Neuroprotection of Retinal Ganglion Cells by Brimonidine in Rats with Laser-Induced Chronic Ocular Hypertension

Elizabeth WoldeMussie, Guadalupe Ruiz, Mercy Wijono, and Larry A. Wheeler

PURPOSE. To examine the neuroprotective effect of the α2 adrenergic agonist brimonidine in a chronic ocular hypertension model.

METHODS. Intraocular pressure (IOP) was elevated by laser photocoagulation of episcleral and limbal veins. Retinal ganglion cell loss was evaluated in wholemounted retinas. Brimonidine or timolol was administered, either at the time of or 10 days after IOP elevation and continued for 3 weeks. Drug-related immunohistochemical changes in glial fibrillary acidic protein (GFAP) were also determined after 3 weeks.

RESULTS. Laser treatment caused a twofold IOP increase over baseline that was maintained for 2 months. A time-dependent loss of ganglion cells occurred with elevated IOP. Systemic administration of brimonidine or timolol caused little decrease in IOP. After 3 weeks of elevated IOP, ganglion cell loss in control rats was 33% ± 3%. Brimonidine reduced the progressive loss of ganglion cells to 26% ± 1% and 15% ± 2% at doses of 0.5 and 1 mg/kg · d, respectively. Timolol had no effect. Ten days of high IOP resulted in 22% ± 4% ganglion cell loss. Brimonidine administration initiated 10 days after IOP elevation prevented any further loss of ganglion cells. In vehicle- or timolol-treated rats, ganglion cell loss continued to 33%. The increase in immunoreactivity of GFAP in ocular hypertensive retinas was attenuated by brimonidine.

CONCLUSIONS. Systemic application of brimonidine or timolol had little effect on IOP. Brimonidine, but not timolol, showed significant protection of retinal ganglion cells when applied at the time of IOP elevation and prevented further cell loss when applied after IOP was elevated. This indicates that brimonidine has a neuroprotective activity unrelated to its effect on ocular hypotension. (Invest Ophthalmol Vis Sci. 2001;42:2849–2855)

Glaucoma-induced ganglion cell degeneration is one of the leading causes of blindness. One of the most well-defined risk factors of glaucomatous ganglion cell damage is chronic elevation of IOP. Recent reports have hypothesized possible mechanisms of this neurodegeneration. Increase in vitreal glutamate in humans with glaucoma and animals with experimental hypertension has suggested that glutamate excitotoxicity may be involved in glaucomatous retinal damage. Others have reported that reduction of transport of neurotrophic factors may be the cause of ganglion cell death. Apoptosis has been reported to be a process of ganglion cell death. Such reports shed light on the mechanisms of ganglion cell injury and suggest the need for a direct therapeutic approach to prevent cell damage or enhance survival.

The conventional treatment of glaucoma has been directed toward controlling IOP. One of the compounds that are currently used in the control of pressure for open-angle glaucoma is the α2-adrenergic receptor agonist brimonidine. It is very effective in lowering IOP. There is also evidence that α2 adrenergic agonists protect neurons from damage in several models of ischemia. For example dexmedetomidine has been reported to be protective in focal cerebral ischemia in rat and rabbit and in incomplete forebrain ischemia in rats. Brimonidine has also been shown to have neuroprotective activity in a variety of neuronal injury models, such as light-induced photoreceptor damage, optic nerve injury, and acute retinal ischemia. In the present study we established laser-induced chronic ocular hypertension in rats and used this model to investigate the neuroprotective effect of brimonidine. We compared the neuroprotective effect of brimonidine to that of timolol, another ocular hypotensive compound used in the treatment of glaucoma.

