Isoforms of Nitric Oxide Synthase in the Optic Nerves of Rat Eyes with Chronic Moderately Elevated Intraocular Pressure

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Purpose. To investigate the hypothesis that nitric oxide (NO) in the optic nerve heads of rats with chronic moderately elevated intraocular pressure (IOP) contributes to neurotoxicity of the retinal ganglion cells, the presence of the three isoforms of nitric oxide synthase (NOS) was determined in the tissue.

Methods. Unilateral chronic moderately elevated IOP was produced in rats by cautery of three episcleral vessels. Histologic sections of optic nerves from eyes with normal IOP and with chronic moderately elevated IOP were studied by immunohistochemistry and by immunoblot analysis. Polyclonal antibodies to NOS-1, NOS-2, NOS-3, and glial fibrillary acidic protein (GFAP) were localized with immunoperoxidase.

Results. In the optic nerve of rat eyes with normal IOP, NOS-1 was constitutively present in astrocytes, pericytes and nerve terminals in the walls of the central artery. NOS-2 was not present in eyes with normal IOP. In these eyes, NOS-3 was constitutively present in the vascular endothelia of large and small vessels. Rat eyes treated with three-vessel cautery had sustained elevated IOP (1.6 fold) for at least 3 months. In these eyes, no obvious changes in NOS-1 or NOS-3 were noted. However, at time points as early as 4 days of chronic moderately elevated IOP, NOS-2 appeared in astrocytes in the optic nerve heads of these eyes and persisted for up to 3 months. Immunoblot analysis did not detect differences in NOS isoforms.

Conclusions. The cellular distributions of constitutive NOS isoforms in the rat optic nerve suggest physiological roles for NO in this tissue. NOS-1 in astrocytes may produce NO as a mediator between neighboring cells. NO, produced by NOS-1 in pericytes and nitrergic nerve terminals and by NOS-3 in vascular endothelia, is probably a physiological vasodilator in this tissue. In eyes with chronic moderately elevated IOP, NOS-2 is apparently induced in astrocytes. The excessive NO production that is associated with this isoform may contribute to the neurotoxicity of the retinal ganglion cells in eyes with chronic moderately elevated IOP. (*Invest Ophthalmol Vis Sci.* 1999;40: 2884–2891)

Although studies of patients with glaucoma indicate that elevated intraocular pressure (IOP) is the single most common finding in glaucoma,[1] the pathophysiological mechanisms by which elevated IOP leads to cellular events that are neurodestructive in the optic nerve head are unknown. Knowledge of cellular mediator pathways that contribute to glaucomatous optic neuropathy may lead to new therapeutic approaches that slow or prevent further damage to the axons of the retinal ganglion cells. Achieving pharmacologic neuroprotection for the treatment of glaucoma may augment or even supersede the current clinical goal of lowering IOP.

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Nitric oxide (NO), a dissolved gas with a half-life of a few seconds,[2] is an important mediator with diverse physiological roles and certain pathologic roles in various tissues, including the central nervous system (CNS) and the eye.[3–7] In the CNS, NO has been implicated in neurodegenerative diseases such as stroke, Alzheimer's disease, multiple sclerosis, and amyotropic lateral sclerosis. In animal models of neurodegeneration, NO can be both neuroprotective and neurodestructive.

Nitric oxide is formed from L-arginine by nitric oxide synthase (NOS). Molecular cloning has identified three distinct genes expressing NOS isoforms: neuronal NOS (nNOS or NOS-1), endothelial NOS (eNOS or NOS-3) and inducible NOS (iNOS or NOS-2). The nomenclature was derived from the tissue in which they were first studied, and the numbering was derived from the order in which they were cloned.[8] NOS-1 and NOS-3 are constitutive, are present physiologically, and are calcium dependent. Under conditions of degeneration and inflammation, both isoforms may be upregulated.[5] In contrast, NOS-2 is not constitutive, is Ca^{2+} independent, and is induced after immunologic challenge and neuronal injury.[5,9]

In the rat CNS, all three NOS isoforms have been well characterized with immunohistochemistry.[10–13] In the retina, NOS-1 has been identified in amacrine cells, nerve fibers of the

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inner and outer plexiform layers, the ganglion cell layer, photoreceptor ellipsoids, bipolar cells, and large choroidal vessels.[10,14,15] However, the optic nerve of normal rats, although not studied in great detail, is reported to be devoid of NOS.[10]

