Reports

Effect of Repeated Anterior Chamber Perfusion on Aqueous Flow in the Cynomolgus Monkey

Paul L. Kaufman and Kristine A. Erickson-Lamy

No differences were found between fluorophotometrically determined aqueous flow rates in cynomolgus monkeys having vs not having undergone prior repeated anterior chamber perfusion. This suggests that: (1) repeated perfusion does not produce a progressive decline in the rate of aqueous formation; and (2) the repeatedly perfused monkey eye can be validly used in studies of aqueous formation. Invest Ophthalmol Vis Sci 26:885–886, 1985

Repeated two-level constant pressure perfusion of the living cynomolgus monkey anterior chamber at 1- to 2-mo intervals causes a progressive decline in total outflow facility, unaccompanied by a rise in intraocular pressure (IOP). The progressive decrease in total facility apparently represents decreased facility across the trabecular meshwork and inner wall of Schlemm’s canal, in contrast to the time-dependent facility increase that occurs during an individual perfusion. The absence of a progressive IOP increase in the presence of decreasing facility implies a progressive decrease in the rate of aqueous humor formation, an increased rate of uveoscleral drainage, or decreased episcleral venous pressure.

We report here the fluorophotometrically determined aqueous flow rates in never perfused and repeatedly perfused living cynomolgus monkey eyes.

Materials and Methods. Twenty cynomolgus monkeys (Macaca fascicularis) of both sexes, weighing 1.6–5.5 kg were studied. None had undergone prior ocular surgery. Ten monkeys had undergone facility determination by a one-needle, two-level constant pressure perfusion technique 13 to 15 times at approximately 2-mo intervals over periods of 26–28 mo; during these perfusions, various drugs had been administered intracameraly or systemically. Three to 4 mo elapsed between the final perfusion and the aqueous flow measurements, at which time the anterior chamber was free of biomicroscopically visible cells and flare. The other 10 monkeys had biomicroscopically quiet virgin eyes.

Mean arterial blood pressure (MAP) and gases; corneal thickness, curvature, and endothelial transfer coefficient for fluorescein (Ka); anterior chamber depth, volume (ACV), and elimination coefficient for fluorescein (Ke); aqueous humor flow rate (F); and IOP were all determined noninvasively, as previously described. Body temperature was monitored rectally and maintained at 37°C by heating pads. All measurements were made with the monkey lying prone in a headholder, so that the eyes were 4–8 cm higher than the heart.

In 18 animals, all ocular measurements were made bilaterally and averaged to give a single value for the individual animal for that experiment, while two animals contributed only one usable eye; thus the statistical unit is the animal.

All animals underwent replicate experiments at biweekly intervals, using a different anesthetic regimen each time, in the following sequence: (1) im methohexitol induction/im pentobarbital maintenance; (2) im ketamine + im diazepam induction/iv ketamine + im diazepam maintenance; (3) inhalation nitrous oxide + oxygen + halothane induction and maintenance; (4) im ketamine induction/iv ketamine maintenance. Dosages and other details of anesthesia have been described previously.

This investigation adheres to the ARVO Resolution on the Use of Animals in Research.

