Nitric oxide: a drug target for glaucoma revisited

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A reduction in intraocular pressure (IOP) is the only recognized therapy for glaucoma. Hence, drugs exhibiting ocular hypotensive effects are important targets for antiglaucomatous drug development. IOP is determined by the equilibrium of aqueous humor production and outflow through either the trabecular meshwork or the uveoscleral outflow pathway. There is increasing evidence that nitric oxide (NO) has a major role in the regulation of IOP by directly acting on the trabecular meshwork and thereby lowering IOP. Taking advantage of this mechanism, newly designed NO-donating drugs combine the IOP-lowering effect of known substances with the trabecular meshwork outflow-increasing effect of NO. Here, we review the molecular mechanism of this new entity of IOP-lowering drugs.

Introduction

Glaucoma is an umbrella term referring to a complex set of ocular diseases characterized by a distinct pattern of neurodegenerative changes in the optic nerve head and the associated retinal ganglion cells [1]. Clinically speaking, these changes are reflected in a characteristic visual field loss, which makes glaucoma one of the leading causes of age-related irreversible vision loss in industrialized countries [2]. From a therapeutic point of view, the reduction of IOP is the only proven intervention leading to a delay of disease progression [3]. Currently, the main drug classes that are used for lowering IOP are prostaglandin analogues, β-adrenergic receptor antagonists, α-adrenergic agonists, and carbonic anhydrase inhibitors [4]. The mechanism of action of these drugs is related to either a decrease in aqueous humor formation or an increase in uveoscleral outflow [5]. The recent introduction of rho-kinase inhibitors, a new class of drugs that lower IOP by a combined mechanism of increasing trabecular meshwork outflow and decreasing aqueous humor production, has renewed interest in drugs modulating aqueous humor outflow resistance [6]. Although currently available medications overall have a good safety profile and offer a considerable decrease in IOP, a significant number of patients do not sufficiently reach their target pressure and require combination therapy or surgical intervention [3]. NO is a potent regulator of IOP by directly targeting the conventional outflow, thereby providing a strong ocular hypotensive effect. Targeting the trabecular outflow system is attractive, because it provides an independent mechanism to the most frequently used treatment options associated with the potential for effective combination therapy [7].

NO

Since the discovery of NO as a ubiquitous signaling molecule during the 1980s, it became evident that it has a key role in a variety of physiological and pathological processes, including the regulation of vascular tone [8,9]. In the human body, NO is synthesized enzymatically by NO synthase (NOS) from L-arginine via three different isoforms of NOS [10]: the two constitutive forms, endothelial NOS (eNOS, NOS3) [11] and neuronal NOS

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(nNOS, NOS1) [12], produce NO under physiological conditions as a paracrine and autocrine signaling molecule with a short half-life of a few seconds [13]. The third form, inducible NOS (iNOS, NOS2), is synthesized under pathological conditions, such as inflammation or ischemia [14].

In the eye, both constitutive isoforms of NOS have been identified in a variety of ocular tissues, including the vascular endothelium of the retinal and choroidal vessels [15], the rat optic nerve head [16], and the porcine trabecular meshwork and the ciliary body [17]. On a molecular basis, the effect of NO is mainly mediated through the soluble guanylate cyclase pathway. Activation of this pathway results in increased levels of intracellular cGMP [18] and a subsequent modulation of the intracellular calcium concentration. Given the ubiquitous occurrence of the NOS enzyme family, it does not come as a surprise that NO also exerts a variety of different biological functions in the eye, including the regulation of vascular tone [19,20], the processing of the visual stimulus [21], immune cytotoxicity [22], and regulation of IOP [23]. For more details, the reader is referred to review articles summarizing the role of NO in ocular health and disease [21,24–26].

Other gaseous neurotransmitters

It has been recognized that carbon monoxide (CO) shares several similarities with NO as an endogenous signaling molecule and gaseous neurotransmitter. Similar to NO, CO cannot be stored *in vivo* but is enzymatically produced by a family of enzymes catalyzing the metabolism of heme to biliverdin, ferrous iron, and CO. Heme oxygenase-2 (HO2) is present in neurons and regulates the local production of CO upon activation. [27] HO2 exerts a variety of different effects *in vivo*, among which smooth muscle cells might be of particular interest as a potential drug target. CO triggers Ca^{2+}-activated K^+ channels, an effect that is also present in smooth muscle cells. However, *in vivo* data regarding a vascular effect of CO are sparse. One study performed in healthy volunteers showed that inhaled CO leads to an increase in retinal and choroidal blood flow [28].

