they were more affected by the hyperglycemia. One STZ-treated SOD1-null mouse was found to have a dense bilateral cataract (digital image analysis/eye; 51.48 and 52.60 AU), even though it did not seem to become diabetic (P-glucose 11.1 mM and U-glucose 5.5 mM). This mouse was excluded from the study, because it could not be ruled out that the cataract was congenital.

**Discussion**

In this study we wanted to explore further the importance of SOD1 in diabetes-induced cataract by using SOD1 knockout mice rendered diabetic by STZ, thus imposing additional oxidative and glycemic stress on lenses already subjected to increased levels of O$_2^{-}$ due to an impaired antioxidant defense. We found that diabetes mellitus accelerated cataract formation in SOD1-null mice more than in wild-type mice, which confirms the results in our previous in vitro glucose-induced cataract studies. Lens clarity in mice has been found to be

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**Figure 1.** Digital image analysis of cataract development as a function of mean plasma glucose levels at 2 and 8 weeks after diabetes mellitus was induced by STZ in SOD1-null and wild-type mice. The SOD1-null mice showed accelerated cataract development compared to the wild-type mice. Linear regression for SOD1-null mice: y = 4.2 ± 1.4x, r = 0.5, and for wild-type mice: y = 12.6 ± 0.4x, r = 0.4. Interaction between slopes, P = 0.002. Multiply millimolar by 18 to convert the plasma glucose level to milligrams per decaliter. (○) Control mice; (●) STZ-treated mice.

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**Table 1. Mouse and Lens Data**

<table>
<thead>
<tr>
<th></th>
<th>SOD1 Null</th>
<th>Wild-Type</th>
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<tr>
<td></td>
<td>STZ</td>
<td>Control</td>
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<tr>
<td>Mouse data</td>
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<tr>
<td>P-Glucose</td>
<td>(n = 13)</td>
<td>(n = 7)</td>
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<tr>
<td>2 Weeks (mmol/L)</td>
<td>12.5 ± 4.7*</td>
<td>8.2 ± 1.0</td>
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<tr>
<td>8 Weeks (mmol/L)</td>
<td>18.4 ± 5.0†</td>
<td>12.6 ± 1.7</td>
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<td>Mean (mmol/L)</td>
<td>15.4 ± 4.4†</td>
<td>10.4 ± 1.1</td>
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<tr>
<td>U-Glucose</td>
<td>(n = 12)</td>
<td>(n = 7)</td>
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<tr>
<td>8 Weeks (mmol/L)</td>
<td>20.6 ± 25.5</td>
<td>5.1 ± 4.7*</td>
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<tr>
<td>Weight</td>
<td>(n = 13)</td>
<td>(n = 7)</td>
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<td>Initial (g)</td>
<td>24.8 ± 5.2*</td>
<td>26.0 ± 4.4</td>
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<td>Change at 2 weeks (g)</td>
<td>-1.2 ± 1.7†</td>
<td>0.2 ± 1.0</td>
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<td>Change at 8 weeks (g)</td>
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<td>0.4 ± 1.6</td>
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<td>Lens data</td>
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<tr>
<td>Wet weight</td>
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<td>(n = 15)</td>
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<td>8 Weeks (mg)</td>
<td>8.6 ± 1.1</td>
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<td>Cataract</td>
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<td>(n = 15)</td>
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<td>8 Weeks (AU)</td>
<td>29.5 ± 5.6‡</td>
<td>14.1 ± 5.6</td>
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<tr>
<td>GSH</td>
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<td>8 Weeks (μmol/g ww)</td>
<td>2.2 ± 0.6§</td>
<td>2.7 ± 0.3</td>
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<tr>
<td>Carboxyls</td>
<td>(n = 19)</td>
<td>(n = 15)</td>
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<tr>
<td>8 Weeks (nmol/mg ww)</td>
<td>7.6 ± 4.5§</td>
<td>5.0 ± 2.0</td>
</tr>
</tbody>
</table>

Data are the mean ± SD. ww, lens wet weight.

* P ≤ 0.05 and † P < 0.001 compared with wild-type mice with STZ.

‡ P < 0.001 compared to all other groups.

§ P ≤ 0.05 compared to control lenses within genotype.
affected within 8 weeks of diabetes establishment, as also shown here, whereas substantial nuclear cataract development has not been observed until after 5 to 6 months. It has actually been suggested that nuclear brunescent cataract in diabetic lenses may be the result of glycation whereas cortical cataract, as seen in our study, may be attributed to oxidation.

Mice of the SOD1-null genotype develop normally to the age of fertility but show a shortened lifespan, mainly due to the increased risk of developing hepatocellular carcinomas. The relatively moderate phenotype compared with wild-type mice has rendered the SOD1 knockout mouse a suitable animal to use when studying the effects of ROS. The mouse model is especially useful when studying diabetic cataract, since the mouse lens resembles the human lens by expressing low activities of aldose reductase, thus rendering the accumulation of GSH in lenses from nondiabetic and STZ-induced diabetic SOD1-null mice. The lenses from the STZ-injected SOD1-null mice showed pronounced cortical cataract without any nuclear component. For each lens, the mean plasma glucose of that mouse, analyzed at 2 and 8 weeks after diabetes induction are shown as well as the lens's opacity in arbitrary units (AU) assessed by digital image analysis of the central 1 mm² of each lens (area marked on each image).

