Influence of Mercurial Sulfhydryl Agents on Aqueous Outflow Pathways in Enucleated Eyes

Thomas F. Freddo,* Mary M. Patterson, Douglas R. Scott, and David L. Epstein

Enucleated calf and primate eyes were perfused with either p-chloromercuribenzenesulfonate (PCMBS) or p-chloromercuribenzoate (PCMB). Both caused a decrease in aqueous outflow that did not result from inhibition of glycolysis in trabecular cells. Morphologic studies of PCMBS-treated primate eyes suggested that the reduction in aqueous outflow occurred as the result of cellular swelling in the trabecular meshwork and, particularly, in the juxtacanalicular connective tissue. These findings suggest that changes in cell volume may influence trabecular outflow resistance and that mercurial-sensitive membrane sulfhydryl groups could participate in the regulation of aqueous outflow. Invest Ophthalmol Vis Sci 25:278–285, 1984

Certain sulfhydryl blocking agents have been shown to alter the resistance to aqueous humor outflow. Two of these, iodoetamide (IA) and N-ethyl maleimide (NEM), have been shown previously to increase the facility of aqueous outflow in enucleated eyes.1,2 This effect could not be attributed to an inhibition of glycolysis in the trabecular meshwork but, instead, a cell membrane site of action was suggested.1,2 A morphologic study of primate eyes perfused with IA or NEM concluded that each caused distinct changes in the tissues of the outflow pathway that might have resulted from different membrane effects.3

To explore further the role of sulfhydryl groups (–SH) in aqueous outflow, the organic mercurials p-chloromercuribenzenesulfonate (PCMBS) and p-chloro-mercuribenzoate (PCMB) have been employed in perfusion studies. These compounds form mercaptide bonds with sulfhydryl groups, and their behavior is quite specific chemically.4 The hydrophilic nature of PCMBS renders it less able to cross cell membranes than PCMB; thus, cytoplasmic and cell membrane effects can be separated to some degree.

In this article, we report the effects of these mercurials on aqueous outflow facility and on the morphology of the trabecular meshwork in primate eyes. In addition, the effects of these agents on the glycolytic pathway in calf trabecular meshwork are reported.

Materials and Methods

Pairs of primate eyes from three species (Macaca mulatta, Macaca fascicularis, Papio papio) were obtained from animals killed upon completion of experiments that did not involve their eyes. Pairs of calf eyes were obtained, freshly enucleated, from a commercial abattoir (Joseph T. Trelegan and Company, Cambridge, MA). All eyes were stored at 4°C in a moist chamber prior to perfusion. All perfusions were performed within 24 hr after enucleation.

The technical aspects of our constant pressure perfusion method have been described elsewhere.1 After insertion of a Grant fitting, eyes were perfused with Dulbecco’s phosphate-buffered saline (GIBCO, Grand Island, NY) and 5.5 mM glucose (basic perfusion medium) for 1 hr at a pressure of 15 mmHg and at room temperature. A baseline measurement of aqueous outflow facility was then recorded.

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Following baseline facility measurements, experimental or control solutions were introduced into the anterior chambers of paired eyes with the fitting removed. Depending upon the experiment, the mercurial was dissolved in perfusion medium to achieve one of the following concentrations: 0.1 mM PCMBS, 5 mM PCMB, 10 mM PCMBS, 0.1 mM PCMB, and 0.4 mM PCMB. Each of these solutions was filtered through a 0.2 μm Nuclepore filter just prior to use. In some experiments, the anterior chamber was simply exchanged with the mercurial solution; in others, reservoirs containing the experimental agent or osmotically matched control medium continued to deliver solutions to the anterior chamber.

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Table 1. Effect of 5 mM PCMBS for 30 min on outflow facility in subhuman primate eyes

<table>
<thead>
<tr>
<th>Number</th>
<th>Species</th>
<th>Experimental eye (µl/min/mmHg)</th>
<th>Control eye (µl/min/mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>1 hr</td>
</tr>
<tr>
<td>1</td>
<td>Baboon</td>
<td>0.31</td>
<td>0.36</td>
</tr>
<tr>
<td>Percent change</td>
<td>+16</td>
<td>−25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Baboon</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td>Percent change</td>
<td>−17</td>
<td>−63</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cynomolgus</td>
<td>0.33</td>
<td>0.32</td>
</tr>
<tr>
<td>Percent change</td>
<td>−3</td>
<td>−25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Cynomolgus</td>
<td>0.38</td>
<td>0.23</td>
</tr>
<tr>
<td>Percent change</td>
<td>−39</td>
<td>−78</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Rhesus</td>
<td>0.85</td>
<td>0.69</td>
</tr>
<tr>
<td>Percent change</td>
<td>−19</td>
<td>−64</td>
<td></td>
</tr>
</tbody>
</table>

