

Nitric Oxide (NO): An Emerging Target for the Treatment of Glaucoma

Megan E. Cavet,¹ Jason L. Vittitow,² Francesco Impagnatiello,³ Ennio Ongini,³ and Elena Bastia³

¹Bausch & Lomb, Inc., Rochester, New York, United States

²Bausch & Lomb, Inc., Bridgewater, New Jersey, United States

³Nicox Research Institute, Bresso, Milan, Italy

Correspondence: Megan E. Cavet, Bausch & Lomb, Inc., 1400 North Goodman Street, Rochester, NY 14609, USA; Megan.Cavet@bausch.com.

Submitted: April 3, 2014

Accepted: June 26, 2014

Citation: Cavet ME, Vittitow JL, Impagnatiello F, Ongini E, Bastia E. Nitric oxide (NO): an emerging target for the treatment of glaucoma. *Invest Ophthalmol Vis Sci.* 2014;55:5005–5015. DOI:10.1167/iovs.14-14515

The predominant risk factor for the progression of glaucoma is an increase in IOP, mediated via a reduction in aqueous outflow through the conventional (trabecular meshwork and Schlemm's canal) outflow pathway. Current IOP lowering pharmacological strategies target the uveoscleral (nonconventional) outflow pathway or aqueous humor production; however, to date no therapy that primarily targets the conventional pathway exists. Nitric oxide (NO) is an intracellular signaling molecule produced by endogenous NO synthases, well-known for its key role in vasodilation, through its action on smooth muscle cells. Under physiological conditions, NO mediates a multitude of diverse ocular effects, including maintenance of IOP. Nitric oxide donors have been shown to mediate IOP-lowering effects in both preclinical models and clinical studies, primarily through cell volume and contractility changes in the conventional outflow tissues. This review is focused on evaluating the current knowledge of the role and mechanism of action of endogenous NO and NO donors in IOP regulation. Data on key additional functions of NO in glaucoma pathology (i.e., ocular blood flow and effects on optic neuropathy) are also summarized. The potential for future therapeutic application of NO in the treatment of glaucoma is then discussed.

Keywords: nitric oxide, intraocular pressure, aqueous outflow

NITRIC OXIDE: AN IMPORTANT CELLULAR SIGNALING MOLECULE

Since the discovery of the role of nitric oxide (NO) as a critical endogenous signaling mediator in 1987, research on this molecule has rapidly flourished and expanded in many directions. Initial seminal studies revealed the key role of NO in the cardiovascular system, as a mediator of smooth muscle relaxation and vasodilation.^{1,2} These discoveries made possible the elucidation of the mode of action of vasodilating nitrates, such as nitroglycerin and isosorbide mononitrate (ISMN) or dinitrate (ISDN), drugs commonly used for treatment of angina.³

With the knowledge that NO and its second messenger cyclic guanosine monophosphate (cGMP) are distributed in most tissues with a diverse array of biological effects it has become evident that the NO pathway is also important for multiple functions in the eye. Under normal conditions, the two constitutive NO synthase (NOS) isoforms, neuronal NOS (nNOS, NOS-1), and endothelial NOS, (eNOS, NOS-3) catalyze the oxidation of the amino acid L-arginine to form NO and L-citrulline. They generate relatively small amounts of NO (picomolar or low nanomolar range) when activated by the calcium/calmodulin complex after an increase of calcium. Moreover, in the vasculature, shear stress generated by blood flow plays an important role in eNOS regulation.⁴ Some of the actions of NO, such as vasodilation are mediated through stimulation of soluble guanylate cyclase (sGC), which leads to elevation of intracellular cGMP levels. Cyclic guanosine monophosphate then interacts with various cyclic-nucleotide-gated channels, protein kinases, and protein phosphodiester-

ases to produce physiological effects. Other biological actions occur through cGMP-independent pathways such as posttranslational modification of proteins by S-nitrosylation⁵ and signaling through NF-κB.⁶ Through S-nitrosylation of proteins, NO has been shown to regulate apoptosis, vascular tone, and inflammatory responses.⁷

In pathological conditions, stimuli such as those generated by infectious diseases, inflammation, or ischemia induce and activate a third NOS isoform, inducible NOS (iNOS; NOS-2). This isoform, whose activity is independent of calcium, generates large amounts of NO (micro to millimolar range) over longer periods.⁸ Reaction of these high NO levels with superoxide free radicals in the local milieu can then lead to the formation of peroxynitrite and other reactive nitrogen species. Under physiological conditions, low levels of peroxynitrite are removed rapidly by endogenous antioxidant mechanisms; however, the high levels formed in conditions of oxidative stress can bind to many molecules including lipids and proteins, thus altering biological function and potentially mediating oxidative damage. For example, increased peroxynitrite levels may lead to protein tyrosine nitration, a covalent modification, which affects protein structure and function.⁹ In the ocular system, reactive nitrogen species may lead to pathophysiological actions such as inflammation and optic nerve degeneration.¹⁰

There are excellent reviews describing the complex role of NO in physiological and pathological conditions.^{11–15} However, very few have addressed the role of NO in ocular function and how it is involved in ocular diseases.^{8,16,17} Glaucoma is a group of severe, sight-threatening diseases, which is the leading worldwide cause of irreversible blindness. People with

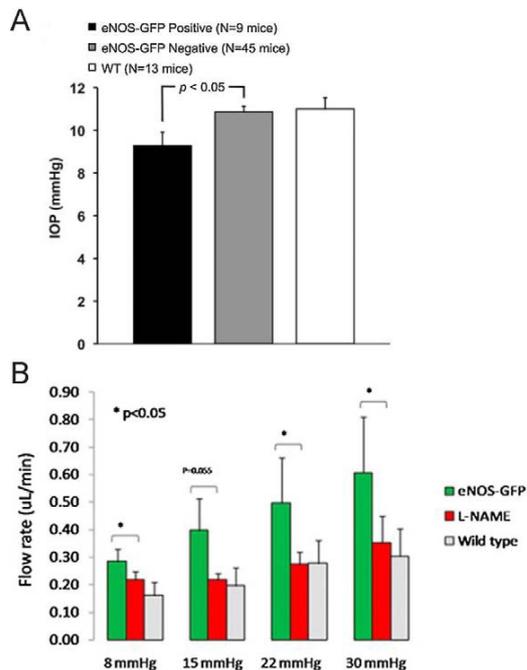


FIGURE 1. Intraocular pressure and outflow facility in transgenic mouse eyes expressing human eNOS-GFP. **(A)** Intraocular pressure measured by rebound tonometry. **(B)** Effect of L-NAME on pressure-flow relationship. Flow rate at constant pressure from transgenic mice perfused with mock aqueous humor \pm L-NAME and wild-type littermate eyes at the same IOPs. *Significantly different from untreated transgenic eyes. Flow rates of L-NAME-treated transgenic eyes were not significantly different from flow rates in wild-type eyes at all pressures tested. Reprinted from Stamer WD, Lei Y, Boussommier-Calleja A, Overby DR, Ethier CR. eNOS, a pressure-dependent regulator of intraocular pressure. *Invest Ophthalmol Vis Sci.* 2011;52:9438-9444.²²

glaucoma often have elevated IOP that is known to be associated with damage to the optic nerve.¹⁸ So far, the reduction of IOP is the only intervention proven to delay the progression of glaucomatous damage.¹⁹ Recently, several interesting research findings have emerged underscoring the important role of NO in the control of IOP.²⁰⁻²² Interestingly, the IOP lowering effects of NO were recently validated in patients with POAG using an NO-donating prostaglandin F (FP) receptor agonist, latanoprostene bunod (LBN). Latanoprostene bunod was more effective than the reference drug, latanoprost, in lowering IOP, presumably via a dual mode of action combining NO donation and FP receptor activation (Katz LJ, et al. *IOVS* 2013;50:ARVO E-Abstract 460).

This review addresses the role of endogenous NO in aqueous humor dynamics (AHD) and IOP control, and with this background then provides an assessment of the current knowledge of the application of NO donors for treatment of glaucoma, focusing on IOP lowering, with a discussion of their potential for a direct action on optic neuropathy.

NO AND REGULATION OF AQUEOUS HUMOR DYNAMICS

Intraocular pressure is regulated by a balance between the production rate of aqueous humor (AH) and its exit through outflow pathways. It has an average value of 15 mm Hg in the

healthy human eye. Under physiological conditions, AH outflow is primarily achieved through the conventional pathway, consisting of the trabecular meshwork (TM) and Schlemm's canal (~60%-90% of outflow in humans and nonhuman primates), while a minor contribution is from the uveoscleral or nonconventional pathway. In older eyes, the contribution of the uveoscleral pathway decreases.²³ Under the pathological condition of elevated IOP, there is increasing evidence that the conventional pathway becomes the limiting factor in AH outflow, with the uveoscleral pathway becoming a more significant contributor.²⁴

Converging evidence indicates that the NO-cGMP signaling pathway is involved in homeostatic processes in the eye, including regulation of AHD and therefore, IOP. In the healthy human eye, the capacity to form NO is found in the anterior ocular tissues. Precisely, eNOS is present in the uveal vascular endothelium, the TM, the Schlemm's canal and the ciliary body.^{8,25} Neuronal NOS is localized in nerve fibers in the limbus, cornea, and lens epithelium.^{26,27} Following cytokine and endotoxin stimulation iNOS is detected in the iris/ciliary body and vessels.⁸ Moreover, the levels of iNOS are reported to be significantly increased in the TM after increasing the perfusion pressure in anterior segments of human donor eyes.²⁸

Support for an important role for endogenous NO in regulating IOP homeostasis comes from a variety of in vitro and ex vivo studies. In vitro studies show that exposure of smooth muscle to inhibitors of NO synthase, with consequent reduction of NO production, increased cell tension, or contraction.^{29,30} The results support the general conclusion that endogenous production and release of NO provides a basal ocular tone, which is important for the regulation of outflow facility, and thus IOP. For example, basal NO production in both human and porcine ciliary processes has been shown to be inhibited by L-NAME (an inhibitor of NOS).²⁹ Isolated strips of bovine TM and ciliary muscle contract in response to L-NAME.³¹ Accordingly, in monkey ciliary muscle, L-arginine (the endogenous substrate of NOS) produces relaxation, whereas L-NAME enhances the contraction induced by carbachol.³² Similarly, Schneemann et al.³⁰ showed that L-NAME decreases flow rate in the TM using a human anterior segment organ culture model. This reduction of the flow was accompanied by a reduction of cGMP levels measured in the perfusate.