NOS-1 mediates early neuronal injury after middle cerebral artery occlusion[16] and is blocked pharmacologically with an NOS-1–selective inhibitor 7-nitroindazole.[17] NOS-1 null transgenic mice sustain reduced infarct volumes, compared with age-matched control animals, after permanent focal middle cerebral artery occlusion.[18] Blockage of neurotoxicity with NOS inhibitors in primary cortical neuronal cultures has also been demonstrated.[5] In contrast, NOS-3, a major regulator of vascular hemodynamics and blood vessel relaxation, is neuroprotective. Transgenic mice without NOS-3 experience increased infarct volumes after middle cerebral artery occlusion.[19] Inducible NOS, which is not normally expressed in the brain, can be induced in astrocytes and microglia after viral infection or trauma. Induction of NOS-2 produces large quantities of NO for sustained periods and results in neuronal damage.[20–22] NOS-2 may contribute to neurodegeneration in several human diseases.[23]

Our laboratory has recently reported the presence of the constitutive isoforms NOS-1 and NOS-3 in the normal human optic nerve head and has demonstrated all three isoforms of NOS in the optic nerve head of patients with primary open-angle glaucoma, suggesting induction of NOS-2 in glaucoma.[24] Excessive NO, synthesized by NOS-1 and/or NOS-2, may be neurodestructive in the optic nerve heads of patients with glaucoma.

Given the findings of NOS isoforms in glaucomatous optic nerve heads and the implication of NO as a neurodestructive agent, we have studied the presence, localization and distribution of the three NOS isoforms in the rat optic nerve. We chose the rat because this species provides a potentially useful model for studying optic neuropathy caused by chronic moderately elevated IOP[25] for the eventual purpose of screening promising pharmacologic compounds.[26] We confirmed the elevated IOP and retinal ganglion cell loss in this model and, using commercially available antibodies for immunohistochemistry and immunoblot analysis, we demonstrated NOS isoforms in the anterior optic nerve of normal rats and rats with chronic moderately elevated IOP. Our results on the NOS-2 isoform are similar to those that we have previously reported for the human optic nerve head.

Materials and Methods

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

AlbinoWistar rats (200–300 g) were fed ad libitum and maintained in temperature-controlled rooms on a 12-hour light–12-hour dark cycle (light period from 6 AM to 6 PM). Animals were anesthetized by intraperitoneal injection of a mixture of xylazine (12 mg/kg) and ketamine (80 mg/kg). IOP was measured with a pneumotonometer (Mentor 30 Classic; BioRad, Santa Ana, CA) under light anesthesia, as previously described. IOP measurements were begun within 5 minutes of inducing anesthesia, and the animals were awake within 30 minutes of the IOP measurement. Rat eyes were anesthetized with one drop of topical 0.5% proparacaine HCl (Bausch & Lomb Pharmaceuticals, Tampa, FL). The mean IOP of four to six consecutive measurements was recorded. IOP was measured before surgery, within 2 hours after surgery, and once or twice a week for up to 3 months after surgery. All IOP measurements were made at the same time of day (10 AM–12 noon) by one of three observers, and the measurements were completed within 5 minutes.

Unilateral, three-vessel cautery for elevating IOP[25] was performed as previously described.[26] Briefly, three of the four to five major trunks formed by limbal-derived veins were exposed at the equator of the eye by incising the conjunctiva. Each vein was lifted with a small muscle hook or forceps and cauterized by direct application of an ophthalmic, disposable cautery (Model RS201; Roboz, Rockville, MD) against the muscle hook. Care was taken to avoid cauterizing sclera directly. Immediate retraction and absence of bleeding of the cauterized ends of the vessels were noted as successful cauterization. All animals had unilateral chronic moderately elevated IOP and the contralateral eye was either untouched or subjected to a sham operation. For each animal, the contralateral eye always served as the comparative control. On a given day, mean ± SD was derived for all control and surgical (three-vessel cautery) eyes. Significant differences between surgical and control eyes were determined by χ^2 analysis with Student's *t*-test for independent means for each day on which measurements were performed.

For immunohistochemistry, after 4, 14, 28, 45, and 84 days of unilateral chronic moderately elevated IOP, groups of four to six animals at each time point were killed with an overdose of the anesthesia mixture described earlier. Enucleation of both eyes was performed with a suture placed at 12 o'clock for proper orientation, and eyes were fixed in fresh 4% paraformaldehyde for 1 hour. Subsequently, the cornea, iris, lens, and vitreous were removed. The posterior segment was fixed in fresh 4% paraformaldehyde for 1 hour and then transferred to 70% ethanol overnight. Fixed tissue was washed in 0.2% glycine in phosphate-buffered saline (PBS; pH 7.4), embedded in paraffin, and oriented for 6-μm sagittal sections. Five sections per optic nerve were examined by use of immunohistochemistry, with one of the sections serving as a negative control.

The isoforms of NOS were identified using a polyclonal antibody to human NOS-1, which recognizes an epitope at the amino terminus (working dilution, 1:300), a polyclonal antibody to human NOS-3, which recognizes an epitope at the carboxyl terminus (working dilution, 1:500), and a polyclonal antibody to mouse NOS-2, which recognizes an epitope at the carboxyl terminus (working dilution 1:25; all polyclonal antibodies to human NOS from Santa Cruz Biotechnology, Santa Cruz, CA). To identify glial cells, a monoclonal antibody (Sigma, St. Louis, MO) to glial fibrillary acidic protein (GFAP) was used (working dilution, 1:400). Slides were preincubated with 5% skim milk for 30 minutes and then incubated with primary antibody overnight at 4°C.

