Summary. The aim of this study was to examine the effects of timolol in an experimental model of elevated intraocular pressure (IOP). Three episcleral veins of rats with normal IOP were cauterized. Three months later we examined the effects on anterograde axonal transport from the retinal ganglion cells (RGCs) to the superior colliculus (SC) as well as on the number of neurons in the retinal ganglion layer (RGL). These parameters were also studied in a group of rats submitted to treatment with timolol after confirming that their IOP was still raised after two weeks. After the surgical procedure, the mean IOP of the experimental eyes increased to 33.5±1.06 mmHg (1.25 fold compared to the control group) and three months later the IOP remained significantly elevated; however, after a long period of treatment with timolol the IOP was 14.05±0.81 mmHg, similar to that of the control group. In the group with normal IOP, labelling with horseradish rabbit peroxidase (HRP) at 120 minutes and 24 hours postinjection showed continuous staining from the retina to the SC. In the experimental group the optic nerve head (ONH) was completely negative, although in the group treated with timolol there was partial block of axonal transport in the ONH, in which the staining was slightly more intense. The number of neurons in the RGL, counted by immunohistochemical labelling with Neu-N, showed that in eyes with normal and elevated IOP there were 423±11 neurons/mm² and 283±10 neurons/mm², respectively. After treatment with timolol the number of neurons (331±10 cells/mm²) increased compared with elevated IOP eyes, although the number did not reach that of the control group. These results indicate that treatment with timolol, started two weeks after the surgical procedure, was partially neuroprotective because the loss of neurons in the RGL was lower than in untreated animals, though not sufficient to re-establish normal axonal transport.

Key words: Glaucoma, Intraocular pressure, Retinal ganglion layer, Anterograde axonal transport

Introduction

Glaucoma is an optic neuropathy associated with elevated intraocular pressure (IOP). Although elevated IOP is the single most common finding in patients with glaucoma, the pathophysiological mechanisms by which an elevated IOP leads to neurodegenerative events are not well understood. The characteristic pathological change in the glaucomatous retina is the loss of retinal ganglion cells (RGCs). No complete explanation exists regarding the cause of the degeneration of RGCs in primary open-angle glaucoma. Whether this selective death of retinal neurons is due to direct compression, to pressure-induced ischaemia, or to other mechanisms remains controversial. Whatever the mechanism, glaucomatous damage to RGCs appears to be secondary to the injury to their axons projecting to the brain. In experimental ocular hypertension, obstruction of axonal transport is seen at the level of the lamina cribrosa (Anderson and Hendrickson, 1974; Quigley and Anderson, 1976; Gaasterland et al., 1978; Hayreh et al., 1979), reducing the delivery of neurotrophic factors necessary for RGCs survival (Lindsay et al., 1994).

The current treatment of glaucoma is based on lowering the elevated IOP to slow or stop the progressive loss of the visual field as a result of optic nerve degeneration and the subsequent loss of RGCs. The β₁-adrenoreceptor antagonists, which comprise selective β₁ (e.g. betaxolol) and non-selective β₁/β₂ (e.g. timolol, carteolol), are some of the most important classes of drugs used clinically to lower raised IOP in glaucoma patients (Hoyng and van Beek, 2000). In clinical studies, timolol has been shown to have preventative actions against visual field-loss progression,
optic-disc cupping, and decreased retinal thickness (Brooks and Gilles, 1992; Zimmerman 1993; Hoyng and van Beek, 2000). Nevertheless, despite treatment with IOP-lowering agents such as timolol, RGCs death continues in many patients (Wood et al., 2003). Timolol exerts a neuroprotective effect against neuronal damage in RGCs by its calcium and sodium channel blocking activities (Wood et al., 2003, Osborne et al., 2004).

The purpose of this study was to investigate the possible protective effects of timolol on neurons in the RGL and its action on axonal transport in an experimental model of elevated IOP in rats. We chose the rat because this species is a useful model for studying this optic neuropathy (Shareef et al., 1995) for the eventual purpose of screening promising pharmacologic compounds.