Nitric oxide also regulates iris sphincter muscle tone. Pianka and colleagues³³ demonstrated that the relaxation of isolated bovine iris sphincter muscle elicited by electrical field stimulation was inhibited by NOS inhibitors L-NAME, N-nitro-L-arginine, and aminoguanidine, suggesting that most of the relaxation is mediated by endogenously generated NO. Moreover, NO has been shown to inhibit the cholinergic-induced contraction of the iris sphincter.^{34,35} In vivo, the NO-dependent effects on the iris sphincter muscle could be a fine tuning mechanism of the muscle contractions. However, any effect of NO on IOP due to relaxation of the iris sphincter has not been demonstrated.

In vitro studies in human Schlemm's canal cells demonstrate that inhibition of endogenous NOS with L-NAME resulted in an increase in cell volume, suggesting that in vivo reduction in NO levels may increase outflow resistance, and thereby elevate IOP.³⁶ These findings are in line with in vivo data, which show that transgenic mice overexpressing eNOS in vascular endothelia including Schlemm's canal have reduced IOP and increased outflow facility compared with wild-type mice (Fig. 1A).²² Moreover, in perfused enucleated eNOS overexpressing mice eyes, L-NAME returned the outflow facility to levels that were indistinguishable from those of wild-type eyes, thus demonstrating the effect to be NO-dependent (Fig. 1B).²² Overall, these findings support the concept that NO is

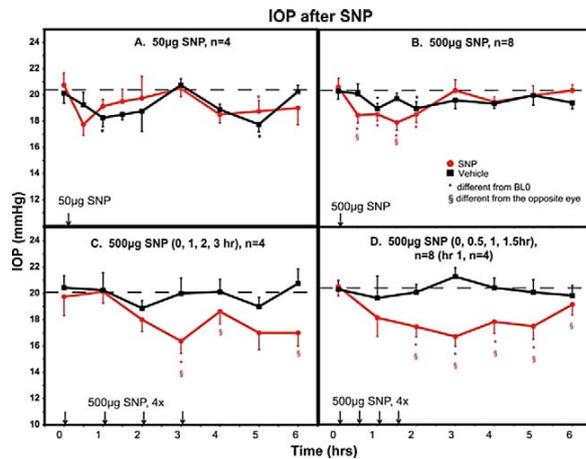


FIGURE 2. Effect of topical SNP on cynomolgus monkey IOP. Following a single treatment with 50 (A) or 500 µg (B) of SNP, IOP was decreased 10% to 15% at various time points compared with vehicle-treated eyes. Four hourly treatments with 500 µg SNP (C) prolonged the IOP reduction. Treatment with 500 µg at four 30-minute intervals (D) produced a significant 15% to 20% reduction in IOP that was sustained for several hours. *Significantly different from baseline prior to the first treatment (BL0) or §compared with the opposite eye, $P < 0.05$. Reprinted from Heyne GW, Kiland JA, Kaufman PL, Gabelt BT. Effect of nitric oxide on anterior segment physiology in monkeys. *Invest Ophthalmol Vis Sci.* 2013;54:5103–5110.²¹

important for modulating the dynamic balance between the rate of secretion (inflow) and drainage (outflow) of AH, and thus IOP regulation.

Evidence indicates that dysregulation of NO generation or resulting downstream signaling may contribute to the elevation of IOP and the development or the progression of glaucoma. Endothelial NOS abundance was decreased in the TM, Schlemm's canal, and ciliary muscle in patients with POAG, suggesting that reduced NO production might contribute to IOP elevation.³⁷ In addition, NO levels were decreased in the AH of patients with POAG.^{38,39} On the other hand, L-arginine levels were significantly increased in vitreous humor of monkeys with experimental glaucoma⁴⁰ and in AH of POAG patients.⁴¹ Interestingly, two studies have identified eNOS gene variants that are associated with POAG in women.^{42,43} A third study that did not find a correlation between eNOS variants and POAG did not provide sex-specific results.⁴⁴

A recent study²⁰ demonstrated that reduction of sGC signaling results in phenotypic changes indicative of the glaucomatous state. Buys et al.²⁰ revealed a decrease in AH outflow in mice lacking the $\alpha 1$ subunit of sGC compared with age-matched, wild-type mice in conjunction with optic neuropathy, increased IOP, and retinal vascular dysfunction. Furthermore, a human genetic association study corroborated the findings in mice by revealing a genetic association between the locus containing genes encoding sGC and a subtype of POAG characterized by initial paracentral vision loss.²⁰ A recent study also found an association between sGC single nucleotide polymorphism score and increased risk for POAG (Qiao C, et al. *IOVS* 2014;55:ARVO E-Abstract 3819). Together, these findings suggest that impaired NO-cGMP signaling can contribute to the etiology of ocular hypertensive glaucoma.

IOP LOWERING ACTIVITY OF NO DONORS

The effects of NO donors on IOP have been studied in multiple species including mouse, rabbit, dog, and monkey. Topical

administration of the NO donors nitroglycerin, ISDN, sodium nitrite, and sodium nitroprusside (SNP) rapidly lowered IOP with a peak effect at 1 to 2 hours in a normotensive rabbit model.⁴⁵ For SNP and nitroglycerin the effect on IOP peaked at 0.1% and 0.03%, respectively, and higher doses were observed to be less effective.⁴⁵ Kotikoski et al.⁴⁶ demonstrated the IOP lowering effects of SNP, S-nitrosothiol, and spermine NONOate in normotensive rabbits after either topical or intravitreal dosing, with a maximal IOP lowering at 2 to 5 hours. In another normotensive rabbit study, intravitreal or intracameral injection of the NO donors 3-morpholinopyridone (SIN-1) or S-nitroacetylpenicillamine (SNAP) induced a dramatic IOP-lowering dependent on the amount of NO delivered to the eye.⁴⁷ Carreiro and coworkers⁴⁸ demonstrated that IOP lowering with the NO donors SNP and SNAP in normotensive rabbits correlated with NO and cGMP levels in the rabbit AH and iris ciliary body after topical administration. With the fast-releasing SNP, maximal cGMP and NO levels in the AH and iris ciliary body were found at 15 and 30 minutes, respectively, whereas slower releasing SNAP elevated cGMP and NO levels maximally at 60 to 180 minutes and 60 minutes post dose in the AH and iris ciliary body. This correlated with a maximal IOP reduction of 9% with SNP at 1 hour post dose and 8% at 2 hours post dose with SNAP.⁴⁸

In a nonhuman primate study, topical administration of nitroglycerin at a dose of 0.1% significantly decreased IOP in normotensive animals after 90 minutes.⁴⁹ Conversely, Wang and Podos⁵⁰ reported that there was no IOP lowering after topical administration of nitroglycerin in either normal or laser-induced glaucomatous monkeys. The reason for the apparent inconsistencies in these observations is uncertain; however, two different protocols of drug administration were used, in the first study multiple drops were applied, in the second a single application was made. A recent study in normotensive nonhuman primates demonstrated that multiple topical treatments of SNP significantly decreased IOP from 2 to 6 hours with a maximal IOP reduction of 20% at 3 hours post dose (Fig. 2).²¹

The hypotensive effect of nipradilol, a β -adrenoceptor blocker with a NO donating nitroxy group, was demonstrated to be dependent on NO donation by pretreating with the NO trapping agent carboxy-PTIO in normotensive rabbits.⁵¹ A later study⁵² demonstrated that nipradilol and the NO donor SNP had IOP lowering ability in both normotensive and hypertensive rabbits, while latanoprost was without significant effect. When either nipradilol or SNP was dosed in conjunction with latanoprost the IOP reduction was significantly greater than that of either of the NO donors administered alone, suggesting a synergistic effect of latanoprost and NO in lowering IOP.⁵²

The efficacy of the released NO of three NO-donating analogs of prostaglandins or prostamides (NCX 139, a NO donating prostamide; NCX 125, and LBN [NCX 116], two NO donating prostaglandin F2 alpha analogs) in IOP lowering has been demonstrated in multiple animal species. Latanoprostene bunod, NCX 125, and NCX 139 were effective in decreasing IOP in a transiently hypertensive rabbit model, while equimolar doses of the corresponding prostamide or prostaglandin alone were ineffective. These three NO-donating compounds were also more efficacious than their respective prostamide or prostaglandin counterparts in glaucomatous dog eyes and in laser-induced hypertensive nonhuman primates.^{53–55}