Based on these observations, it was hypothesized that CO also has a role in the regulation of trabecular meshwork cells and is involved in the regulation of IOP. This concept is supported by the fact that the NO and HO2 system show numerous important interactions. There is evidence that NO can upregulate HO expression through a cGMP-dependent pathway [29]. Given this crosstalk, NO-releasing molecules that are also capable of increasing HO are currently under development [30]. For further information on the biological role of CO as an endogenous neurotransmitter, readers are referred to an excellent review covering the biological functions of CO, its interactions with NO, and its potential role in glaucoma treatment [31].

In addition to NO and CO, hydrogen sulfide (H_2S), another endogenously produced neurotransmitter in gas form, has attracted much interest [32]. Enzymatically produced in several tissues of the eye, such as the cornea and the retina, it has shown to exert a variety of physiological effects, such as vasodilatation or regulation of oxidative stress [33]. There is evidence that H_2S-releasing agents reduce IOP via an increase outflow facility [34]. Dysregulation of endogenous production of H_2S might be involved in the pathogenesis of in several age-related neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [32,35]. However, little is known about a potential role of H_2S in the pathophysiology of ocular neurodegenerative diseases, such as glaucoma.

NO and IOP

Recent evidence underlines the importance of NO as a regulator of IOP [26]. IOP is determined by the equilibrium between aqueous humor production in the ciliary body and the drainage through the two main outflow systems of the eye: the uveoscleral outflow pathway and the trabecular meshwork/Schlemm's canal pathway, the latter of which is also referred to as the conventional outflow pathway [36,37]. Under physiological conditions, aqueous humor is, to a large degree, drained via the pressure-dependent conventional outflow pathway through the trabecular meshwork and subsequently into Schlemm's canal, collector channels, and adjacent veins [36]. It is well accepted that most of the resistance to flow is in the juxtacanalicular region of the trabecular meshwork [37]. Under pathological conditions, such as ocular hypertension or glaucoma, outflow resistance in the conventional outflow pathway is increased, in turn leading to increased IOP values [38].

There is accumulating evidence that NO and its second messenger, cGMP, contribute to the regulation of outflow resistance by directly acting on trabecular meshwork cells [26,38–40]. Histological studies of human and animal tissue indicate that NO synthases are present in the trabecular meshwork and Schlemm's canal [41,42], supporting a direct regulatory role at these sites. Indeed, it has been shown experimentally that NO is produced endogenously in Schlemm's canal cells, thereby regulating cell volume [43]. On a molecular level, free NO activates soluble guanylate cyclase, leading to an increase in cGMP. High cGMP concentrations, in turn, activate and modulate different downstream enzymes, such as cGMP-dependent protein kinases or cGMP-mediated ion channels [26,43]. A more detailed review on the role of the NO–guanylate cyclase pathway in glaucoma is available elsewhere [26].

In vitro studies indicate that trabecular meshwork cells share similarities with smooth muscle cells and are highly reactive in response to NO and endothelin-1, a potent vasoconstrictor. NO leads to a relaxation of both bovine and human trabecular meshwork cells and the ciliary muscle [44,45]. Interestingly, Schlemm's canal endothelial cells respond to shear stress in a similar way to other vascular endothelia. *In vitro* data showed that increased shear stress triggers the production of NO, thus increasing outflow, which might be a feedback loop that normalizes IOP when increased [46]. In contrast to NO, endothelin-1 leads to a pronounced constriction of trabecular meshwork cells, indicating that trabecular meshwork cells are able to rapidly change their contractility status over a wide dynamic range and, therefore, might actively participate in the regulation of outflow resistance [45].