Material and methods

Subjects

Twenty-two adult, male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 250-300 g at the beginning of the experiment were used. The rats were divided into three groups: control (n=6), experimental (n=8) and treated with timolol (n=8). They were housed in individual home cages in an air-conditioned room (21±1°C with 66±3% humidity) with a 12-hour light-dark diurnal cycle. They had free access to food (dry pellets) and tap water. To minimize animal suffering and the number of animals used, the experiments were carried out in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals, and were approved by the Scientific Committee of Malaga University.

Surgical procedure

One group of rats was not submitted to the surgical procedure and was used as a control group. The remainder of the animals underwent unilateral ocular surgical manipulation, with the contralateral eye remaining untouched. These rats were deeply anaesthetized by intraperitoneal injection of 8% chloral hydrate (0.1 ml per 30 g body weight). Right eye limbus-draining veins were exposed by incising the conjunctiva and three of the four veins were cauterized using a small vessel cautzerizer (Ophthalmic Cautery-Cautere, Moria, Antony, France) (García-Valenzuela et al., 1995; Shareef et al., 1995; Laquis et al., 1998). After surgery, the eyes were treated topically with an antibiotic (Tobrex®, Alcon Cusí S.A., Barcelona, España) during recovery. The rats were then divided into two groups, an untreated group and a group treated with timolol (Timotol®, MSD de España S.A.) and were caged individually. Timolol treatment was started two weeks after inducing elevated IOP. We considered this period sufficient to simulate in our experimental animals the conditions usually found in the human eye, because an elevated IOP is not usually diagnosed until it has been present for some time. After determining that the IOP remained high during this period, we began to instil in the operated eye two drops per day of timolol for three months. All ocular tissues, including the cornea, lens and sclera appeared normal throughout the experiment.

Measurement of intraocular pressure

The IOP of both eyes was measured using a calibrated Tono-Pen XL tonometer (Mentor Ophthalmics, Inc., Norwell, MA, USA) before and immediately after catherization and every two weeks for the following three months, as well as immediately before perfusion. A drop of topical anaesthetic (proparacaine hydrochloride; Alcon Inc., Mississauga ON, Canada) was instilled and, with the eye under good illumination, the Tono-Pen was oriented perpendicular to the cornea and, using a swift and steady stroke, the tip was brought into contact with the cornea (Moore et al., 1993). Each IOP registered was an average of three consecutive measurements made at the same time of day (10am-12pm), as well as immediately just before killing by perfusion.

Tracer injection

Animals were anaesthetized by chloral hydrate 8% and a small incision was made through the sclera to the vitreous humour. Injections of the anterograde axonal tracer wheat germ agglutinin-horseradish peroxidase (1.5 µl of 5% WGA-HPA saline; HRP type VI in fast green, Sigma Chemical, St. Louis, MO, USA) were made into the right eye using a Hamilton syringe with a 0.3 mm needle. The procedure was based on the introduction of the Hamilton needle through the sclera at the level of the pars plana towards the vitreous cavity, under control of a surgical microscope. After confirming the correct position of the needle, we injected the peroxidase until we saw the enzyme dissolve in the vitreous cavity, after which we immediately withdrew the needle. No loss of vitreum was observed due to perforation of the sclera.

At 120 minutes and 24 hours after tracer injection, animals (control, experimental and timolol-treated) were deeply anaesthetized as described above and perfused through the heart with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1M phosphate buffer, followed by 500 ml of the same fixative with 0.1M phosphate buffer in 10% sucrose and finally, with 0.1M phosphate buffer in 10% sucrose. The whole brain and eyeballs and optic nerve were dissected and cryopreserved in 0.1M phosphate buffer pH 7.4 containing 30% sucrose at 4°C. Serial 20 µm-thick sagittal sections of the eyeballs and optic nerve, and serial 50 µm-thick coronal sections of the lateral geniculate nucleus (LGN) and superior colliculus (SC) were cut in a cryostat. Slide-mounted sections were reacted with a sodium nitroprusiate solution in acetate buffer pH 3.3, with 3,3’-5,5’-tetramethylbenzidine dihydrochloride and 0.03% H₂O₂.
(modified standard procedure of Mesulam, 1978) and examined with a Leitz microscope.

