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The Nitric Oxide-Guanylate Cyclase Pathway and Glaucoma

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Abstract

Glaucoma is a prevalent optic neuropathy characterized by the progressive dysfunction and loss of retinal ganglion cells (RGCs) and their optic nerve axons leading to irreversible visual field loss. Multiple risk factors for the disease have been identified, but elevated intraocular pressure (IOP) remains the primary risk factor amenable to treatment. Reducing IOP however does not always prevent glaucomatous neurodegeneration, and many patients progress with the disease despite having IOP in the normal range. There is increasing evidence that nitric oxide (NO) is a direct regulator of IOP and that dysfunction of the NO- Guanylate Cyclase (GC) pathway is associated with glaucoma incidence. NO has shown promise as a novel therapeutic with targeted effects that: 1) lower IOP; 2) increase ocular blood flow; and 3) confer neuroprotection. The various effects of NO in the eye appear to be mediated through the activation of the GC- guanosine 3:5′-cyclic monophosphate (cGMP) pathway and its effect on downstream targets, such as protein kinases and Ca^{2+} channels. Although NO-donor compounds are promising as therapeutics for IOP regulation, they may not be ideal to harness the neuroprotective potential of NO signaling. Here we review evidence that supports direct targeting of GC as a novel pleiotrophic treatment for the disease, without the need for direct NO application. The identification and targeting of other factors that contribute to glaucoma would be beneficial to patients, particularly those that do not respond well to IOP-dependent interventions.

1. Introduction

Glaucoma is a neurodegenerative disease characterized by progressive degeneration of retinal ganglion cells (RGCs) and subsequent irreversible loss of vision. Over 60.5 million people worldwide are affected by primary open angle glaucoma (POAG) – a figure projected to increase to 79 million in 2020 and 111.8 million by 2040 [1, 2]. Glaucoma is often associated with elevated intraocular pressure (IOP), termed ocular hypertension. However, at least a third of patients with glaucomatous vision loss have normotensive IOP (normotensive glaucoma; NTG) [3–6] and disease incidence increases with age, regardless of IOP. This suggests that ocular hypertension is only one mechanism for glaucoma etiology and progression [7]. Despite these indications, ocular hypertension remains the only target of current glaucoma therapeutics. Current strategies to lower IOP include topical application of

eye drops and surgical intervention. Unfortunately, successful reduction of IOP via these therapies only serves to slow progression of the disease.[8] Thus, the identification of novel therapeutics that target other disease mechanisms is important for the evolution of glaucoma treatment.

Nitric oxide (NO) is an endogenous signaling molecule that is emerging as a novel target for therapeutic lowering of IOP [8]. NO is produced endogenously in various ocular tissues in both the anterior and posterior segments of the eye and is a potent activator of soluble guanylate cyclase (termed GC, formerly known as sGC). Recent evidence implicates the NO-GC-cyclic guanosine monophosphate (cGMP) pathway in both IOP regulation (see section 6.1) and retinal pathophysiology of glaucoma (see section 6). In this review, we will discuss the evidence that the NO-GC-cGMP pathway may contribute to glaucoma pathophysiology as well as its potential as a novel multi-target approach for glaucoma therapeutics.

2. Pathophysiology of Glaucoma

Glaucoma is a group of optic neuropathies defined by progressive degeneration of RGCs and their axons in the optic nerve, which leads to irreversible loss of vision [3, 8, 9]. RGC degeneration is often significantly advanced before changes in visual acuity and evidence of optic nerve cupping are detected in the clinic [10–12]. Although the pathogenesis of glaucoma is not well understood, progression correlates with IOP, regardless of whether IOP is normotensive or hypertensive [13]. Several clinical trials indicate that IOP-lowering drugs are effective in delaying progression of the disease. In particular, the Early Manifest Glaucoma Trial (EMGT) indicates that the risk of progression decreases by approximately 10% with each 1 mmHg IOP reduction from baseline [4]. Similarly, the Ocular Hypertension Treatment study indicates that a 20% reduction in IOP is effective in delaying or preventing the onset of POAG in patients with ocular hypertension [14]. Thus, lowering IOP remains the primary course of treatment for glaucoma patients as well as for those with ocular hypertension deemed at-risk for glaucoma.