Primary antibodies were localized by immunoperoxidase staining using commercially available reagents (Vector, Burlingame, CA). The biotinylated secondary antibody was incubated on the sections for 30 minutes, washed with PBS, and reacted with the streptavidin-peroxidase conjugate for 30 minutes. After washing, sections were incubated with the substrate mixture (1.5 mg 3,3-diaminobenzidine tetrahydrochloride and 50 μl 30% hydrogen peroxide in 0.1 mM Tris phosphate [pH 7.6]). The sections were reacted until brown

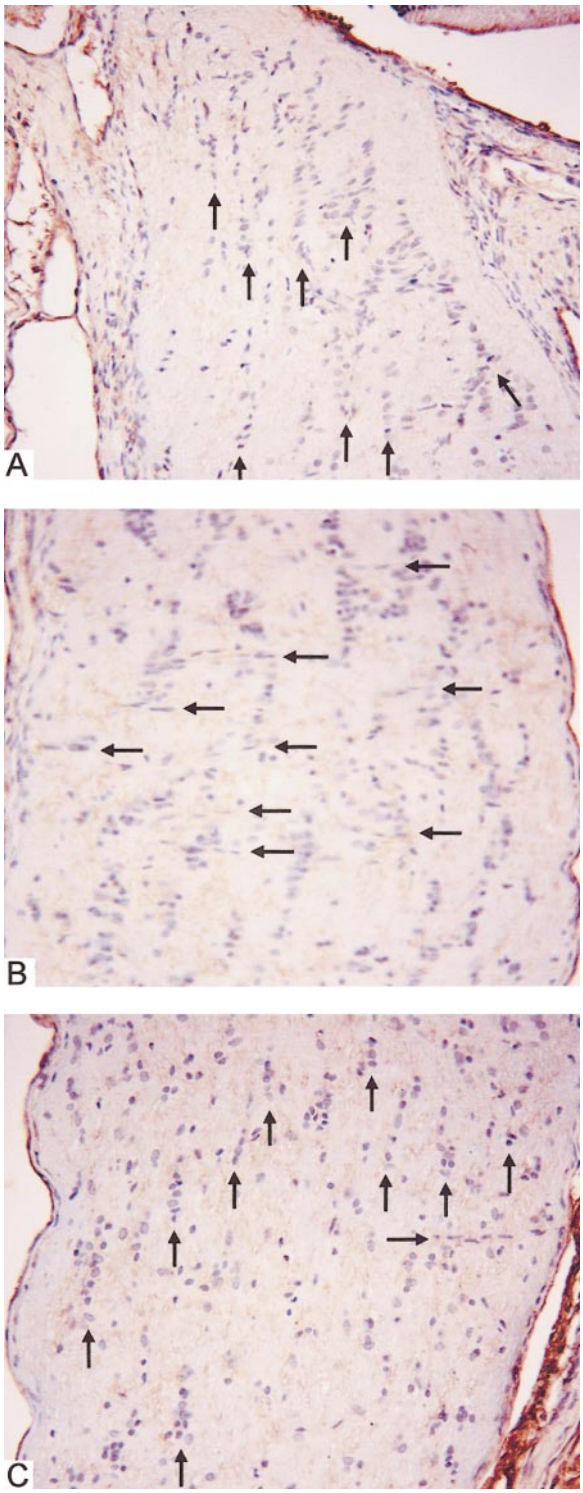

FIGURE 1. Anatomy of the rat optic nerve head. In the neck region (**A**), *vertical arrows* indicate glial columns. In the transition region (**B**), transverse linear arrays of cells are indicated by *horizontal arrows*. In the posterior region (**C**), where the nerve becomes myelinated, *vertical arrows* indicate glial columns. Magnification, ×200.

staining appeared, in approximately 1 to 3 minutes, washed in PBS, counterstained with hematoxylin, and dehydrated. A coverslip was mounted on the slide with sealer (Cytoseal 60; Fisher Scientific, Pittsburgh, PA).

Representative sections of all samples were stained simultaneously to control variation in the reactions. Negative controls were performed by eliminating primary antibody from the incubation medium, using the antibody preabsorbed with the appropriate antigenic peptide, or by replacing the primary antibody with nonimmune serum followed by immunoperoxidase staining. Slides were examined by microscope (BH2; Olympus, Tokyo, Japan), and images were recorded by digital photography and stored as a computer file.