Results. Table 1 presents the data. Specific parameters demonstrated the anticipated anesthetic dependence: F and MAP were lowest and PO2 highest under halothane; IOP was lowest and pH highest under pentobarbital; Ka was highest under the ketamine regimens. IOP in the repeatedly perfused eyes tended to be higher than in the virgin eyes under ketamine but lower under the other three anesthetic regimens; the difference was statistically significant only under pentobarbital. F and Ke were not lower in the repeatedly perfused eyes than in the virgin eyes under any regimen; indeed, there was an opposite tendency under ketamine. Ka in the repeatedly perfused eyes tended to be higher than in the virgin eyes under pentobarbital, ketamine, and ketamine + diazepam; the difference was statistically significant.
Table 1. Ocular and systemic physiology of repeatedly perfused vs nonperfused cynomolgus monkey eyes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pentobarbital</th>
<th>Ketamine</th>
<th>Diazepam</th>
<th>Halothane</th>
<th>Ketamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (μl × min⁻¹)</td>
<td>1.30 ± 0.17 (10.7)</td>
<td>1.25 ± 0.11 (10.7)</td>
<td>0.87 ± 0.16 (7.7)</td>
<td>1.37 ± 0.13 * (8.6)</td>
<td>0.98 ± 0.17</td>
</tr>
<tr>
<td>Ka (min⁻¹ × 10⁻⁵)</td>
<td>3.73 ± 0.79 (10.7)</td>
<td>4.12 ± 0.84 (7.7)</td>
<td>2.20 ± 0.59 (7.7)</td>
<td>7.44 ± 0.55 † (8.6)</td>
<td>4.08 ± 1.29</td>
</tr>
<tr>
<td>Ke (min⁻¹ × 10⁻⁵)</td>
<td>1.38 ± 0.18 (7.7)</td>
<td>1.41 ± 0.14 (7.7)</td>
<td>0.90 ± 0.13 (7.7)</td>
<td>1.46 ± 0.14 (7.7)</td>
<td>1.13 ± 0.21</td>
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<tr>
<td>ACV (μl)</td>
<td>101.6 ± 4.2 (10)</td>
<td>106.0 ± 3.2 (10)</td>
<td>104.1 ± 4.6 (10)</td>
<td>106.1 ± 5.8 (10)</td>
<td>—</td>
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<tr>
<td>IOP (mmHg)</td>
<td>6.45 ± 0.52 † (7.7)</td>
<td>10.50 ± 0.74 (7.7)</td>
<td>9.86 ± 1.17 (7.7)</td>
<td>13.31 ± 0.81 (7.7)</td>
<td>—</td>
</tr>
<tr>
<td>pH</td>
<td>7.42 ± 0.01 (10)</td>
<td>7.38 ± 0.01 (10)</td>
<td>7.37 ± 0.02 (10)</td>
<td>7.37 ± 0.01 (10)</td>
<td>—</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>94.3 ± 2.4 (10.5)</td>
<td>92.3 ± 2.1 (10.5)</td>
<td>140.6 ± 5.6 (10.5)</td>
<td>98.0 ± 8.5 (10.5)</td>
<td>—</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>40.7 ± 1.0 (10.5)</td>
<td>40.4 ± 1.3 (10.5)</td>
<td>40.6 ± 1.8 (10.5)</td>
<td>36.7 ± 1.7 (10.5)</td>
<td>—</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>19.2 ± 6.0 (10.5)</td>
<td>99.9 ± 6.2 (10.5)</td>
<td>45.7 ± 2.3 (10.5)</td>
<td>90.6 ± 4.4 (10.5)</td>
<td>—</td>
</tr>
</tbody>
</table>

† P < 0.05.
* P < 0.10.
‡ P < 0.005.

Discussion. Our data show that the fluorophotometrically determined rate of aqueous humor flow is similar in monkeys that have undergone prior repeated anterior chamber perfusion and in monkeys never previously perfused; and, therefore, suggest that the progressive decrease in outflow facility that occurs with repeated perfusion is not accompanied by a decreased aqueous formation rate.

The comparison between repeatedly perfused and never perfused monkeys is perhaps not ideal since it requires the unprovable, albeit reasonable, assumption that the two groups had similar aqueous formation rates before perfusions of one group were begun. The alternative and perhaps more desirable approach would have been to measure the aqueous formation rate before, during, or after each perfusion, to determine the longitudinal trend in the perfused animals. This latter approach was applied in the facility/IOP experiments, and clearly revealed the progressive facility decrease in the absence of an IOP increase even though the last, lowest facility values remained in the "normal" range. However, no methodology for determining aqueous flow in monkeys was available in our laboratory at that time.

