Nitric oxide synthase in retina and optic nerve head of rat with increased intraocular pressure and effect of timolol

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Abstract

We investigated the expression of nitric oxide synthase (NOS) isoforms -1, -2 and -3 in the retina and optic nerve head (ONH) in an experimental rat model of elevated intraocular pressure (IOP) before and after treatment with timolol, to assess whether its neuroprotective action is associated with the activity of these enzymes. Episcleral vein cauterization in unilateral eyes of Wistar rats was performed to produce elevated IOP. Histological sections of retina and ONH from animals with normal IOP, with elevated IOP, and elevated IOP treated with timolol, were studied by immunohistochemistry with antibodies to NOS-1, NOS-2, and NOS-3. In the control rats, NOS-1 was localized to photoreceptor inner segments, amacrine cells and bipolar cells in the retina, and in astrocytes, pericytes and vascular nitrergic terminals in the ONH. NOS-3 immunostaining localized to the endothelial cells. The rats with elevated IOP showed increased expression of NOS-1 in the plexiform layers of the retina and reactive astrocytes in the ONH. These cells also showed NOS-2 positivity. The rats treated with timolol showed reduced expression of NOS-1 in the retina and ONH. NOS-2 was only detected in a few groups of astrocytes in the ONH. NOS-3 was unchanged in both elevated IOP and timolol-treated groups. These results show that excessive levels of NO synthesized by the NOS-1 and -2 isoforms, considered neurotoxic, might contribute to the progressive lesions of retinal ganglion cell axons. Their reduction after treatment suggests a possible neuroprotective effect of timolol in neurons exposed to excessive amounts of NO.

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1. Introduction

Glaucoma is a slowly progressive optic neuropathy that causes loss of retinal ganglion cells (RGC), changes in the optic nerve head (ONH) and a characteristic visual field defect. Elevated intraocular pressure (IOP) is considered to be the main risk factor for the development of the changes characteristic of glaucomatous optic neuropathy in the retina and in the ONH. The mechanisms that lead to the neurodegenerative lesions are unclear [4,36,37]. The most accepted possibilities are mechanical compression of the axons of the RGC at the level of the cribiform lamina [49] and alterations in vascular perfusion causing ischemia in the ONH [43].

Several changes might have a neurodegenerative effect in glaucoma, including molecular changes in the components of the extracellular matrix of the cribiform plates [22,26], apoptosis of the RGC due to lack of neurotrophic factors [16,27] and excitotoxicity from an increased glutamate concentration [12]. Likewise, astrocytes, the main cell type in the ONH [2], have been suggested as possible initiating agents of the lesions associated with glaucomatous optic neuropathy [58]. These cells are vital for maintenance of the normal physiology of the RGC. They are activated in response to mechanical and ischemic factors [13,58] and in the initial stages of their activation they may have a direct toxic effect on the axons of the RGC, producing elevated levels of nitric oxide (NO) [38–41].

Nitric oxide acts as a regulator of different physiological processes in numerous tissues. In the eye it is involved in the regulation of IOP. A small amount of NO production is beneficial to the retina as it can up-regulate the blood circulation and
thus facilitate the flow of metabolites [45,55]. In pathological conditions, however, such as human neurodegenerative diseases, and in animal models of neurodegeneration, NO can have a neurodestructive or a neuroprotective action, or both [44,53].

Nitric oxide is produced by the enzyme nitric oxide synthase (NOS), for which three isoforms have been identified: neuronal NOS (nNOS or NOS-1), endothelial NOS (eNOS or NOS-3) and inducible NOS (iNOS or NOS-2). The first two isoforms are constitutive and are normally expressed. They produce low levels of NO, which is thought to have homeostatic and regulatory functions. NOS-2 is not expressed under normal conditions, and it is primarily involved in inflammatory processes [9,33,62].

Several different studies in the retina of humans, rats and other mammals have shown immunoreactivity for NOS-1 in diverse types of amacrine cells [4,6,20,40], as well as in horizontal and interplexiform cells [48,61], ganglion cells [48] and bipolar cells [21,28,48]. Positivity has also been seen in the inner segments of the photoreceptors [40].

The constitutive isoforms of NOS are present under normal conditions in the ONH of humans [38] and rats [53]. Studies have shown variation in the expression of the three isoforms in primary open angle glaucoma in humans [38] and in an experimental rat model [53].

