

# Inducible Nitric Oxide Synthase-Mediated Alteration of Mitochondrial OPA1 Expression in Ocular Hypertensive Rats

Yi Dai,<sup>1,2</sup> Robert N. Weinreb,<sup>1</sup> Keun-Young Kim,<sup>3</sup> Duy Nguyen,<sup>1</sup> Sangwoo Park,<sup>1</sup> Xinghuai Sun,<sup>2</sup> James D. Lindsey,<sup>1</sup> Mark H. Ellisman,<sup>3</sup> and Won-Kyu Ju<sup>1</sup>

**PURPOSE.** To investigate how OPA1 expression and distribution are altered by increased nitric oxide (NO) and whether aminoguanidine, a relative selective NO synthase (NOS)-2 inhibitor, can restore OPA1 expression and subsequently increase retinal ganglion cell (RGC) survival in ocular hypertensive rats.

**METHODS.** Elevated intraocular pressure was induced unilaterally by translimbal laser photocoagulation of the trabecular meshwork in Sprague-Dawley rats. Aminoguanidine (100 mg/kg) was administered by intraperitoneal injection for 3 consecutive days in rats after laser treatment. Preservation of fluorochrome-labeled RGCs was assessed 2 weeks later. GFAP, NOS-2, or OPA1 protein expression and distribution were assessed by Western blot analysis and immunohistochemistry. OPA1 mRNA was measured by qPCR.

**RESULTS.** OPA1 mRNA and protein expression were significantly increased in the vehicle-treated hypertensive rat retina. Aminoguanidine treatment significantly reduced expression of the 90- and 65-kDa OPA1 isoforms but did not significantly change the 80-kDa OPA1 isoform in hypertensive retina. In addition, the increases in NOS-2 and GFAP protein expression were blocked by aminoguanidine treatment in the hypertensive retina. NOS-2 immunoreactivity was induced in cells of the ganglion cell layer in the vehicle-treated hypertensive retina. Aminoguanidine treatment significantly increased RGC survival at 2 weeks after IOP elevation.

**CONCLUSIONS.** Although NOS-2/NO induction may contribute to hypertensive retinal cell death, an increase in mitochondrial OPA1 may provide an important cellular defense mechanism against pressure-mediated retinal damage. These findings suggest that mitochondrial preservation after inhibition of NOS-2 may be useful for protecting RGCs against glaucomatous

damage. (*Invest Ophthalmol Vis Sci.* 2011;52:2468–2476)  
DOI:10.1167/iovs.10-5873

Glaucoma is a leading cause of irreversible blindness. Elevated intraocular pressure (IOP) is a major, and perhaps the most significant, risk factor for glaucomatous optic nerve (ON) damage and retinal ganglion cell (RGC) loss.<sup>1</sup> Emerging evidence indicates pressure-related mitochondrial dysfunction and axonal degeneration in RGCs of the glaucomatous ON or retina.<sup>2,3</sup> However, the precise mechanisms underlying these are poorly understood.

Growing evidence indicates that the free radical nitric oxide (NO) plays a role in mitochondrial fission-mediated mitochondrial dysfunction in the central nervous system by triggering mitochondrial fission, synaptic loss, and neuronal cell death.<sup>4–7</sup> The inducible, calcium-independent isoform of NO synthase, termed iNOS or NOS-2, is expressed in cells of various origins (e.g., macrophages, microglia cells, and reactive astrocytes) when these cells are activated. NO neurotoxicity mediated by NOS-2 contributes to RGC damage in experimental rat models of glaucoma.<sup>8,9</sup> In contrast to these reports, recent studies argued that NOS-2 is not associated with glaucomatous neurodegeneration in the retina, ON of the glaucomatous DBA/2J mouse, or hypertonic saline-induced glaucomatous rat model.<sup>10,11</sup> Nevertheless, the pathophysiological relationship between NO-mediated mitochondrial dysfunction and RGC damage in glaucoma remains unknown. In this regard, it has been suggested that OPA1, the human ortholog of Mgm1p/Msp1p, may play an important role in mitochondrial dysfunction-mediated glaucomatous RGC degeneration.

OPA1 is required for mitochondria fusion, and increased OPA1 expression protects cells from apoptosis by preventing cytochrome *c* release and by stabilizing the shape of mitochondrial cristae.<sup>12,13</sup> Recent studies indicated that OPA1 is expressed in the soma and axons of the RGCs and in horizontal cells in the normal mouse and rat retina.<sup>3,14</sup> Further, elevated IOP alters OPA1 expression and triggers the release of OPA1 and cytochrome *c* in the retina or ON of the glaucomatous mouse model.<sup>2,3</sup> Moreover, the relationship between NO induction and OPA1 expression is unknown.

The present study was undertaken to investigate whether protection from NO toxicity caused by increased NOS-2 alters mitochondrial OPA1 expression and increases RGC survival in the experimental hypertensive retina.

## MATERIALS AND METHODS

### Animals

All procedures concerning animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under protocols approved by institutional IACUC

From the <sup>1</sup>The Sophie and Arthur Brody Optic Nerve Laboratory, Hamilton Glaucoma Center, University of California San Diego, La Jolla, California; the <sup>2</sup>Eye and ENT Hospital, Fudan University, Shanghai, China; and the <sup>3</sup>Center for Research on Biological Systems, National Center for Microscopy and Imaging Research and Department of Neuroscience, University of California San Diego School of Medicine, La Jolla, California.

Supported by National Institutes of Health Grants EY018658 (WKJ) and NCRP P41 RR004050 (MHE) and an unrestricted research grant to the Sophie and Arthur Brody Laboratory for Optic Nerve Biology.

Submitted for publication May 12, 2010; revised September 19 and November 3, 2010; accepted November 7, 2010.

Disclosure: **Y. Dai**, None; **R.N. Weinreb**, None; **K.-Y. Kim**, None; **D. Nguyen**, None; **S. Park**, None; **X. Sun**, None; **J.D. Lindsey**, None; **M.H. Ellisman**, None; **W.-K. Ju**, None

Corresponding author: Won-Kyu Ju, The Sophie and Arthur Brody Optic Nerve Laboratory, Hamilton Glaucoma Center, University of California San Diego, La Jolla, CA 92037; danielju@glaucoma.ucsd.edu.

TABLE 1. IOP Exposure in the Experimental Glaucoma and Contralateral Control Eyes

Treatment Group (n)	Mean IOP (mm Hg)		Peak IOP (mm Hg)	
	Hypertensive	Control	Hypertensive	Control
1-day saline (n = 8)	36.7 ± 3.1*	10.1 ± 0.5	36.7 ± 3.1	10.1 ± 0.5
3-day saline (n = 15)	32.3 ± 5.5*	10.2 ± 1.8	37.3 ± 2.0	11.4 ± 1.5
3-day AG (n = 15)	31.8 ± 6.1*	10.3 ± 1.6	37.4 ± 2.1	11.2 ± 1.4
7-day saline (n = 8)	25.9 ± 9.5*	9.9 ± 1.9	36.8 ± 2.0	11.4 ± 1.4
2-week saline (n = 6)	26.8 ± 8.8*	11.0 ± 2.1	35.9 ± 2.7	10.8 ± 1.1
2-week AG (n = 6)	26.2 ± 9.4*	10.5 ± 1.8	36.8 ± 2.4	11.0 ± 1.8

Mean IOP is the average IOP after induction of experimental glaucoma. Peak IOP is the IOP of the experimental and control eyes on the day on which the IOP was most elevated after laser treatment.

