Effect of Ocular Hypertension on Retinal Nitridergic Pathway Activity

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Purpose. Understanding the mechanisms of neuronal cell death in glaucoma is important for devising new treatments. Excitatory amino acids, excessive Ca^{2+} influx, and formation of nitric oxide (NO) via NO synthase (NOS)-1 could be involved in glaucomatous neuropathy. The purpose of the present study was to examine the retinal nitridergic pathway activity in rats exposed to experimentally elevated intraocular pressure.

Methods. Weekly injections of HA were performed unilaterally in the rat anterior chamber, whereas the contralateral eye was injected with saline solution. At 3 or 6 weeks of treatment, retinal NOS activity was assessed through the conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline, whereas NOS-1, -2, and -3 levels were assessed by Western blotting. L-Arginine uptake was measured using ^{3}H-L-arginine, whereas mRNA levels of L-arginine transporters were determined by semiquantitative RT-PCR. In addition, cyclic guanosine monophosphate (cGMP) levels were quantified by radioimmunoassay.

Results. At both 3 and 6 weeks of treatment, NOS activity significantly increased in HA-injected eyes although no changes in retinal NOS-1, -2, or -3 levels were observed in eyes injected with HA. L-Arginine influx and mRNA levels of cationic amino acid transporter type (CAT)-1 and -2 significantly increased in retinas from hypertensive eyes. Retinal cGMP levels significantly increased in eyes injected with HA for 3 but not 6 weeks.

Conclusions. These results suggest a significant activation of the retinal nitridergic pathway in hypertensive eyes. (*Invest Ophthalmol Vis Sci.* 2007;48:2127–2133) DOI:10.1167/iovs.06-1229

Glaucoma is a leading cause of blindness worldwide, characterized by specific visual field defects due to the loss of retinal ganglion cells and damage to the optic nerve head. Elevated intraocular pressure (IOP) is one of the most important risk factors for development of glaucoma. However, the underlying mechanisms that link elevated IOP to ganglion cell death are not fully understood. An experimental model of pressure-induced optic nerve damage would greatly facilitate the understanding of the cellular events leading to ganglion cell death and how they are influenced by ocular hypertension and other risk factors. We have developed a new model of glaucoma in rats through intracameral injections of hyaluronic acid (HA).[1,2] Several advantages support the usefulness of this model: (1) A highly consistent hypertension may be achieved; (2) daily variations of IOP persist in HA-injected eyes; (3) in contrast to other models, HA does not impede the blood flow out of the eye; (4) it is easy to perform; and (5) histologic and functional alterations induced by HA mimic central features of human glaucoma.[2] In addition, since no loss of the midretinal architecture (the area supplied by retinal arteries) was evident,[2] it seems improbable that HA-induced hypertension could provoke significant retinal ischemia.

Besides the increase of IOP, several concomitant factors like elevation of glutamate levels, disorganized NO metabolism, and oxidative damage, could significantly contribute to the neurodegeneration.[3] We have demonstrated a significant decrease of the retinal antioxidant defense system activity in eyes with ocular hypertension induced by HA,[4] which suggests the involvement of oxidative stress in glaucomatous cell death. In addition, we showed significant alterations of the glutamate–glutamine cycle activity that support an increase in retinal glutamate synaptic concentrations in HA-treated eyes.[5] *N*-methyl-aspartate (NMDA) subtype glutamate receptor plays a central role in retinal glutamate neurotoxicity.[6,7] In neuronal glutamate excitotoxicity, calcium is taken up through the NMDA receptor-gated channel and then acts as a messenger for excitotoxicity in brain[8] and retina.[9] In cultured retinal neurons, it was demonstrated that calcium influx resulting from stimulation of NMDA receptor activates NOS and that an excess amount of NO produced by activated NOS mediates glutamate neurotoxicity.[10] NO-mediated cytotoxicity and the capacity of NO to induce apoptosis have been documented in several systems including macrophages,[11] astrocytes,[12] and neurons.[13]

As for the link between NO and glaucoma, an increased presence of 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).[14,15] In rats whose extraocular veins were cauterized to produce chronic ocular hypertension and retinal damage, expression of NOS-2 but not NOS-1 increases in ONH astrocytes.[16] Moreover, elevation of hydrostatic pressure in vitro upregulates the expression of NOS-2 in human astrocytes derived from the ONH.[17] Most important, inhibition of NOS-2 by aminoguanidine or L-*N*(6)-(1-iminoethyl)lysine 5-tetrazole amide protect against ganglion cell loss in the rat cautery model of glaucoma.[18,19] These data support that activation of NOS, especially NOS-2, may play a significant role in glaucomatous optic neuropathy. However, in a recent report, Pang et al.[20] showed that chronically elevated IOP in the rat induced by episcleral injection of hypertonic saline does not increase NOS-2 immunoreactivity in the optic nerve, ONH, or ganglion cell layer. Moreover, retinal and ONH NOS-2 mRNA levels did not correlate with either IOP level or severity of optic nerve injury. In addition, there was no difference in

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NOS-2 immunoreactivity in the optic nerve or ONH between POAG and nonglaucomatous eyes,[20] and aminoguanidine treatment did not affect the development of pressure-induced optic neuropathy in rats.[20] In view of these contradictory results, we considered it worthwhile to analyze the retinal nitridergic pathway activity in an experimental model of glaucoma induced by HA.

