Superoxide Dismutase and Catalase of Calf Trabecular Meshwork

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Superoxide dismutase and catalase activities have been measured in cell-free extracts of calf trabecular meshwork, and for comparison, in calf iris, retina, lens, liver, and erythrocytes. Gel electrophoresis has been used to identify isozymes of each enzyme. The superoxide dismutase and catalase activities per milligram wet weight of calf trabecular meshwork, 0.184 and 0.884 U/mg wet wt, respectively, were comparable to those found in iris and retina, and much higher than those found in lens. Three isozymes of superoxide dismutase were identified in trabecular meshwork. Two of these presumably correspond to cytoplasmic superoxide dismutase, while the third corresponds to a mitochondrial isozyme. A presumably mitochondrial superoxide dismutase activity was also observed in iris and retina, but not in lens. A single catalase isozyme was found in all tissues examined. At physiologic H₂O₂ concentrations, catalase may have similar levels of activity to glutathione peroxidase. Superoxide dismutase, catalase, and glutathione peroxidase may constitute an important defense mechanism of trabecular meshwork against the toxic O₂⁻ and H₂O₂ to which it must be continuously exposed from endogenous production as well as from the aqueous humor. Invest Ophthalmol Vis Sci 26:1330-1335, 1985

The trabecular meshwork (TM), which forms the major route for aqueous outflow from the anterior chamber of the eye, has been shown to be a metabolically active, respiring tissue in the calf. The cells of TM, therefore, would be expected to possess defense mechanisms against toxic byproducts of oxidative metabolism, such as the superoxide radical (O₂⁻) and H₂O₂. In addition, TM cells probably need to be equipped to withstand extracellular exposure to these same oxidants in the aqueous humor: H₂O₂ is a normal constituent of the aqueous humor in many species, and light entering the anterior chamber of the eye may result in the in vivo photochemical generation of O₂⁻ in the aqueous humor. Since O₂⁻ and H₂O₂ are apparently among the agents primarily responsible for oxidative damage to cells, and since oxidative damage to the lens has been implicated in cataractogenesis, it seemed important to ask: how do TM cells defend against the O₂⁻ and H₂O₂ to which they are exposed both intra- and extra-cellularly?

Superoxide dismutase (SOD), a superoxide radical scavenger, and the two H₂O₂-metabolizing enzymes—catalase and glutathione peroxidase (GSH-Px)—may constitute an important defense mechanism against oxidative damage in cells. These enzymes have been found in several ocular tissues of a variety of mammals and glutathione reductase activity have recently been examined in the trabecular meshwork of calf. In this communication, we measured the activities of superoxide dismutase and catalase in cell-free extracts of this same tissue, using gel electrophoresis to identify isozymes of each. For comparison, we also determined superoxide dismutase (SOD) and catalase activities and electrophoretic patterns in calf iris, retina, lens, liver, and erythrocytes.

Materials and Methods

Chemicals

Horse heart cytochrome c (type III), xanthine oxidase (grade IV), xanthine, hydrogen peroxide, horse radish peroxidase (type VI), bovine erythrocyte superoxide dismutase, and bovine liver catalase were obtained from Sigma Chemical Corporation (St. Louis, MO). Xanthine oxidase was dialysed for 8 hr against 0.05 M potassium phosphate buffer, pH 7.8, 0.4 mM EDTA at 4°C prior to use, and was stored at 4°C for up to 1 mo. Ammonium persulfate (electrophoretic purity), Biorad protein assay kit and bovine serum albumin standard were from Biorad Laboratories (Richmond, CA). All other chemicals were reagent grade.
Tissues

Calf eyes, whole blood, and liver were obtained from Joseph Trelegan and Company (Cambridge, MA). The animals were killed by exsanguination. Whole blood was collected into a heparinized test tube, which was placed on ice. Shortly after death, the eyes and liver were removed and placed in iced saline for transportation. The trabecular meshwork from 20–40 eyes was obtained by the method of Anderson et al., substituting Dulbecco’s phosphate buffered saline for 0.9% NaCl in the washing step.13 Pellets of TM tissue were blotted dry and used immediately or stored at −80°C. Trabecular meshwork visibly contaminated with blood was discarded. This procedure produces clean TM1 free of any trace of hemoglobin.13 Iris, lens, and retina were each collected from two eyes in a similar fashion, with care being taken not to contaminate the individual tissues. Liver was similarly rinsed in physiologic saline prior to use. Whole blood was used upon receipt as described below.

