Evaluation of Inducible Nitric Oxide Synthase in Glaucomatous Optic Neuropathy and Pressure-Induced Optic Nerve Damage

Iok-Hou Pang,1 Elaine C. Johnson,2 Lijun Jia,2 William O. Cepurna,2 Allan R. Shepard,1 Mark R. Hellberg,1 Abbot F. Clark,1 and John C. Morrison2

PURPOSE. To determine whether inducible nitric oxide synthase (NOS-2) is involved in glaucomatous optic neuropathy.

METHODS. Chronic elevation of rat intraocular pressure (IOP) leading to optic nerve damage was induced by episcleral injection of hypertonic saline, which caused sclerosis and blockade of aqueous humor outflow pathways. Expression of NOS-2 in the retina and optic nerve head (ONH) was evaluated by immunohistochemistry, gene array analysis, and quantitative PCR (Q-PCR). Immunohistochemistry was also used to assess the NOS-2 level in the ONH from primary open-angle glaucoma (POAG) and nonglaucomatous human eyes. Finally, an NOS-2 inhibitor, aminoguanidine, administered orally in the drinking water, was tested for its effect on optic nerve injury in rats with ocular hypertension.

RESULTS. Chronically elevated IOP in the rat produced optic nerve damage that correlated with pressure change ($r^2 = 0.77$), but did not increase NOS-2 immunoreactivity in the optic nerve, ONH, or ganglion cell layer. Retinal and ONH NOS-2 mRNA levels did not correlate with either IOP level or severity of optic nerve injury. Similarly, there was no difference in NOS-2 immunoreactivity in the optic nerve or ONH between POAG and nonglaucomatous eyes. Furthermore, aminoguanidine treatment did not affect the development of pressure-induced optic neuropathy in the rat.

CONCLUSIONS. As demonstrated by several independent methods, glaucomatous optic neuropathy was not associated with a significant change in the expression of NOS-2 in the retina, ONH, or optic nerve. (Invest Ophthalamol Vis Sci. 2005;46:1313-1321) DOI:10.1167/iovs.04-0829

Approximately 70 million people worldwide have glaucoma. Over the course of their lives, 10% of these patients will be bilaterally blind.4 The blindness is a result of progressive glaucomatous optic neuropathy and retinopathy.2-4 Many hypotheses have been proposed to describe the biological events involved in the pathologic course of this disease.3-7 The notable theories are ocular hypoperfusion and ischemia,8,9 disruption of the supply of trophic factors to the retinal ganglion cells (RGCs),10-12 and retinal excitotoxicity.13,14 Although there is circumstantial evidence supporting each of these theories, none has been unequivocally proved.

Recently, activation of nitric oxide synthase (NOS) has been reported as another potential mechanism of glaucomatous damage to the retina and optic nerve. This enzyme was associated with the death of RGC caused by ischemic injury, since the NOS inhibitors, aminoguanidine and $N^\text{G}$-nitroarginine, protect against retinal-ischemia–induced RGC loss.15,16 This observation prompted Geyer et al.17 to speculate that NOS inhibitors may be useful agents in the treatment of glaucoma. Subsequently, an increased presence of the NOS isoforms, neuronal NOS (NOS-1 or nNOS) and inducible NOS (NOS-2 or iNOS), was reported in astrocytes of the lamina cribrosa and optic nerve head (ONH) of patients with primary open-angle glaucoma (POAG).15,18 In rats whose extraocular veins were cauterized to produce chronic ocular hypertension and retinal damage, expression of NOS-2, but not NOS-1, was increased in ONH astrocytes.19 Elevation of hydrostatic pressure in vitro was sufficient to upregulate expression of NOS-2 in cultured rat RGCs20 and human astrocytes derived from the ONH.21 Most important, inhibition of NOS-2 by aminoguanidine or 1-N6(1-iminoethyl)lysine 5-tetrazole amide was shown to protect against RGC loss in the rat cautery model of retinopathy.22-23 These data suggest that activation of NOS, especially NOS-2, may play a significant role in glaucomatous optic neuropathy and retinopathy.

However, as indicated earlier, the preclinical evidence for NOS-2 involvement in optic neuropathy has been shown only in one specific rat model where retinal injury was generated by cauterization of extraocular blood vessels. Occlusion of these vessels may induce damage other than that related to elevated intraocular pressure (IOP), such as ocular ischemia,24-26 which may confound the conclusion that NOS-2 is critical to glaucoma- or ocular-hypertension–induced RGC loss. Furthermore, a recent preliminary report has argued that NOS-2 is not involved in the optic neuropathy occurring in a mouse model of pigmentary glaucoma (Libby RT, et al. JOVS 2005;44:ARVO E-Abstract 145).