Materials and Methods

Animals and Tissues

Male Wistar rats (average weight, 200 ± 40 g) were housed in a standard animal room with food and water ad libitum under controlled conditions of humidity and temperature (21 ± 2°C), under a 12-h light–dark lighting schedule (lights on at 0700 hours). The study protocol complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (0.5 mg/kg) administered intraperitoneally. With a syringe (Hamilton, Reno, NV) and a 30-gauge needle, under a surgical microscope with coaxial light, 25 μL of HA (catalog H1751, molecular weight ~400 kDa, 10 mg/mL in saline solution; Sigma-Aldrich, St. Louis, MO), were injected into one eye of anesthetized rats, and an equal volume of vehicle (saline solution) was injected into the fellow eye, as previously described.[1,2]

After IOP assessment, animals were killed by decapitation at 1200 hours. Eyeballs were quickly enucleated after death and the corneas removed. The lens and vitreous were dissected under a surgical microscope, and the retinas were detached by blunt dissection. The retinas were examined to eliminate possible choroidal tissues. Immediately after dissecting, retinas were homogenized. At least two people performed this operation. The delay between harvesting and homogenization was between 30 and 45 seconds. A total of 144 animals were used for the experiments as follows: 32 for NOS activity assay, 24 for NOS activity assessment in the presence of 25 mM valine, 24 for Western blot analysis, 24 for L-arginine uptake assessment, 16 for RT-PCR studies, and 24 for cGMP assessment. In addition, a group of 46 animals, handled and anesthetized once a week for 6 weeks but not injected, was used as the control.

IOP Assessment

A tonometer (TonoPen XL; Mentor, Norwell, MA) was used to assess IOP in conscious, unsedated rats, as previously described.[1] All IOP determinations were assessed by operators who were blind to the treatment applied to each eye. Five IOP readings were obtained from each eye by using firm contact with the cornea and omitting readings obtained as the instrument was removed from the eye. The mean of these readings was recorded as the IOP for that eye on that day. IOP was assessed between 1100 and 1200 hours, to correct for diurnal variations in IOP.[1]

NOS Activity Assessment

Retinal NOS activity was assessed as previously described.[21] Each retina was homogenized in 100 μL of buffer solution containing 0.32 M sucrose and 0.1 mM EDTA (adjusted to pH 7.4 with Tris base). Reaction mixtures contained 50 μL of the enzyme source and 50 μL of a buffer stock solution (final concentrations: 10 mM HEPES, 3 mM $CaCl_2$, 1 mM NADPH, 5 μM FAD, 1 mM β-mercaptoethanol, ^{3}H-L-arginine (5 μCi/mL, purity greater than 98%), and 1 μM L-arginine, with or without 25 mM L-valine. After incubation at 37°C for 30 minutes, the reaction was stopped by adding 200 μL of stop buffer (50 mM HEPES, 10 mM EDTA, and 10 mM EGTA [pH 5.5]) and cooling the tubes for 5 minutes. The solution was mixed with 600 μL of Na^+ resin (Dowex AG50W-X8 Dow Chemical Co., Midland, MI) to remove L-arginine and centrifuged at 10,000*g* for 5 minutes. ^{3}H-L-citrulline in the supernatant was quantified by liquid scintillation counting. Nonenzymatic conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline was tested by adding buffer instead of the enzyme source.

Western Blot Analysis

Rat retinas were homogenized with a homogenizer (Polytron; Glen Mills, Clifton, NJ) in cold lysis buffer (20 mM Tris-HCl [pH 7.4], 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 10 μg/mL leupeptin, 1 mM polymethylsulfonyl fluoride [PMSF] and 2 μg/mL pepstatin A). Samples were mixed 1:1 with loading buffer (1.2 mL 1 M Tris [pH 6.8], 2 mL of glycerol, 4 mL of 10% sodium dodecyl sulfate, 2 mL of 1 M dithiothreitol, and 1% bromphenol blue), boiled for 5 minutes, and electrophoresed (80 μg proteins/lane) on 12% polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membranes for 45 minutes at 15V in a commercial system (Trans-Blot SD system; Bio-Rad, Hercules, CA) in transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 1.3 mM SDS [pH 9.2]). PVDF membranes were blocked in TBST buffer (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl and 0.05% Tween 20) and 1% BSA for 60 minutes at room temperature and then incubated for 1 hour in a 1:1000 dilution of the respective anti-NOS antisera (obtained from Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. Membranes were washed with TBST buffer and then incubated for 1 hour with a 3:10,000 dilution of a goat anti-rabbit IgG antibody-horseradish peroxidase conjugate. The membranes were washed, and the bands were visualized by chemiluminescence (ECL Western Blotting Analysis System; GE Healthcare, Amersham, UK).