Cell-free Extracts

Tissue (except whole blood) was combined at a ratio of 1 g/5 ml ice-cold potassium phosphate buffer, 0.05 M, pH 7.8, containing 0.1 mM EDTA, homogenized for 1 min with Tekmar Tissumizer, Model SDT 100U (Tekmar; Cincinnati, OH), and sonicated for 30 sec with a Branson Sonifier (Branson; Danbury, CT). Sonicates were centrifuged at 10,000 × g for 10 min at 4°C to pellet cell debris, and the supernatants were collected and kept at 0°C for same day use or stored at −20°C for use within 1 wk. No change in enzyme activities was observed following such storage; enzyme activities were likewise stable to repeated freezing and thawing of extracts. The protein content of each tissue extract was determined by a dye-binding method with a bovine serum albumin standard, using a kit from Biorad Laboratories (Biorad Technical Bulletin No. 1051). A 2-ml vol of whole blood was diluted to 8 ml with ice-cold 0.9% NaCl and erythrocytes were pelleted by centrifuging at 1000 × g for 5 min in an analytic centrifuge. Packed erythrocytes were resuspended in 8 ml fresh saline and repelleted twice more. Then cells were lysed by adding 5 ml deionized water; lysate was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was collected and stored in the same way as the other tissue extracts.

Enzyme Assays

Optical densities were measured on a Cary 210 spectrophotometer equipped with a thermostatted cell compartment (Varian Instruments; Palo Alto, CA).

**Superoxide dismutase:** Activity was determined by the technique of McCord and Fridovich.14 Briefly, xanthine and xanthine oxidase were used to generate superoxide which in turn reduced cytochrome c. The rate of reduction of cytochrome c was followed spectrophotometrically. The activity of superoxide dismutase (SOD) was measured by determining the amount by which it inhibited this reaction. The reaction mixture had a total volume of 2.5 ml. Potassium cyanide was included at 5 × 10^-5 M in all reaction mixtures, in order to eliminate possible interference by contaminating cytochrome oxidase in cell-free extracts; this level of cyanide did not detectably alter the control rate of cytochrome reduction nor the activity of commercial SOD. The method met the following criteria: heating extracts to 100°C for 10 min completely eliminated SOD activity; the rate of cytochrome c reduction with xanthine and xanthine oxidase present was 95% inhabitable by commercial superoxide dismutase; there was no detectable cytochrome c reduction in the absence of xanthine oxidase. One unit of SOD activity was defined as the amount of enzyme which reduced the rate of cytochrome c reduction to 50% that of control under the specified conditions.

**Catalase:** Activity was determined at 25°C according to the technique of Beers and Sizer,15 by following the absorbance of hydrogen peroxide at 240 nm. One unit of catalase activity was defined as the amount of enzyme which decomposes 1 μmol H2O2/min at an initial H2O2 concentration of 30 mM at pH 7.0 and 25°C. Addition of 0.1 mM sodium azide or 1.0 mM KCN to a complete reaction mixture, completely inhibited H2O2 decomposition. Extraxts heated to 100°C for 10 min showed no enzyme activity. The initial rate of H2O2 decomposition was linear with respect to: (1) initial H2O2 concentration (at fixed catalase) up to 50 mM; and (2) the amount of enzyme present (at fixed initial concentration of H2O2).

