Retinal ganglion cell neuroprotection in a rat model of glaucoma following brimonidine, latanoprost or combined treatments

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Abstract

The aim of the present study is to evaluate the neuroprotective effect of two antiglaucomatous substances, regardless of their hypotensive effect in the eye. Brimonidine, which does not reduce IOP when administered intraperitoneally, and latanoprost, which has a renowned hypotensive effect topically. We examined rat retinal ganglion cell (RGC) survival and size distribution in experimental glaucoma in response to different glaucomatous agents. IOP was elevated by episcleral vein cauterization (EVC) prior to the application of different treatments: (I) PBS application (control group), (II) intraperitoneal administration of brimonidine (a general hypotensive agent), (III) topical application of latanoprost (an ocular hypotensive agent), and (IV) latanoprost combined with brimonidine. After 12 weeks, RGCs were retrogradely labeled with fluorogold and RGC density was analyzed. EVC caused a significant increase (42%) in IOP in each group before drug treatment. After 12 weeks of EVC, RGC survival in control vs. EVC rats was 78.9 ± 3.2%. No IOP reduction was observed in brimonidine injected rats, but RGC survival at 12 weeks was total (103.7 ± 2.7%). In latanoprost treated rats, IOP dropped by around 22% and 94.7 ± 3.7% of the RGC population survived. Finally in the latanoprost + brimonidine combined group, IOP was significantly reduced by 25% and 94.4 ± 2.2% of RGCs survived. Surprisingly, whereas EVC led to a 6% increase in RGC soma size, brimonidine treatment was associated with a 9% reduction in the soma size of RGCs at 12 weeks. We conclude that brimonidine exerts a neuroprotective effect via a mechanism which is independent of IOP reduction. These findings indicate that cell survival in glaucoma may be enhanced by neuroprotective strategies which are independent of IOP reduction. No synergistic neuroprotective effect was observed when both treatments were applied simultaneously.

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1. Introduction

Primary open angle glaucoma (POAG) is an optical neuropathy characterized by the gradual and progressive loss of retinal ganglion cells (RGCs). Elevation of intraocular pressure (IOP) is the principal risk factor associated with the progression of this disease (Quigley et al., 1994). As a consequence, current treatments are primarily focused on reducing IOP (Heijl et al., 2002; Leske et al., 2003).

Hypotensive drugs which are used daily in clinical practice include alpha-2 adrenergic agonists such as brimonidine and prostaglandin analog such as latanoprost. Brimonidine reduces the production of aqueous humor and facilitates its exit via the trabecular meshwork, while latanoprost promotes the drainage of aqueous humor via the uveoscleral pathway.

The neuroprotective effect of brimonidine has been studied in a variety of experimental models of optic neuropathy including optic nerve injury (Yoles et al., 1999; Ruiz et al., 2000), transitory ischemia (Donello et al., 2001; Lafuente...
et al., 2001) and experimental glaucoma (Ahmed et al., 2001; Wheeler and Woldemussie, 2001; Wheeler et al., 2003). However, the intraperitoneal application of brimonidine in animals has not been found to reduce IOP. The pharmacological profile of brimonidine fulfills three of four criteria associated with retinal neuroprotectors: (i) to have a specific target (receptors) in the retina; (ii) to exhibit neuroprotective activity and have a measurable effect on RGC survival; and (iii) to reach the retina in neuroprotective concentrations after clinical dosing. Nevertheless, clinical assays to evaluate the utility of this drug in optical neuropathies related to glaucoma (4th criteria) have not been carried out heretofore.

Prostaglandin analogs, such as latanoprost, are the antiglaucoma drugs which are reported to present the largest hypotensive effect in clinical practice. Thus, they are considered to be first line drugs for the treatment of glaucoma (Camras, 1996; Bernard et al., 2003; Lee and Higginbotham, 2005), promoting the drainage of aqueous humor via the uveoscleral pathway (Gabelt and Kaufman, 1989; Nilsson et al., 1989; Richter et al., 2003; Weinreb et al., 2004). Latanoprost has been reported to have a hypotensive effect in animal models including the mouse (Aihara et al., 2002; Crowston et al., 2004), rat (Benozzi et al., 2002; Pang et al., 2005) and monkey (Gagliuso et al., 2004). However, no studies have yet been published on RGC survival in experimental glaucoma following various weeks of treatment, since only functional aspects, such as the absence of progression of the campimetric defect, have been measured.

