Effects of Pilocarpine, Salbutamol, and Timolol on Aqueous Humor Formation in Cynomolgus Monkeys

Hiroaki Miichi and Shigetoshi Nagataki

The rate of aqueous humor formation was determined in the cynomolgus monkey eyes by a tracer dilution technique. One 25-gauge needle was inserted into the posterior chamber and a solution of fluorescein labeled dextran with a molecular weight of 40,000 was infused at a constant rate. The aqueous humor was collected through a needle inserted into the anterior chamber, while the intraocular pressure (IOP) was maintained at a constant level. The aqueous humor formation rate and the dye distribution volume were calculated from the time profile of the dye concentration in the effluent aqueous humor. By means of this technique, the effects of pilocarpine, salbutamol, and timolol on the aqueous humor formation rate were studied. The test drug solution was administered into the conjunctival reservoir during a 90-min period before measurements and also during the measurement so that a steady-state drug concentration was maintained in the anterior chamber during the measurement. Pilocarpine 0.1% reduced the aqueous humor formation rate to approximately 50% of the control without significantly changing the IOP or the distribution volume. Salbutamol 0.5%, a β-adrenergic agonist, increased the rate by about 38%, but timolol 0.1%, a β-adrenergic antagonist, reduced the rate by an average of 36%. Timolol caused a statistically significant lowering of the IOP by about 2 mmHg. Simultaneous administration of salbutamol 0.5% and timolol 0.2% caused no change in the aqueous humor formation rate or the IOP. The barrier function of the blood aqueous barrier was not altered by these drugs as revealed by aqueous protein determinations. Invest Ophthalmol Vis Sci 24:1269-1275, 1983

There has been increasing interest in the mechanism of action of β-adrenergic drugs, agonist and antagonist, on the rate of aqueous humor formation. Fluorophotometric studies in the human eye have demonstrated that aqueous humor formation is suppressed by topical instillation of β-adrenergic agonists1-4 and increased by topical instillation of epinephrine, an α- and β-adrenergic agonist,5-6 or metaproterenol, a selective β-adrenergic agonist.3 The increase in the aqueous humor formation with epinephrine can be enhanced slightly by pretreatment of thymoxamine, a selective α-adrenergic antagonist,7 but blocked by pretreatment of timolol, a selective β-adrenergic antagonist.8,9 These results indicate that the acute effect of β-adrenergic agonists is to increase the aqueous humor formation and that β-adrenergic antagonists have the opposite effect. In humans, however, the determination of drug effects takes place under circumstances in which the experimental conditions cannot be rigidly controlled. In addition, the measurement of aqueous humor flow in human eyes with fluorescein is based on certain assumptions, some of which have not been proved to be correct.10-13

We felt, therefore, it would be better to study a subhuman primate and to determine aqueous humor formation by measuring the anterior chamber clearance of a tracer with sufficiently high molecular weight that it would leave the system only by flow. In this context, the extensive studies of Bill are pertinent, studies in which the aqueous humor formation was determined in the monkey eye by the clearance of albumin.14-18 Bill has demonstrated that the intracameral infusion of isoproterenol, a β-adrenergic agonist, increases aqueous humor formation and that the increase can be blocked by intravenous injection of propranolol, a β-adrenergic antagonist.17 However, he did not observe a difference in aqueous humor formation between propranolol injected and control animals and attributed the lack of effect of propranolol on aqueous humor formation to anesthesia with barbiturate. Bill and Walinder also demonstrated a reduction of aqueous humor formation by intracameral infusion of pilocarpine.15-16 The opposite effect of topical pilocarpine, however, has been observed in the normal human eye by fluorophotometry.19

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In this study, a method of measuring aqueous humor formation in anesthetized animals was developed based on the anterior chamber clearance of a fluorescent macromolecule, dextran labeled with fluorescein. Using this technique, the rate of aqueous humor formation was determined in the cynomolgus monkey eye where pilocarpine, timolol, and salbutamol, a selective β-adrenergic agonist, were administered into the conjunctival reservoir in order to maintain a steady-state drug concentration in the anterior chamber. In addition, the rate of aqueous humor formation was determined either with pentobarbital or urethane anesthesia, and the effects of these anesthetic agents were compared.

Materials and Methods

General Procedures

Adult female cynomolgus monkeys, weighing 2.4 to 3.3 kg, were anesthetized either with intravenous sodium pentobarbital (40 mg kg⁻¹) or with intraperitoneal urethane (1.5 g kg⁻¹). A conjunctival reservoir was formed by pulling up the upper and lower eyelids with 5-0 nylon sutures, the lacrimal puncta being closed with a heat-sealed polyethylene tube (Intramedic PE50, Clay Adams). This reservoir was filled with the test solution and refilled at 15-min intervals.