In view of these uncertainties and contradictory results, we decided to determine whether NOS-2 is upregulated in another rat model of chronic ocular hypertension, in which hypertonic saline was injected into the aqueous humor outflow pathway to cause sclerosis and blockade of aqueous outflow pathways.27,28 In this model, pressure elevation is produced by obstruction of aqueous outflow, the mechanism thought to occur in most patients with glaucoma. Initially, we used immunohistochemistry to assess levels of NOS-2 in both the normal and glaucomatous rat retina, optic nerve, and ONH. We followed this by using more sensitive methods to evaluate the expression of NOS-2 mRNA in the same tissues of glaucoma-
tous rats. We then confirmed these observations by immuno- 
histochemical evaluation of human ONH and optic nerve from 
normal individuals and patients with POAG. Finally, we treated 
rats having experimentally elevated IOP with a NOS-2 inhibi-
tor, aminoguanidine, to determine whether NOS-2 inhibition 
could provide neuroprotection in this model of pressure-in-
duced optic neuropathy.

METHODS

Rat Glaucoma Model

Adult male Brown Norway rats weighing 300 to 400 g were used in the 
study. All animal procedures were performed in compliance with the 
ARVO Statement for the Use of Animals in Ophthalmic and Vision 
Research. Animals were housed in a constant low-light (40–90 lux) 
environment, which minimized IOP circadian oscillations and facil-
tated accurate IOP history determination.29 Elevated IOP was induced 
unilaterally by an injection of hypertonic saline into one of the epis-
cleral veins, as previously described.30 The daily IOPs of each animal 
throughout the experimental period of 5 weeks were averaged and 
recorded as the mean IOP. Furthermore, the daily difference in IOP 
between the injected and contralateral control eye summed for the 
experimental duration (35 days) is reported as the cumulative IOP 
increase (mm Hg/day).

Induction of NOS-2 Expression

To obtain a positive control for NOS-2 mRNA analysis, intraperitoneal 
injection of lipopolysaccharide (6 mg/kg) was used to induce liver 
NOS-2 expression in the rat, as previously described.31 In addition, rat 
eyes with endotoxin-induced uveitis (a kind gift from the laboratory of 
Tammy Martin, Oregon Health and Science University) were used as 
a positive control for NOS-2 immunohistochemistry.32 In this case, eyes 
were collected 24 hours after subcutaneous injection of endotoxin 
(200 μg at 1 μg/μL).

Optic Nerve Transection

Optic nerve transection was performed under general anesthesia (in-
tramuscular injection of ketamine 55 mg/kg, xylazine 5 mg/kg, and 
aicetamide 1 mg/kg) with additional topical anesthesia (1–2 drops 
of 0.5% proparacaine HCl), as previously described.33 In brief, the 
eye and sheath were accessed, an incision made in the superior 
aspect of the meningeal sheath, and the optic nerve proper gently 
lifted up and away from the underlying ophthalmic artery. The optic 
nerve was then transected approximately 2 mm behind the globe 
without damaging the ocular blood supply. Completeness of transec-
tion was confirmed by visual examination of the posterior end of the 
stump. Normal retinal perfusion was verified with direct ophthalmos-
copy immediately after the operation. Postoperative analgesia was 
provided with intramuscular injections of buprenorphine hydrochlo-
ride (0.075 mg/kg, Buprenex; Reckitt & Colman Products, Richmond, 
VA) every 8 hours for 2 to 3 days. Eyes were also dressed with a 
broad-spectrum antibiotic ointment (bacitracin and polymyxin B sul-
phate [AK-POLY-BAC]; Akorn, Inc., Buffalo Grove, IL), which was reap-
plied twice daily for 4 days. Tissues were collected 10 days after 
transection.

Rat Tissue Collection

All animals were deeply anesthetized with isoflurane before tissue 
collection. For mRNA analysis, animals were decapitated and the eyes 
quickly enucleated and chilled in iced phosphate-buffered saline. Ret-
inas were dissected on ice and quickly frozen in dry ice or liquid N₂ 
and stored at ~70°C. Optic nerve heads were dissected from the 
surrounding sclera with a 1-mm trephine. The optic nerve sheath and 
adjacent sclera were then removed and the length of the tissue short-
ened to 1 mm from the optic disc. Approximately half this length 
represents the unmyelinated portion of the optic nerve. Dissected 
ONH were frozen on dry ice and stored at ~80°C until use. Liver 
samples from the lipopolysaccharide-treated rat were collected at 6 
hours after injection and frozen for use as a positive control for NOS-2 
mRNA induction.