Along this line of thought, it was shown that pharmacological alterations of NO production can alter trabecular outflow and, in turn, IOP. Inhibition of NOS in a perfused organ model using NG-nitro-L-arginine methyl ester citrate (L-NAME) reduced trabecular outflow, indicating that local production of NO contributes to the regulation of basal trabecular outflow and, therefore, might have a role in IOP control [47]. Furthermore, L-NAME reduces IOP in a

dose-dependent manner when applied topically in a rabbit model of increased IOP, whereas no effect was observed in animals with normal IOP [48]. It was hypothesized that this IOP-lowering effect is mainly attributed to a decrease in ciliary blood flow followed by a flow-dependent reduction in aqueous production [49]. Data from a rat model of chronic glaucoma indicated that inhibition of inducible NOS by oral administration of aminoguanidine did not affect IOP [50].

Furthermore, there is evidence that NO has a dilatory effect also distal to the trabecular meshwork. In particular, it has been shown that myosin-containing cells are located adjacent to the collector channels distal to the outer wall of the Schlemm's canal in the aqueous outflow pathway [51]. There is evidence that significant resistance to outflow is located distal to the trabecular meshwork [52]. Recent studies indicate that NO dilates structures of the distal outflow tract and increases outflow facility in a trabecular meshwork-independent fashion in human and porcine eyes [53,54]. However, the degree to which this contributes to the ocular hypotensive effects of NO *in vivo* is currently not known.

There is longstanding evidence that the NO system is impaired in patients with glaucoma. Data from cross-sectional studies published more than 15 years ago showed that aqueous humor nitrite levels are significantly reduced in patients with glaucoma compared with control subjects [55]. This is compatible with the finding that serum levels of two dimethylarginines, ADMA and SDMA, both of which interfere with the production of NO, are associated with advanced glaucoma [56]. Furthermore, using the NO-indicator marker NADPH-diaphorase, histological studies in human donor eyes showed marked differences in the distribution and amount of ocular NO production in the anterior segment of glaucomatous eyes compared with healthy eyes, indicative of an alteration of NOS [57]. This is compatible with data obtained in patients with glaucoma, where decreased cGMP and NO_2– concentrations in the plasma and in aqueous humor have been reported [58]. Data from genome-wide association studies (GWAS) indicate that several genes that are involved in the regulation of the NO system, including those encoding Caveolin 1 and 2, are altered in glaucoma [59].

Recent results from animal models showed that, in transgenic mice overexpressing eNOS, IOP is lower and pressure-dependent drainage is higher compared with wild-type animals [23]. This indicates that increased basal secretion of NO leads to increased drainage, thereby lowering IOP. By contrast, mice lacking the α1 subunit of soluble guanylate cyclase, which mediates the NO effect on a molecular basis, showed increased outflow resistance and increased IOP, confirming that cGMP has an important role in the modulation of trabecular outflow resistance [60]. This finding is also supported by studies in guanylyl cyclase-deficient mice that showed that inhaled NO decreased IOP in the presence of cGMP, whereas mice with low cGMP levels did not exhibit an ocular hypotensive effect [61]. In summary, these results strongly indicate that an altered NO system leads to an increase in IOP and might be involved in glaucoma pathogenesis.

NO-donating drugs for the treatment of glaucoma

There is longstanding evidence from both *in vitro* and *in vivo* experiments that NO has the potential to lower IOP in animals as well as humans [62]. Data from several animal models showed that local intracameral injections and systemic as well as topical administration of NO donators increased trabecular meshwork outflow and in turn decreased IOP [63,64]. As such, it was shown in a rabbit model that topically applied nitroglycerine at a concentration of 0.003–0.1 g % instantly lowered IOP in a dose-dependent fashion, with a peak effect at 1–2 h after instillation on the ocular surface [63]. NO donors, such as isosorbide dinitrate, sodium nitrite, hydralazine, minoxidil, and sodium nitroprusside, mimic the ocular hypotensive actions of nitroglycerin after topical administration [63]. Studies in nonhuman primates confirmed these results. As such, it was shown that the nitrovasodilators nitroglycerin and hydralazine decreased IOP in monkeys, an effect that was paralleled by a 92% increase in outflow facility [64].

