Alterations of Ocular Nitric Oxide Synthase in Human Glaucoma

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Purpose. The authors recently reported that sites of outflow resistance and regulation in the human eye are highly enriched in the endothelial isoform of nitric oxide (NO) synthase (ecNOS). In vasculature, ecNOS activation is associated with altered vascular resistance and, in the stomach, defects in NO are associated with pathologic gastric hypertension. Because human glaucoma sometimes is associated with an increase in intraocular pressure and resistance changes in the aqueous outflow pathway (OP), the authors have investigated the possibility that alterations in NO or defects in NO-synthesizing tissues might exist in glaucomatous eyes.

Methods. Occurrence, distribution, and extent of sites of ocular NO production in the anterior segments of 16 normal eyes (10 patients) and 17 eyes (12 patients) with a history of primary open-angle glaucoma (POAG) were determined using the NO-indicator marker, NADPH-diaphorase (NADPH-d), which is known to colocalize with ecNOS immunoreactivity. Analysis of NADPH-d reactivity in tissues was combined with examination of overall cell distribution and use of neuron-specific markers.

Results. The ciliary muscle (CM) and OP of glaucomatous eyes showed marked differences in the amount and distribution of NADPH-d and alterations in gross structure. NADPH-d reactivity was decreased in trabecular meshwork (TM) and Schlemm's canal, and there was a marked reduction of anterior longitudinal CM fibers that insert near (and may normally regulate resistance in) the TM.

Conclusion. Abnormalities in NO or NO-containing cells occur in POAG. These abnormalities may be causally related to glaucoma or may be a manifestation of the disease or its treatment. In either case, such alterations, together with recent pharmacologic studies showing that NO-mimicking nitrovasodilators alter IOP, indicate that NO has relevance to the course, treatment, or both, of some forms of this disease. Invest Ophthalmol Vis Sci. 1995;36:1774–1784.

Although the precise relationship between ocular hypertension and retinal damage in glaucoma remains unclear, several studies have suggested that increased intraocular pressure (IOP) is a significant risk factor for visual impairment in this disease. In many patients with glaucoma, IOP elevation is associated with an increase in resistance to fluid leaving the eye, an abnormality thought to occur primarily at the level of fluid passage through the trabecular meshwork (TM) and Schlemm's canal. In some instances, the increase in outflow resistance is caused by restricted access of aqueous humor to the TM (angle closure or narrow-angle glaucoma) or to a mechanical clogging of the meshwork (e.g., pigmentary glaucoma). However, in most cases (primary open-angle glaucoma [POAG]), the angle appears normal and the cause for the increase in outflow resistance through the outflow pathway (OP), especially the cell- and connective tissue-filled lattice work of the TM, has remained obscure.

In blood vessels, a key regulator of resistance changes is the intercellular (and sometimes intracellular) gaseous modulator, nitric oxide (NO). Production of vascular NO is regulated in several ways: by circulating hormones acting on endothelial cell NO

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Proprietary interest category: N.
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synthase (ecNOS); by nonadrenergic, noncholinergic, perivascular nerves acting through neuronal NOS (bNOS; NOS-1); by blood flow acting through mechanical distortion of vessels (shear stress); and by cytokines and perhaps other factors acting on inducible NOS (iNOS; macNOS) present in vascular smooth muscle. Increased synthesis of NO leads to smooth muscle relaxation, increase in luminal diameter, and a reduction in vascular resistance.

Interestingly, NO also regulates pressure and alters resistance to the outflow of stomach contents through the pylorus. Although the stomach is anatomically unrelated to blood vessels or to the ocular OP of the eye, NO acts functionally in a similar manner to decrease intraluminal pressure by reducing smooth muscle tone and resistance. NO-induced relaxation of the pylorus allows the exit of gastric contents from the stomach, and failure of this system to function properly results in greatly increased intraluminal pressure. Such loss of NO action has been observed experimentally in “knockout” mice lacking the NO synthase (NOS)-1 gene and clinically in patients with infantile hypertrophic pyloric stenosis who lack NO-containing, nonadrenergic, noncholinergic, pyloric nerves.5,6

Although studies have reported that rats demonstrate little NOS-1 activity in their ocular OP, we have recently shown an enrichment (relative to other ocular tissues) of ecNOS (NOS-3) in the human outflow system (TM, Schlemm’s canal, and collecting channels) and in the ciliary muscle (CM), especially its anterior longitudinal portion (long.CM). The long.CM is known to send tendinous insertions into the TM and, by contraction and relaxation, to affect the complex TM anatomy and to alter outflow resistance.9-11 Direct topical or intracameral application of NO agonists has been reported to alter outflow facility,12,15 and some clinical reports have indicated that systemic administration of the NO-mimicking nitrovasodilators can lower IOP at doses that do not alter systemic blood pressure. Interestingly, the magnitude of the IOP decrease observed in such cases has sometimes been observed to be greater in patients with POAG than in patients with ocular normotension or in those with angle-closure glaucoma.