For immunohistochemistry, animals were transcardially perfused 
with 4% phosphate-buffered paraformaldehyde and globe sections 
prepared as previously described.34 The optic nerves from these animals 
were postfixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 
phosphate buffer. For the aminoguanidine experiment, animals were 
perfusion fixed with 5% buffered glutaraldehyde and prepared as 
previously described.35

Evaluation of Optic Nerve Injury

Fixed rat optic nerves were embedded in Spurr's resin, sectioned, and 
stained with toluidine blue.36 Optic nerve injury was graded as previ-
ously reported.37 In brief, sections from approximately 2 mm behind 
the globe were evaluated under light microscopy by five masked 
observers based on the Optic Nerve Injury Grading (ONIG) system, 
which grades each optic nerve from 1 (normal) to 5 (near total 
degeneration). The ONIG of each optic nerve is reported as the mean 
value of all observers' grades.

Immunohistochemistry on Rat and 
Human Tissues

Fixed rat eyes were embedded in paraffin and longitudinal sections 
through the ONH prepared.28 POAG (n = 8) and control (n = 11) 
human eyes were obtained through the Glaucoma Research Founda-
tion and the Oregon Lions Eye Bank, respectively. Clinical histories 
were obtained for POAG eyes to confirm diagnosis. POAG eyes had a 
mean cup-to-disc ratio of 0.7 ± 0.1 (mean ± SD; range, 0.5 to 0.9, 
similar to a previous study38) and a history of elevated IOP (>21 mm 
Hg). Six histories contained records to document visual field defects, 
and all donors were taking at least one glaucoma medication and four 
had undergone surgical intervention to control IOP. Control eyes had 
no evidence of glaucoma or retinal disease. The ONH, with attached 
nasal and temporal regions of the retina, were removed and frozen in 
optimal cutting temperature (OCT) compound, and longitudinal sec-
tions were prepared. There was no significant difference between 
the two groups in either the mean age (77 and 75 years, respectively) or 
the mean interval between death and freezing of the tissues (23 and 21 
hours, respectively). As a positive control, human benign prostate 
hyperplasia samples (a generous gift from Victor K. Lin, Department 
of Urology, University of Texas Southwestern Medical Center, Dallas, TX) 
were included. They were collected from a location within 5 mm lateral 
to the proximal urethra, OCT embedded, and sectioned at 6 μm in 
thickness.

Three antibodies were used in the study: (1) NOS2 polyclonal 
(SC-650), 1 and 4 μg/mL (Santa Cruz Biotechnology, Santa Cruz, CA); 
(2) iNOS-OX polyclonal, 1:1000 and 1:4000 dilution (Oxford Biomed-
ical Research, Oxford, MI), and (3) iNOS-BD, 4 μg/mL (Transduction 
Laboratories, San Diego, CA). Purified IgG served as a negative control. 
Immunohistochemistry was performed by the avidin-biotin complex 
(ABC) technique with the 3,3′-diaminobenzidine (DAB) chromogen, 
as previously described.28 The intensity of staining in the indicated 
regions was graded by two masked observers on a scale of 0 to 4, where 
0 = no staining, 1 = pale orange-brown; 2 = orange-brown, 3 = dark 
orange-brown, and 4 = intensely dark brown-black staining. Grades 
reported are the mean of the scores given by the two observers. Two 
complete globe sections (including the entire length of the retina) per 
rat eye and three adjacent sections of optic nerve and ONH from each 
human eye were evaluated.

Gene Expression Profiling

Total RNA, isolated from rat retina and ONH (TRizol reagent; Invitro-
gen, Carlsbad, CA) from 18 hypertonic saline-injected eyes, was di-

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vided into three groups and pooled (n = 6 in each group) according to the severity of optic nerve injury (group 1: ONIG < 1.5; group 2: ONIG = 1.5–4.5; group 3: ONIG > 4.5). The pooled study samples were then compared by a rat gene microarray analysis (GeneChip; Affymetrix, Santa Clara, CA) to those obtained from uninjected eyes. Reverse transcription, second-strand cDNA synthesis, and biotin-labeling of amplified RNA, as well as hybridization, washing, and scanning of the arrays were performed according to standard Affymetrix protocols. Hybridized GeneChip arrays were scanned (GeneArray scanner; Agilent Technologies, Palo Alto, CA). Raw data were collected and analyzed on computer (Microarray Suite software, ver. 5.0; Agilent Technologies, Palo Alto, CA). Relative intensity of retinal RNA was confirmed by gel electrophoresis, while the amount of ONH RNA (~100 ng per eye) was too low for this analysis.

