Effects of Ethacrynic Acid on Intraocular Pressure of Anesthetized Rats (44362)

ROBIN V. SEARLES,* MARTINIQUE JOHNSON,* VLADIMIR SHIKHER,† CAREY D. BALABAN,† AND WALTER B. SEVERS*,[1]

Department of Pharmacology, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033; and Department of Otolaryngology,† Eye & Ear Institute of Pittsburgh, Pittsburgh, Pennsylvania 15213*

Abstract. **Ethacrynic acid (ECA) lowers intraocular pressure (IOP) by an effect usually ascribed to increased drainage of aqueous humor by the trabecular meshwork. Here, we describe the effects of a continuous 2-hr intracameral infusion of balanced salt solution (BSS), with or without 2 m*M* ECA (sodium salt), on IOP of pentobarbital anesthetized rats. The infusion was divided into a constant (0.05 μl/min) and a periodic (0.25 μl/min) component that cycled 4 min on then 4 min off. This permitted the calculation of dynamic changes in resistive (trabecular and uveoscleral drainage) and nonresistive (aqueous synthesis, episcleral venous pressure) components of IOP by fitting a second-order transfer function to the responses. ECA markedly blunted the BSS-induced rise in IOP ($P < 0.01$). The rise in resistive mechanisms (ocular impedance) was transiently blunted by ECA ($P < 0.05$) during the third and fourth 8-min cycles, and nonresistive mechanisms were reduced by ECA from cycles 3–10 ($P < 0.05$). Then, at the end of the infusion, the control and ECA dynamic values were similar ($P > 0.05$), although IOP of ECA-treated rats was still slightly reduced ($P < 0.05$). The most likely explanation is a summation of small changes in both resistive and nonresistive components of IOP dynamics. Systemic blood pressure was unchanged within either group. The well-known effects of ECA on the trabecular meshwork, alone, are insufficient to explain the dynamic changes in IOP observed in this model.**

[P.S.E.B.M. 1999, Vol 220]

Ethacrynic acid (ECA), a diuretic drug that inhibits the renal Na^+-K^+-$2Cl^-$ symporter of the thick ascending loop of Henle, also inhibits some tissue Na^+-K^+ ATPases, phosphodiesterases, and glutathione transferases (1). ECA is known to lower intraocular pressure (IOP), including IOP of patients with glaucoma (2).

ECA decreased IOP in perfused organ-cultured human eyes (3) and after topical application or injection to the eyes of animals and humans (4–7). Direct (intracameral) injection of ECA (3 m*M*) into the eyes of living monkeys lowered IOP without adverse effects and caused breaks in the endothelium of the inner wall of the canal of Schlem, resulting in a "pharmacological trabeculocanalotomy" (5, 6). In cell cultures derived from human trabecular meshwork, ECA produced reversible changes in cell shape after 2 hr (8), supporting the hypothesis that ECA-induced changes in cytoskeletal structure of the trabecular meshwork were related to effects of ECA on ocular dynamics. However, the drug is a sulfhydryl reactive reagent, and it caused corneal edema at high doses, thereby limiting its clinical utility (4).

Nevertheless, ECA does decrease IOP in patients with glaucoma (2), thereby warranting studies to determine the underlying mechanism(s) of action. We tested ECA in a newly developed *in vivo* model using anesthetized rats receiving direct, cyclic infusions that permit separate calculations of resistive and nonresistive mechanisms that participate in regulating IOP (9). The model makes use of the "washout" effect associated with direct intraocular infusions of low volumes of BSS. Such infusions cause, after a delay, a gradual rise in IOP, perhaps by "washout" of a substance needed for maintenance of the extracellular matrix (10–12).

Materials and Methods

Animals. The animals used for this research were male Sprague-Dawley rats (300–400 g) that were cared for

Supported in part by NASA Award NGT-UMF-93018.

[1] To whom requests for reprints should be addressed at College of Medicine–H078, Pennsylvania State University, Hershey, PA 17033-0850. E-mail: wbs2@psu.edu

Received August 7, 1998. [P.S.E.B.M. 1999, Vol 220]
Accepted November 18, 1998.