However, the clinical use of nitrovasodilators is limited by the short half-life of NO in tissue, which makes it difficult to obtain sufficient drug concentrations at the level of the target tissue. One way to overcome this is to use NO-donating substances, which are metabolized within the eye and release NO within the tissue of interest. Here, we review some of the most promising NO-donating substances and their potential use in glaucoma treatment.

Latanoprostene bunod is a NO-donating drug that is based on a prostaglandin analog backbone with a terminal butyl nitrate ester functional group that acts a as a NO-donating moiety [65,66]. Latanoprostene bunod was approved in the USA in November 2017 for the reduction of IOP in patients with primary open-angle glaucoma under the brand name VYZULTA™ (Bausch and Lomb, USA) at a concentration of 0.024%. Latanoprostene bunod is the first US Food and Drug Administration (FDA)-approved prostaglandin analog, and also acts as a NO donor in the tissue [67]. Chemically speaking, latanoprostene bunod is a single molecule that is enzymatically processed in different ocular compartments, including the cornea, and split into its two active compounds (Fig. 1) [65,68]: first, to the prostanoid F2α receptor agonist latanoprost acid and then to butanediol mononitrate (BMT) [65]. When further enzymatically reduced, BMT splits into the inactive metabolite 1,4 butanediol and NO. Latanoprost acid is a potent and selective agonist at PGF2α-sensitive FP receptors, exerting its IOP-lowering effect by increasing uveoscleral outflow [69]. Data from different animal models showed that exposure to latanoprost acid in different ocular tissues was comparable between either topical administration of latanoprostene bunod or latanoprost, indicating a comparable penetration rate into ocular compartments [65].

Given that latanoprostene bunod comprises two pharmacological active compounds, its clinical effect is based on two different mechanisms of action [67]. First, the prostaglandin analog latanoprost acid lowers IOP mainly by its well-described mechanism of increasing uveoscleral outflow [70]. Second, NO increases trabecular meshwork outflow by activating cGMP-mediated relaxation of trabecular meshwork cells, as described in detail previously [67].

Therefore, the IOP-lowering efficacy of latanoprostene appears superior to that of latanoprost. Indeed, preclinical models in nonhuman primates, dogs, and rabbits with increased IOP showed that latanoprostene bunod is more effective in lowering IOP compared with the prostaglandin analog alone [65]. This is also reflected in human data. A dose-finding study in patients with primary open-angle glaucoma and ocular hypertension showed that latanoprostene at a concentration of 0.024% led to