A role for NO in reducing IOP has been demonstrated in humans after administration via various routes. A seminal study carried out by Wizemann and Wizemann⁵⁶ demonstrated that intravenously administered nitroglycerin lowered IOP in humans in a dose-dependent manner without altering systemic blood pressure. Dinitrate given orally (2×40 mg per day) also

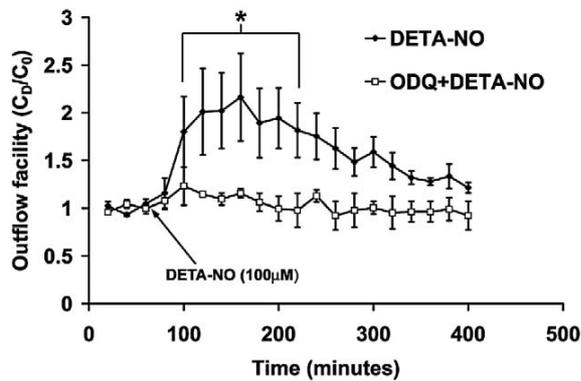


FIGURE 3. The NO donor DETA-NO induces a cGMP dependent increase in outflow facility. The anterior chamber perfusate was replaced with an acute treatment of DETA-NO (100 μ M) or ODQ for 50 minutes, followed with DETA-NO (100 μ M). *Significantly different from baseline values, $P < 0.05$. Reprinted from Ellis DZ, Dismuke WM, Chokshi BM. Characterization of soluble guanylate cyclase in NO-induced increases in aqueous humor outflow facility and in the trabecular meshwork. *Invest Ophthalmol Vis Sci.* 2009;50:1808-1813.⁶⁵

had a potent lowering effect on IOP.⁵⁶ Moreover, topically applied ISMN (2%) significantly reduced IOP within 3 hours after dosing.⁵⁷ In addition, intravenous administration of the NO precursor L-arginine in humans significantly decreased IOP, resulting in an average drop of 2 mm Hg in healthy subjects.⁵⁸ Nipradilol is approved for clinical IOP lowering in Japan and clinical studies have demonstrated that it is effective in IOP lowering in normal-tension glaucoma over a 5-year period.⁵⁹ In patients with ocular hypertension twice daily dosing with 0.25% nipradilol decreased IOP and also reduced the aqueous flow rate.⁶⁰

The NO-donating FP receptor agonist LBN, is a new molecular entity that delivers two active moieties. After exposure to esterases present in the anterior eye, LBN is cleaved to latanoprost acid and butanediol mononitrate, an NO-donating moiety. Latanoprostene bunod Phase 3 clinical trials commenced in 2013 to evaluate the reduction of elevated IOP in patients with open-angle glaucoma or ocular hypertension. In a previous Phase 2 study, LBN, dosed once a day in the evening, was shown to be efficacious in treating POAG at multiple concentrations, with dose-dependent IOP reductions over the 28-day treatment period. Statistically significant greater reduction in mean IOP at day 28 (primary endpoint) was observed with LBN at 0.024% and 0.040% compared with latanoprost 0.005%. While the LBN 0.006% and 0.012% groups showed numerically greater reductions in IOP as compared with the latanoprost 0.005% group, these differences were not statistically significant at any time point. In addition to the primary endpoint, statistically significant differences in IOP between the LBN 0.024% and latanoprost 0.005% groups were reported for a majority of secondary endpoints (Katz LJ, et al. *IOVS* 2013;50:ARVO E-Abstract 460). The safety assessment indicated that LBN at concentrations from 0.006% to 0.040% was well tolerated (Katz LJ, et al. *IOVS* 2013;50:ARVO E-Abstract 460).

While improvements in IOP reduction were seen with increasing concentrations of LBN, it seems that the maximal IOP lowering cannot simply be attributed to increased doses of latanoprost acid. Work by Eveleth et al.⁶¹ has demonstrated that increasing the dose of Xalatan (latanoprost) above the clinical dose of 0.005% does not result in additional IOP lowering effects. Rather than being a dose-related effect

associated with an increase in latanoprost acid exposure, the improvements in IOP reduction seen with LBN 0.024% compared with latanoprost 0.005% suggest that the NO-donating active moiety contributes to the additional IOP lowering effect.

MECHANISM OF ACTION OF NO IN IOP LOWERING

Current therapies for IOP lowering include β -adrenergic receptor blockers, α -adrenergic receptor agonists, and carbonic anhydrase inhibitors, which target AH formation (AHF), and prostaglandin analogs that are understood to lower IOP through effects on the uveoscleral pathway.⁶² There is evidence that NO induction may mediate IOP lowering effects through a predominant increase in the aqueous outflow, mainly with effects on the conventional pathway. An activity through reduction in AHF (inflow) has also been reported.

Effects of NO Donors on the Conventional Outflow Pathway

Animal studies indicate that NO-induced IOP lowering is mediated predominantly via an increase in conventional outflow facility. In nonhuman primates, nitroglycerin caused a 92% increase in outflow facility when administered by intracameral bolus injection.⁴⁹ In another primate study, the cGMP analog 8-Br-cGMP increased outflow facility by approximately 30% after intracameral infusion.⁶³ In rabbit eyes, topical administration of nitroglycerin or intracameral administration of SNP increased conventional outflow facility.^{45,64} More recently, perfusion of SNAP significantly increased conventional outflow facility by 64% over the inactive N-acetylpenicillamine control, which lacks the NO-donating moiety, in ex vivo mouse eyes (Chang J, et al. *IOVS* 2013; 54:ARVO E-Abstract 2002). Likewise, 1 mM SNP, administered intracamerally, increased outflow facility by 77% in the cynomolgus monkey.²¹

There is increasing evidence for a primary role of the TM and Schlemm's canal as the cellular targets for NO in IOP lowering. Organic nitrates ISDN, ISMN, as well as SNP and SNAP have been shown to relax isolated bovine TM strips, either at resting tension or after contraction using carbachol, pointing to this tissue as the site mediating an increased conventional outflow and IOP lowering in vivo.³¹ SNP increased flow rate in human donor eye anterior segments maintained in an organ culture perfusion system.³⁰ In addition, recent studies have demonstrated that diethylenetriamine (DETA)-NO increased outflow facility in isolated porcine anterior segments, mediated by increased sGC activity and cGMP production⁶⁵ (Fig. 3). Further investigation into the cellular signaling responsible for the increase in outflow facility used TM cells and demonstrated that DETA-NO-mediated activation of large conductance Ca^{2+} activated K^{+} (BKCa) channels resulting in a decrease in TM cell volume.^{65,66}

Alterations in the cytoskeletal network resulting in TM relaxation are also likely involved in the increase in outflow facility induced by NO. Trabecular meshwork cells are known to be highly contractile in nature, analogous to vascular smooth muscle cells (VSMC), in which the role of NO-cGMP signaling in endothelium dependent relaxation is well understood.⁶⁷ In VSMC, the NO-cGMP signaling pathway primarily mediates its effects through activation of protein kinase G (PKG), which has an inhibitory effect on the Rho kinase cascade leading to inhibitory effects on myosin light chain (MLC)-2 phosphorylation. In addition, effects of NO on potassium channels, including the BKCa channels lead to membrane hyperpolarization and inhibition of calcium influx

though L-type calcium channels. These and other pathways lead to alterations in cytoskeletal proteins, preventing the interaction of actin and myosin, and ultimately result in VSMC relaxation.⁶⁸⁻⁷¹ In the TM, pharmacological inhibition of MLC-2 phosphorylation, actomyosin organization, and cell contractility has been demonstrated using Rho-kinase inhibitors, and this in turn has been demonstrated to increase aqueous outflow facility.⁷²⁻⁷⁴ In addition, TM relaxation induced by the multidrug resistance-associated protein-4 is predominantly mediated via induction of cGMP and the downstream PKG signaling pathway.⁷⁵ Recently, DETA-NO and SNP were demonstrated to relax TM cells in a cGMP dependent manner using a gel contraction assay. The effect of DETA-NO and SNP was biphasic in that higher concentrations (>200 μ M) were not effective in eliciting cell relaxation.⁷⁶

The Schlemm's canal consists of endothelial cells and connective tissue, similar in structure to a vein.⁷⁷ Contractility of these cells plays a role in the modulation of aqueous outflow and therefore these cells are a potential site of action for NO. Inhibition of Rho kinase, which is downstream of cGMP in the NO/sGC/cGMP pathway, has been demonstrated to regulate actin dynamics and cell contractility in cultured Schlemm's canal cells.^{74,78} By analogy to vascular endothelial cells, where the vasodilatory effects of NO are well understood,⁷⁹ NO would be expected to mediate its IOP lowering effects by targeting these cells. Indeed, cell-based studies demonstrated that the NO donor DETA-NO regulates Schlemm's canal cell volume via the NO/cGMP pathway.³⁶ Therefore, effects of NO on outflow facility mediated through the NO/cGMP signaling pathway resulting in TM and Schlemm's canal cell relaxation is likely a major mechanism involved in the IOP lowering activity of NO.

