Biochemical and Biophysical Research Communications **272,** 1–5 (2000)
doi:10.1006/bbrc.2000.2728, available online at http://www.idealibrary.com on IDEAL®

Antithrombin III, a Serpin Family Protease Inhibitor, Is a Major Heparin Binding Protein in Porcine Aqueous Humor

P. Vasantha Rao, R. Rand Allingham, Leon W. Herndon, and David L. Epstein
Department of Ophthalmology, Duke University Medical Center, Box 3802, Durham, North Carolina 27710

Received March 28, 2000

Our hypothesis is that the proteins in aqueous humor may be involved in the regulation of outflow facility through the trabecular meshwork and uveoscleral meshwork. In this study, we analyzed the profile of heparin-binding proteins present in porcine aqueous humor to identify and characterize secretory proteins with a binding affinity for heparin. A single step involving heparin-sepharose affinity chromatography of porcine aqueous humor yielded a ≈60 kDa protein as the major heparin-binding species. This protein was specifically eluted from the column by heparin. The N-terminal sequence and immunological cross reactivity of this protein confirmed its identity as antithrombin III. Aqueous humor from different species, as well as cells from human trabecular meshwork, Schlemm's canal, and lens epithelium, contained detectable amounts of antithrombin III. Based on its known anticoagulative function in endothelial cells and effects on the production of prostacyclin, it is reasonable to speculate that antithrombin III present in aqueous humor might influence the physiology of the trabecular and uveoscleral meshwork and thereby regulate intraocular pressure. © 2000 Academic Press

Key Words: **antithrombin III; aqueous humor; prostaglandins; trabecular meshwork; Schlemm's canal; intraocular pressure and heparin.**

Aqueous humor is secreted by the nonpigmented ciliary body epithelium of the eye and its protein composition has been found to be qualitatively and quantitatively different from that of serum (1). Several secretory and regulatory proteins have been documented to be present in the aqueous humor (2–6). Interestingly, ocular conditions such as inflammation and glaucoma, and certain ocular medications have been shown to change the protein composition of the aqueous humor (7–9). However, the role of aqueous humor proteins in the maintenance of intraocular pressure is not well understood at the present (10–13).

The extracellular matrix of the trabecular meshwork, and its turnover are thought to have a critical role in the regulation of outflow facility (14–16). Members of several families of cell surface and secretory proteins bind glycosaminoglycans (GAGs), and this binding is thought to be important for the cellular effects of these proteins (17, 18). A number of secretory proteins including growth factors, fibronectin, thrombospondin, hormone receptor, SERPIN family protease inhibitors, interleukins, and other cell surface proteins are recognized to have affinity for heparin (17–20). Additionally, heparin, a highly sulfated glycosaminoglycan, has been found to influence intraocular pressure when injected intracamerally (21). The molecular mechanism(s) mediating this effect of heparin however, is not understood. Here we attempted to identify the heparin-binding proteins present in aqueous humor with the intention of elucidating their potential involvement in modulation of aqueous humor outflow facility. Using heparin-sepharose affinity chromatography of porcine aqueous humor, we identified and characterized antithrombin III as a major heparin binding protein present in aqueous humor.

MATERIALS AND METHODS

Heparin Sepharose CL-6B was purchased from Pharmacia Biotech Company. Heparin (low molecular weight) and antithrombin III polyclonal antibody were obtained from Sigma Chemical Company. All other reagents were of analytical grade.

Collection of aqueous humor. Enucleated pig and bovine eyes were obtained from a local abattoir. Fresh rhesus monkey (Macaca mulatta) eyes were obtained from the Center for Biological Evaluation and Research, U.S. Food and Drug Administration (Bethesda, MD). Eyes from these different species were transported to the laboratory on ice and aqueous humor was collected within 2 to 6 hours. Aqueous humor from these enucleated eyes was collected by paracentesis (perforation of the cornea), using a 30 ga. Precision Glide needle. Fresh human aqueous humor was obtained from patients who underwent either cataract or glaucoma surgery at the Duke University Eye Center. Human aqueous samples were obtained after informed and written consent from patients. All the aqueous humor samples were stored at −80°C until use.