The combination of distinct treatments with a view to obtaining a larger therapeutic effect is a common practice in patients with glaucoma. Nevertheless, few experimental studies have examined the effectiveness of these combined treatments (Ishii and Araie, 2000; Wang et al., 2000). Thus, the aim of the present study was to evaluate: (i) the neuroprotective effect of two substances; one of which does not reduce IOP (brimonidine) when it is injected, but has been demonstrated to have a neuroprotective effect, vs. (ii) a substance which reduces IOP (latanoprost) but its neuroprotective effect is unknown, and (iii) the putative synergistic effect of co-administration of both antiglaucoma drugs. To study the neuroprotective effect of these three treatments we measured: (1) the RGC survival, and (2) the RGCs soma size and its localization in the retina in a rat model of chronic EVC-induced glaucoma.

2. Materials and methods

2.1. Animals

Fifteen female Sprague–Dawley rats weighing 250 g were used. Animals were housed with food, water, constant temperature (21 °C) and under a 12-h light/dark cycle. All animal experimentation adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Episcleral vein cauterization (EVC)

IOP increase was induced in 15 rats by cauterizing three episcleral veins on the left eyes of the animals following the method previously described (Shareef et al., 1995; Urcola et al., 2006). The right eyes of all groups were not operated in order to be considered as control eyes. Animals were kept alive for 12 weeks.

2.3. IOP measurements in awake animals

IOP was measured weekly with an applanation tonometer (TonoPen XL, Mentor, Norwell, MA) in awake animals at the same time of day (3 p.m.) in order to avoid circadian IOP changes following the method previously described (Urcola et al., 2006).

2.4. Pharmacological treatments

Three days after surgery, when the IOP was elevated, animals were treated and distributed into different groups: (I) control rats (n = 3) were treated with 10 μl of PBS topically applied daily in the left eye and with a weekly intraperitoneal injection of 150 μl of PBS; (II) four rats were injected with brimonidine (Alphagan 0.2%, Allergan Laboratories, Madrid, Spain) (150 μl; 1 mg/kg body weight, intraperitoneal) once a week for 12 weeks; (III) four rats were topically administered with a 10 μl drop of latanoprost (Xalatan 0.005%, Pharmacia Laboratories, Barcelona, Spain) daily, applied to the left eye for 12 weeks; (IV) four rats received both treatments: daily, topical latanoprost plus weekly intraperitoneal brimonidine at the same dose for the same period as described above.

2.5. Side effects of treatments

Brimonidine has side effects, such as sedation and blood pressure reduction when administered systemically (WoldeMussie et al., 2001). After injecting brimonidine intraperitoneally, animals resumed their standard locomotor activity in approximately the next 60 min. No significant differences in weight increase were observed between rats from these four groups.

2.6. Retrograde labeling of RGCs

After the 12 weeks of the treatment, RGCs were retrogradely labeled with 3% fluorogold (Fluorochrome, Inc.; Englewood, CO, USA) as previously described (Urcola et al., 2006). Animals were kept alive for 24 h to allow fluorogold to fill the entire population of RGCs. Then, animals were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The eyes were enucleated and the lens and vitreous were extracted by cutting the anterior chamber at the level of the ora serrata. The eyecups were postfixixed in the same fixative for 2 h. Then, retinas were removed and flat-mounted, with the retinal ganglion cell layer being uppermost using PBS/glycerol (1/1).

2.7. Image capture

Images of each retina were obtained using an epifluorescence microscope (Axioskop 2; Zeiss, Jena, Germany)
coupled to a digital camera (Coolsnap, RS Photometrics, Tucson, USA). The images were systematically captured using the optic disc as a reference point for three eccentricities (centre, middle and periphery) from nasal, temporal, dorsal and ventral areas of each retina. A total of 24 fields were obtained for each retina (0.08 mm²). The number and size of the RGCs were compared between comparable areas from control (right) and glaucomatous (left) retinas, as previously described (Urcola et al., 2006). Since some RGCs were placed out of the focal plane of the majority of the RGCs, especially in the central retina, several images were captured from the same area at different focal planes. This was the way to ensure that the total population of RGCs in the designated fields was counted, and its size correctly measured.