**Electrophoresis**

Disc gel electrophoresis was performed essentially according to Davis,16 using 7.5% polyacrylamide gels in 0.38 M Tris-HCl buffer, pH 8.8, 3.75% stacking gels in 0.12 M Tris-HCl buffer, pH 6.8, running buffer 0.025 M Tris-glycine, pH 8.3. Gels were cast in 15 × 150 mm tubes, loaded with up to 200 μl extract, 0.002% bromphenol blue tracking dye in 20% glycerol, and run in sets of 18 gels at 160 V (~2 mA/gel) until tracking dye approached bottom of gel. Superoxide dismutase activity stains were performed according to Beauchamp and Fridovich,17 and yielded blue gels with achromatic zones marking enzyme positions on gels. Control gels processed without extract.
or other enzyme sources attained blue coloration throughout. Cyanide-sensitive superoxide dismutase could be differentiated from cyanide-insensitive SOD by including 3 mM cyanide in the developing reagents. Gels were scanned spectrophotometrically at 560 nm in an attempt to quantify the various isozymes, but nonuniformity of background staining allowed only crude quantitation.

Catalase activity stains were performed according to the method of Gregory and Fridovich with the following modifications: samples of extract or commercial catalase containing 5-10 units enzyme activity were run on gels. Gels were then soaked for 40 min at 25°C in test tubes containing 0.25 mg/ml horse radish peroxidase in 0.05 M potassium phosphate buffer, pH 7.0. To each tube was added hydrogen peroxide to a final concentration of 5 mM. After 10 min in the above solution, gels were rinsed twice in deionized water, then placed into diaminobenzidine 0.5 mg/ml in 0.05 M solution, gels were rinsed twice in deionized water, then placed in 5% acetic acid prior to photographing.

Results

Superoxide Dismutase

Cell-free extracts prepared from calf trabecular meshwork, as well as those from calf iris, retina, lens, liver and erythrocytes all showed SOD activity, ranging from 0.02 (lens) to 1.3 (liver) U/mg wet wt (Table 1). The enzyme activity per milligram wet weight in trabecular meshwork was 0.184, which is similar to that found in iris and retina, but was almost 10 times higher than that in calf lens. Specific activities of SOD were likewise of comparable magnitude in trabecular meshwork, iris, and retina, while much lower values were found in the lens (14.4, 21.6, 7.2, and 0.08 U/mg soluble protein, respectively). Disc gel polyacrylamide electrophoresis was performed using extracts as well as commercial bovine erythrocyte SOD. Gels were stained for SOD activity in the presence and in the absence of 3 mM potassium cyanide, and for protein as shown in Figure 1. Liver extract showed four distinct bands of enzyme activity in the absence of cyanide (gel IIx); these have been labeled a, b, c, d in order of increasing mobility. Band a was unaffected by 3 mM cyanide, whereas bands b, c, and d were eliminated (gel IIy). Commercial bovine erythrocyte SOD clearly showed bands b and c (band d was faint and did not photograph well) in the absence of cyanide (gel IIx), while there was no activity detectable in the presence of cyanide (gel IIy). Bands a, b, and c were seen on gels of trabecular meshwork, iris, and retina in the absence of cyanide (gels IIIx, IVx, Vx, respectively), while band a alone remained in the presence of cyanide for all three tissues (gels IIy, IVy, Vy, respectively). In the case of lens, three distinct bands of cyanide sensitive activities were visible (compare gels VIx and y), which may correspond to bands b, c, and d on the other gels. The achromatic zone above presumptive band b on the lens gel (VIx) was accompanied by tracking dye and was likely due to protein including SOD which did not migrate freely because of the protein overloading of these gels which was needed to visualize SOD by activity stain. From the position of the achromatic bands, it appears that trabecular meshwork, iris, retina, and lens each contain at least three different isozymes of SOD. In the former three tissues, the cyanide-insensitive activity (band a) was found, corresponding in mobility to the cyanide-sensitive activity band of calf liver and presumably representing the mitochondrial enzyme, a manganese-containing metalloprotein. Bands b and c, which appear on gels of trabecular meshwork, iris, and retina in the absence of cyanide, correspond to the activity bands b and c of bovine erythrocyte SOD, and hence presumably represent isozymes of cytoplasmic SOD, a Cu-Zn metalloprotein.

