Neuroprotective effects of brimonidine treatment in a rodent model of ischemic optic neuropathy

Nataliya O. Danylkova a, Sandra R. Alcala a,b, Howard D. Pomeranz a,1, Linda K. McLoon a,b,*

a Department of Ophthalmology, University of Minnesota, Minneapolis, MN 55455, USA
b Department of Neuroscience, University of Minnesota, Minneapolis, MN 55455, USA

Received 30 May 2006; accepted in revised form 2 October 2006
Available online 16 November 2006

Abstract

Ischemic optic neuropathy (ION) is a common disorder caused by disruption of the arterial blood supply to the optic nerve. It can result in significant loss of visual acuity and/or visual field. An ischemic optic nerve injury was produced in rats by intravenous injection of Rose Bengal dye followed by argon green laser application to the retinal arteries overlying the optic nerve, causing a coagulopathy within the blood vessels and disruption of optic nerve and retinal perfusion. The effect of brimonidine tartrate eye drops on survival of retinal ganglion cell axons in this experimental paradigm was studied. One eye was treated and the contralateral eye served as a control. Four groups of animals were used for this study. Group 1 received 7 days of treatment with 0.15% brimonidine tartrate eye drops twice a day prior to the ischemic injury. Group 2 animals received 0.15% brimonidine tartrate eye drops twice a day for 14 days after photocoagulation injury. Animal groups 3 and 4 received eye drops of 0.9% NaCl twice a day either daily for 7 days before injury or daily for 14 days, respectively. All rats were sacrificed 5 months after the injury to ascertain long-term optic axon survival. Coagulopathy-induced optic nerve ischemia resulted in a 71% loss of optic axons. Treatment with brimonidine daily for the 7 days prior to the injury resulted in a greater survival of optic axons, with only a 56.1% loss compared to control. Brimonidine treatment every day for 14 days after the ischemic injury did not result in a significant rescue of optic axons compared to injury alone. In summary, the application of brimonidine eye drops for one week prior to an ischemic injury resulted in a statistically significant increase in survival of optic axons within the injured optic nerves. Brimonidine treatment of the eye after the ischemic injury did not result in axon rescue, and axon loss was similar to the injured optic nerves treated with saline only. These results suggest that brimonidine may have potential use for prevention of ION in at-risk patients.

Keywords: neuroprotection; optic nerve; ischemia; hypoxia; α2-agonists

1. Introduction

Ischemic optic neuropathy is one of the most common optic nerve disorders in the elderly. It is characterized by disruption of blood supply to the optic nerve by branches of the posterior ciliary arteries (Hayreh, 1985, 1996). The resultant transient ischemia leads to an alteration of retinal cell metabolism, including changes in extracellular ion concentrations, depletion of growth factors, altered release of neurotransmitters, and increases in free radicals. These processes lead to axonal degeneration and progressive neuronal cell loss via apoptosis, which ultimately results in significant and permanent vision loss (Levin and Louhab, 1996; Salazar et al., 2000). Due to the complexity of the pathologic processes in the development of ischemic optic neuropathy (ION), many different treatment approaches have been advocated. However, all of the current...
treatment methods fail to result in any significant improvement in vision in patients with ION.

Alpha 2-adrenergic receptors play an important role in vascular autoregulation (Faber and Meininger, 1990; McGillivray-Anderson and Faber, 1991). Activation of alpha 2-adrenergic receptors inhibits adenylyl cyclase activity (Jakobs, 1979; Osborne, 1991), inhibits calcium channels (Han and Wu, 2002), activates opening of the potassium channels in the cells (Debock et al., 2003), and inhibits pro-apoptotic mitochondrial signaling (Tatton et al., 2001a,b). Alpha 2-adrenergic receptors are present in the retina (Elena et al., 1989; Matsuo and Cynader, 1992), specifically localized in rat retina to the inner plexiform and ganglion cell layers (Zarbin et al., 1986; Wheeler et al., 2001).