Heparin-sepharose affinity chromatography. A Bio-Rad polyprep chromatography column was packed with heparin-sepharose gel (5

0006-291X/00 $35.00

ml bed volume), in accordance with the protocol provided by the manufacturer. Before loading aqueous humor samples, the column was equilibrated at room temperature (≈23°C) with 10 mM phosphate buffer pH 7.3 containing 0.15 M NaCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.2 mM mercaptoethanol (buffer A). Porcine aqueous humor (20 ml) was dialyzed overnight against buffer A, prior to centrifugation at 12000 RPM for 20 minutes at 4°C. The supernatant was loaded onto the heparin sepharose column and elution performed by gravity. The eluant was reloaded onto the column, following which the column was washed with 20 ml of buffer A (4 volumes of the gel bed volume). Subsequently, the column was washed with 4 volumes of buffer A containing 1 M NaCl. Heparin-bound proteins were finally eluted using heparin (3 mg/ml) in buffer A, and the eluant (20 ml) was concentrated down to 0.3 ml using a Millipore Ultrafree centrifugal filter with a 10 kDa cutoff. This sample was then analyzed by SDS–PAGE (under reducing condition) to evaluate the profile/composition of heparin-bound proteins present in the aqueous humor.

Electrophoresis and Western blot analysis. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were performed as described earlier (22). The 12.5% polyacrylamide gels used to separate heparin-bound proteins were visually examined after staining with Coomassie Blue-250. For N-terminal sequence determination, protein(s) was transferred to polyvinylidene difluoride membrane (Bio-Rad Sequi-Blot PVDF, 0.2 μm) and visualized with Ponceau S. A portion of the PVDF membrane containing the major heparin-bound protein of 60 kDa molecular weight was excised and sequenced by the Howard Hughes Protein Sequence Facility at Duke University Medical Center. Immunoblots were developed according to the Bio-Rad protocol using an antibody (polyclonal) raised against purified anti-human antithrombin III (Sigma Chemicals), and protein bands were visualized using a peroxide-linked secondary antibody and ECL detection reagent (Amersham Pharmacia Biotech).

Immunocytochemistry. Human trabecular meshwork and Schlemm's canal primary cell cultures were generated and cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum as described earlier (23). The human lens epithelial cell line (SRA01/04) was provided by V. N. Reddy from Kelloggs Eye Institute, Michigan. Cells were plated on gelatin (2%)-coated glass cover slips, fixed with 3.7% formaldehyde and stained for antithrombin III using anti-human antithrombin III polyclonal antibody and anti rabbit IgG (whole molecule) conjugated to a FITC secondary antibody (Sigma), as described earlier (23). Control cells were incubated with preimmune serum instead of primary antibody. Cells were viewed and photographed using a Zeiss Axioplan 2 microscope.

RESULTS AND DISCUSSION

Porcine aqueous humor samples applied to a column of heparin sepharose CL-6B were eluted sequentially using Buffer A alone, Buffer A plus 1 M NaCl, and Buffer A plus heparin (3 mg/ml), respectively. SDS–PAGE analysis of heparin-eluted fractions revealed a single protein of 60–63 kDa molecular weight, as visualized with Coomassie Blue-250 staining (Fig. 1). The 63 kDa protein was then transferred onto PVDF membrane, visualized with Ponceau S, excised from the membrane and subjected to N-terminal sequencing at the Howard Hughes Protein Sequencing Facility, Duke University Medical Center. The N-terminal sequence confirmed presence of a single species in the 60–63 kDa heparin-bound protein fraction, and yielded information on a total of 19 amino acid residues

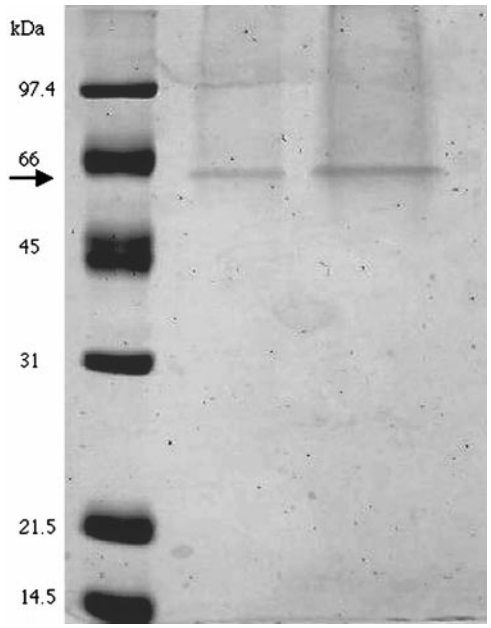

FIG. 1. Coomassie Blue-stained SDS–polyacrylamide gel profile of the heparin-bound protein fraction of porcine aqueous humor. The heparin-sepharose column was sequentially eluted with buffer A alone, buffer A plus 1 M NaCl, and finally buffer A containing heparin (3 mg/ml). The heparin-bound protein fraction eluted in the last elution step was then concentrated down to 0.3 ml, of which 10 and 15 μl aliquots were analyzed by SDS–PAGE (lanes 1 and 2). Arrow indicates location of the 60 kDa heparin-binding protein from porcine aqueous humor.