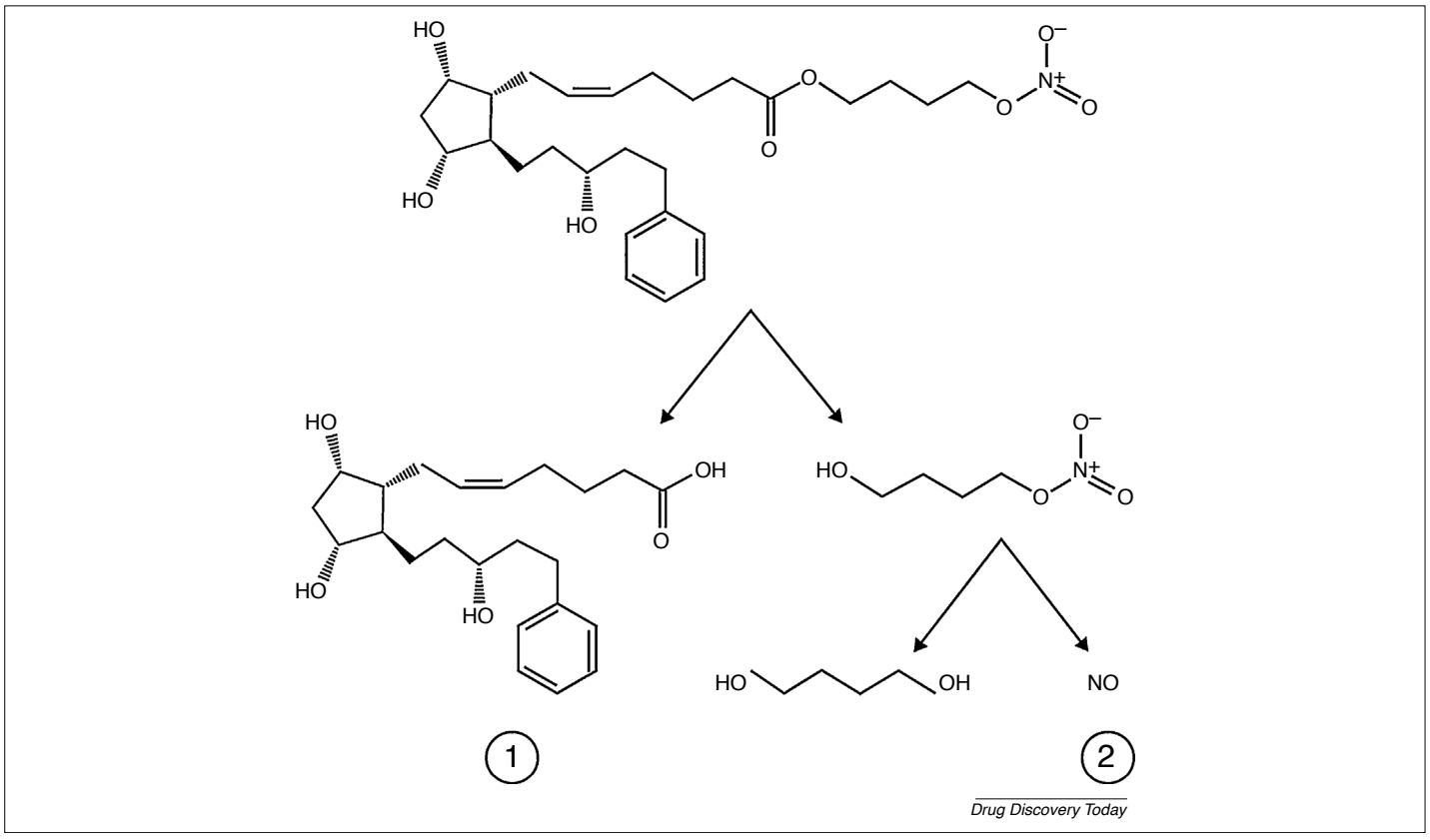

FIGURE 1

Metabolism of latanoprostene bunod to latanoprost acid (1) and butanediol mononitrate with subsequent release of nitric oxide (2) and 1,4-butanediol, an inactive metabolite. Reproduced, with permission, from [68].

a significantly more pronounced reduction in diurnal IOP compared with latanoprost alone [71]. Three Phase III trials have been performed to assess the safety and tolerability of the drug as a basis for regulatory drug approval [68,72,73]. A pooled analysis of the two pivotal studies performed in the USA and Europe including more than 700 subjects showed that latanoprostene 0.024% provided a stronger IOP-lowering effect compared with timolol 0.5% twice daily and maintained IOP reduction through 12 months (Fig. 2) [74]. Hyperemia was the most common adverse effect of latanoprostene bunod treatment and occurred in ~6% of patients, although the most of the cases of hyperemia was mild in severity. Based on the data of the pivotal trial, the authors of the study concluded that the overall safety profile of latanoprostene bunod 0.024% was comparable to that of prostaglandin analogs.

A comparable approach uses the modified prostaglandin F2α analog bimatoprost to carry a NO moiety for extending the IOP-lowering properties of bimatoprost alone. Data from preclinical models in monkeys, rabbits, and dogs showed that the NO-donating bimatoprost, which is also referred to as NCX 470, lowers IOP superior to bimatoprost alone at an equimolar dose [75]. This effect has been attributed to the release of NO, thereby leading to enhanced drainage of aqueous humor via the trabecular meshwork and the Schlemm's canal, as described for latanoprostene bunod [75]. Whether these findings can be translated to patients with glaucoma or ocular hypertension needs to be investigated. Finally, two other compounds, NCX 139, a NO-donating latanoprost amide [76], and NCX 125, a NO-donating latanoprost-free acid [77], have been reported to lower IOP in several animal models. However, as yet, no clinical data have been published regarding their IOP lowering capacity in humans.