Based on the above anatomic and physiological observations, we have investigated postmortem human eyes to determine if defects might exist in the NO system or NO-synthesizing tissues in POAG. Such alterations, should they exist, would have considerable relevance to the treatment of this disease and to the pathophysiology of elevated IOP. Investigation of the NO system in POAG is of additional interest because of NO’s possible role in modulating the neurotoxic response of excitatory amino acid neurotransmitters and NO’s ability to affect the survival of injured and metabolically stressed cells, including retinal neurons, as well as recent evidence that NO itself can alter the energy metabolism of neuronal and nonneuronal cells. These studies were approved by the Massachusetts General Hospital Human Studies Committee and were consistent with ARVO guidelines.

METHODS

Tissue Preparation

Tissue was obtained from donor eyes (New England Eye Bank, Boston, MA) that had been enucleated and cooled to 4°C within 8 hours of death (average time, 4.8 hours). (See below for detailed selection criteria and patient population.) One milliliter of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, was injected intravitreally through two 4-mm scleral slits made on opposite sides of the ocular equator, and eyes were fixed by immersion in additional paraformaldehyde overnight at 4°C. After rinsing in buffer, eyes were hemisected on a pupil–optic (p.o.) axis and cryoprotected in serial 10%, 20%, and 30% sucrose in phosphate buffer. They were then embedded and stored at −50°C until frozen-sectioning. Tissue analysis was carried out without personal identifiers using coded specimens.

NADPH-d

Localization of NO synthetic activity was determined using NADPH-diaphorase (NADPH-d), distribution of which, with only a few exceptions, is known to be highly correlated with that of NOS. Furthermore, by using NADPH-d coupled with immunocytochemical localization of all three NOS isoforms, we have directly demonstrated that in the human eye NADPH-d colocalizes identically to specific sites of NOS immunoreactivity in the anterior segment (see ref. 8). Twenty-micron air-dried p.o. sections were incubated (40' at 37°C) in a solution of 0.3% Triton X-100, 0.5 mM nitroblue tetrazolium, 10 mM sodium phosphate (pH 7.4), and 1.25 mM NADPH. In recent studies of normal human eyes, immunocytochemical evaluation with isotype-specific monoclonal antibodies indicated that NADPH-d staining observed both in the human CM and OP colocalizes almost exclusively with the endothelial form of NOS (ecNOS). However, in our experience, section-to-section reproducibility of labeling and anatomic resolution have been superior using NADPH-d rather than using immunocytochemistry. In addition, background levels of staining (in the absence of NADPH) have been lower with the diaphorase method than background levels of fluorescence observed in control immunocytochemical sections. These factors, which substantially reduce statistical variability, have led us to use the NADPH-d
method for comparisons between control and POAG eyes.

Controls for diaphorase staining included slides incubated without NADPH, with NADH instead of NADPH, in the presence of the calmodulin antagonist calmidazolium (10 to 100 μM), and preincubated (30’ at 37°C) with diphenyliodonium chloride (DPI) (Fluka) (1 to 1000 mM), a potent inhibitor of NOS, and then incubated in the presence of NADPH and DPI. For comparison of NADPH-d with normal anatomy, adjacent p.o. sections were stained with hematox- ylin and eosin using standard techniques.

Finally, to determine if certain sites of ocular NADPH-d and NO synthesis correspond to neuronal projections, other sections were immunostained for the neuronal markers (Dako, Carpinteria, CA) tau, neurofilament protein, and synaptophysin. Discussion of other controls and the association of NADPH-d with the enzymatic production of NO in human CM and TM is described in detail elsewhere.