2.8. Morphometric analysis

For each recorded retinal field, we quantified the number of RGCs (Table 1) and soma area of each RGC (Table 2) as previously described (Urcola et al., 2006). Analysis of these parameters was performed using a digital palette (EasyPen, Genius) in combination with image analysis software (Scion Image; Scion, Frederick, MD). Each RGC soma was manually filled out directly on the computer screen and values obtained were transferred to a data sheet for subsequent statistical analysis. In order to identify a possible subpopulation of RGCs which is more sensitive to neuroprotective treatments, we measured the size of each of the total counted RGCs. As described above, several pictures were taken from the same areas of the retina, in order to capture the RGCs located at different focus levels. The number and size of the cells in those images were manually superposed, in order to obtain the exact number of cells from a given area and avoid the double counting of the cells. We calculated the mean values of RGC density and RGC soma area in regions located at different eccentricities from the optic disc as described above.

We elected to do the counting and size measurements of the RGCs manually, since the automatic method interfered with the labeled axons in the central retina, and the displaced ganglion cells had not enough sensitivity to be measured (Darias, personal communication, 2007).

Moreover, we analyzed the distribution of different sized RGCs by means of their soma diameter as a function of eccentricity. We counted the number of RGCs with a given major axis length value, and frequencies were represented by histograms. Statistical analysis was performed using SPSS software (SPSS Sciences, Chicago, IL). RGC density and soma area were expressed as mean ± SEM. Mean data from different retinas, regions and subregions were compared with one-way ANOVA followed by the Scheffé test. The minimum level of significant difference was defined as p < 0.05.

2.9. Retinal structure

In order to analyze the preservation of the retinal layers, the retina was cryoprotected in 30% sucrose overnight after capture of fluorogold images, and 14 µm frozen sections were collected. Nuclei were stained with DAPI (Molecular Probes) and images from equivalent areas of control and treated retinas were taken.

3. Results

3.1. Effects of drug treatments on intraocular pressure

The mean basal IOP of the control right eye during the 12 weeks of this study was 22.2 ± 0.5 mmHg. EVC of the experimental left eye resulted in a significant increase in IOP (p < 0.01), leading to a mean IOP of 31.6 ± 0.7 mmHg. Treatment with topical and intraperitoneal PBS did not induce significant changes in IOP in EVC eyes with respect to EVC eyes which were not further treated (Urcola et al., 2006) (Fig. 1A). Weekly injection of brimonidine did not significantly alter the mean IOP of EVC eyes. Thus, the mean IOP of these eyes following treatment was similar to that observed on the days prior to brimonidine treatment (30.7 ± 1.8 mmHg vs. 31.8 ± 1.0 mmHg, p = 0.163, respectively) (Fig. 1B). Daily application of latanoprost produced a significant IOP reduction in EVC eyes. Thus, the mean IOP of these eyes following glaucoma induction was reduced to normal values following latanoprost treatment (24.8 ± 0.8 mmHg vs. 32.8 ± 0.6 mmHg, p < 0.001) (Fig. 1C). Similar results were observed in the group which combined both treatments. Thus, we observed a mean IOP reduction of 26%, and the mean IOP of the cataract eye following combined

Table 1
Percentage of RGC survival in distinct retinal zones in each experimental group

<table>
<thead>
<tr>
<th>Group</th>
<th>Central retina</th>
<th>Middle retina</th>
<th>Peripheral retina</th>
<th>Total average</th>
</tr>
</thead>
<tbody>
<tr>
<td>EVC</td>
<td>78.6 ± 3.7</td>
<td>77.8 ± 4.7</td>
<td>80.5 ± 7.7</td>
<td>78.9 ± 3.2</td>
</tr>
<tr>
<td>BMD</td>
<td>101.7 ± 4.1**</td>
<td>105.9 ± 4.2**</td>
<td>103.4 ± 6.1</td>
<td>103.7 ± 2.7**</td>
</tr>
<tr>
<td>LT</td>
<td>92.3 ± 3.8</td>
<td>93.4 ± 6.3</td>
<td>98.3 ± 8.9</td>
<td>94.7 ± 3.7*</td>
</tr>
<tr>
<td>LT + BMD</td>
<td>94.1 ± 2.0**</td>
<td>92.7 ± 3.3</td>
<td>96.4 ± 5.5</td>
<td>94.4 ± 2.2**</td>
</tr>
</tbody>
</table>

Results from statistical analysis are represented as: *p < 0.05, **p < 0.01 significant differences with respect to each control eye (right eye). Abbreviations: EVC, episcleral vein cauterization; BMD, brimonidine; LT, latanoprost; LT + BMD, combination of both treatments.

