Influence of Ethacrynic Acid on Outflow Facility in the Monkey and Calf Eye

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Living monkeys were perfused via the anterior chamber by the two step constant pressure technique with the sulfhydryl (SH)-reactive diuretic, ethacrynic acid (ECA) at dosages from 0.1 to 1.0 mM. Above 0.37 mM a reproducible increase in outflow facility (C) was observed. At 0.5 mM C increased 115% with ECA compared to a 22% increase in control eyes (n = 9; P < 0.01). In three monkeys simultaneous perfusion of ECA with cysteine in 10% excess prevented this effect. In enucleated calf eyes at dosages ranging from 0.1 to 0.5 mM ECA a similar increase in outflow facility was demonstrated. Simultaneous perfusion of 0.1 mM ECA with 0.5 mM cysteine blocked this increase in facility. At high dosages (6 mM) of ECA a decrease in C of 35% was observed (P < 0.01, n = 8).

Electron microscopic studies of the perfused monkey eyes did not readily explain the observed facility effects of ECA, except for the occurrence of a small number of breaks in the inner wall endothelium of Schlemm's canal. The juxtacanalicular meshwork appeared normal. Moderate to profound trabecular endothelial cell swelling was observed with ECA alone and with simultaneous cysteine. Morphologic studies performed 4 and 11 weeks after repetitive acute exposure to ECA indicated that the tissues of the outflow pathway appeared normal. The importance of this work is that it points to a new class of drugs that may be used someday to treat glaucoma. Also, the good correlation of the in vivo monkey and in vitro enucleated calf eye data substantiates the usefulness of the latter as an alternative method to study compounds which affect the aqueous humor outflow process. Invest Ophthalmol Vis Sci 28:2067-2075, 1987.

Our previous studies have indicated that sulfhydryl (SH) reactive compounds can influence aqueous humor outflow facility in freshly enucleated calf and monkey eyes.1-3 It would be of obvious therapeutic importance to extend these observations to living animals. However, one of the difficulties with previously studied -SH-reactive compounds such as iodoacetamide and N-ethylmaleimide is that these agents are more chemicals than drugs and have significant potential toxicity.

Ethacrynic acid (ECA) is a -SH-reactive diuretic that was originally introduced to replace mercurial agents.4 It was subsequently learned that the active site for the diuretic effect was not the -SH-reactive ligand, and ECA was eventually replaced as a diuretic by non-SH-reactive drugs such as furosemide.5 Yet ECA was given safely to humans for several years and is still available for use.6

We wondered whether ECA, because of its -SH reactivity, might be able to influence aqueous humor outflow facility in living monkeys. We report here that ECA substantially increases outflow facility in living monkeys with good correlation to similar effects in excised calf eyes.

Materials and Methods

The animals in these studies were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Nine adult cynomolgus monkeys (Macaca fascicularis) of either sex weighing 2 kg to 3.5 kg were used for intracameral perfusion studies. At least 6 weeks' time was allowed between perfusion experiments. In each animal one eye was randomly assigned to either the experimental or control group. All animals were examined gonioscopically and angle photos taken with a Kowa fundus camera (Kowa Company, Ltd., Tokyo, Japan) and a pediatric Koepppe lens (Ocular Instruments, Inc., Redmond, WA) prior to use in perfusion experiments. Intraocular pressure (IOP) was measured using a Digilab pneumotonometer (Digilab, Inc., Cambridge, MA). For these noninva-
sive procedures, anesthesia consisted of proparacaine hydrochloride 0.5% topically and ketamine hydrochloride (10 mg/kg i.m.). If necessary, acepromazine maleate (0.5–2 mg/kg i.m.) was also used.

The protocol for the intracameral perfusion experiments was as follows. The constant pressure perfusion apparatus and instruments were sterilized overnight with 70% ethanol. The perfusion fluid, Dulbecco’s PBS with added 5.5 mM glucose (Grand Island Biological Co., Grand Island, NY) was placed in a 31°C water bath overnight to degas. The next morning the perfusion apparatus was flushed with distilled water and filled with the perfusion fluid; all tubing and reservoirs were inspected for air bubbles. The machine was then calibrated with a 250 μl Gil-\textsuperscript{mont} calibration pipet (Gilmont Instruments, Inc., Great Neck, NY).