^{3}H-L-arginine Uptake Assessment

L-Arginine uptake was examined as previously described.[21] Retinas were homogenized (1:9 wt/vol) in 0.32 M sucrose containing 1 mM $MgCl_2$, and centrifuged at 900*g* for 10 minutes at 4°C. Nuclei-free homogenates were further centrifuged at 30,000*g* for 20 minutes. The pellet was immediately resuspended in buffer Tris-HCl, and aliquots (100–300 μg protein/100 μL) were incubated with ^{3}H-L-arginine (10 μM, 800,000–1,000,000 dpm/tube, specific activity 53.4 Ci/mmol). After 10 minutes, ^{3}H-L-arginine uptake was terminated by adding 4 mL of ice-cold Tris-HCl buffer. The mixture was immediately poured onto filters (GF/B; Whatman, Florham Park, NJ) under vacuum. The filters were washed twice with 4-mL aliquots of ice-cold buffer, and the radioactivity on the filters was counted in a liquid scintillation counter. Nonspecific uptake of ^{3}H-L-arginine was assessed by adding an excess of L-arginine (10 mM).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from rat retinas (TRIzol Reagent; Invitrogen-Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions and stored in diethyl pyrocarbonate (DEPC)-treated water at −20°C. For each time point, two retinas were homogenized in 1 mL of the reagent (TRIzol; Invitrogen-Life Technologies), and the experiment was repeated four times with different animals. Before the reverse-transcription (RT) step, RNA was subjected to DNase I treatment (DNase I Amplification Grade; Invitrogen-Life Technologies), to eliminate any possible DNA contamination. RT was then performed on total RNA (2 μg). Briefly, cDNA synthesis was performed using 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI), 100 ng/μL random primers (Promega), dNTPs 0.5 mM each, and 25 U of RNase inhibitor (rRNAsin) in a total reaction volume of 25 μL. The temperature profile was 37°C for 50 minutes with a termination step at 70°C for 15 minutes. PCR reactions were performed in a thermocycler (Tpersonal; Biometra biomedizinische Analytik GmbH, Göttingen, Germany) with 5 μL of cDNA for the amplification of rat *Cat* gene products. The cDNA was added to 20 μL of the following reaction mixture: 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 400 nM of each specific oligonucleotide primer, and 1.25 units of *Taq* polymerase (Life Technologies, Bs As, Argentina). The sequences for the oligonucleotide primers were based on published sequences for *Cat-1*, *-2*, and *-3* (GenBank, L03290.1 and NM_007515,

TABLE 1. IOP of Rats Injected with HA or Vehicle for 3 or 6 Weeks

Treatment Length	Vehicle	HA
3 weeks	11.80 ± 0.24	23.2 ± 0.41*
6 weeks	12.30 ± 0.18	22.5 ± 0.38*

IOP (in mm Hg) was assessed with a handheld tonometer in rats injected with HA in one eye and vehicle in the contralateral eye for 3 or 6 weeks. At all these intervals, the injection of HA induced a significant increase in this parameter. In animals handled and anesthetized once a week for 6 weeks but not injected (control), IOP was 12.10 ± 0.20 mm Hg (n = 38 animals). No differences were observed between noninjected and vehicle-injected eyes at 3 or 6 weeks. Data are expressed as the mean ± SE (n = 48 eyes/group).

* $P < 0.01$, by Tukey test.

respectively; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The oligonucleotide upstream and downstream primer sequences and the size of the expected products were as follows: *Cat-1*: 5′-GCCATCGTCATCTCCTTCCTG-3′ and 5′CCCTCCCTCACCG-TATTTCAC-3′, (530 bp); *Cat-2*: 5′-AACGTGCTTTTATGCCTTTGT-3′ and 5′-GGTGACCTGGGACTCGCTCTT-3′, (612 bp); *Cat-3*: 5′-GCCTTT-TGGGCT CTATGTTTC-3′, and 5′TGCGGTTCTGTGGTTGTCT-3′ (560 bp), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 5′-TC-CCTCAAGATTGTCAGCAA-3′; and 5′-AGATCCACAACGGATACATT-3′ (309 bp). The identity of the obtained amplicons was confirmed by sequencing. Preliminary experiments were performed to determine the number of cycles to achieve a linear range of amplification. PCRs were performed with a first step at 94°C for 2 minutes, and then 27 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute plus a final incubation at 72°C for 10 minutes. Reaction products were electrophoresed on a 1.5% agarose gels in 40 mM Tris-acetate and 2 mM EDTA (pH 8); stained with ethidium bromide, photographed, and quantitated by a video documentation system (GelPro Imager, Media Cybernetics, Silver Spring, MD).