0037-9727/99/2203-0184$10.50/0

in accordance with the *Guide for the Care and Use of Laboratory Animals,* DHEW/NIH Publication number 86-23, 1985. The protocol was approved by the Institutional Animal Care and Use Committee of the College of Medicine at Pennsylvania State University.

Animal Preparation. General anesthesia was induced by the ip injection of 45 mg/kg pentobarbital sodium. A femoral artery was catheterized for arterial blood pressure recording, and a femoral vein was catheterized for maintenance of anesthesia. When needed, additional pentobarbital was slowly infused until blood pressure stabilized near 80 mmHg. Rats rested on their ventral surface with the head fixed in the normal upward position. The anterior chamber of the eye was cannulated by procedures described in detail elsewhere (9). Briefly, the tip of a 27-gauge needle was inserted into the anterior chamber and fixed in place. The other end of the needle was fixed to a tubing/manifold assembly that interfaced with two infusion pumps and permitted continuous, direct recording of IOP. The entire infusion system was filled with BSS (9) with or without 2 m*M* ECA and filtered with a 0.22-μ Millipore filter immediately before use. Body temperature of the animals was maintained with incandescent lamps.

Protocol. After surgery, the rats were allowed a 20-min baseline period to ensure the stability of the blood and intraocular pressure recordings. Then, two infusion pumps were activated. One pump continuously infused BSS into the anterior chamber at a rate of 0.05 μl/min. The second pump, automatically controlled by a timer, cycled 4 min on—then 4 min off. When on, the pump infused the eye with 0.25 μl/min. Thus, on average, the eye was infused with 0.175 μl/min for 2 hr. This volume approximates about 25% of the normal synthesis rate for aqueous humor in rats (11), and the cycling component of the infusion provided a sufficient magnitude of oscillation for calculation of outflow impedance. Two infusates were compared: BSS alone, and BSS with 2 m*M* ECA as the sodium salt (Edecrin). Preliminary experiments showed that, under our conditions, low concentrations (0.2 m*M*) were inactive, and higher concentrations (up to 8 m*M*) caused observable corneal edema. Thus, our rat model appeared to be responsive to ECA at concentrations similar to those described for living monkeys (i.e., intracameral administration up to 3 m*M* without adverse effects even though our infusions were longer (5, 6).

Data Acquisition and Analysis. Blood and intraocular pressures were recorded from Harvard ultralow volume transducers connected to a Keithley Model 570 data acquisition system and stored on the hard drive of a computer that sampled the transducer outputs at a rate of 1/sec during the experiment. A cathode ray screen allowed real-time visual monitoring. Data were analyzed off-line.

Blood pressure (BP) and IOP data for each animal were partitioned into 10 min time bins and evaluated by two-way analysis of variance with time as a repeated measure. Significant F ratios (P = 5% or less) were evaluated with Tukey's Test.

To derive estimates of dynamic changes in IOP, the IOP data from the computer were synchronized to the time when the pumps were turned on. The data were then assessed by a standard complex demodulation approach (9, 13) to determine the magnitude and phase angle of the IOP responses at the fundamental frequency of 0.125 cycles/min (1 cycle = 8 min). Next, the transfer function (2nd order) of cycle-by-cycle responses to the periodic infusion was calculated (9). The analyses were implemented in MATLAB-386 on a computer with a 66-MHz, 80486 microprocessor. MATLAB Tool kit functions were used for least squares optimization (leastsq.m), digital filtering (fir 1.m) and Runge-Kutta solutions of systems of differential equations (ode45.m). Two assumptions are implicit in the process. First, it is assumed that the IOP control system is linear to a first approximation, such that the IOP responses to the mean infusion rate and the IOP responses to the periodic component are linearly superimposed. Second, it is assumed that the coefficients of the transfer function are constant within a given cycle of stimulation. The validity of these assumptions, the data filtering routine, and the specific equations have been described in detail (9, 13). The gain parameter of the transfer function, A_o, (units = $mmHg\mu l^{-1}min^{-1}$) reflects passive resistance to outflow of aqueous humor (i.e., drainage by the trabecular meshwork and uveoscleral pathways). The residual pressure (RP, mmHg) that is unexplained by the transfer function reflects non-resistive changes in aqueous humor synthesis and/or episcleral venous pressure. Changes in Ao and RP were evaluated over 13 consecutive 8-minute cycles by repeated measures analysis of variance and least significant difference (LSD) tests using Systat®. A_o data were square-root transformed before analysis to reduce heterogeneity. F ratios of with probabilities of 5% or less were regarded as significant.