A third possible mechanism for the increased rate of conventional outflow by NO is via regulating episcleral blood flow and thus lowering episcleral venous pressure (EVP; i.e., the pressure that must be overcome for the AH to leave the eye via the trabecular outflow pathway). The effect of NO on EVP has not been investigated extensively, though reports of either an increase or decrease in EVP exist in the literature. Topical administration of SNP is reported to decrease EVP 30 minutes after administration of 0.2 mg⁸⁰ or increase EVP after 0.5 mg in rabbits.⁸¹ In both of these studies, however, there was a concomitant increase in IOP. Funk et al.⁸² demonstrated a dose dependence of SNP on EVP, in that a high dose of SNP (5 mg) increased EVP (and also IOP), while a low dose (0.5 mg) had no effect on EVP, and a tendency to reduce IOP.

Effects of NO Donors on the Uveoscleral Pathway

The NO donors ISDN, ISMN, SNP, and SNAP have been shown to relax isolated bovine ciliary muscle at resting tension, though to a lesser extent than isolated TM strips.³¹ Isosorbide dinitrate and SNP were effective in producing pronounced relaxation of cynomolgus and rhesus monkey longitudinal carbachol-contracted ciliary muscle in vitro, which was partially reversible with the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ).³² Relaxation of the ciliary muscle increases uveoscleral outflow since flow through the ciliary muscle is the rate limiting step. On the other hand, ciliary muscle relaxation may have the effect of decreasing conventional outflow through contraction of the TM and Schlemm's canal. As described above, however, multiple in vivo studies have demonstrated that NO increases outflow facility, suggesting the TM/Schlemm's canal as a predominant site of NO action.

Effects of NO Donors on AHF

Several research reports indicate that NO plays a role in reducing AHF. The NO donor SNP has been demonstrated to

reduce AH secretion using a fluorescein dilution method, and to lower IOP in both bovine and porcine ex vivo intact eye preparations.^{83,84} This reduction in AHF was accompanied by an increase in cGMP in the ciliary epithelia.⁸⁵ Further investigations elucidated the signaling pathways mediating this AHF reduction. Sodium nitroprusside and SNAP were demonstrated to inhibit Na/K ATPase activity via the NO-sGC-cGMP pathway, and this inhibition was dependent on Src family kinase activation.^{84,86-88} Contrary to this, it has been reported that SNP and SNAP, via activation of cGMP signaling pathways, lead to membrane potential depolarization in isolated porcine ciliary epithelial processes, suggesting that NO may stimulate AHF.^{89,90} Differences in the model system used (isolated ciliary processes versus ex vivo intact eye) may underlie the observed disparity between studies.

OTHER IMPLICATIONS OF NO AS A TARGET FOR TREATMENT OF GLAUCOMA

Glaucoma is characterized by progressive optic nerve damage, loss of retinal ganglion cells, nerve fiber layers thinning, and extensive remodeling of the surrounding tissues.⁹¹ In addition to targeting the conventional outflow pathway to reduce IOP, NO has been reported to have effects on the posterior eye structures relevant to glaucoma, namely optic nerve and ocular blood vessels.

Upregulation of nNOS and iNOS has been reported in activated astrocytes in the optic nerve head (ONH) from glaucomatous eyes, as compared with healthy eyes.⁹² Other reports detected high expression of iNOS in the retina after ischemia⁹³ and after cauterization of the episcleral veins to elevate IOP.⁹⁴ In the vein cauterization rat model it was demonstrated that inhibition of iNOS prevented loss of retinal ganglion cells (RGCs).^{95,96} These reports indicate that the glaucomatous ONH is exposed to enhanced concentrations of NO, particularly from upregulation of iNOS, which might play a role in chronic neurodegeneration due to formation of high levels of peroxynitrite.⁹⁷ However, the role of iNOS in optic neuropathy is controversial, in that other animal studies have not demonstrated a link between iNOS and neurodegeneration. In a rat model of glaucoma in which IOP was elevated by episcleral injection of hypertonic saline, there was no increase in iNOS expression and iNOS inhibition with aminoguanidine did not affect the development of neuropathy.⁹⁸ In addition, iNOS levels from POAG and nonglaucomatous human eyes were similar.⁹⁸ In the DBA/2J mouse glaucoma model, neither genetic deficiency of iNOS nor inhibition of iNOS using aminoguanidine had any detectable effect on glaucomatous optic nerve damage.⁹⁹ However, in the same mouse model, nNOS was elevated in the retina and L-NAME was effective in preventing RGC apoptosis though it did not attenuate the morphologic lesion of the retina.¹⁰⁰ Therefore, further studies are necessary to fully understand the role of endogenous NO through upregulation of NOS in optic neuropathy.

The glaucoma drug nipradilol has been demonstrated to have neuroprotective effects after topical administration, due to its NO donating action. In a rat optic nerve crush model, 0.25% nipradilol (BID) was demonstrated to reduce RGC death as compared with control.¹⁰¹ Studies in rabbits also demonstrated that topical nipradilol reaches the retina-choroid in effective concentrations after topical application.^{102,103} Other studies have also shown neuroprotective effects of intravitreal administration of nipradilol and SNP after N-methyl-D-aspartate (NMDA)-induced retinal damage in rats¹⁰³ and in an axotomized cat model.¹⁰⁴ NMDA-induced neurotoxicity was also significantly reduced by 1-hydroxy-2-oxo-3,3-bis (2-aminoethyl)-1-triazene (NOC18) in rat retina.¹⁰⁵ A clinical study found

that risk of optic nerve degeneration was lower in glaucoma patients receiving nitroglycerin for nonophthalmic reasons than in the control group.¹⁰⁶ On the other hand, intravitreal administration of the NO releasing compound N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazinoethanamine (NOC12) induced retinal neurotoxicity in rats.¹⁰⁷ Therefore, effects appear to depend on the type of NO donor (presumably due to differences in amount and duration of NO release) and the animal model employed.

In vitro studies in cultured retinal neuronal cells have demonstrated that NO can have either neuroprotective or neurodegenerative properties depending on the dose. Low concentrations of the NO donor SNAP ($\leq 1 \mu\text{M}$) or S-nitrosocysteine ($50 \mu\text{M}$) increased survival of retinal neuronal cells, while SNAP at greater than or equal to $100 \mu\text{M}$ or $500 \mu\text{M}$ of S-nitrosocysteine decreased neuronal cell survival.^{108,109} Inhibiting endogenous NO using L-NAME or the NO scavenger c-PTIO also concentration-dependently decreased RGC survival.¹⁰⁹ Nipradilol has been shown to mediate NO-dependent S-nitrosylation of regulatory proteins involved in antioxidant and protective effects in RGCs. Nipradilol was neuroprotective against oxidative stress-induced cell death in RGCs and mediated S-nitrosylation of Kelch-like ECH-associated protein (Keap1), which regulates the transcriptional activation of the antioxidant protein heme oxygenase-1.¹¹⁰ Nipradilol also caused S-nitrosylation and inhibition of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), leading to increased protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling, a pathway for axon regeneration.¹¹¹

Overall, the literature on the role of NO in optic neuropathy is suggestive of a dual action, with low levels exhibiting neuroprotective effects and high levels (from induction of iNOS and/or upregulation of nNOS) mediating neurodegenerative effects. In the case of topical NO therapy, data is lacking; however, studies using nipradilol demonstrate that this particular NO donor reaches the retina at levels that can mediate neuroprotective effects.¹⁰¹⁻¹⁰³ Interestingly, it is reported that NCX 2057 (an NO-releasing derivative of ferulic acid) reduced iNOS levels in macrophages as well as reducing microglia activation¹¹² in an NO-dependent manner,¹¹³ the NO donors (\pm)-(E)-ethyl-2-[(E)-hydroxyamino]-5-nitro-3-hexeneamide (NOR3) and SNP decreased iNOS expression in VSMC,¹¹⁴ and the NO donors SNP and SNAP inhibited iNOS activity in intestinal epithelial cells,¹¹⁵ raising the possibility that exogenous NO could mitigate the increased NOS levels observed in the optic nerve head in glaucoma.

Reduced ONH blood flow in POAG has been shown in a number of investigations.¹¹⁶⁻¹¹⁸ According to the vascular concept of glaucoma,^{119,120} RGC damage is in part caused by ocular blood flow impairment and instability resulting in ischemia-reperfusion injury. The retinal, ONH, and choroidal endothelium play a key role in maintaining the homeostasis of vascular tone and regulation of blood flow by releasing vasoactive mediators including NO.^{17,121} A number of studies in animals and humans show that the vascular endothelium of the retina and choroid express NOS.¹²²⁻¹²⁴ The importance of NO-mediated control of basal ocular vascular tone is demonstrated by studies in which NOS inhibition results in vasoconstriction of isolated porcine and human ophthalmic artery segments.^{125,126} In agreement with this finding, inhibition of sGC results in contraction of isolated bovine retinal arteries.¹²⁷ Likewise, in in vivo studies inhibition of NOS reduces basal blood flow in the choroid of dogs, rabbits, pigs, and rats.¹²⁸⁻¹³¹ The majority of the studies report a lack of significant alteration in retinal blood flow. This regional difference could be the result of a finer autoregulatory system present in the blood vessel of the retina and/or technical difficulties in assessing the relatively low retinal blood flow.