As in the present study, lower blood glucose values in SOD1-null compared with wild-type mice have been found after the multidose STZ regimen. The mechanisms responsible for this reduced effect of STZ on glucose levels in the SOD1-null strain are not known. However, we suggest that the discrepancy may be attributed to the fact that SOD1-null mice are known to have lower body weights, also found in the present study. The differences in body weights may reflect reduced food intake which subsequently would give lower nonfasting glucose levels. Alternatively, the absence of SOD1 may cause an increased metabolic rate that increases glucose consumption. Although plasma and urine glucose samplings were performed by professional animal caretakers, the han-

![Digital image analysis of cataract development as a function of GSH in lenses from nondiabetic and STZ-induced diabetic SOD1-null and wild-type mice. Lenses with cataract show lower levels of GSH. Linear regression for SOD1-null lenses: y = 45.2 - 8.9x, r² = 0.3, P = 0.003, and for wild-type lenses: y = 28.2 - 3.4x, r² = 0.1, P = 0.002. (C) Lenses from control mice; (O) lenses from STZ-treated mice.](image)

![Representative lenses photographed on a grid after 8 weeks of STZ-induced diabetes mellitus in wild-type and SOD1-null mice and in nondiabetic control mice. The degree of lens opacification was staged on a scale from 0 to 5 by three independent evaluators, and the median value of the assessments was used as the resulting stage. According to this subjective quantification system, the lenses from the STZ-injected SOD1-null mice showed pronounced cortical cataract without any nuclear component.](image)
dling of unanesthetized animals may trigger stress responses that generate an increase in plasma glucose. This explanation is a plausible one for the somewhat elevated plasma glucose levels found in the nondiabetic mice, especially at 8 weeks when the blood was drawn after the urine sampling which inevitably would trigger a greater stress response in the animal. To compensate for this effect, we used the mean value of the plasma glucose at 2 and 8 weeks as a measure of hyperglycemia. A more accurate way to define diabetes is needed, however. Unfortunately, the SOD1-null genotype has shown an accelerated turnover rate of erythrocytes, thus making glycated hemoglobin as a measure of the past glycemic state impossible to use in this mouse strain.32

GSH is especially abundant in the lens where it plays a vital role as a first line of defense against ROS.33 The assessment of GSH has therefore become an appreciated method for evaluating oxidative damage in the lens. Lenses from diabetic animals show a loss of GSH34 that is aggravated by exposure to further oxidative stress.33 This decrease may be attributable not only to the consumption of GSH but also to its decreased synthesis due to the lower levels of ATP found in diabetics, to the glycation and inactivation of enzymes essential for GSH recycling,35 and to a loss of GSH regeneration due to a leakage of GSSG and protein-thiol mixed disulfides out of the lens.33 In the present study, we found lower GSH levels in the SOD1-null lenses from the STZ-induced diabetic mice but not in the diabetic wild-type lenses compared with the nondiabetic control subjects. The finding in the wild-type lenses is thus contrary to that of Hegde et al.33 who showed a 50% reduction in lens GSH levels in mice 5 weeks after diabetes was induced by STZ. However, the unaffected GSH levels in the diabetic wild-type mice in this study agree well with the limited cataract development seen in these lenses. It has been shown that increased carbonyl formation suggestive of protein oxidation is related to the rate of diabetic cataract development40 which we can confirm. The fact that cataract was more advanced in the diabetic SOD1-null mice despite equal increases of carbonyls in the diabetic lenses of both genotypes suggests that the oxidants causing cataract may be in part different from those causing protein carbonylation. In addition, the glycation of proteins may have contributed to the formation of carbonyls in the diabetic lenses.40 Before this study, protein carbonylation has not been explored in SOD1-null lenses. However, it has been suggested in studies on liver tissue, that oxidized proteins accumulate only in highly damaged tissues40 in this genotype. In view of the mild injury in the lenses in the present study, it may thus be somewhat misleading to compare the lens carbonyls between SOD1-null and wild-type mice. The accelerated cataract formation in the diabetic SOD1-null mice as well as the reduced lens GSH levels, suggest the involvement of O$_2^{•-}$ in diabetes-induced cataract development. However, O$_2^{•-}$ shows a relatively low reactivity with most biological molecules but can react with transition metal ions or reactive nitrogen species40 to generate more damaging compounds. These reactions are facilitated in the diabetic lens where levels of copper and iron ions38 as well as NO12 are elevated. We have previously also shown that NO may be reduced by ascorbate which actually produces twice as much H$_2$O$_2$ as in SOD-catalyzed reactions.16 Already in the wild-type lens, one fourth of the O$_2^{•-}$ radicals can be calculated to react with ascorbate and three-fourths with SOD1,19 thus rendering ascorbate an important alternative elimination pathway. Furthermore, in most tissues examined, the glutathione peroxidase activity is halved in SOD1 knockouts, and could well be reduced also in the lens.27 We therefore suggest that SOD1-null lenses may be exposed to increased levels of H$_2$O$_2$ which may enhance the formation of the toxic OH$^-$ radical through superoxide-assisted Fenton reactions facilitated in the diabetic lens due to increased levels of transition metals.7,38 Nevertheless, additional studies are needed to further explore the exact mechanisms for the superoxide-derived lens toxicity found in our studies. In conclusion, the findings in the present study further emphasize the importance of SOD1 in the protection against cataract development in the presence of diabetes mellitus.

References