For immunoblot analysis, 12 animals were killed after 28 days of unilateral chronic moderately elevated IOP. Immunoblots were performed on lysates of individual rat optic nerves that were dissected from surrounding tissues. Protein (10 μg) was loaded and run on to 10% Tris-glycine gels (Novex, San Diego, CA) and then transferred to a nitrocellulose membrane (Hybond-c; Amersham, Arlington Heights, IL). Membranes were blocked with 2% milk and treated with 0.5% Tween-20 before application of the primary antibodies (working dilution, 1:1000). After 1 hour, the membranes were washed, and secondary antibody with conjugated horseradish peroxidase (Santa Cruz) was applied (working dilution, 1:10,000). After further washing, the blots were developed with enhanced chemiluminescence western blot detection reagents (Amersham).

RESULTS

Anatomy of the Rat Optic Nerve Head

The rat optic nerve head is somewhat analogous to the human optic nerve head. The area of the optic nerve head in the rat is well demarcated between the vitreal surface, the retinal nerve fiber layer, the scleral canal, and the beginning of the myelination of the axons of the optic nerve (Fig. 1). The optic nerve head region in the rat is relatively elongated compared with human tissue. Morrison[27,28] described two anterior regions, the neck and the transition. In the neck region, there are glial columns in linear arrays of single cells with round nuclei that run parallel to the nerve bundles (Fig. 1A). These glial columns are similar to glial columns in the prelaminar region in the human. Below this region, many cells, presumably glia, have a transverse orientation with the long axis of their flat nucleus running perpendicular to the nerve bundles (Fig. 1B). Within this region, there are many, adjacent collections of flat nuclei running perpendicular to the nerve bundles, suggesting rudimentary, individual, cribriform, platelike structures. These transverse plates stained weakly for collagen, indicating that they are composed of connective tissue (data not shown). The individual cribriform plates in the rat optic nerve head are spread over a greater relative distance than in the human optic nerve head where they form the lamina cribrosa. In the region posterior to the transverse plates where the axons become myelinated, glial cells form the glial columns and the glia limitans (Fig. 1C), as in the human.

Chronic Moderately Elevated IOP

After three-vessel cautery, IOP was consistently elevated approximately 1.6 fold and remained elevated for at least 3 months compared with contralateral control eyes (Table 1). No further treatment was necessary after the initial three-vessel cautery. Immediately after surgery, IOP was never higher than 35 to 40 mm Hg, and there was no indication of retinal ischemia by histologic examination.

TABLE 1. IOP Levels

	1 Month	2 Months	3 Months
Three-vessel cautery eyes	19.6 ± 1.9*	18.6 ± 1.7*	17.6 ± 1.0*
Contralateral eyes	11.5 ± 0.7	11.5 ± 0.9	11.7 ± 0.9

Data are expressed as mean mm Hg ± SD. Number of eyes examined: 1 month, 105; 2 months, 30; 3 months, 14.

* $P < 0.01$ versus contralateral eyes.

Immunohistochemistry for NOS Isoforms

Figure 2 demonstrates the cellular localizations of NOS-1 and NOS-3 in the optic nerve head of the rat. In the anterior portion of the optic nerve head, corresponding to the neck region, and in the transition area, containing cells organized transversely across the nerve bundles, NOS-1–positive, granular staining appeared to be distributed throughout the cytoplasm of individual cells (Fig. 2A). The pattern of distribution of NOS-1–positive cells was similar to the pattern of cells that were positive for GFAP, a cytoskeletal marker for astrocytes (Fig. 2B). However, many more cells were positive for GFAP than for NOS-1, suggesting that not all astrocytes contain NOS-1. In the glial columns that were posterior to the transition area, most cells were positive for NOS-1 (Fig. 2C).

Occasionally, cells in the transition zone, closely wrapped around a capillary wall and abutting the nerve fiber bundles, were positive for NOS-1 (Fig. 2D). This NOS-1–positive immunoreactivity was not localized to the endothelial cells of the capillary and did not correspond to GFAP staining (compare to Fig. 2B). This pattern suggests that NOS-1 may be present in pericytes in the rat optic nerve head.