A number of studies demonstrated the efficacy of alpha 2-agonists in reducing the negative effects of brain ischemia. Alpha 2-agonists such as dexmedetomidine (Maier et al., 1993), and clonidine (Yuan et al., 2001; Zhang, 2004) provided neuroprotection in animal models of CNS ischemia. Another promising candidate for therapeutic neuroprotective effects following transient ischemia is the alpha 2-agonist, brimonidine. Currently, brimonidine tartrate is used clinically as a topical ocular hypotensive agent in glaucoma patients (Gandolfi et al., 2003), as well as in postoperative patients, in order to control intraocular pressure (Katsimpris et al., 2003). Brimonidine is thought to lower intraocular pressure by a combination of reducing aqueous humor production and increasing uveoscleral outflow (Toris et al., 1995; Greenfield et al., 1997). Topical application of brimonidine can achieve a concentration sufficient to activate alpha2-adrenergic receptors within ocular tissues (Acheampong et al., 2002).

A number of studies have demonstrated the efficacy of brimonidine in increasing survival of retinal ganglion cells after various types of injury. Intraportal pretreatment with brimonidine tartrate significantly increased ganglion cell survival and retinal function after optic nerve crush (Yoles et al., 1999; Wheeler et al., 1999; Lafuente et al., 2001). Prevention of an early loss of retinal ganglion cells was demonstrated after topical administration of brimonidine prior to transient retinal ischemia induced by ophthalmic vessel ligation for 60–90 min (Lafuente et al., 2001). The mechanism for this protection is unclear, but brimonidine appears to result in inhibition of glutamate and aspartate accumulation (Donello et al., 2001), the up-regulation of anti-apoptotic genes such as bcl-2 and bcl-xl and neuroprotective molecules such as fibroblast growth factor (Lai et al., 2002).

The main objective of this study was to determine the effect of brimonidine treatment on the survival of the optic axons and neurons within the retinal ganglion cell layer in the ischemia-compromised eyes. Brimonidine eye drops were applied topically before and/or after induction of ischemia-reperfusion damage to the optic nerve and retina using a coagulopathy method that is a rodent model of ION (Bernstein et al., 2003; Danylkova et al., 2006). Optic nerve survival was determined by morphometric analysis of the optic nerve 5 months after injury and treatment.

2. Methods

All procedures were approved by the Animal Care Committee of the University of Minnesota and conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement on the Use of Animals in Ophthalmic Research. Adult Long Evans rats, 250–300 g, were housed with Research Animal Resources at the University of Minnesota on a 12-h light/dark cycle with food and water ad libitum. Rats were anesthetized with 0.224 g/kg tribromoethanol (Sigma—Aldrich, St. Louis, MO) and kept on a heating pad for the duration of anesthesia and any experimental manipulations.

2.1. Induction of the ION

Pupils were dilated by instillation of 2 drops of a 2% cyclogyl and 2.5% phenylephrine hydrochloride ophthalmic solution (Bausch & Lomb, Tampa, FL) 5 min before laser application. GenTeal, a lubricant gel (CibaVision, Duluth, GA), was applied to the rat’s right eye to prevent eye irritation and increase the optical power of the lens. A transparent contact lens was placed on the animal’s right eye.

Transient ischemia was induced as described previously (Bernstein et al., 2003; Danylkova et al., 2006). Briefly, 0.25 ml of a 2.5 mM solution of Rose Bengal dye, (Sigma—Aldrich, St. Louis, MO) was injected into the rat’s tail vein, followed immediately by application of a 500 μm laser beam covering the entire optic disc area and emerging vessels. Laser application was within 30 s of Rose Bengal application, and thus primarily in the arteries. However, both arteries and veins at the optic nerve head were subjected to laser application. Twelve pulses of 1 s duration each were applied with an intensity of 100 μW from an argon green laser at a 514 nm wavelength (Coherent Novus 2000). The opposite eye served as a control.