**Patient Population**

Seventeen glaucomatous eyes from 12 patients, all with POAG (average duration ± SEM; 10.3 ± 1.7 years), and 16 eyes from 10 patients with no known history of glaucoma, were evaluated (Fig. 1). Patients with secondary and congenital glaucoma were excluded, as were any patients with sepsis, ocular infection, ocular trauma, or human immunodeficiency virus. Eyes were obtained through the New England Eye Bank and its exchange program for corneal transplantation from a geographic area that primarily included New England, exclusive of Connecticut. Eyes were enucleated in a standardized manner by the Eye Bank’s technical staff after review of the patient’s medical record. Eyes were immediately cooled, transported to Boston by the Eye Bank’s staff, evaluated by slit lamp for suitability for corneal transplantation, and then stored at 4°C until use. Eyes used for the current study were those excluded for use in corneal transplantation. Usually this was because of the patient’s history of glaucoma or because the patient age was older than 79 years (both automatic exclusion criteria for transplantation). In six patients, eyes were excluded for transplantation because of corneal damage observed by slit lamp. Eyes were fixed at the Eye Bank (usually within 24 hours of cooling) and transported on ice to the laboratory for histochemical processing.

Each patient’s ophthalmologist (or the physician of record if there was no ophthalmologist) completed a standardized written questionnaire (with follow-up, if necessary) concerning history of glaucoma, ocular and systemic disease, details of terminal illness, and systemic and topical drug use. Eyes for which sufficient information could not be obtained were excluded from the study, as were any eyes for which time from death to enucleation exceeded 8 hours. Selection criteria were the same for controls and POAG eyes. Six of the 16 controls were used in the previous study. These had been picked because their NADPH-d staining was typical of that seen in the control group.

Median age of glaucomatous eyes was 73 years, and of control eyes it was 75 years. Eight of 17 glaucomatous eyes had undergone laser surgery. However, as noted in the Results section, group differences were not affected by a history of such surgery. Drugs to reduce chronic ocular pressure administered before death to the group with glaucoma included timolol (eight eyes), pilocarpine (seven eyes), betaxolol (two eyes), levobunolol (one eye), acetazolamide (one eye), epinephrine (one eye), dipivefrin (one eye), none (three eyes). (Some eyes received dual drug therapy; hence, the total of 24 drug treatments.) As noted in the Results section, history of taking any particular drug was not correlated with the alterations in NADPH-d observed in the group with POAG. When suitable, both eyes from a single patient were included in the study. Accordingly, statistical analysis was carried out in two ways, with N = number of eyes as well as N = number of patients. In the latter case, ratings for left and right eyes were averaged to obtain a single patient value. Group differences described in the Results section were significant using either method; that is, with N of either 16 and 17 (control versus POAG patients) or 10 and 12 (control versus POAG patients).

In addition to these postmortem eyes, specimens of glaucomatous TM were obtained from six other patients at the time of trabeculectomy surgery (courtesy of Johnson D and Brubaker R, Mayo Clinic). These surgical specimens were immediately cooled on ice, fixed overnight, and frozen as described above, then sectioned and stained for NADPH-d.

**Rating of Eyes**

NADPH-d reactivity was quantitated, in a masked manner, through evaluation of coded sections and comparison with reference sections from known normal eyes. In the CM, intensity of overall NADPH-d reactivity and extent and prominence of long CM fiber fascicles extending toward the TM were rated on a scale of 1 to 5 (5 = normal). In a typical score of 5, the anterior portion of the long CM stained dark blue or black and extended to a point equal to or just anterior to the angle. In addition, the muscle fibers were arranged clearly in fascicles. In a typical score of 1, the staining was a light to medium blue, and the anterior part of the long CM failed to extend as far as the angle. In addition, the muscle fibers were diffuse and not arranged in fascicles. Except for the long CM, overall size and configuration of the CM were not considered in the CM ratings used for intergroup com-
comparisons. This was done to avoid the possible confounding effects of any cholinergic drugs taken before the onset of disease. In the monkey, acute administration of such drugs has been reported to alter the shape of the circular and posterior longitudinal parts of the CM but not the area of anterior longitudinal fibers held by tendons to the scleral spur, cornea, and TM\textsuperscript{22,23} (see also discussion of chronic drug effects). For long.CM, an example of a score of 5 is shown in Figure 2C, a score of 3.5 in Figure 3C, a score of 2 in Figure 2A, and a score of 1 in Figure 3B. In the TM and adjacent Schlemm’s canal, together termed the outflow pathway in this article, the extent and intensity of NADPH-d also were assessed on a scale of 1 to 5. Both the intensity of NADPH-d (dark blue or black versus light to medium blue) and the relative number of cells that reacted were used as the criteria for rating. As with the CM, typical sections, rated as 1, 3, and 5, were used for comparison during the rating process to enhance consistency. Because collecting channels were rarely sectioned in a consistent manner in the p.o. plane, they were not included in the OP rating.