Next, the fasted animal was anesthetized, as recommended by Kaufman and Bárány\textsuperscript{7} with methohexital sodium (Brevital sodium, Lilly Research Labs, Indianapolis, IN; 15 mg/kg, i.m.) and pentobarbital sodium (Lemmon Co., Seilersville, PA; 35 mg/kg i.m.). Anesthesia was supplemented with pentobarbital sodium 10 mg/kg/hr as needed. The animal was placed supine on a heating pad in a contoured restraining device and intraocular pressures were measured. For each eye, one 23 gauge needle was inserted by hand near the limbus into the anterior chamber parallel to the iris. The two-step constant pressure perfusion method of Bárány\textsuperscript{8} was used to obtain facility measurements. Pressures for the perfusion were chosen by adding 2 mmHg and 12 mmHg to the animal’s resting intraocular pressure to yield a PI and P2 perfusion pressure value respectively. A usual set of perfusion pressures for an animal with a resting intraocular pressure of 18 mmHg would be 20 mmHg for PI and 30 mmHg for P2. Corneas were kept moist throughout the experiment by intermittent applications of 1% methylcellulose (Bausch and Lomb, Tewksbury, MA).

Three sets of 6 min recordings (indicating flow) were collected at PI and P2 to yield six values. At this point, a 10 μl bolus of fluorescein solution was injected with a microliter syringe (Hamilton Co., Reno, NV) through a T-shaped connector piece in the infusion line (courtesy of Paul Kaufman, MD, University of Wisconsin, Madison). Five minutes were allowed for the anterior chamber contents to mix once the infusion tubing was clear of all fluoresceinated solution. Post-drug recordings were then obtained in the same manner as for the baseline measurements. These recordings were converted to flow in µl/min and then aqueous humor outflow facility values were calculated. Any ocular effects of the drug infusion were noted during the experiment.

At the conclusion of the perfusion, needles were removed with tamponading and subconjunctival gentamicin sulfate (Ivenex Laboratories, Chagrin Falls, OH; 20 mg/0.25 ml OU) was injected. Topical erythromycin ointment (E. Fougera and Co., Melville, NY) was also applied.

The animals were observed with a penlight the next morning, and then intraocular pressure measurements and slit-lamp examinations were performed beginning several days later.

Eyes from calves were obtained, freshly enucleated and chilled, from a commercial abattoir (Joseph T. Trelegan and Co., Cambridge, MA) and were perfused within 24 hr (usually within 6 hr). Our standard constant-pressure perfusion technique was followed using a Grant stainless steel corneal fitting.\textsuperscript{1,2} The medium for perfusion was Dulbecco’s phosphate-buffered salt solution (Grand Island Biological Co.) with added 5.5 mM glucose. All solutions were filtered through a 0.2 μm Nuclepore filter (Nuclepore Corporation, Pleasanton, CA).

The baseline facility was determined after the eye had been perfused at 15 mmHg and 22°C for 1 hr to achieve a steady-state flow value. The anterior chamber was then gently emptied and refilled with the same kind of perfusion medium containing ECA. A second facility value was determined after the eye was perfused with medium containing ECA for 2 additional hr. Fellow eyes were used in control experiments in which an osmotically equivalent amount of sodium chloride was added in place of the ECA. Mannitol and thimerosal were added to the control solutions in equivalent concentrations to those present in the ECA solutions.

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.

**Drug Preparation**

Ethacrynic acid (ethacrynate sodium MSD, Merck, Sharp and Dohme, West Point, PA) was prepared fresh daily. Each vial contained ethacrynate sodium powder equivalent to 50 mg of ethacrynic acid; inactive ingredients were 62.5 mg mannitol and 100 μl of 0.1% thimerosal (a preservative). The powder was diluted at room temperature with Dulbecco’s PBS with added 5.5 mM glucose to yield the desired concentration. Fluorescein was added and the pH measured Radiometer A/S, Copenhagen, Denmark; PHM 62 Standard pH meter); the value was between 7.0–7.2.
Prior to use, the solution was filtered with a 0.2 \( \mu m \) Nuclepore filter.