Results

IOP and BP Effects of Infusion. Figure 1 presents the IOP and BP results obtained after BSS and ECA infusion into the anterior chamber. The F ratios for treatment, time, and interaction were all significant ($P < 0.05$) for IOP. The following are the main comparisons that emerge. IOP, from the beginning of the experiment to the last 40 min of the post-infusion time, was lower in ECA rats. The lower baseline IOPs were likely due to immediate diffusion of ECA into aqueous humor at the time the cannula was inserted into the eye, since BSS and ECA rats were usually done in pairs. IOP increased within each group during the infusion with the following differences. In the case of BSS, the rise in IOP first increased significantly ($P < 0.05$) 30 min into the infusion, rose further by 60 min, and was unchanged during the second hr of infusion. A partial ($P <$ 0.05) recovery toward baseline IOP occurred after the infusion. In the case of ECA-treated rats, the within group IOP rise first became signficant 60 min into the infusion,

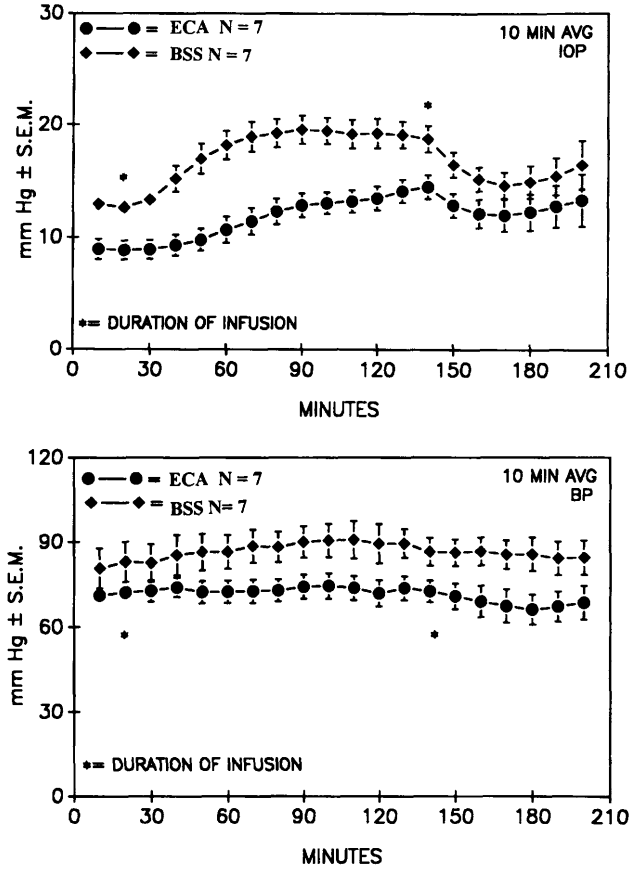

Figure 1. (Top panel) Intraocular pressure and (Lower panel) blood pressure of rats receiving dynamic infusions of balanced salt solution (BSS) alone, or with ethacrynic acid (ECA) into the anterior chamber of the eye. The asterisks indicate the start and stop times of the infusion. The rise in IOP after BSS infusion was markedly blunted by ECA ($P < 0.05$). Within group analysis of BP data indicated that BP was stable during the experiment.