Cyclic GMP Levels Assessment

Retinal cell suspensions obtained by mechanical disruption (100 μL, 250–350 μg protein/tube) were incubated for 30 minutes at 37°C in buffer Tris-HCl with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). The cell suspensions were centrifuged at 800*g* for 5 minutes, and the pellets were resuspended in 0.4 mL of water and boiled for 2 minutes. The content of cGMP in the rat retinas was assessed as previously described.[21] Briefly, the suspensions were centrifuged at 5000*g* for 5 minutes at 4°C. Cyclic GMP content was measured in the supernatants by RIA after acetylation. For this purpose, aliquots of samples or standards were acetylated with acetic anhydride/triethylamine. The acetylated samples and the standard curve were mixed with ^{125}I-cyclic GMP (15,000–20,000 dpm, specific activity 140 mCi/mmol) and a rabbit antiserum (Chemicon International, Inc., Temecula, CA) diluted 1:150 and incubated overnight at 4°C. The antibody complex was precipitated with ethanol at 4°C, with 2% bovine serum albumin used as a carrier, centrifuged at 2000*g* for 30 minutes, and separated by aspirating supernatants. The radioactivity was measured in a gamma counter. The range of the standard curves was 10–5000 fmol of cGMP.

Protein concentration was determined by the method of Lowry et al.,[22] with bovine serum albumin as a standard.

Statistical analysis of results was made by a two-way analysis of variance (ANOVA), followed by the Tukey test, as stated.

RESULTS

Table 1 shows the average IOP of rats weekly injected with HA in one eye and vehicle in the other for 3 or 6 weeks. A significant increase in IOP was observed in eyes injected with HA compared with the respective control eyes. No differences in IOP in vehicle-injected eyes were detected between these time points or between noninjected and vehicle-injected eyes.

Retinal NOS activity was assessed in retinas from eyes injected with vehicle or HA for 3 or 6 weeks. As shown in Figure 1A, a significant increase in the conversion of arginine to citrulline was observed in HA-injected eyes during both periods, although the effect at 3 weeks of treatment with HA was significantly higher than after six injections of HA. No differences in this parameter were observed between noninjected and vehicle-injected eyes. Similar results were observed when retinal conversion of arginine to citrulline was assessed in the presence of an arginase inhibitor (25 mM valine; Fig. 1B). The arginase inhibitor slightly, but significantly decreased the conversion of ^{3}H-arginine to ^{3}H-citrulline in each experimental group ($P < 0.01$, Student's t-test). In addition, the percentage

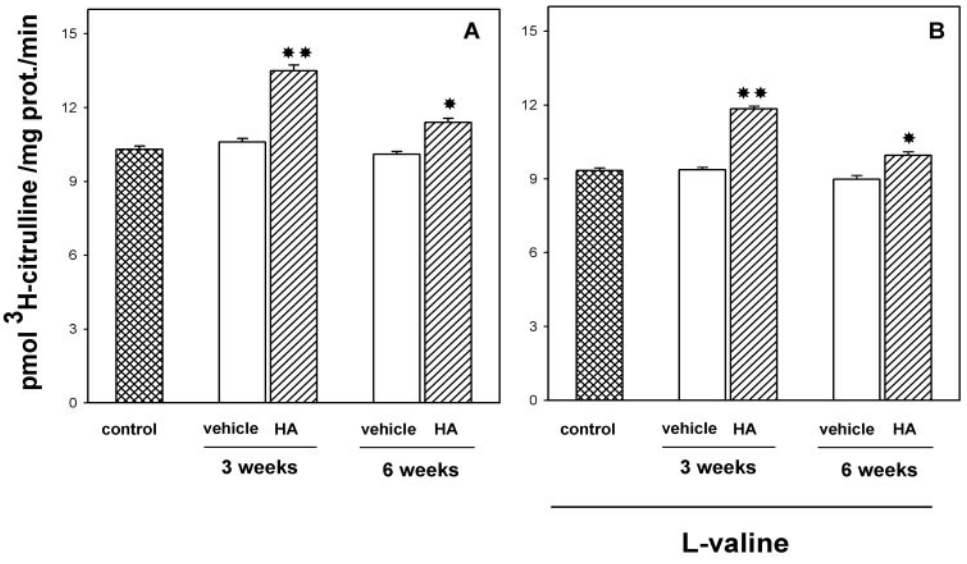

FIGURE 1. Effect of intracameral injections of HA on retinal NOS activity. Retinal conversion of ^{3}H-arginine to ^{3}H-citrulline was assessed, in the absence (**A**) or in the presence of 25 mM valine (**B**). In both cases, a significant increase of this parameter was observed in retinas of eyes injected with HA for 3 and 6 weeks. No differences in this parameter were observed between animals handled and anesthetized once a week for 6 weeks but not injected (control) and animals injected with vehicle for the same period (vehicle). Both in the absence or presence of valine, the retinal conversion of ^{3}H-arginine to ^{3}H-citrulline was significantly higher at 3 than at 6 weeks of treatment with HA ($P < 0.01$), but no changes in this parameters were observed between both periods of vehicle treatment. In each experimental group (control, vehicle for 3 or 6 weeks, HA for 3 or 6 weeks), valine significantly decreased the conversion of ^{3}H-arginine to ^{3}H-citrulline ($P < 0.01$, Student's t-test). Data are the mean ± SEM (for **A**, n = 1516 animals per group, for **B**, n = 10–12 animals per group). *$P < 0.05$, **$P < 0.01$ versus vehicle-injected eyes for the same period, by the Tukey t-test.