For reverse transcription of total RNA, 150 ng retinal or 40 ng of ONH RNA was incubated in 20 μL of reverse transcription buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl2, 8 mM dithiothreitol, 0.1 mM each deoxyribonucleoside triphosphates, 0.8 U/mL RNAsin RNase inhibitor, and 8 ng/mL oligo-dT12-18) and incubated at 37°C for 2 hours with 8 U/mL M-MLV reverse transcriptase (Invitrogen-Gibco, Rockville, MD). Control experiments for genomic DNA contamination, such as RNase treated of isolated RNA, were negative.

Retinal and ONH cDNA were amplified with a thermocycler (Light-Cycler with LightCycler Software, ver. 3.5, and DNA Master SYBR Green 1 kit; Roche, Indianapolis, IN), according to the manufacturer’s protocol with a final concentration of 4 mM MgCl2 and 0.15 μM each primer. Each assay of experimental samples included the relevant standard curve for relative quantitation, as well as a standard curve of lipopolysaccharide-treated liver cDNA as a positive control. PCR was followed by the generation of melting curves for the amplified products to verify amplification specificity. Quantitation of PCR product was made by the fit point method, according to the manufacturer’s manual.

The primers used for each cDNA were designed on computer (Primer Designer 3 software for Windows; Sci-ed Software, State Line, PA) using sequences for rat mRNAs available in the NIH National Center for Biotechnology Information databases. NOS-2 forward primer was 5’GAT ATC TTC GGT GCC GTC TT and the reverse primer was 5’GGC CAG ATG CTG TTA CTC TT, producing a 105-bp product that was confirmed by sequencing. The housekeeping gene for the retina and ONH was glyceraldehyde phosphate dehydrogenase (GAPDH), whose forward primer was 5’CAT CAA GAA GGT GGT GAA GCC GG and the reverse primer was 5’CCA CCA CCC TGT TGC TGT AGC CA to yield a product of 206 bp. The GAPDH mRNA in the retina and ONH showed no significant correlation to either IOP or ONIG. Assay for the housekeeping gene was performed in triplicate on cDNA from each experimental sample and the mean values used for normalization of NOS-2 data for each sample. For the GAPDH PCR reaction, 0.4 μM primers were used.

According to the study in vivo effects of an NOS-2 inhibitor on chronic pressure-induced optic neuropathy, drinking water containing aminoguanidine (2 g/L; Sigma-Aldrich, St. Louis, MO) was freshly provided to rats three times a week for 42 days starting 7 days before hypertonic saline injection. Control rats received untreated drinking water. For all animals, IOP was measured daily before and after hypertonic saline injection. Throughout the experiment, individuals measuring IOP were masked as to which animals received the aminoguanidine-treated water. Optic nerve injury was also evaluated in a masked manner.

### Statistical Analysis

To compare data between two groups, Student’s t test was used. To compare data among three or more groups, one-way analysis of variance (ANOVA) followed by the Dunnett or Bonferroni test was used. Results with P < 0.05 were considered as statistically significant.

### Results

#### IOP and Optic Nerve Injury in the Rat Glaucoma Model

As reported previously, mean IOP for rats housed in low level, constant light stabilized at 28.6 ± 0.1 mm Hg (mean ± SEM, n = 44). Mean IOP in hypertonic saline-injected eyes during the 5-week postinjection period ranged from 26.8 to 44 mm Hg and correlated highly with ONIG (r² = 0.77). Figure 1 illustrates the relationship between mean IOP and ONIG for 44 un.injected and 80 injected eyes sorted into groups with different levels of nerve injury. For injected eyes with ONIG < 1.5, mean IOP was not significantly elevated at 30.0 ± 0.5 mm Hg, compared with fellow eyes. Their cumulative IOP increase (as defined by the difference in IOP between the injected and contralateral control eye multiplied by duration) was 37 ± 17 mm Hg·d. The IOPs of all other groups were significantly different from one another (P < 0.01). For optic nerves with moderate damage (ONIG = 1.5–4.5), the mean IOP was 33.3 ± 0.5 mm Hg (cumulative IOP increase = 165 ± 19 mm Hg·d), whereas the mean IOP for optic nerves with ONIG > 4.5 was 38.2 ± 0.5 mm Hg (cumulative IOP increase = 355 ± 18 mm Hg·d). Therefore, the optic nerves from injected eyes in this study represent a full range of response due to the elevated IOP: from no apparent injury to optic nerves with active degeneration across the entire nerve cross-section.
Immunohistochemical Localization of NOS-2 in the Rat

Initially, we used this glaucoma model to look for changes in NOS-2 levels in the ONH and retina after IOP elevation, ex-

pecting results similar to those reported in another pressure-
induced injury model. The NOS2 (SC-650) antibody (1 µg/ 

ml) resulted in a low-level, relatively even distribution of label in both 

groups, as illustrated by the control 

retina (A) and ONH (D), compared with the retinas (B, C) and ONH (E, 

F) from elevated-IOP eyes with an 

ONIG of 2.5 and 4.9, respectively. 

