Expression of Nitrotyrosine and Oxidative Consequences in the Trabecular Meshwork of Patients with Primary Open-Angle Glaucoma

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PURPOSE. To evaluate the possible correlation between the visual field defects in patients with primary open-angle glaucoma (POAG) and the expression and enzymatic activity of nitric oxide synthase (NOS) isoenzymes and nitrotyrosine in trabecular meshwork (TM) samples.

METHODS. TM specimens were collected from 146 patients with POAG by using standard filtration surgery. Visual field defects were evaluated by perimeter. Expression of endothelial (e)NOS and inducible (i)NOS were evaluated by quantitative RT-PCR. Constitutive (Ca2+/calmodulin-dependent) and iNOS (Ca2+-independent) activities were measured by the conversion of l-[14C]-arginine to l-[14C]-citrulline. In four TM specimens from POAG-affected eyes and in three human donor control eyes, 3-nitrotyrosine was localized by immunohistochemistry. The marker of lipid peroxidation malondialdehyde (MDA) was measured by the thiobarbituric acid test in samples of aqueous humor (AH) from 48 patients with either POAG or cataracts.

RESULTS. The results showed an upregulation of iNOS and a downregulation of calcium-dependent NOS correlated with visual field defects. Expression and activity of iNOS increased in parallel with visual field defects. However, constitutive activity decreased as the visual field defect increased. Nitrotyrosine was observed only in the cells of the TM specimens from eyes with severe POAG.

CONCLUSIONS. The increased expression and activity of iNOS in the TM of patients with POAG are proportional to the visual field defect and could lead to the increased nitrotyrosine levels which may serve as marker of oxidative stress in the progression of cell death of the TM in POAG. (Invest Ophthalmol Vis Sci. 2008;49:2506–2511) DOI:10.1167/iovs.07-1363

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Supported by Fondo de Investigaciones Sanitarias Grant FIS 04/0251 (RF-D), Spanish Ministry of Science and Technology Grant SAF00027-2004 (JCL), and CIBERSAM (JCL). BGB and BGP-N are recipients of a Formación de Personal Investigador/Formation of Profesorado Universitario fellowship (Ministerio de Educación y Ciencia, Spanish Government).

Accepted for publication October 23, 2007; revised December 4, 2007; accepted March 28, 2008.

Disclosure: R. Fernández-Durango, None; A. Fernández-Martínez, None; J. García-Feijoo, None; A. Castillo, None; J. Martínez de la Casa, None; B. García-Bueno, None; B.G. Pérez-Nievas, None; A. Fernández-Cruz, None; J.C. Leza, None

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Glaucoma, a progressive optic neuropathy, is characterized by retinal ganglion cell death and a distinctive pattern of visual field defects. It is one of the most common causes of blindness in the world. Primary open-angle glaucoma (POAG), the most common form of the disease, is more closely associated with intraocular ocular pressure (IOP) resulting from an abnormal resistance to the outflow of aqueous humor (AH) through the conventional outflow pathway. This pathway, consisting of trabecular meshwork (TM) and Schlemm’s canal (SC), modulates the outflow of AH from the anterior chamber to the venous system. The longitudinal ciliary muscle (CM), by its tendinous insertion into the TM alters the outflow resistance. Both TM and longitudinal CM are actively involved in the regulation of AH outflow and IOP acting as functional antagonists: contraction of ciliary muscle leads to a distortion of the TM, with subsequent reduction in outflow; however, contraction of the TM leads to the opposite effect. Thus, the TM is the key region in the pathogenesis of glaucoma and therefore the target of possible therapeutic interventions.

The physiological mechanisms by which the TM–SC outflow pathway regulates the outflow of AH, as well as the cause of the increase in resistance leading to elevated IOP in POAG remain partially unknown. Architectural features and cell contractility characteristics of TM cells are thought to be crucial aspects in IOP regulation. Special interest has been directed toward endothelial cell function. In samples from patients with POAG, the population of TM endothelial cells is markedly decreased compared with that of age-matched healthy subjects.