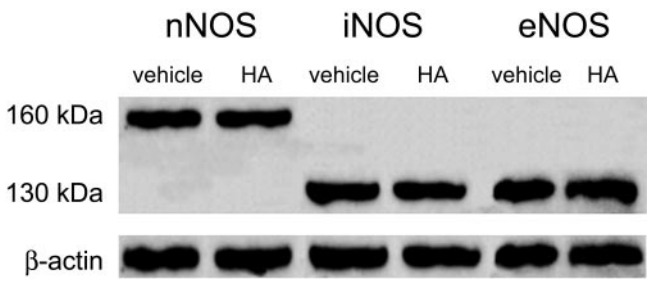

FIGURE 2. Assessment of retinal NOS-1, -2, and -3 levels in eyes injected with HA or vehicle for 3 weeks. Representative gels for NOS isoforms are shown. Each lane was loaded with 80 μg of proteins. In all the samples, bands of ~160, ~130, and ~134 kDa were identified in blots for NOS-1, -2, and -3, respectively. Scanning densitometry of the bands revealed no significant overall differences between groups (n = 4–6 retinas per group).

of valine-induced decrease (~10%) in this parameter was similar among groups.

Figure 2 shows a representative Western blot analysis of NOS isoform levels in retinas of eyes injected with vehicle or HA for 3 weeks. In agreement with the molecular mass reported for NOS isoenzymes in other tissues, bands of ~160, ~130, and ~134 kDa were identified for NOS-1, -2, and -3 (endothelial NOS [eNOS]), respectively. Scanning densitometry of the bands revealed no significant differences in the levels of these isoenzymes between control (not shown), vehicle, and HA-injected eye groups for both intervals. No changes in NOS isoforms levels were obtained in retinas from noninjected eyes and eyes injected with vehicle or HA for 6 weeks (data not shown).

Besides NOS activity, another limiting step in the regulation of NO biosynthesis is the availability of the precursor L-arginine. Therefore, the effect of ocular hypertension on L-arginine uptake was assessed. As shown in Figure 3, the influx of L-arginine significantly increased at 3 and 6 weeks of treatment with HA. The increase in L-arginine uptake was significantly higher at 3 weeks than at 6 weeks of HA-treatment, whereas no differences in L-arginine uptake were observed between noninjected and vehicle-injected eyes.

Results of semiquantitative RT-PCR using primers for CAT-1 and -2 are shown in Figure 4. CAT-1 and -2 mRNAs levels were significantly higher in eyes injected with HA than in vehicle-injected eyes. CAT-3 mRNA was not detected in both vehicle- and HA-treated eyes (data not shown). For CAT-2 mRNA levels, the increase was higher at 6 than at 3 weeks of treatment with HA, whereas no changes were detected for CAT-1 mRNA levels between these groups.

Figure 5 shows the effect of ocular hypertension on retinal cGMP content. At 3 weeks of treatment, this parameter significantly increased in HA-injected compared with vehicle-injected eyes, whereas no significant differences were observed at 6 weeks of treatment with HA or in control retinas.

DISCUSSION

The foregoing results indicate a significant activation of the retinal nitridergic pathway in rats exposed to experimentally elevated IOP. NO is a ubiquitous signaling molecule that participates in a variety of cellular functions. However, in concert with reactive oxygen species, NO can be transformed into a highly potent and effective cytotoxic entity of pathophysiological significance. NO may also signal through the interaction with reduced cysteines of proteins changing protein function.[23] As an intracellular signaling molecule, NO modulates the activity of various proteins that contribute to apoptosis.[24] Furthermore, it was recently demonstrated that an extracellular proteolytic pathway in the retina contributes to retinal ganglion cells death via NO-activated metalloproteinase-9.[25]

Several studies, most of them based on Western blot or immunohistochemical analysis, have addressed NO involvement in human or experimental glaucoma; however, they did not assess changes in the functional capacity of the retinal nitridergic pathway. Although no changes in the levels of NOS isoforms were observed in HA-treated eyes, the present results support a significant increase in the retinal arginine-to-citrulline conversion in hypertensive eyes. The presence of arginase was detected in the retina of several species, suggesting the existence of a retinal pathway through which L-arginine could be hydrolyzed to urea and ornithine, without synthesis of NO.[26,27] However, the fact that the increase of the arginine-to-citrulline conversion in HA-treated eyes persisted in the presence of an inhibitor of arginase activity, supports the notion that ocular hypertension provokes an increase in retinal NO biosynthesis.

