The role of glutathione metabolism in the detoxification of $H_2O_2$ in rabbit lens

Frank J. Giblin, Janet P. McCready, and Venkat N. Reddy

Aqueous humor is known to contain a significant level of $H_2O_2$, but the mechanisms by which ocular tissues protect against oxidative damage are not well understood. With the use of C-1-, C-2-, and C-6-labeled glucose, the contribution of glutathione (GSH) metabolism and the hexose monophosphate shunt (HMS) to the detoxification of peroxide in the lens has been evaluated. It was observed that $H_2O_2$ in the culture medium disappeared rapidly (0.5 μmol $H_2O_2$/lens/hr) upon incubation of a rabbit lens at 37° C. At 0° to 3° C, however, the rate of disappearance of $H_2O_2$ was only one fifth of that observed at the higher temperature. In the absence of a lens or after pretreatment of the lens with methyl mercuric hydroxide, the rate of disappearance of peroxide from the medium was reduced to nearly zero. When a nearly constant level of $H_2O_2$ (0.05 to 0.07 mM) was maintained in the medium by means of a peristaltic pump, the amount of CO$_2$ liberated by the HMS at 37° C was found to be three times that liberated from lenses cultured in the absence of peroxide. No change was noted in the level of GSH in the $H_2O_2$-treated lenses at 37° C. A significant decrease in GSH was observed, however, at 0° to 3° C, suggesting nonenzymatic oxidation of the tripeptide at the lower temperature. The results indicate that GSH metabolism and the HMS pathway contribute significantly to the detoxification of $H_2O_2$ in the lens. (INVEST OPHTHALMOL VIS SCI 22:330-335, 1982.)

Key words: hydrogen peroxide, hexose monophosphate shunt, lens, glutathione, aqueous humor, glucose oxidation, glutathione peroxidase, nicotinamide-adenine dinucleotide phosphate, glutathione reductase

Significant levels of hydrogen peroxide ($H_2O_2$) have been found to be present in normal aqueous humor of various species. The concentration in the human has been reported to be approximately 0.03 mM (Spector, A: personal communication), whereas that in rabbit is in the range 0.05 to 0.07 mM. 1,2 It is believed that peroxide is formed in aqueous humor during the oxidation of ascorbic acid,3 which is normally present in high concentration in this fluid. This oxidation reaction has been found to be catalyzed by light and riboflavin. The mechanism by which ocular tissues such as the lens, which are continuously exposed to aqueous humor, are able to prevent $H_2O_2$-induced oxidative damage is not completely understood and is of great importance. Evidence is accumulating which suggests that in older human lenses $H_2O_2$ may be a common factor in the development of nuclear cataract. 4-6

It has been suggested by Pirie7 that the lens is able to detoxify $H_2O_2$ enzymatically by means of coupled reactions involving glutathione peroxidase, glutathione reductase, and...
the hexose monophosphate shunt (HMS) pathway. However, this hypothesis has never been substantiated. In this article, measurements have been made of the rate of disappearance of $H_2O_2$ from media during the incubation of rabbit lenses, and evidence is presented to show that a significant portion of the disappearance of peroxide can be attributed to increased activity of the HMS pathway.

Methods

Albino rabbits weighing between 1.8 and 2.2 kg were employed throughout the study. $H_2O_2$ was obtained as a 30% solution from Mallinckrodt, Inc. (St. Louis, Mo.). Glucose labeled with $^{14}C$ in either the 1, 2, or 6 position of the molecule (2 to 10 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, Mass.). Methylmercuric hydroxide (97%) was purchased from Ventron Alfa Products (Beverly, Mass.).

Lenses were cultured in 5 ml of Tyrode's medium (5.5 mM glucose) in Merriam-Kinsey culture tubes. In certain experiments a nearly constant concentration of $H_2O_2$ (0.05 to 0.07 mM) was maintained in the culture media. This was accomplished by continuously pumping a solution of $H_2O_2$ into the medium to replace peroxide, which had been detoxified by the lens. For this purpose, either a syringe pump (Model 352; Sage Instruments) with two 5 ml plastic syringes, 26-gauge by $\frac{3}{4}$ inch needles, and polyethylene tubing (0.015 inch inner diameter, 0.043 inch outer diameter; Intramedic) or a peristaltic pump (Multichannel Cassette Pump; Manostat Corp.) with silicone rubber tubing (0.015 inch inner diameter, $\frac{1}{32}$ inch inch wall; Manostat Corp.) was employed. The tubing extended from the pump through a cap placed in the rear gas inlet port of the culture tube and then down into the medium. The rate of flow of $H_2O_2$ solution was measured after each experiment and was found to be approximately 0.2 ml/hr.