In contrast, several in vivo and in vitro studies have demonstrated a role of the free radical nitric oxide (NO) in the regulation of ocular blood flow and IOP. NO is synthesized from l-arginine by a family of nitric oxide synthase (NOS) isoenzymes: neuronal (n)NOS (NOS1), endothelial (e)NOS (NOS3), and inducible-NOS (i)NOS (NOS2). nNOS and eNOS are constitutive, Ca2+/calmodulin-dependent enzymes and are tightly controlled by mechanisms regulating physiological intracellular Ca2+ levels. However, iNOS is Ca2+-independent, and it is induced in response to immunologic or inflammatory stimuli, leading to the production of large amounts of potentially cytotoxic NO.

In TM–SC outflow pathway, immunohistochemical studies have revealed that human TM expresses mainly the eNOS isoform with a much smaller amount of nNOS. eNOS physiologically regulates aqueous outflow in the eye by maintaining vascular endothelial cell function. This physiological, low concentration dependent aspect of NO has also been demonstrated pharmacologically: Drugs acting as NO donors applied topically to the eyes of rabbits and monkeys lower IOP. In humans, intravenous administration of the precursor l-arginine also lowers IOP.

On the other hand, in vitro studies of perfused anterior segments of human donor eyes have shown an increase in NO production (~66%) after elevation of the pressure gradient over the TM, accompanied by an upregulation of iNOS gene.
TABLE 1. Demographic Characteristics of Patients Enrolled in the Study

<table>
<thead>
<tr>
<th>Control</th>
<th>POAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>AH 48</td>
</tr>
<tr>
<td>Mean age, y (±SD)</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>IOP (mm Hg; mean ± SD)</td>
<td>14.2 ± 2.1*</td>
</tr>
</tbody>
</table>

AH, aqueous humor; TM, trabecular meshwork.
* P < 0.05.

expression. Similar effects were observed in bovine TM subjected to pressure.
It has been shown that cytotoxicity associated with high levels of NO are due to the formation of the powerful oxidant peroxynitrite, (ONOO−) by its interaction with superoxide anion (O2·−). ONOO− can cause cell damage or death by lipid peroxidation of cell membranes, oxidation of sulphhydril groups, and oxidation or nitration of various amino acid functional groups, such as tyrosine. A major reaction with proteins is the formation of nitrotyrosine (NT). Studies in patients with POAG have provided evidence of an increase in oxidative DNA damage, in relationship with the lack of genes regulating the function of antioxidant, free-radical neutralizing defenses.

We hypothesized that in vivo iNOS overexpression in the chronic progress of POAG could contribute to TM cell damage, through protein nitration by reactive peroxynitrite. This process can be an important link in the chain of events leading to the oxidative damage observed in severe POAG. Therefore, the purpose of this study was to evaluate the possible correlation between the visual field defects in patients with POAG and the expression and enzymatic activity of NOS isoenzymes and NT in TM samples.

METHODS

Study Population
Human tissue was handled according to the Declaration of Helsinki and the Local Committee on Ethics in experimentation with human samples. Institutional review and approval were obtained, and all enrolled patients provided informed written voluntary consent. One hundred forty-six consecutive patients with POAG, aged 55 to 85 years (mean ± SD, 71 ± 12) were included. Table 1 summarizes the demographic distribution. Only one eye per patient was included in the study. The duration of the disease was 4 to 12 years. We included all patients who fulfilled the following inclusion criteria: a diagnosis of POAG, patients older than 50 years, access to at least three reliable baseline preoperative visual fields, and no retinal or neurologic disease that may have affected the visual field. Exclusion criteria included ocular disease other than POAG, normal-tension glaucoma, pseudoexfoliation or pigmented syndrome, previous eye surgery or laser trabecuoplasty (ALT or SLT), diabetes mellitus, uveitis, systemic collagenopathy, and objective neurologic signs.