Different mechanisms may modulate NOS activity, including changes in substrate supply,[28] protein phosphorylation,[29] acylation,[30] and subcellular localization,[31] among others. The intracellular events triggered by ocular hypertension that could explain the effect described herein and the identification of the isoform(s) of NOS, the activity of which is augmented by ocular hypertension remain to be established. However, since glutamate acting through NMDA receptors is one of the most conspicuous activators of NOS-1 activity, the raise in glutamate synaptic levels in HA-treated eyes[5] could account for an increase in nNOS activity in this experimental model. Thus, the activation of NOS in hypertensive eyes may be linked to glutamate levels that, in turn, may be elevated to such an extent that they are toxic for ganglion cells. In this sense, it has been shown that retinal ganglion cells in the nNOS-deficient mouse are relatively resistant to NMDA, whereas damage in the retina of the eNOS-deficient mouse is not distinguishable from that observed in control animals.[32] Moreover, it has been demonstrated that intravitreal injection of NMDA in rats induces

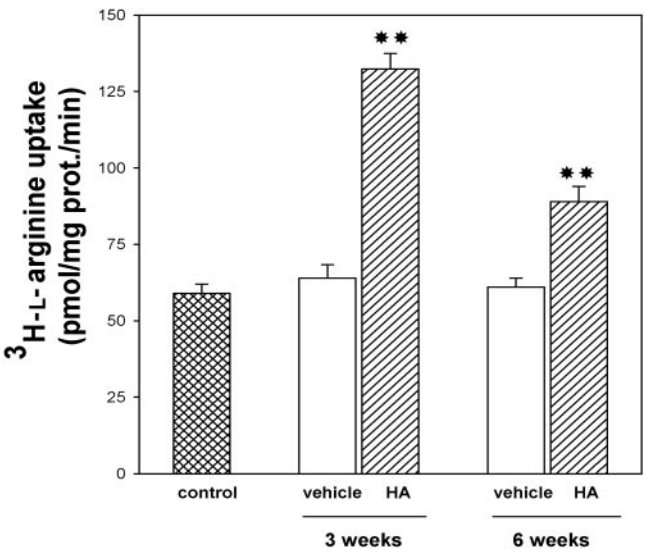

FIGURE 3. Effect of experimental ocular hypertension on retinal L-arginine uptake. The treatment with HA for 3 and 6 weeks significantly increased L-arginine influx. The increase in this parameter was significantly higher at 3 than at 6 weeks of treatment with HA ($P < 0.01$), whereas no difference between noninjected and saline-injected animals was observed. Data are mean ± SEM (n = 10–12 animals per group). $^{**}P < 0.01$ versus vehicle-injected eyes for the same period; Tukey t-test.

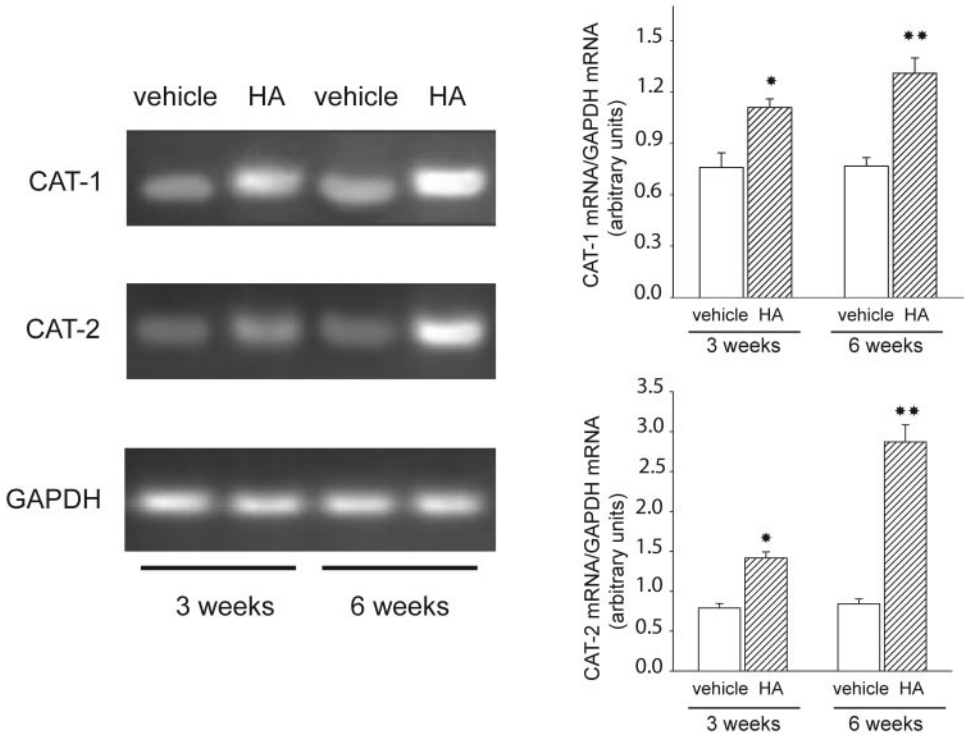

FIGURE 4. CAT-1 and -2 mRNA levels in retinas of eyes injected with vehicle or HA for 3 and 6 weeks. Total RNA was assayed by semiquantitative RT-PCR by use of gene-specific primers. A 309-bp fragment for GAPDH was amplified from cDNA generated by the RT for each sample to verify cDNA input in each PCR reaction. *Left*: both CAT-1 and -2 mRNAs levels were significantly higher in HA-treated eyes for 3 and 6 weeks. For CAT-2 mRNA levels, the increase was higher at 6 than at 3 weeks of treatment with HA ($P < 0.01$), whereas no changes were detected for CAT-1 mRNA levels between these groups. Results are representative of four independent RT-PCR analyses. *Right*: data are the mean ± SEM of densitometric values of *Cat* mRNA levels relative to GAPDH mRNA band intensity. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-injected eyes for the same period, the Tukey *t*-test ($n = 4$).