**Immunohistochemical procedure**

Under deep anaesthesia, as described above, we performed perfusion through the heart in control, experimental and timolol-treated animals with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, the eyeballs were removed and postfixed in the same fixative for four hours, transferred into ethanol 70°, embedded in paraffin and cut in 6 µm-thick sagittal sections. The sections were mounted onto pre-treated glass slides. They were deparaffinized in xylene and rehydrated with distilled water through the conventional ethanol scale, preincubated in citrate buffer (pH 6.0) in a pressure cooker and treated with 0.06% H₂O₂ for 15 min. The sections were then incubated overnight with the primary monoclonal neuronal nuclei (NeuN) antibody (MAB377, Chemicon, USA; dilution 1:500). The slides were rinsed in phosphate buffer, incubated with biotinylated anti-mouse IgG (1:200) for one hour and treated with the avidin-biotin peroxidase complex (Vectastain-ABC Kit, Vector Lab Burlingam, CA, USA) for 60 minutes and 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO, USA) as the peroxidase substrate for five minutes. Finally, the slides were counterstained with haematoxylin, dehydrated and coverslipped with Entellan.

**Number of neurons in the retinal ganglion layer**

Sagittal sections of eye cups through the optic disc were prepared at 6 µm-thick, and used for the immunohistochemical study. The number of cells in the RGL was measured to evaluate retinal damage. For this we used an Analysis Image System (Visilog, Noesis, France). The equipment included a microscope (Eclipse E400, Nikon, Tokyo, Japan) with a 20x objective lens, a digital colour camera (Polaroid Corp, Waltham, MA), and an image-processing and analysis software (version 5.2, Visilog).

We measured the number of neurons in the RGL at 200x magnification in six fields in each eye at a total distance of ~1.2 mm either side of the centre of the optic nerve, the regional area which is more susceptible to glaucomatous damage. The values used were the average of six measurements in each eye (Goto et al., 2002) and expressed as NeuN immunoreactive neurons per mm² of retina.

**Statistical analysis**

The quantitative values obtained for each animal were used to calculate the mean and the standard error of the mean (SEM). The statistical analysis was performed using the commercially available software SPSS 10-0. Comparison between groups was made using either analysis of variance (ANOVA) or the non-parametric Tukey test. Where significant differences were found, a multiple comparison test was carried out. Differences were considered to be statistically significant at p<0.05.

**Results**

**Intraocular pressure**

The average IOP in the control eyes was 14.85±0.65 mmHg (Figs. 1, 5A). Immediately after the surgical procedure, the mean IOP in the experimental eyes increased to 33.5±1.06 mmHg (p<0.001). Measurements of the IOP each two weeks for the following three months showed that the IOP remained significantly raised for the entire duration of the experiment (p<0.001) (Figs. 1, 5A). The IOP in the treated group was elevated immediately after surgery (32.27±0.98 mmHg) and remained so two weeks later (34.91±1.12 mmHg) (Fig. 1). After starting treatment the IOP fell to normal values (Fig. 1, 5A), with a mean value at the end of the treatment phase of 14.05±0.81 mmHg.

**Tracer injection**

Intense staining of the retina, especially of the inner layers, was seen in the control group with normal IOP 120 minutes after the injection of the anterograde axonal tracer (Fig. 2A). No important differences were observed 24 hours after the injection compared with the observations at 120 minutes. In the animals submitted to elevated IOP, the retina was weakly stained 120 minutes after tracer injection (Fig. 2B). Almost no further differences were detected 24 hours later and only a very small amount of spotty but intense staining in the group...
treated with timolol (Fig. 2C).

In the control group, the ipsilateral optic nerve, in its whole extension, showed intense staining (Fig. 3A), whereas in the animals with elevated IOP, this nerve was completely negative (Fig. 3B), and timolol ONH showed fewer instances of spotty staining (Fig. 3C).

In the control group, the contralateral SC was also stained with clear delimitation of the superficial layers (Fig. 3D). In the animals submitted to elevated IOP and treated with timolol, the SC was virtually unstained even 24 hours after the intravitreous injection of HRP (Fig. 3E,F).