POAG was defined as the presence of a reproducible visual field defect consistent with glaucoma and the appearance of the optic disc, along with a pretreatment IOP of 21 mm Hg or more, and an open angle with no signs of secondary causes of glaucoma.

Stratification of Patients
Perimetry was performed during the 4 weeks before surgery (Octopus tG1; Interzeag AG, Schlieren, Switzerland). We divided the patients into four groups based on mean defect (MD): MD better than −6 dB (MD > −6 dB; mild visual field loss; n = 35), MD between −6 and −12 dB (MD −6 to −12 dB; moderate visual field loss; n = 35), MD between −13 and −20 dB (MD −13 to −20 dB; severe visual field loss; n = 38) and MD worse than −20 dB (MD < −20 dB; blinding; n = 38). IOP was determined using the Goldmann applanation tonometer (GAT; Haag-Streit, König, Switzerland; Table 2).

TM Specimen Collection
The experimental protocol required removal of the TM specimens during trabeculectomy. The surgical technique has been described elsewhere. A large flap was used (scleral flap size: 8:10 × 5:6 mm, flap thickness: two thirds scleral thickness; excised inner block size: 6.5 × 4 mm) allowing us to obtain a large specimen. The TM specimens were obtained according to standard surgical procedures: to remove all specimens, a 45° knife was used to cut a 6.5 × 4 mm button of corneoscleral tissue. The TM was then dissected under microscopic control. TM specimens were either snap frozen in liquid nitrogen and stored at −80°C until assayed or fixed in 10% neutral-buffered formalin.

NO Synthase Activity
Calcium dependent and independent NOS activities were measured by the conversion of L-[14C]-arginine to L-[14C]-citrulline, as previously described. Each TM specimen was assayed independently. Frozen tissues were homogenized by sonicaton (Vibracell; Sonics & Materials, Inc., Newtown, CT) in an ice-cold buffer (pH 7.4) containing Tris-HCl (50 mM), sucrose (320 mM), dithiothreitol (1 mM), leupeptin (10 µg/mL), soybean trypsin inhibitor (10 µg/mL), and aprotinin (2 µg/mL), followed by centrifugation at 10,000g for 20 minutes 4°C. NOS activity was determined in cell extracts under conditions (substrate and calcium concentration) of maximal activity, to assess indirectly the amount of enzyme. The samples (40 µL of supernatant) were incubated at 37°C for 10 minutes in a buffer (KH2PO4, 50 mM; MgCl2, 1 mM; CaCl2, 0.2 mM; L-valine, 50 mM; L-citrulline, 1 mM; L-arginine, 20 mM; and dithiothreitol 1.5 mM) containing L-[14C]-arginine (0.1 mM/L; GE Healthcare, Iberica, Spain). The reaction was terminated by removing the substrate by the addition of 1 mL of 1:1 H2O resin (Dowex AF 500W-8; Bio-Rad Laboratories SA, Madrid Spain). The activity of the calcium-dependent NOS was calculated from the difference between L-[14C]-citrulline produced from control samples and samples containing ethylene glycol-bis(aminoethyl ether) N,N,N′-tetraacetic acid (EGTA, 1 mM). The activity of the calcium-independent isoform was determined from the difference between samples with EGTA and samples containing 1 mM N′-monomethyl-arginine (L-NMMA). The L-[14C]-bound radioactivity was counted using a liquid scintillation counter (LS-6500; Beckman Instruments, Fullerton, CA).