\* P < 0.01 compared with contralateral control eyes.

committees at the University of California-San Diego. Female Sprague-Dawley rats (250–300 g in weight; Harlan Laboratories, Indianapolis, IN) were housed in covered cages, fed with a standard rodent diet ad libitum, and kept on a 12-hour light/12-hour dark cycle.

**Experimental Glaucoma**

Elevated intraocular pressure (IOP) was induced by translimbal laser photocoagulation of the trabecular meshwork.<sup>15,16</sup> Animals were anesthetized with a mixture of ketamine (50 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (5 mg/kg, TranquiVed;

Vedeco, Inc., St. Joseph, MO) by intraperitoneal (IP) injection. Rat eyes were also treated with 1% proparacaine drops. Laser treatment (532-nm diode laser, 320 mW power, 0.4-seconds duration, 50-mm diameter spot size) was delivered to the right eye of each rat. Approximately 45 to 55 trabecular burns were evenly distributed around the limbus. The treatment was repeated after 1 week for all rats except those killed at 1, 3, and 7 days. IOP was measured in each eye under anesthesia with a hand-held tonometer (TonoLab; Tiolat Oy, Helsinki, Finland). Readings were taken just before treatment and 1, 3, and 7 days after each treatment. Mean or peak IOPs were calculated. Rats

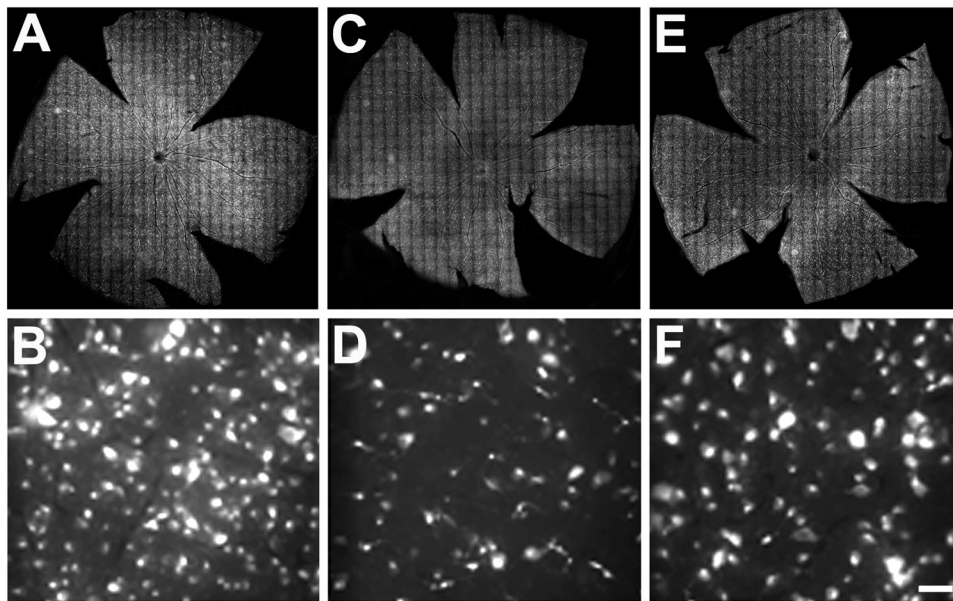
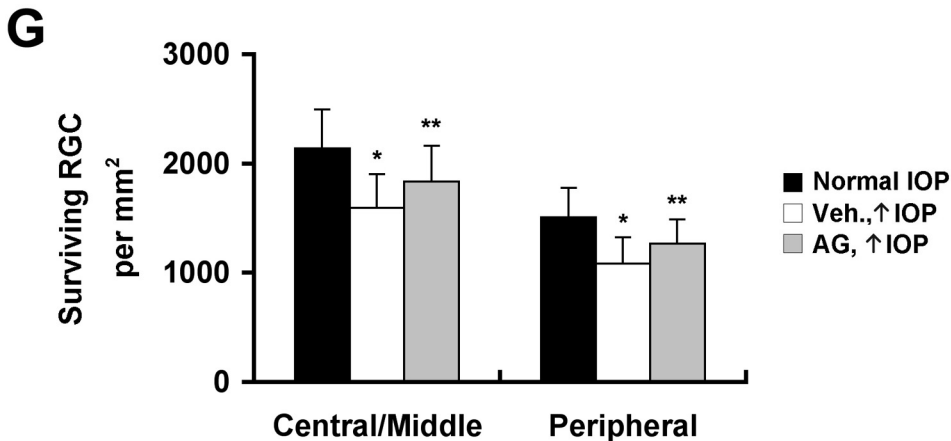


FIGURE 1. RGC survival in the hypertensive rat retina after aminoguanidine treatment. Retinal flatmounts of normal rat (A, B), vehicle-treated hypertensive rat (C, D), and aminoguanidine-treated hypertensive rat (E, F) at 2 weeks after IOP elevation. \*P < 0.01 compared with the normal retina. \*\*P < 0.05 compared with the vehicle-treated hypertensive retina. n = 6 retinal flatmounts/rats/group. Veh, vehicle; AG, aminoguanidine. Scale bar, 50 μm.



were killed at 1, 3, and 7 days or 2 weeks after the first laser treatment. At 1 day after surgery, slit lamp ophthalmoscopy was used to examine the rat eyes. If anterior chamber inflammation was observed, usually because of the overly elevated IOP, the rat was discarded from further experiment.

### Pharmacologic Treatment

Two groups of rats were studied after translimbal laser photocoagulation: one group was treated with vehicle (0.9% saline, IP injection,  $n = 37$  rats/group), and the other group was treated with aminoguanidine (100 mg/kg in saline, IP injection,  $n = 21$  rats/group) given immediately after and then 1 and 2 days after each laser treatment. The dose and delivery route for aminoguanidine was modified based on previous reports for aminoguanidine treatment.<sup>17,18</sup>