For each eye, the diaphorase reactivity in two representative p.o. sections, each containing two angles, was evaluated and averaged to obtain separate CM and OP scores for that eye. The degree of reactivity between two sections from the same eye tended to be similar. However, because the whole eye was not serially sectioned, we could not determine if there was variability in staining in different areas of CM and TM within the same eye.

Ratings were also made for NADPH-d reactivity in iris and neural retina. When statistical comparison by standard $t$-test was performed using more than a single criterion (e.g., both CM and OP ratings as in Fig. 1A), appropriate downward adjustment of the $P$ value was made for the multiple comparison. In addition to direct microscopic viewing, assessment of eyes was made through use of coded photomicrographs taken of all sections at $\times 40$ and $\times 100$ magnifications using color film and including the area of the TM, Schlemm’s canal, and long.CM, as well as the wedge of anterior long.CM fibers that normally insert into the TM. Angle photographs were also used for additional blind rating by an independent observer who was not associated with the study who had not seen the sections. This outside rating was achieved by comparison of angle photographs to a series of photographic standards of diaphorase-stained angles with scores of 1, 3, and 5. Despite efforts to minimize variability, it should be kept in mind when comparisons are made later that these data were semi-quantitative because they were derived from a qualitative assessment, albeit a reproducible one.

**RESULTS**

In control CM and OP,\textsuperscript{16} NADPH-d gave higher resolution labeling of NO-related anatomy than did immunocytochemistry, and, given its colocalization with NOS immunoreactivity, NADPH-d was used as a probe for comparative studies of the distribution of NOS in POAG versus control eyes. Observer-blind assessment of diaphorase-labeled sections indicated that there were group differences in NADPH-d reactivity and in NADPH-d-containing cells apparent in the CM and OP between eyes with and without a history of glaucoma.

A plot (Fig. 1A) of CM and OP scores for each of the 33 eyes (CM, x-axis; OP, y-axis) demonstrated a highly significant separation of control group versus group with glaucoma based on either criterion (for CM NADPH-d, Student’s $t$-test = 5.53, $P < 0.0001$; for TM, Student’s $t$-test = 5.28, $P < 0.0001$). (Please note that some symbols that overlap one another on the graph are not visible.) Within the group with glaucoma, the CM and OP scores for each eye were correlated with each other ($r = 0.78$; $P < 0.0001$).

In addition, CM and OP values for each eye were averaged to obtain a single CM–OP score, and these combined scores were plotted for each eye on a single axis (Fig. 1B). Group separation based on this combined CM–OP score was highly significant (mean control $\pm$ SEM = 3.99 $\pm$ 0.17; mean glaucoma = 2.07 $\pm$ 0.17; Student’s $t$-test = 6.14; $P < 0.0001$). If comparisons were made on the basis of individual patients instead of individual eyes (when more than a single eye had been evaluated from a given patient), then intergroup differences ($N = 10$ for control group, and $N = 12$ for POAG group) remained highly significant.