Table 2
RGC density and soma size in the four different experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Glaucoma</th>
<th>Control</th>
<th>Glaucoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGC density (RGCs/mm²)</td>
<td>2258 ± 65</td>
<td>1713 ± 64**</td>
<td>1371 ± 2.1</td>
<td>1452 ± 2.0**</td>
</tr>
<tr>
<td>Soma area (µm²)</td>
<td>2511 ± 58</td>
<td>2536 ± 63</td>
<td>1275 ± 2.3</td>
<td>1162 ± 1.7**</td>
</tr>
<tr>
<td>LT</td>
<td>2409 ± 82</td>
<td>2186 ± 67**</td>
<td>1239 ± 2.6</td>
<td>1224 ± 1.3</td>
</tr>
<tr>
<td>LT + BMD</td>
<td>2289 ± 51</td>
<td>2093 ± 44**</td>
<td>1298 ± 1.9</td>
<td>1284 ± 1.4</td>
</tr>
</tbody>
</table>

RGC density values are expressed as mean (number of RGCs per mm²) ± SEM. RGC soma area values are expressed as µm² ± SEM. Results from statistical analysis are represented as: *p < 0.05, **p < 0.01 significant difference with respect to the corresponding control eye (right eye). Abbreviations: EVC, episcleral vein cauterization; BMD, brimonidine; LT, latanoprost; LT + BMD, combination of both treatments.
treatment was reduced to 23.7 ± 0.4 mmHg (p < 0.001) (Fig. 1D).

3.2. Effect of drug treatments on RGC survival

In the present study, EVC induced the death of 21.1% of the RGCs by week 12. The extent of RGC death following IOP elevation induced by cautery was slightly higher in the more peripheral areas of the retina; however, we did not detect statistically significant differences between the different regions (data not shown). The same results were obtained when PBS was applied to the EVC retinas (Fig. 2A,B). Brimonidine injection increased RGC survival in EVC eyes. Thus, the extent of survival of eyes treated with brimonidine (103.7 ± 2.8%) was significantly higher (p < 0.01) than that observed in EVC eyes treated with PBS (78.9 ± 3.21%) (Fig. 2C,D). Daily treatment with latanoprost also produced a significant increase in RGC survival in EVC eyes with respect to EVC eyes treated with PBS (94.7 ± 3.8% vs. 78.9 ± 3.21%, p < 0.05) (Fig. 2E,F). Finally, treatment with latanoprost and brimonidine produced a mean RGC survival of 94.4 ± 2.2%, which is significantly higher than that observed in EVC eyes treated with PBS (p < 0.01) (Fig. 2G,H). Statistical analysis of these results revealed that there were no significant differences in the increase of RGC survival in cauterized eyes following treatment with brimonidine, latanoprost or both. The increase in RGC survival in cauterized eyes in each group was similar for the different regions of the retina analyzed (Table 1, Fig. 3). The density of RGCs comparing the control and the glaucomatous eyes for each treatment are summarized in Table 2 and Fig. 4.

3.3. Effect of drug treatments on the size of RGC somata

The mean area of RGC somata following EVC increased by 7.2 ± 1.8%, increasing from 137.1 ± 2.1 μm² in control eyes, to 145.2 ± 2.0 μm² in cauterized eyes (p < 0.01). This increase in soma size was significant in each of the three regions of the retina analyzed (data not shown). Regarding the effect of the different treatments on RGC soma size, we observed that the administration of brimonidine led to a significant reduction (8.9 ± 1.3%) in the mean RGC soma area. Thus, the mean RGC soma area of the cauterized eye was 127.5 ± 2.3 μm² and 116.2 ± 1.7 μm² in the brimonidine eye (p < 0.01) (Table 2, Fig. 5).