Spectrophotometric scanning of gels suggested that the manganese-containing SOD of trabecular meshwork accounts for between 10% and 20% of the total SOD activity observed in cell-free extracts of this tissue.

Catalase

Cell-free extracts prepared from calf trabecular meshwork, as well as those from calf iris, retina, liver and erythrocytes all displayed catalase activity (Table 2). The enzyme activity per milligram wet weight in

Table 1. Superoxide dismutase activity of bovine tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Soluble protein (mg/ml)</th>
<th>Enzyme content (units/mg wet wt)</th>
<th>Specific activity (units/mg soluble protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular meshwork (6)</td>
<td>2.77 ± 0.30</td>
<td>0.184 ± 0.021</td>
<td>14.4 ± 1.8</td>
</tr>
<tr>
<td>Iris (2)</td>
<td>1.88 ± 0.56</td>
<td>0.28 ± 0.10</td>
<td>21.6 ± 9.0</td>
</tr>
<tr>
<td>Retina (3)</td>
<td>4.76 ± 0.54</td>
<td>0.166 ± 0.008</td>
<td>7.2 ± 1.1</td>
</tr>
<tr>
<td>Lens (2)</td>
<td>54.7 ± 0.9</td>
<td>0.0218 ± 0.0044</td>
<td>0.080 ± 0.013</td>
</tr>
<tr>
<td>Liver (2)</td>
<td>26.9 ± 1.0</td>
<td>1.30 ± 0.08</td>
<td>9.7 ± 1.0</td>
</tr>
<tr>
<td>Erythrocyte (1)</td>
<td>33.4</td>
<td>—</td>
<td>3.38</td>
</tr>
</tbody>
</table>

Values are given as units/mg wet wt (enzyme content) and units/mg soluble protein (specific activity). Numbers in parentheses are number of preparations. Results are means of preparations ± SEM. Values were determined for each preparation 2-4 times. Assays were performed by the method of McCord and Fridovich, with the addition of 5 × 10⁻³ M KCN to each reaction mixture.
Fig. 1. Electrophoretic patterns of superoxide dismutase and total soluble protein of bovine tissue. Extracts were electrophoresed on 7.5% polyacrylamide disc gels, and stained for superoxide dismutase activity (all “x” and “y” gels) and soluble protein (all “z” gels). SOD staining was performed in the presence of 3 mM CN⁻ on all “y” gels. The gels in each case contain: (!) purified bovine erythrocyte SOD, and (II) liver, (III) trabecular meshwork, (IV) iris, (V) retina, and (VI) lens extracts. SOD activity bands are lettered a, b, c, d, in order of increasing mobility.

Trabecular meshwork is similar to that found in iris and retina (0.884, 0.648, and 0.450 U/mg wet wt, respectively). The specific activity of catalase, while similar in trabecular meshwork and iris, appears somewhat lower in retina (70, 106, and 21 U/mg soluble protein, respectively). Calf lens catalase could not be quantitated in this assay system (see footnote, Table 2).

Disc gel polyacrylamide electrophoresis was performed using both tissue extracts and commercial bovine liver catalase. Catalase activity staining of gels re-
Table 2. Catalase activity of bovine tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme content</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular meshwork (7)</td>
<td>0.884 ± 0.020</td>
<td>70.2 ± 4.9</td>
</tr>
<tr>
<td>Iris (2)</td>
<td>0.638 ± 0.002</td>
<td>106 ± 37</td>
</tr>
<tr>
<td>Retina (3)</td>
<td>0.450 ± 0.075</td>
<td>21.0 ± 2.3</td>
</tr>
<tr>
<td>Liver (2)</td>
<td>63.8 ± 1.8</td>
<td>476 ± 32</td>
</tr>
<tr>
<td>Erythrocyte (1)</td>
<td>—</td>
<td>211</td>
</tr>
</tbody>
</table>

Values are units/mg wet wt (enzyme content) and units/mg soluble protein (specific activity). Numbers in parentheses are number of preparations. Results are means of preparations ± SEM. Values were determined 2–4 times for each preparation. Assays were performed according to the method of Beers and Sizer, using H2O2 at an initial concentration of 30 mM. Lens catalase activity could not be detected in this assay system because of high concentrations of soluble protein in lens extract (see Materials and Methods). Fecondo and Augusteyn report values of 0.30 ± 0.10 and 0.19 ± 0.03 for lens cortex and soluble protein in lens extract (see Materials and Methods).