Non-IOP-related effects of NO-donating drugs

Apart from its previously discussed IOP modulating effect, NO exerts a variety of other, non-IOP-related physiological functions that might be of importance with respect to glaucoma pathophysiology and treatment. As stated previously, NO is an important regulator of vascular tone in the human body, including the eye [24]. Inhibition of NOS leads to a pronounced reduction of ocular blood flow in animal models [78,79] as well as in humans [80–82], indicating that NO is a major determinant of basal ocular blood flow. Beside regulating basal ocular blood flow, there is compelling evidence that NO is also involved in the autoregulatory response of the ocular vascular system. Autoregulation (the response of the ocular vasculature to keep blood flow constant despite changes of perfusion pressure) is an important intrinsic mechanism to maintain stable tissue blood flow. In a rabbit model, inhibition of NOS modified the choroidal pressure–flow relationship, indicating a a role of NO also in the vascular autoregulatory response [83]. This also appears to hold true for humans: administration of a NOS inhibitor modified the response of the ocular vasculature to changes in ocular perfusion pressure [84,85]. Interestingly, patients with primary open-angle glaucoma showed an abnormal blood flow response to systemic NOS inhibition, indicating abnormal vascular NO levels [86].

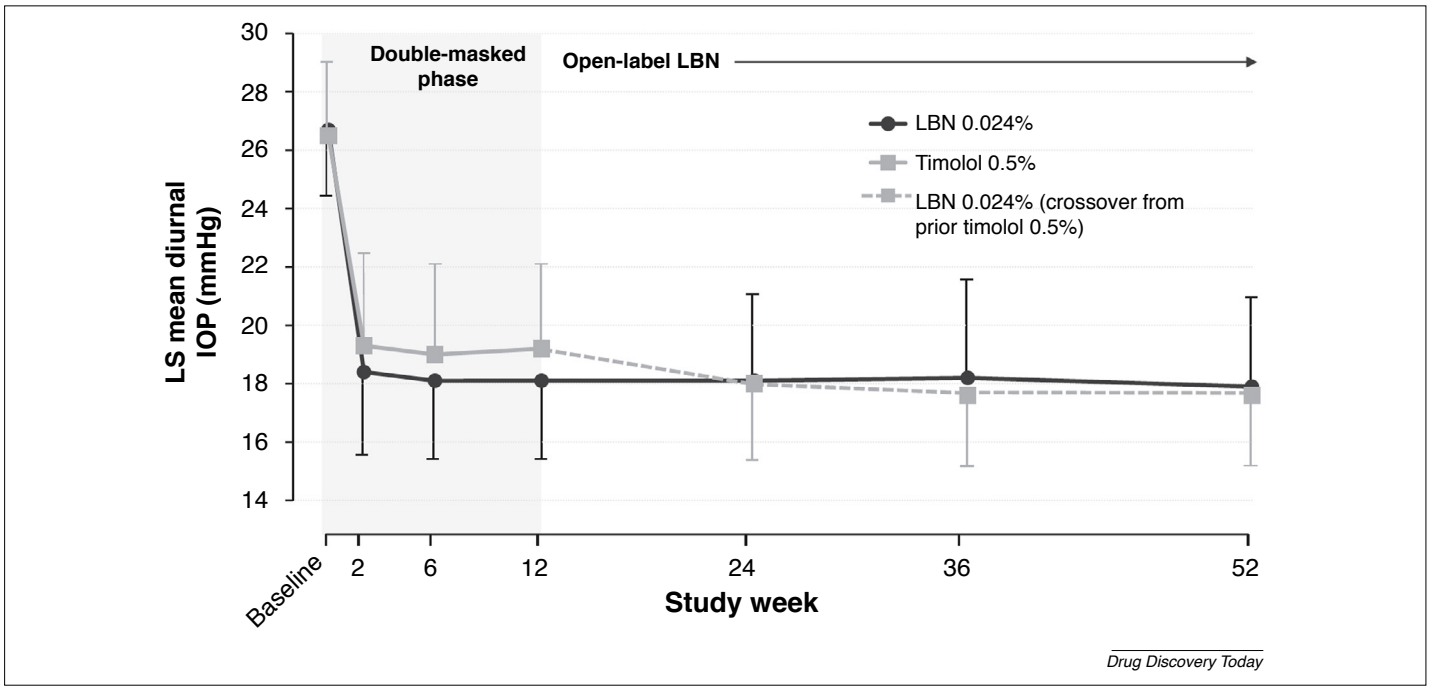

FIGURE 2

Mean diurnal intraocular pressure (IOP) for subjects receiving latanoprostene bunod (LBN 0.024%) or timolol 0.5% in the double-masked efficacy phase. Patients then crossed over to LBN 0.024% in the open-label safety extension phase (ITT population; data as observed). LS, least squares. *$P \leq 0.009$ versus month 3 for subjects randomized to timolol 0.5% in the efficacy phase. Reproduced, with permission, from [74].