(of which 2 were undetermined). A BLAST search conducted against the NCBI protein data bank to detect sequence homology with known proteins, confirmed identity of the 60–63 kDa heparin-bound protein as porcine antithrombin III, with 16 of the 17 amino acid residues matching those present in the porcine and sheep antithrombin III sequence. The identity of this aqueous humor protein as porcine antithrombin III is also in agreement with the reported molecular weight range (58–63 kDa), strong affinity for heparin, and unblocked N-terminal residue of antithrombin III.

The identity of this 60–63 kDa heparin-binding protein was further confirmed by analysis of immunological cross reactivity. An anti-human antithrombin III polyclonal antibody displayed a very strong immunopositive reaction with the heparin-bound protein as assessed by Western blot analysis (Fig. 2). This protein was also confirmed to be present in the aqueous humor of other species by subjecting aqueous humor (20 μl) samples from human, monkey, bovine, and porcine eyes to Western blot analysis, as described above. Human and porcine samples exhibited clearly positive immunopo-reactive bands with a molecular weight corresponding to that of molecular weight purified antithrombin III from porcine aqueous humor. Monkey and bovine aqueous humor samples also yielded a positive reaction upon longer exposure of the ECL blots to film

```
1 SPVEDIXTAKPXDIPVNGM 19   (N-terminal sequence of heparin-sepharose bound protein)
  |||||| |||| ||||| |
1 SPVEDICTAKPRDIPVNPM 21   (Porcine antithrombin III)
```

FIG. 2. Alignment of partial N-terminal sequence of the heparin sepharose bound 60 kDa protein of porcine aqueous humor. 16 out of 17 residues of the 60 kDa heparin bound protein are identical to the sequence of porcine antithrombin III.

(Fig. 3). Longer exposures also revealed some nonspecific signals however, which most likely can be attributed to cross-reactivity of albumin, a major protein present in the aqueous humor of various species. In support of our data, Schmut and Hofmann *et al.* (24) has reported the presence of immunologically detectable antithrombin III in aqueous humor.

The vast majority of aqueous humor proteins are believed to be blood-derived, entering the anterior chamber by simple filtration or seepage through the ciliary body (1, 25). Alternatively, tissues of the anterior chamber of the eye could contribute to the protein make-up of the aqueous humor. To explore whether the antithrombin III found in aqueous humor of different species could potentially be contributed by the tissues of anterior chamber of the eye, primary cell cultures isolated from human trabecular meshwork (HTM) and Schlemm's canal (SC), and a human lens epithelial cell line (SRA01/04) were analyzed for the presence of antithrombin III by immunocytochemistry using the antithrombin III polyclonal antibody and a fluorescein (FITC)-conjugated anti-rabbit secondary antibody. Both confluent HTM and SC cells, as well as lens epithelial cells exhibited very distinct positive staining as compared to control cells treated with preimmune rabbit serum. The immunostaining revealed a vesicular pattern which is typical of many secretory proteins (Fig. 4). Furthermore, these data are consistent with previously reported work (26) which documented antithrombin III expression in HTM cells, based on results of PCR amplification strategies. Among the three different cell types tested, SC cells were found to have a relatively higher concentration of antithrombin III. This difference might be related to lumenal platelet aggregation on the endothelium of Schlemm's canal as has been reported under various conditions (27).

Our study convincingly demonstrates the presence of antithrombin III as a major heparin-binding protein in the aqueous humor. While this is the only protein detectable by Coomassie Blue staining in our experiments, it does not exclude the presence of other minor heparin-binding proteins in aqueous humor, which might be visible upon silver stain analysis. α1-antitrypsin, another serpin protease inhibitor, α- and β-lipoproteins and thrombospontin, which also have an affinity for heparin, have been reported to be present in the aqueous humor of human and other species (2–5).