### Tissue Preparations

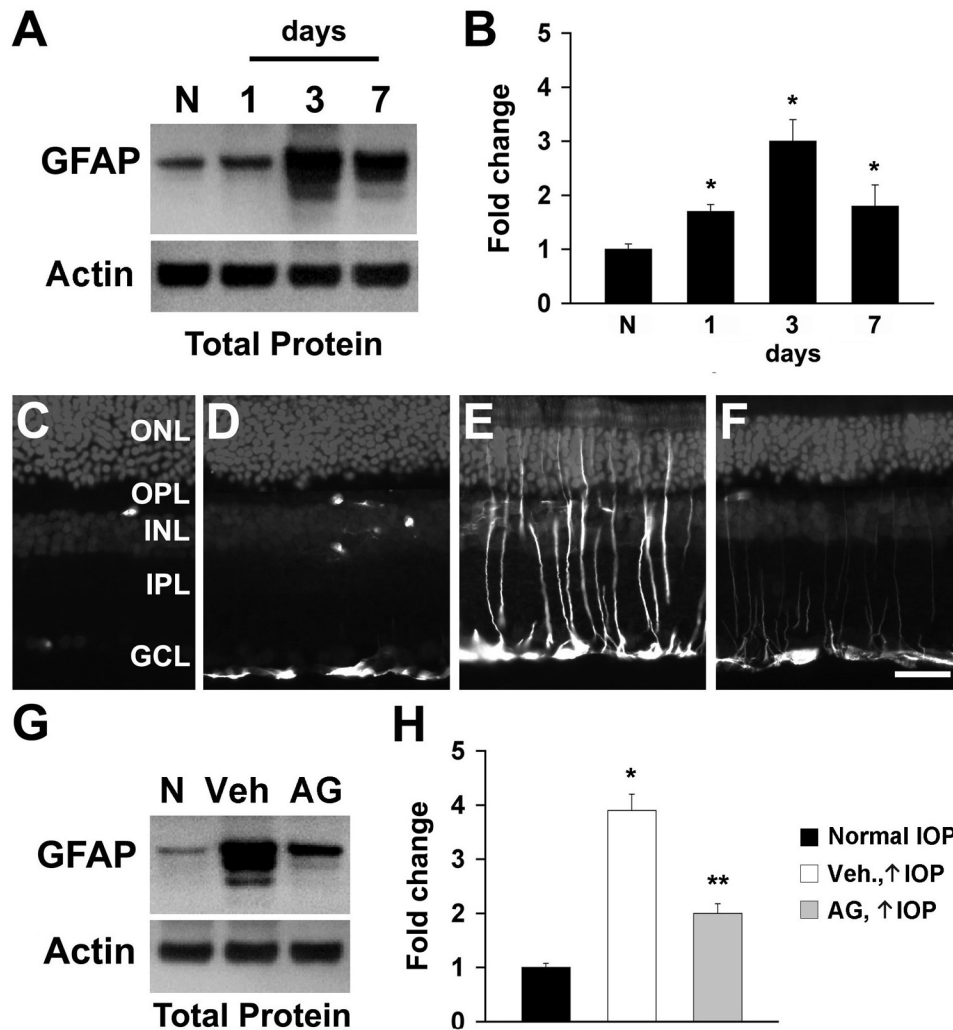
Light-adapted rats were anesthetized with IP injection of a mixture of ketamine/xylazine, as described, and then rats were killed by CO<sub>2</sub> inhalation. The rats were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). Both eyes were enucleated and postfixed in 4% paraformaldehyde in 0.1 M PBS for 4 hours at 4°C. Retinas were dehydrated through graded ethanols and then embedded in polyester wax, as described previously.<sup>2</sup> For Western blot analyses, both eyes were enucleated immediately after kill. Whole retinas were used immediately or stored at -80°C until use.

### Retrograde Labeling of RGCs

Four days before the first laser treatment, fluorochrome (FluoroGold 4% diluted in saline; Fluorochrome Inc., Denver, CO) was microinjected bilaterally into the superior colliculi of anesthetized rats (2.4  $\mu$ L/injection) immobilized in a stereotaxic apparatus, as previously described.<sup>14</sup> Images were captured with a spinning-disc confocal microscope (Olympus America Inc., Center Valley, PA) equipped with a high-precision, closed-loop x-y stage and closed loop z control with commercial mosaic acquisition software (MicroBrightField; MBF Bioscience, Inc., Williston, VT).<sup>19,20</sup> The microscope was equipped with a high-resolution, high-sensitivity CCD camera for high-speed mosaic acquisition. Images were stored on a computer (Photoshop files; Adobe Systems Inc., San Jose, CA). RGC counting was conducted in the central/middle retina at a distance of 1000 to 2000  $\mu$ m from the optic disc and in the peripheral retina (3000–4000  $\mu$ m from the optic disc) by two investigators in a masked fashion, and the counts were averaged. A counting frame (0.344 mm<sup>2</sup>) was placed in 16 distinct areas (two areas at center/middle and two areas at periphery in each retinal quadrant). Care was taken to avoid counting microglia according to different morphologic appearances.

### Western Blot Analysis

Retinas ( $n = 4$  per group) were homogenized in a glass tissue grinder (Teflon Potter homogenizer in lysis buffer [20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5% CHAPS, complete protease inhibitors]; Roche Biochemicals, Indianap-



**FIGURE 2.** GFAP expression in the hypertensive rat retina. (A, B) The GFAP protein showed a major band at 50 kDa in the normal rat retina. GFAP expression was significantly increased in the vehicle-treated hypertensive retina at 1 day after surgery, peaked by 3 days, and decreased at 7 days. (C–F) When the primary antibody for GFAP was omitted, there was limited nonspecific labeling at the OPL by the secondary antibody in the retina of the normal rat (C). Compared with the normal rat retina (D), GFAP immunoreactivity was induced in the Müller cells of the vehicle-treated hypertensive retina (E). Aminoguanidine treatment decreased GFAP immunoreactivity in Müller cells of the hypertensive retina (F). (G, H) Aminoguanidine treatment significantly decreased GFAP protein expression in the hypertensive retina at 3 days after IOP elevation. \* $P < 0.05$  compared with normal rats. \*\* $P < 0.01$  compared with vehicle-treated hypertensive retinas. N, normal; Veh, vehicle; AG, aminoguanidine; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 20  $\mu$ m.

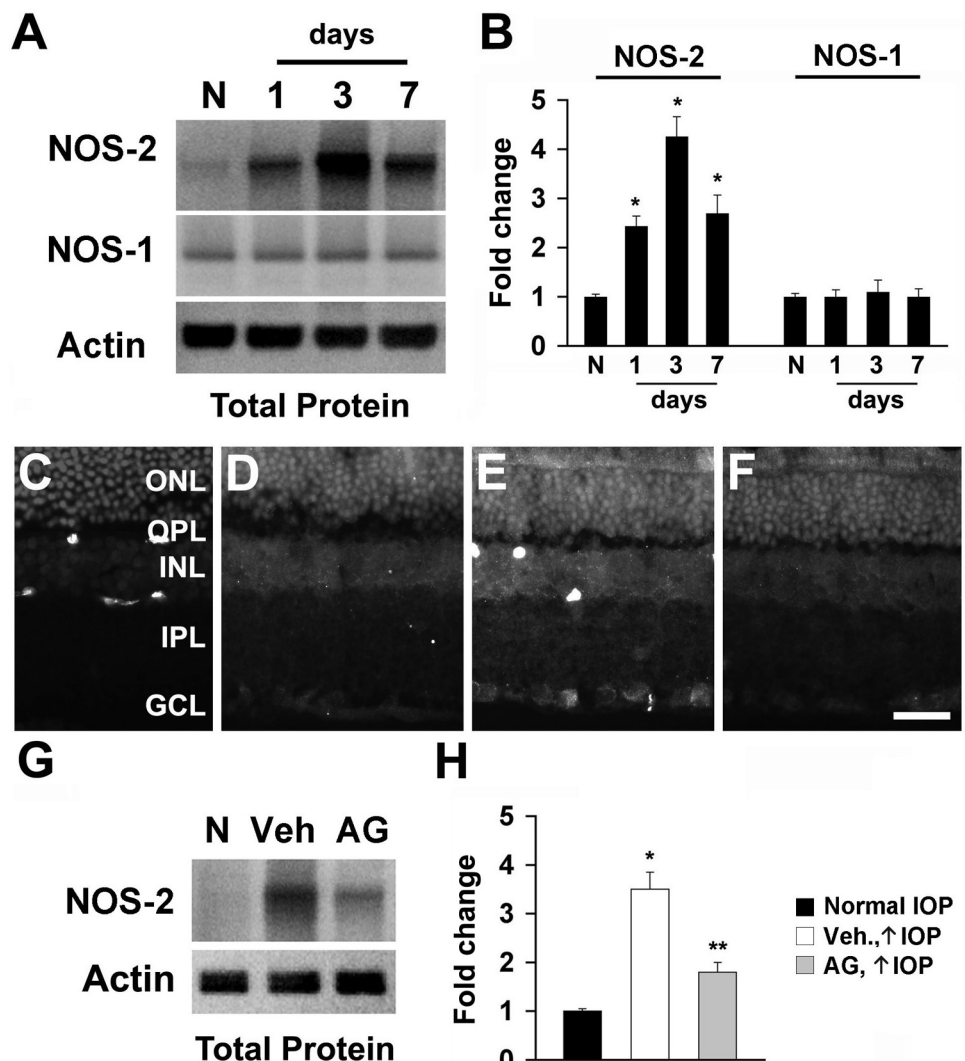
olis, IN). Each sample (10  $\mu$ g) was separated by PAGE and electrotransferred to polyvinylidene difluoride membranes. The membrane was blocked with 5% nonfat dry milk and 0.05% in Tween-20 in phosphate-buffered saline (PBS), incubated with monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:3000; Sigma, St. Louis, MO), monoclonal mouse anti-inducible NOS (NOS-2) antibody (1:2000; BD Transduction Laboratories, San Diego, CA), polyclonal rabbit anti-brain NOS (NOS-1) antibody (1:5000; Sigma), monoclonal mouse anti-OPA1 antibody (H-300/1:2000; BD Transduction Laboratories), or monoclonal mouse anti-actin antibody (Ab-1/1:5000; Calbiochem, La Jolla, CA), rinsed with 0.05% Tween-20 in PBS, incubated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:2000; Bio-Rad, Hercules, CA), and developed using chemiluminescence detection (ECL Plus; GE Healthcare Bio-Sciences, Piscataway, NJ). Images were analyzed by digital fluorescence imager (Storm 860; GE Healthcare Bio-Sciences), and band densities were normalized with actin used as a calibrator (ImageQuant TL; GE Healthcare Bio-Sciences).