METHODS

Elevation of IOP

Experiments were conducted in strict accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and strict adherence to internal animal care and use committee guidelines. Male Wistar rats weighing 350 to 450 g were used. IOP was elevated by laser photocoagulation with a blue-green argon laser (Coherent, Palo Alto, CA). Rats were anesthetized with a mixture of ketamine (50 mg/kg), acepromazine (1 mg/kg), and xylazine (25 mg/kg). Laser treatment was performed on the episcleral veins within 0.5 to 0.8 mm from the limbus and on the veins in the limbus. Treatment was applied in two parts, 1 week apart. The amount of energy used was 1 W for 0.2 seconds, delivering a total of 130 to 150 spots (50–100 μm spot size) with both laser treatments.

IOP was measured by tonometer (Tono-Pen; Mentor, Norwell, MA) as described previously, with some modification. Rats were injected with 3.0 mg/kg acepromazine intramuscularly, enough to keep them calm but not to sedate them. Proparacaine (0.5%) was applied topically on the eyes to anesthetize the cornea. Fifteen readings were taken and averaged to give one measurement. IOP measurements were taken three times for the first 2 weeks and then once a week for the remainder of the experimental period. In drug-treated rats, IOP was measured once a week. To determine the correlation of elevated IOP and ganglion cell loss, IOP was elevated for different periods ranging from 4 to 60 days, and ganglion cell loss was evaluated.

Drug Treatment

Drugs were administered continuously using an osmotic pump that was inserted subcutaneously on the back. They were administered in two different paradigms. In one group, brimonidine (0.5 or 1 mg/kg · d, n = 10), timolol (1 or 2 mg/kg · d, n = 10), or phosphate-buffered...
TABLE 1. Time Course of Laser-Induced Increase in IOP and Ganglion Cell Loss

<table>
<thead>
<tr>
<th>Days after Laser</th>
<th>IOP (mm Hg)</th>
<th>RGC Loss (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.8 ± 0.6</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>25.0 ± 0.4</td>
<td>61 ± 5.4</td>
<td>11</td>
</tr>
<tr>
<td>10</td>
<td>30.9 ± 0.5</td>
<td>19 ± 4.7</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>33.1 ± 0.4</td>
<td>26.5 ± 7.2</td>
<td>8</td>
</tr>
<tr>
<td>21</td>
<td>32.6 ± 0.6</td>
<td>35.5 ± 4.7</td>
<td>8</td>
</tr>
<tr>
<td>30</td>
<td>32.3 ± 0.3</td>
<td>36.8 ± 3.6</td>
<td>6</td>
</tr>
<tr>
<td>43</td>
<td>29.9 ± 0.3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>29.2 ± 0.6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>27.9 ± 0.6</td>
<td>44.2 ± 4.9</td>
<td>16</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

saline (PBS) vehicle (n = 10) was administered at the time of initial IOP elevation. Age-matched naive control rats (n = 5) were used to determine baseline ganglion cell number. In another group, IOP was elevated for 10 days before drug treatment with 1 mg/kg · d brimonidine (n = 15) or 2 mg/kg · d timolol (n = 15), or PBS vehicle (n = 15). As before, an age-matched group (n = 9) was used to determine baseline. In both paradigms treatment was continued for 5 weeks after drug administration.

Because the drug treatment was continuous for 3 weeks, it was important to establish that the high dose of brimonidine did not cause sedation. To determine this, rats were treated with brimonidine at 1 mg/kg · d (n = 5) for 9 days, and locomotor activity was measured on days 5 and 9 and compared with that of vehicle-treated rats. Activity was measured in photocell cages where horizontal and vertical movements were recorded with an activity monitor (Digiscan Animal Activity Monitor; Omnitech Electronics, Columbus, OH). Locomotor activity was defined as the total number of horizontal and vertical beam interruptions over a 5-minute period.