Despite this shortcoming, the data seem reasonably persuasive that a progressive decline in the rate of aqueous formation does not occur to account for the lack of IOP elevation in the face of a progressive decline in outflow facility in repeatedly perfused monkey eyes. Apparently, increased uveoscleral drainage, decreased episcleral venous pressure, or other factors are responsible. This indicates that the repeatedly perfused monkey eye can be validly used in studies of aqueous formation.

Key words: anterior chamber perfusion, anterior segment fluorophotometry, aqueous humor flow, Macaca fascicularis, monkey eye

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References
Glutathione Reductase of Calf Trabecular Meshwork

Khiem P. V. Nguyen, Howard Weiss, Levon N. Karageuzian, P. John Anderson, and David L. Epstein

Hydrogen peroxide has been found in both calf and human aqueous humor at a level of 25 μM. It is likely, therefore, that trabecular meshwork possesses mechanisms for detoxifying H₂O₂ both to protect itself and other more distal structures of the outflow pathway from oxidative damage. We have recently demonstrated an active glutathione peroxidase in calf trabecular meshwork. In this study, we have characterized the complementary enzyme, glutathione reductase. The activity was present at a level of 0.120 units/min/g wet of tissue (0.005 units/min/mg soluble protein). The enzyme quickly lost activity in crude extracts but could be stabilized by heating at 60°C for 30 min. Denatured protein was removed by centrifuging at 43,000 × g. Heating at 80°C for 10 min destroyed all enzyme activity. Addition of 1 mM GSSG protected the enzyme completely from heat denaturation; NADPH⁺ and GSH offered some protection but NADPH provided none. The supernatant from the 60°C heat treatment was further purified by affinity chromatography on 2',5'-ADP-agarose. Overall purification was 200-fold with a yield of 80%. The pH optimum of the purified enzyme was 7.0. The Kₘ for NADPH and GSSG were 19 μM and 78 μM, respectively. The heat inactivation properties of the purified enzyme were identical to those in the crude extract. An enzyme activity stain on disc gel electrophoresis showed that the enzyme exists in only one form. Invest Ophthalmol Vis Sci 26:887-890, 1985

Materials and Methods.

GSH, GSSG, NADPH, NADPH⁺, 2',5'-ADP-agarose, peptatin and trypsin inhibitor were obtained from Sigma Chemical Corp. (St. Louis, MO). All other chemicals were reagent grade. Protein determinations were done with a dye-binding method using a globulin standard. A kit from Biorad Laboratories (Richmond, VA) was used (Biorad Technical Bulletin, No. 1051). Calf TM was harvested as described previously.

Enzyme assay: The activity of GR was measured by following the decrease in NADPH absorbance at 340 nm. The final assay mixture had a volume of 0.5 ml, with 0.1 M potassium phosphate buffer, pH 7.0, 2.5 mM GSSG, 0.15 mM NADPH, 1 mM EDTA, and 0.02 to 0.1 ml of enzyme extract. To correct for minimal, nonenzymatic oxidation of NADPH, blanks were prepared by either omitting GSSG, or by replacing enzyme extract with distilled water. One unit of GR was defined as 1 μmol of NADPH oxidized per minute at 25°C at pH 7.0.

Enzyme purification: All manipulations were performed at 0–4°C unless otherwise indicated. Tissue homogenates were prepared by combining tissue at a ratio of 1 g/5 ml with ice cold 5 mM potassium phosphate buffer, pH 7.0, 140 mM KCl, 1 mM EDTA, 1 mM DTT, homogenizing in a Tissumizer (Model SDT 100N, Tekmar; Cincinnati, OH), then sonicating for 30 sec (Ultrasonic model 8849-00, Cole Parmer; Shelton, CT). The homogenate was centrifuged for 10 min at 1100 × g to remove collagen debris and unbroken cells. The supernatant was transferred to 1.3 ml capped plastic vials (Eppendorf, Brinkmann Instruments, Westbury, NY), and was heated in a water bath at 60°C for 30 min. The material was then centrifuged at 43,000 × g for 1 hr. The supernatant was collected and kept at 0°C for same day use or stored in the freezer for use within 1 wk. There was no appreciable loss of enzyme activity following such storage.

