Nitric Oxide Mediates Excitotoxic and Anoxic Damage in Rat Retinal Ganglion Cells Cocultured With Astroglia

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Background: Nitric oxide has been implicated in the process of retinal ganglion cell death in glaucoma.

Objective: To investigate the role of nitric oxide in mediating retinal ganglion cell death in a culture system that models glial-neuronal interactions at the level of the optic nerve head.

Methods: Dissociated retinal ganglion cells from neonatal rats were plated on monolayers of astroglia and identified by retrograde labeling with the fluorescent marker 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate. Two days after dissociation, cocultures of retinal ganglion cells and glia were treated with graded concentrations of the nitric oxide synthase inhibitor N-nitro-L-arginine (NNA), and exposed to either anoxia for 1 to 24 hours or excitatory amino acids for 6 hours. Surviving retinal ganglion cells were counted with fluorescence microscopy and expressed as a percentage of retinal ganglion cells surviving in control cultures.

Results: Cell survival after anoxia increased in a dose-dependent fashion with exposure to NNA. Mean ± SD survival rate of retinal ganglion cells after 6 hours of anoxia was 57% ± 10% with NNA treatment compared with 31% ± 3% without treatment (P < .01). When treated with excitatory amino acids, cell survival was 31% ± 6% after administration of N-methyl-D-aspartate, 500 µmol/L, and 27% ± 8% after administration of sodium glutamate, 500 µmol/L. Survival was increased in cultures with exposure to NNA, 100 µmol/L, to 53% ± 11% and 69% ± 11%, respectively (P < .01).

Conclusion: In this coculture of retinal ganglion cells and astroglia, reduction of the glial source of nitric oxide through nitric oxide synthase inhibition provided partial but significant protection against the lethal effects of anoxia and excitatory amino acids on retinal ganglion cells.

Clinical Relevance: Neuroprotective agents may play a role in patients with glaucoma who have progressive visual field loss, despite satisfactory control of intraocular pressure. Inhibition of nitric oxide synthase at the level of the optic nerve head may contribute to a clinically significant level of neuroprotection.

Retinal ganglion cell death is the pathologic end point in glaucoma and is the basis for loss of visual function. For decades, clinical practice has focused on the reduction of intraocular pressure, but the realization that this is not always sufficient to halt disease progression has prompted research into the basic mechanisms of glaucomatous retinal ganglion cell death.

Recent advances in elucidating the mechanisms of cell death in ischemic and neurodegenerative central nervous system diseases are relevant to mechanisms of cell death in glaucoma and have highlighted some of the metabolic pathways that may be involved. In the cerebral cortex, excitatory amino acids (EAAs) play a key role in the neuronal cell death that occurs after ischemia. Similar effects of EAAs have been seen in the eye, and the effects of short-term retinal anoxia can be diminished with administration of N-methyl-D-aspartate (NMDA) antagonists. The finding that levels of EAAs may be elevated in the vitreous of glaucomatous human and primate eyes supports the role of these agents in glaucoma. Although marked elevation of extracellular levels of EAAs will cause rapid cell death, mild, long-term elevation causes a pattern of neuronal toxic effects similar to that seen in glaucoma. Excitatory amino acids can also initiate programmed cell death, which may account for most retinal ganglion cell loss in experimental glaucoma.

Results of initial studies of the neuroprotective effect of NMDA antagonists in models of glaucomatous retinal ganglion cell death show either no or only...
MATERIALS AND METHODS
PREPARATION OF RETINAL GANGLION CELLS AND GLIAL MONOLAYERS

These preparations have been described in detail elsewhere and are summarized here. All procedures involving animals conform to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Five- to 7-day-old Sprague-Dawley rats were anesthetized by chilling on ice for 10 minutes. One microliter of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, Ore), dissolved as 2.5 mg in 100% ethanol, was injected into both superior colliculi. The rats were decapitated 48 hours later and both eyes were enucleated and placed in Hank’s balanced salt solution (HBSS; Gibco Laboratories, New York, NY) containing sodium penicillin, 100 U/mL, and streptomycin sulfate, 100 μg/mL (Sigma-Aldrich Corp, St Louis, Mo). Retinae were dissected out and incubated for 40 minutes at 37°C in HBSS containing papain, 26 U/mL (Sigma-Aldrich Corp), Dl-cysteine, 0.2 mg/mL (Sigma-Aldrich Corp), and bovine serum albumin, 0.2 mg/mL (Gibco). The tissue was rinsed with HBSS and replaced with 4 mL of Dulbecco modified Eagle medium (Gibco) and triturated through a 1-mL pipette. The supernatant (containing approximately 3 × 10⁶ cells) was dispersed in 35-mm dishes containing 1.5 mL of culture medium (Dulbecco modified Eagle medium, glutamine [580 mg/L], glucose [1000 mg/L], rat serum [5%], sodium penicillin [100 U/mL], and streptomycin sulfate [100 mg/mL]) over a monolayer of rat cortical astrocytes. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.