2.2. Brimonidine administration

Four groups of animals were prepared for this study. The first group (n = 6) received topical treatment with two drops (5 μl) of 0.15% brimonidine tartrate twice a day for 7 days before the injury. The second group (n = 6) received topical treatment of 0.15% brimonidine tartrate twice a day for 14 days following the induction of optic nerve ischemia. Groups 3 (n = 3) and 4 (n = 3) received a sham topical treatment of 0.9% NaCl, twice a day, either 7 days before or 14 days after injury.

2.3. Histology

Five months after the injury, a time when both the acute and chronic phases of degeneration are complete (Danylkova et al., 2006), rats were deeply anesthetized with tribromoethanol and intracardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Globes with optic nerves attached were removed and post-fixed in 4% paraformaldehyde overnight at room temperature. After 12 h, the tissues were rinsed...
in PBS, and optic nerves were carefully separated 1 mm from the
globe with a scalpel. The globes were embedded in paraf-
fin, sectioned at 10 μm, and stained with cresyl violet for mor-
phological analysis. Optic nerves were placed in 2% osmium
tetroxide in saline for 1 h, rinsed in PBS, dehydrated in alco-
hol, and embedded in epoxy resin. Subsequently, 1-μm thick
cross-sections were cut on an ultramicrotome and stained
with toluidine blue for light microscopic examination and
morphometric analysis.

2.4. Morphometric analysis

Axonal loss in the optic nerve was determined by exam-
ining 3–6 optic nerve cross-sections from each collected
optic nerve at 100× using oil immersion. This results in
very clear visualization of even thinly myelinated optic
axons. Axonal counts were performed by using Bioquant
Nova Prime software (Nashville, TN). Axonal counts were
performed by counting all the myelinated axons in a contin-
uous strip across the entire diameter of each optic nerve us-
ing the Bioquant Nova Prime software (Nashville, TN).
Total numbers of axons in the area counted were deter-
mimed, and these were recalculated as total axon number/
μm² (Danylkova et al., 2006). We counted approximately
20,000 axons in each control optic nerve; thus, the area
counted represented approximately 20% of the total optic
axons (Cepurna et al., 2005). It is possible that there is
some experimental error in these counts due to the patchy
loss in the injured optic nerves. In order to control for this,
we counted 3–6 sections for each nerve and averaged the
counts for each experimental animal. We have previously
shown that laser alone and Rose Bengal administration alone
do not cause optic nerve injury (Danylkova et al., 2006), so
those controls are not included. All data are presented as
mean ± SEM. Significance was assessed using Student’s
paired two-tailed t-test and Dunn’s multiple comparison tests
aided by the Prism and Statmate software (Graphpad, San
Diego, CA), and data were considered statistically signifi-
cant if p < 0.05.

3. Results

3.1. No systemic side effects of brimonidine

The rats showed no evidence of adverse effects from the
brimonidine treatment. We had no animals die, nor did any of
the treated rats show signs of pain or discomfort as manifested
by changes in activity level, changes in eating patterns and the
like.

3.2. Morphology of the optic nerve

Changes of the optic axons within the optic nerve were
evaluated 5 months after induction of the ischemic injury.
Compared to the normal optic nerve (Fig. 1A), the vehicle-
treated groups of rats displayed a severe loss of optic axons
(Fig. 1B). There was a tendency toward increased loss in the
more central regions of the injured optic nerves, with sparing
in the more peripheral portions of the optic nerve cross-
sections.

