Experimental Model of Ocular Hypertension in the Rat: Study of the Optic Nerve Capillaries and Action of Hypotensive Drugs

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PURPOSE. To investigate quantitatively the effect of elevated intraocular pressure (IOP) on the microvasculature of the optic nerve with and without topical treatment with two hypotensive drugs, timolol and latanoprost.

METHODS. Three groups of rats underwent cauterization of three episcleral veins to produce elevated IOP in the right eye. Two of these groups were treated with timolol or latanoprost for 3 months. Eyeballs were incubated with anti–GLUT-1 polyclonal antibody. GLUT-1-positive capillaries of the optic nerve head (ONH) and optic nerve exit (ON) were examined and analyzed for their number per square millimeter, volume fraction, length per unit volume, surface area per unit volume, and mean diameter.

RESULTS. An increase in IOP resulted in a significant decrease in microvesSEL density in the laminar region (LR) and postlaminar region (PR) and ON compared with the control group. The other parameters fell significantly in all regions of the optic nerve. Topical treatment with timolol or latanoprost did not modify the density of the capillaries, although the other parameters increased significantly compared with the untreated experimental group. Additionally, the mean diameter of the capillaries in the LR and the PR recovered after treatment.

Conclusions. The results indicate that the capillaries of the LR and the PR of the ONH are the most susceptible to IOP elevation. The authors suggest that timolol and latanoprost have a certain vascular action by increasing the available blood volume, surface area per unit volume, length per unit volume, and diameter of the capillaries of the ONH in these two regions. (Invest Ophthalmol Vis Sci. 2010;51:946–951) DOI: 10.1167/iovs.09-3667

Glaucoma is an optic neuropathy characterized by progressive loss of the visual field as a result of optic degeneration and subsequent loss of retinal ganglion cells (RGCs). Although elevated intraocular pressure (IOP) is considered the main risk factor in patients with glaucoma, this alone cannot explain all the clinical and experimental observations. Two theories, not mutually exclusive—alteration in ocular blood flow and mechanical compression of the axons of the RGCs at the level of the lamina cribrosa—have been postulated to explain the mechanisms that lead to the neurodegenerative lesions.1–3

The β-adrenoceptor antagonist timolol and the prostaglandin F2α analog latanoprost are two of the most important drugs used clinically to lower IOP in patients with glaucoma.4 Betaxolol, levobunolol, and timolol all increase blood velocities in retinal and epipapillary capillaries, but their ocular hemodynamic effects are not well known.5 It has been suggested that they might increase the perfusion of the optic nerve head (ONH) by an indirect hemodynamic mechanism.6 Other studies have shown that treatment with latanoprost increases the blood flow at the ONH7 and has neuroprotective effects that may contribute to its efficacy in glaucoma therapy.8

Several different markers have been used to study the characteristics of the blood-brain barrier (BBB), including those of the glucose transporters gene family (GLUT). The brain GLUTs are divided into class 1 (GLUTs 1–4), class 2 (GLUT-5), and class 3 (GLUT 6–8 and 10).9,10 The GLUT-1 isoform can be used as an immunohistochemical marker11 because of its presence on the endothelial cell membrane (55-kDa isosform),11 and the astrocyte feet surrounding the capillaries and astrocyte cell bodies (45-kDa isoform).10,12

The purpose of this study was to use an experimental rat model of elevated IOP to examine the changes in the microvessels of the optic nerve after elevation of the IOP. GLUT-1 (45-kDa isoform) was used as an immunohistochemical marker of the capillaries. After evaluating stereologic microvascular parameters in the ONH and ON in this model of elevated IOP, we undertook a comparative examination of the microvascular network of the optic nerve of rats treated topically for 3 months with 1 of 2 hypotensive drugs, timolol or latanoprost.