Our current understanding of the relationship between IOP and RGC degeneration indicates that IOP elevation leads to a corresponding increase in pressure exerted posteriorly at the optic nerve head, where the optic nerve exits the globe of the eye [15, 16]. The lamina cribrosa, a band of extracellular matrix in the optic nerve head, marks the beginning of the optic nerve and is prone to compression, deformation, and remodeling induced by mechanical strain related to IOP. This compressive deformation is transferred to RGC axons, which pass through perforations in the lamina cribrosa as they exit the globe. Studies in animal models of glaucoma indicate that ocular hypertension results in the disruption of both anterograde and retrograde transport in RGC axons, particularly near the optic nerve head [17]. These studies are corroborated by structural changes in RGC axons of the optic nerve head from human donors with glaucoma [10]. Interestingly, studies in animal models indicate that deficits in axon transport occur early in glaucoma progression, prior to structural degeneration of RGC axons and soma [3, 18]. These studies suggest that the interval between changes in axon transport and structural degeneration of RGCs may constitute a window for therapeutic intervention. While glaucoma is typically diagnosed in patients already exhibiting 40–50%

visual field loss [19, 20], the cellular process of degeneration is occurring at various rates throughout the RGC population. If targetable, this therapeutic window provides the opportunity to interrupt degeneration in RGCs within glaucomatous retina that have not yet progressed to structural degeneration. Thus, there is the possibility of preserving RGCs and preventing further vision loss, independent of or in addition to IOP management.

3. Why the need for new medications?

IOP is established by the balance of AqH production and elimination from the anterior chamber. Two independent pathways regulate AqH dynamics: the conventional pathway and the unconventional pathway. In humans, the majority of AqH drainage occurs via the trabecular meshwork (TM) and Schlemm's canal (SC), which constitute the conventional pathway [21] (Figure 1). However, it has been estimated that around 3–82% of AqH drainage can also occur via the uveoscleral tract of the unconventional pathway across different species [22–27]. The first course of treatment to lower IOP is usually through topical application of drugs that modulate AqH dynamics by: 1) reducing AqH production 2) increasing uveoscleral outflow or 3) increasing flow through the conventional pathway via contraction of the ciliary muscle (CM) [8]. Issues with patient compliance and side effects can reduce efficacy of topical drugs. Accordingly, sustained delivery platforms, such as the bimatoprost intracemeral slow-release implant, are already in phase III clinical trials [28]. Alternative strategies for IOP management are currently surgical, i.e. laser trabeculoplasty or incisional glaucoma surgery [29].

As indicated by the wide variety of pharmaceuticals for IOP management (Table 1), each case of glaucoma is unique and requires a unique treatment regimen to effectively lower IOP. This often results in patients utilizing several medications at once and/or combining medications with surgical intervention. Over the long-term, the likelihood of preserving functional vision diminishes and the risk of significant blindness is considerably high. This is likely attributable to both poor patient compliance and the unilateral targeting of only one facet of the disease.

The identification and targeting of other factors that contribute to glaucoma would be beneficial to patients, particularly those that do not respond well to IOP-dependent interventions. Neuroprotection for glaucoma could be an effective strategy, but studies aimed at protecting RGCs have thus far failed to demonstrate efficacy in clinical trials [30]. However, recent evidence supports the notion that targeting both neurobiological and IOP regulatory aspects of the disease may be more effective as a treatment strategy. For example, in a study comparing two adrenergic blockers timolol (beta-adrenergic) and brimonidine (alpha-adrenergic), brimonidine was more effective than timolol in stabilizing visual fields [31]. Both timolol and brimonidine reduce IOP by decreasing AqH production at the level of the non-pigmented ciliary epithelium [9, 32] and display similar IOP-lowering efficacy [31]. However, brimonidine also has neuroprotective qualities, as demonstrated by its use in Alzheimer's disease and other cognitive impairments [33, 34]. Thus, the identification of other pathways that could potentially target both IOP and neurodegeneration is intriguing and potentially beneficial.

NO is emerging as a potential therapeutic target that could impact both IOP regulation and RGC neurodegeneration. Here, we will summarize the potential implications of NO signaling for glaucoma pathophysiology and advocate the NO-GC-cGMP pathway as a putative candidate for a new class of multi-target therapeutics.