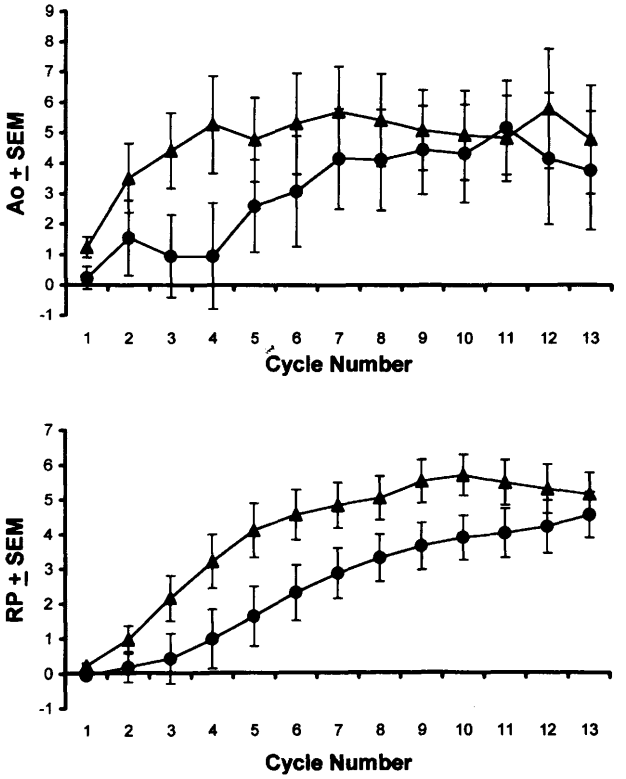

Figure 2. Dynamic changes in IOP control mechanisms. The upper panel depicts changes in passive resistance to outflow (trabecular and uveoscleral drainage, A_o, units = $mmHg\mu l^{-1}min^{-1}$) and the lower panel depicts changes in nonresistive mechanisms (aqueous humor synthesis and episcleral venous pressure, RP, units = mmHg). The cycle numbers represent 13 consecutive 8-min infusion cycles starting when the pumps were turned on. The triangles illustrate the effects of BSS, and the circles illustrate the effects of ECA. The A_o of BSS and ECA rats differed ($P < 0.05$) at Cycles 3 and 4. The RP differed ($P < 0.05$) from cycles 3 through 10.

and rose slightly but significantly ($P < 0.05$) during the second hr of infusion. A partial recovery toward baseline ($P < 0.05$) occurred. The ANOVA for mean arterial blood pressure (Fig. 1, lower panel) revealed a borderline treatment effect (12%, 2-tail) and no time or interaction effects. Separate one-way repeated measure ANOVA confirmed that blood pressures within the separate groups did not change during the entire experiment ($P > 0.2$). During the course of the experiments, ECA did not produce frank corneal edema, although preliminary studies showed edema when higher concentrations were used.

Dynamic Changes During Infusion. Figure 2 displays the changes in passive outflow resistance (A_o) and nonresistive dynamic changes (RP) during the first consecutive 13 8-min cycles of the infusion. The ANOVA of the A_o data revealed a strong F ratio for the cycle, and treatment x cycle interaction ($P < 0.001$). Resistance rose rapidly in both groups, and A_o was reduced by ECA at cycles 3 and 4. The ANOVA of the RP data generated a borderline significant F ratio for treatment (8%, two-tail), and significant F ratios for cycle ($P < 0.001$), and treatment x cycle interaction ($P < 0.04$). As shown in the lower panel of Figure 2, the nonresistive components of aqueous humor dynamics increased in both groups, the rise in the ECA group was less than BSS-infused rats at cycles 3–10 (inclusive), and the BSS group reached an apparent peak by cycle 7, as there was no within-group difference at later cycles. There was a within-group rise in the ECA rats between cycles 7 and 13. There were no ECA-induced differences ($P > 0.05$) from cycles 11–13 in either A_o or RP.