In order to examine further the cell mechanisms that contribute to glaucomatous optic neuropathy we used an experimental rat model of elevated IOP, based on the cauterization of three episcleral veins, similar to that developed by Shareef et al. [52]. We have previously shown that chronic IOP elevation causes a 33% loss of cells in the ganglion layer of the retina [11]. Submitting these rats to topical treatment with timolol, a non-selective beta-blocker, significantly reduced the IOP, as well as resulting in a lower cell loss in the ganglion cell layer, as compared with untreated animals, although partial blockade of the axoplasmic transport persisted in the ONH [11].

In this study we attempted to determine whether the neuroprotective action of timolol is associated with NOS activity. Accordingly, we examined the expression of the different isoforms of NOS in the retina and the ONH of rats submitted to experimental elevation of the IOP for 3 months and after topical treatment with timolol under the same experimental conditions.

2. Material and methods

2.1. Subjects

We used 38 adult, male Wistar rats (Charles River Laboratories, Barcelona, Spain), weighing 250–300 g at the start of the experiment. The rats were divided into three groups: control (n = 14), experimental (n = 14) and experimental treated with timolol (n = 10). They were housed in individual home cages in an air-conditioned room (21 ± 1 °C with 66 ± 3% humidity) with a 12 h 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.

2.2. Surgical procedure

All ocular surgical procedures were unilateral, with the contralateral eye remaining untouched. Rats were deeply anesthetized 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 cauterizer (Ophthalmic Cautery-Cautere, Moria, Antony, France) [16,32,52]. After ocular surgery, the eyes were treated topically with an antibiotic (Tobrex®; Alcon Cusi S.A., Barcelona, Spain) during recovery. The rats were immediately divided into two groups, an untreated group and a group treated with timolol (Timofitol®, MSD de España, S.A.), and were caged individually. In the treated group, after verifying that the IOP remained elevated for 2 weeks, treatment was started by instilling in the operated eye two drops per day (every 12 h) of timolol for 10 weeks, thus completing the period of 3 months after the surgical procedure. All ocular tissues, including the cornea, lens and sclera, appeared normal throughout the experiment.

2.3. 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 cauterization and every 2 weeks for 3 months, as well as immediately before perfusion. After instillation of a drop of topical anesthetic (proparacaine hydrochloride; Alcon Inc., Mississauga ON, Canada) 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 [35]. Each IOP registered was the average of three consecutive measurements made at the same time of day (10 a.m. to noon). The average IOP in the control eyes was 14.85 ± 0.65 mmHg. Immediately after the surgical procedure, the mean IOP in the experimental eyes increased to 33.5 ± 1.06 mmHg. The IOP remained significantly elevated for the entire duration of the experiment. Before starting treatment in the treated group, the IOP was 34.91 ± 1.12 mmHg; after starting treatment the IOP fell to normal, with a mean value at the end of the experiment phase of 14.05 ± 0.81 mmHg (Fig. 1).

2.4. Immunohistochemical procedure

Under deep anesthesia, as described above, we performed perfusion through the heart in control, experimental and timolol-treated animals with 0.1 M phosphate buffer (pH 7.4), and 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 h; transferred into ethanol 70°C and processed following the protocol for embedding in paraffin. Longitudinal sections 6 μm thick were prepared. The sections were mounted onto pre-treated glass slides. They were deparaffinized in xylene and rehydrated with distilled water through the conventional ethanol scale, reincubated 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 primary NOS-1 (Santa Cruz Biotechnology, CA, USA; dilution 1:300), NOS-2 (BD Transduction Laboratories, CA, USA; dilution 1:25), NOS-3 (Santa Cruz Biotechnology, CA, USA; dilution 1:500) and GFAP (Glial fibrillary acidic protein) (DAKO, Denmark; dilution 1:1000) antibodies. The slides were rinsed
in phosphate buffer and incubated with biotinylated anti-mouse IgG for NOS-2 (Vector Lab Inc., Burlingame, CA, USA) and goat anti-rabbit (Dako A/S, Denmark) for NOS-1, NOS-3 and GFAP for 1 h (dilution 1:600) and treated with the avidin–biotin peroxidase complex (Vectastain-Abc Kit, Vector Lab Inc., Burlingame, CA, USA) for 60 min and 3,3′-diaminobenzidine tetrahydrochloride (Sigma chemical, St. Louis, MO, USA) as the peroxidase substrate for 5 min. Finally, the sections were counterstained with hematoxylin, dehydrated, mounted with Entellan and examined with a Leitz microscope.