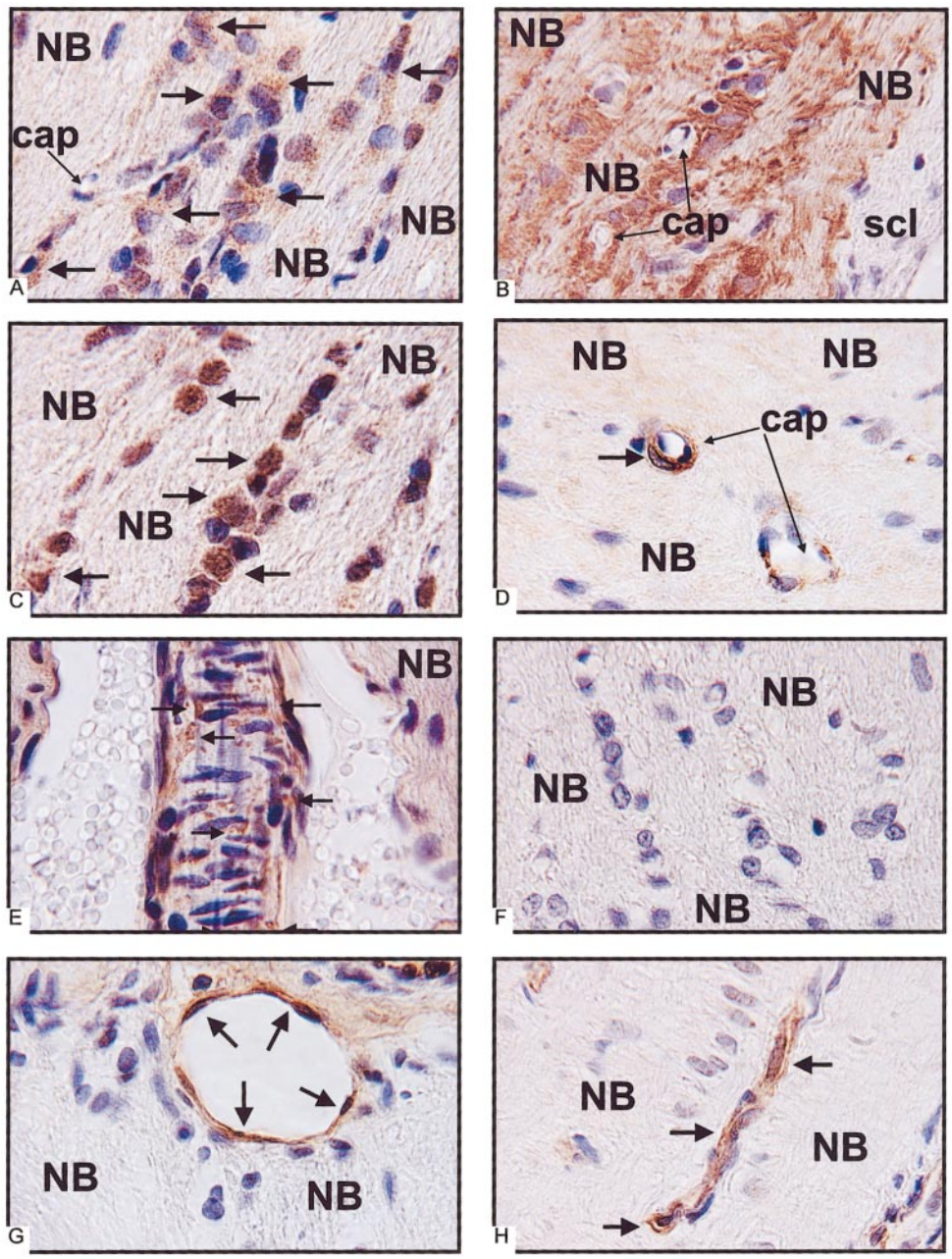

FIGURE 2. Immunohistochemistry for NOS-1 and NOS-3 in the rat optic nerve head. In the transition region (**A**), many but not all cells were positive for NOS-1. The location of NOS-1–positive cells is the same as the location of GFAP-positive cells (**B**). In the posterior region of the optic nerve (**C**), most cells in the glial columns were positive for NOS-1. NOS-1–positive cells, presumably pericytes, also surrounded small blood vessels (**D**). In the walls of large arterial vessels, punctate staining for NOS-1 was present (**E**), perhaps associated with nitrergic nerve terminals. In the absence of primary antibody for NOS-1, there was no specific staining of optic nerve head tissue (**F**). In large vessels near the vitreous surface (**G**) and in capillaries (**H**), the vascular endothelia are positive for NOS-3. *Arrows* point to specific NOS staining. NB: nerve bundles; cap: capillary; scl: sclera. Magnification, ×1000.