**Number of neurons in the retinal ganglion layer**

Neurons in the rat RGL were identified immunohistochemically by using an antiserum against neuron-specific protein NeuN, which is present in most neuronal cell types of vertebrates. Almost all ganglion cells revealed strong immunoreactivity for NeuN in nuclei, perikarya, and some proximal neuronal processes, whereas more distal axon and dendritic ramifications were not stained (Fig. 4).

The number of Neu-N positive neurons in normal and elevated IOP eyes was 423±11 cells/mm² and 283±10 cells/mm², respectively (Fig. 5B). Thus, the elevated IOP eyes showed a significant reduction in the number of neurons in the RGL compared with the normal eyes (p<0.001), equivalent to a 33% loss of cells. After treatment with timolol, an increased number of neurons (331±10 cells/mm²) was found compared with the number in untreated elevated IOP eyes (p<0.001) (Fig. 5B), equivalent to a 17% increase compared with the untreated elevated IOP eyes. However, the mean number of neurons in the RGL in timolol-treated eyes failed to reach the same density as in normal eyes, with a
21.74% loss compared with normal eyes (p<0.001).

Discussion

Glaucoma is a slowly progressive neuropathy, whose main risk factor is increased IOP. Glaucoma is the second leading cause of preventable blindness worldwide (Goto et al., 2002). The end result of this neuropathy is death of RGCs (Levin, 1997) associated with changes in the ONH, visual field and visual acuity.

Animal models of elevated IOP have been developed, although these were initially restricted to the rabbit and monkey. However, the anatomy of the rabbit aqueous outflow system is very different to that of the primate, and availability of the monkey in sizeable numbers for research is impractical (Cabrera et al., 1999), thereby limiting their usefulness for the detailed study of chronic glaucomatous damage to the optic nerve. Up until the early 1990s the rat proved impractical for the study of IOP because of limitations in measuring methods. However, the Tono-Pen, a tonometer widely used in clinical practice, has been used successfully to make non-invasive IOP measurements in anaesthetized rats.

The surgical procedure used in this study, which followed the protocol of Shareef et al. (1995) with cauterization of the three episcleral veins, prevents normal outflow of the aqueous humour and produces a constant, prolonged increase in the IOP. The IOP remains consistently elevated in cauterized eyes for at least three months, and results in cupping of the optic nerve and loss of RGCs (Naskar et al., 2002).

Fig. 4. Representative photomicrographs of NeuN-stained retinal sections obtained from normal (A), elevated intraocular pressure (B) and timolol-treated eyes (C). The monoclonal antibody NeuN labelled almost all the neurons in the GCL and some neurons in the INL. OS: outer segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGL: retinal ganglion layer. Scale bars: 25 µm.

Fig. 5. Graphs showing the mean±SEM corresponding to the intraocular pressure (A) and number of neurons/mm² in the retinal ganglion layer (B). A. An important increase is seen in the IOP in the group submitted to the surgical procedure. However, the timolol-treated animals recovered normal IOP values. B. Timolol treatment prevents loss of neurons in the retinal ganglion layer in albino rat eyes with elevated IOP. When no drug was administered there was an important loss of neurons in the retinal ganglion layer over a 3-month period in eyes with elevated IOP. *p<0.001, experimental group compared to control group; **p<0.001, treated group compared to control group, and *** p<0.001, treated group compared to experimental group.
Treatment of glaucoma involves the use of topically applied pharmacological agents which are known to reduce IOP (Hoyng and van Beek, 2000) by reducing the production of aqueous humour and/or by increasing outflow from the eye (Sugrue, 1997). One class of compound used in this treatment includes the β-adrenoceptor antagonists, which are either selective for the β₁-receptor or are non-selective (Brooks and Gillies, 1992; Zimmerman, 1993). Timolol, used in our study, is a non-selective β₁/β₂ adrenoceptor antagonist and one of the most important drugs used in clinical practice to lower elevated IOP in glaucoma patients (Zimmerman, 1993, Osborne et al., 1999a; Hoyng and van Beek, 2000, Wood et al., 2003).