**FIGURE 1.** (A) Plot of ratings of NADPH-d reactivity in long.CM (x-axis) and OP (y-axis) for each of 33 eyes with (open squares) and without (filled circles) a history of primary open-angle glaucoma (POAG). Note that some symbols, marked with an M (for multiple) overlap and represent two eyes. For this reason, fewer than 33 symbols are visible. (B) Averaged NADPH-d rating for both long.CM and OP for each of 33 eyes, showing significant separation ($P < 0.0001$) of the two groups. As noted in text, there was no significant correlation between patient age or duration of POAG and NADPH-d in either long.CM or OP. CM = ciliary muscle; OP = outflow pathway.
FIGURE 2. NADPH-d reactivity in CM and OP of glaucomatous eye, age 82 years (a, top left). Note loss of reactivity in the area in which, in a normal 83-year-old eye (c, top right), a wedge of anterior long.CM fibers is typically seen. In the normal eye, long.CM fibers originate from an area to the right of the figure and extend to the left as the prominent dark staining band that tapers to a point at the approximate center of the figure. (The reticular and circular parts of the CM involved in the accommodation response are on the right above the long.CM band.) In the glaucomatous eye (a), long.CM fibers originate from an area to the left of the figure but, as they extend to the right, appear to fade and disappear, and the prominent band and wedge-shaped areas are not seen. The reticular and circular portions of the muscle, however, appear largely preserved. In the glaucomatous eye (a), NADPH-d is decreased in the area of the trabecular meshwork (TM) (arrow), compared with the scattered dark staining of NADPH-d-positive TM cells seen in the normal eye (c) at right (arrow). The few dark spots remaining in the TM of the glaucomatous eye represent pigment granules. It should be noted that the particular glaucomatous eyes shown here and in Figure 3 are especially affected (with scores of 1.0 to 1.5) and were chosen to illustrate more clearly the defects found in the group with glaucoma. The average glaucomatous eye had a similar distribution of NADPH-d loss, but the degree of loss was quantitatively less (i.e., had a higher score). Below the figure of each NADPH-d-stained section is a figure of an adjacent section stained with a routine histologic stain (hematoxylin and eosin). Glaucomatous eye (b, lower left); normal eye (d, lower right). Note that, in the area of the TM, many cells still remain in the glaucomatous eye (b), indicating that there is not a total loss of cells but a selective loss of NADPH-d cells. In the long.CM of the glaucomatous eye (b), there may be an actual loss of fibers as they approach the TM compared to the normal eye (d). However, note that even in the normal eye, hematoxylin and eosin does not stain the wedge-shaped area of the long.CM well compared to the intense NADPH-d reactivity seen in (c). This situation may account for the fact that earlier studies using routine histologic stains have not reported the marked changes in the long.CM that can be seen easily by NADPH-d reactivity. Marker = 100 μm. CM = ciliary muscle; OP = outflow pathway.

(Student's t-test = 4.92, P < 0.0001 for combined CM-OP score).

Using a CM-OP score of 3.0 as a cutoff between groups, 29 of 33 eyes showed no overlap between groups. Based on NADPH-d reactivity alone, only three eyes would have been misclassified concerning history of glaucoma.

Figures 2 and 3 illustrate the observed histochemical alterations that contributed to the ratings differential. (The particular eyes shown are especially affected [with scores of 1 to 1.5] and were chosen to illustrate more clearly the defects found in the group with glaucoma.) The most dramatic and frequently observed abnormality in the group with glaucoma (11 of 17 eyes) was a selective reduction or complete absence of the wedge of NADPH-d-reactive long.CM fibers that normally extend toward and insert into the TM (Figs. 2A, 3B, 3D). Associated with this reduction, and sometimes seen separately (4 of 17 eyes), was a loss of the prominent fasciculation normally seen in the long.CM fibers of controls; instead, this was replaced by a more diffuse staining (compare Figs. 3B, 3D with Figs. 3A, 3C). An examination of hematoxylin and eosin-stained sections adjacent to those above revealed that the alterations in NADPH-d activity observed in the long.CM were caused not only by a decrease in cyto-
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**FIGURE 3.** Additional examples of NADPH-d reactivity in anterior segments of eyes with no history of glaucoma (a, top left; c, top right) in patients 83 and 84 years of age, and from patients with primary open-angle glaucoma (POAG) (b, bottom left; d, bottom right) in patients 82 and 73 years of age. Glaucomatous eye in b shows a more general decrease in NADPH-d reactivity throughout the CM and TM. In glaucomatous eye seen at lower right (d), loss is restricted primarily to the anterior portion of the long CM. Note that this anterior long CM appears wedge-shaped and well-preserved in the two normal eyes shown in a and c. Note also the prominent staining of scattered TM cells in the two normal eyes. Marker = 100 μm. Note that orientation of control versus POAG is different from that in Figure 2. CM = ciliary muscle; OP = outflow pathway.

plasmic staining of fibers that persisted but also by an actual loss of long CM muscle fibers. Sometimes changes in long CM were accompanied by an overall decrease in NADPH-d reactivity in other parts of the ciliary muscle. In these eyes, examination at higher magnification revealed that reductions in NADPH-d were caused by a decrease in muscle cytoplasmic staining and a decrease in the frequency of intensely positive fiber profiles (see ref. 8).