The preparation of glial monolayers has been described in detail elsewhere.

DETERMINATION OF CELL VIABILITY

Cell survival was based on the ability of a cell to exclude trypan blue. Cells were observed with phase contrast microscopy and counted as surviving only if they excluded trypan blue and remained firmly attached to the substrate. Sixty fields were sampled from each culture plate at ×200 magnification, and the number of viable cells in a plate exposed to anoxia or EAAs were expressed as a percentage of the cells surviving in control plates. All values are given as mean ± SD percentages compared with controls, in which n is the number of plates in each group (matched for experimental and control groups). Statistical comparisons were made using unpaired, 2-tailed t tests.

partial neuroprotective effects. This finding underscores the importance of considering other factors such as events at the optic disc, where the process of glaucomatous retinal ganglion cell death is thought to initiate. Recently, Neufeld and coworkers pointed to the role that nitric oxide (NO) may play in this process by demonstrating the presence of the enzyme nitric oxide synthase (NOS) in the optic nerve head. Neuronal and inducible isoforms of NOS were found in astrocytes of the lamina cribrosa, whereas the endothelial isofrom was detected only in the endothelium of small vessels of the prelaminar region. All 3 isoforms were present in greater amounts in patients with glaucoma compared with nonglaucomatous controls, consistent with the idea that retinal ganglion cells—at least at the level of the optic nerve head—can be exposed to neurotoxic levels of NO. Nitric oxide can be beneficial in its role as a vasodilator, but it can also act as a potent neurotoxic agent, primarily by inhibiting mitochondrial function, disrupting DNA, and overstimulating pathways involved in DNA repair. Results of in vivo studies demonstrated that intravitreal injection of the NO donor (S-nitroso-N-acetyl-DL-penicillamine) can cause retinal ganglion cell and photoreceptor loss. Reduction of NO levels by the systemic inhibition of NOS reduces retinal ganglion cell loss in a rat model of retinal ischemia.

The possible involvement of NOS suggests novel therapeutic approaches to the prevention of glaucomatous retinal ganglion cell death. To prepare for exploring the role of NO inhibition in an animal glaucoma model, we investigated the effect of NO on retinal ganglion cell survival in a culture system that permits the quantitative assessment of the cytotoxic effects of various agents, with results of previous work suggesting that NO inhibition may be neuroprotective for retinal ganglion cells. This system has the advantage of allowing assessment of NO inhibitor action without the confounding vasodilator effects of NO in the affected area. Cortical astroglial cells provided the substrate in this culture system as a correlate of astroglia in the optic nerve head. In contrast to retinal ganglion cells, these glial cells possess NOS and act as the source of NO in this system. We demonstrate the cytotoxic effects of anoxia and EAAs in this culture system and examine the role of a
NOS inhibitor in the prevention of retinal ganglion cell death.

RESULTS

RETINAL GANGLION CELL SURVIVAL IN CULTURE

Retinal ganglion cells were easily identified by the red fluorescence of DiI. The tracer was restricted to the cytoplasm and plasma membrane without evidence of spread to adjacent cells. After a week of culture, DiI labeling was more punctate and restricted to the cell body but still allowed ready identification of retinal ganglion cells. The ratio of retinal ganglion cells to other retinal cells was approximately 1:200 after dissociation.

Retinal ganglion cell survival depended significantly on the presence of an underlying glial monolayer. With a monolayer in place, cells remained viable for up to 7 days in culture. Figure 1 shows the number of viable cells, expressed as a percentage of the population immediately after plating, at various times during the 7 days after dissociation. Even without a metabolic or excitotoxic insult, more than 50% of cultured cells died within the first 24 hours. However, after this, the population was more stable, with an equivalent decrease in cell numbers taking up to 6 days. Our studies of the effects of anoxia and EAA were started 24 hours after dissociation to take advantage of this relatively stable phase in the cell population.

EFFECT OF ANOXIA AND NNA

Anoxia resulted in a dramatic reduction in the number of viable cells. After 24 hours of exposure, the viable cell population decreased to about 10% of the control population. However, the greatest cell loss occurred during the first 6 hours of anoxia (to approximately 30% of controls). We therefore monitored the effect of NNA on cell death during this period to determine an optimal concentration of NNA to rescue cells.