Control solutions were mixed at room temperature and consisted of 9.5 mg sodium chloride (to osmotically equal the experimental solution), 62.5 mg mannitol and 100 \( \mu l \) of 0.1% thimerosal dissolved in Dulbecco's PBS with added 5.5 mm glucose. Fluorescein was added and the pH read prior to filtering; the value was between 7.0–7.2.

For the monkey perfusion experiments, dosage concentrations were calculated assuming an anterior chamber volume of 200 \( \mu l \). For example, to achieve a final concentration of 0.5 mM ethacrynic acid (ECA), 10 \( \mu l \) of 10 mM ethacrynic acid was infused.

For morphologic examination, five pairs of primate eyes were fixed by switching the perfusion fluid to a mixture of 3% gluteraldehyde 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. Two of the primate eyes were fixed 4 and 11 weeks after last exposure to ECA (after two and three separate ECA experiments, respectively).

Following enucleation, the eyes were opened equatorially, immersed in fixative fluid for an additional 3 hr and washed overnight in cold cacodylate buffer. Subsequently, the crystalline lens was removed and the anterior segment of each eye was divided into four quadrants. From each quadrant a series of radially-oriented wedges was cut prior to further processing.

Specimens were postfixed in 1% OsO\(_4\) and 1.5% potassium ferrocyanide in distilled water, dehydrated and embedded in an Epon-Araldite mixture. One micron sections were stained with toluidine blue and examined with a Leitz-Orthophan photomicroscope (Rockleigh, NJ). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope (Mahwah, NJ).

**Results**

Living monkeys were perfused via the anterior chamber by the two-step constant pressure technique with ethacrynic acid (ECA) at dosages from 0.1 to 1.0 mM. Above 0.37 mM a reproducible increase in outflow facility (C) was observed (Fig. 1). At 0.5 mM C increased 115% with ECA compared to a 22% increase in control eyes (washout effect) (n = 9, \( P < 0.01 \)). The outflow facilities were 0.24 \( \pm \) 0.06 and 0.23 \( \pm \) 0.04 increasing to 0.28 \( \pm \) 0.07 and 0.46 \( \pm \) 0.09 respectively for control and 0.5 mM ECA-treated eyes. In three monkeys simultaneous perfusion of 0.5 mM ECA with 0.55 mM cysteine prevented this increase in outflow facility.

Post-perfusion slit-lamp examination revealed moderate corneal striae and increased corneal thickness that was symmetrical in both control and experimental eyes, and resolved within the first week. A small amount of cells and flare were likewise present in both eyes. There were no sustained corneal or any lenticular changes in either group of eyes. At dosages above 0.25 mM some of the animals developed a dilated pupil in the ECA-treated eye. Intraocular pressure could not be reliably taken until a few days after the perfusion experiments and at that time IOP was symmetrical in both eyes, except at 0.5 mM where a possible effect on IOP was observed (16.0 \( \pm \) 2.8 versus 18.7 \( \pm \) 2.3, 17.4 \( \pm \) 1.2 versus 16.3 \( \pm \) 1.4, and 15.6 \( \pm \) 2.9 versus 19.7 \( \pm \) 2.0—experimental versus control eyes for 0.1, 0.25, and 0.50 mM ECA respectively).

**Microscopy**

In light microscopic sections of control eyes the trabecular meshwork and canal of Schlemm appeared normal except for the presence of a small amount of extracellular debris consistent with constant pressure perfusion. Only rarely was a break in the continuity of the inner wall lining observed. The trabecular beams were covered by thin processes of endothelial cells. The nuclei of these cells appeared...
either fusiform or polygonal in shape (Fig. 2). The juxtacanalicular region (JCT) was composed of a loose cellular matrix, and along the inner wall of Schlemm's canal giant vacuoles were commonly seen.

In similar sections of experimental eyes several differences were apparent. Trabecular endothelial cells exhibited moderate to profound cytoplasmic and nuclear swelling. Trabecular endothelial cells situated beneath the operculum were unaffected, however (Fig. 3). Similarly, the cells of the JCT were not swollen.