The oxidation of glucose in control and experimental lenses was measured by determining the amount of $^{14}CO_2$ liberated in the presence of $^{14}C$-labeled glucose. The method employed has been described in detail previously. At the end of the culture period, the experimental tube was unstoppered briefly to sample for measurement of $H_2O_2$ concentration and to insert a vial of methyl benzethonium hydroxide for absorption of $CO_2$. Blanks were run both with and without $H_2O_2$ in the medium. Experiments were conducted which showed that nearly 100% of labeled $CO_2$ in the medium was subsequently recovered in the methyl benzethonium hydroxide.

The concentration of $H_2O_2$ in the medium was measured by the spectrophotometric method described by Pirie. The absorbance at 610 nm of a 1 ml solution of 0.04 mM 2,6-dichlorophenolindophenol in 50 mM phosphate buffer, pH 6.6, was lowered to approximately 0.18 by the addition of ascorbic acid (0.01 ml of a 20% solution in 4% metaphosphoric acid). After addition of 0.1 ml of sample (diluted if necessary to <0.06 mM $H_2O_2$) the absorbance was again recorded. Five microliters of 5 mg/ml horseradish peroxidase solution (Type VI, Sigma Chemical Co., St. Louis, Mo.) was then added and the increase in absor-

![Fig. 1. Graph showing the disappearance of $H_2O_2$ from Tyrode's medium in the presence of rabbit lens. •, Control, medium maintained at either 0° to 3° C or 37° C without the presence of a lens; ▲, medium maintained at 37° C and containing a lens that was preincubated for 5 hr at 37° C in medium containing 8.5 mM methyl mercuric hydroxide (MeHg$^+$); ▼, medium maintained at 0° to 3° C and containing a normal lens that was preincubated for 1 hr at 0° to 3° C; ○, medium maintained at 37° C and containing a normal lens. Each curve represents a typical result for at least four experiments. The volume of medium was 5 ml and the mean lens weight was 300 mg.](image-url)
Table I. Detoxification of H$_2$O$_2$ by rabbit lens in vitro

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>$H_2O_2$ pumped into medium in 3 hr (μmol)</th>
<th>$H_2O_2$ present in medium (μmol)</th>
<th>Disappearance of $H_2O_2$ in 3 hr (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C (14)</td>
<td>1.44 ± 0.12</td>
<td>0.30 ± 0.06</td>
<td>0.28 ± 0.11 0.02 1.46</td>
</tr>
<tr>
<td>0°C to 3°C (7)</td>
<td>0.30 ± 0.06</td>
<td>0.35 ± 0.05</td>
<td>0.34 ± 0.11 0.01 0.31</td>
</tr>
</tbody>
</table>

*a Lenses were cultured for 3 hr in a medium in which $H_2O_2$ concentration was maintained constant (0.05 to 0.07 mM). (see Methods.)

*b Results are expressed as mean ± S.D. The number of experiments is in parentheses.

Table II. Concentration of GSH in rabbit lenses cultured for 3 hr in medium in which the concentration of $H_2O_2$ was maintained constant (0.05 to 0.07 mM)*

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Experiment</th>
<th>GSH* (μmol/lens)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>Control</td>
<td>3.5 ± 0.2 (6)</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>3.4 ± 0.2 (6)</td>
</tr>
<tr>
<td>0°C to 3°C</td>
<td>Control</td>
<td>3.2 ± 0.1 (6)</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td>2.8 ± 0.1 (6)</td>
</tr>
</tbody>
</table>

*a For experimental details refer to Methods and Table I.

*b Results are expressed as mean ± S.D. The number of experiments is in parentheses.

Results

Fig. 1 shows the rate of disappearance of $H_2O_2$ from the medium when lenses were cultured in Tyrode's solution under various conditions. In each case the initial concentration of $H_2O_2$ in the medium was 0.06 to 0.07 mM, approximating the physiologic level present in aqueous humor of the rabbit. It was found that the concentration of $H_2O_2$ in the control medium (without a lens) remained nearly constant during the 80 min incubation period. However, a significant decrease occurred in the level of $H_2O_2$ when a lens was present in the medium, both at 0°C and 37°C. The initial rates of disappearance of $H_2O_2$ were 0.5 μmol/lens/hr at 37°C and 0.1 μmol/lens/hr at 0°C to 3°C.

The decrease in the level of $H_2O_2$ in the medium was also studied in the presence of lenses that had previously been incubated for 5 hr in medium containing 8.5 mM methyl mercuric hydroxide (MeHg$^+$). Pretreatment with the mercurial decreased the concentration of free GSH in the lenses by 75% (data not shown). The medium containing the MeHg$^+$-treated lenses exhibited a slower rate of disappearance of $H_2O_2$ equal to about 0.02 μmol/lens/hr.