A 2',5'-ADP-agarose affinity chromatography column (0.5 × 2 cm) was equilibrated with 0.05 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) and 1 mM EDTA. Approximately 700 μg of protein from the heated supernatant were applied to the column, which was then washed with 2 ml of the equilibration buffer. The elution buffer was the same as the equilibration buffer, but adjusted to pH 7. GR was eluted with 0.5 mM NADPH in elution buffer, at room temperature, in 0.5-ml fractions. To stabilize the enzyme, GSSG was...
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Fig. 2. Kinetics of glutathione reductase from calf trabecular meshwork. Substrate NADPH. Intercept \( s/v = 32.06 \), slope \( = 1863.76 \). Estimate of \( K_m = 19 \pm 1 \mu M \). Graph for GSSG is not shown.

Table 2. Comparison of glutathione reductase from different tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity</th>
<th>GSSG</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf TM</td>
<td>5</td>
<td>78</td>
<td>19</td>
</tr>
<tr>
<td>Rat lens</td>
<td>6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Human lens*</td>
<td>16</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cortex</td>
<td>4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nucleus</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rat brain</td>
<td>7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Human erythrocyte</td>
<td>4</td>
<td>65</td>
<td>3</td>
</tr>
<tr>
<td>Calf liver</td>
<td>120</td>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

Units of activity are nmoles/min/mg soluble protein.
* Lens enzyme activity measured at 37°C.

Thermal inactivation: The purified enzyme could be heated to 60°C for at least 1 hr without loss of activity. However, it lost all activity if heated at 80°C for 10 min. GSSG 1 mM offered 100% protection against heat inactivation at 80°C. NADP⁺ 1 mM offered 70% protection; GSH 1 mM, 30% protection; and NADPH 0.20 mM, none.

Gel electrophoresis: After staining with phenazine methosulfate and nitroblue tetrazolium, one hypochromatic band with relative mobility (rm) value of 0.26 was seen. A faint, more anodal band, with rm = 0.36 could also be seen. However, this band is not specific for GR since it is also present on control zymograms where GSSG was omitted.

Discussion. H₂O₂, which is present at a high concentration in the anterior chamber, may exert toxic effects on intraocular tissues. As aqueous humor leaves the eye principally via the TM, one may expect that this tissue would require an efficient system for removing H₂O₂ in order to protect itself.

Kahn et al.⁹ have found that calf TM is very resistant to acute damage, even from high levels of H₂O₂. However, if TM GSH was removed by prior perfusion with diamide and BCNU, H₂O₂ caused an appreciable increase in outflow resistance.

Scott et al.⁴ calculated that, through glutathione peroxidase, calf TM could remove all the anterior chamber H₂O₂ in 2 min. At 0.4 μmol/g wet weight, the GSH content of calf TM was also calculated to be depleted in 5 min without replenishment.

In this study, we have demonstrated that calf TM contains the companion enzyme to glutathione peroxidase, glutathione reductase. There seems to be more GSH-Px per milligram protein than there is GR (0.026 vs 0.005). However, the \( K_m \) of GR for GSSG is much lower than the \( K_m \) of GSH-Px for GSH (78 nM vs 9890 nM). The high affinity of GR for GSSG is consistent with the observation that in most tissues, including calf TM, glutathione exists mainly in its reduced form.⁹ The level of GR from calf TM is comparable to that in lens, brain and erythrocyte, but less than in liver (Table 2).