The alterations in long CM were distinct enough to allow categorization of most eyes into one of two types—type A, with dark staining, distinct fasciculation, and a wedge-shaped appearance, and type B, with loss of wedge shape, loss of fasciculation, and/or marked loss of overall staining. Of 33 eyes, 24 were clearly either type A (13 eyes) or type B (11 eyes), whereas 9 were intermediate in character. Using the above criteria, and assuming that type B corresponds to a history of glaucoma, two observers independently were able to correctly differentiate between a group of cycled normal and glaucomatous tissue sections with an average 91% accuracy. In addition, the interobserver correlation based on comparison of the matrix of specific numeric ratings for each eye (on the standard scale of 1 to 5) was highly significant (r = 0.83; P < 0.0001).

In the OP, alterations observed in glaucomatous eyes ranged from partial to total absence of NADPH-d reactivity (Figs. 2A [arrow], 3B, 3D) in the TM and in Schlemm's canal. Hematoxylin and eosin staining of adjacent sections revealed that this loss of diaphorase reactivity was out of proportion to the small loss in overall TM cell number sometimes observed in POAG specimens. For example, even in eyes that totally lacked NADPH-d staining, numerous hematoxylin and eosin-stained trabecular cells (as well as endothelial cells in Schlemm's canal) could be seen (Fig. 2B) (see also Discussion).

To determine if the alterations observed in the long CM and OP were part of a more general ocular decrease in NADPH-d reactivity was rated in the iris (vasculature and nerve fibers) and, separately, in the neural retina. Although the staining of individual glaucomatous eyes was reduced, overall group differences in iris (control, 2.65 ± 1.05; glaucoma, 2.5 ± 1.35) and retinal (control, 3.58 ± 1.15; glaucoma, 2.94 ± 1.3) NADPH-d reactivity between the control group and the group with glaucoma was not statistically significant. Similarly, within groups there was no statistically significant correlation between NADPH-d activity in OP or CM and that in retina or iris.

To determine whether laser trabeculoplasty (performed in 8 of 17 glaucomatous eyes) could have contributed to the observed defects in NADPH-d, reactivity was rated in the iris (vasculature and nerve fibers) and, separately, in the neural retina. Although the staining of individual glaucomatous eyes was reduced, overall group differences in iris (control, 2.65 ± 1.05; glaucoma, 2.5 ± 1.35) and retinal (control, 3.58 ± 1.15; glaucoma, 2.94 ± 1.3) NADPH-d reactivity between the control group and the group with glaucoma was not statistically significant. Similarly, within groups there was no statistically significant correlation between NADPH-d activity in OP or CM and that in retina or iris.

To determine whether laser trabeculoplasty (performed in 8 of 17 glaucomatous eyes) could have contributed to the observed defects in NADPH-d, the lasered subgroup was analyzed separately, as was the subgroup of nonlasered glaucomatous eyes. Both subgroups were compared to one another, and each subgroup was compared to control eyes. Statistical analysis revealed that there was no significant difference in CM–TM NADPH-d reactivity between lasered...
and nonlasered glaucomatous eyes (mean laser = 2.06 ± 0.30; mean nonlaser = 2.10 ± 0.33; NS) and that both subgroups with glaucoma had significantly altered NADPH-d reactivity relative to control eyes (mean = 3.99 ± 0.17). These results do not preclude the possibility that laser injury or the ocular response to laser injury could alter NADPH-d, but they do indicate that lasering did not contribute as an independent variable to explain the alterations in NADPH-d observed between eyes with and without a history of POAG.

Because of varied, often multiple drug treatments and because of inadequate group size, it was not possible manner to analyze statistically the effects of particular pharmacologic treatments as factor(s) contributing to the alterations observed in NADPH-d. As discussed below, qualitative analysis of drug history revealed no obvious association of any particular ocular hypotensive medication with changes in NADPH-d. Furthermore, the three glaucomatous eyes that were reported to have received no pharmacologic treatment at all included two with severe alterations in NADPH-d. Conversely, there was no obvious association between NADPH-d severity and the use of multiple drugs. Finally, in the group with glaucoma, we did not find a statistically significant correlation between NADPH-d and severity of the disease as measured by duration, IOP, visual acuity, or overall physician assessment of severity (see Table 1).