METHODS

Animals

We used male, adult Wistar rats (Charles Rivers Laboratories, Barcelona, Spain), each weighing 250 to 300 g at the start of the study. The rats were divided into four groups: control (n = 6), experimental (n = 8), treated with timolol (n = 8) or treated with latanoprost (n = 6). They were housed in individual home cages in an air-conditioned room (21 ± 1°C with 66 ± 3% humidity) with a 12-hour light/12-hour 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 in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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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 anesthetized by intraperitoneal injection of 8% chloral hydrate (0.1 mL/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 cautizer (Ophthalmic Cautery-Cautere, Moria, Antony, France). After surgery, the eyes were treated topically with an antibiotic (Tobrex; Alcon Cusí S.A., Barcelona, Spain) and were caged individually. Timolol and latanoprost were instilled. With the eye under good illumination, the eye limbus-draining veins were exposed by incising the conjunctiva, and three of the four veins were cauterized using a small vessel cautizer (Ophthalmic Cautery-Cautere, Moria, Antony, France).13–15

IOP Measurements

The IOP of each eye was measured using a calibrated tonometer (Tono-Pen XL; Mentor Ophthalmics, Inc., Norwell, MA) before and immediately after cauterization and every 2 weeks for the following 3 months as well as immediately before perfusion. One drop of topical anesthetic (proparacaine hydrochloride; Alcon Inc., Mississauga ON, Canada) was instilled. With the eye under good illumination, the tonometer (Tono-Pen XL; Mentor Ophthalmics, Inc.) was oriented perpendicularly to the cornea and, using a swift and steady stroke, the tip was brought into contact with the cornea.16 Each IOP registered was an average of three consecutive measurements made at the same time of day to correct for diurnal variations in IOP (10 am-12 pm) and immediately before killing by perfusion. Mean IOP in the control eyes was 14.85 ± 0.65 mm Hg. Immediately after the surgical procedure, the mean IOP increased to 33.5 ± 1.06 mm Hg and remained significantly elevated at 15 days and for the entire length of the experiment (Fig. 1). In the groups of rats treated with timolol or latanoprost, IOP at the start of the treatment was similar to that of the experimental group. Measurements of the IOP taken every 2 weeks for 3 months showed a decrease to normal values, with mean values of 14.05 ± 0.81 and 14.11 ± 0.72 mm Hg, respectively, at 3 months (Fig. 1).

Immunohistochemical Procedure

Under deep anesthesia, as described, we performed perfusion through the heart in control, experimental, timolol-, and latanoprost-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 4 hours, transferred to ethanol 70°, embedded in paraffin, and cut into 6-μm-thick sagittal sections. The sections were mounted onto pretreated 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 minutes. The sections were then incubated with the primary antibody overnight at 4°C. We used rabbit polyclonal antibody anti-GLUT-1 (AB 1340; Chemicon International, Temecula, CA; dilution 1:1000) as the primary antibody. Slides were rinsed in phosphate buffer, incubated with biotinylated goat anti-rabbit (Dako A/S, Denmark) for 1 hour (dilution 1:600), and treated with the avidin-biotin peroxidase complex (Vectorstain-ABC Kit; Vector Laboratory Inc., Burlingame, CA) for 60 minutes and 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) as the peroxidase substrate for 5 minutes. Finally, the slides were lightly counterstained with hematoxylin, dehydrated, mounted with mounting medium (Entellan; Merck, Whitehouse Station, NJ), and examined under a microscope (H550L; Nikon, Tokyo, Japan).

Also included as a negative control was one section from each animal processed according to the same protocol but with omission of the primary antibody.

Morphometric Procedure

To ensure a representative sample of the capillaries, we examined and counted at least three representative sections of optic nerve per animal per group. We used the regional classification system reported by Morrison et al.,17 which distinguishes three regions in the ONH, the prelaminar region (PLR), laminar region (LR), and postlaminar region (PR). The PLR is at the level of the sclera. The LR is an expanded zone below the PLR, whose posterior limit marks the beginning of the PR intraorbital optic nerve, in which most of the nerve fibers are fully myelinated9 (Fig. 2).