Retinal Ganglion Cell Count

The effect of elevated IOP on retinal ganglion cell loss was examined histologically in wholemounted retinas. At the end of each experimental period, the ganglion cells were labeled by retrograde transport using crystals of 3000 molecular weight (MW) dextran tetramethylrhodamine (DTMR; Molecular Probes, Eugene, OR). This dye traveled by diffusion at the rapid rate of 2 mm/hour and did not require active transport.11 Thus, we assumed that all the ganglion cells would be labeled regardless of health, as long as they were present. Rats were deeply anesthetized and the optic nerve was exposed. A longitudinal incision was made on the meninges, and the optic nerve was completely sectioned at approximately 2 to 3 mm from the globe. Crystals of DTMR were applied to the cut end of the optic nerve. To determine whether IOP elevation impeded the transport of the dye, we compared effectiveness of labeling in normotensive eyes (n = 5), with eyes in which IOP was raised twofold by laser treatment for 1 day (n = 5). Twenty-four hours after application of DTMR, the rats were killed, eyes enucleated, and fixed with 4% paraformaldehyde. The retinas were wholemounted, and labeled ganglion cells in eight central (0.66–1.103 mm from the edge of the optic disc) and four to eight peripheral (1.98–2.43 mm) areas in four quadrants of the retina were counted under 400× magnification. These areas represent 2.54% to 3.12% of the ganglion cells in the retina. Non-laser-treated, age-matched retinas were counted in the same areas to estimate total ganglion cell number. Ganglion cell loss in experimental eyes was calculated as percentage of cell loss compared with the non-laser-treated control. Percentage protection of ganglion cells by test compounds was calculated by comparing to vehicle-treated rats.

Immunohistochemistry

We had shown that elevation of IOP resulted in increase in immunoreactivity of intermediate filament glial fibrillary acidic protein (GFAP) in Müller cells.12 In this study we examined whether treatment with brimonidine affects the increase in this protein. At the end of the experimental period, fixed retinas were cryoprotected with sucrose (10%–30%) at 4°C for 2 days. Cuts were made at the horizontal meridian, and retinas were frozen in optimal cutting temperature compound (OCT; Tissue Tek, Torrance, CA). Cross sections of 15 μm were made from the upper half of the retina, collected on gelatin-coated slides, and air dried. The sections were then rehydrated and incubated with 10% normal goat serum in PBS for 30 minutes to block nonspecific sites. The sections were incubated overnight with the primary antibody; polyclonal rabbit anti-cow GFAP (Dako, Carpinteria, CA) diluted at 1:500, at 4°C in a humidified chamber. The following day they were rinsed with PBS and incubated with biotinylated secondary antibody for 60 minutes followed by indocarbocyanine (Cy 2)-conjugated avidin (Jackson ImmunoResearch, West Grove, PA.), rinsed, and cover-slipped. Control samples were incubated without primary antibodies. Quantitative analysis of the immunostaining was performed by measuring fluorescence intensity by computer (Image Pro software; Media Cybernetics, Silver Spring, MD) using the “count-measure” function. Several sections were made, and five to six samples (every five sections) were immunostained. Images from these sections from all the rats were captured on camera (Spot; Diagnostic Instruments Inc., Sterling Heights, MI), with the same magnification and exposure period on the same day, and stored in the computer for further processing. Using the same size of sampling area in different layers of the retina, fluorescence intensity was measured in pixels and the numbers recorded. The absolute intensity values were normalized to a mean value from non-laser-treated control retinas. The control value was expressed as 1. Changes in intensity in laser-treated eyes were calculated in relation to the normalized control.

Statistical Analysis

The data are expressed as mean ± SEM. Statistical comparison was made using unpaired Student’s t-test. P < 0.05 was considered significant.