Figure 2 shows the percentage of cells, relative to controls, that survived 6 hours of anoxia with the addition of graded concentrations of NNA. Each data point represents mean ± SD of surviving cells counted from 10 cultures (60 fields counted per culture). Degree of cell loss decreased in a dose-dependent fashion, with the effect peaking at 100 µmol/L NNA. At no concentration used did cell counts match those for the control cultures. Above 100 µmol/L NNA, the propagation of surviving cells decreased. We therefore took 100 µmol/L as the optimal concentration of NNA for cell survival and used this concentration in subsequent studies.

Figure 3 shows the percentage of cell survival against time for cells exposed to anoxia, with and without treatment with N-nitro-L-arginine (NNA) (100 µmol/L). Values are expressed as percentages of healthy cultured cells. Counts of surviving cells were made after removal from anoxia, and represent the mean ± SD of 60 counts per culture from 10 cultures.

EFFECT OF EEAs AND NNA

To explore the interaction between the excitotoxic effects of the amino acids glutamate and NNA, we exposed retinal ganglion cells to 6 hours of either glutamate or NMDA (100 µmol/L), with and without treatment
with NNA (100 µmol/L) (Figure 4). The increase in cell death was similar after glutamate or NMDA exposure. In both cases, treatment with NNA rescued significant numbers of cells (P < .001), although this clearly offers only partial protection.

**COMMENT**

We described a culture model of neuroglial interactions in the optic nerve head in which the inhibition of NOS increases retinal ganglion cell survival after exposure to EAAs or ischemia. With the data from this model, we conclude that the mechanism of protection is conferred at the cellular level, and is not secondary to changes in vascular perfusion. Cortical astrocytes, rather than retinal ganglion cells or any contaminant glial cells, are the most likely source of NO. We cannot exclude the possibility that some contaminant retinal glial cells may also have produced NO, but this is unlikely to have been a significant factor. Levels of nicotinamide adenine dinucleotide phosphate diaphorase, a marker for NOS, are very low in the retinal ganglion cell layer of the rat, whereas NOS activity has been demonstrated in astrocytes, and microglia-derived NO has been shown to induce cell death in cocultures of cerebellar granule neurons and microglia. In excitative cell death, increased NOS activity is secondary to calcium influx after activation of astroglial NMDA receptors. In hypoxia, this influx is most likely mediated by a decrease in membrane potential, which lowers the threshold toward activation of the NMDA.

Nitric oxide is highly reactive and can cause intracellular damage in several ways, the most important of which involve depletion of intracellular energy stores. Nitric oxide inactivates enzymes involved in mitochondrial electron transport by complexing with their iron-sulfur centers. In addition, it stimulates adenosine diphosphate–riboseylation of glyceraldehyde-3-phosphate, depressing glycolysis and decreasing adenosine triphosphate production. It can also directly damage DNA, which results in activation of poly (adenosine diphosphate–ribose) synthetase and the further reduction of adenosine triphosphate stores. Cellular energy depletion can lead to a reduction in membrane potential, bringing the NMDA receptors into the range for magnesium extrusion and calcium influx. In the chick retina, intracellular energy depletion precedes excitotoxic damage and is not associated with an increase in extracellular glutamate. The importance of these mechanisms is stressed by the finding that the inhibition or complete removal of poly (adenosine diphosphate–ribose) synthetase activity alone can protect against NMDA-induced cytotoxic effects.

Our results are consistent with a role for NO in the induction of retinal ganglion cell death in glaucoma, but should be applied with caution to events at the optic nerve level because our model deals with axotomized cells. The levels of inducible and neuronal NOS are increased in the optic nerve head astrogia of glaucomatous eyes, which would increase the levels of NO in this region. It is possible that the induction of NOS activity in astrogia is an early response to increased intraocular pressure. In cultured rat astrocytes, lipopolysaccharide-induced NOS activation is associated with reduced gap junction permeability, and one of the earliest changes in the astroglial population of the optic nerve head in the rat ocular hypertension model is a reduction in labeling of the gap junction protein connexin-43.

We have not assessed the effect of EAAs or hypoxia on astroglial survival. As with other neurons that possess NOS activity, they are likely relatively resistant to the effects of EAAs, possibly because NO nitrosylates the glutamate receptor and down-regulates its activity. Some NOS neurons also have high levels of superoxide dismutase that prevent local formation of reactive peroxynitrite and ensure protection from the toxic effects of NO. Results of histological studies of glaucomatous human optic nerve heads show that the preservation of astrocytes as retinal ganglion cell axons are lost, consistent with their metabolic resilience.