The elevation of IOP that we obtained in our experimental model of glaucoma (1.25 fold compared with the control group) is consistent, though lower than the values obtained at Sharma’s laboratory of 1.5-1.8 fold (García-Valenzuela et al., 1995; Shareef et al., 1995; Laquis et al., 1998; Naskar et al., 2000) applying the same methods that we used, and the values from Morrison’s laboratory of 1.4-2.6 fold (Johnson et al., 1996; Morrison et al., 1997), using the injection of hypertonic saline into limbal vessels of brown Norway rats. These differences in the IOP could be due to the different methods used to raise the pressure or to the different measuring instruments used. The IOP fell after treatment with timolol and returned to normal values.

Our interest in the experimental model developed in this study was to determine whether the elevated IOP might influence the axonal transport in RGCs, whose axons comprise nerve fibers, especially in the regional area where they transverse the optic nerve head and exit from the eye. This transport moves organelles and membrane-bound vesicles from the cell body towards the nerve terminal by an active process involving kinesin hydrolysis of ATP. Endothelin-1 (ET-1), a neuroactive and vasoactive peptide present in the normal eye (MacCumber et al., 1991), has been attributed with a general role as an effector molecule able to locally modulate anterograde fast axonal transport (Stokely et al., 2005). Furthermore, experimental studies have shown that axonal transport is blocked by anoxia (Rogers, 1964; Byers et al., 1973) and by inhibitors of phosphorylation and/or of glycolysis (Rogers, 1964). In glaucoma, movement of selected components of anterograde axonal transport, specifically, the transport of mitochondria, is seriously compromised as axons traverse the peripapillary region of the ONH (Holland et al., 1995).

In this study the intravitreal injection of HRP in rats with a normal IOP showed that the anterograde tracer reached all the components of the optic pathway after 120 minutes and 24 hours, with an intense reaction in the inner layers of the retina, in all the regions of the ipsilateral ONH as well as in the contralateral SC. These observations agree with those of Wakakura and Uga (1987), who saw staining of ganglion cells, Müller cells and horizontal cells at just one hour and, over longer times, in the optic nerve. However, in the experimental group the axonal transport appeared blocked by elevation of the IOP. Thus, 120 minutes after the intravitreal injection of HRP the only structures which stained were the retina, and only lightly. This interruption was determined because the contralateral SC was not labelled 24 hours after the tracer injection. This blockage of axonal transport in the ONH could be due to a mechanical compression of the axonal bundles during IOP elevation. In an experimental glaucoma model similar to ours, at three months postoperation, many axons and axon terminals in the retina, especially in the ganglion cell layer and inner plexiform layer, were found to be abnormal (Wang et al., 2002).

In the group treated with timolol, a slightly more intense staining of the retina was visible at 120 minutes, especially of the ganglion layer, compared to animals with elevated IOP. This difference is compatible with the increase in the number of neurons in the RGL expressing NeuN and, possibly with an increase in the number of ganglion cells which stained with HRP. Nevertheless, this transport was partly blocked in the ONH and had not reached the SC 24 hours after the intravitreal injection of HRP. Whether this blockade is due to a partial effect on all axons or to a total effect on some axons and no effect on others is unknown. Different studies have shown that the ET-1 is elevated in aqueous humour of primary open-angle glaucoma patients (Noske et al., 1997; Tezel et al., 1997), and in aqueous humour and ONH in a rat model of elevated IOP (Prasanna et al., 2005), and that this increase can produce an extended period of aberrant anterograde axonal transport within the optic nerve (Stokely et al., 2002).

Regarding our model of elevated IOP and topical treatment with timolol, our study confirms the effectiveness of this pressure-lowering drug, although it had no significant effect on axonal transport under the experimental conditions used. Recently, some reports have suggested that topically instilled ocular pressure-lowering drugs can attenuate the ET-1 induced constriction of retinal arteries by local penetration (Ishii et al., 2003); however, timolol had no significant effect (Okada et al., 2004).