In healthy subjects, systemic administration of a NOS inhibitor reduces blood flow in the choroid, retina, and ONH.¹³²⁻¹³⁴ Interestingly, in POAG patients, Polak and colleagues¹³⁵ showed less pronounced decrease of blood flow in the ONH and the choroid after systemic NOS inhibition with L-NMMA as compared with healthy controls, whereas the systemic blood pressure response was the same. This is in accordance with the hypothesis of local alteration of the NO signaling pathway at the level of the ocular vasculature in glaucoma. In glaucoma patients, while iNOS and nNOS upregulation in the ONH may contribute to the oxidative stress resulting from repeated ischemia-reperfusion injury, elevated eNOS in vascular endothelium may be neuroprotective through causing vasodilation and increased ONH blood flow.⁹²

A number of studies support the vasodilatory role of NO on ocular blood vessels. For example, in isolated bovine retinal and ciliary arteries SNP elicits relaxation.^{127,136} Likewise, nipradilol produces retinal arterial dilation in isolated canine arterial strips and it dilates arterioles in anesthetized dogs following intra-arterial injections.¹³⁷ Similarly, nitroglycerin and SNP cause vasodilation of the choroidal and retinal blood vessels in anesthetized dogs and in a model of branch vein occlusion in pigs.^{138,139} Another study demonstrated that NOS inhibition using intravenous L-NMMA decreased rat retinal blood vessel diameter while SNP and NOR3 resulted in vessel dilation.¹⁴⁰ On the other hand, Granstam and colleagues¹²⁹ showed that intravenously administered SNP does not significantly alter choroidal blood flow in rats, probably due to an overall decrease in systemic blood pressure and consequently reduction of ocular perfusion pressure. Similarly, a clinical study demonstrated that intravenously administered nitroglycerin, ISDN, and SNP decreased vascular resistance but did not affect ocular hemodynamics, thought to be due to a reduction in blood pressure.¹⁴¹ In contrast, in healthy subjects, oral administration of ISMN increased optic nerve blood flow, but not blood flow in the choroid or retina.^{142,143} Moreover, in healthy volunteers topical nipradilol significantly increased retinal arterial blood flow and ONH blood velocity.^{144,145} The effects of NO donors on ocular blood flow in nonclinical glaucoma models and in humans with POAG remains to be evaluated. Overall, the literature on NO and ocular blood flow suggest that NO plays a major role in controlling basal ocular vascular tone and that exogenous NO delivery can modulate ocular blood flow, with effects dependent on numerous factors including the specific NO donor used, its route of administration and the ocular vascular region studied.

COMMENTARY AND CONCLUSIONS

Overall, the wealth of experimental data from a variety of models coupled with recent clinical studies strongly point to an important role of NO in modulating IOP via an increase in conventional outflow facility, through actions on the TM/Schlemm's canal directly and also possibly by changes in EVP. Other mechanisms such as reduction in AHF and modulation of uveoscleral outflow may also play a role. Nitric oxide could also exert additional positive effects on cell health and survival in ocular tissues. Physiologic low concentrations of NO provide cell sparing activities because of its antiinflammatory and antiapoptotic properties.¹⁴⁶ For instance, NO inhibits the activation of NF- κ B as well as of various pro-inflammatory proteins including iNOS, TNF- α , and other cytokines.^{113,147} In terms of posterior effects, potential mechanisms of neuroprotection, either directly on the optic nerve axons or through alterations to blood flow in the choroid, optic nerve, and retina have been reported.^{106,148,149} Further investigations are required to answer the question of whether such properties

confer additional clinical therapeutic benefits and could improve the progression of disease in patients with glaucoma.

An important consideration for exogenous NO delivery as a glaucoma therapy appears to be dosing. As is the case for many drugs or biological effectors, the biphasic nature of NO releasing agents in numerous cellular responses is well known.¹⁵⁰ Of direct relevance to glaucoma therapy, both in vitro and in vivo studies have demonstrated that IOP lowering effects may be reduced at higher NO levels with the NO donor SNP.^{45,76} In the case of optic neuropathy, on one hand, NO is reported to have a neuroprotective effect on isolated RGCs at low doses, but on the other, a neurodegenerative effect at higher pathological levels^{108,109}; however, actual NO levels mediating these effects in vivo are yet to be defined.

Despite the availability of various treatments to reduce elevated IOP, the most commonly prescribed glaucoma drugs do not target the compromised function of the trabecular outflow pathway. In adults, approximately 60% to 90% of outflow works its way through the TM, the eye's primary draining tissue, which is compromised in glaucoma patients and continues to deteriorate over time.^{151,152} In addition, this could explain why a considerable proportion of glaucoma patients require more than one class of glaucoma medication and some of them do not find an appropriate medical therapy, thus requiring surgical intervention. Therapeutic use of NO has the potential to benefit glaucoma patients by reducing IOP via targeting the diseased conventional outflow pathway.

Acknowledgments

The information presented on latanoprostene bunod concerns a use that has not been approved by the US Food and Drug Administration.

Disclosure: M.E. Cavet, Bausch & Lomb (E); J.L. Vittitow, Bausch & Lomb (E); F. Impagnatiello, Nicox (E); E. Ongini, Nicox (E); E. Bastia, Nicox (E)

References

- Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288:373-376.
- Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. 1987;327:524-526.
- Moncada S, Higgs A, Furchgott R. International Union of Pharmacology Nomenclature in Nitric Oxide Research. *Pharmacol Rev*. 1997;49:137-142.
- Boo YC, Jo H. Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *Am J Physiol Cell Physiol*. 2003;285:C499-C508.
- Handy DE, Loscalzo J. Nitric oxide and posttranslational modification of the vascular proteome: S-nitrosation of reactive thiols. *Arterioscler Thromb Vasc Biol*. 2006;26:1207-1214.
- Colasanti M, Suzuki H. The dual personality of NO. *Trends Pharmacol Sci*. 2000;21:249-252.
- Hara MR, Agrawal N, Kim SF, et al. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat Cell Biol*. 2005;7:665-674.
- Becquet F, Courtois Y, Goureau O. Nitric oxide in the eye: multifaceted roles and diverse outcomes. *Surv Ophthalmol*. 1997;42:71-82.
- Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. 2007;87:315-424.
- Goldstein IM, Ostwald P, Roth S. Nitric oxide: a review of its role in retinal function and disease. *Vision Res*. 1996;36:2979-2994.
- Bryan NS, Bian K, Murad F. Discovery of the nitric oxide signaling pathway and targets for drug development. *Front Biosci*. 2009;14:1-18.
- Kuo PC, Schroeder RA. The emerging multifaceted roles of nitric oxide. *Ann Surg*. 1995;221:220-235.
- Moncada S. Nitric oxide in the vasculature: physiology and pathophysiology. *Ann N Y Acad Sci*. 1997;811:60-67, discussion 67-69.
- Stewart AG, Phan LH, Grigoriadis G. Physiological and pathophysiological roles of nitric oxide. *Microsurgery*. 1994;15:693-702.
- Thomas DD, Ridnour LA, Isenberg JS, et al. The chemical biology of nitric oxide: implications in cellular signaling. *Free Rad Biol Med*. 2008;45:18-31.
- Chiou SH, Chang CJ, Hsu WM, et al. Elevated nitric oxide level in aqueous humor of patients with acute angle-closure glaucoma. *Ophthalmologica*. 2001;215:113-116.
- Toda N, Nakanishi-Toda M. Nitric oxide: ocular blood flow, glaucoma, and diabetic retinopathy. *Prog Retin Eye Res*. 2007;26:205-238.
- Sommer A. Intraocular pressure and glaucoma. *Am J Ophthalmol*. 1989;107:186-188.
- Kass MA, Heuer DK, Higginbotham EJ, et al. The Ocular Hypertension Treatment Study: a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch Ophthalmol*. 2002;120:701-713.
- Buys ES, Ko YC, Alt C, et al. Soluble guanylate cyclase alpha1-deficient mice: a novel murine model for primary open angle glaucoma. *PLoS One*. 2013;8:e60156.
- Heyne GW, Kiland JA, Kaufman PL, Gabelt BT. Effect of nitric oxide on anterior segment physiology in monkeys. *Invest Ophthalmol Vis Sci*. 2013;54:5103-5110.
- Stamer WD, Lei Y, Boussommier-Calleja A, Overby DR, Ethier CR. eNOS, a pressure-dependent regulator of intraocular pressure. *Invest Ophthalmol Vis Sci*. 2011;52:9438-9444.
- Alm A, Nilsson SF. Uveoscleral outflow—a review. *Exp Eye Res*. 2009;88:760-768.
- Wan Z, Woodward DE, Stamer WD. Endogenous bioactive lipids and the regulation of conventional outflow facility. *Expert Rev Ophthalmol*. 2008;3:457-470.
- Nathanson JA, McKee M. Identification of an extensive system of nitric oxide-producing cells in the ciliary muscle and outflow pathway of the human eye. *Invest Ophthalmol Vis Sci*. 1995;36:1765-1773.
- Chen HB, Yamabayashi S, Ou B, Tanaka Y, Ohno S, Tsukahara S. Histochemical study on rat tear film and ocular surface epithelial cells. *Curr Eye Res*. 1998;17:642-649.
- Osborne NN, Barnett NL, Herrera AJ. NADPH diaphorase localization and nitric oxide synthetase activity in the retina and anterior uvea of the rabbit eye. *Brain Res*. 1993;610:194-198.
- Schneemann A, Leusink-Muis A, van den Berg T, Hoyng PE, Kamphuis W. Elevation of nitric oxide production in human trabecular meshwork by increased pressure. *Graefes Arch Clin Exp Ophthalmol*. 2003;241:321-326.
- Haufschild T, Tschudi MR, Flammer J, Luscher TF, Haefliger IO. Nitric oxide production by isolated human and porcine ciliary processes. *Graefes Arch Clin Exp Ophthalmol*. 2000;238:448-453.
- Schneemann A, Dijkstra BG, van den Berg TJ, Kamphuis W, Hoyng PE. Nitric oxide/guanylate cyclase pathways and flow in anterior segment perfusion. *Graefes Arch Clin Exp Ophthalmol*. 2002;240:936-941.
- Wiederholt M, Sturm A, Lepple-Wienhues A. Relaxation of trabecular meshwork and ciliary muscle by release of nitric oxide. *Invest Ophthalmol Vis Sci*. 1994;35:2515-2520.