The pH optimum and the Michaelis–Menten constants of calf TM GR are comparable to those of GR from calf liver⁶ (\( K_ms \) of 20 μM and 100 μM for NADPH and GSSG, respectively). The heat inactivation properties of calf TM GR are similar to those observed for mouse liver GR by Lopez-Barea et al.⁷

On gel electrophoresis, we noted a non specific band in addition to the main, single activity band of GR. Other workers have observed a similar phenomenon and coined the term “nothing reductase” to describe it.¹⁰

Oxidative damage to TM from products in the aqueous humor remains a provocative hypothesis for the mechanism of certain forms of glaucoma.⁴,⁹,¹¹ To evaluate this hypothesis, other enzymes that may be involved in protecting the TM must be investigated. We have recently completed a study in this laboratory on superoxide dismutase and catalase of calf trabecular meshwork. For the future, it will be of interest to characterize γ-glutamylcysteine synthase, glutathione synthase, and enzyme components of the hexose monophosphate shunt. These enzymes need to be examined in normal and diseased human trabecular meshwork.

† Data was plotted as s/v versus s, where s is the substrate concentration in mM and v is the rate of reaction in μmol/min. The \( K_m \) is given by the intercept of the line on the s axis: \( K_m = -\text{intercept}_s / -\text{intercept}_v / \text{slope} \). This plot yields a valid, unweighted fit to the data.⁴ Straight lines were fitted using routines from PROPHET, a national computer resource sponsored by the Division of Research Resources, NIH. The SDs for the \( K_ms \) were estimated using the approximation: \( CV^2(A/B) \) or \( A \times B \) = \( CV^2(A) + CV^2(B) \), where \( CV = SD/\text{mean} \).
Key words: glutathione reductase, affinity chromatography, disc gel electrophoresis, kinetics, calf, trabecular meshwork


References

Defects in Cortisol-Metabolizing Enzymes in Primary Open-Angle Glaucoma

Bernard I. Weinstein, Pedda Munnoangi, Gary G. Gordon, and A. Louis Southren

Assays of cortisol-metabolizing enzymes in homogenates of human trabecular meshwork cells under optimal conditions revealed two defects in primary open-angle glaucoma (POAG): one is a marked increase in Δ4-reductase and the other is a decrease in 3-oxidoreductase. Experiments indicated that the differences in enzyme activities seen between POAG and nonPOAG trabecular meshwork derived cell homogenates were due to altered amounts of enzymes rather than to alterations in cofactor availability, pH, or endogenous activators or inhibitors. This clearly demonstrates an enzymatic defect(s) in POAG which may be the basis for the ocular hypertension and sensitivity to exogenous glucocorticoids seen in this disorder. Invest Ophthalmol Vis Sci 26: 890–893, 1985

We reported earlier that cells cultured from trabecular meshwork specimens obtained from patients with primary open-angle glaucoma (TMPOAG cells) exhibited two major differences in cortisol metabolism when compared to similar cells from nonglaucomatous patients (TMnonPOAG cells).1 One is a marked increase in Δ4-reductase activity and the other is a decrease in 3-oxidoreductase activity leading to an accumulation of 5α (5β)-dihydrocortisol, intermediates not found with TMnonPOAG cells.

In order to determine whether the differences in cortisol metabolism found in these cells were due to changes in the amount of enzymes as opposed to alterations in cofactor availability, pH, or the presence or absence of inhibitors or activators, we studied the kinetic parameters of cortisol Δ4-reductase and 3-oxidoreductase in homogenates of these same TMPOAG and TMnonPOAG cells where these factors can be experimentally controlled. Defining the enzymatic basis for the altered metabolism of cortisol in TMPOAG cells is important in view of the recent findings that 5β-dihydrocortisol can potentiate threshold levels of topically applied cortisol and dexamethasone in causing nuclear translocation of the cytosolic glucocorticoid receptor in rabbit iris–ciliary body tissue,2 an early and necessary event in steroid hormone action and potentiate threshold levels of dexamethasone in elevating intraocular pressure in young rabbits.3

Materials and Methods. Culture of cells and preparation of cell homogenates: Cells cultured from trabecular meshwork from two nonPOAG patients (autopsy) and two POAG patients (surgical trabeculectomy) were grown to confluence in 75-cm² tissue culture flasks. The TMPOAG cells, used in the present