**DISCUSSION**

The current study demonstrates a deficit in NOS-like reactivity in the CM and OP (consisting of the TM and Schlemm's canal) of many of the eyes with POAG. However, it should be emphasized that at present we cannot say whether this defect is associated with the cause of POAG or whether it is a result of the disease (see below).

In the OP, NADPH-d is substantially and disproportionately reduced relative to a modest loss in overall TM cell number seen by hematoxylin and eosin staining and also reported by others. This raises the possibility that the decrease in NADPH-d observed in POAG eyes may be caused by a preferential loss of cells in the subset of NADPH-d-positive TM cells. Whether this does, in fact, occur or whether there is a loss of NADPH-d reactivity independent of cell viability cannot be answered by the current studies and must await the development of reliable independent markers for NADPH-d-reactive TM cells.

In the CM, the deficit observed in POAG eyes was twofold. There was a decrease in NADPH-d reactivity, indicative of an alteration in NOS, but there was also a frank structural loss in long CM fibers made visible by the fact that the diaphorase reaction intensely stains the muscle, making it possible to visualize structural alterations that by traditional staining were much less apparent. Once this second abnormality is known, reexamination of sections stained with hematoxylin and eosin (Figs. 2, 3) confirms that there is a loss of long CM fibers. Although it is not possible to conclude from the current studies whether this structural defect is primary or secondary to the loss in NADPH-d, it has, in either case, important implications for CM function.

The prominent localization of NADPH-d in long CM fibers is of interest because, although the circular portion of the CM is presumably involved in accommodation, the long CM represents a late evolutionary development, seen in primates, that links the CM to the OP. There is also evidence that the long CM differs biochemically, has a different distribution of muscarinic receptor subtypes, and shows a differential effect of denervation than other parts of the CM. Specific localized changes of the long CM in POAG have not been described previously, although there are reports of plaque accumulation in long CM tendons and at least one report, in four patients, of a more widespread compaction of the uveal meshwork associated with hyalinization and generalized atrophy of the adjacent CM and iris root.

It has been shown that chronic treatment of monkeys with cholinergic agonists can cause morphologic changes in the CM and secondary changes in the TM, including discontinuity between CM bundles and TM beams. However, such monkeys also show marked anterior displacement of the mobile, internal, circular portion of the CM, something not observed in the current study. The macroscopic configuration of the long CM, with its firm attachment to the scleral spur, TM, and cornea, is not easily altered by exposure to cholinergic agents, making it unlikely that the NADPH-d alterations we observed were caused drug-induced muscle contraction before the onset of disease (see later discussion). The restricted distribution of the abnormality provided an internal control of sometimes normal-appearing circular and reticular CM fibers, making it unlikely that the alterations in NADPH-d resulted from a nonspecific staining artifact common to glaucomatous eyes. Consistent with this were the lack of significant group differences in NADPH-d reactivity observed in iris and neural retinas.

Although age-related degenerative changes have been observed in primate CM and OP, this does not appear to have been a factor in the current studies because the median age of the group with glaucoma was not significantly different from the median age of controls (see Methods). Also, within groups, there was no statistically significant correlation between age and degree of NADPH-d in long CM or OP, and, as noted
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TABLE 1. Descriptive Parameters of Glaucosomatous Eyes Used

<table>
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<th>Code Number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Duration of POAG (years)</th>
<th>Visual Acuity</th>
<th>IOP* (mm Hg)</th>
<th>Severity†</th>
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<td>7</td>
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<td>19</td>
<td>Mild</td>
<td>2.6</td>
</tr>
<tr>
<td>14</td>
<td>84</td>
<td>M</td>
<td>NA</td>
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<tr>
<td>16</td>
<td>72</td>
<td>F</td>
<td>2</td>
<td>20/50</td>
<td>18</td>
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<td>1.5</td>
</tr>
<tr>
<td>18</td>
<td>68</td>
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<td>4</td>
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<tr>
<td>22</td>
<td>82</td>
<td>M</td>
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<tr>
<td>23</td>
<td>82</td>
<td>M</td>
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<td>20/60</td>
<td>11</td>
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<tr>
<td>27</td>
<td>64</td>
<td>M</td>
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<td>NA</td>
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<td>2.5</td>
</tr>
<tr>
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<td>84</td>
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<td>10+</td>
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<td>NA</td>
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<td>3.25</td>
</tr>
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NA = data not available; POAG = primary open-angle glaucoma; IOP = intraocular pressure.