Discussion

The mechanism by which the "washout" effect raises IOP is not fully described when employed either *in vitro* or *in vivo* (10, 14–16). Under the described *in vivo* conditions, the BSS-induced IOP rise is due partly to resistance increases and partly due to a nonresistive component, presumably vascular, because pretreatment with acetazolamide had minimal effects (9). This enigmatic phenomenon is associated with a delayed rise in IOP after low-volume infusions of BSS, which are benign to tissue and administered at rates that should not physically damage tissue (10, 11). The "washout" of a material from the extracellular matrix, perhaps a glycoprotein (14), or changes in tPA in ocular tissue may be contributory mechanisms (15, 16, 17).

Typically, "washout" experiments in living eyes employ larger species and evoke a delayed rise in IOP and an increased outflow facility. However, these experiments often hold pressure constant and allow volume to vary, whereas our experiments employ infusions at a constant rate. The usual calculation of $\Delta IOP/\Delta flow$ over a fixed experimental interval to estimate outflow facility (reciprocal of resistance) may not fully segregate ocular impedance from nonresistive components (14, 17). Thus, our experiments are not directly comparable to studies of living eyes in large animals where increased outflow facility is often observed. Croft and Kaufman (17) provided an analysis of ECA and outflow facility under various experimental conditions.

Most previous studies on the ocular effects of ECA suggest that it lowers IOP by altering the trabecular meshwork (2, 5, 6). It is likely that the Na^+-K^+-Cl^- transporters of trabecular cells are affected by ECA, and the possibility exists that transport mechanisms in the ciliary epithelium could also be sensitive to ECA (18). The present study made use of the "washout" effect of a low-volume infusion of BSS to raise IOP. IOP is, at any point, in dynamic equilibrium between resistive (trabecular and uveoscleral drainage) and nonresistive (aqueous synthesis and episcleral venous pressure) components (11).

The utility of the rat model described herein as a surrogate for aqueous drainage mechanisms in humans has been discussed previously (9, 11). It is noteworthy that the trabecular meshwork of the rat eye displays the same morphological changes to IOP increases as primates (19). The cyclic infusion used to challenge dynamic equilibrium herein, after a baseline period, has the advantage that IOP does not have to be at steady-state (9). Thus, changes in dynamics are easily partitioned into the resistive and nonresistive components by standard complex demodulation and transfer function analysis (9, 13).

Using these conditions and calculation methods, the results revealed that ECA, after intracameral administration, markedly lowered IOP. When challenged by low-volume infusions, BSS produced a "washout"-induced rise in IOP by increasing both resistive and nonresistive components of IOP control. The presence of ECA in the infusate blunted ($P < 0.05$) the resistive component for a relatively brief time, (until the fifth 8-min cycle), whereas the blunted rise ($P < 0.05$) in nonresistive mechanisms continued until the eleventh cycle. Also, the within group temporal pattern revealed that BSS rats first displayed a significant rise in IOP ($P < 0.05$) at 30 min, whereas the IOP increase in ECA-infused rats was delayed for 60 min.

The data document that ECA affects multiple mechanisms to defend IOP against a constant volume stimulus. While it is likely that decreased trabecular meshwork resistance was contributory under our conditions, effects on aqueous synthesis and/or episcleral venous pressure seem probable because of the pronounced effects on nonresistive mechanisms. The renal effects of ECA on sodium transport mechanisms and the dependence of aqueous synthesis on sodium transporters raise the potential for a reduction in aqueous synthesis by ECA (1). However, even if there was no reduction in synthesis, the total volume load of the eye, endogenous plus exogenous, was only about 125% of the normal aqueous synthesis rate in rats (11). Thus, we believe the nonresistive component that we observed includes, at least in part, an ECA-induced fall in episcleral venous pressure.

In summary, the "washout" phenomenon coupled with cyclic infusions offer an excellent tool to partition underlying effects of ocular hypotensive drugs into resistive and nonresistive components *in vivo*. Tingey *et al.* (4, 5) suggest that topical ECA application to the eye results in corneal edema, and that an ECA-sulfhydryl adduct might penetrate the cornea and release free drug to reduce this side effect. The *in vivo* efficacy of ECA as an ocular hypotensive, including the present data, suggest that this goal should be pursued.