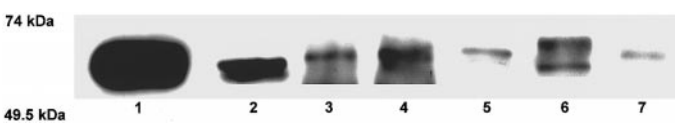

FIG. 3. Immunological identification of the heparin-bound 60 kDa protein as antithrombin III (lanes 1 and 2) and a Western blot showing the immunoreactivity of antithrombin III in the aqueous humor from different species (lanes 3 to 7) with a polyclonal antibody raised against human antithrombin III. The heparin bound protein fraction (lanes 1 and 2) and aqueous humor (20 μl aliquots) derived from human (3 and 4), pig (5), monkey (6), and bovine eyes (7) were analyzed by SDS–PAGE, followed by electrophoretic transfer to nitrocellulose. Nitrocellulose blots were subsequently probed using a polyclonal antibody generated against human antithrombin III and a peroxidase-conjugated secondary antibody. Blots were developed using enhanced chemiluminescence reagents.

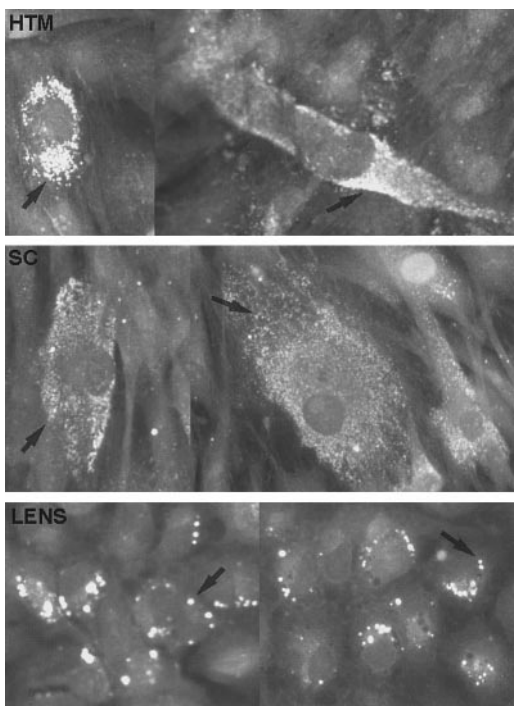

FIG. 4. Immunolocalization of antithrombin III in human trabecular meshwork (HTM), Schlemm's canal (SC) primary cells and in a human lens epithelial cell line (LENS). Cells were plated on gelatin-coated glass cover slips and grown to confluence in DMEM medium containing fetal bovine serum. Cells were fixed with formaldehyde and stained for antithrombin III using an antithrombin III polyclonal antibody and a fluorescein-conjugated secondary antibody. Control cells treated with preimmune serum and stained with secondary antibody showed no immunoreactivity (not shown). All three different cells used for this analysis exhibited a distinctly vesicular pattern of immunoreactivity against the anti-human antithrombin III antibody.

Antithrombin III, which has a very high binding affinity for heparin, is reported to undergo both conformational change and activation as a result of the binding interaction (28, 29). Evolutionarily this protein belongs to the serine protease inhibitor family (SERPIN) and it inactivates thrombin as a result of an irreversible binding interaction with the latter protein (29). Its main physiological role as an anticlotting agent is well understood. Antithrombin III also blocks other serine proteases besides thrombin, in the clotting cascade, namely, XIIa, XIa, IXa, and Xa (28). Although the aqueous humor and anterior chamber of the eye are avascular in nature, blood leakage due to trauma and clot formation that might occur under certain conditions such as hyphema, have the potential to block aqueous humor outflow through the trabecular meshwork and Schlemm's canal (30). Under such situations, aqueous humor antithrombin III may play a critical role in preventing blood clot formation, which could otherwise block aqueous humor outflow.

Its presence in the aqueous humor and its possible secretion by ciliary processes, HTM, SC cells and lens cells all suggest a role for antithrombin III in the anterior chamber of the eye. Further in addition to its role as an anti-clotting agent. Intracameral heparin perfusion of the rabbit eye has been reported to reduce the intraocular pressure elevation induced by sodium hyaluronate (21). Since antithrombin III is a major protein that binds to heparin (28), this heparin effect could conceivably be mediated through its antithrombin III function.