The intensity of this label was graded 

in a masked fashion and is presented 

in Table 1. In contrast, strong perinu-

clear localization of NOS2 was visible 

in limbal cells of uveitic rat eyes (G, 
arrows). No labeling was seen with 

the iNOS-BD antibody (4 µg/mL) in 

an uninjected eye (H) and elevated- 

IOP eye with ONIG of 2.5 (I), in 

contrast to positive staining in a uve-

itic eye limbus (J). Similar lack of 

labeling was found with the iNOS-OX 

antibody (1:4000 dilution) in the ret-

ina and ONH of an uninjected (K) 

and an elevated-IOP eye with ONIG 

of 3.4 (L). Note the disorganization 

of glial columns in the ONH of eyes 

with elevated IOP (E, F, I, L), indi-

cating glial responses to the injury. 

All sections (except G, J) were ori-

ented with the vitreous up. In Brown 

Norway rats, dark brown pigment 

was present in the nerve sheath (S) 

and choroid in ONH sections. Scale 

bars: (A–C, G, J) 10 µm; (D–F, H, I, 

K, L) 50 µm.
were disorganized, indicating glial responses to the injury (Figs. 2E, F, I, L). Three different NOS-2 antibodies were tested, including two referenced in previous rat studies. As illustrated in Figure 2, none of the three antibodies detected NOS-2 in either the ONH or retinas in both the control and hypertensive eyes. However, the same antibodies resulted in strong cellular staining of NOS-2 in the limbus of uveitic rat eyes (Figs. 2G, 2J). More concentrated antibody solutions resulted in faint and diffused labeling of neural tissues in both control and hypertensive eyes. When the intensity of immunoreactivity of each sample stained with the NOS2 (SC-650) antibody at 1 μg/mL titer was graded by two masked observers, there was no difference (P > 0.05) between the hypertensive and normal rat eyes (Table 1). The staining scores of the ONH, optic nerve, and ganglion cell layer among the three study groups were all 1.2 or below, on a scale ranging to 4. In contrast, scleral cells of uveitic eyes evaluated under the same conditions had a significantly higher mean staining score of 2.7.

### Immunohistochemical Localization of NOS-2 in Human Eyes

Based on immunohistochemical staining, NOS-2 induction has been reported in ONH from humans with POAG. Therefore, we used the same NOS-2 antibody to evaluate sections of frozen ONH and optic nerve from both POAG (n = 8) and nonglaucomatous (n = 11) human eyes. Similar to the above rat results, we found no difference in NOS-2 levels between the two groups (Fig. 3, Table 2) in all regions of the ONH and optic nerve examined. All staining scores were 1.1 or lower. In addition, we found no significant correlation between the intensity of immunoreactivity in any region of the POAG eyes and their respective cup-to-disc ratio (\( r^2 < 0.1, P > 0.5 \)). The immunohistochemistry technique we used in the human ocular tissue was able to detect expression of NOS-2 in the ducal epithelium of human benign prostate hyperplasia tissue (Fig. 3E), which had a mean staining score of 3.0.

### NOS-2 mRNA in the Rat Eye

Because immunohistochemical analysis failed to demonstrate any differences in NOS-2 in response to IOP elevation and optic nerve injury in either rat or human specimens, we used additional and more sensitive methods to assess NOS-2 expression after increased IOP in the rat eye. Initially, expression of NOS-2 mRNA was analyzed by gene microarray (GeneChip; Affymetrix), using both the rat genome U34A GeneChip, which contains five probe sets for NOS-2, and the Rat Expression Set 230A GeneChip, which contains two additional NOS-2 probe sets. For all seven NOS-2 probe sets, NOS-2 mRNA levels in the ONH and retina of control or the three groups of hypertensive eyes with different severities of optic nerve damage were all flagged as below detectable level (data not shown), suggesting that NOS-2 mRNA was not upregulated in the glaucoma eyes.