32. Gabelt BT, Kaufman PL, Rasmussen CA. Effect of nitric oxide compounds on monkey ciliary muscle in vitro. *Exp Eye Res.* 2011;93:321-327.
33. Pianka P, Oron Y, Lazar M, Geyer O. Nonadrenergic, noncholinergic relaxation of bovine iris sphincter: role of endogenous nitric oxide. *Invest Ophthalmol Vis Sci.* 2000;41:880-886.
34. Chuman H, Chuman T, Nao-i N, et al. Different responsiveness to nitric oxide-cyclic guanosine monophosphate pathway in cholinergic and tachykinergic contractions of the rabbit iris sphincter muscle. *Invest Ophthalmol Vis Sci.* 1997;38:1719-1725.
35. Chuman T, Chuman H, Nao-i N, Sawada A, Yamamoto R, Wada A. Nitric oxide-sensitive and -insensitive contractions of the isolated rabbit iris sphincter muscle. *Invest Ophthalmol Vis Sci.* 1996;37:1437-1443.
36. Ellis DZ, Sharif NA, Dismuke WM. Endogenous regulation of human Schlemm's canal cell volume by nitric oxide signaling. *Invest Ophthalmol Vis Sci.* 2010;51:5817-5824.
37. Nathanson JA, McKee M. Alterations of ocular nitric oxide synthase in human glaucoma. *Invest Ophthalmol Vis Sci.* 1995;36:1774-1784.
38. Doganay S, Evereklioglu C, Turkoz Y, Er H. Decreased nitric oxide production in primary open-angle glaucoma. *Eur J Ophthalmol.* 2002;12:44-48.
39. Galassi F, Renieri G, Sodi A, Ucci F, Vannozzi L, Masini E. Nitric oxide proxies and ocular perfusion pressure in primary open angle glaucoma. *Br J Ophthalmol.* 2004;88:757-760.
40. Carter-Dawson L, Crawford ML, Harwerth RS, et al. Vitreal glutamate concentration in monkeys with experimental glaucoma. *Invest Ophthalmol Vis Sci.* 2002;43:2633-2637.
41. Hannappel E, Pankow G, Grassl F, Brand K, Naumann GO. Amino acid pattern in human aqueous humor of patients with senile cataract and primary open-angle glaucoma. *Ophthalmic Res.* 1985;17:341-343.
42. Kang JH, Wiggs JL, Rosner BA, et al. Endothelial nitric oxide synthase gene variants and primary open-angle glaucoma: interactions with sex and postmenopausal hormone use. *Invest Ophthalmol Vis Sci.* 2010;51:971-979.
43. Magalhaes DS, Rocha AV, Lacchini R, et al. Association of polymorphisms of endothelial nitric oxide synthase (eNOS) gene with the risk of primary open angle glaucoma in a Brazilian population. *Gene.* 2012;502:142-146.
44. Weiss J, Frankl SA, Flammer J, et al. No difference in genotype frequencies of polymorphisms of the nitric oxide pathway between Caucasian normal and high tension glaucoma patients. *Mol Vis.* 2012;18:2174-2181.
45. Nathanson JA. Nitrovasodilators as a new class of ocular hypotensive agents. *J Pharmacol Exp Ther.* 1992;260:956-965.
46. Kotikoski H, Alajuuja P, Moilanen E, et al. Comparison of nitric oxide donors in lowering intraocular pressure in rabbits: role of cyclic GMP. *J Ocul Pharmacol Ther.* 2002;18:11-23.
47. Behar-Cohen FF, Goureau O, D'Hermies F, Courtois Y. Decreased intraocular pressure induced by nitric oxide donors is correlated to nitrite production in the rabbit eye. *Invest Ophthalmol Vis Sci.* 1996;37:1711-1715.
48. Carreiro S, Anderson S, Gukasyan HJ, Krauss A, Prasanna G. Correlation of in vitro and in vivo kinetics of nitric oxide donors in ocular tissues. *J Ocul Pharmacol Ther.* 2009;25:105-112.
49. Schuman JS, Erickson K, Nathanson JA. Nitrovasodilator effects on intraocular pressure and outflow facility in monkeys. *Exp Eye Res.* 1994;58:99-105.
50. Wang RF, Podos SM. Effect of the topical application of nitroglycerin on intraocular pressure in normal and glaucomatous monkeys. *Exp Eye Res.* 1995;60:337-339.
51. Sugiyama T, Kida T, Mizuno K, Kojima S, Ikeda T. Involvement of nitric oxide in the ocular hypotensive action of nipradilol. *Curr Eye Res.* 2001;23:346-351.
52. Orihashi M, Shima Y, Tsuneki H, Kimura I. Potent reduction of intraocular pressure by nipradilol plus latanoprost in ocular hypertensive rabbits. *Biol Pharm Bull.* 2005;28:65-68.
53. Borghi V, Bastia E, Guzzetta M, et al. A novel nitric oxide releasing prostaglandin analog, NCX 125, reduces intraocular pressure in rabbit, dog, and primate models of glaucoma. *J Ocul Pharmacol Ther.* 2010;26:125-132.
54. Impagnatiello F, Borghi V, Gale DC, et al. A dual acting compound with latanoprost amide and nitric oxide releasing properties, shows ocular hypotensive effects in rabbits and dogs. *Exp Eye Res.* 2011;93:243-249.
55. Krauss AH, Impagnatiello F, Toris CB, et al. Ocular hypotensive activity of BOL-303259-X, a nitric oxide donating prostaglandin F₂alpha agonist, in preclinical models. *Exp Eye Res.* 2011;93:250-255.
56. Wizemann AJ, Wizemann V. Organic nitrate therapy in glaucoma. *Am J Ophthalmol.* 1980;90:106-109.
57. Diestelhorst M, Hinzpeter B, Krieglstein GK. The effect of isosorbide-mononitrate eye drops on the human intraocular pressure and aqueous humor dynamics. *Int Ophthalmol.* 1991;15:259-262.
58. Chuman H, Chuman T, Nao-i N, Sawada A. The effect of L-arginine on intraocular pressure in the human eye. *Curr Eye Res.* 2000;20:511-516.
59. Inoue K, Noguchi K, Wakakura M, Tomita G. Effect of five years of treatment with nipradilol eye drops in patients with normal tension glaucoma. *Clin Ophthalmol.* 2011;5:1211-1216.
60. Kanno M, Araie M, Koibuchi H, Masuda K. Effects of topical nipradilol, a beta blocking agent with alpha blocking and nitroglycerin-like activities, on intraocular pressure and aqueous dynamics in humans. *Br J Ophthalmol.* 2000;84:293-299.
61. Eveleth D, Starita C, Tressler CA. 4-week, dose-ranging study comparing the efficacy, safety and tolerability of latanoprost 75, 100 and 125 mug/mL to latanoprost 50 mug/mL (xalatan) in the treatment of primary open-angle glaucoma and ocular hypertension. *BMC Ophthalmol.* 2012;12:9.
62. Sambhara D, Aref AA. Glaucoma management: relative value and place in therapy of available drug treatments. *Ther Adv Chronic Dis.* 2014;5:30-43.
63. Kee C, Kaufman PL, Gabelt BT. Effect of 8-Br cGMP on aqueous humor dynamics in monkeys. *Invest Ophthalmol Vis Sci.* 1994;35:2769-2773.
64. Kotikoski H, Vapaatalo H, Oksala O. Nitric oxide and cyclic GMP enhance aqueous humor outflow facility in rabbits. *Curr Eye Res.* 2003;26:119-123.
65. Ellis DZ, Dismuke WM, Chokshi BM. Characterization of soluble guanylate cyclase in NO-induced increases in aqueous humor outflow facility and in the trabecular meshwork. *Invest Ophthalmol Vis Sci.* 2009;50:1808-1813.
66. Dismuke WM, Mbadugha CC, Ellis DZ. NO-induced regulation of human trabecular meshwork cell volume and aqueous humor outflow facility involve the BKCa ion channel. *Am J Physiol Cell Physiol.* 2008;294:C1378-C1386.
67. Arnal JF, Dinh-Xuan AT, Pueyo M, Darblade B, Rami J. Endothelium-derived nitric oxide and vascular physiology and pathology. *Cell Mol Life Sci.* 1999;55:1078-1087.
68. Bolz SS, Vogel L, Sollinger D, et al. Nitric oxide-induced decrease in calcium sensitivity of resistance arteries is attributable to activation of the myosin light chain phosphatase and antagonized by the RhoA/Rho kinase pathway. *Circulation.* 2003;107:3081-3087.
69. Etter EF, Eto M, Wardle RL, Brautigan DL, Murphy RA. Activation of myosin light chain phosphatase in intact arterial