accumulation of nitrite-nitrate and lipid peroxidation.[33] In agreement, we have reported a significant increase of lipid peroxidation in HA-treated eyes.[4] These findings notwithstanding, the involvement of e-NOS and/or iNOS in the increased biosynthesis of NO in retinas treated with HA cannot be ruled out.

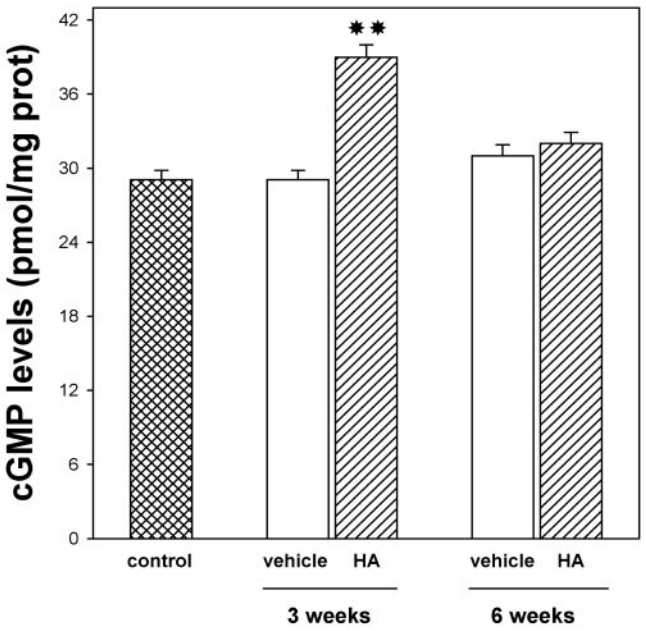

FIGURE 5. Effect of intracameral injections of HA for 3 and 6 weeks on retinal cGMP accumulation. A significant increase of cGMP accumulation was observed in retinas from eyes injected with HA for 3 weeks compared with eyes injected with saline solution. No significant differences in eyes injected with vehicle or HA for 6 weeks, and between not injected and saline-injected animals were observed. Data are mean ± SEM ($n = 10$–12 retinas per group). $^{**}P < 0.01$ by Tukey *t*-test.

The present results showed an increase in retinal L-arginine as well as CAT-1 and -2 mRNA levels in HA-treated eyes. Because the assay of NOS activity involves the incubation of retinal homogenates in the presence of controlled concentrations of L-arginine, one can assume that the higher NOS activity observed in hypertensive eyes is not merely a consequence of an increase in L-arginine. The coexistence of NOS and arginase could indicate that both enzymes may compete for intracellular L-arginine. However, whereas the K_m of NOS isoenzymes for L-arginine is in the 2- to 20-μM range, the K_m of mammalian arginases is ~2 to 20 mM,[34,35] indicating that the affinity of NOS for L-arginine is approximately 1000-fold greater than that of arginase. Thus, it seems likely that extracellular L-arginine could be more preferentially delivered to NOS than to arginases.

Purified NOS from different sources has been reported to have a low half-saturating L-arginine concentration (EC_{50}, 10 μM). Since high levels of intracellular L-arginine ranging from 0.1 1 mM have been measured in many systems,[36] it is expected that endogenous L-arginine would support the maximum activation of NOS. However, a number of in vivo and in vitro studies indicate that NO production under physiological conditions can be increased by extracellular L-arginine, despite saturating intracellular L-arginine concentrations. This has been termed "the arginine paradox."[37] One explanation could be that intracellular L-arginine is sequestered in one or more pools that are poorly, if at all, accessible to NOS, whereas extracellular L-arginine transported into the cells is preferentially delivered to NO biosynthesis.[37] Accordingly, it has been demonstrated that L-arginine availability controls NMDA-induced NO synthesis in the rat central nervous system.[38] Therefore, it seems likely that to induce the activation of NOS, an obligatory influx of L-arginine is required. The coordination between NOS activity and L-arginine uptake has been demonstrated in several systems such as rat brain,[39] and diabetic rat retina.[40] A similar coordination between NO biosynthesis and intracellular L-arginine availability seems to occur in hypertensive eyes. Recently, it has been demonstrated that activation of NMDA receptors in

cultured retinal cells promotes an increase of the intracellular L-arginine pool available for NO synthesis.[41] This way, the increase in both NOS activity and L-arginine influx could be triggered by higher levels of synaptic glutamate levels in retinas from eyes injected with HA.