4. NO-GC Pathway

NO is a ubiquitous and endogenous signaling molecule. Since its discovery as an endothelium-derived relaxing factor (EDRF) in 1987 [35], NO has been implicated in a myriad of physiological processes, including smooth muscle relaxation and vasodilation [35, 36], blood pressure regulation, antimicrobial defense and vascular homeostasis [37, 38]. Nitric oxide synthase (NOS) is the enzyme that produces endogenous NO from l-arginine in a two-step oxidation process that also yields l-citrulline [39–41]. Molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) are co-substrates (reviewed in [42]).

There are three isoforms in mammals: neuronal NOS1 (nNOS), endothelial NOS3 (eNOS) and inducible NOS2 (iNOS) [43, 44]. Under normal physiological conditions, NO is produced by the two constitutive, Ca^{2+}/calmodulin-regulated isoforms of the enzyme (nNOS and eNOS), which generate relatively small amounts of NO (picomolar to nanomolar range) in response to a variety of stimuli, including elevated calcium and shear stress [45]. In pathological conditions (e.g. infection, inflammation or ischemia), there is induction of the third transcriptionally-regulated isoform of NOS (iNOS), which produces higher concentrations NO (micro to millimolar levels) over longer time periods [46]. The differential isoforms of NOS, paired with its widespread distribution in most tissues, allows for an array of diverse biological functions of NO.

The classic NO pathway starts with the binding of a ligand, i.e. a hormone or first messenger, to its receptor that then induces production of NO by NOS. NO is a lipophilic molecule capable of traversing the phospholipid membranes of cells, where it has numerous targets, reacting typically via thiol groups or transition metal centers [47–50]. A major target of NO is the enzyme soluble guanylate cyclase (GC-1 and GC-2, formerly known as sGCα1β1 and sGCα2β1 respectively), the only known receptor of NO [51–53]. The GC enzyme is a heme-containing heterodimeric protein, consisting of one α and one β subunit (Figure 2) [52]. The GC-α1 and GC- β1 subunits that make up the GC-1 isoform are expressed in most cell types and tissues; however, two other subunits of GC, α2 and β2, have also been identified [54]. Although GC-1 is the most abundantly expressed form, other mixed heterodimer combinations of the protein have been identified, such as GC-2 (formerly sGCα2β1), which is expressed in the brain, placenta, spleen, and uterus [54, 55]. This review will focus on GC-1, which converts guanosine triphosphate into the secondary messenger cGMP (Figure 2) [56–58]. Upon NO binding, the activity of GC-1 increases more than 200-fold [59, 60] producing high concentrations of cGMP that then modulate functions of numerous downstream enzymes, such as cyclic nucleotide phosphodiesterases (PDEs), cGMP-dependent protein kinases and cGMP-gated ion channels [61, 62] (reviewed in [63]; Figure 2). Downstream signaling cascades produce different biological effects depending on the location of NO release and the site of cGMP production.

4.1. NO-GC-1 Pathway in the Eye

GC-1, the downstream target of NO, is expressed widely in the retina of multiple species, including human [64, 65], rabbit [66], rat [67], turtle [68], and mouse [64, 69]. GC-1 expression is evident in RGCs, photoreceptor cells and cells in the vascular smooth muscle layer of retinal arterioles [64, 69] (Table 2). *In vitro* studies also indicate GC-1 expression in human TM cells, and both human and mouse ciliary muscle (CM) [7064]. Tissues in both the anterior and posterior segments of the eye express the three isoforms of NOS (Table 2). We will review the functional implications of these expression patterns for each isoform.

4.1.a. eNOS—The role of eNOS in the cardiovascular system includes regulating vascular tone by inhibiting smooth muscle contraction. In the eye, eNOS is constitutively expressed in sites that are important in the regulation of AqH outflow in the eye, including the endothelium of ciliary and retinal vessels [71, 72], and the ciliary muscle of the uveoscleral pathway [71, 73–75]. In the conventional outflow pathway, the trabecular meshwork (TM) was also thought to endogenously produce NO through the activity of eNOS. However, recent data suggests that, in murine eyes, eNOS expression is predominantly found in cells of the SC [76–78]. It therefore comes as no surprise that eNOS is an important regulator of IOP through physiological regulation of outflow facility [78]. Elevated eNOS expression in eNOS-GFPtg mice leads to reduced IOP and increased outflow facility [78] and conventional AqH outflow is impaired and IOP is increased in eNOS knockout mice [79]. Furthermore, eNOS gene polymorphisms are associated with increased risk of developing POAG, including both ocular hypertensive and NTG forms of the disease [80–83].

eNOS also has a central role in ocular blood flow.: NO produced in the endothelium acts as an important physiological mediator to exert vascular smooth muscle relaxation in the eye, as seen in other organs and tissues of mammals. Studies investigating the involvement of endogenous NO on the ocular circulation of healthy subjects show strong evidence for the involvement of endogenous NO derived from either endothelial cells or perivascular nitrergic neurons in the control of vascular smooth muscle tone under resting and stimulated conditions (reviewed extensively in [84]).