Experiments were also conducted in which $H_2O_2$ was continuously pumped into the medium during lens culture (see Methods) for 3 hr to maintain a concentration of 0.05 to 0.07 mM $H_2O_2$ in the medium (Table I). Thus, as $H_2O_2$ disappeared from the medium containing a lens, more $H_2O_2$ was pumped into the medium to maintain a nearly constant concentration. The rate of disappearance of $H_2O_2$ from the medium over the 3 hr period was calculated on the basis of the pumping rate, the concentration of $H_2O_2$ in the pumping medium, and the difference between the initial and final concentrations of $H_2O_2$ in the medium. $H_2O_2$ was detoxified by the lens at rates of 1.46 μmol/lens/hr at 37°C and 0.31 μmol/lens/hr at 0°C to 3°C. These values were nearly identical to the initial rates of disappearance of $H_2O_2$ determined previously without the use of a pump (Fig. 1). When control experiments were also done, in which $H_2O_2$ was pumped for 3 hr...
Table III. Oxidation of glucose in rabbit lenses cultured for 3 hr in medium in which the concentration of H₂O₂ was maintained constant (0.05 to 0.07 mM)*

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Experiment</th>
<th>CO₂ production (μmol CO₂/lens/3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose-1-¹⁴C</td>
</tr>
<tr>
<td>37° C</td>
<td>Control (9)</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ (9)</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>0° to 3° C</td>
<td>Control (6)</td>
<td>0.01 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ (6)</td>
<td>0.01 ± 0.005</td>
</tr>
</tbody>
</table>

*For experimental details, refer to Table I. Results are expressed as means ± S.D. The number of experiments is in parentheses. The average lens weight was 300 mg.

Table IV. Effect of H₂O₂ on the activity of the HMS and production of NADPH in rabbit lenses cultured at 37° C^A

<table>
<thead>
<tr>
<th>CO₂ production (μmol CO₂/lens/3 hr)</th>
<th>A (1-¹⁴C) – (6-¹⁴C)</th>
<th>B (2-¹⁴C) – (6-¹⁴C)</th>
<th>A + B Shunt activity</th>
<th>NADPH produced^c (μmol/lens/3 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.08</td>
<td>0.01</td>
<td>0.09</td>
<td>0.18</td>
</tr>
<tr>
<td>H₂O₂^b</td>
<td>0.25</td>
<td>0.04</td>
<td>0.29</td>
<td>0.58</td>
</tr>
</tbody>
</table>

^A Determined from the data in Table III.
^bThe concentration of H₂O₂ in the medium was maintained at 0.05 to 0.07 mM (Table I).
^c Based on the production of two molecules NADPH per molecule CO₂ liberated from the HMS pathway.

into medium without a lens, it was found that the increase of H₂O₂ in the control medium based on analysis of H₂O₂ was nearly 95% of that expected on the basis of the pumping rate and the concentration of H₂O₂ in the pumping medium (data not shown).

It was also of interest to determine the concentration of GSH in lenses that had been incubated for 3 hr in medium maintained at 0.05 to 0.07 mM H₂O₂. Table II shows that no change was observed in the concentration of GSH when the experiment was carried out at 37° C. However, during exposure to H₂O₂ at a lower temperature (0° to 3° C), the level of GSH decreased significantly (0.4 μmol/lens).

To gain a clue to the mechanism of detoxification of H₂O₂ in the lens, the effect of peroxide on the oxidation of glucose was studied. Again, in these experiments the concentration of H₂O₂ in the incubation medium was maintained at 0.05 to 0.07 mM. The data in Table III show that there was a threefold increase in the amount of CO₂ liberated from the C-1 carbon of glucose when the lenses were cultured in the presence of H₂O₂ at 37° C. H₂O₂-induced increases in CO₂ production also occurred during the oxidation of C-2- and C-6-labeled glucose, but the levels were considerably less than that from the C-1-labeled compound. The C-1:C-6 ratio for lenses exposed to H₂O₂ was 24 compared with 18 for the control lenses. When the incubation was carried out at 0° to 3° C very little oxidation of glucose occurred in either the control or H₂O₂-treated lenses.

From the data on the oxidation of C-1-, C-2-, and C-6-labeled glucose (Table III) it is possible to approximate the amount of CO₂ that arises directly from the HMS pathway. The results of such calculations are shown in Table IV and include the subtraction of values for the oxidation of C-6-labeled glucose from those obtained for oxidation at the C-1 position. Because oxidation at the C-2 position of glucose also contributes to shunt activity, apparently after reconversion of excess pentose phosphate to hexose phosphate, values for oxidation at the C-2 position are also included in the calculations. The data demonstrate that exposure of lenses to 0.05 to 0.07 mM H₂O₂ stimulates the release of CO₂, i.e., 0.29 μmol...
CO₂/lens/3 hr for the H₂O₂-treated lenses compared with 0.09 μmol CO₂/lens/3 hr for the controls.