Transmission electron microscopy clearly confirmed the presence of both cytoplasmic and nuclear swelling of trabecular endothelial cells (Fig. 4). Most endothelial cells remained attached to their respective beams along their entire course and despite their swollen appearance there was little evidence of cellular disruption. Changes in the size and appearance of the cores of trabecular beams were not observed.

The JCT was little changed from its appearance in control specimens. The cells were not swollen and ample extracellular space remained (Fig. 5). Giant vacuoles were commonly observed along the inner wall of Schlemm's canal. The size and frequency of the vacuoles were not remarkably different from those seen in control specimens. A small number of openings were observed in the inner wall of Schlemm's canal. The maximum diameter of these openings was 0.2 \( \mu \)m and it was not uncommon to observe these openings in close proximity to vacuoles (Fig. 6).

The corneal endothelium appeared normal in ECA-treated eyes (Fig. 7). Similarly, no abnormalities were evident in the iris, ciliary body or in the collector channels of the outflow pathway.

In one monkey eye perfused with 0.5 mM ECA and cysteine in 10% excess, no facility effect was demonstrated. It was remarkable that trabecular cell swelling, however, was observed in a pattern indistinguishable from that seen with ECA alone.

Finally, specimens examined from eyes fixed after outflow facility had returned to normal (4 and 11 weeks after last ECA exposure) demonstrated resolution of all morphologic changes. Trabecular endothelial cells were no longer swollen and the continuity of

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**Fig. 2.** Light micrograph from control eye. Trabecular meshwork appears normal. Trabecular endothelial cells are not swollen and giant vacuoles are evident facing Schlemm's canal (asterisks). AC; anterior chamber. Original magnification X275.

**Fig. 3.** Light micrograph from experimental eye. Trabecular endothelial cells appear swollen except under the operculum (to right of vertical line). Cells of the juxtacanalicular region are not swollen and giant vacuoles are evident facing Schlemm's canal (asterisks). AC; anterior chamber. Original magnification X275.
the inner wall of Schlemm's canal had been reestablished.

Calf Eye Perfusion Experiments

In enucleated calf eyes at dosages ranging from 0.1 to 0.5 mM a similar increase in outflow facility was demonstrated with ECA (Fig. 8). Simultaneous perfusion of 0.1 mM ECA with 0.5 mM cysteine blocked this increase in facility. At high dosages (6 mM) of ECA in calf eye perfusions, a decrease in C of 35% was observed (P < 0.01, n = 8).

Discussion

It is known that aqueous humor is not actively transported out of the eye by the outflow pathway, but it seems likely that this process may be modified by cellular metabolic events. Among the trabecular cellular influences on aqueous outflow that include the action of corticosteroids, adrenergic agents, cytochalasins, and calcium chelators, should be added that of various sulfhydryl agents. Our current study indicates that -SH-reactive agents can alter trabecular fluid outflow in living animals as well as in enucleated eyes. It may therefore be possible in the future to develop a -SH drug for the treatment of glaucoma.

It is known that cellular -SH groups are involved in passive fluid movement in many different tissues. As one conceptualizes the movement of aqueous humor through the trabecular outflow pathways into the juxtacanalicular meshwork and somehow through the inner wall endothelium in the vacuolization process, there are multiple potential sites where -SH agents might be acting to alter this passive fluid movement. The dimensions of the extracellular outflow pathway could be influenced by changes in cell size and shape and cell to cell attachment. Our previous studies using -SH-reactive compounds suggested that an increase in cell volume in the juxtacanalicular meshwork (JCT) might narrow the outflow channel and increase the resistance to flow, whereas widening of this space by loss of cell-to-cell attachments, or cell-to-cell separation in the inner wall endothelium, or possibly an alteration of the vacuolization process, itself, might act to increase outflow facility. ECA may be acting to pharmacologically alter the tissue in only one of these ways. Alternately, from the observed diverse influences of these different -SH agents, it is possible that cellular -SH groups in the TM may be intimately involved in the normal fluid egress process.