### Immunohistochemical Analysis

Immunohistochemical staining of 7- $\mu$ m wax sections of full-thickness retina was performed with an immunofluorescence method, as previously described.<sup>2</sup> Five sections per wax block from each group ( $n = 4$  retinas/group) were used for immunohistochemical analysis. The primary antibodies were monoclonal mouse anti-GFAP antibody (1:200; Sigma), monoclonal mouse anti-iNOS antibody (1:100; BD Transduc-

tion Laboratories), or polyclonal rabbit anti-mouse OPA1 antibody (1:1000, a gift of Takumi Misaka [University of Tokyo, Tokyo, Japan] and Yoshihiro Kubo [National Institute for Physiological Sciences, Aichi, Japan]). To prevent nonspecific background, tissues were incubated with 1% bovine serum albumin (BSA)/PBS for 1 hour at room temperature and then with the primary antibodies against NOS-2, GFAP, or OPA1 for 16 hours at 4°C. After several wash steps, the tissue was incubated with the secondary antibody, AlexaFluor 488 dye-conjugated goat anti-mouse IgG, or goat anti-rabbit IgG (1:100; Invitrogen-Molecular Probes, Eugene, OR) for 4 hours at 4°C and then washed with PBS.

To enhance OPA1 immunoreactivity, immunohistochemistry was performed (Tyramide Signal Amplification Kit; Molecular Probes, Eugene, OR). Tissue sections were permeabilized with 0.1% Triton X-100 in PBS, incubated with quenching buffer (amplification buffer and 0.0015% H<sub>2</sub>O<sub>2</sub>) for 1 hour at room temperature, blocked with 1% BSA/PBS, and then incubated with antibody against OPA1 for 16 hours at 4°C. After several washings, tissues were incubated with peroxidase-conjugated goat anti-rabbit IgG for 4 hours at 4°C, washed, and incubated with tyramide working solution for 10 minutes at room temperature. The sections were counterstained with the nucleic acid stain Hoechst 33342 (1  $\mu$ g/mL; Invitrogen-Molecular Probes) in PBS. Images were captured by fluorescence microscopy (Eclipse E800; Nikon Instruments Inc., Melville, NY) equipped with a digital camera (Spot; Diagnostic Instrument, Sterling Heights, MI). Image expo-



**FIGURE 3.** Differential expression of NOS-1 and NOS-2 in the hypertensive rat retina. (A, B) NOS-1 and NOS-2 proteins showed a major band at 130 kDa and 150 kDa in the rat retina. Elevated IOP significantly increased NOS-2 protein expression in retina at 1 day after surgery, peaked by 3 days, and decreased at 7 days. No significant difference was observed for NOS-1 expression in the hypertensive retina. (C-F) When the primary antibody for NOS-2 was omitted, there was limited nonspecific labeling between OPL and IPL by the secondary antibody in the retina of the normal rat (C). Compared with the normal rat retina (D), NOS-2 immunoreactivity was induced in the GCL of the vehicle-treated hypertensive retina (E). Aminoguanidine treatment decreased NOS-2 immunoreactivity in these layers of hypertensive retina (F). (G, H) Aminoguanidine treatment significantly decreased NOS-2 protein expression in hypertensive retinas at 3 days after IOP elevation. \* $P < 0.05$  compared with normal rats. \*\* $P < 0.01$  compared with vehicle-treated hypertensive retinas. N, normal; Veh, vehicle; AG, aminoguanidine; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 20  $\mu$ m.

ures were the same for all tissue sections and were acquired with commercial software (Simple PCI version 6.0; Compix Inc., Cranberry Township, PA).

For retinal flat mount immunohistochemistry ( $n = 3$  retinas/group), the primary antibody for NOS-2 (1:500; BD Transduction Laboratories) was incubated for 3 days at 4°C. After several wash steps, retinal flat mounts were incubated with the secondary antibody, AlexaFluor 488 dye-conjugated goat anti-mouse IgG (1:100; Invitrogen-Molecular Probes), for 4 hours at 4°C and then washed with PBS.

### Quantitative PCR

Total RNA from retinal tissues ( $n = 4$  retinas/group) was extracted with Trizol (Invitrogen, Carlsbad, CA), purified on RNeasy mini columns (Qiagen, Valencia, CA), and treated with RNase-free DNase I (Qiagen). RNA purity was verified by confirming that the OD260 nm/280 nm absorption ratio exceeded 1.9. cDNA was synthesized using a first-strand RT-PCR kit (SuperScript II; Invitrogen). *OPA1* gene expression was measured by qPCR (MX3000P; Stratagene, La Jolla, CA) using 25 ng cDNA from retinas and 2× universal PCR master mix (Applied Biosystems, Foster City, CA) with a one-step program (95°C for 10 minutes, 95°C for 30 seconds, and 60°C for 1 minute for 50

cycles). Primers for *OPA1* and *GAPDH*, as well as probe (TaqMan) for *GAPDH*, were designed with sequence detection software (Primer Express 2.0; Applied Biosystems) obtained from Biosearch Technologies (Novato, CA). The probe for *OPA1* was obtained from the Roche Universal Probe Library (Roche Diagnostics, Mannheim, Germany), and the optimal concentrations for probe and primers were determined using heart tissue. Standard curves were constructed using nine twofold dilutions (50–0.195 ng) for both the target (*OPA1*) and the endogenous reference (*GAPDH*). Samples were run in duplicate for each target and endogenous *GAPDH* control.