In addition, several other cellular effects of this protein might be of physiological relevance. For instance, antithrombin III has been shown to induce the production of prostacyclin in a dose-dependent manner in vascular endothelial cells (31, 32). This effect has been thought to be important in PGI2 induced vasodilation of blood vessels (31–34). Interestingly, various prostaglandins including prostacyclin exhibit ocular hypotensive effects (35–39). Prostaglandin analogs are currently used in clinical practice to lower intraocular pressure (37–39), and the uveoscleral outflow pathway has been identified as the tissue target for this effect (37–39). Therefore, the known effects of antithrombin III on the production of PGI2 in endothelial cells could be involved in such unconventional outflow pathway regulation.

Cultured HTM cells have been shown to produce prostacyclin, PGE2, PGF2 and other eicosanoid metabolites and the production of these metabolites is influenced by serum, thrombin, and bradykinin (40, 41). Both the HTM and SC cells are thought to be endothelial in nature and found to express several endothelial cell surface markers (23, 40). Therefore, antithrombin III secreted by HTM and SC as well as that is present in aqueous humor might also influence conventional aqueous humor outflow facility through the HTM and SC.

Recently, antithrombin III was also shown to stimulate nitric oxide production in macrophages through an as yet uncharacterized signaling pathway(s) (42). This aspect may also be relevant to the regulation of intraocular pressure. Nitric oxide producing agents have been found to enhance aqueous outflow facility, an effect presumably involving the known endothelial relaxation property of nitric oxide (43, 44).

Thus, based on the collective understanding of these known effects of antithrombin III, it is reasonable to speculate that its presence in aqueous humor could be of significance in the regulation of aqueous humor outflow. Studies designed to explore antithrombin-mediated regulation of prostacyclin production in HTM and SC cells could provide better understanding of the potential role(s) of antithrombin III in regulating intraocular pressure.

ACKNOWLEDGMENTS

The authors thank Dr. Samuel Zigler from the National Eye Institute, NIH, for providing aqueous humor from monkey and bovine eyes and Dr. V. N. Reddy from Kelloggs Eye Institute, Michigan, for providing the human lens epithelial cell line. We thank Pei-Feng Deng for technical assistance. Grants from Research to Prevent Blindness and the National Glaucoma Research Foundation, a Program of the American Health Assistance Foundation and NIH Grant EY01894 (D.L.E.) support this work.

REFERENCES

1. Pavao, A. F., Lee, D. A., Ethier, C. R., Johnson, M. C., Anderson, P. J., and Epstein, D. L. (1989) *Invest. Ophthalmol. Vis. Sci.* **30,** 731–738.
2. Zirm, M. (1980) *Adv. Ophthalmol.* **40,** 100–172.
3. Dernouchamps, J. P. (1982) *Doc. Ophthalmol.* **53,** 193–248.
4. Tripathi, R. C., Millard, C. B., and Tripathi, B. J. (1989) *Exp. Eye Res.* **48,** 117–130.
5. Sheibabi, N., Sorenson, C. M., Cornelius, L. A., and Frazierm, W. A. (2000) *Biochem. Biophys. Res. Commun.* **267,** 257–261.
6. Salzmann, J., Flitcroft, D., Bunce, C., Gordon, D., Wormald, R., and Migdal, C. (1998) *Br. J. Ophthalmology* **82,** 830–834.
7. Herschler, J., and Litin, B. S. (1987) *Ophthalmic. Surg.* **18,** 792–795.
8. Tripathi, R. C., Li, J., Chan, W. F., and Tripathi, B. J. (1994) *Exp. Eye Res.* **59,** 723–727.
9. Stur, M., Grabner, G., Huber-Spitzy, V., Schreiner, J., and Haddad, R. (1986) *Arch. Ophthalmol.* **104,** 899–900.
10. Sit, A. J., Gong, H., Ritter, N., Freddo, T. F., Kamm, R., and Johnson, M. (1997) *Exp. Eye Res.* **64,** 813–821.
11. Russell, P., Koretz, J., and Epstein, D. L. (1993) *Med. Hypothesis* **41,** 455–458.
12. Gual, A., Llobet, A., Gilabert, R., Borras, M., Pales, J., Bergamini, M. V., and Belmonte, C. (1997) *Invest. Ophthalmol. Vis. Sci.* **38,** 2165–2171.
13. Erickson-Lamy, K., Schroeder, A. M., Bassett-Chu., S., and Epstein, D. L. (1990) *Invest. Ophthalmol. Vis. Sci.* **31,** 2384–2388.
14. Yue, B. Y. (1996) *Surv. Ophthalmol.* **40,** 379–390.