As a control, one section from each animal was processed with the same protocol but with the omission of the primary antibodies (NOS-1, NOS-2, NOS-3 and GFAP).

3. Results

3.1. Retina

Immunohistochemical study of the rat retina from the control group demonstrated NOS-1 positivity in the inner segments of the photoreceptors (Fig. 2A, asterisk) and in numerous soma cells in the inner nuclear layer, which may correspond to bipolar cells and horizontal cells situated in the outermost part (Fig. 2A, white arrow) and especially to amacrine cells in the innermost part (Fig. 2A, arrow). The immunoreactivity was weakest in the ganglion cell layer (GCL) and in the plexiform layers. Occasionally visible in the inner plexiform layer were isolated positive NOS-1 cells that could correspond to displaced amacrine cells (Fig. 2A, arrow). The retina also showed positivity for the NOS-3 isoform in the endothelium of the capillary vessels.

The rats with chronic elevated IOP showed immunoreactivity for NOS-1 in the same places as in the control group, although the staining was more intense in the nerve fibers of the plexiform layers, especially the inner layer (Fig. 2B). NOS-3 positivity in the vascular endothelium showed no differences with respect to the controls. No positive NOS-2 cells were visible in the retina after 3 months with elevated IOP.

In the group treated with timolol, staining for NOS-1 was very weak in all the retinal layers, although it was conserved intensely and well-defined in the cytoplasm of isolated amacrine cells in the internal part of the inner nuclear layer (Fig. 2C, arrow). Intense NOS-1 positivity was also retained in the vessel walls (Fig. 2C, white arrow).

No positive NOS-2 cells were detected in the treated rats (Fig. 2D) and immunoreactivity for NOS-3 was conserved in the endothelium of the retinal vessels.

3.2. Optic nerve head

The rat ONH shows several important anatomic similarities to the human ONH. We used the regional classification reported by Morrison [37] which distinguishes three regions in the ONH. The neck region is located at the level of the sclera; in this region there are glial columns in linear arrays of single cells that run parallel to the nerve bundles. The transition region is an expanding zone below the neck region; glial cells have an orientation
perpendicular to the nerve bundles. Scattered patches of connective tissue are seen throughout this zone that may represent a rudimentary lamina cribrosa, which are spread over a greater relative distance than in the human ONH [37,53]. In the posterior region, the axons become myelinated and glial cells form the glial columns and the glia limitans, as in the human.

In the control group, immunostaining for NOS-1 was visible in the perinuclear cytoplasm of a few astrocyte-like cells, either isolated or in small groups, situated in the three regions of the ONH, especially in the neck region (Fig. 3A). This area also showed positivity for NOS-1 in fiber-like structures arranged in a parallel fashion, which at some points showed continuity with the stained soma cells; they probably corresponded to prolongations of the astrocytes (Fig. 3A, arrow). The capillary walls in the neck region showed positivity in the pericytes (Fig. 3A, arrowhead). The artery walls showed a punctate-like pattern that was suggestive of nitrergic nerve terminals (Fig. 3B).

In the group with chronic elevated IOP, we saw NOS-1 expression in the neck and posterior regions, whereas the transition region showed weak positivity (Fig. 4A). The pattern of distribution of NOS-1 was similar to the pattern of GFAP positive cells (Fig. 4B) suggesting that positivity for NOS-1 was located in astrocytes. A greater number of stained cells, especially cell processes, were visible as compared with those of the control group (Fig. 4C). These stained cells were situated between the nerve fascicles, always in locations associated with glia, and they probably corresponded to reactive astrocytes. NOS-1 was also visible in the vessel walls (Fig. 4C, arrow).

Some NOS-2 positive cells, presumably astrocytes, were visible in the ONH after 3 months of chronic moderately elevated IOP. These cells were few and isolated, mainly in the posterior and transition zones (Fig. 4D, arrows). Positivity was also visible in fiber-like structures forming fascicles parallel to the nerve bundles and with a subpial location, probably corresponding to astrocyte processes and their end-feet, formers of the glia limitans separating the nervous components from the pial elements. No NOS-2 positive cells were present in control eyes with normal IOP.

The timolol-treated animals showed a reduction in the expression of NOS-1 in all parts of the ONH, whereas positivity was seen in the nerve endings of the vessel walls (Fig. 4E, arrow).