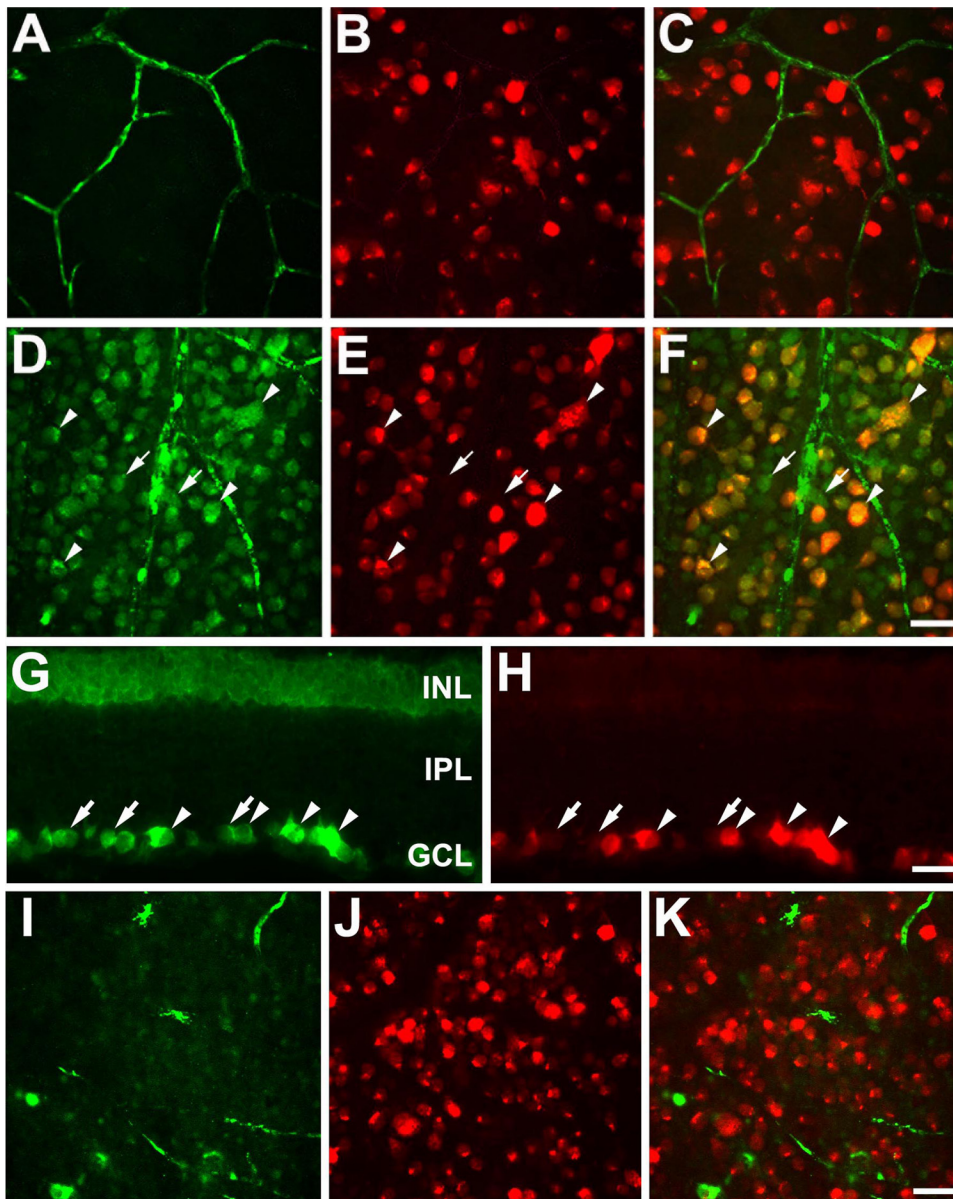
### Statistical Analysis

Data are expressed as mean  $\pm$  SD. One-way analysis of variance and the Bonferroni *t*-test were used to evaluate study results.  $P < 0.05$  was considered statistically significant.

## RESULTS

### IOP Exposure

All laser-treated eyes had significantly elevated IOP compared with their contralateral control eyes (Table 1). There was no



**FIGURE 4.** NOS-2 immunoreactivity in fluorochrome-prelabeled RGCs of the hypertensive rat retina. NOS-2 immunoreactivity (A, green) was present in the vessels but not colabeled by fluorochrome (B, red) in the normal rat retina and merged image (C). Neurons containing NOS-2 immunoreactivity (D, G, green) were colabeled by fluorochrome (E, H, red, arrowheads) in the vehicle-treated hypertensive retina at 3 days after surgery and merged images (F). (G, H) NOS-2 immunoreactivity was also present in GCL cells negative for fluorochrome (arrows). (I–K) Aminoguanidine treatment decreased NOS-2 immunoreactivity in the GCL of the hypertensive retina. INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars, 20  $\mu$ m.

significant difference in mean or peak IOP between vehicle- and aminoguanidine-treated groups ( $P > 0.05$ ).

### Effect of NOS-2 Inhibition on RGC Survival and Müller Glial Activation

As shown in Figure 1, the normal rat retina has an average density of 2140 RGCs/mm<sup>2</sup> in the central and middle and 1506 RGCs/mm<sup>2</sup> in the peripheral areas ( $n = 6$  retinas; Figs. 1A, 1B, 1G). Compared with the contralateral control eyes, the vehicle-treated hypertensive retina had a 26% RGC loss in the central and middle retina and a 28% loss in the peripheral retina 2 weeks after IOP elevation ( $n = 6$  retinas,  $P < 0.01$ ; Figs. 1C, 1D, 1G). Aminoguanidine treatment significantly increased RGC survival by 15% in the central and middle retina and by 17% in the peripheral retina compared with the vehicle-treated hypertensive retina ( $n = 6$ ,  $P < 0.05$ ; Figs. 1E, 1F, 1G).

After IOP elevation, GFAP protein expression was significantly increased at 1 day, peaked by 3 days, and decreased at 7 days in vehicle-treated hypertensive retinas (Fig. 2A). Aminoguanidine treatment decreased GFAP protein expression by  $0.51 \pm 0.07$ -fold in hypertensive retinas at 3 days ( $P < 0.01$ ; Fig. 2B). Compared with the normal rat retina (Fig. 2D), GFAP immunoreactivity was increased in the Müller cells of the hypertensive retina (Fig. 2E). Aminoguanidine treatment decreased GFAP immunoreactivity in Müller cells of hypertensive retina at 3 days (Fig. 2F). In addition, Western blot analysis showed that aminoguanidine treatment significantly decreased GFAP protein expression in the hypertensive retina ( $P < 0.01$ ; Figs. 2G, 2H).