RESULTS

IOP and Ganglion Cell Loss

Laser photocoagulation of limbal and episcleral veins performed close to the limbus resulted in increased IOP. The baseline IOP in acepromazine-treated non-laser-treated rats was 16 ± 0.4 mm Hg (n = 25). The first laser treatment increased IOP to 25 to 27 mm Hg, approximately 1.6-fold over baseline (Table 1). The second laser treatment increased IOP to a maximum of approximately 32 to 35 mm Hg (approximately twofold). This level of IOP was sustained for longer than 2 months, with a slight decrease. Elevated IOP-induced ganglion cell loss was evaluated by labeling the ganglion cells with

Table 2. Regional Variation of Ganglion Cell Loss after Elevation of IOP

<table>
<thead>
<tr>
<th>Days after Laser</th>
<th>RGC Loss (Superior)</th>
<th>RGC Loss (Inferior)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8.2 ± 5.9</td>
<td>5 ± 5 (11)</td>
</tr>
<tr>
<td>10</td>
<td>27.1 ± 5.5</td>
<td>7.2 ± 2.7 (6)</td>
</tr>
<tr>
<td>15</td>
<td>31.6 ± 9.4</td>
<td>18.5 ± 3.8 (8)</td>
</tr>
<tr>
<td>21</td>
<td>42.1 ± 7.5</td>
<td>29.1 ± 4.2 (8)</td>
</tr>
<tr>
<td>30</td>
<td>51.2 ± 7.5*</td>
<td>23.2 ± 5 (6)</td>
</tr>
<tr>
<td>60</td>
<td>56.6 ± 7.2†</td>
<td>32.9 ± 3.5 (16)</td>
</tr>
</tbody>
</table>

Data are from Table 1. Percentage of ganglion cell loss in the upper and lower retina was determined separately. More cell loss was evident in the upper retina at all time points. Data are expressed as mean ± SEM. Numbers in parenthesis indicate number of animals.

* P < 0.02.
† P < 0.001.
DTMR at the optic nerve 1 day before the end of each time point. To determine whether IOP elevation did not obstruct the transport of the dye, we compared quantity of labeling in normotensive eyes, with eyes in which IOP had been increased twofold for 1 day. At normal IOP of 16.4 ± 0.1 mm Hg, ganglion cell count was 1393 ± 47/mm² (n = 5). At the high IOP of 30.2 ± 0.5 mm Hg, the number of cells was 1207 ± 73/mm² (n = 5). These numbers were not significantly different from each other (P > 0.05), indicating that the transport of DTMR was not hindered by elevated IOP.

Elevation of IOP by laser treatment caused a time-related loss of ganglion cells (Table 1). The loss of ganglion cells was linear for the first 3 weeks, resulting in a calculated cell loss of 12% per week and slowed to 2% per week from 3 weeks to 2 months. The average cell loss over the 2-month period was at 5% per week. By examining the ganglion cell loss in the different quadrants, we noted a regional variation in which there were more cells lost in the superior half than in the inferior half (Table 2). Although this difference was evident at all time points, it was significant after 1 and 2 months of elevated IOP only.

Effect of Brimonidine or Timolol on IOP

The effect of timolol or brimonidine was evaluated after systemic administration. These drugs have side effects, such as slowing of the heart rate (timolol) and sedation and a decrease in blood pressure (brimonidine), when administered systemically. Sedation was a big concern, because it would interfere with the rats’ eating or drinking. Therefore, we tested the highest dose (1 mg/kg · d) of brimonidine for its sedative effect, using as a measure locomotor activity on days 5 and 9 after placing the osmotic pump. Total activity was 3360 ± 301 (vehicle) and 3159 ± 341 (brimonidine) on day 5 (P > 0.66) and 2177 ± 452 (vehicle) and 2511 ± 388 (brimonidine) on day 9 (P > 0.66). These values were not significantly different from each other, indicating that there was no side effect from this dose of brimonidine. In addition, these animals appeared active when routinely observed in their cages.

In the first treatment paradigm, the compounds were administered immediately after initial laser treatment. Systemic administration of either brimonidine or timolol lowered IOP very slightly by 5% to 10% at both doses tested (Fig. 1). In the second treatment paradigm, in which drug treatment com-
menced 10 days after elevation of IOP, neither brimonidine nor timolol showed any effect on IOP (Fig. 2).