Quantitative Real-Time RT-PCR
eNOS and iNOS expression was detected by real-time RT-PCR. Three TM specimens from each group of patients were pooled. Total mRNA was extracted and purified (RNasy Mini Kit; Qiagen, Crayle, UK), according to the manufacturer’s protocol with residual DNA removed by DNase digestion. cDNA was synthesized using a high capacity kit (Applied Biosystem. Inc. [ABI], Foster City, CA). PCR amplification (Assays-on-Demand Gene Expression Products; ABI) and the inventory gene-specific primers: β-actin: HS99999903; eNOS: HS00167257; and iNOS: HS00167166. The cycling conditions were

<table>
<thead>
<tr>
<th>MD (dB)</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>−6 to −12</td>
<td>35</td>
<td>20.6 ± 1.8</td>
<td>18–23</td>
</tr>
<tr>
<td>−13 to −20</td>
<td>38</td>
<td>25.1 ± 2.9</td>
<td>20–30*</td>
</tr>
<tr>
<td>&lt; −20</td>
<td>38</td>
<td>28.3 ± 4.0</td>
<td>22–40*</td>
</tr>
</tbody>
</table>

n, number of patients.
* P < 0.05 vs. −6 dB group.
Lipid Peroxidation

Aqueous humor was collected from 48 POAG eyes. Forty-eight eyes that underwent cataract operation served as the control. AH was withdrawn from the anterior chamber by paracentesis before any other surgery, with special care taken to avoid blood contamination. The samples were stored at −80°C until tests were performed. The POAG samples were taken from patients classified according to their MD. Twelve patients were included in each MD group.

In AH samples (70 μL), lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde (MDA) after the method described by Das and Ratty,20 with some modifications. Samples were sonicated in 10 volumes 50 mM phosphate buffer and deproteinized with 40% trichloroacetic acid, and 5 M HCl, followed by the addition of 2% (wt/vol) thiobarbituric acid in 0.5 M NaOH. The reaction mixture was heated in a water bath at 90°C for 15 minutes and centrifuged at 12,000g for 10 minutes. The pink chromogen was measured at 532 nm in a spectrophotometer (DL-7500; Beckman). The results were expressed as the index level with respect to the group of patients with MD better than −6 dB (MD > −6 dB; mild visual field loss). A value of 1.0 was assigned to the expression of each gene in the group of patients with MD better than −6 dB (MD > −6 dB; mild visual field loss), which served as a calibrator. The expression values for all other group of patients were calculated as change in expression level with respect to the group of patients with MD > −6 dB.

Immunohistochemistry for 3-NT

Peroxynitrite formation can be estimated immunohistochemically by using anti-NT antibody, because NT is a major product of the attack of peroxynitrite on proteins17 and it has subsequently been used as a marker of ONOO− formation.20 Immediately after trabeculectomy, six TM specimens from six donors (three with MD between −13 and −20 dB [IOP: 22.6 ± 0.8 mm Hg] and three with MD < −20 dB [IOP: 24.2 ± 1.2 mm Hg]) were immediately fixed in 10% neutral-buffered formalin for 24 hours, embedded in paraffin, sectioned at 4 μm, and dried on slides (Snowcoat X-tra; Surgipath; Winnipeg, Manitoba, Canada). Three donor human eyes (age range, 65–85 years) without ocular diseases, provided by the Tissue Bank (Hospital Clínico San Carlos, Madrid, Spain) were enucleated within 2 to 3 hours after death.

Immunohistochemical staining was performed as previously described.25 Mouse monoclonal antibody against 3-NT (sc-32,757; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:75 dilution. Briefly, deparaffinized and hydrated sections were incubated in blocking solution TBT (Tris-buffered saline [TBS] 0.5 M [pH 7.4], containing 3% [wt/vol] BSA and 0.05% [vol/vol] Triton X-100) for 30 minutes at room temperature, to reduce nonspecific binding. The sections were incubated overnight at 4°C in a humidified chamber. The slides were washed for 5 minutes in TBS. Immunodetection was performed with biotinylated anti-mouse immunoglobulins followed by streptavidin conjugated with alkaline phosphatase and a fuchsin chromogen for red staining (LSAB2 kit; Dako Corp., Carpinteria, CA). The sections were lightly counterstained with Mayer’s hematoxylin. Final mounting was in water-soluble medium (Glicergel; Dako). Negative controls were treated in the same manner but with the omission of the primary antibodies. With the observer blinded to the experimental conditions, a series of three images of equal surface of 3NT-stained TMs were delineated with an operator-controlled cursor. Sections were analyzed with an image analyser (Image processing and analysis in Java Image; National Institutes of Health, Bethesda, MD).