4.1.b. nNOS—nNOS is constitutively expressed in both the anterior and posterior chambers of the eye. Anteriorly, nNOS is expressed in the ciliary non-pigmented epithelium and is a key factor in controlling ocular blood flow [74, 85]. Posteriorly, nNOS is expressed across species in pigment epithelium, optic nerve head and in the neural retina by amacrine cells, rod and cone photoreceptors and RGCs [66, 69, 72, 73, 86–94]. It has been suggested that NO production by nNOS may serve as a molecular messenger between cells in the inner layers of the retina (e.g. amacrine cells), astrocytes and cells in the RGC layer [95].

4.1.c. iNOS—iNOS is not constitutively expressed in the eye under physiological conditions. However, upregulation of iNOS expression has been detected in human eyes in macrophages of the stroma and astrocytes [74, 96] and in chicken retinal pigment epithelium (RPE) [94]. iNOS activity was discovered in patients with POAG and visual field loss [75]. *Ex vivo* analysis of human donor eyes revealed that iNOS expression in the TM is induced by increasing perfusion pressure in the anterior chamber [97]. This increase in iNOS expression is

accompanied by NO production, suggesting a functional role for iNOS in mediating pressure-induced NO release [97]. Similarly, *in vitro* studies of ocular tissues and cells indicate that iNOS can also be induced by inflammatory and hypoxic stressors, like those associated with glaucoma [8973].

5. NO-GC-1 Pathway and Implications for Glaucoma

Direct *in vivo* measurement of NO in the eye is not yet feasible. However, measurement of nitrate and nitrite levels are routinely used as markers for the activity of NOS and the production of NO radicals [98]. Several studies in human glaucoma patients suggest that various components of the NO-GC-1-cGMP pathway and its associated outcomes are linked to disease progression. Glaucoma patients exhibit decreased NO metabolite (nitrate/nitrite) and cGMP levels in AqH and plasma compared to patients without glaucoma [99, 100]. This is accompanied by corresponding increases in the AqH level of l-Arginine, the amino acid precursor of NO [101], and serum levels of l-arginine analogs, which are endogenous inhibitors of NOS or l-arginine uptake [102]. In eyes harvested posthumously from POAG patients, NADPH-diaphorase (NADPH-d) reactivity, a marker for NO production, is decreased in TM, SC and anterior longitudinal CM fibers [85].

NO production by cells of the SC may have a homeostatic function during IOP elevation, i.e. when the SC narrows and shear stress increases. Cells respond to increased IOP through increases in NO, which increases the permeability of the SC inner wall and decreases contractility of the juxtacanalicular TM in order to normalize IOP levels [77]. SC cells isolated from glaucomatous eyes are unresponsive to shear stress [77]. This suggests that the homeostatic feedback loop controlling NO synthesis is impaired in glaucoma and may contribute to elevations in IOP.

Finally, NOS inhibition impairs blood flow in the optic nerve head of POAG patients to a greater extent than in healthy controls [103]. Taken together, these studies suggest an important role for the NO-GC-1 pathway and its downstream effector, cGMP, in glaucoma pathophysiology [64].

5.1. NO-GC-1-cGMP Pathway as an Etiological Factor in Glaucoma

In recent years, GWAS have identified several genetic loci linked to POAG (reviewed recently in [104]). Amongst the genes identified, three are associated with the NO-GC-1-cGMP signaling pathway.

5.1.a. Caveolin 1 and 2 (CAV1/CAV2)—Variants in genes encoding CAV1 and CAV2 are associated with ocular hypertension [104–110]. Caveolins are involved in controlling the production of NO by NOS enzymes [111].