After treatment with timolol, immunoreactivity for NOS-2 was detected in the cytoplasm of a few isolated cells in the posterior and transition zones (Fig. 4F and G), weaker than in the groups with untreated elevated IOP.

In the control group, the NOS-3 isoform was detected in the cytoplasm of the endothelial cells in the large arteries and veins of the ONH, and in the capillary vessels in the three areas of the ONH (Fig. 5A). No positivity was seen in the remaining structures of the vessel walls. No differences were detected in NOS-3 expression in the ONH from rats with chronic elevated IOP (Fig. 5B) or in the group treated with timolol (Fig. 5C) as compared with the controls.

4. Discussion

We analyzed the expression of the NOS-1, -2 and -3 isoforms in the retina and the ONH of rats submitted to chronic elevated IOP before and after treatment with timolol, a non-selective beta-blocker and one of the most important drugs used in clinical practice to lower elevated IOP in glaucoma patients [24,46,60,65]. For this purpose, we used an experimental model of glaucoma, which followed the protocol of Shareef et al. [52], with cauterization of three episcleral veins to reduce the flow of aqueous humor from the anterior chamber and produce a constant, prolonged increase in the IOP. We have previously verified the efficacy of this model, in which the IOP remains consistently elevated by 1.25-fold in cauterized eyes for up to 3 months after the surgical procedure, as compared with a control group [11].

The results in the retina of the control group showed immunoreactivity of the NOS-1 isoform in the inner segments of the photoreceptors, in bipolar and amacrine neurons of the inner nuclear layer and weaker staining in the ganglion cell layer and in both plexiform layers, sites that have been previously reported by others [6,14,40,53]. In the inner segments of photoreceptors NOS-1 could be closely associated with the membranes of mitochondria [14,29]. NO synthesized at this level may be able to increase cGMP levels [30,31]. Others have found NOS-1 mainly in amacrine cells and in displaced amacrine cells [8,29], which are thought to be the major source of NO in the mammalian retina. NO released by amacrine cells could modulate RGC activity by activating GMP-gated cation channels [1]. NO
could also influence both amacrine cells and RGC, modulating the activity of GABA_A and NMDA receptors [29].

In the ONH, we found positivity in the soma and in the prolongations of a few astrocytes. A similar distribution was reported by Shareef et al. [53] in the rat and by Neufeld et al. [38] in the human ONH. The release of NO by these cells is likely to have a physiological function, acting as a mediator between astrocytes or between astrocytes and axons [53].

Immunohistochemical study of the vascular structures of the ONH showed punctiform staining in the walls of the large arteries of the anterior zone, near the vitreous body, similar to that shown by the choroid arteries, in which NO produces vasodilatation [61]. These points could correspond to parasympathetic nitrergic terminals, in which the NO has a vasodilator function regulating blood flow [53]. In the capillaries of the ONH, NOS-1 was detected in the pericytes, whereas the endothelium was positive for NOS-3. The NO produced by NOS-1 probably contributes with NOS-3 to the vasodilatation and autoregulation of the blood flow in the capillary network [53].

In the group with elevated IOP, NOS-1 was expressed in the same sites as in the control group, although the staining was more intense in the plexiform layers of the retina. In these layers, NO may be involved in synaptic transmission, modifying the electrical coupling in horizontal cells and decreasing dopamine release [29]. Consequently, increased synthesis of NOS-1 may produce changes in the synaptic activity of the retina.

We also saw increased expression of NOS-1 in the neck and posterior regions, compatible with the presence of reactive astro-
cytes. This finding may be because this enzyme is subject to regulation and its level of expression changes in response to different physiological and pathological stimuli [64]. Changes in the distribution and characteristics of these cells, with hypertrophy, hyperplasia and increased expression of GFAP, have been seen in patients with primary open angle glaucoma [58]. These patients have increased expression of NOS-1 in astrocytes, which by producing excess NO are probably responsible for the glaucomatous changes in the ONH [39]. Other authors, however, using this same experimental model of elevated IOP in rats, have failed to detect changes in the expression of NOS-1 [53]. We did not detect the NOS-2 isoform in any type of retinal cell or in the ONH in the control animals. Although this enzyme is induced in disease states, we did not detect it in the retina after 3 months of elevated IOP, probably because the glial reaction, typical during the first 3 post-operative weeks, is not maintained at 3 months [59]. An ultrastructural study undertaken by us (unpublished results) also failed to show during this period either microglial cells or reactive astrocytes, which are the cells that predominantly express NOS-2 [10].