The anterior chamber was cannulated with two 25-gauge needles: one needle was inserted into the posterior chamber through the pupil and the other needle was placed in the anterior chamber near the iridocorneal angle. The posterior chamber needle was connected to a syringe-type infusion pump (Model 940, Harvard Apparatus) and the anterior chamber needle to a teflon three-way valve (Omnifit, Biolab, Inc.). One way of the valve was connected to a pressure transducer (Model P-50, Statham-Gould) located at the level of the eye, and the other way of the valve to a polyethylene tube (Intramedic PE20, Clay Adams), the end of which was placed approximately 30 cm above the eye. The effluent from the anterior chamber was collected through the outlet of this tube. The total volume of the tube from the needle to the outlet was approximately 110 μl.

The intraocular pressure (IOP) was measured before starting the infusion, and this IOP level was maintained subsequently by adjusting the height of the outlet of the anterior chamber tube. The left femoral artery was cannulated with a polyethylene tube (Intramedic PE90, Clay Adams) and the systemic blood pressure was monitored continuously with a pressure transducer (Model MPU-0.5, Nihon Koden).

Drug Preparations

Solutions of pilocarpine hydrochloride (Junsei Chemical Co., Ltd.), salbutamol sulfate (Sankyo Co., Ltd.), and timolol maleate (Nippon Merck-Banyu Co., Ltd.), were prepared with a bicarbonate-buffered Ringer's solution containing glutathion (GBR) at the following concentrations: pilocarpine 1 mg ml⁻¹, salbutamol 5 mg ml⁻¹, timolol 1 mg ml⁻¹, and a combination of salbutamol 5 mg ml⁻¹ and timolol 2 mg ml⁻¹. All these solutions were warmed to a body temperature before use. The test drug solution or the placebo solution, ie, GBR solution alone, was administered into the conjunctival reservoir 5 min after the cannulation and thereafter at 15-min intervals.

Infusion of Fluorescein-Labeled Dextran

Dextran labeled with fluorescein isothiocyanate (FD-40, Sigma Chemical Co.), with a molecular weight of approximately 40,000, was dissolved in GBR solution at various concentrations and intensity of fluorescence was measured with a spectrofluorophotometer (Model 204, Hitachi Ltd.). Monochromators were set at 490 nm for the excitation and 520 nm for the emission; a bandwidth of 10 nm was used. Since a linear relationship was observed between fluorescent intensity and the dye concentration of 0.1 to 100 μg ml⁻¹, the GBR solution with 2 mg ml⁻¹ fluorescein-dextran was chosen as the perfusate.

Ninety minutes after the cannulation, the fluorescein-dextran solution was infused into the posterior chamber at a constant rate. The infusion rate which was measured every time with a calibrated capillary micropipette was in a range of 5.1 to 7.0 μl min⁻¹. The effluent was collected through the anterior chamber tube at 10-min-intervals and diluted 100-fold by adding distilled water. The infusion was continued for 2½ hrs, and the time course of the dye concentration changes in the effluent was determined by measuring the fluorescent intensity of the diluted effluent.

Calculation of the Rate of Aqueous Humor Formation

Fluorescein-dextran infused into the posterior chamber is diluted by the posterior chamber aqueous humor and enters the anterior chamber through the pupil. The dye is mixed with the aqueous humor in the anterior chamber and leaves the eye through the normal aqueous outflow channels and through the anterior chamber tube. The dye concentration in the aqueous humor (Ca, μg ml⁻¹) is given by
where Cin is the dye concentration in the infused solution (μg μl⁻¹), Rin is the infusion rate (μl min⁻¹), Rout is the outflow rate (μl min⁻¹), and Va is the dye distribution volume of the chambers (μl). Rout is equal to the sum of Rin and the aqueous humor formation rate (F, μl min⁻¹) when the IOP is maintained at a constant level. Under the assumption that Va stays constant, one can express Ca by

\[ Ca = \frac{Cin \cdot Rin}{Rin + F \left(1 - \exp\left(-\frac{Rin + F}{Va} \cdot t\right)\right)} \]  (1)

Thus, the concentration in the aqueous is expected to approach a steady level with an exponential rate.20-24

Since the anterior chamber tube contains a considerable amount of aqueous humor and this aqueous humor may not mix with the aqueous humor in the anterior chamber, the lag time was calculated by dividing the volume in the tube by the infusion rate. The aqueous humor formation rate and the distribution volume were then calculated using a curve fitting program based on a Gauss-Newton method.