### Table 1. Comparison of NOS-2 Immunoreactivity in Control and Ocular Hypertensive Rat Eyes

<table>
<thead>
<tr>
<th>Control Eyes (n = 7)</th>
<th>Moderate Damage (n = 7)</th>
<th>Severe Damage (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optic nerve</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Optic nerve head</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Ganglion cell layer</td>
<td>0.7 ± 0.2</td>
<td>0.7 ± 0.4</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM. IOP of control eyes = 28.8 ± 0.2 mm Hg; moderate damage eyes = 35.8 ± 1.7 mm Hg (cumulative IOP increase = 175 ± 60 mm Hg · d); severe damage eyes = 39.7 ± 0.5 mm Hg (cumulative IOP increase = 382 ± 18 mm Hg · d). ONIG of control eyes = 1.0 ± 0.0; moderate damage eyes = 3.0 ± 0.2; severe damage eyes = 5.0 ± 0.0. NOS-2 immunoreactivity was evaluated by masked readers who used a grading system ranging from 0 (no stain) to 4 (very heavy stain) on tissues stained with the NOS2 (SC-650) antibody at 1 μg/mL. There is no statistically significant difference in staining intensities among the three groups in any region. There is no statistically significant correlation between ONIG and staining intensity in any region.

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**Figure 3.** Lack of NOS-2 immunoreactivity (orange-brown label) in control (A, C) and POAG (B, D) human ONHs. (B) Cup-to-disc ratio was 0.9, with visual field limited to a central island; (D) cup-to-disc ratio was 0.8, with an inferior neuroretinal rim notch. The NOS2 (SC-650) antibody at 1 μg/mL was used on these sections. Arrows: glial nuclei; in both POAG ONHs, the glial columns appeared disrupted, indicating tissue damage. No consistent difference in NOS-2 immunoreactivity in ONH between control and POAG was detected (see Table 2). Sections were oriented with the vitreous up. Arrows: glial nuclei (blue counterstain); arrowheads: marking the Bruch’s membrane and (S) signifying the sclera. Under the same experimental conditions, positive NOS-2 immunostaining (arrow) was clearly visible in the ducal epithelium of human benign prostate hyperplasia tissue (E). Scale bars: (A–D) 50 μm; (E) 5 μm.
To confirm these findings, ocular hypertension and optic nerve damage were induced in other rats and retinal and ONH mRNA extracted separately. Quantitative Q-PCR was used to quantify the mRNA content encoding NOS-2 in each of the samples, and the results were compared with those in untreated fellow eyes. It is evident that, although retinal NOS-2 mRNA levels tended to increase slightly in ocular hypertensive eyes, these changes were not statistically significant ($P > 0.05$), and they did not correlate with the increase in mean IOP (Fig. 4). Similarly, no consistent or statistically significant changes ($P > 0.05$) in NOS-2 mRNA contents were observed in the ONH of treated rats though there was a tendency of decrease in NOS-2 mRNA in the moderately and severely damaged samples (Fig. 5). There was no significant correlation between ONIG and NOS-2 mRNA levels in the retina ($r^2 = 0.02$) or the ONH ($r^2 = 0.05$).

These observations agreed with the gene array study that ocular hypertension and the consequent optic nerve damage did not significantly upregulate NOS-2 gene transcription in the retina or ONH. In addition to elevated IOP, optic nerve transection also did not significantly affect the NOS-2 mRNA level in the ONH compared with the control samples ($P > 0.05$; Fig. 5).

**In Vivo Inhibition of NOS-2 Activity by Aminoguanidine**

Because aminoguanidine, an NOS-2 inhibitor, has been reported to be protective in a rat model of retinopathy, we tested whether the same treatment regimen could protect against optic nerve damage in the hypertonic saline injection model. Oral administration of rats with aminoguanidine solution did not affect the basal IOP of control eyes (IOP = 28.6 ± 0.1 mm Hg, $n = 13$, in the water-treated group; and 28.5 ± 0.1 mm Hg, $n = 17$, in the aminoguanidine-treated group). In addition, there was no significant difference between the groups in the mean level of IOP reached in the hypertonic saline-injected eyes (IOP = 34.4 ± 1.1 mm Hg, $n = 21$, in the water-treated group eyes; and 34.9 ± 0.9 mm Hg, $n = 18$, in the aminoguanidine-treated group eyes; Fig. 6). Most impor-

**FIGURE 4.** Lack of correlation between rat optic nerve damage and mRNA levels of NOS-2 in the retina. There was no statistically significant difference among the groups ($P > 0.05$, one-way ANOVA). In addition, there was no significant correlation between injury grade and NOS-2 mRNA levels ($r^2 = 0.02$). Data are expressed as the mean ± SEM.