Amino acid transport systems have been classified considering two main criteria: (1) substrate specificity (i.e., which amino acids or groups of amino acids are transported by the system); and (2) sodium dependence of the transport mechanism, generally defined with reference to the rate measured in the presence of choline salt. Four amino acid transport systems (denoted by y^+, $b^{o,+}$, $B^{o,+}$ or y^+L) have been defined on the basis of substrate specificity and sodium dependence (for a review, see Ref. 42). Only one of them (y^+) is selective for cationic amino acids and is sodium independent. It has been demonstrated that uptake of L-arginine in retinas of rats occurs through a transporter resembling the y^+ system.[43] The same amino acid transport system has been identified in a wide variety of tissues, including the hamster retina.[44] This transport system encompasses three homologous proteins (CAT-1, -2, and -3) that have been characterized in several tissues. *Cat-1*, the first amino acid transporter gene to be cloned, is widely expressed, and the protein product exhibits the characteristics originally assigned to system y^+. A subsequently identified cationic amino acid transporter gene, *cat-2*, shares high sequence homology with *cat-1* and also exhibits the same characteristics assigned to system y^+.[45] RT-PCR analysis using primers for the aforementioned isoforms demonstrated an increase of mRNAs for both CAT-1 and -2 in retinas from hypertensive eyes, suggesting that ocular hypertension could induce an upregulation of L-arginine transporters. CAT-2 mRNA levels were significantly higher at 6 than at 3 weeks of treatment with HA, whereas changes in CAT-1 mRNA did not reach statistical significance. At present, we do not have any explanation for the lack of correlation between mRNA levels and arginine uptake in HA-injected eyes. An insight into this problem could be obtained by assessing protein levels of arginine transporters. However, specific antibodies against these proteins are not commercially available. Besides, postransductional regulation mechanisms cannot be ruled out.

The binding of NO to the heme iron of soluble guanylyl cyclase. and its consequent activation. have been well characterized. If both L-arginine influx and its conversion to NO were higher in HA-injected eyes, higher levels of retinal cGMP would be expected in retinas exposed to experimentally induced hypertension. Although a significant increase in cGMP accumulation was observed in retinas of eyes injected with HA for 3 weeks, no differences were detected after 6 weeks of treatment. There is no ready explanation for the fact that cGMP levels did not increase after 6 weeks of ocular hypertension. However, in agreement with this result, the increase in both NOS activity and L-arginine uptake was significantly lower at 6 than at 3 weeks of treatment with HA. Moreover, because cGMP plays a critical role in the phototransduction cascade, in all likelihood, retinal levels of this nucleotide are regulated by other mechanisms besides NO. In fact, it has been demonstrated that atrial natriuretic peptide increases retinal synthesis of cGMP through a NO-insensitive particulate guanylate cyclase.[46] In addition, other regulators of retinal cGMP content than NO might slow its catabolism, accounting for an increase in the levels of this nucleotide.

Since nitridergic parameters were assessed in the whole retina, we could not ascertain the precise locus of the observed phenomena. The distribution of NOS isoforms among the different retinal cell types is not yet known in detail, but the available results indicate that they are present in at least some members of each basic type of retinal cell. In both rat and human retinas, NOS-1 is expressed in the inner segments of photoreceptors and in cells in the inner nuclear layer (INL), particularly amacrine cells and retinal ganglion cells,[47] whereas NOS-2 has been detected in cell somas localized in the inner nuclear layer (INL), ganglion cell layer (GCL), Müller cell processes along the inner plexiform layer (IPL) and GCL, and also in the inner photoreceptor segments (IPS).[48] In addition, the presence of eNOS immunoreactivity has been demonstrated in Müller glia and horizontal cells.[49] Assuming the hypothesis that NMDA receptors are involved in the overactivation of the nitridergic pathway described herein, it is tempting to speculate that the increase in NO production is restricted to those cell types that coexpress NMDA receptors and NOS. The demonstration of a strong NMDA activation restricted to subsets of the amacrine and ganglion cell populations,[50] together with the fact that intravitreal injection of NO donors provokes a significant decrease in cell density in the ganglion cell layer and the thinning of the IPL,[51] support the thought that the phenomena described herein could occur in the inner (but not outer) retina.

In a previous report, injections of HA for 6 weeks induced a significant decrease in electroretinographic activity[2] and a significant loss of ganglion cells after 10 weeks of ocular hypertension induced by HA.[2] Thus, the changes in nitridergic pathway activity described herein preceded functional and histologic alterations induced by ocular hypertension. Therefore, it is possible that an overactivation of the retinal nitridergic system contributes to the ocular hypertension-induced neuropathy.

Although the current management of glaucoma is mainly directed at the control of IOP, a therapy that prevents the death of ganglion cells should be the main goal of treatment. As previously suggested,[19] these results further support that a decrease in the retinal nitridergic pathway activity may be a therapeutic strategy to prevent glaucomatous cell death.

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