With the use of a Nikon light microscope with a color videocamera (CM 715; Hitachi, San Jose, CA) connected to a computer (Visilog...
Image Analysis; Noesi S.A., Courtadoeus, France), all GLUT-1–positive cross-sectioned microvessel profiles in each microscopic field were drawn on an area of reference of 49,152 \( \mu m^2 \) (two fields per section in the PLR and PR regions, four in the LR, and three in the ON; Fig. 2). Vessels with a cross-section diameter of 3 to 10 \( \mu m \) were considered to be capillaries, and vessels with larger diameters were excluded. Vessels that had been cut longitudinally or tangentially were also excluded. In the different regions of the ONH and the ON, we analyzed the mean capillary diameter \( d \) and the number of capillaries within the measured tissue area (49,152 \( \mu m^2 \)). From these measurements, we calculated the capillary profile density, expressed as the number of capillaries per square millimeter of tissue; the volume fraction (Vv); the ratio between the total area occupied by capillaries and the reference area in percentage; the length per unit volume of capillaries, according to the volume fraction and the capillary diameter \( Lv = Vv/\pi (d/2)^2 \times 10^3 \) and the surface area per unit volume of capillaries \( Sv = \pi (d)(Lv) \times 10^{-3} \).

**Statistical Analysis**

Quantitative values obtained for each parameter were used to calculate mean values, expressed as the mean \( \pm \) SEM. Statistical analysis was performed using SPSS 13.0. Comparison between groups was made using the nonparametric Kruskal-Wallis test. Significant post hoc comparisons are shown after performing Bonferroni correction. Differences were considered to be statistically significant at \( P < 0.05 \).

**RESULTS**

Figures 2 and 3 show the light morphology of optic nerve microvessels in the ONH and the ON, stained intensely for GLUT-1.

**Capillary Density**

The GLUT-1–positive capillary density (number of capillaries/mm\(^2\)) for each group is shown in Figure 4A. The capillary density in the PLR was less than in the other regions of the optic nerve. No changes were found in this parameter in the PLR in the experimental group compared with the control group. Densities in the LR, PR, and ON were 39%, 41.5%, and 24.7%, respectively, less than in the control group \( (P < 0.05) \). Treatment with timolol or latanoprost failed to modify the values compared with the experimental group (Fig. 4A).

**Capillary Volume Fraction**

In the control group, the Vv in the various regions studied accounted for 0.16% in the PLR, 0.23% in the LR, 0.41% in the PR, and 0.24% in the ON of the tissue volume. Chronic elevation of the IOP resulted in a significant reduction of the Vv in all the regions, 52% in the PLR, 78% in the LR, 88% in the PR, and 65% in the ON compared with the control group \( (P < 0.05) \). Treatment with timolol or latanoprost failed to modify the values compared with the experimental group (Fig. 4B).

**Length of Capillary per Unit Volume**

The Lv fell significantly in the experimental group in all the regions studied: 73.5% in the PLR, 90% in the LR, 93.5% in the PR, and 76% in the ON compared with the control group \( (P < 0.05) \). Treatment with timolol or latanoprost produced a significant increase in this parameter in the LR, in the PR, and in the ON compared with the experimental group \( (P < 0.05) \). However, the differences in the LR and the ON were not significant when compared with the control group (Fig. 4C).

**Surface Area of Capillary per Unit Volume**

The Sv fell significantly after chronic elevation of the IOP in all the regions studied, especially in the PR (96%). Although topical treatment with timolol or latanoprost returned the Sv values to those of the control group in the initial part of the optic nerve, it was onefold to twofold less in all the regions of the ONH after the two drugs (Fig. 4D).

**Mean Capillary Diameter**

The capillaries with the largest diameters were found in the PLR, in both the control group (8.09 \( \pm \) 0.2 \( \mu m \)) and the
effects of IOP on optic nerve fibers are generally assumed to manifest itself mainly in the lamina cribrosa, which could explain the significant reduction in capillary density noted in this region. A previous study in humans with primary open-angle glaucoma showed that capillary density in the PR of the optic nerve also decreases in parallel with axon loss and with increased connective tissue thickness in the septa. Our findings in the same region in rodents show similarities with the results in humans, with a significant decrease in capillaries per square millimeter (41.5%) in the experimental group compared with the control group.

