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

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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.5

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 phosphodiesterases to produce physiological effects. Other biological actions occur through cGMP-independent pathways such as posttranslational modification of proteins by S-nitrosylation3 and signaling through NF-kB.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
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. 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.

Converging evidence indicates that the NOcGMP 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. Neuronal NOS is localized in nerve fibers in the limbus, cornea, and lens epithelium. Following cytokine and endotoxin stimulation iNOS is detected in the iris/ciliary body and vessels. 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.

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. The results support the general conclusion that endogenous NO 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). Isolated strips of bovine TM and ciliary muscle contract in response to L-NAME. Accordingly, in monkey ciliary muscle, L-arginine (the endogenous substrate of NO synthase) enhances the contraction induced by carbachol. Similarly, Schneemann et al. 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 colleagues 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. 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. 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). 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).

Overall, these findings support the concept that NO is
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. In addition, NO levels were decreased in the AH of patients with POAG. On the other hand, L-arginine levels were significantly increased in vitreous humor of glaucomatous state. Buys et al. revealed a decrease in AH of patients with POAG. On the other hand, L-arginine levels were significantly increased in vitreous humor of glaucomatous state. Buys et al. revealed a decrease in AH of patients with POAG. On the other hand, L-arginine levels were significantly increased in vitreous humor of glaucomatous state. Buys et al. revealed a decrease in AH of patients with POAG.

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. 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. Komatsubara et al. demonstrated the IOP lowering effects of SNP, nitrosothiol, and spermene 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 5-morpholinolsydnonimine (SIN-1) or S-nitroacetylpenicillamine (SNAP) induced a dramatic IOP lowering dependent on the amount of NO delivered to the eye. Carreiro and coworkers 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 SNP 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.

In a nonhuman primate study, topical administration of nitroglycerin at a dose of 0.1% significantly decreased IOP in normotensive animals after 90 minutes. Conversely, Wang and Podos 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 5 hours post dose (Fig. 2).

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. A later study 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.

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. Latanoprostone 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.

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 Wizemann 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
had a potent lowering effect on IOP.\textsuperscript{56} Moreover, topically applied ISMN (2\%) significantly reduced IOP within 3 hours after dosing.\textsuperscript{57} In addition, intravenous administration of the NO precursor Larginine in humans significantly decreased IOP, resulting in an average drop of 2 mm Hg in healthy subjects.\textsuperscript{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.\textsuperscript{59} In patients with ocular hypertension twice daily dosing with 0.25\% nipradilol decreased IOP and also reduced the aqueous flow rate.\textsuperscript{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 active 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 resting tension or after contraction using carbachol, pointing to this tissue as the site mediating an increased conventional outflow and IOP lowering in vivo.\textsuperscript{51} SNAP increased flow rate in human donor eye anterior segments maintained in an organ culture perfusion system.\textsuperscript{30} In addition, recent studies have demonstrated that diethylenetriamine (DETA)-NO increased outflow facility by 77\% in the anterior segments, mediated by increased sGC activity and cGMP production\textsuperscript{65} (Fig. 3). Further investigation into the cellular signaling responsible for the increase in outflow facility mediated predominantly via an increase in conventional outflow facility and 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 \(\beta\)-adrenergic receptor blockers, \(\alpha\)-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.\textsuperscript{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.\textsuperscript{49} In another primate study, the cGMP analog 8-BrcGMP increased outflow facility by approximately 30\% after intracameral infusion.\textsuperscript{53} In rabbit eyes, topical administration of nitroglycerin or intracameral administration of SNP increased conventional outflow facility.\textsuperscript{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 vivo mouse eyes (Chang J, et al. IOVS 2013: 54;ARVO E-Abstract 2002). Likewise, 1 mM SNP, administered intracameraly, increased outflow facility by 77\% in the cynomolgus monkey.\textsuperscript{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.\textsuperscript{31} SNP increased flow rate in human donor eye anterior segments maintained in an organ culture perfusion system.\textsuperscript{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 production\textsuperscript{65} (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\textsuperscript{2+}-activated K\textsuperscript{+} (BKCa) channels resulting in a decrease in TM cell volume.\textsuperscript{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.\textsuperscript{57} 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 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.
through 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. In the TM, pharmacological inhibition of MLCK-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. 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. 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 μM) were not effective in eliciting cell relaxation.

The Schlemm's canal consists of endothelial cells and connective tissue, similar in structure to a vein. 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. By analogy to vascular endothelial cells, where the vasodilatory effects of NO are well understood, 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 vasodilatory effects of NO are well understood, 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.