* IOP represents the most recent reading before death.
† Severity is the attending ophthalmologist’s subjective opinion as to whether the severity of the glaucoma was mild, moderate, or severe.
‡ Macular degeneration.
tile cells is supported by studies demonstrating the presence of cellular actin and myosin in the TM, Schlemm's canal, and collecting channels, and by experiments showing that tissue strips of isolated TM undergo contraction in response to cholinergic agonists and high potassium, and relaxation in response to NO agonists. As noted, OP NOS reactivity is present in giant endothelial cell vacuoles in Schlemm's canal, and nonmechanical effects of NO on fluid transport are possible, given recent observations showing that NO can alter fluid transport and Na,K-ATPase activity in kidney.

As noted, it is not possible to determine from the current studies whether the described alterations in CM and OP NADPH-d reactivity are a consequence or a cause of POAG. For example, the observed loss of long CM fibers occurs in the same area as the secondary or so-called uveoscleral (non trabecular) OP. Although uveoscleral flow is thought to be pressure independent, it is nonetheless possible that such flow, in the context of chronic IOP elevation, could lead to anatomic changes in the CM. We also cannot rule out the possibility that chronic use of antiglaucoma drugs could bring about pathologic changes through direct toxic effects (distinct from the changes from chronic CM contraction, discussed earlier). On the other hand, drug use would not explain the often focal nature of the alterations observed in CM and the fact that examination of patient history revealed no consistent association of any particular drug with the degree of long CM change observed.

A degeneration of the CM similar to that seen in the current studies has been reported in the rhesus monkey after chronic denervation of the CM by experimental lesioning of the parasympathetic ciliary ganglion. Because parasympathetic ganglia innervate the CM and TM with NOS-containing neurons, it is of interest that the disorder of infantile hypertrophic pyloric stenosis (and increased gastric outflow resistance), noted earlier, is associated with a marked deficiency of NADPH-d-staining nerves and is mimicked by a loss of the NOS-1 gene. Thus, although much of the alteration in ocular NADPH-d staining observed in the current study was primary to CM and OP cells (involving areas of ecNOS immunoreactivity), we cannot rule out the possibility that the changes seen in POAG could be associated with a defect in innervation by NOS-containing nerves, affecting all or part of the CM and OP.

We also cannot, at present, determine whether the alterations seen in NADPH-d are a cause or an effect (direct or indirect) of POAG. However, whether cause or result, the observed alterations in ocular NADPH-d-staining described in this study—if proven to be present in larger populations of patients with glaucoma—may have potential consequences for the treatment of this disease. For example, it has been reported that the nitrovasodilator-induced decrease in outflow resistance observed in rabbit eyes (which have a small or absent CM) is not always seen in normotensive human eyes. This species difference is thought to result from the fact that, in humans, the effects of nitrovasodilators on lowering resistance in the TM and distal drainage system are offset by a nitroglycerine-induced relaxation of the CM, which tends to collapse the TM and secondarily to increase its resistance. The current findings suggest that, in POAG, a loss of long CM fibers may reduce the opposing effects of nitrovasodilators mediated through the CM and could result in greater efficacy of these compounds, making them particularly useful for lowering outflow resistance in POAG. As noted, at least one study has reported an increased responsiveness of some POAG eyes to systemically administered nitrovasodilators. Also of consequence is the report that a number of patients with low tension POAG have increased systemic vasoreactivity. These patients show an exaggerated vasoconstrictive response to cold, suggesting either increased smooth muscle tone or, relevant to the current findings, a disordered vasodilatory system such as would occur if, in addition to an ocular deficit, there were an abnormality in systemic ecNOS. For such patients, ocular use of a nitrovasodilator or other smooth muscle relaxant warrants further investigation. Finally, relevant to the issue of retinal ganglion cell death in glaucoma, it may be of interest to note recent findings indicating that certain nitrovasodilators, through modulation of NMDA receptor sensitivity, might improve survival of injured or ischemically challenged neurons.

**Key Words**
ciliary muscle, glaucoma, NADPH-diaphorase, nitric oxide, nitric oxide synthase, trabecular meshwork

**Acknowledgments**
The authors thank R. Brubaker and D. Johnson for trabeculectomy specimens, the staff of the New England Eye Bank (Boston, MA), and P. Bhide for evaluation of data, as well as K. Erickson and J. Schuman for helpful advice.

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