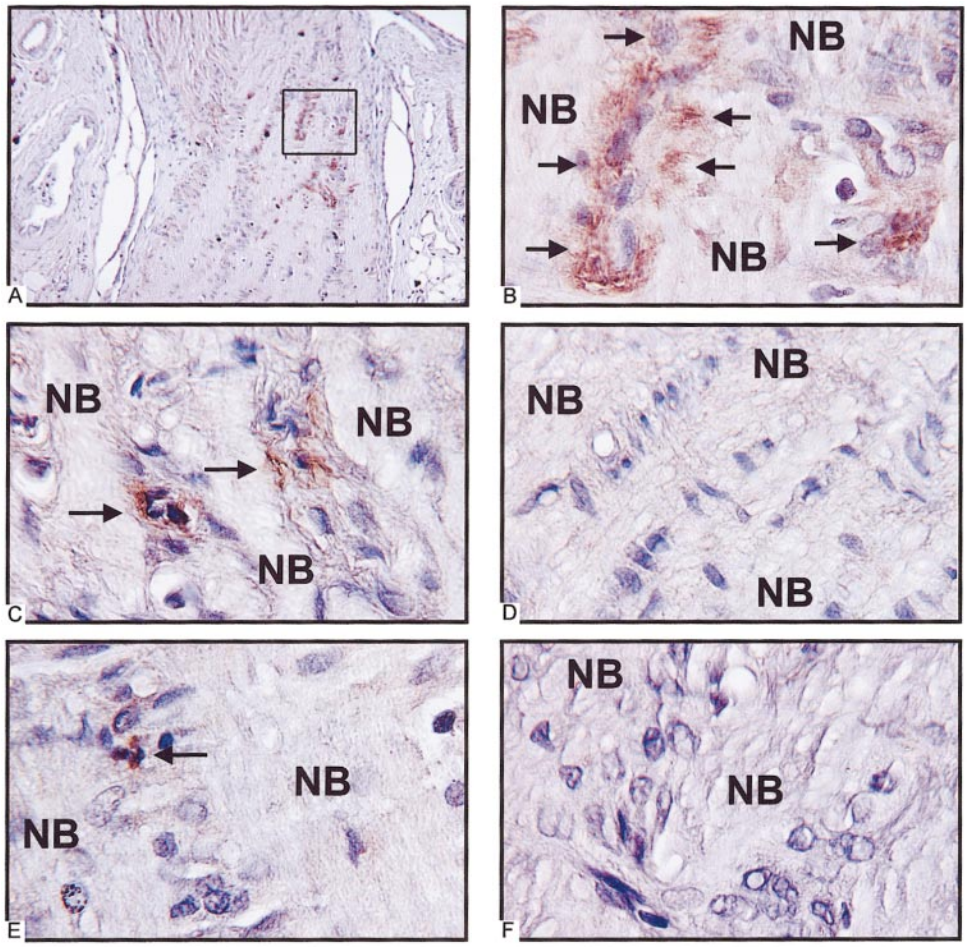

FIGURE 3. Immunohistochemistry for NOS-2 in the rat optic nerve heads of rats with chronic moderately elevated IOP. (**A**) Low-power magnification of optic nerve head demonstrating clusters of NOS-2–positive cells in an eye with chronic moderately elevated IOP for 4 days. (**B**) High-power magnification of region in *inset* in (**A**) demonstrating NOS-2–positive cells, presumably astrocytes. NOS-2–positive cells were present in the optic nerve heads of eyes after 28 days (**C**) and 84 days (**E**) of chronic moderately elevated IOP, but not in the contralateral eyes (**D**, **F**, respectively). *Arrows* point to specific NOS-2 staining. NB: nerve bundles. Magnification, (**A**) ×100, (**B** through **F**) ×1000.

In the optic nerve head near the vitreal surface, staining for NOS-1 was also associated with the arterial vasculature, with a punctatelike pattern in the outer walls of the vessel that was suggestive of nerve terminals (Fig. 2E). This type of staining was not apparent around capillaries or veins. Of note, the endothelial cells bordering the vascular lumen were not positive for NOS-1.

Eyes with chronic moderately elevated IOP were evaluated for NOS-1–positive cells on days 4, 14, 28, and 45. No difference in the density of positive cells could be discerned by inspection.

Figure 2 also demonstrates the presence of cells that were positive for NOS-3. These cells were localized primarily to the vasculature. NOS-3–positive cells are present in both the arterial and venous systems in the optic nerve head (Fig. 2G). The staining pattern was diffuse throughout the cell body and localized to the endothelial cells with no evidence of NOS-3 in the surrounding perivascular cells. Throughout the optic nerve, including the neck region, transition zone and the area of myelination, numerous capillaries were present, with positive NOS-3 immunoreactivity in the endothelial cells (Fig. 2H).

Eyes with chronic moderately elevated IOP for 4, 14, 28, and 45 days were evaluated for NOS-3–positive cells. No difference in the density of positive cells could be discerned by inspection.

NOS-2–positive cells were present in the optic nerve heads of rat eyes with chronic moderately elevated IOP but were not present in contralateral control eyes with normal IOP (Fig. 3). Examination at low power of optic nerve head tissue from rat eyes with 4 days of chronic moderately elevated IOP demonstrated clusters of cells in the transition zone that were positive for NOS-2 (Fig. 3A). Not all cells were positive for NOS-2, and not all regions contained NOS-2–positive cells. At high power, NOS-2–positive cells had the distribution of GFAP-positive cells or astrocytes (Fig. 3B). At 28 and 84 days of chronic moderately elevated IOP, NOS-2–positive cells, presumably astrocytes, were identified in the transition region of optic nerve heads (Figs. 3C, 3E, respectively) but not in contralateral control eyes with normal IOP (Figs. 3D, 3F). Usually, several cells could be identified per tissue section. NOS-2–positive cells were always in locations associated with glia. The vascular endothelia of some capillaries may have been NOS-2–positive in these eyes. Cells that were positive for NOS-2 were not present in the optic nerve proper or the retina. No loss of nerve fibers or disorganization of the optic nerve head tissue was histologically apparent after 3 months of chronic moderately elevated IOP.