### NOS-2 Induction in Hypertensive RGCs

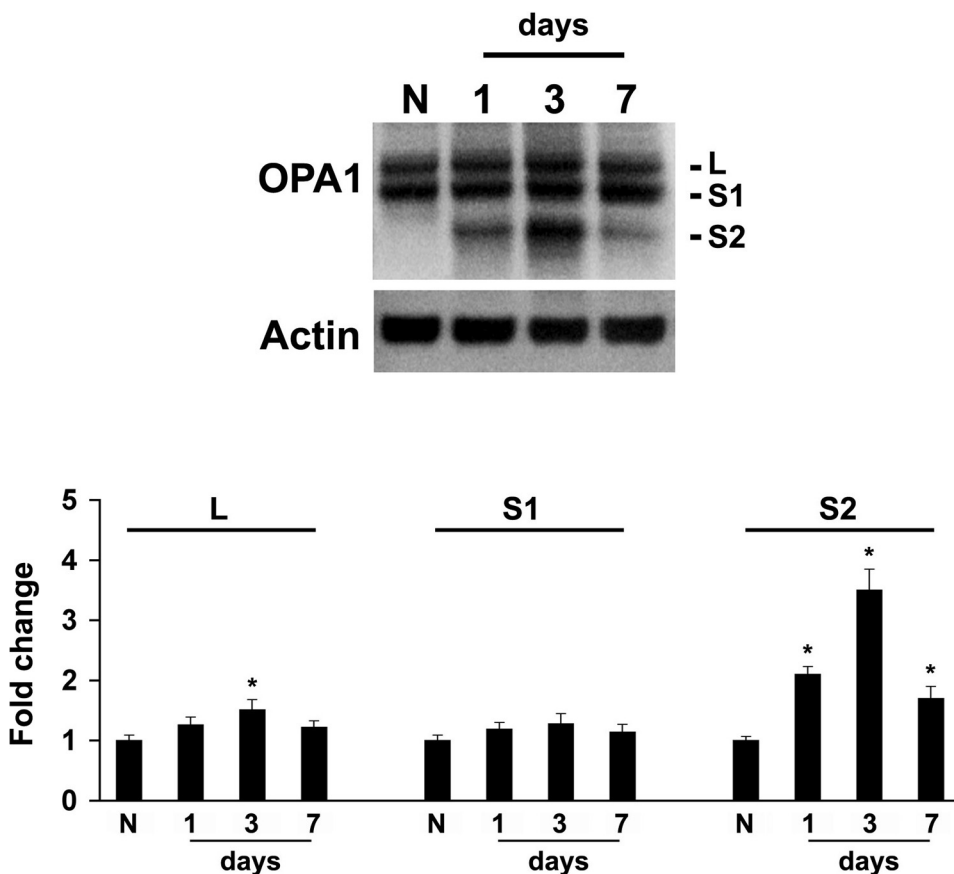
After IOP elevation, retinal NOS-2 was significantly increased at 1 day, peaked by 3 days, and decreased at 7 days (Figs. 3A, 3B).

There was no significant change of NOS-1 expression in the hypertensive retina (Figs. 3A, 3B). Compared with the normal rat retina (Fig. 3D), NOS-2 immunoreactivity was greater in the ganglion cell layer (GCL) of the vehicle-treated hypertensive retina (Fig. 3E). Aminoguanidine treatment blocked this NOS-2 induction in the hypertensive retina (Fig. 3F). In addition, Western blot analysis showed that aminoguanidine treatment decreased NOS-2 protein expression by  $0.50 \pm 0.05$ -fold in hypertensive retinas at 3 days ( $P < 0.01$ ; Figs. 3G, 3H).

When these experiments were repeated in rats in which RGCs had been prelabeled by fluorochrome, approximately 89% of the labeled RGCs were immunoreactive for NOS-2 at 3 days after IOP elevation. Compared with the normal rat retina (Figs. 4A-C), NOS-2 was induced in the RGCs of the hypertensive retina (Figs. 4D-F). In addition, immunoreactivity for NOS-2 was also present in cells of the GCL that were negative for fluorochrome (Figs. 4G, 4H). Aminoguanidine treatment decreased NOS-2 immunoreactivity in the GCL of the hypertensive retina (Figs. 4I-K). Consistently, NOS-2 protein expression by a single injection of aminoguanidine was higher than triple injections of aminoguanidine in the hypertensive retina (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5873/-/DCSupplemental>). However, no protection of RGCs was observed when aminoguanidine was delivered every day for the entire 2-week study (data not shown).

### NOS-2 Inhibition-Mediated OPA1 Restoration

The OPA1 antibody recognized three major OPA1 isoforms in the total protein extracts of the retina in normal rats (90 kDa, L; 80 kDa, S1; ~75 kDa, S2; Fig. 5). Quantitative PCR analysis showed that OPA1 mRNA expression was increased by 52% in the vehicle-treated hypertensive retina at 3 days compared



**FIGURE 5.** OPA1 protein expression in the hypertensive rat retina. The OPA1 protein bands showed three major isoforms (90 kDa, L; 80 kDa, S1; and ~75 kDa, S2) in the normal rat retina. After IOP elevation, isoforms (L and S2) of OPA1 protein expression were increased in the retina at 1 day, peaked by 3 days, and decreased at 7 days. \* $P < 0.05$  compared with normal rats.

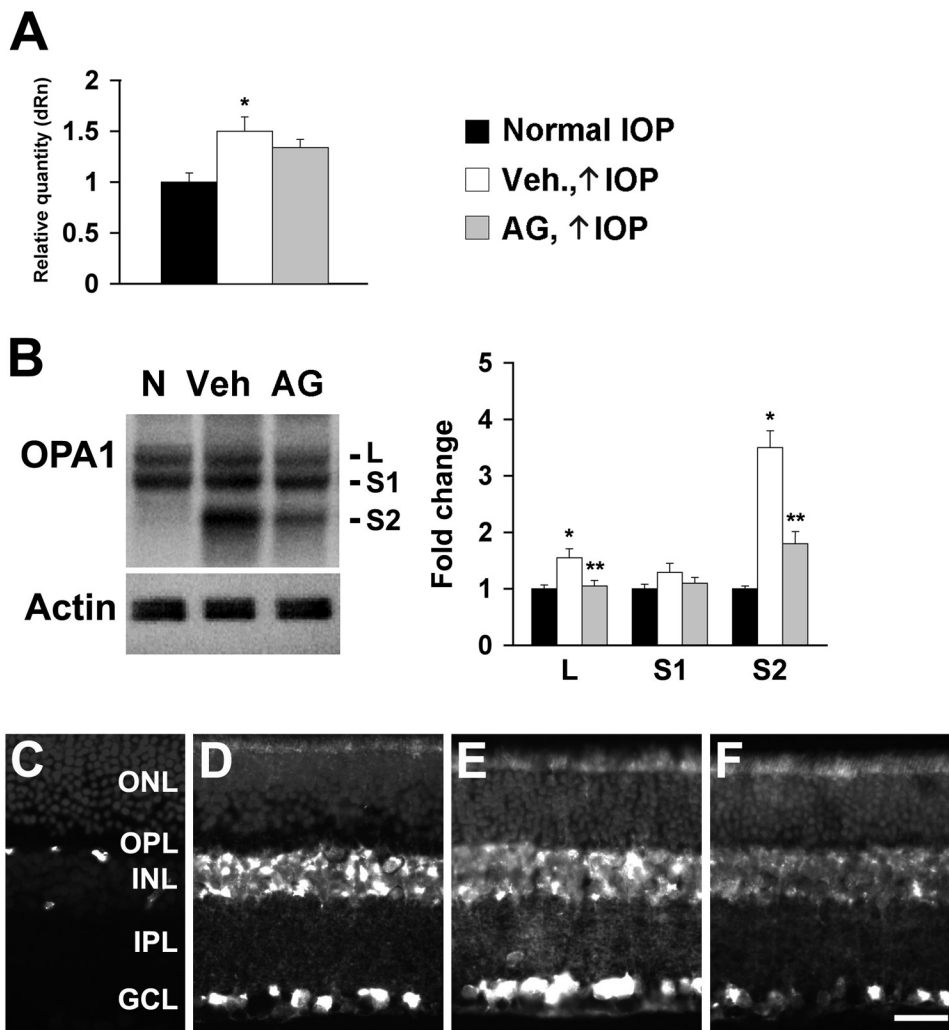
with the retinas of normal rats ( $P < 0.01$ ). Aminoguanidine treatment did not show a significant change in OPA1 mRNA expression by 14% at 3 days compared with the vehicle-treated hypertensive retina (Fig. 6A). After IOP elevation, isoforms S2 of OPA1 protein were increased at 1 day, peaked by 3 days, and decreased at 7 days in the hypertensive retina ( $P < 0.05$ ); isoforms L of OPA1 protein were also increased at 3 days ( $P < 0.05$ ), but no significant change was observed for isoform S1 in the hypertensive retina. In addition, aminoguanidine treatment significantly decreased two isoforms (L and S2) of total OPA1 protein expression in the hypertensive retina at 3 days ( $0.67 \pm 0.08$ -fold, L;  $0.51 \pm 0.05$ -fold, S2;  $P < 0.05$ , Fig. 6B). Compared with the normal rat retina (Fig. 6D), OPA1 immunoreactivity was increased in the GCL of vehicle-treated hypertensive retina (Fig. 6E). Aminoguanidine treatment blocked this increase of OPA1 immunoreactivity (Fig. 6F).