### Drug Concentration in the Aqueous Humor

The drug concentration in the aqueous humor was determined in two monkeys using ¹⁴C-timolol maleate (Merck, Sharp and Dohme). The radioactive timolol, specific activity 1.44 μCi mg⁻¹, was purified by thin-layer chromatography and dissolved in GBR solution at a concentration of 1 mg ml⁻¹. The solution was administered into the conjunctival reservoir 5 min after the anterior chamber cannulation. Ninety minutes later, GBR solution was infused into the posterior chamber and the effluent from the anterior chamber was collected. Fifty microliters of the effluent was mixed with 5 ml of a scintillation cocktail and samples were stored in a dark room (13 C) for 24 hrs. The count for each sample was measured with a liquid scintillation counter (Model 3330, Packard Instrument Co.).

### Determination of Protein Leakage

The protein concentration in the effluent was determined in all animals with the method of Lowry et al. The rate of protein leakage into the aqueous humor (L, μg min⁻¹) was calculated from the steady-state protein concentration in the effluent aqueous humor (Cp, μg μl⁻¹) using the following equation:

\[ L = Cp(F + Rin) \]  (2)

### Results

#### Effect of the Anesthetic Agent on Aqueous Humor Formation

The time course of the mean dye concentration in the effluent is illustrated for seven monkeys with urethane anesthesia (Fig. 1). The dye was not observed in the initial 10-min effluent and a large variance of the concentration among animals was observed in the early period, probably due to the unmixed aqueous humor existing in the tube and to poor dye mixing in the anterior chamber, but the variance became fairly small in the later period.

The IOP and aqueous humor formation rate in two groups of animals, ie, under pentobarbital or urethane anesthesia, are given in Table 1. The aqueous formation rate was 1.68 ± 0.52 (±SD) μl min⁻¹ under pentobarbital anesthesia and 1.93 ± 0.61 μl min⁻¹ under urethane anesthesia; the difference was not statistically significant. The IOP averaged 9.7 ± 1.6 mmHg under pentobarbital anesthesia and 13.6 ± 1.7 mmHg under urethane anesthesia. With pentobarbital, the IOP was significantly lower than with urethane. Since pentobarbital anesthesia resulted in a significantly lower level of IOP and the aqueous formation rate tended to be lower than with urethane (although the difference was statistically insignificant), urethane anesthesia was thought to be the better choice for testing drug effects.
Table 1. Effect of anesthetic agents on aqueous humor formation

<table>
<thead>
<tr>
<th></th>
<th>Pentobarbital anesthesia</th>
<th>Urethane anesthesia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous humor formation</td>
<td>IOP*</td>
</tr>
<tr>
<td>Eye</td>
<td>(µl min⁻¹)</td>
<td>(mmHg)</td>
</tr>
<tr>
<td>1</td>
<td>2.3 (6.1)†</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>2.0 (4.0)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>1.3 (6.9)</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>1.0 (5.0)</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>2.1 (8.1)</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>1.4 (8.6)</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.68</td>
<td>9.7</td>
</tr>
<tr>
<td>SD</td>
<td>0.52</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Measured before infusion and this IOP level was maintained during infusion.
† Standard error of estimate expressed as a percent of the value.
‡ Significantly different from pentobarbital anesthesia (P < 0.05).

**Effects of Drugs on Aqueous Humor Formation**

The effects of pilocarpine, salbutamol, timolol, and a combination of salbutamol with timolol on the rate of aqueous humor formation are summarized in Table 2. A reduction in the rate of aqueous humor formation was observed using 1 mg ml⁻¹ pilocarpine in the conjunctival reservoir. The aqueous humor formation rate averaged 1.29 ± 0.49 µl min⁻¹ which was significantly lower than the rate in the placebo eyes. In contrast, the IOP, which averaged 12.6 ± 1.7 mmHg, was not affected by pilocarpine. With salbutamol, the rate averaged 2.66 ± 0.56 µl min⁻¹ which was significantly greater than the rate in the placebo eyes. The IOP was not affected. Timolol, on the other hand, significantly reduced aqueous humor formation giving an average rate of 1.24 ± 0.50 µl min⁻¹. Timolol lowered the IOP significantly, ie, the mean IOP was lower by about 2 mmHg than that in the placebo eyes. With the combined solution of salbutamol and timolol, the mean rate of aqueous humor formation was 1.86 ± 0.51 µl min⁻¹, and the mean IOP was 12.3 ± 1.6 mmHg: these values were not at variance from those found in the placebo eyes.