**FIGURE 5.** Lack of correlation between rat optic nerve damage and mRNA levels of NOS-2 in the optic nerve head. There was no statistically significant difference among the groups ($P > 0.05$, one-way ANOVA). In addition, there was no significant correlation between injury grade and NOS-2 mRNA in the elevated IOP groups ($r^2 = 0.05$). NOS-2 mRNA level in ONH of the optic-nerve–transected group was also not significantly affected. Data are expressed as the mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 11)</th>
<th>POAG (n = 8)</th>
<th>Control IgG (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prelamina: astrocytes in glial columns</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Prelamina: nerve fiber bundles</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Lamina: astrocytes associated with lamina beams</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Lamina: nerve fiber bundles</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Optic nerve: septal-associated glial cells</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Optic nerve: nerve fiber bundles</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SEM. NOS-2 immunoreactivity was evaluated by masked readers who used a grading system ranging from 0 (no stain) to 4 (very heavy stain) on tissues stained with the NOS2 (SC-650) antibody at 1 μg/mL. There is no statistically significant difference in staining scores between the two groups in any region. Control IgG were conducted in three nonglaucoma and two glaucomatous tissues. There was no difference between the two sets of samples; hence, the combined results are reported.

**TABLE 2.** Comparison of NOS-2 Immunoreactivity in Human Control and POAG Ocular Tissues

Data are presented as the mean ± SEM. NOS-2 immunoreactivity was evaluated by masked readers who used a grading system ranging from 0 (no stain) to 4 (very heavy stain) on tissues stained with the NOS2 (SC-650) antibody at 1 μg/mL. There is no statistically significant difference in staining scores between the two groups in any region. Control IgG were conducted in three nonglaucoma and two glaucomatous tissues. There was no difference between the two sets of samples; hence, the combined results are reported.

**FIGURE 4.** Lack of correlation between rat optic nerve damage and mRNA levels of NOS-2 in the retina. There was no statistically significant difference among the groups ($P > 0.05$, one-way ANOVA). In addition, there was no significant correlation between injury grade and NOS-2 mRNA levels ($r^2 = 0.02$). Data are expressed as the mean ± SEM.

**FIGURE 5.** Lack of correlation between rat optic nerve damage and mRNA levels of NOS-2 in the optic nerve head. There was no statistically significant difference among the groups ($P > 0.05$, one-way ANOVA). In addition, there was no significant correlation between injury grade and NOS-2 mRNA in the elevated IOP groups ($r^2 = 0.05$). NOS-2 mRNA level in ONH of the optic-nerve–transected group was also not significantly affected. Data are expressed as the mean ± SEM.
In this study, we used several techniques to determine whether NOS-2 expression or its activation correlates with ocular hypertension and the consequent optic neuropathy in a rat model of glaucoma. The rat model used in the present study is a well-characterized experimental model of glaucoma, in which there is a good correlation between IOP elevation and optic nerve damage (Fig. 1). In addition, IOP elevation in this model produces selective loss of RGCs and deposition of extracellular matrix in the ONH, similar to glaucoma in humans.\(^{36}\)

We showed, using multiple antibodies, that the intensity and distribution of immunoreactivity of NOS-2 in the retina, ONH, and optic nerve of normal and glaucomatous eyes were indistinguishable. These results in the rat corroborated those from human ocular tissues. In our study, NOS-2 expression was not different between human glaucomatous and control eyes.

We further demonstrated that NOS-2 mRNA levels in the retina and ONH were not significantly changed regardless of the mean IOP elevation or severity of optic nerve injury. The negative results were not due to the lack of sensitivity of the techniques used. The gene expression profiling was sufficiently sensitive to demonstrate damage-dependent changes in the transcription of other genes in this same model of ocular hypertension.\(^{37,38}\)

The present study also demonstrated that the relatively selective NOS-2 inhibitor aminoguanidine was not efficacious in protecting against the optic neuropathy induced by ocular hypertension. Although we did not directly assay rat ocular tissues to find out if the treatment schedule produces a pharmacologically effective concentration of aminoguanidine in the retina or optic nerve, similar dose regimens have been shown by others to be sufficient to generate in vivo pharmacological effects.\(^{39,40}\) Most important, the preparation of aminoguanidine solution, dosage, and route of administration used in this study were identical with those in a previous report,\(^{22}\) which showed that the treatment was equivalent to an oral dose of 60 mg/kg per day in the rat and was protective against nerve injury. It is therefore logical to assume that the same treatment schedule was appropriate for the present study. The lack of effect of aminoguanidine in reducing optic nerve degeneration suggests that NOS-2 does not play a critical role in glaucomatous damage in this rat glaucoma model, in which IOP elevation is produced by aqueous humor outflow obstruction.