Control slides, obtained by omitting the primary antibody or by preincubating the primary antibody with specific antigenic peptides, were all negative for specific NOS staining. Figure 2F demonstrates the absence of specific staining when the primary antibody for NOS-1 was omitted. Additional figures demonstrating the absence of specific staining under control conditions are not presented. However, because we can demonstrate different cellular localizations for the NOS isoforms in the rat optic nerve, comparisons can be made between the

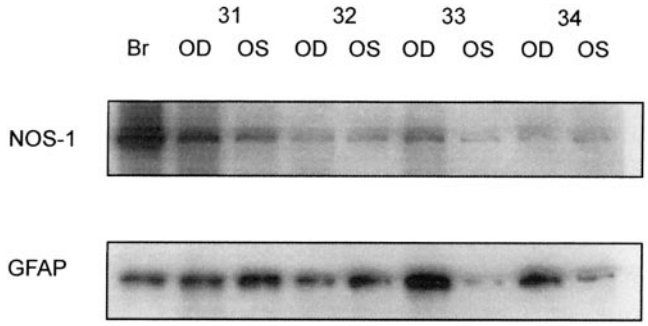

FIGURE 4. Immunoblots for NOS-1 and GFAP in rat optic nerve tissue. Rats 31, 32, 33, and 34 had chronic moderately elevated IOP for 28 days in the right eye (OD) and normal IOP in the left eye (OS). Rat cerebellar tissue (Br) was run as a positive control. From standard, molecular weight markers, which were run in parallel, the bands for NOS-1 corresponded to an approximate molecular weight of 160 kDa and the bands for GFAP corresponded to an approximate molecular weight of 55 kDa.

figures presented for each primary antibody used for each isoform to demonstrate the specificity of any one primary antibody.

Immunoblots for NOS Isoforms

Immunoblots for NOS isoforms and GFAP were performed on individual optic nerve heads from normal rat eyes paired with contralateral eyes with chronic moderately elevated IOP for 4, 14, 28, and 45 days (Fig. 4). NOS-1 and GFAP were clearly detected in rat optic nerve heads, but no consistent difference was evident comparing tissues from eyes with normal IOP and tissues from eyes with chronic moderately elevated IOP. NOS-3 was detected, but not consistently. NOS-2 was not detectable in normal optic nerve heads or in tissue from eyes with chronic moderately elevated IOP. The difficulties in detecting NOS-2 and NOS-3 in individual rat optic nerve heads by immunoblots were probably due to the small amount of tissue and the low levels of these NOS isoforms in the tissue.

DISCUSSION

The distribution of constitutive isoforms of NOS in rat optic nerve heads suggests a physiological role for NO in this tissue. NOS-1 is present in many but not all astrocytes throughout the rat optic nerve. Specifically, based on the classification by Johnson et al.[27] and Morrison et al.[28] NOS-1–positive astrocytes are in the glial structures of the neck region (prelaminar), transition zone (laminar), and postlaminar region and in the glia limitans. The cytoplasmic staining pattern of each cell is consistent with localization of NOS-1 to the cytosol. Similar sections stained for GFAP suggest that the NOS-positive cells are astrocytes. The presence of NOS-1 in astrocytes throughout the optic nerve indicates that this isoform is constitutive in certain glial cells and that NO may be functioning physiologically as a mediator between astrocytes, astrocytes, and axons and/or astrocytes and capillaries. These findings are similar to our previous demonstration of NOS-1 in astrocytes of the human optic nerve head[24] and are consistent with the presence of constitutive NOS in glial elements in the CNS.[23]

NOS-1 staining is also associated with the vascular system in the rat optic nerve head, particularly with arteries in the neck region and capillaries in the transition zone. The punctate staining pattern in the large-caliber arteries in the optic nerve head is similar to that described in the choroidal vessels,[10] and is most likely the nitrergic nerve endings of nerve fibers, primarily parasympathetic in nature, that are derived from the pterygopalatine ganglion. Nitric oxide causes choroidal vasodilation and may have a similar function in the optic nerve arterial system. In dogs,[29] a rich plexus of NOS-containing fibers in the media and adventitia of the central retinal artery has been described as arising from the pterygopalatine ganglion.[30] Elevated NO levels were detected at the surface of the optic disc in cats with vasodilation of optic disc vessels,[31] and humans given NO donor drugs have increased blood flow in the optic disc vessels.[32] Thus, NO released from nitrergic nerve terminals in the wall of the retinal artery at the level of the optic disc regulates vasodilation and, consequently, blood flow in this region.