- smooth muscle during nitric oxide-induced relaxation. *J Biol Chem.* 2001;276:34681-34685.
70. Sauzeau V, Le Jeune H, Cario-Toumaniantz C, et al. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca²⁺ sensitization of contraction in vascular smooth muscle. *J Biol Chem.* 2000;275:21722-21729.
 71. Thoonen R, Sips PY, Bloch KD, Buys ES. Pathophysiology of hypertension in the absence of nitric oxide/cyclic GMP signaling. *Curr Hypertens Rep.* 2013;15:47-58.
 72. Ramachandran C, Patil RV, Combrink K, Sharif NA, Srinivas SP. Rho-Rho kinase pathway in the actomyosin contraction and cell-matrix adhesion in immortalized human trabecular meshwork cells. *Mol Vis.* 2011;17:1877-1890.
 73. Rao PV, Deng P, Maddala R, Epstein DL, Li CY, Shimokawa H. Expression of dominant negative Rho-binding domain of Rho-kinase in organ cultured human eye anterior segments increases aqueous humor outflow. *Mol Vis.* 2005;11:288-297.
 74. Wang JW, Woodward DF, Stamer WD. Differential effects of prostaglandin E2-sensitive receptors on contractility of human ocular cells that regulate conventional outflow. *Invest Ophthalmol Vis Sci.* 2013;54:4782-4790.
 75. Pattabiraman PP, Pecan PE, Rao PV. MRP4-mediated regulation of intracellular cAMP and cGMP levels in trabecular meshwork cells and homeostasis of intraocular pressure. *Invest Ophthalmol Vis Sci.* 2013;54:1636-1649.
 76. Dismuke WM, Liang J, Overby DR, Stamer WD. Concentration-related effects of nitric oxide and endothelin-1 on human trabecular meshwork cell contractility. *Exp Eye Res.* 2013;120C:28-35.
 77. Goel M, Picciani RG, Lee RK, Bhattacharya SK. Aqueous humor dynamics: a review. *Open Ophthalmol J.* 2010;4:52-59.
 78. Kameda T, Inoue T, Inatani M, et al. The effect of Rho-associated protein kinase inhibitor on monkey Schlemm's canal endothelial cells. *Invest Ophthalmol Vis Sci.* 2012;53:3092-3103.
 79. Tousoulis D, Androulakis E, Papageorgiou N, Stefanadis C. Novel therapeutic strategies in the management of arterial hypertension. *Pharmacol Ther.* 2012;135:168-175.
 80. Krupin T, Weiss A, Becker B, Holmberg N, Fritz C. Increased intraocular pressure following topical azide or nitroprusside. *Invest Ophthalmol Vis Sci.* 1977;16:1002-1007.
 81. Zamora DO, Kiel JW. Episcleral venous pressure responses to topical nitroprusside and N-Nitro-L-arginine methyl ester. *Invest Ophthalmol Vis Sci.* 2010;51:1614-1620.
 82. Funk RH, Gehr J, Rohen JW. Short-term hemodynamic changes in episcleral arteriovenous anastomoses correlate with venous pressure and IOP changes in the albino rabbit. *Curr Eye Res.* 1996;15:87-93.
 83. Millar JC, Shahidullah M, Wilson WS. Intraocular pressure and vascular effects of sodium azide in bovine perfused eye. *J Ocul Pharmacol Ther.* 2001;17:225-234.
 84. Shahidullah M, Yap M, To CH. Cyclic GMP sodium nitroprusside and sodium azide reduce aqueous humour formation in the isolated arterially perfused pig eye. *Br J Pharmacol.* 2005;145:84-92.
 85. Miller TL, Willis AM, Wilkie DA, Hoshaw-Woodard S, Stanley JR. Description of ciliary body anatomy and identification of sites for transscleral cyclophotocoagulation in the equine eye. *Vet Ophthalmol.* 2001;4:183-190.
 86. Ellis DZ, Nathanson JA, Rabe J, Sweadner KJ. Carbachol and nitric oxide inhibition of Na,K-ATPase activity in bovine ciliary processes. *Invest Ophthalmol Vis Sci.* 2001;42:2625-2631.
 87. Shahidullah M, Delamere NA. NO donors inhibit Na,K-ATPase activity by a protein kinase G-dependent mechanism in the nonpigmented ciliary epithelium of the porcine eye. *Br J Pharmacol.* 2006;148:871-880.
 88. Shahidullah M, Wei G, Delamere NA. DIDS inhibits Na-K-ATPase activity in porcine nonpigmented ciliary epithelial cells by a Src family kinase-dependent mechanism. *Am J Physiol Cell Physiol.* 2013;305:C492-C501.
 89. Fleischhauer JC, Beny JL, Flammer J, Haefliger IO. NO/cGMP pathway activation and membrane potential depolarization in pig ciliary epithelium. *Invest Ophthalmol Vis Sci.* 2000;41:1759-1763.
 90. Wu R, Yao K, Flammer J, Haefliger IO. Role of anions in nitric oxide-induced short-circuit current increase in isolated porcine ciliary processes. *Invest Ophthalmol Vis Sci.* 2004;45:3213-3222.
 91. Johnson EC, Morrison JC. Friend or foe? Resolving the impact of glial responses in glaucoma. *J Glaucoma.* 2009;18:341-353.
 92. Neufeld AH, Hernandez MR, Gonzalez M. Nitric oxide synthase in the human glaucomatous optic nerve head. *Arch Ophthalmol.* 1997;115:497-503.
 93. Neufeld AH, Kawai S, Das S, et al. Loss of retinal ganglion cells following retinal ischemia: the role of inducible nitric oxide synthase. *Exp Eye Res.* 2002;75:521-528.
 94. Shareef S, Sawada A, Neufeld AH. Isoforms of nitric oxide synthase in the optic nerves of rat eyes with chronic moderately elevated intraocular pressure. *Invest Ophthalmol Vis Sci.* 1999;40:2884-2891.
 95. Neufeld AH, Das S, Vora S, et al. A prodrug of a selective inhibitor of inducible nitric oxide synthase is neuroprotective in the rat model of glaucoma. *J Glaucoma.* 2002;11:221-225.
 96. Neufeld AH, Sawada A, Becker B. Inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma. *Proc Natl Acad Sci U S A.* 1999;96:9944-9948.
 97. Flammer J, Mozaffarieh M. What is the present pathogenetic concept of glaucomatous optic neuropathy? *Surv Ophthalmol.* 2007;52(suppl 2):S162-S173.
 98. Pang IH, Johnson EC, Jia L, et al. Evaluation of inducible nitric oxide synthase in glaucomatous optic neuropathy and pressure-induced optic nerve damage. *Invest Ophthalmol Vis Sci.* 2005;46:1313-1321.
 99. Libby RT, Howell GR, Pang IH, et al. Inducible nitric oxide synthase, Nos2, does not mediate optic neuropathy and retinopathy in the DBA/2J glaucoma model. *BMC Neurosci.* 2007;8:108.
 100. Chen C, Xu Y, Zhang J, et al. Altered expression of nNOS/N1DD in the retina of a glaucoma model of DBA/2J mice and the intervention by nNOS inhibition. *J Mol Neurosci.* 2013;51:47-56.
 101. Karim MZ, Sawada A, Mizuno K, Kawakami H, Ishida K, Yamamoto T. Neuroprotective effect of nipradilol [3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran] in a rat model of optic nerve degeneration. *J Glaucoma.* 2009;18:26-31.
 102. Mizuno K, Koide T, Shimada S, Mori J, Sawanobori K, Araie M. Route of penetration of topically instilled nipradilol into the ipsilateral posterior retina. *Invest Ophthalmol Vis Sci.* 2009;50:2839-2847.
 103. Mizuno K, Koide T, Yoshimura M, Araie M. Neuroprotective effect and intraocular penetration of nipradilol, a beta-blocker with nitric oxide donative action. *Invest Ophthalmol Vis Sci.* 2001;42:688-694.
 104. Watanabe M, Tokita Y, Yata T. Axonal regeneration of cat retinal ganglion cells is promoted by nipradilol, an anti-glaucoma drug. *Neuroscience.* 2006;140:517-528.
 105. Kitaoka Y, Kumai T, Isenoumi K, et al. Neuroprotective effect of nitric oxide against NMDA-induced neurotoxicity in the rat retina is associated with tyrosine hydroxylase expression. *Brain Res.* 2003;977:46-54.
 106. Zurakowski D, Vorwerk CK, Gorla M, et al. Nitrate therapy may retard glaucomatous optic neuropathy, perhaps through