Vascular dysregulation as well as reduced ocular perfusion pressure have been implicated in the pathogenesis of glaucoma [87–95]. NO could be an interesting target to tackle non-IOP-related risk factors for glaucoma, such as ocular blood flow impairment. There is evidence from a variety of animal and human trials that, in principle, exogenous administration of NO-releasing drugs leads to retinal vasodilatation corresponding to a decrease in ocular vascular resistance [96,97]. However, this approach is usually limited by the fact that strong vasodilatations when applied systemically lead to a decrease in ocular perfusion pressure, which counteracts the local vasodilatation in the eye. Whether topically administered NO donors can influence blood flow at the level of the retina and optic nerve head is unclear and has yet to be investigated. Recent evidence indicates that latanoprostene bunod causes a more pronounced increase of ocular perfusion pressure compared with timolol via a stronger IOP-lowering effect [98]. Whether this translates into improved blood flow and improved vascular regulation has yet to be investigated. However, there is evidence that a latanoprost-induced decrease in IOP leads to improved vascular regulation because of the decrease in venous ocular pressure [99].

NO can also exhibit neuroprotective effects and promote survival in cultured neuronal cells [100]. The neuroprotective effects of NO are also partially mediated by the activation of the soluble guanylate cyclase pathway and Ca^{2+} channels [101,102]. Apoptosis in cultured neuronal cells can be prevented by either NO or nitrite via cellular elevation of cGMP [102,103]. Neuroprotective effects of the NO-releasing β-receptor antagonist nipradilol are well documented and mediated via NO [104,105]. These data indicate that NO, if delivered in sufficient concentrations to the back of the eye, can at least in principle promote retinal ganglion cell survival. For more detailed reviews of the role of NO in neuroprotection, see [106,107].

Gaps in knowledge and future directions

Whereas the double action of latanoprostene bunod and related drugs on uveoscleral and trabecular outflow has attracted much interest, its relative contribution *in vivo* is not well characterized. One way to gain more insight into this issue is to use ultrahigh-resolution optical coherence tomography imaging of the Schlemm's canal and the collector channels [108,109] to visualize dilatation. Such studies could provide insight into the relative importance of trabecular outflow in the ocular hypotensive effects.

Non-IOP-related effects of latanoprostene bunod, such as the increase in ocular blood flow or neuroprotection, have not been proven *in vivo*. Thus, it is crucial to determine whether NO reaches the posterior pole in a concentration sufficient to elicit such effects. In addition, high NO concentrations can be neurotoxic and induce oxidative stress via reactive nitrogen species, such as peroxynitrite [110]. In a rat model of glaucoma, increased expression of retinal nNOS was associated with retinal ganglion cell degeneration that could be prevented by NOS inhibition [111]. It might be unlikely that such high concentrations reach the posterior pole after topical administration, given the short half-life of NO *in vivo*. By contrast, given these potential toxic effects, it will be important to monitor potential oxidative damage at anterior segment tissues, such as the trabecular meshwork in particular, after long-term treatment with NO-releasing substances.

Concluding remarks

There is evidence that NO has an important role in the regulation of IOP in healthy as well as diseased eyes. Given that NO is able to modulate the conventional outflow facility by directly acting on the trabecular meshwork, NO donors could be a valuable option to lower IOP by targeting a complementary outflow compared with

drugs that are currently most widely used for the treatment of glaucoma. Recently, latanoprostene bunod, the first NO donating drug, was approved by the FDA, and several others are currently under clinical development. Whether NO-donating drugs exert effects other than lowering IOP, such as vasodilatation or direct neuroprotection, needs to be investigated.

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