Perivascular cells surrounding capillaries are NOS-1 positive and may be pericytes. Because pericytes are the site of local autoregulation in the CNS, NOS-1 may contribute to autoregulation of optic nerve head blood flow in small vessels. Bovine retinal pericytes relax in response to NO, suggesting autoregulation at the level of the capillary bed.[33] The findings of NOS-1 in presumed pericytes and NOS-1–containing, perivascular nerve fibers suggest that NO-mediated vasodilation in the optic nerve head can be accomplished by NOS-1 in addition to NOS-3 in the vascular endothelium (see later discussion).

NOS-3 is constitutively present throughout the vasculature of the rat optic nerve head, specifically localized to vascular endothelia. NOS-3–positive endothelial cells presumably have a physiological role in increasing blood flow. In addition, this isoform may serve a neuroprotective function. Transgenic mice with no NOS-3 experience increased infarct volumes after mid–cerebral artery occlusion.[19] Although infarct volume in NOS-1 knockout mice is reduced after permanent focal ischemia, subsequent treatment with an NOS inhibitor causes increased infarct volume, because of inhibition of NOS-3.[19] NOS-3 is upregulated during the acute phase of cerebral ischemia.[34] These studies provide evidence for a neuroprotective role of NOS-3 in CNS tissue by maintaining regional blood flow.

The localization of NOS-3 in the optic nerve head of rats is similar to the localization in human vessels.[24] NO from NOS-3 contributes to the vasodilatory action in the optic nerve head, when measured by laser Doppler flowmetry.[31] Thus, vascular endothelial NOS-3 may cause vasodilation under physiological conditions and contribute to increased blood flow offering neuroprotection to the optic nerve in glaucoma.

Under pathologic conditions, the NOS-2 isoform can be induced in most tissues, including neurons, astrocytes, and endothelial cells.[35,36] When present in the CNS, the NOS-2 isoform is associated with neurotoxicity. In cerebral ischemia in NOS-2 knockout mice, infarct volumes are significantly reduced compared with those in wild-type controls.[37] In neural tissue, induction of NOS-2 in vitro results in the secondary phase of neuronal cell death.[38] After cerebral ischemia, NOS-2 activity peaks at 48 hours and returns to baseline at 7 days.[39]

The activity of NOS-2 is not regulated by calcium as are the activities of NOS-1 and NOS-3.[40] When present in a tissue, NOS-2 synthesizes excessive quantities of NO, which combines

chemically with superoxide to form the highly reactive and destructive peroxynitrite.[41] Nitrosylation by peroxynitrite of cellular proteins, lipids, and DNA triggers apoptosis.[42] NOS-2 is implicated in neurodegeneration in several human diseases[23] and may drive progressive neuropathy such as glaucoma.[43] The isozyme is induced in reactive astrocytes and microglia in the CNS of patients with multiple sclerosis, amyotropic lateral sclerosis, Alzheimer's disease, and acquired immune deficiency syndrome.[44,45]

The appearance of inducible NOS (NOS-2) in the transition zone of the optic nerve in experimental eyes with chronic moderately elevated IOP, compared with that in contralateral control eyes with normal IOP, is suggestive of NO neurotoxicity. Relating to NOS-2, our results in rats with chronic moderately elevated IOP are similar to our results in human glaucomatous eyes.[24] These findings are consistent with the concept that the optic nerve head is an early site of focal neuronal degeneration of the axons of the retinal ganglion cells in glaucoma. The NOS-2 isoform is apparently induced in the rat optic nerve head as early as 4 days after elevation of IOP in experimental eyes, persists for at least 3 months in these eyes, and is not present at all in normal tissue. In a 6-month pharmacologic experiment using this rat model of chronic moderately elevated IOP, aminoguanidine, an inhibitor of NOS-2, significantly blocked the loss of retinal ganglion cells.[46]

Clusters of NOS-2–positive cells have been found in human glaucomatous optic nerve heads.[24] Our current finding of NOS-2 in rat eyes throughout the experimental period of chronic moderately elevated IOP may indicate that sustained, excessive NO levels in the optic nerve head cause progressive damage to the axons of the retinal ganglion cells. We hypothesize that the NOS-2–positive cells in the optic nerve head are reactive astrocytes that are participating in neurodestruction.

We postulate that the excessive NO produced by astrocytes is neurodestructive and contributes to the focal degeneration of axons in the optic nerve head that is characteristic of chronic, progressive, glaucomatous optic neuropathy.[43] Conversely, local physiological synthesis of NO by vascular endothelia in the optic nerve head may be neuroprotective by causing vasodilation and increased blood flow in the tissue. Pharmacologic agents that are selective NOS inhibitors, targeted against NOS-2 but sparing NOS-1 and NOS-3, may be useful for treatment of various optic nerve degenerations, such as glaucoma, based on neuroprotection.

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