In the current study the morphological findings did not clearly demonstrate changes which could readily account for the increase in outflow facility. It is possible that the infrequently observed "breaks" in the inner wall of Schlemm's canal may give a hint as to the mechanism, but the findings are inconclusive. Of significance is the fact that the swelling of trabecular endothelial cells in the corneal-scleral meshwork did not preclude a net increase in outflow facility. When ECA was perfused with cysteine, the effect on aqueous outflow was blocked and yet the endothelial cell swelling remained. This indicates that non-sulfhydryl-reactive sites of the ECA molecule may be possibly responsible for the cellular swelling. Additionally however, these results suggest that significant alterations in trabecular endothelial cell volume seem to have little impact on outflow facility.

It must be pointed out that standard morphological techniques are not well suited to demonstrate small changes in cell shape or changes in most components of the extracellular matrix, especially with regard to the dimensions of the extracellular outflow pathway in the JCT. It is conceivable that there is more dynamic modulation of such deeper flow channels than we currently understand, and it is likewise possible that cellular -SH groups in the region may be intimately involved in this process.

Although it would seem most likely that alterations...
of cell membrane -SH groups cause these pharmacologically induced changes in aqueous outflow function, it is also possible that intracellular glutathione, which has been demonstrated in the trabecular meshwork, may be somehow involved, as it is with fluid movement in other tissues.

It is again quite remarkable that dramatic morphological alterations of the trabecular meshwork tissue which might explain the large facility increases were not, in fact, observed with ECA. From a toxicity point of view, the moderate to profound cellular swelling of the trabecular endothelium observed acutely would seem worrisome. Yet 4 and 11 weeks after last ECA exposure the entire trabecular meshwork appeared totally normal, despite repetitive intracameral exposure to ECA. Also, since simultaneous perfusion with cysteine blocks the facility-increasing effect of ECA but not this induced cellular swelling, it may prove possible in the future to chemically alter the ECA molecule to separate these actions.

In the monkey perfusion experiments, an anterior chamber volume of 200 μl was assumed, based on previous unpublished studies involving anterior chamber filling of enucleated monkey eyes. It is possible that such measurements were overestimates due to scleral expansion, and recent studies indicate that anterior chamber volume in this species is approxi-
Fig. 6. Electron micrograph from experimental eye again demonstrates lack of swelling in cells of the juxtanacalicular region (JCT) and demonstrates a focal break in the inner wall of Schlemm's canal (arrow). Magnification ×11,200.

approximately 105–110 μl. Therefore the reported concentrations in the live monkey may actually be constantly two-fold higher.

A curious finding in certain monkeys with the higher dosages of ECA was the observed pupillary dilatation; yet morphological examination of the iris was normal, suggesting a neuropharmacologic effect. Of additional importance is the fact that -SH sites that might be affected by ECA exist on the corneal endothelium, but no biomicroscopic or morphological abnormalities were observed. Similarly, biomicroscopic studies gave no evidence of crystalline lens changes. These findings are not surprising, for -SH-reactive mercurial diuretics have been used systemically in previous years. The apparent clinical safety of these agents must relate to the number, accessibility, and different characteristics of the cellular sulfhydryl groups in different organs, as well as to their affinity, reversibility, etc. with different -SH agents.

Our observation that the trabecular endothelial but not the JCT cells demonstrated cytoplasmic swelling in these experiments might suggest that there are potentially important biochemical differences between these two cell types in the trabecular meshwork. Alternatively, it is possible that these effects simply reflect concentration differences of the drug at the two sites, although it is not clear why this should occur.

Another important finding in this study was the good correlation of aqueous humor outflow facility effects in living monkeys with those in enucleated calf...
eyes. The results suggest that the latter preparation, which is more plentiful and less expensive than that of the living animals, may be useful as an alternative method to screen other -SH compounds. However, unlike the calf eye, there was no trend in the monkey for a reversal of the facility improvement with higher dosages of ECA. Perhaps then, the excised eye may in fact be more sensitive to adverse influences of such agents, eg, cell swelling.

The importance of this work is that it points to a new class of drugs that might be used someday to treat glaucoma. Obviously it would be important to develop a topical preparation of such a class of compound. Alternatively, a drug that truly enhances the trabecular outflow process may not necessarily require repetitive dosing.

Key words: sulfhydryl, ethacrynic acid, trabecular meshwork, glaucoma, alternative experimentation

References