## DISCUSSION

These results demonstrate that OPA1 gene and protein expression in the rat retina are increased in response to pressure. Further, pressure triggers NOS-2 induction in the RGCs of the hypertensive retina. Aminoguanidine, a selective NOS-2 inhibitor, protects against RGC loss and restores normal OPA1 expression in the hypertensive rat retina induced by laser photocoagulation.

There is growing evidence that NO neurotoxicity is linked to mitochondrial dysfunction-mediated neuronal cell death in neurodegenerative diseases, including Alzheimer's and Parkinson's diseases.<sup>5-7,21,22</sup> Although excessive NO has been reported to induce neuronal cell damage in the ischemic or hypertensive retina,<sup>8,23-28</sup> the relationships among elevated IOP, NO toxicity, and mitochondrial dysfunction in glaucoma remain unknown. In the present study, we provide the first *in vivo* evidence that elevated IOP induces NOS-2 protein expression in RGCs and, possibly, displaced amacrine cells in early neurodegenerative events (within 3 days) in the hypertensive rat retina. In addition, inhibition of NOS-2 by aminoguanidine significantly increases RGC survival. Taken together, it is possible that NO neurotoxicity induced by NOS-2 may exacerbate pressure-mediated damage by autocrine or paracrine injury of RGC mitochondria.

In addition to protecting RGCs, we found that aminoguanidine treatment significantly reduced the activation of Müller cells in the hypertensive retina. Astroglia or Müller cell activation coincides with RGC degeneration in the hypertensive retina of the human, rat, or mouse.<sup>29-33</sup> Although NOS-2 expression in Müller cells has been reported in retinal degeneration after several insults,<sup>34-39</sup> NOS-2 expression in Müller cells was not found in the hypertensive retina. Hence, the present results suggest that aminoguanidine treatment may indirectly reduce the activation of Müller cells as a result of increased RGC survival. On the other hand, aminoguanidine is



**FIGURE 6.** OPA1 gene and protein expression in the hypertensive rat retina. **(A)** OPA1 mRNA expression was significantly increased by 52% in the vehicle-treated hypertensive retina compared with normal rat retinas. Aminoguanidine treatment did not show a significant change in OPA1 mRNA expression by 14% compared with the vehicle-treated hypertensive retina. **(B)** Two isoforms of total OPA1 protein expression were significantly decreased in the aminoguanidine-treated hypertensive retina. **(C-F)** When the primary antibody for NOS-2 was omitted, there was limited nonspecific labeling between OPL and IPL by the secondary antibody in the retina of the normal rat **(C)**. OPA1 immunoreactivity was localized in the GCL, IPL, INL, and OPL in the normal rat retina **(D)**. OPA1 immunoreactivity was increased in the GCL of the vehicle-treated hypertensive retina **(E)**. These increases were blocked by aminoguanidine treatment **(F)**. \* $P < 0.01$  compared with normal rats. \*\* $P < 0.05$  compared with vehicle-treated hypertensive retinas. N, normal; Veh, vehicle; AG, aminoguanidine; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 20 μm.

also an inhibitor of advanced glycation end product (AGE). In light of evidence for the potential involvement of AGE in glaucoma,<sup>40</sup> the mechanism of the protective effect of aminoguanidine in the hypertensive rat retina should be further explored.

It is possible that baseline levels of NO provided by normal amounts of NOS is beneficial for RGCs, whereas excess NO from elevated NOS-2 is stressful for RGCs. After laser treatment, NOS-2 expression peaked at 3 days in the hypertensive rat retina. Aminoguanidine treatment for 3 days immediately after each laser surgery provided significant RGC protection compared with the vehicle-treated hypertensive rat retina at the end of the 2-week study. However, no protection of RGCs was observed when aminoguanidine was delivered every day for the entire 2-week study (data not shown). This suggests that effective management with aminoguanidine may be time-dependent in our ocular hypertensive rat model. Thus, our results raise the possibility that a time-dependent design for aminoguanidine treatment may be an important strategy for RGC protection against glaucomatous damage.

In the present study, OPA1 protein expression was significantly increased in the vehicle-treated hypertensive rat retina at 3 days, and aminoguanidine treatment reduced the magnitude of increased OPA1 protein expression. Overexpression of retinal OPA1 after injection of an adenoassociated virus serotype 2 construct containing wild-type mouse OPA1 increased RGC survival in glaucomatous DBA/2J mice,<sup>41</sup> and increased OPA1 protects cells against apoptotic cell death.<sup>12,13,41</sup> Together, although there is no direct evidence, the difference between these findings reflects the possibility that increased OPA1 expression may be a cellular compensatory response that may protect retinal cells against hypertensive injury in our model. In addition, it is possible that aminoguanidine may indirectly regulate an upstream signaling event that leads to the modulation of OPA1 protein expression in the hypertensive rat retina. Thus, further insight into the functional mechanism of OPA1 protein may be obtained by studying the overexpression of OPA1 in the hypertensive rat retina. Nevertheless, our findings suggest that increased OPA1 expression may be an important cellular defense mechanism against NO toxicity in early hypertensive retinal damage and support the view that OPA1 may protect against pressure-induced mitochondrial damage in the hypertensive retina.

In summary, our findings demonstrate that elevated IOP can trigger NOS-2 induction, and increased OPA1 expression may provide a cellular defense mechanism for RGC survival against the early hypertensive retinal degeneration. The biochemical relationship between NO induction and increased OPA1 expression in hypertensive RGCs remains unknown. Hence, the present results justify further studies to explain this relationship and to clarify how limiting deleterious effects of excessive local NO production could protect RGC mitochondria in glaucoma.