Discussion

The trabecular meshwork of calf eye has been found to contain both superoxide dismutase and catalase activities comparable to those found in iris and retina, and much higher than those found in lens. Catalase activity in lens was not measurable by our method because the high protein content of the extracts caused high background absorption at 240 nm. Fecondo and Augusteyn, using an oxygen electrode assay, have reported an activity of 0.30 for lens cortex and 0.19 for lens nucleus. Gel electrophoresis reveals the presence in trabecular meshwork of three distinct SOD activities, the slowest-moving of which presumably corresponds to a mitochondrial, manganese-containing SOD, (which accounts for 10–20% of the tissue’s total SOD activity); the remaining two activity bands likely represent copper-zinc-containing cytoplasmic SOD iso-enzymes. A presumably mitochondrial SOD activity of the same mobility was also observed in iris and retina. Previous investigators have failed to report a mitochondrial SOD activity in iris and retina.3-4 The presence of this isozyme is not unexpected, however, given that all three tissues have been shown by electron microscopy to contain mitochondria. No mitochondrial (ie, cyanide-insensitive) SOD activity has been detected for the lens in this study.

There has been much discussion concerning the relative effectiveness of catalase and glutathione peroxidase in detoxifying H2O2. It has been suggested that in the lens GSH-Px is more important when levels of H2O2 in the tissues are low, while catalase becomes more important when concentrations of H2O2 far exceed the physiologic level.20 Bovine aqueous humor contains about 25 μM H2O2.2 Given that enzyme activity varies linearly with changing H2O2 concentration over wide ranges,2 one may estimate that at 25 μM H2O2, catalase activity should be 59.2 ± 6.0 nmol H2O2 decomposed/min/mg soluble protein, or 0.74 ± 0.02 nmol H2O2 decomposed/min/mg wet weight of TM. Using the assumptions of Scott et al,16 one TM could decompose H2O2 at a maximum rate of ~15 nmol/min compared to ~12 nmol/min for GSH-Px; on the other hand, H2O2 is presented to the meshwork at about 0.75 nmol/min. As H2O2 is about as diffusible as water, it is unlikely that any H2O2 is left in the aqueous humor by the time it reaches Schlemm’s canal.

Our interest in these “defensive enzymes” has been prompted by a question: could a lifetime’s insult from H2O2 damage the TM of humans to a degree sufficient to cause glaucoma, at least in those more susceptible?22,23 We know that in the calf eye, the TM is well protected against the deleterious effect of H2O2 by two equally vigorous detoxification systems, catalase and GSH-Px and, in addition, has protection against other highly reactive oxygen species through the superoxide dismutase. Preliminary data from Polansky et al24 would suggest that TM cells from human eyes are similarly defended. They found SOD levels of 23.5 U/mg protein in the cells grown from young eyes. This is similar to the level we find in calf TM. In the absence of data on the H2O2 levels at which they determined their catalase activity, it is not possible to make a strict comparison. However, if one assumes a starting concentration in the region of 25–30 mM, then their measured levels in human TM (28.3 U/mg protein) are not very different from those we measure in calf (70 U/mg protein). If the catalase activity of these cultured cells was inhibited by 3-aminotriazole, acute damage could be demonstrated but only at levels of H2O2 well above physiological. This does not mean that the TM may not be damaged by chronic exposure to a lifetime’s H2O2, especially as with age, the levels of the protective enzymes may decrease. More data, both from animal and human eyes is needed to pursue this question, including information on possible targets of oxidative damage. Currently we are investigating enzymes in plasma membranes, prepared from calf TM, and will be studying their susceptibility to H2O2 and other reactive derivatives of oxygen.

Key words: trabecular meshwork, superoxide dismutase, catalase, calf, hydrogen peroxide

References