Knowledge of the amount of CO₂ produced by the HMS also permits a reasonable estimate to be made of the amount of nicotinamide-adenine dinucleotide phosphate (NADPH) that is generated by this pathway (Table IV). Since two molecules of NADPH are produced for each molecule of CO₂ formed, it can be determined that exposure of the rabbit lens to 0.05 to 0.07 mM H₂O₂ yields 0.58 μmol NADPH/lens/3 hr, which is 0.40 μmol/lens higher than the control value.

Discussion

The present study has shown that exposure of lenses to H₂O₂ results in an increased production of NADPH via the HMS pathway. This suggests a role for the enzymes glutathione peroxidase and glutathione reductase in the detoxification of H₂O₂ in the lens through the following sequence of reactions, where GSSG is oxidized glutathione, as originally suggested by Pirie⁷:

\[
\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

Such a mechanism is consistent with many previous findings that lens epithelium contains unusually high concentrations of GSH and phosphorylated pyridine nucleotides as well as significant activity of glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase.¹², ¹⁴ The shunt activity that was observed under the experimental conditions of this study was one half of the maximum possible activity that has been determined previously for rabbit.⁹

The contribution of the above process to the overall detoxification of peroxide in the cultured lens can be estimated by comparing the amount of H₂O₂ that disappeared from the incubation medium to the corresponding increase in the formation of NADPH.* These data indicate that the increase in NADPH could detoxify 27% of the total H₂O₂ that disappeared. This figure must be considered an approximation, since it is not known whether the use of labeled glucose leads to isotopic mixing in the lens, as has been found in other tissues.¹⁵

The fact that exposure of the lens to H₂O₂ at 37° C did not affect the level of GSH is not surprising, since increased HMS activity should prevent loss of the reduced tripeptide. At a temperature of 0° to 3° C, where shunt activity was found to be negligible, a decrease of GSH did occur in H₂O₂-treated lenses. The loss of GSH at the lower temperature was probably the result of nonenzymatic oxidation of GSH and would explain, at least in part, the disappearance of H₂O₂ from the medium in the cold. At least 65% of the H₂O₂ lost from the medium at 0° to 3° C can be accounted for by the coincident decrease in the level of GSH. Further support for the role of GSH in detoxifying H₂O₂ comes from the methyl mercury hydroxide experiment, in which disappearance of H₂O₂ from the incubation medium was nearly completely prevented by initial treatment of the lenses with the mercurial, which bound 75% of the -SH groups of GSH.

Another mechanism for detoxification of H₂O₂ is the enzyme catalase, which is active in lens epithelium¹⁶ and would be expected to contribute to the protection of the lens against oxidative damage. An inhibitor of catalase, aminotriazole, has been shown to significantly increase the level of H₂O₂ in aqueous humor after injection of the compound into rabbits.¹⁶ It may be that both catalase and glutathione peroxidase protect the lens against oxidative damage from H₂O₂, as suggested in the case of the red blood cell.¹⁷

The concentration of H₂O₂ used in this study was chosen because it equals the normal level found in rabbit anterior aqueous humor. However, it is possible that the lens

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*It was found that 1.46 μmol H₂O₂ disappeared from the medium in 3 hr (Table I), which corresponded to an increased formation of 0.40 μmol NADPH (Table IV). Based on the oxidation of two molecules of GSH per H₂O₂, a decrease in GSH of 0.4 μmol/lens at 0° to 3° C (Table II), and a disappearance of H₂O₂ of 0.31 μmol/lens (Table I).
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In situ is actually exposed to a lower level of $H_2O_2$, since only the anterior surface interacts with aqueous humor, whereas in vitro the lens is totally immersed in the culture medium. Also, it is not known whether the concentration of $H_2O_2$ in posterior aqueous humor is as high as that in the anterior chamber. Thus the rate of detoxification of $H_2O_2$ by the lens in vivo may be lower than that determined in this study.

In conclusion we have demonstrated that when lenses are cultured in the presence of $H_2O_2$ in concentrations that are usually present in aqueous humor, the peroxide is removed from the medium by a mechanism involving GSH metabolism and HMS reactions. It is evident that the lens is able to detoxify a significant amount of $H_2O_2$ and is thus able to protect itself against oxidative damage that might otherwise result.

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REFERENCES