15. Alexander, J. P., Samples, J. R., Van Buskirk, E. M., and Acott, T. S. (1991) *Invest. Ophthalmol. Vis. Sci.* **32,** 172–180.
16. Wordinger, R. J., and Clark, A. F. (1999) *Prog. Retin. Eye. Res.* **18,** 629–667.
17. Ruoslathi, E. (1988) *Ann. Rev. Cell Biol.* **4,** 229–255.
18. Jackson, R. L., Busch, S. J., and Cardin, A. D. (1991) *Physiol. Rev.* **71,** 481–539.
19. Lee, M. K., and Lander, A. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2768–2772.
20. McKay, E. J., and Laurell, C. B. (1980) *J. Lab. Clin. Med.* **95,** 69–80.
21. Kondo, H., Hayashi, H., and Oshima, K. (1994) *Nippon Ganka Gakkai Zasshi.* **98,** 423–428.
22. Rao, P. V., Garrow, T. A., John, F., Garland, D., Millian, N. S., and Zigler, J. S. (1998). *J. Biol. Chem.* **273,** 30669–30674.
23. Stamer, W. D., Roberts, B. C., Howell, D. N., and Epstein, D. L. (1998) *Invest. Ophthalmol. Vis. Sci.* **39,** 1804–1812.
24. Schmut, O., and Hofmann, H. (1979) *Albrecht Von Graefes arch Klin Exp. Ophthalmol.* **210,** 219–221.
25. Barsotti, M. F., Bartels, S. P., Freddo, T. F., and Kamm, R. D. (1992) *Invest. Ophthalmol. Vis. Sci.* **33,** 581–595.
26. Fukuchi, T., Sawaguchi, S., Hanyuu, T., and Abe, H. (1997) *Nippon Ganka Gakkai Zasshi.* **101,** 265–271.
27. Hamanaka, T., and Bill, A. (1994) *Exp. Eye Res.* **59,** 249–256.
28. Ersdal-Badju, E., Lu, A., Zuo, Y., Picard, V., and Bock, S. C. (1997) *J. Biol. Chem.* **272,** 19393–19400.
29. Travis, J., and Salvesen, G. S. (1983) *Ann. Rev. Biochem.* **52,** 655.
30. Leet, D. M. (1977) *Am. J. Ophthalmol.* **84,** 79–84.
31. Yamuchi, T., Umeda, F., Inoguchi, T., and Nawata, H. (1989) *Biochem. Biophys. Res. Commun.* **163,** 1404–1411.
32. Uchiba, M., and Okajima, K. (1997) *Semin. Thromb. Hemost.* **23,** 583–590.
33. Kaji, T., Itoh, F., Hayakawa, Y., Oguma, Y., and Sakuragawa, N. (1989) *Thromb. Res.* **56,** 99–107.
34. Harada, N., Okajima, K., Kushimoto, S., Isobe, H., and Tanaka, K. (1999) *Blood* **93,** 157–164.
35. Bito, L. Z. (1984) *Exp. Eye Res.* **38,** 181–194.
36. Nilsson, S. F. E., Samuelsson, M., Bill, A., and Stjernschantz, J. (1989) *Exp. Eye Res.* **48,** 707–716.
37. Camras, C. B. (1996) *Ophthalmology* **103,** 138–147.
38. Linden, C., and Alm, A. (1999) *Drug Aging.* **14,** 387–398.
39. Hoyng, P. F., and Groeneboer, M. C. (1989) *Prog. Clin. Biol. Res.* **312,** 369–378.
40. Weinreb, R. N., Mitchell, M. D., and Polansky, J. R. (1983) *Invest. Ophthalmol. Vis. Sci.* **24,** 1541–1545.
41. Weinreb, R. N., Polansky, J. R., Alvarado, J. A., and Mitchell, M. D. (1988) *Invest. Ophthalmol. Vis. Sci.* **29,** 1708–1712.
42. Kwak, J. Y., Park, S. Y., Han, M. K., Lee, H. S., Sohn, M. H., Kim, U. H., McGregor, J. R., Samlowski, W. F., and Yim, C. Y. (1998) *Cell Immunol.* **188,** 33–40.
43. Nathanson, J. A. (1993) *J. Glaucoma* **2,** 206–210.
44. Schuman, J. S., Erickson, K., and Nathanson, J. A. (1994) *Exp. Eye Res.* **58,** 99–105.