Brimonidine treatment induced a similar reduction in RGC soma area in each region of the retina. Treatment with latanoprost or with latanoprost + brimonidine did not induce significant changes in the mean RGC soma area with respect to control eyes. The mean RGC area of cauterized eyes treated with latanoprost was 122.4 ± 1.3 μm², whereas this value was 128.4 ± 1.4 μm² in eyes treated with both latanoprost and brimonidine (Table 2). These values are similar to those observed in control eyes: 123.9 ± 2.6 μm² (p = 0.56) and 129.8 ± 1.9 μm² (p = 0.44) respectively. Latanoprost or the combined treatment did not induce a significant change in the mean RGC area in the different analyzed regions of the retina.
3.4. Retinal structure

No changes in the structure of the outer retina were found after any of the treatments. Thus, quantification of the number of photoreceptor rods in control vs. EVC retinas in the same area revealed the preservation of the retinal layers. No differences were detected when comparing right and left eyes for each treatment studied (Fig. 6).

4. Discussion

It is currently unclear if reducing IOP in glaucoma is the only way of preventing RGC death, thereby providing a therapeutic effect. In the present study, we found that latanoprost, an agent which lowers IOP in glaucoma by around 25%, does indeed enhance RGC survival. However, intraperitoneal injection of brimonidine does not lower glaucoma IOP but...
exerts an even stronger neuroprotective effect on RGCs, indicating that RGC death in glaucoma could be due to factors other than increased IOP.

4.1. Effects of glaucomatous drugs on IOP

IOP reduction is the therapy most commonly employed in clinical practice to reduce the risk of glaucoma progression. In this regard, the Early Manifest Glaucoma Trial (EMGT) and the Advanced Glaucoma Intervention Study (AGIS, 2000) are large scale randomized clinical trials which have provided scientific evidence for the benefit of IOP reduction in cases of progressive glaucoma (WoldeMussie et al., 2001; Heijl et al., 2002; Leske et al., 2003). The hypotensive efficacy of the topical administration of agonists of alpha-2 adrenergic receptors, such as brimonidine, has been studied in different clinical assays (Serle, 1996; Schuman et al., 1997; Reitsamer et al., 2004) and experimental models of glaucoma (Wang et al., 2000). However, brimonidine when applied systemically in rat models of experimental glaucoma has been reported to have no hypotensive effect (Ruiz et al., 2000; Ahmed et al., 2001; WoldeMussie et al., 2001). Thus, we compared the neuroprotective effect of a non-hypotensive treatment (systemic brimonidine) vs. a hypotensive treatment (latanoprost) and a possible synergistic effect when both drugs are applied together, at the moment when damage has just been initiated and IOP begins to rise. In this regard, a previous study on the distribution of brimonidine when applied intraperitoneally in rats has revealed that this drug can reach elevated concentrations in plasma, retina and vitreous humor (Acheampong et al., 2002). However, the same study did not specify the concentration of the drug in tissues of the anterior segment. Thus, as has been demonstrated in rabbits, monkeys and humans,

Fig. 3. Percentage of RGC survival in middle, central and peripheral retinal zones of the retina for each experimental treatment group. We observed no significant differences in the increase in RGC survival in cauterized eyes following treatment with brimonidine (BMD), latanoprost (LT) or both. Results from statistical analysis are represented as: *p < 0.05, **p < 0.01 significant differences with respect to each control eye (right eye). Abbreviations: EVC, episcleral vein cauterization; BMD, brimonidine; LT, latanoprost; and LT + BMD, combination of both treatments.

Fig. 4. Comparison of RGC density in control and glaucomatous eyes in different experimental treatment groups. Results from statistical analysis are represented as: *p < 0.05, **p < 0.01 significant differences with respect to each control eye (right eye). Abbreviations: EVC, episcleral vein cauterization; BMD, brimonidine; LT, latanoprost and LT + BMD, combination of both treatments.