In the group of animals pretreated topically with brimoni-
dine 7 days before injury, then subjected to photocoagula-
tion-induced ischemia of the optic nerve, there was a
significant reduction in axonal loss compared to the vehi-
cle-treated group (Fig. 1C and D). The central area of the optic
nerve contained scattered myelinated axons and the periphery
of the optic nerve had large areas with preservation of normal-
appearing axons. In contrast, in animals that received a 14-day
treatment of topical brimonidine following ischemic injury,
there appeared to be a modest reduction in the axonal degen-
eration in the periphery of the optic nerve (Fig. 1E) and
absence of myelinated axons in the central portion of the optic
nerve, similar to the control group of optic nerves treated with
saline only. Photomicrographs at higher power demonstrate
the clarity with which even thinly myelinated axons can be
visualized in the plastic-embedded optic nerves (Fig. 2A–C)
from control animals (Fig. 2A), animals treated with brimoni-
dine 7 days prior to injury (Fig. 2B), animals treated with bri-
monidine for 14 days after injury (Fig. 2C), and animals that
received an ischemic injury and were treated with saline only
(Fig. 2D).

3.3. Axonal counting

Normal control optic nerves of rats in our study had an op-
tic axon density of 0.362 ± 0.011 axons per μm² (n = 6) (Fig. 3).
In rats subjected to the coagulopathy injury, there was
a significant loss of optic axons, with 0.087 ± 0.018 axons
per μm² (n = 3). This represents a survival of only 23.5%
of the axons after the ischemic injury. The number of axons
per sampled area in cross-sections from rats treated with top-
ical brimonidine daily for 7 days prior to optic nerve ischemia
was 0.203 ± 0.016 per μm² (n = 6), which represents a 56.1%
long-term axonal survival compared to control eyes (Fig. 3).
Topical treatment with brimonidine prior to ischemic insult
to the optic nerve and retina resulted in significant rescue of
optic axons compared to injury alone when examined 5
months after the injury.

In contrast, the optic nerves of rats treated with topical
brimonidine for 14 days after the photocoagulation-induced
optic nerve ischemia contained 0.115 ± 0.008 axons per
μm² (n = 6) while optic nerves from control eyes contained
0.365 ± 0.008 per μm² (n = 6) (Fig. 3). This corresponds to
30.7% axonal survival in the injured optic nerves treated
with brimonidine compared to control optic nerves. No signi-
ficant difference was observed between groups of animals
treated with 0.9% NaCl either 7 days prior to induction of
the ION or 14 days following the photocoagulation injury.
Specifically, there were 0.073 ± 0.018 axons per μm²
(n = 3) in the group of animals treated with 0.9% NaCl
for 14 days after the injury, which is a survival rate of
19.7% when compared to normal control (0.370 ± 0.010
per μm²; n = 6).
3.4. Cresyl violet staining of the retina

Based on qualitative analysis only, there were more neurons present in the ganglion cell layer in the retinas of rats pretreated with brimonidine eye drops (Fig. 4). There was no apparent difference in neuronal density in the retinas of animals that received the 14-day brimonidine treatment after induction of the ischemic injury when compared to the ischemic-injured tissue from the vehicle-treated group.

4. Discussion

The present study demonstrates that transient ischemia of the optic nerve produced by experimental coagulopathy results in long-term neuronal and axonal loss which was significantly reduced by topical treatment with brimonidine tartrate for 7 days prior to the ischemic insult. The degree of neuroprotection of topical brimonidine application when given after the ischemic injury was limited and variable, with pretreatment significantly more effective than treatment immediately post-injury. However, application of brimonidine after the ischemic injury did result in moderate rescue of optic axons from injury as evidenced by the morphometric analysis of optic axon number.