Although the case for NOS inhibition as a neuroprotective strategy in glaucoma is compelling, the value of this approach requires careful consideration. In other systems, NOS inhibition does not always provide effective neuroprotection. For example, Zeevak and Nicklas showed in chick retina cultures that NOS inhibition with NNA did not prevent the cytotoxic effects of EAAs, although the concentration of NNA (100 µmol/L) completely suppressed cyclic guanosine monophosphate (cGMP) production (indicating complete NOS inhibition). Interaction with astroglial cells is an important factor in determining the effects of NOS inhibition. In hippocampal neuronal cultures that were relatively free of astrocytes, NOS inhibition also failed to prevent the cytotoxic effects of administered EAAs. Efficacy of NOS inhibition may be further limited when used in vivo because endothelial and neuronal NOS will be inhibited, leading to increased blood pressure and possibly reduced blood flow. In the present study, neuronal survival was reduced when the concentration of NNA was greater than 100 µmol/L. The possibility that this reflects a toxic effect of NNA is difficult to distinguish experimentally from the more likely explanation that the preservation of some NOS activity may be beneficial for
retinal ganglion cell survival. This is not unexpected because NO increases cellular cGMP by the activation of guanylate cyclase, which has been shown to be neuroprotective in several systems. It is also possible that a low level of NO acts by closing the NMDA receptor-gated ion channel because treatment of retinal ganglion cell cultures with NO generators will protect against NMDA-mediated excitotoxic effects. Further support for a moderate level of NOS inhibition comes from work on focal cerebral ischemia in the mouse, in which low doses of NNA are neuroprotective and higher doses are ineffective. Consistent with this, complete inhibition of cGMP production by NOS inhibition with NNA treatment did not prevent glutamate-induced neurotoxic effects in rat hippocampal cultures. By contrast, cortical cultures from NOS-negative mice have shown significant resistance to the effects of NMDA-induced toxic effects. However, although the NMDA-induced increase in cGMP is abolished, the basal levels of cGMP in NOS-negative mice were still 60% of wild-type levels, which might have conferred some level of neuroprotection. Because a certain level of NO is likely to improve cell survival, it would be useful in further studies to refine the dose of NNA required to rescue cells from NMDA agonist-induced cell death.

Two other factors should be considered if NOS inhibition is to be incorporated into a neuroprotective strategy. First, the cellular redox state will affect the cytotoxic effects of NO. Thus, although retinal ganglion cell death is the common end point in glaucoma and ischemic retinal disease, it may be associated with different redox states. In glaucoma, less of a shift in the redox state to the reduced form is anticipated compared with vascular occlusive disease. Inhibition of NOS might, therefore, be more effective in the former than in the latter. Second, the efficacy of NOS inhibition may depend on the timing of its administration. Early in the disease, it may be harmful in reducing blood supply to affected regions. Later, NOS inhibition may be more beneficial because NO plays a greater role in inducing cell death.

The role of NOS inhibition—to promote retinal ganglion cell survival—is not addressed in this study and is open to debate. Nitric oxide is produced in the retina, with the highest activity of nicotinamide adenine dinucleotide phosphate diaphorase occurring in the inner nuclear layer, with lighter labeling in the amacrine cells. Retinal Muller cells do not normally express NOS activity, but can be induced to do so with the addition of suitable cytokines, producing the noncalcium-dependent (inducible) form of the enzyme. Although possibly an important source of NO in inflammatory disease, this is not likely in chronic noninflammatory ophthalmic diseases such as glaucoma, suggesting that this would not be an important site of neuroprotection with NOS inhibition.

The results of this study show that NOS inhibition confers partial but significant neuroprotection against the effects of anoxia and EAs on rat retinal ganglion cells cocultured with astrocytes. The data are based on a single NOS inhibitor that, even in this simple model system, might have other (possibly detrimental) actions in addition to NOS inhibition. In future work, other NOS inhibitors should be considered to determine whether a greater neuroprotective effect can be obtained. Our results suggest that NOS inhibition may play a role in the protection of retinal ganglion cells at the optic disc. The finding that NOS inhibition potentiates the neurotoxic effects of brain-derived neurotrophic factor on the survival of axotomized retinal ganglion cells suggests ways in which this approach could be a useful adjunctive treatment for glaucoma and emphasizes the importance of further research in this area.

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REFERENCES


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