- modulation of glutamate receptors. *Vision Res.* 1998;38:1489-1494.
107. Takahata K, Katsuki H, Kume T, et al. Retinal neuronal death induced by intraocular administration of a nitric oxide donor and its rescue by neurotrophic factors in rats. *Invest Ophthalmol Vis Sci.* 2003;44:1760-1766.
 108. Kashii S, Mandai M, Kikuchi M, et al. Dual actions of nitric oxide in N-methyl-D-aspartate receptor-mediated neurotoxicity in cultured retinal neurons. *Brain Res.* 1996;711:93-101.
 109. Kashiwagi K, Iizuka Y, Tanaka Y, et al. Dual action of nitric oxide on purely isolated retinal ganglion cells. *Curr Eye Res.* 2001;23:233-239.
 110. Koriyama Y, Kamiya M, Takadera T, et al. Protective action of nipradilol mediated through S-nitrosylation of Keap1 and HO-1 induction in retinal ganglion cells. *Neurochem Int.* 2012;61:1242-1253.
 111. Koriyama Y, Kamiya M, Arai K, Sugitani K, Ogai K, Kato S. Nipradilol promotes axon regeneration through S-nitrosylation of PTEN in retinal ganglion cells. *Adv Exp Med Biol.* 2014;801:751-757.
 112. Wenk GL, McGann-Gramling K, Hauss-Wegrzyniak B, et al. Attenuation of chronic neuroinflammation by a nitric oxide-releasing derivative of the antioxidant ferulic acid. *J Neurochem.* 2004;89:484-493.
 113. Ronchetti D, Impagnatiello F, Guzzetta M, et al. Modulation of iNOS expression by a nitric oxide-releasing derivative of the natural antioxidant ferulic acid in activated RAW 264.7 macrophages. *Eur J Pharmacol.* 2006;532:162-169.
 114. Katsuyama K, Shichiri M, Marumo F, Hirata Y. NO inhibits cytokine-induced iNOS expression and NF-kappaB activation by interfering with phosphorylation and degradation of I-kappaB-alpha. *Arterioscler Thromb Vasc Biol.* 1998;18:1796-1802.
 115. Cavicchi M, Gibbs L, Whittle BJ. Inhibition of inducible nitric oxide synthase in the human intestinal epithelial cell line, DLD-1, by the inducers of heme oxygenase 1, bismuth salts, heme, and nitric oxide donors. *Gut.* 2000;47:771-778.
 116. Findl O, Rainer G, Dallinger S, et al. Assessment of optic disk blood flow in patients with open-angle glaucoma. *Am J Ophthalmol.* 2000;130:589-596.
 117. Michelson G, Langhans MJ, Harazny J, Dichtl A. Visual field defect and perfusion of the juxtapapillary retina and the neuroretinal rim area in primary open-angle glaucoma. *Graefes Arch Clin Exp Ophthalmol.* 1998;236:80-85.
 118. Grunwald JE, Piltz J, Hariprasad SM, DuPont J. Optic nerve and choroidal circulation in glaucoma. *Invest Ophthalmol Vis Sci.* 1998;39:2329-2336.
 119. Flammer J. The vascular concept of glaucoma. *Surv Ophthalmol.* 1994;38(suppl):S3-S6.
 120. Flammer J, Orgul S. Optic nerve blood-flow abnormalities in glaucoma. *Prog Retin Eye Res.* 1998;17:267-289.
 121. Haefliger IO, Flammer J, Beny JL, Luscher TF. Endothelium-dependent vasoactive modulation in the ophthalmic circulation. *Prog Retin Eye Res.* 2001;20:209-225.
 122. Chen Z, Gu Q, Kaufman PL, Cynader MS. Histochemical mapping of NADPH-diaphorase in monkey and human eyes. *Curr Eye Res.* 1998;17:370-379.
 123. Hashitani H, Windle A, Suzuki H. Neuroeffector transmission in arterioles of the guinea-pig choroid. *J Physiol.* 1998;510(pt 1):209-223.
 124. Yamamoto R, Bredt DS, Snyder SH, Stone RA. The localization of nitric oxide synthase in the rat eye and related cranial ganglia. *Neuroscience.* 1993;54:189-200.
 125. Haefliger IO, Flammer J, Luscher TF. Nitric oxide and endothelin-1 are important regulators of human ophthalmic artery. *Invest Ophthalmol Vis Sci.* 1992;33:2340-2343.
 126. Yao K, Tschudi M, Flammer J, Luscher TF. Endothelium-dependent regulation of vascular tone of the porcine ophthalmic artery. *Invest Ophthalmol Vis Sci.* 1991;32:1791-1798.
 127. Benedito S, Prieto D, Nielsen PJ, Nyborg NC. Role of the endothelium in acetylcholine-induced relaxation and spontaneous tone of bovine isolated retinal small arteries. *Exp Eye Res.* 1991;52:575-579.
 128. Deussen A, Sonntag M, Vogel R. L-arginine-derived nitric oxide: a major determinant of uveal blood flow. *Exp Eye Res.* 1993;57:129-134.
 129. Granstam E, Granstam SO, Fellstrom B, Lind L. Endothelium-dependent vasodilation in the uvea of hypertensive and normotensive rats. *Curr Eye Res.* 1998;17:189-196.
 130. Seligsohn EE, Bill A. Effects of NG-nitro-L-arginine methyl ester on the cardiovascular system of the anaesthetized rabbit and on the cardiovascular response to thyrotropin-releasing hormone. *Br J Pharmacol.* 1993;109:1219-1225.
 131. Jacot JL, O'Neill JT, Scandling DM, West SD, McKenzie JE. Nitric oxide modulation of retinal, choroidal, and anterior uveal blood flow in newborn piglets. *J Ocul Pharmacol Ther.* 1998;14:473-489.
 132. Dorner GT, Garhofer G, Kiss B, et al. Nitric oxide regulates retinal vascular tone in humans. *Am J Physiol Heart Circ Physiol.* 2003;285:H631-H636.
 133. Luksch A, Polak K, Beier C, et al. Effects of systemic NO synthase inhibition on choroidal and optic nerve head blood flow in healthy subjects. *Invest Ophthalmol Vis Sci.* 2000;41:3080-3084.
 134. Schmetterer L, Krejcy K, Kastner J, et al. The effect of systemic nitric oxide-synthase inhibition on ocular fundus pulsations in man. *Exp Eye Res.* 1997;64:305-312.
 135. Polak K, Luksch A, Berisha F, Fuchsjaeger-Mayrl G, Dallinger S, Schmetterer L. Altered nitric oxide system in patients with open-angle glaucoma. *Arch Ophthalmol.* 2007;125:494-498.
 136. Delaey C, Van de Voorde J. The effect of NO donors on bovine retinal small arteries and posterior ciliary arteries. *Invest Ophthalmol Vis Sci.* 1998;39:1642-1646.
 137. Okamura T, Kitamura Y, Uchiyama M, Toda M, Ayajiki K, Toda N. Canine retinal arterial and arteriolar dilatation induced by nipradilol, a possible glaucoma therapeutic. *Pharmacology.* 1996;53:302-310.
 138. Donati G, Pournaras CJ, Tsacopoulos M. Effect of nitroprusside on arteriolar constriction after retinal branch vein occlusion. *Invest Ophthalmol Vis Sci.* 1998;39:1910-1917.
 139. Kitamura Y, Okamura T, Kani K, Toda N. Nitric oxide-mediated retinal arteriolar and arterial dilatation induced by substance P. *Invest Ophthalmol Vis Sci.* 1993;34:2859-2865.
 140. Ogawa N, Mori A, Hasebe M, et al. Nitric oxide dilates rat retinal blood vessels by cyclooxygenase-dependent mechanisms. *Am J Physiol Regul Integr Comp Physiol.* 2009;297:R968-R977.
 141. Schmidl D, Polska E, Kiss B, Sacu S, Garhofer G, Schmetterer L. Ocular hemodynamic effects of nitrovasodilators in healthy subjects. *Clin Pharmacol Ther.* 2010;87:87-92.
 142. Grunwald JE, Iannaccone A, DuPont J. Effect of isosorbide mononitrate on the human optic nerve and choroidal circulations. *Br J Ophthalmol.* 1999;83:162-167.
 143. Iannaccone AE, DuPont J, Grunwald JE. Human retinal hemodynamics following administration of 5-isosorbide mononitrate. *Curr Eye Res.* 2000;20:205-210.
 144. Kida T, Sugiyama T, Harino S, Kitanishi K, Ikeda T. The effect of nipradilol, an alpha-beta blocker, on retinal blood flow in healthy volunteers. *Curr Eye Res.* 2001;23:128-132.
 145. Mizuno K, Koide T, Saito N, et al. Topical nipradilol: effects on optic nerve head circulation in humans and periorbital distribution in monkeys. *Invest Ophthalmol Vis Sci.* 2002;43:3243-3250.
 146. Keeble JE, Moore PK. Pharmacology and potential therapeutic applications of nitric oxide-releasing non-steroidal anti-

- inflammatory and related nitric oxide-donating drugs. *Br J Pharmacol.* 2002;137:295-310.
147. Mariotto S, Suzuki Y, Persichini T, Colasanti M, Suzuki H, Cantoni O. Cross-talk between NO and arachidonic acid in inflammation. *Curr Med Chem.* 2007;14:1940-1944.
148. Guimaraes C, Assreuy J, Linden R. Paracrine neuroprotective effect of nitric oxide in the developing retina. *J Neurochem.* 2001;76:1233-1241.
149. Mejia-Garcia TA, Portugal CC, Encarnacao TG, Prado MA, Paes-de-Carvalho R. Nitric oxide regulates AKT phosphorylation and nuclear translocation in cultured retinal cells. *Cell Signal.* 2013;25:2424-2439.
150. Calabrese EJ. Nitric oxide: biphasic dose responses. *Crit Rev Toxicol.* 2001;31:489-501.
151. Stamer WD, Acott TS. Current understanding of conventional outflow dysfunction in glaucoma. *Curr Opin Ophthalmol.* 2012;23:135-143.
152. Toris CB, Yablonski ME, Wang YL, Camras CB. Aqueous humor dynamics in the aging human eye. *Am J Ophthalmol.* 1999;127:407-412.