To assess the vulnerability of neurons in the RGL to the elevation of the IOP in our experimental model and the potentially beneficial effect of the use of β-adrenoceptor antagonists, an automated quantitative method was employed. Neurons in the RGL were labelled with a monoclonal antibody against the nuclear protein NeuN (Wolf et al., 1996) and a count was made of these cells on either side of the centre of the optic nerve. The results of our study using NeuN indicate a significant loss of these cells three months after surgery (33% lower compared to the control group). This loss is a feature of a number of diseases which decrease visual function, including primary open-angle glaucoma (Nickells, 1996; Quigley, 1999) and anterior ischaemic optic neuropathy (Anderson and Quigley, 1992).

Although accumulated evidence suggests that RGCs die by apoptosis in open-angle glaucoma and in experimental animal models of optic nerve damage...
(García-Valenzuela et al., 1995; Kerrigan et al., 1997; Nickells, 1999), the exact mechanisms leading to neuronal loss have not been resolved. Intraocular pressure and a variety of additional factors, including oxidative stress, intracellular ion and electrolyte disturbances, and increased extracellular glutamate levels, together with excessive stimulation of excitatory amino acid receptors (excitotoxicity), and pathologic increases in intracellular calcium ([Ca^{2+}]_i) ion concentrations have all been implicated in RGCs loss (Nickells, 1996; Osborne et al., 1999b).

The study by García-Valenzuela et al. (1995) of retinas flatmounted on a glass slide where the RGCs were stained with Fast-blue showed that the reduction in the number of these cells in retinas maintained at 44.8 mmHg, as compared to control retina, was proportional to the increase in IOP. Other reports have also noted a loss of RGCs in rats with experimental glaucoma (Laquis et al., 1998; Wang et al., 2000; Bakalash et al., 2002; Fortune et al., 2004).

The exact cause and mechanisms involved in ganglion cell death in glaucoma remain a matter of speculation. In addition to its vasodilatory actions for lowering IOP, timolol, like other ß-adrenoceptor antagonists, has been proposed as a neuroprotective agent against ganglion cell death in glaucoma, mainly because it blocks calcium and sodium channels. Timolol may exert its neuroprotective action by suppression of a glutamate-induced intracellular calcium increase in retinal ganglion cells (Zhang et al., 2003).

Timolol treatment in our study resulted in an attenuation of the detrimental effects in neurons in the RGL (17% better compared to the untreated elevated IOP group), although the number did not reach that of the control group. Thus, treatment with timolol started two weeks after IOP elevation was partially neuroprotective, perhaps because the events associated with cell degeneration had begun before starting treatment. In fact, ultrastructurally, neuronal degeneration is discernible in all different layers of the retina at both one and three weeks post-operation, as well as many degenerating axons and axon terminals between neurons (Wang et al., 2002). There is evidence that ganglion cell death in glaucoma is due to an initial ischaemia-like insult to the cell axon and a later insult to the ganglion cell body caused by elevated extracellular glutamate (Zhang et al., 2003; Osborne et al., 2004). A sustained stimulation of glutamate receptors in ganglion cells will lead to an uncontrolled rise in [Na^+]_i and [Ca^{2+}]_i, which will trigger a cascade of events leading to cell death.

Goto et al. (2002) demonstrated that timolol had a direct neuroprotective effect in experimental models of retinal injury. First, in the in vitro study, timolol showed its protective effects against glutamate-induced neurotoxicity in both primary retinal cell and RGCs cultures, suggesting that it has direct actions on neuronal cells. Second, in the in vivo study, topically applied timolol showed its neuroprotective effects on ischaemia-induced retinal damage in rats, suggesting that sufficient timolol had reached the retina. We verified that treatment with timolol reduces the expression of the constitutive and inducible isoforms of the nitric oxide synthase enzyme in the ONH, which suggests that timolol could exert a neuroprotective effect on the RGCs (unpublished data).

In summary, our study shows that the surgical procedure used causes a consistent elevation of IOP, with an important loss of neurons in the RGL and partial blockade of axonal transport. Treatment with the ß-blocker timolol was partially neuroprotective, because the loss of neurons in the RGL was lower in treated than in untreated animals, though normal axonal transport was not restored.

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