Fig. 5. Analysis of the soma mean area of RGCs (µm²) in control and glaucomatous eyes in different experimental treatment groups. Administration of brimonidine (BMD) led to a significant reduction in the mean RGC soma area. Results from statistical analysis are represented as: *p < 0.05, **p < 0.01 significant differences with respect to each control eye (right eye). Abbreviations: EVC, episcleral vein cauterization; BMD, brimonidine; LT, latanoprost and LT + BMD, combination of both treatments.

Fig. 6. DAPI staining in retinal layers from control (A) and EVC retinas (B). The scale bar is the same for both figures and represents 50 µm. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
topical application is the most appropriate way to obtain optimal concentrations of brimonidine in the aqueous humor, ciliary processes and iris, which are the targets for IOP reduction (Acheampong et al., 2002).

The hypotensive efficacy of prostaglandin analogs, such as the synthetic agonist of the PGF2 alpha receptor, when applied in a single daily dose has been widely demonstrated in clinical trials (Camras, 1996; Mishima et al., 1997; Alm et al., 1997). In contrast, there is little consensus regarding the real hypotensive effect of prostaglandin analogs in experimental studies. Thus, whereas in control mice they provoke IOP reductions of the order of 15% (Aihara et al., 2002), in rats subjected to experimental glaucoma by means of hyaluronic acid injection, IOP reduction is around 25% only during the first 24 h following its application (Benozzi et al., 2002). A recent study using pigmented rats (Brown Norway) reported a first acute hypertensive effect during the first 6 h of topical application; with a subsequent IOP decrease 24 h later (Pang et al., 2005).

The results of the present study demonstrate that latanoprost produces IOP reduction of the order of 25% when applied topically and daily to eyes subjected to EVC. Any obstacle to drainage independent of the aqueous pathway, for example, an increase in episcleral vein pressure, would lead to a reduced hypotensive effect of this drug. In this regard, the experimental glaucoma model employed here may better reflect the type of glaucoma which is secondary to the increase in episcleral vein pressure. Indeed, reduced hypotensive efficacy of prostaglandin analogs has been reported in similar cases, for example in cases of encephalotrigeminal angiomaticis or Sturge–Weber syndrome (Altuna et al., 1999).

Regarding the hypotensive effect of the combined brimonidine + latanoprost treatment, we found that IOP reduction in EVC eyes was similar to that observed when treatment was with latanoprost alone. Thus, brimonidine contributes no additional neuroprotective effect when applied together with latanoprost, suggesting that its mechanism of action is somehow preferentially occupied by latanoprost when IOP is reduced.

4.2. RGC counts

The number and size of the surviving RGCs have been evaluated using a manual method. We used this method mainly for two reasons: (1) to avoid the error in the central areas of the retina where automatic counting can incorrectly identify labeled RGC axons with RGCs somata, and (2) to count the RGCs somata that were out of focus and measure their size. The manual method we have used in the present study is currently accepted and has been used in this field to establish the neuroprotective effect of different molecules in RGCs (Li et al., 2006; MacLaren et al., 2006; Schuetttauf et al., 2006; Jeng et al., 2007). The automatic method of counting RGCs (Danias et al., 2002, 2006; personal communication from Danias), on the other hand, is a valid one, since it allows the measurement of a larger number of RGCs. Nevertheless, for individual cases, as in the displaced RGCs, special optics should be used or, as we have done in the present study, the most reliable method is to count them manually.

4.3. RGC survival

Treatment with intraperitoneal brimonidine leads to a total rescue of the RGCs in glaucomatous eyes. Moreover, the neuroprotective effect of this drug does not depend on reducing IOP, but may rather be due to direct activation of RGC α-2 adrenergic receptors. Due to their high hypotensive efficiency and their lack of secondary effects, prostaglandin analogs are considered to be the first choice of drugs for the therapeutic treatment of glaucoma (Camras, 1996; Lee and Higginbotham, 2005; Bernard et al., 2003). Nevertheless, this is the first study which has analyzed RGC survival following application of latanoprost in a model of experimental glaucoma. The daily administration of topically applied latanoprost increased RGC survival in EVC eyes. It is likely that the hypotensive effect of latanoprost favors RGC survival in glaucomatous eyes probably by improving blood flow in the optic nerve head (Ishii et al., 2001). Therefore, latanoprost exerts a neuroprotective effect via a mechanism which involves lowering the IOP; this may be via the induction of the synthesis of specific matrix metalloproteinases (MMPs) which results in the reduction of the interstitial extracellular matrix, thus widening the spaces between the fibers of the ciliary muscle and promoting drainage of aqueous humor (Richter et al., 2003; Weinreb et al., 2004). The combination of topically applied anti-glaucoma drugs, each of which acts via a distinct mechanism, is common in clinical practice (Lee and Gornbein, 2001; Lafuente et al., 2002; Higginbotham et al., 2002; Fechter and Realini, 2004). The present results show that treatment consisting of intraperitoneal brimonidine + topical latanoprost has a similar effect to that observed when topical latanoprost is applied alone, and slightly inferior, in terms of RGC survival, to that observed with intraperitoneal brimonidine. These differences were not statistically significant and our study reveals the absence of a synergy between these drugs, despite the fact that they act via independent mechanisms.