Significance of experimental effect in first hour after treatment: \( P < 0.2 \)

Significance of experimental effect in second hour after treatment: \( P < 0.01 \)

flow to the eyes for 30 min following the initial exchange. Eyes then were reconnected to the original apparatus without further manipulation and perfused for several hours with basic medium. Aqueous outflow facility measurements were taken hourly during this period.

For each hour, the experimental effect was calculated to be the percent change in facility of the experimental eye, minus the percent change in the facility of the paired control eye.

For morphologic examination, five pairs of primate eyes were fixed by switching the perfusion fluid to a mixture of 3% glutaraldehyde and 0.1 M cacodylate buffer, upon completion of the facility measurements. This occurred without any interruption of flow and at the normal perfusion pressure of 15 mmHg.

The eyes then were opened equatorially, immersed in fixative fluid for an additional 2 hr, and washed overnight in cold buffer. Subsequently, the anterior segment of each eye was divided into four quadrants: half of each quadrant was processed for light and transmission electron microscopy and half for scanning electron microscopy.

Specimens for light and transmission electron microscopy were sectioned meridionally with a Smith-Farquhar tissue chopper (200 µm). The specimens were postfixed in 1% OsO₄ and 1.5% potassium ferrocyanide in distilled water, dehydrated and embedded in an Epon–Araldite mixture. An LKB Ultratome III was used to cut thick (1 µm) and silver-gray thin sections. Thick sections were stained with toluidine blue and examined with a Leitz-Orthoplan photomicroscope. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL-100S electron microscope.

For scanning electron microscopy, specimens were trimmed to facilitate observation of the drainage angle and postfixed as noted above. The specimens were dehydrated in a graded series of acetones, critical-point dried with CO₂, and coated with gold–palladium alloy in a Polaron E-5000 sputter coater. Coated specimens were examined in an ISI-60 scanning electron microscope operated either at 15 or 30 kV.

The biochemical status of the glycolytic pathway in calf trabecular meshwork, exposed to PCMBS or PCMB, was assessed as reported previously.¹²⁻⁵ Six pairs of calf eyes were perfused simultaneously under conditions identical to those described above. One mM dithiothreitol (DTT), in basic perfusion medium, was placed into the anterior chamber of each eye after the desired time interval and perfused for 20 min to combine with any residual mercurial. In previous studies, DTT had been observed not to reverse mercurial-induced glycolytic enzyme inhibition under meshwork lactate assay conditions (Anderson and Epstein, unpublished data). Trabecular meshwork was harvested from experimental and control eyes, and the level of lactate produced from added glucose by tissue homogenate was assayed as previously described.¹²⁻⁵ The data for each assay were averaged, and the percent inhibition was calculated from the ratio of experimental lactate production to control lactate production.²

Data from aqueous outflow facility and lactate production measurements are reported with their respective standard errors. Appropriate paired t tests were performed.

Results

All primate eyes that received a 30-min perfusion with 5 mM PCMBS showed substantial declines in outflow facility after 2 hr (\( P < 0.01 \)) (Table 1). No association existed between species of primate (rhesus, cynomolgus, or baboon), time postmortem, or the final magnitude of the decrease. However, the three eyes (#2, 4, 5) that showed the most change with PCMBS...
in the first hour after treatment also showed the largest effect in the next hr, compared with their own controls.

In light microscopic sections (1 μm) of control eyes, the trabecular meshwork and Canal of Schlemm appeared normal. The trabecular beams were covered by thin endothelial cell processes with slender, polygonal, or fusiform nuclei (Fig. 1). The juxtacanalicular connective tissue (JCT) was represented by a loose cellular network, and along the inner wall of Schlemm’s canal, giant vacuoles were clearly visible (Fig. 1).