**Volume of Distribution**

The volume of dye distribution in the aqueous chambers was determined in each group (Tables 1 and 2). The mean volume of distribution in placebo-treated eyes was 153 ± 47 µl in animals anesthetized with pentobarbital and 135 ± 33 µl in animals with urethane. No statistically significant change in the volume of distribution was noticed with all the drugs used.

**Concentration of Timolol in Aqueous Humor**

The timolol concentration in the aqueous humor was determined in two monkeys by radioactivity measurement, and the time course of the concentra-

Table 2. Effects on aqueous humor formation of pilocarpine, salbutamol and timolol

<table>
<thead>
<tr>
<th></th>
<th>Pilocarpine</th>
<th>Salbutamol</th>
<th>Timolol</th>
<th>Salbutamol and timolol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous humor formation (µl min⁻¹)</td>
<td>IOP* (mmHg)</td>
<td>Distribution volume (µl)</td>
<td>Aqueous humor formation (µl min⁻¹)</td>
</tr>
<tr>
<td>Eye</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.2 (9.5)†</td>
<td>15</td>
<td>142 (11.3)</td>
<td>2.4 (4.2)</td>
</tr>
<tr>
<td>2</td>
<td>1.4 (8.7)</td>
<td>11</td>
<td>174 (11.2)</td>
<td>3.2 (6.3)</td>
</tr>
<tr>
<td>3</td>
<td>1.3 (5.4)</td>
<td>14</td>
<td>129 (6.2)</td>
<td>2.8 (8.2)</td>
</tr>
<tr>
<td>4</td>
<td>0.7 (10.0)</td>
<td>11</td>
<td>118 (5.9)</td>
<td>2.9 (6.2)</td>
</tr>
<tr>
<td>5</td>
<td>1.5 (10.0)</td>
<td>11</td>
<td>79 (19.0)</td>
<td>2.3 (7.0)</td>
</tr>
<tr>
<td>6</td>
<td>1.0 (11.0)</td>
<td>14</td>
<td>124 (12.1)</td>
<td>1.7 (5.9)</td>
</tr>
<tr>
<td>7</td>
<td>0.9 (13.3)</td>
<td>12</td>
<td>105 (11.4)</td>
<td>3.3 (5.5)</td>
</tr>
<tr>
<td>Mean</td>
<td>1.29‡</td>
<td>12.6</td>
<td>124</td>
<td>2.66‡</td>
</tr>
<tr>
<td>SD</td>
<td>0.49</td>
<td>1.7</td>
<td>30</td>
<td>0.56</td>
</tr>
</tbody>
</table>

* Measured before infusion and this IOP level was maintained during infusion.
† Standard error of estimate expressed as a percent of the value.
‡ Significantly different from the placebo-treated eye (P < 0.05).
tion is illustrated in Figure 2. A steady level of the drug concentration, approximately 0.8 \( \mu g \)/ml\(^{-1}\), was observed throughout the experiments.

**Leakage of protein**

In all animals tested, the protein content in the effluent aqueous humor was measured and the rate of protein leakage was calculated (Table 3). No statistically significant difference was noticed in the rate of protein leakage between the eyes with placebo and with the drugs.

**Discussion**

Fluorescein-dextran has been known to be very stable both in vitro and in vivo,\(^{25}\) and the usefulness of this dye as a tracer for studying the aqueous humor dynamics has been reported by Cole and Monro.\(^{26}\) Their study showed that the dye was scattered diffusely throughout the iris stroma but the diffusional loss was within a few percent of the total loss from the anterior chamber. In the present study, the mean volume of dye distribution was 153 \( \mu l \) for pentobarbital anesthetized and 135 \( \mu l \) for urethane anesthetized animals. The method used here for estimating the distribution volume did not have suitable precision to make an accurate measurement of the aqueous volume in the chambers, but our mean values were inconsistent with the volume of aqueous humor in the cynomolgus monkeys determined by emptying the chambers.\(^{15}\)

It is possible that our calculations of distribution volume may have been influenced by poor mixing of the dye with aqueous humor in the early period since the distribution volume was calculated from the initial slope of the concentration curve. The large variance in the distribution volume was, thus, due probably to poor mixing during the early period as expected from the large fluctuation of concentrations in the early period (Fig. 1). A constant volume of distribution was assumed for solving the differential equation, and this condition appeared to be satisfied since the IOP was maintained at a constant level during the infusion.