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The Vv, a parameter useful for estimating the blood volume available in the capillary bed, decreased significantly in all the regions of the ONH in the experimental group compared with the control group (52% in the PLR, 78% in the LR, and 88% in the PR), which suggests there is less blood supply for these regions. This reduction probably also correlates with the changes observed in the mean capillary diameter and the total length of the capillaries.

The total length of the capillaries was significantly reduced per unit volume of tissue in the experimental group compared with the control group in all the regions studied, especially in the LR and the PR (90% and 93.5%, respectively). These data can be considered to confirm that the increase in the IOP compresses the capillaries of the ONH, as shown ultrastructurally. Additionally, the Sv, which represents the capillary surface area available for metabolic exchange, also fell significantly in all regions in this same group, again markedly in the LR and the PR (93% and 96%, respectively). This may reflect changes in the permeability in the capillaries of these regions, failing to ensure an adequate supply of nutrients after IOP elevation. Of note, too, is the fact that progressive glaucoma is associated with decreased blood flow velocities in the small retrobulbar vessels supplying the ONH.

Results concerning the mean capillary diameter in the control group showed values of 8 μm in the PLR and slightly lower values in the other regions (7.1 μm in the LR, 6.9 μm in the PLR, and 6.1 μm in the initial part of the ON). Mean capillary size in the experimental group fell significantly in the different regions of the ONH (28% in the PLR; 34% in the LR, and 36% in the PR), which corroborates the occlusion of the capillary wall as a consequence of IOP elevation. These changes could be related to the mechanism of self-regulation of the blood flow of the ONH in response to an increase in the IOP, such that when the ONH is compressed by elevated IOP, a reduction in blood flow is produced, causing the vessels to undergo vasoconstriction.

To determine the effects on the capillaries of the optic nerve of two hypotensive drugs, timolol and latanoprost, in eyes submitted to elevated IOP, we analyzed all these parameters after 3 months of treatment. Timolol, a nonselective β-adrenoreceptor antagonist, lowers IOP by decreasing the formation of aqueous humor in the ciliary epithelium. It has a hypotensive effect in the rat, decreasing [Ca2+] by acting as a Ca2+ channel blocker, and a neuroprotective effect on the RGCs and other neurons of the retina. Applied topically, timolol reaches the retina-choroid at concentrations within the effective pharmacologic range. The other drug used, a prostaglandin F2α analog, latanoprost, exerts its hypotensive action by increasing uveoscleral outflow, although the exact mechanism of action is not known. Its hypotensive effect has also been shown, as has its neuroprotective effect in the rat.

Our data show that timolol and latanoprost do not change the GLUT1-positive capillary density in the different regions of the optic nerve. However, both drugs cause a significant increase in Vv, especially in the PR (approximately 73%), without reestablishing the values to those of the control group. The Lv also rises in the LR, the PR, and the initial part of the ON, ranging from 78% to 87% depending on the exact region. Notably, treatment with latanoprost reestablished the Lv in the LR and the ON to levels of the control group.

Treatment with the drugs produced no changes in Sv in the PLR compared with the experimental group. In the three regions, however, there was a significant increase (85%–90%) in the exchange surface, although without reaching the control group values. Tamaki et al. showed in the human ONH and retinal circulation that blood velocity is increased, at least temporarily, with a single instillation of latanoprost.
a comparative study with timolol and latanoprost in patients with glaucoma found no substantial hemodynamic changes in the retrobulbar vessels.45

Finally, our data on the mean capillary diameter show an increase in the mean size of the LR and the PR after treatment with the drugs, which we attribute to their possible vasodilator action.

In summary, this is the first study in rodents to analyze quantitatively the effects of experimental elevation of the IOP on the capillaries of the ONH and the initial part of the ON. The results indicate that the ONH capillaries are susceptible to IOP elevation. We also suggest that the hypotensive effects of timolol and latanoprost are associated with a certain degree of vascular action by increasing the blood volume available in the capillary bed, the capillary surface area available for metabolic exchange, the length of the capillaries per unit volume of tissue, and the capillary diameter, especially in the LR and the PR.

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References