Statistical Analysis

Results were expressed as the mean ± SE. Statistical analysis was performed with Student’s t-test. Correlations between IOP and iNOS expression were evaluated by using the Spearman and simple regression tests (SPSS ver. 14; SPSS, Chicago, IL). The results were considered statistically significant at P < 0.05.

RESULTS

Expression of iNOS mRNA and Calcium-Independent NOS Activity in Samples from Patients with POAG

iNOS mRNA expression were significantly higher in the TM specimens from the MD < −13 to −20 dB and MD < −20 dB groups compared with those of the MD > −6 dB group (Fig. 1A).

Ex vivo analysis of calcium-independent NOS activity in the soluble fraction of these samples paralleled these findings (Fig. 1B). POAG caused the appearance of iNOS expression and activity in TM, which increased with the degree of severity. As shown in Figure 1, iNOS appeared when MD reaches severe visual field loss −13 to −20 dB (300 ± 30 pg/min per mg protein) and < −20 dB (400 ± 15 pg/min per mg protein). Spearman ρ showed significant correlation among iNOS expression and IOPs (Fig. 2) in patients with terminal (MD < −20 dB; mild visual field loss). A value of 1.0 was assigned to the expression of each gene in the group of patients with MD better than −6 dB (MD > −6 dB; mild visual field loss).
The reported results demonstrate that iNOS expression and activity increase in TM of patients with POAG, in parallel with visual field defects. Of interest, the expression of eNOS and activity of Ca\(^{2+}\)-dependent NOS decreases as the visual defect increases. The induction of the cytotoxic, high-output enzymatic source of NO, and the functional decrease in the physiological NOS leads to the accumulation of MDA in AH, a marker of lipid damage by free radicals. Finally, this is the first study, to our knowledge, that shows the expression of the powerful

**FIGURE 2.** Scatterplots of the relationship between the relative expression of iNOS in TM of patients with severe POAG and their IOPs. Spearman \(\rho\) showed significant correlation \((r = 0.41, P = 0.15)\) in patients with MD between 0 and 6 (MD = −6 dB; Fig. 4). The levels of MDA in AH of cataract patients were similar to those in the other MD groups.

**Expression of eNOS mRNA and Calcium-Dependent NOS Activity in Samples from Patients with POAG**

eNOS mRNA expression was significantly lower in the TM when MD reached severe visual field loss at −13 to −20 dB (6.3 ± 1.5 pg/min per mg protein) in comparison with that in the MD < −6 dB (140 ± 20 pg/min per mg protein) and MD = −6 to −12 (120 ± 15 pg/min per mg protein) eyes.

**Oxidative Consequences in AH of Patients with POAG**

POAG caused the accumulation of the mediator of lipid peroxidation malondialdehyde (MDA) in AH of patients with POAG with terminal disease (MD < −20 dB; Fig. 4). The levels of MDA in AH of cataract patients were similar to those in the other MD groups.

**Localization of Peroxynitrite by 3 NT Staining**

In TM specimens from eyes with MD between −13 and −20 dB (24.2 ± 1.2 mm Hg; Fig. 5A) and with MD < −20 dB (IOP: 27 ± 1.9 mm Hg; Fig. 5B), moderate to intense labeling intensity was found in many but not all cells of the uveal, corneoscleral meshwork, and juxtacanalicular tissue. Only a few cells of the inner wall endothelium of SC were stained. In the control section (IOP: 15.3 ± 0.9 mm Hg), no signal was detected (Fig. 5C).

The 3-NT immunostaining quantification obtained from image processing and analysis were: control \((n = 3)\): 54.4 ± 2 (AU, arbitrary units; Fig. 5C); MD between −13 and −20 dB \((n = 3)\): 80.7 ± 4 AU \((P < 0.05\) vs. control, Fig. 5A); and MD ≤ −20 dB \((n = 3)\): 151.8 ± 17 AU \((P < 0.05\) vs. MD between −13 and −20 dB and control, Fig. 5B). A correlation between the results of the immunostaining quantification and the IOP in each group was observed (Table 3).