4.4. Effect of brimonidine on RGC soma size

Analysis of soma size in the control and glaucomatous RGCs showed that the soma of RGCs in glaucomatous retinas is about 6% bigger than that of normal RGCs. This increase was significant in EVC retinas following 12 weeks of IOP. Indeed, an increase in RGC soma area has already been reported following EVC (Ahmed et al., 2001; Ruiz-Ederra et al., 2005). It has been postulated that the increase in the soma area may be related to the progressive loss of RGCs (Urcola et al., 2006). Nevertheless, in EVC retinas subjected to brimonidine or latanoprost treatments, we found that the soma size of RGCs is up to 9% smaller than that of control retinas. This decrease is statistically significant in retinas treated with intraperitoneal brimonidine.

Brimonidine may activate a variety of neuroprotective mechanisms which could explain the reduction in soma size. Thus, brimonidine could activate an anti-apoptotic pathway in RGCs by inducing the expression of anti-apoptotic genes such as bcl2 and bcl-xl (Lai et al., 1999). Similarly, activation
of RGC alpha-2 adrenergic receptors may inhibit pro-apoptotic mitochondrial signaling (Wheeler et al., 2001a,b) and thus reduce soma size. Brimonidine-mediated neuroprotection could also be due to the inhibition of glutamate-mediated excitotoxicity (Vorwerk et al., 1996). Thus, at least in hippocampal slices, it has been demonstrated that excitotoxic activation of glutamate receptors increases the intracellular Cl⁻ within the pyramidal cell soma, and this precedes an increase in cell volume (Inglefield and Schwartz-Bloom, 1998). It is possible that brimonidine inhibits elevated glutamate levels since RGCs express both glutamate receptors (Grunder et al., 2000) and alpha-2 adrenergic receptors (Wheeler et al., 2003). In this sense, it is possible that brimonidine-mediated neuroprotection is due to a direct effect on RGCs, being independent to IOP. Thus, the decrease in RGC soma size in brimonidine treated rats may well be representative of a neuroprotective response.

Brimonidine could also exert its beneficial effects via neurotrophins. Thus, intravitreal injection of brimonidine has been shown to upregulate brain-derived neurotrophin factor (BDNF) expression in rat RGCs (Gao et al., 2002), demonstrating the neuroprotective capacity of this drug in neurodegenerative diseases. In this regard, intravitreal injection of BDNF significantly inhibited increases in RGCs soma size following axotomy in the rat optic nerve (Ota et al., 2002). Thus, the decrease of RGC soma size which we observed in brimonidine treated rats with respect to the control group could be a result of the upregulation of endogenous BDNF by RGCs (Vecino et al., 2002).

In summary, we have found that antiglaucomatous agents typically used in clinical practice exert neuroprotective effects on RGCs via distinct mechanisms. Brimonidine treatment may exert its neuroprotective effect through inhibition of the apoptotic cascade, reduction of glutamate toxicity, and/or enhancing the expression of BDNF.

In contrast, latanoprost treatment exerts its neuroprotective effect by progressively lowering IOP.Surprisingly, we did not find a synergistic effect on neuroprotection when both treatments were applied simultaneously. Further experiments are warranted in order to study in more detail the mechanisms of action of brimonidine on RGCs. Elucidating the precise mechanisms of neuroprotective action of brimonidine on RGC survival, may open up novel, IOP-independent therapeutic approaches for the successful treatment of human glaucoma.

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