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 mg or increase EVP after 0.5 mg in rabbits. In both of these studies, however, there was a concomitant increase in IOP Funk et al. 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. Isosorbide dinitrate and SNP were effective in producing pronounced relaxation of cynomolgus and rhesus monkey longitudinal cibachol-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). 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. This reduction in AHF was accompanied by an increase in cGMP in the ciliary epithelium. 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. 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. 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

Glucoma is characterized by progressive optic nerve damage, loss of retinal ganglion cells, nerve fiber layers thinning, and extensive remodeling of the surrounding tissues. 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. Other reports detected high expression of iNOS in the retina after ischemia and after catarterization of the episcleral veins to elevate IOP. In the vein cataraterization rat model it was demonstrated that inhibition of iNOS prevented loss of retinal ganglion cells (RGCs). 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. 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. In addition, NO levels from POAG and nonglaucomatous human eyes were similar. In the DBA/2 mouse glaucoma model, neither genetic deficiency of iNOS nor inhibition of iNOS using aminoguanidine had any detectable effect on glaucomatous optic nerve damage. 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. Therefore, further studies are necessary to fully understand the role of endogenous NO through upregulation of NOS in optic neuropathy.

The glaucoma drug nepadrilol 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% nepadrilol (BID) was demonstrated to reduce RGC death as compared with control. Studies in rabbits also demonstrated that topical nepadrilol reaches the retina–choroid in effective concentrations after topical application. Other studies have also shown neuroprotective effects of intravitreal administration of nepadrilol and SNP after N-methyl-D-aspartate (NMDA)–induced retinal damage in rats and in an axomotized cat model. NMDA-induced neurotoxicity was also significantly reduced by 1-hydroxy-2-oxo-3,3-bis (2-aminoethyl)-1-triazene (NOC18) in rat retina. A clinical study found...
that risk of optic nerve degeneration was lower in glaucoma patients receiving nitroglycerin for nonphthisalnic reasons than in the control group.\textsuperscript{106} On the other hand, intravitreal administration of the NO releasing compound N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrososilazanethane (NOC12) induced retinal neurotoxicity in rats.\textsuperscript{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 (≤ 1 μM) or S-nitroscysteine (50 μM) increased survival of retinal neuronal cells, while SNAP at greater than or equal to 100 μM or 500 μM of S-nitroscysteine decreased neuronal cell survival.\textsuperscript{108,109} Inhibiting endogenous NO using L-NAME or the NO scavenger cPTIO also concentration-dependently decreased RGC survival.\textsuperscript{108,109} 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 activation\textsuperscript{112} in an NO-dependent manner,\textsuperscript{113} the NO donors (≥)E-ethyl-2-[E-hydroxyaminol]-S-nitro-3-hexenamide (NOR3) and SNP decreased iNOS expression in VSMC.\textsuperscript{114} The NO donors SNP and SNAP inhibited iNOS activity in intestinal epithelial cells,\textsuperscript{115} raising the possibility that exogenous NO could mitigate the increased NO levels observed in the optic nerve head in glaucoma.

Reduced ONH blood flow in POAG has been shown in a number of investigations.\textsuperscript{116-118} According to the vascular concept of glaucoma,\textsuperscript{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.\textsuperscript{117,121} A number of studies in animals and humans show that the vascular endothelium of the retina and choroid express NOS.\textsuperscript{122-124} The importance of NO-mediated control of basal ocular vascular tone is demonstrated by studies in which NO inhibition results in vasoconstriction of isolated porcine and human ophthalmic artery segments.\textsuperscript{125,126} In agreement with this finding, inhibition of sGC results in contraction of isolated bovine retinal arteries.\textsuperscript{127} Likewise, in in vivo studies inhibition of NOS reduces basal blood flow in the choroid of dogs, rabbits, pigs, and rats.\textsuperscript{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.\textsuperscript{132-134} Interestingly, in POAG patients, Polak and colleagues\textsuperscript{135} showed less pronounced decrease of blood flow in the ONH and the choroid after systemic NOS inhibition with LNMMA 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 endothelia may be neuroprotective through causing vasodilatation and increased ONH blood flow.\textsuperscript{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.\textsuperscript{127,136} Likewise, nipradilol produces retinal arterial dilation in isolated canine arterial strips and it dilates arterioles in anesthetized dogs following intra-arterial injections.\textsuperscript{137} Similarly, nitroglycerin and SNP cause vasodilatation of the choroidal and retinal blood vessels in anesthetized dogs and in a model of branch vein occlusion in pigs.\textsuperscript{138,139} Another study demonstrated that NOS inhibition using intravenous L-NMMA decreased retinal blood vessel diameter while SNP and NOR3 resulted in vessel dilation.\textsuperscript{140} On the other hand, Granstam and colleagues\textsuperscript{129} 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.\textsuperscript{141} In contrast, in healthy subjects, oral administration of ISMN increased optic nerve blood flow, but not blood flow in the choroid or retina.\textsuperscript{142,143} Moreover, in healthy volunteers topical nipradilol significantly increased retinal arterial blood flow and ONH blood velocity.\textsuperscript{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.\textsuperscript{146} For instance, NO inhibits the activation of NF-kB as well as of various pro-inflammatory proteins including iNOS, TNF-α, and other cytokines.\textsuperscript{145,147} 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.\textsuperscript{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. 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. 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, 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 drainage tissue, which is compromised in glaucoma patients and continues to deteriorate over time. 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.

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