In sections of experimental eyes, the most salient feature present was profound intracytoplasmic and nuclear swelling throughout the trabecular meshwork (Fig. 2). While not all cells were affected equally, no differential response was evident between trabecular endothelial cells and those of the JCT or between either of these populations and the cells lining the inner wall of Schlemm’s canal. In contrast, however, cells lining the outer wall of Schlemm’s canal and the collector channels remained unaffected. In many sections the extracellular outflow pathway, particularly in the juxtacanalicular region, was occluded almost entirely by the swollen cells.

Scanning electron microscopy demonstrated that the uveal meshwork was essentially unaltered by the constant pressure perfusion. In control eyes, the trabecular beams were neither grossly distorted nor were they stripped of their normally smooth endothelial cell covering (Fig. 3). In experimental eyes, however, micrographs revealed that cellular swelling had led to lysis.
Fig. 3. Scanning electron micrograph from a control eye following constant pressure perfusion without PCMBS. The trabecular beams appear normal, without loss of endothelial cells (×500).

Fig. 4. Scanning electron micrograph from an experimental eye following perfusion with 5 mM PCMBS for 30 min. Cellular swelling has led to endothelial cell lysis with baring of some trabecular beams. The swollen nucleus of a disrupted endothelial cell is present within the circle (×500).
of some trabecular cells, with scattered baring of trabecular beams (Fig. 4). Because an understanding of the relationship between facility change and extent of meshwork alteration was desirable, it was important to rule out the possibility that the decreased facility was due to accumulation of cell debris from lysed cells. For this reason, a sixth pair of rhesus monkey eyes was perfused following the usual protocol except that the experiment was terminated early, as soon as a moderate facility reduction (28%) was achieved. This occurred 30 min after the experimental eye had received a 30-min perfusion of 5 mM PCMBS. The trabecular cells in the experimental eye of this pair were swollen uniformly but not lysed.

In the cells of the JCT, transmission electron micrographs confirmed the presence of intracellular swelling. Within these cells, mitochondria appeared distended and the normal organization of their cristae was lost (Fig. 5). Similar changes were evident in cells lining the inner wall of Schlemm’s canal, but despite the swelling in these cells, giant vacuoles were still present, and disruptions in the continuity of the inner wall were not observed (Fig. 5).

Endothelial cells lining the trabecular beams were affected similarly, and the usual complement of intracytoplasmic filaments present in these cells was replaced by material having a granular, amorphous appearance (Fig. 6). In addition, the cores of the trabecular beams in experimental eyes appeared to contain more wide-spacing collagen than did the contralateral control eyes (Fig. 6, inset).

In calf eyes, both PCMBS and PCMB caused a statistically significant decrease in the outflow facility, with the exception of the lower concentration of PCMB. The results are illustrated in Figures 7 and 8. In the first hour after 10 mM PCMBS was placed in the anterior chamber of experimental eyes, the facilities of outflow decreased 29.2 ± 2% (percent change in experimental eye minus percent change in control eye, P < 0.001) relative to the controls.
Fig. 8. Effects of 0.1 mM and 0.4 mM PCMB on aqueous outflow facility. Only the higher dose of PCMB caused a reduction in aqueous outflow facility (calculated as percent change in experimental eye minus percent change in control eye).

Table 2. Percent inhibition of lactate production in calf trabecular meshwork after exposure to mercurials

<table>
<thead>
<tr>
<th>Agent and method</th>
<th>Mean % inhibition</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCMBS, 10 mM in anterior chamber.</td>
<td>-16 ± 13</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>Perfused 1.5 hr after treatment. N = 5.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCMBS, 10 mM in anterior chamber.</td>
<td>14 ± 3</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Perfused 3 hr after treatment. N = 2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCMBS, 0.1 mM for 30 min. Perfused 1 hr</td>
<td>60 ± 4</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>after treatment. N = 3.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCMB, 0.4 mM for 30 min. Perfused 1 hr</td>
<td>9 ± 13</td>
<td>&gt;0.2</td>
</tr>
<tr>
<td>after treatment. N = 3.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0.4 mM PCMB was succeeded by a 25 ± 9% (P < 0.05) further decline at the end of the second hr.