1. Jackson EK. Diuretics. In: Hardman JG, Limbird LE, Eds. Goodman & Gilman's The Pharmacological Basis of Therapeutics. New York: McGraw-Hill, 9th Ed., p685, 1996.
2. Melamed S, Kotas-Neumann R, Barak A, Epstein DL. The effect of intracamerally injected ethacrynic acid on intraocular pressure in patients with glaucoma. Am J Ophthalmol **113:**502–512, 1992.
3. Johnson DH, Tschumper RC. Ethacrynic acid: Outflow effects and toxicity in human trabecular meshwork in organ perfusion culture. Curr Eye Res **12:**385–396, 1993.
4. Tingey DP, Schroeder A, Epstein MPM, Epstein DL. Effects of topical ethacrynic adducts on intraocular pressure in rabbits and monkeys. Arch Ophthalmol **110:**699–702, 1992.
5. Tingey DP, Ozment R, Schroeder A, Epstein DL. The effect of intracameral ethacrynic acid on the intraocular pressure of living monkeys. Am J Ophthalmol **113:**706–711, 1992.
6. Croft MA, Hubbard WC, Kaufman PL. Effect of ethacrynic acid on aqueous outflow dynamics in monkeys. Invest Ophthalmol Vis Sci **35:**1167–1175, 1994.
7. Epstein DL, Hooshmand LB, Epstein MPM. Thiol adducts of ethacrynic acid increase outflow facility in enucleated calf eyes. Curr Eye Res **11:**253–258, 1992.
8. Erikson-Lamy K, Schroeder A, Epstein DL. Ethacrynic acid induces reversible shape and cytoskeletal changes in cultured cells. Invest Ophthalmol Vis Sci **33:**2631–2640, 1992.
9. Balaban CD, Palm DE, Shiker V, Searles RV, Keil LC, Severs WB. Mechanisms for vasopressin effects on intraocular pressure in anesthetized rats. Exp Eye Res **65:**517–531, 1997.
10. Johnson M, Chen A, Epstein DL, Kamm RD. The pressure and volume dependence of the rate of washout in the bovine eye. Curr Eye Res **10:**373–375, 1991.
11. Searles RV, Balaban CD, Severs WB. Interaction between head-down tilt and anterior chamber infusions on intraocular pressure of anesthetized rats. Exp Eye Res **62:**621–626, 1996.
12. Davson H. The Physiology of the Ocular and Cerebrospinal fluids. New York: Academic Press, p3, 1980.
13. Bloomfield P. Fourier Analysis of Time Series: An Introduction. New York: Wiley, p118, 1976.
14. Gaasterland DE, Pederson JE, MacLellan HM. Perfusate effects upon resistance to aqueous humor outflow in the rhesus monkey eye: A

comparison of glutathione-bicarbonate Ringers solution to pooled aqueous humor as perfusate. Invest Ophthalmol Vis Sci **17:**391–397, 1978.
15. Smalley DM, Fitzgerald JE, Taylor DM, Cone RE, O'Rourke JO. Tissue plasminogen activator in human aqueous humor. Invest Ophthalmol Vis Sci **35:**48–53, 1994.
16. Shuman MA, Polansky JR, Merkel C, Alvarado JA. Tissue plasminogen activator in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci **29:**401–405, 1988.
17. Croft MA, Kaufman PL. Effect of daily topical ethacrynic acid on aqueous humor dynamics in monkeys. Curr Eye Res **14:**777–781, 1995.
18. O'Donnell ME, Brandt JD, Curry FE. Na-K-Cl transporter regulates intracellular volume and monolayer permeability of trabecular meshwork cells. Am J Physiol **268:**C1067–1074, 1995.
19. McMenamin PG, Al-Shakarchi MJ. The effects of various levels of intraocular pressure on the rat aqueous outflow system. J Anatomy **62:**67–82, 1989.