In the ONH we saw an irregular pattern of positivity for NOS-2 in small groups of cells, which we interpreted as reactive astrocytes, and in the glia limitans, which is in agreement with the characteristic zonal involvement of the optic nerve in glaucoma. According to Shareef et al. [53], this isoform appears in the ONH 4 days after elevation of the IOP and remains for at least 3 months. Our results are similar to those seen in the ONH of human glaucomatous eyes, which have been shown to have groups of NOS-2 positive reactive astrocytes, responsible for the production of massive amounts of NO with a powerful neurodestructive effect on neighboring cells [38,39,42].

Our results concerning the NOS-1 and NOS-2 isoforms in the retina and in the ONH of rats submitted to prolonged periods of elevated IOP suggest that excessive levels of NO, considered neurotoxic, could be the cause of the progressive lesions in the axons of the RGC. NO generated from NOS-3, however, is protective [25]. Low levels exert a vasodilatory function regulating blood flow. In the retina and the ONH of both control animals and animals with elevated IOP, we saw that this enzyme was mainly located in the endothelial cells of the arteries, veins and capillaries, chiefly in the neck region of the ONH and in the choriovascular layer. These results coincide with those of Neufeld et al. [39] in healthy persons and in glaucomatous patients. The same author, nevertheless, found positive astrocytes in the prelaminar region and the cribrous lamina of the ONH.

Finally, we studied the effect of timolol, a non-selective beta-adrenoceptor antagonist, submitting a group of animals with elevated IOP to topical treatment with this drug for 10 weeks. Timolol and betaxolol (selective beta1-adrenoceptor antagonist) are the most important drugs used clinically in patients with glaucoma [24,56,65]. For 10 years clinical trials have shown that timolol prevents progression of visual field loss, optic-disc cupping and decreased retinal thickness [50] and results in decreased IOP by reducing the production of aqueous humor [15]. We have also seen this pressure-lowering effect in the experimental model of elevated IOP [11].

Timolol has a neuroprotective action on the RGC and other neurons of the retina [3,60]. In a previous study, we reported a lower cell loss in the ganglion cell layer in rats submitted to topical treatment with timolol as compared with untreated animals. Timolol treatment in our study resulted in an attenuation of the detrimental effects in neurons in the GCL (17% better compared to the untreated elevated IOP group), although the mean number of neurons did not reach the same density as in normal eyes (21.74% loss compared with normal eyes) [11].

It is unclear whether the action is direct or indirect, as a consequence of the reduction in IOP. Applied topically, timolol
reaches the retina-choroid at a concentration within the effective pharmacological range and it is effective against retinal neuron damage both in vitro and in vivo [18, 57].

The mechanism of action of beta-blockers on the cells of the retina is not well known. Some evidence exists concerning the presence of beta-adrenergic receptors in the RGC [54] and they have been clearly shown in astrocytes [7]; they also appear to be expressed by Müller cells in culture [17]. Their neuroprotective action seems to be related with their ability to reduce the flow of calcium and sodium towards the interior of the neurons by blocking the ion channels [3, 5, 19, 23, 34, 47, 60] or by modulating the intracellular concentration of calcium via the glutamate receptors [63]. It has recently been suggested that beta-blockers might exert their neuroprotective effect in the retina via induction of the expression of endogenous brain-derived neurotrophic factor (BDNF), a strong neuroprotector of the RGC [51].

The rats treated with timolol showed decreased expression of NOS-1 in the retina, as compared with the other two groups, with only a few isolated amacrine cells showing positivity. The expression of this enzyme was also reduced in all areas of the ONH and NOS-2 was only noted in the cytoplasm of a few isolated cells. These results suggest that timolol may lower the expression of these enzymes and the synthesis of NO, acting as a neuroprotector in neurons exposed to an excessive amount of extracellular NO. Whether this effect is due to direct action on beta-adrenergic receptors of the astrocytes [3] or through their ability to attenuate neuronal calcium and sodium influx by blocking the ion channels [60] is unknown.

Finally, it is important to note that the persistence of NOS-1 staining after treatment with timolol in nerve terminals and smooth muscle cells of artery walls, together with NOS-3 staining in the vascular endothelia, indicates that the vasodilator function and regulation of blood flow exerted by NO in the ONH could be conserved after treatment, contributing to the neuroprotective effect of timolol.

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