**Effect of Brimonidine or Timolol on Ganglion Cell Loss**

After 3 weeks of elevated IOP, there was a considerable decrease in labeled ganglion cells in retinas of vehicle-treated eyes compared with those treated with brimonidine during the same period (Fig. 3). The ganglion cell loss, in vehicle-treated rats was 33% after 3 weeks of elevated IOP (Fig. 4). Brimonidine treatment during this 3-week period resulted in a dose-related protection of ganglion cells in which cell loss was reduced to 26% ± 1% and 16% ± 3% with 0.5- and 1-mg/kg·d doses, respectively (Fig. 4). Timolol treatment did not have any protective effect with either dose (Fig. 4).

After 10 days of IOP elevation there was a 22% ± 4% decrease in ganglion cell number (Fig. 5). Drug treatment was initiated at this time and continued for 3 weeks. Ganglion cell loss in brimonidine-treated rats remained at 22% ± 3.8%, whereas those treated with vehicle or timolol continued to 33% ± 5.6% or 35% ± 3.3%, respectively (Fig. 5).

**Immunohistochemistry**

In normal retina, GFAP immunoreactivity is restricted to astrocytes and end feet of the Müller cells at the inner limiting membrane. However, its expression increases after different types of retinal injury. After elevation of IOP, GFAP immunoreactivity increased throughout the length of the Müller cells (Fig. 6). Treatment with brimonidine caused a significant decrease in GFAP expression compared with vehicle-treated eyes (Fig. 6). This decrease spanned the length of the Müller cells (from ganglion cell to photoreceptor layers). Timolol did not show any decrease in immunoreactivity of GFAP.

**DISCUSSION**

Glucoma-induced vision loss is the result of degeneration of ganglion cells and their axons. Although it is well established...
that elevated IOP is one of the risk factors in this disease process the mechanism of degeneration is unclear. The continued loss of vision in eyes with glaucoma, despite control of IOP, emphasizes the need for neuroprotective therapy. The development of a chronic ocular hypertension model in the rat provides a valuable means of evaluating the mechanism and process of injury and can be used to evaluate compounds with neuroprotective activity.4,10,15–18 In this study, the neuroprotective effect of brimonidine was examined using rats with laser-induced chronic ocular hypertension. All the rat models reported by others and the one described in this study have shown that elevation of IOP causes ganglion cell or optic nerve fiber loss.4,10,15–18 It has also been shown that the level of IOP obtained determines the extent of ganglion cell loss.4 In this study, IOP elevation of twofold caused ganglion cell loss of 44% after 2 months, averaging 5.5% per week. This result is comparable to previous reports showing that cell losses of approximately 37% and 47% were obtained after 6 and 10 weeks, averaging 6% and 5% per week, respectively.4,15 Other investigators have observed cell loss at a slower rate of 1.4% per week when the average cell loss was calculated over a longer period of 6 months and the increase in IOP was 1.6-fold or less.17 We noted a biphasic rate of ganglion cell loss with a fast rate of 12% per week for the first 3 weeks of IOP elevation, followed by a slower rate of 2% per week for the remainder of the experimental period. The rate of ganglion cell loss in the other studies is linear.4,15,17

In prior studies cautery was used once to occlude two or three major episcleral veins, 2 to 3 mm from the limbus, whereas laser treatment in the present study was performed twice, on several veins within 1 mm from the limbus. Thus, in addition to a difference in the technique of vessel occlusion, the discrepancy in rate of ganglion cell loss could also be due to the rate at which the target IOP was reached and main-

**Figure 6.** Immunohistochemical staining of retinas for GFAP. Drugs were administered at the time of IOP elevation. Three weeks later, eyes were fixed and cross-sectioned retinas were stained with anti-GFAP antibody. (A) GFAP immunostaining in retinas with normal IOP was limited to the end feet of Müller cells in the fiber layer. (B) Elevation of IOP increased GFAP staining. (C) Brimonidine caused a significant decrease in GFAP staining (*P < 0.025). (D) Timolol caused a slight, non-significant decrease. (E) Quantitative analysis of fluorescence intensity of GFAP, measured in all layers of the retina. Values represent mean ± SEM of measurements in three retinas. RGC, retinal ganglion cells; IPL, inner plexiform layer; INL, inner nuclear layer; PR, photoreceptor.