Our findings are consistent with results from Kasmala et al., who reported that treatment of the rat by another NOS inhibitor, SC-51, also did not protect against optic nerve axon loss after saline-injection-induced ocular hypertension (Kasmala LT, et al. IOVS 2004;45:ARVO E-Abstract 904). However, our results do not agree with other published studies. For example, Shareef et al.\(^{19}\) described enhanced NOS-2 expression in ONH astrocytes in rats with cautery-induced chronic ocular hypertension and retinal damage. Neufeld et al.\(^{22,23}\) indicated that NOS-2 inhibitors, such as aminoguanidine, protected against RGC loss in the same cautery-induced rat model of retinopathy. Currently, the exact explanation(s) for these discrepancies is not known. However, findings in both Shareef et al.\(^{19}\) and Neufeld et al.\(^{22,23}\) were based on an animal model in which the ocular hypertension was induced by the cauterization of extraocular veins. Some of these extraocular veins in rats receive venous blood from the ciliary body and choroid, as well as the episcleral veins.\(^{41-43}\) Cauterization of these blood vessels may produce additional biological effects unrelated to ocular hypertension. Localized ocular ischemia and ocular congestion, as well as abnormal production of cytokines and other angiogenic factors may be induced, which could lead to an upregulation of NOS-2 expression, consequently damaging the retina and optic nerve. In contrast, the principal cause of the retina and optic nerve injuries produced by hypertonic saline injection is the raised IOP. Even though this hypertonic saline injection may activate other neurodegenerative mechanisms, evidence shows that these mechanisms, if present, are not essential in

**DISCUSSION**

In this study, we used several techniques to determine whether NOS-2 expression or its activation correlates with ocular hypertension.
the ensuing retinopathy and optic neuropathy, because simply lowering IOP by topical administration of betaxolol or apraclonidine was sufficient to minimize the glaucoma damage seen in this model.\textsuperscript{44} In addition, Shareef et al.\textsuperscript{19} evaluated retina damage by labeling the RGC with fluorescent gold label (Fluorogold; Fluorochrome, Englewood, CO). Fluorogold at high concentration by itself can induce cellular damage. It is not clear whether this potential toxicity was additive to or synergistic with the cautery-induced insult. Hence, there is a slight possibility that the fluorescent gold labeling detected RGCs with defective axonal transport that was sensitive to the aminoguanidine treatment, while the ONH grading did not have such complications. At this time, we propose that the technical differences in the elevation of IOP and morphologic evaluation of damage in these two models explain the difference in the observations of the involvement of NOS-2. In our study, as well as in previous neuroprotective studies, only morphologic evidence of retinal or optic nerve injury have been evaluated. It is essential to perform functional testing, such as electroretinography or visual-evoked potentials, to determine whether pharmacological NOS-2 inhibition affects ocular-hypertension–induced functional changes in the retina or optic nerve.

Our observations of NOS-2 expression in ocular tissues from patients with POAG also disagree with a previous published study,\textsuperscript{18} as we found no difference in labeling intensity or distribution between the glaucoma and control specimens. In both studies, the same antibody source and concentration were used. Although unlikely, the slight differences in experimental details, such as tissue preparation, visualization technique, and quantification of immunoreactivity, may have contributed to the dissimilarity in the results. In addition, the limited supply of glaucoma donor eyes resulted in small sample sizes in both studies (8 in the present study, 15 in the previous study), which could contribute to these different results and conclusions.

In our results, NOS-2 upregulation does not seem essential in glaucomatous damage of the retina and optic nerve head. Nonetheless, we cannot absolutely exclude the possibility that there may be a low level of increase in NOS-2 expression in the glaucoma tissues. We, however, demonstrated that this potential increase, if present, was very small and clearly quite different from the changes reported previously.

Though NOS-2 may not be involved in glaucoma, nitric oxide or other NOS isozymes may play a role in this disease. In the rat, when IOP was increased by thermal blockade of the perilimbal/episcleral drainage vessels and anterior angle by laser irradiation, nitric oxide levels were increased in the reti-

In summary, we have demonstrated that in a rat model of aqueous humor outflow obstruction, NOS-2 expression was not affected by chronically elevated IOP and did not correlate with the severity of optic nerve damage. We also found that NOS-2 immunoreactivity was not significantly changed in the optic nerve or ONH of patients with glaucoma. Furthermore, treatment of animals with a selective NOS-2 inhibitor did not ameliorate pressure-induced injury to the optic nerve. Although further investigation into the role of NOS-2 in other glaucoma models is warranted, the results presented herein support the conclusion that NOS-2 does not play an essential role in glaucomatous optic neuropathy.

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References

Involvement of NOS-2 in Glaucoma