It is well established that acute ischemic insult to the optic nerve results in a significant loss of retinal ganglion cells and their axons. Ganglion cell death occurs in two waves after injury, with the initial wave occurring within the first week (Sievers et al., 1987). This suggests that there is a window of time during the initial phases of injury and cell death when neuroprotective strategies could be applied to provide some rescue of injured neurons. In the period immediately following 60 or 90 min of ischemia followed by reperfusion, significant retinal edema develops, with concomitant inflammatory cell infiltrate (Szabo et al., 1991), which begins to abate during the first 24 h. Edema at the optic nerve head occurs in our coagulopathy injury model, suggesting similar histological changes immediately following injury in both this model of injury and in patients. Using this same model of coagulopathy injury, c-fos was shown to rapidly elevate in oligodendrocytes within the injured optic nerves, followed in turn by demyelination (Goldenberg-Cohen et al., 2005). Brimonidine would seem to have a protective effect against these early changes induced by ischemia. In a small-scale clinical trial, brimonidine was effective in improving visual acuity and decreasing micro-aneurysm formation when administered topically to patients in the very early stages of type 2 diabetes mellitus (Mondal et al., 2004). It was also effective in improving contrast sensitivity when administered to newly diagnosed, previously untreated glaucoma patients (Evans et al., 2003). In ocular hypertensive patients, patients treated with brimonidine showed less retinal fiber layer damage than those treated with timolol (Tsai and Chang, 2005). While brimonidine has an excellent safety profile, there are some reported side effects, particularly in children (Al-Shahwan et al., 2005). These include lethargy and burning of the eyes. However, the most recent study in adult ischemic neuropathy patients did not show

---

**Fig. 1.** Histological changes in the optic nerve cross-sections 5 months after a photocoagulation injury. (A) Cross-section of a normal optic nerve from a rat that received saline only. (B) Cross-section of an injured optic nerve treated with saline only. (C and D) Cross-section of optic nerves in rats that received brimonidine 7 days prior to production of the ischemic optic nerve injury. Significant optic nerve axon preservation is apparent. (E) Cross-section of an optic nerve in a rat that received 14 days of brimonidine treatment following the ischemic optic nerve injury. The bar is 50 μm.
any harmful effects in the brimonidine-treated patients (BRAION Study Group et al., 2006).

Ischemia results in long-term loss of both optic axons and retinal ganglion cells (Berkelaar et al., 1994). In the present study, only brimonidine pretreatment had a significant rescue effect when analyzed by survival of optic axons at 5 months, when maximal optic axon loss would have occurred. In previous studies, topical administration of brimonidine 1 h before experimentally-produced retinal ischemia protected against ganglion cell loss (Vidal-Sanz et al., 2001) and ischemia-induced degeneration of the retinotectal projection (Aviles-Trigueros et al., 2003), supporting the effectiveness of brimonidine in preventing optic axon loss. In another study, topical brimonidine applied before treatment reduced collateral damage caused by laser photocoagulation to treat choroidal neovascularization (Ferencz et al., 2005). The mechanism(s) for the neuroprotective effects of pretreatment are unclear, but they may be related to the need to up-regulate survival factors such as fibroblast growth factor or anti-apoptotic proteins. These would require sufficient time for protein synthesis to occur. Further studies are needed to address these questions.

Application of brimonidine, an alpha 2-agonist, is a common treatment for glaucoma and causes reduction of intraocular pressure by decreasing ciliary blood flow (Reitsamer et al., 2005), lowering aqueous humor production and increasing uveoscleral outflow (Greenfield et al., 1997; Toris et al., 1995). However, it is unclear whether any of these changes would be the primary mechanism of its neuroprotective effect in this study. Brimonidine, however, affects more than just the vasculature within the orbit. While brimonidine treatment decreases intraocular pressure, it does not appear to change retinal capillary blood flow in patients with ocular hypertension (Lachkar et al., 1998; Carlsson et al., 2000). In addition, long-term application of brimonidine does not appear to affect the blood flow or vasomotor activity of the anterior part of the optic nerve in rabbits (Bhandari et al., 1999). In a model of chronic ocular hypertension, systemic administration of brimonidine and timolol, a non-selective beta-adrenergic receptor blocking agent used for treatment of intraocular hypertension, showed little effect on intraocular pressure. The neuroprotective effect of brimonidine does not appear to be related to its ability to lower intraocular pressure, because timolol, a similar glaucoma medication, does not result in significant protection of retinal ganglion cells after ischemic injury (WoldeMussie et al., 2001; Wheeler and WoldeMussie, 2001). Brimonidine activation of alpha2-adrenoreceptors at clinical doses does, however, produce nitric oxide-dependent vasodilation in larger caliber retinal arterioles and vasoconstriction in smaller caliber arterioles (Rosa et al., 2006). This suggests that brimonidine may play a role in retinal blood flow regulation.