**DISCUSSION**

The reported results demonstrate that iNOS expression and activity increase in TM of patients with POAG, in parallel with the accumulation of MDA in AH, a marker of lipid damage by free radicals. Finally, this is the first study, to our knowledge, that shows the expression of the powerful
oxidant peroxynitrite (by 3-NT staining) in the TM of patients with severe to blinding POAG.

The induction of iNOS in the TM from severe POAG along with MDA accumulation in the AH is concomitant with extensive and prolonged release of NO metabolites, which finally lead to a production of other oxidant species such as peroxynitrite.  

The decrease in eNOS function is in agreement with previously published data in POAG, where the population of TM endothelial cells is markedly decreased compared with that of age-matched healthy subjects. Taking into account the cytotoxic effects of iNOS and particularly of ONOO⁻, we hypothesized that the increased production of NO by iNOS present in the TM of patients with POAG could contribute to the death of TM cells. The double-faced effect of NO also appears to increase in this disease. The fact that the TM of patients with POAG contains less eNOS than that of normal control subjects suggests that the NO pathway is affected. Of interest, some studies suggest that eNOS gene polymorphisms act as an additional risk factor in the development of endothelial dysfunction in glaucoma.  

We have demonstrated the presence of ONOO⁻ by showing positive NT immunoreactivity in cells from uveal, corneoscleral meshwork, and juxtacanalicular tissues. Such a distribution within the TM from patients with severe POAG indicates a long-term exposure to peroxynitrite.  

Mechanisms by which POAG induces iNOS on the human TM remain to be elucidated. In vitro studies of perfused anterior segments of human donor eyes have shown that NO production increases after elevation of the pressure gradient over the TM accompanied by an upregulation of iNOS gene expression. In agreement, our study demonstrated a statistically significant correlation between iNOS expression and IOP values in patients with terminal POAG. Indeed, we observed an increase in this disease. The fact that the TM of patients with POAG donors demonstrated downregulated expression of the antioxidants paraoxonase 3 and ceruloplasmin.  

The membrane lipid peroxidation due to an oxyradical attack on membrane fatty acids results in the formation of several cytotoxic aldehyde products, such as MDA and has been described to play an important role in the pathogenesis of several degenerative diseases.  

This process is of particular interest, since the high-output isoform of NO synthase (iNOS) has been implicated in cellular toxicity in many cell systems. Once iNOS is expressed, the formation of large amounts of oxygen and nitrogen reactive species may account for the oxidation of cellular components—membrane lipid peroxidation due to the oxyradical attack on membrane fatty acids resulting in the formation of several cytotoxic aldehyde products, such as MDA.  

In conclusion, the expression of iNOS and the activity of the inducible Ca²⁺-independent iNOS in the TM of patients with POAG are proportional to the visual field defect as well as the loss of constitutive eNOS activity. These changes lead to the increased NT in severe POAG, which may serve as a marker of oxidative stress in the progression of cell death in POAG. Further studies should determine whether specific inhibition of iNOS would be of therapeutic benefit in this condition of vast overproduction of NO.

<table>
<thead>
<tr>
<th>Group of Patients</th>
<th>IOP (mm Hg)</th>
<th>3-NT (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.3 ± 0.9</td>
<td>54.4 ± 2</td>
</tr>
<tr>
<td>MD &lt;–13 and –20 dB</td>
<td>24.2 ± 1.2</td>
<td>80.7 ± 4*</td>
</tr>
<tr>
<td>MD &lt;–20 dB</td>
<td>27.0 ± 1.9*</td>
<td>151.8 ± 17†</td>
</tr>
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</table>

* P < 0.05 vs. control.
† P < 0.05 vs. control and MD <–13 and –20 dB.
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References