## References

- Weinreb RN, Khaw PT. Primary open-angle glaucoma. *Lancet*. 2004;363:1711-1720.
- Ju WK, Kim KY, Lindsey JD, et al. Intraocular pressure elevation induces mitochondrial fission and triggers OPA1 release in glaucomatous optic nerve. *Invest Ophthalmol Vis Sci*. 2008;49:4903-4911.
- Ju WK, Kim KY, Angert M, et al. Memantine blocks mitochondrial OPA1 and cytochrome c release and subsequent apoptotic cell death in glaucomatous retina. *Invest Ophthalmol Vis Sci*. 2009;50:707-716.
- Barsoum MJ, Yuan H, Gerencser AA, et al. Nitric oxide-induced mitochondrial fission is regulated by dynamin-related GTPases in neurons. *EMBO J*. 2006;25:3900-3911.
- Yuan H, Gerencser AA, Liot G, et al. Mitochondrial fission is an upstream and required event for bax foci formation in response to nitric oxide in cortical neurons. *Cell Death Differ*. 2007;14:462-471.
- Westermann B. Nitric oxide links mitochondrial fission to Alzheimer's disease. *Sci Signal*. 2009;2:pe29.
- Cho DH, Nakamura T, Fang J, et al. S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. *Science*. 2009;324:102-105.
- Neufeld AH, Sawada A, Becker B. Inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma. *Proc Natl Acad Sci U S A*. 1999;96:9944-9948.
- Neufeld AH. Pharmacologic neuroprotection with an inhibitor of nitric oxide synthase for the treatment of glaucoma. *Brain Res Bull*. 2004;62:455-459.
- Pang IH, Johnson EC, Jia L, et al. Evaluation of inducible nitric oxide synthase in glaucomatous optic neuropathy and pressure-induced optic nerve damage. *Invest Ophthalmol Vis Sci*. 2005;46:1313-1321.
- Libby RT, Howell GR, Pang IH, et al. Inducible nitric oxide synthase, Nos2, does not mediate optic neuropathy and retinopathy in the DBA/2J glaucoma model. *BMC Neurosci*. 2007;8:108.
- Cipolat S, Rudka T, Hartmann D, et al. Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. *Cell*. 2006;126:163-175.
- Ishihara N, Fujita Y, Oka T, Mihara K. Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. *EMBO J*. 2006;25:2966-2977.
- Ju WK, Misaka T, Kushnareva Y, et al. OPA1 expression in the normal rat retina and optic nerve. *J Comp Neurol*. 2005;488:1-10.
- Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophthalmol Vis Sci*. 2002;43:402-410.
- Pease ME, Zack DJ, Berlinicke C, et al. Effect of CNTF on retinal ganglion cell survival in experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2009;50:2194-2200.
- Bostanci MO, Bagirci F. Neuroprotective effect of aminoguanidine on iron-induced neurotoxicity. *Brain Res Bull*. 2008;76:57-62.
- Iadecola C, Zhang F, Xu X. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am J Physiol*. 1995;268:R286-R292.
- Chow SK, Hakozaki H, Price DL, et al. Automated microscopy system for mosaic acquisition and processing. *J Microsc*. 2006;222:76-84.
- Price DL, Chow SK, Maclean NA, et al. High-resolution large-scale mosaic imaging using multiphoton microscopy to characterize transgenic mouse models of human neurological disorders. *Neuroinformatics*. 2006;4:65-80.
- Bossy-Wetzel E, Talantova MV, Lee WD, et al. Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K<sup>+</sup> channels. *Neuron*. 2004;41:351-365.
- Nakamura T, Lipton SA. Redox regulation of mitochondrial fission, protein misfolding, synaptic damage, and neuronal cell death: potential implications for Alzheimer's and Parkinson's diseases. *Apoptosis*. 2010;15:1354-1363.
- Ju WK, Kim KY, Park SJ, et al. Nitric oxide is involved in sustained and delayed cell death of rat retina following transient ischemia. *Brain Res*. 2000;881:231-236.
- Geyer O, Almog J, Lupu-Meir M, Lazar M, Oron Y. Nitric oxide synthase inhibitors protect rat retina against ischemic injury. *FEBS Lett*. 1995;374:399-402.
- Yucel I, Akar Y, Yucel G, Ciftcioglu MA, Keles N, Aslan M. Effect of hypercholesterolemia on inducible nitric oxide synthase expression in a rat model of elevated intraocular pressure. *Vision Res*. 2005;45:1107-1114.
- Polak K, Luksch A, Berisha F, Fuchsjaeger-Mayrl G, Dallinger S, Schmetterer L. Altered nitric oxide system in patients with open-angle glaucoma. *Arch Ophthalmol*. 2007;125:494-498.



27. Park SH, Kim JH, Kim YH, Park CK. Expression of neuronal nitric oxide synthase in the retina of a rat model of chronic glaucoma. *Vision Res.* 2007;47:2732-2740.
28. Aslan M, Cort A, Yucel I. Oxidative and nitrative stress markers in glaucoma. *Free Radic Biol Med.* 2008;45:367-376.
29. Tezel G, Chauhan BC, LeBlanc RP, Wax MB. Immunohistochemical assessment of the glial mitogen-activated protein kinase activation in glaucoma. *Invest Ophthalmol Vis Sci.* 2003;44:3025-3033.
30. Schuettauf F, Rejdak R, Walski M, et al. Retinal neurodegeneration in the DBA/2J mouse—a model for ocular hypertension. *Acta Neuropathol.* 2004;107:352-358.
31. Schmidt JF, Agapova OA, Yang P, Kaufman PL, Hernandez MR. Expression of ephrinB1 and its receptor in glaucomatous optic neuropathy. *Br J Ophthalmol.* 2007;91:1219-1224.
32. Bosco A, Inman DM, Steele MR, et al. Reduced retina microglial activation and improved optic nerve integrity with minocycline treatment in the DBA/2J mouse model of glaucoma. *Invest Ophthalmol Vis Sci.* 2008;49:1437-1446.
33. Ergorul C, Ray A, Huang W, et al. Hypoxia inducible factor-1alpha (HIF-1alpha) and some HIF-1 target genes are elevated in experimental glaucoma. *J Mol Neurosci.* 2010;42:183-191.
34. Jacquemin E, de Kozak Y, Thillaye B, Courtois Y, Goureau O. Expression of inducible nitric oxide synthase in the eye from endotoxin-induced uveitis rats. *Invest Ophthalmol Vis Sci.* 1996;37:1187-1196.
35. Koeberle PD, Ball AK. Nitric oxide synthase inhibition delays axonal degeneration and promotes the survival of axotomized retinal ganglion cells. *Exp Neurol.* 1999;158:366-381.
36. Kobayashi M, Kuroiwa T, Shimokawa R, Okeda R, Tokoro T. Nitric oxide synthase expression in ischemic rat retinas. *Jpn J Ophthalmol.* 2000;44:235-244.
37. Abu El-Asrar AM, Meersschaert A, Dralands L, Missotten L, Geboes K. Inducible nitric oxide synthase and vascular endothelial growth factor are colocalized in the retinas of human subjects with diabetes. *Eye (Lond).* 2004;18:306-313.
38. Walker RJ, Steinle JJ. Role of beta-adrenergic receptors in inflammatory marker expression in Muller cells. *Invest Ophthalmol Vis Sci.* 2007;48:5276-5281.
39. Kaur C, Sivakumar V, Foulds WS, Luu CD, Ling EA. Cellular and vascular changes in the retina of neonatal rats after an acute exposure to hypoxia. *Invest Ophthalmol Vis Sci.* 2009;50:5364-5374.
40. Tezel G, Luo C, Yang X. Accelerated aging in glaucoma: immunohistochemical assessment of advanced glycation end products in the human retina and optic nerve head. *Invest Ophthalmol Vis Sci.* 2007;48:1201-1211.
41. Ju WK, Kim KY, Duong-Polk KX, Lindsey JD, Ellisman MH, Weinreb RN. Increased optic atrophy type 1 expression protects retinal ganglion cells in a mouse model of glaucoma. *Mol Vis.* 2010;16:1331-1342.