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tained. In this study, target IOP was reached by two laser treatments within 1 week, which may have initiated a faster rate of initial ganglion cell loss. The consistent and significant loss of ganglion cells after 3 weeks of elevated IOP provided us with an opportunity to evaluate neuroprotective activity of pharmacologic agents within a relatively short period.

In this study, we provide evidence that the α2-adrenergic agonist brimonidine is neuroprotective of ganglion cells in eyes with laser-induced chronic ocular hypertension. Brimonidine is currently used to lower IOP in the treatment of open-angle glaucoma.19–21 The neuroprotective effect of brimonidine was evaluated in two different ways. When brimonidine was applied at the time of IOP elevation, before pressure-induced ganglion cell injury began, it attenuated ganglion cell loss by 50% (cell loss with vehicle 33% and with brimonidine 15%), in spite of the sustained increase in IOP. More interesting was that when brimonidine was administered 10 days after IOP was elevated, it prevented any further loss of ganglion cells. In both treatment paradigms systemically applied brimonidine (400 µg or 17 µg/kg to each rat) reached the retina in sufficient amount to exert neuroprotection but had little or no effect on IOP. However, topically applied brimonidine (0.2%, 10 µg/5 µl) was effective in lowering IOP by 40% (data not shown) and was neuroprotective of retinal neurons in a rat acute retinal ischemia–reperfusion injury model.22 Moreover a recent study has shown that vitreal levels of brimonidine after topical application of a 0.2% dose in phakic eyes with planned vitrectomy was 9.3 ± 8 nM.23 This concentration is more than adequate to activate the α2 receptors in the retina. The median effective dose (EC50) of brimonidine for activating α2 receptors is approximately 2 nM.24

The neuroprotective effect of brimonidine was compared with that of timolol. Both brimonidine and timolol are currently used for the treatment of glaucoma, and they lower IOP significantly. It is thought that lowering of IOP reduces injury and preserves the visual field. In this study, brimonidine, but not timolol, was neuroprotective. Similar to brimonidine, topical timolol (0.5%, 5 µl) caused a decrease in IOP by 27% (data not shown). Similarly, the amount of timolol that reached the eye when delivered by osmotic pump appeared to be lower than required to decrease IOP. This emphasizes the direct neuroprotective effect of brimonidine.

In several models of retinal injury, stress, or degeneration, expression of GFAP increases in Müller cells.25–28 The increase in immunoreactivity of GFAP in this ocular hypertensive model may be a response to the stress and pathologic process that leads to degeneration of ganglion cells. The decrease in GFAP immunoreactivity by brimonidine suggests that activation of the selective α2-adrenergic receptors reduces this injury in the retina.

Several mechanisms underlying the neuroprotective activity of α2-adrenergic agonists have been proposed. Brimonidine has been shown to increase neurotrophic factors and thus may be neuroprotective by enhancing the survival of ganglion cells in this hostile environment. In addition, activation of presynaptic α2 receptors results in inhibition of transmitter release.29,30 It is possible that brimonidine treatment attenuated the release of glutamate in the eyes with elevated IOP. Increase in vitreal glutamate has been implicated in excitotoxicity of ganglion cells in glaucoma.31 Although we did not measure vitreal glutamate in this study, there is a report showing that brimonidine inhibits accumulation of glutamate in the vitreous after acute retinal ischemia.32

In summary, we have shown that laser photocoagulation of limbal and episcleral veins in the rat caused chronically elevated IOP, resulting in significant loss of ganglion cells. Systemic treatment with the α2 agonist brimonidine provided neuroprotection to ganglion cells in this model.

Acknowledgments

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