The aqueous humor formation and the IOP of pentobarbital anesthetized animals were in good agreement with the data of Bill, in which the same anesthetic agent was used.\(^{18}\) With pentobarbital anesthesia, however, the IOP was significantly lower than with urethane, and a slight lowering of aqueous humor formation was observed with pentobarbital although the difference was not statistically significant. On the other hand, the mean IOP in urethane anesthetized animals was in accord with the IOP measured by a pneumotonometer under ketamine cata-

lepsia.\(^{27}\) Thus, the present results indicate that aqueous humor dynamics in monkeys are influenced by anesthetic agents, and urethane was thought to be a better anesthetic agent for testing the drug effects in the monkey eye as in the cat eye.\(^{28}\)

A significant reduction in aqueous humor formation was observed following topical application of pilocarpine. This finding is in agreement with the reports of Bill and Walinder.\(^{15-16}\) They observed that the IOP was higher in the pilocarpine treated eye and concluded, on the basis of their flow data and facility of outflow data, that the uveo-scleral drainage sites were blocked by pilocarpine.\(^{15}\) Later they demonstrated that pilocarpine produced a significant reduction in aqueous humor formation when the IOP was kept at a constant level.\(^{16}\) In our experiment, we did not observe a rise in the IOP with pilocarpine. Rather, a slight decrease in spontaneous IOP was observed but the drop was not sufficient to be statistically significant. Our results in conjunction with Bill's data indicate that pilocarpine exerts some direct effect on aqueous humor formation in monkeys.

Timolol caused a significant reduction in the aqueous humor formation, 36% lower as compared to the placebo eyes. In these eyes, the observed IOP was significantly lower than in the placebo eyes. The reduction in aqueous humor formation by timolol was consistent with the studies in the human eye following instillation of this drug and other beta-antagonists,\(^{14,16}\) but inconsistent with the data of Bill in which no significant reduction was observed with intravenous injection of propranolol.\(^{17}\) It cannot be
The concentration of protein in the effluent aqueous humor and the rate of protein leakage

Table 3. The concentration of protein in the effluent aqueous humor and the rate of protein leakage

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Pilocarpine</th>
<th>Salbutamol</th>
<th>Timolol</th>
<th>Salbutamol and timolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>Protein conc. (μg ml⁻¹)</td>
<td>Leakage rate (μg min⁻¹)</td>
<td>Protein conc. (μg ml⁻¹)</td>
<td>Leakage rate (μg min⁻¹)</td>
<td>Protein conc. (μg ml⁻¹)</td>
</tr>
<tr>
<td>1</td>
<td>0.40</td>
<td>3.4</td>
<td>0.38</td>
<td>4.1</td>
<td>0.26</td>
</tr>
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<td>2</td>
<td>0.26</td>
<td>2.3</td>
<td>0.67</td>
<td>5.0</td>
<td>0.53</td>
</tr>
<tr>
<td>3</td>
<td>0.84</td>
<td>6.6</td>
<td>1.09</td>
<td>8.1</td>
<td>0.51</td>
</tr>
<tr>
<td>4</td>
<td>0.67</td>
<td>4.2</td>
<td>0.38</td>
<td>2.2</td>
<td>0.42</td>
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<tr>
<td>5</td>
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<td>1.4</td>
<td>0.63</td>
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<td>0.25</td>
</tr>
<tr>
<td>6</td>
<td>0.53</td>
<td>4.1</td>
<td>0.61</td>
<td>3.9</td>
<td>0.40</td>
</tr>
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<td>7</td>
<td>0.29</td>
<td>2.0</td>
<td>0.47</td>
<td>2.8</td>
<td>0.25</td>
</tr>
<tr>
<td>Mean</td>
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<td>3.4</td>
<td>0.60</td>
<td>4.3</td>
<td>0.40</td>
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<tr>
<td>SD</td>
<td>0.24</td>
<td>1.8</td>
<td>0.24</td>
<td>2.0</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* Calculated from the equation (2).

was approximately five times higher if dilution of the concentration with perfusate was taken into account. Thus, it should be pointed out that the observed drug effects were not necessarily extrapolated back to the eye with normal blood-aqueous barrier. However, our analysis of the protein concentration would suggest that none of the drugs used in this experiment exerts its primary effect on aqueous humor formation by causing a dramatic change in the blood-aqueous barrier. We realize that a subtle change in blood-aqueous barrier permeability might not have been detected by measuring the protein leakage rate, but a significant change in permeability to protein would have been detected by our method.

Key words: aqueous humor formation, distribution volume, fluorescein-labeled dextran, cynomolgus monkey, pilocarpine, salbutamol, timolol

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