The results of the lactate production study are given in Table 2. After perfusing under the conditions described, lactate production from added glucose was measured in homogenates of trabecular meshwork. Only the 30-min perfusion with 0.1 mM PCMBS had any significant effect, producing a 60% reduction in glycolytic activity. The 10 mM doses of PCMBS failed to produce a comparable effect, even after 3 hr of posttreatment perfusion. Perfusion with 0.4 mM PCMB for 30 min was also without effect.

Discussion

In the present studies, we have demonstrated that organic mercurial-sulfhydryl agents produce a decrease in aqueous humor outflow facility in enucleated calf and primate eyes. We attribute this reduction primarily to the cellular swelling that was observed, particularly in the juxtacanalicular tissue. It was reported previously that perfusion of the anterior chamber of monkey eyes with a hypotonic medium (ie, distilled water) caused a marked swelling of trabecular cells and a commensurate reduction in aqueous outflow.6

The effect of PCMB and PCMBS on outflow facility is opposite that reported previously for other sulfhydryl agents including iodoacetamide (IA) and N-ethyl mal-lemide (NEM).12 This seeming paradox is less sur-
prising when the literature regarding PCMB and PCMBS is reviewed. It has been demonstrated that these organic mercurials induce a marked increase in the volume of several types of cells including red blood cells, Ehrlich ascites tumor cells, and the cells lining renal tubules. Mercurial-induced changes in cell volume may be due to interaction with membrane —SH ligands involved with ATPase activity or the cellular sodium channels. Indeed, this mechanism was suggested to explain the profound swelling and eventual lysis observed in rabbit corneal endothelial cells exposed to PCMB and PCMBS. Although this seems a reasonable explanation, the precise mechanisms by which these —SH blocking agents cause their effects remain unclear.

PCMB is more hydrophilic than PCMB and, thus, it less readily permeates cell membranes. For example, the uptake of PCMB by human red blood cells in one hr at 25°C was reported to be 30 x 10^{-17} moles/cell whereas for PCMBS, a one-hundred times lower concentration (0.25 x 10^{-17}) was found.

Because of its slower penetration, PCMBS has been used as a cell membrane —SH probe. Although dose and exposure time are critical factors, the findings that both compounds produced similar effects suggest that their major site of action in the trabecular meshwork is on cell membranes and not on cytoplasmic proteins.

The failure of the 10 mM PCMBS dose to inhibit glycolysis when 0.1 mM continuous perfusion does is puzzling, and its cause remains unclear. Perhaps the high dose renders the plasma membrane less permeable to further PCMBS penetration. It is possible that the glycolytic rate as measured using the 0.1 mM PCMBS may represent a maximal capacity. Normally the cell would need much less than the uninhibited 40% of this capacity. Therefore, 60% inhibition of glycolysis alone probably could not account for the observed effects, but might impaire the cell’s ability to respond to other stress. Even in this case, however, the morphologic and rheologic effects were so similar to the case where glycolysis was essentially unimpaired that a common mechanism seems reasonable.

In this study, another aspect of the importance of sulfhydryl groups in the trabecular meshwork has been demonstrated. Like IA and NEM, PCMB and PCMBS appear to owe their effect upon aqueous outflow to interaction with membrane sulfhydryl groups. However, their effects are quite different. IA and NEM have been shown to enlarge the “subendothelial space” within the juxtacanalicular meshwork and disrupt the continuity of the inner wall of Schlemm’s canal. On the other hand, the mechanism of organic mercurial perturbation appears to involve cellular swelling. While alternative mechanisms could be proposed to account for the present findings, cellular swelling as a cause for the reduction in outflow facility is the most reasonable hypothesis.

Particularly in the juxtacanalicular meshwork, cellular swelling could decrease the dimension of the extracellular outflow pathway. Alternatively, cellular swelling of the inner wall endothelium could retard fluid movement into Schlemm’s canal. It is possible that alterations in trabecular cell volume could be involved in the mechanism of certain secondary glaucomas and that cell volume regulation may be an important function of trabecular cells.

The fact that certain —SH agents increase, and others decrease outflow resistance suggests that these —SH effects may represent perturbations of a naturally occurring mechanism for control of aqueous outflow. The diverse effects on aqueous outflow caused by these agents may result from selective blockade of different populations of sulphydryl groups in the aqueous drainage pathways.

Key words: glaucoma, aqueous humor, trabecular meshwork, sulfhydryl groups, electron microscopy, monkey, calf

Acknowledgments

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References