Ischemic injury has many sequelae, and brimonidine may affect a number of these processes. N-methyl-D-aspartate apparent. (C) Cross-section of an optic nerve in a rat that received 14 days of brimonidine treatment following the ischemic optic nerve injury. (D) Cross-section of an injured optic nerve treated with saline only.
(NMDA) antagonists protect retinal neurons from toxicity induced by increased exogenous glutamate, aspartate, and hypoxic damage (El-Asrar et al., 1992). Alpha 2- agonist activation also prevents accumulation of extracellular glutamate and aspartate, and this may play a role in the ability of brimonidine to reduce ischemic retinal and optic nerve injury (Donello et al., 2001). Topical application results in a high concentration of brimonidine in tissues with high ocular melanin (Acheampong et al., 1995). Hence, ocular structures such as the iris, choroid, and retina retain brimonidine compared to non-pigmented eye structures. In addition, there is a carrier-mediated transport of brimonidine in the retinal pigment epithelium (Zhang et al., 2006). This high affinity of brimonidine to retinal pigment epithelium would increase its concentration near the tissue damaged by ischemic injury, and this may, in part, also explain its effectiveness in preserving retina and optic nerve axons from ischemia-reperfusion damage. In both human and animal studies, vitreous concentrations resulting from topically applied brimonidine reached levels above 2 nM, a concentration previously shown to activate alpha 2- receptors (Kent et al., 2001; Acheampong et al., 2002). These levels were slightly higher in aphakic patients; nonetheless topical application of brimonidine results in vitreal levels that are physiologically relevant.

Brimonidine in the retina and optic nerve is associated with up-regulation of bcl-2 and bcl-xl, resulting in the inhibition of neuronal apoptosis (Tatton et al., 2001a,b; Lai et al., 2002). Brimonidine also preserves anterograde axonal transport after transient ischemia of the retina (Lafuente Lopez-Herrera et al., 2002), and this may result in the retention of neurotrophic factors needed for maintenance of the injured pathway. Brimonidine application results in an up-regulation of various growth factors that are known to protect neurons from injury. Exogenous administration of basic fibroblast growth factor (bFGF) can protect photoreceptors in rats exposed to constant light (Faktorovich et al., 1990, 1992), and stimulation of alpha 2-adrenergic receptors in the rat retina results in the up-regulation of bFGF mRNA (Wen et al., 1996) and protein expression.

Fig. 3. (A) Axon count of the optic nerves from animals that received 7 days treatment with brimonidine before ION induction. (B) Axon count of the optic nerves from animals that received 14 days treatment with brimonidine following ION induction.

Fig. 4. Cresyl violet staining of the retina 5 month after ION induction. Arrows indicate neurons in the ganglion cell layer. (A) Retina from animals receiving 7-day brimonidine treatment prior to the injury shows moderate loss of neurons in the ganglion cell layer; (B) Retina from animals receiving 14 day brimonidine treatment following the injury demonstrates severe loss of neurons in the retinal ganglion cell layer; (C) Control retina shows the normal ganglion cell layer. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. The bar is 50 μm.
in rats resulted in a significant increase in axonal survival rate compared to brimonidine treatment given after the induction of optic nerve ischemia. While the clinical trials in human patients have been equivocal, the prophylactic use of brimonidine is worth further investigation. These results suggest that brimonidine may be useful as a preventive measure for patients with a high risk of developing ION and for those developing such a condition in the fellow eye.

Acknowledgements

Supported by the Neuro-ophthalmology Research Fund, the Minnesota Lions and Lionesses, Lew Wasserman Mid-Career Research to Prevent Blindness Merit Award (LKM) and an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness Inc.

References