5.1.b. NOS3/eNOS—More directly, *eNOS* gene variants associated with ocular hypertension in females are thought to induce differential expression or modulation of eNOS that effects NO expression in the eye [82]. The promoter-region polymorphism T-786C of eNOS may lower local NO concentrations by reducing promoter activity to influence gene transcription [112]. This functional polymorphism is associated with POAG and links age and

gender with risk of POAG development [113]. Similarly, variants in the promoter region of *eNOS* were identified in 20% of familial POAG patients [114] and a recent study identified the *eNOS* variant *rs2070744* as a significant genetic risk factor for developing disc hemorrhage in NTG patients [83]. In the NTG population, additional studies suggest that NO dysregulation may impact blood supply to the optic nerve as well as aqueous humor outflow [100]. This is supported by the presence of altered vasodilatory responses in forearm microcirculation of NTG patients [80], and well-documented vascular abnormalities in POAG patients generally (reviewed in [115]).

5.1.c. GC-1—A gene candidate association study in the GLAUGEN cohort identified a variant (rs11722059) in *GUCY1A3/GUCY1B3,* which encodes the α_1/β_1 subunits of the GC-1 enzyme; in POAG individuals that develop early paracentral visual field loss [10864]. Interestingly, loss of early paracentral visual field loss is predominant in a subsection of POAG patients associated with vascular dysregulation [116]. This link between GC-1 and POAG etiology is further confirmed in animal studies, where mice lacking the α_1-subunit of GC-1 develop optic neuropathy associated with moderate ocular hypertension [64]. This optic neuropathy is accompanied by both retinal and systemic vascular dysfunction [117, 118] and decreased AqH outflow resistance [64].

Together, these studies suggest that genetic mutations leading to impaired NO-GC-1-cGMP signaling are risk factors for glaucoma and reinforce the connection between NO signaling and glaucoma pathogenesis. Furthermore, these studies suggest that the NO-GC-1-cGMP pathway, specifically NO metabolites and cGMP, could be potential biomarkers of glaucoma pathophysiology [64, 98,–99] where early detection could expand the therapeutic window and improve patient outcomes.

6. NO-GC-1-cGMP Pathway and Disease Mechanisms

6.1. IOP regulation

There is increasing evidence that NO is a direct regulator of IOP and that dysfunction of the NO-GC-1 pathway is associated with glaucoma. In healthy human eyes, tissues capable of producing NO include: the ciliary body, the TM, and the SC [119]. In many cases, the effect of NO on IOP is linked to the action of its downstream messenger cGMP, particularly in the conventional outflow pathway (see Figure 1). Stimulation of the NO-GC-1-cGMP pathway via administration of NO donor compounds lowers IOP through relaxation of the TM, alteration of TM volume, and an increase in the permeability of cells in the SC [76, 120–127]. In addition, increased iNOS is observed following increased perfusion pressure in the anterior segments of human donor eyes [128]. In rabbits, a NO donor and a cGMP analogue both decrease IOP [129] and increase outflow facility [130]. In mice, *eNOS* overexpression lowers IOP by increasing pressure-dependent drainage [78]. This concurs with previous work suggesting that the ability of NO to lower IOP is mediated by a decrease in the AqH resistance rather than changing the rate of AqH secretion [74]. Interestingly, eNOS expression and NO levels are both decreased in the CM, TM, and SC of POAG patients [85, 99, 100].

Intravitreal or intracameral injection of 8-Br-cGMP, a cGMP analog, increased AqH outflow facility in a dose-dependent manner [131]. 8-Br-cGMP is cell-permeable, activates cGMP-

dependent kinases, and is more resistant to hydrolysis by phosphodiesterases than native cGMP [132]. At lower doses (10–30 μg), intravitreal 8-Br-cGMP decreases AqH flow, but does not affect outflow facility [131]. In contrast, higher doses of 8-Br-cGMP (100–300 μg), increases outflow facility [131]. These results suggest that cGMP may be an important factor in the regulation of IOP by facilitating AqH outflow via the TM and decreasing AqH production by the ciliary epithelium. One potential mechanism that mediates the suppressive effect of NO on AqH production involves inhibition of the Na,K-ATPase pump. In the eye, the Na,K-ATPase is the primary active transporter involved in the establishment of ion gradients that drive AqH formation [133]. Inhibition of this pump by ouabain decreases AqH secretion by ~62% [134]. Studies indicate that NO donors, such as sodium nitroprusside (SNP), and 8-Br-cGMP reduce AqH secretion and therefore, reduce IOP in bovine and porcine eyes through cGMP- and PKG-dependent inhibition of Na,K-ATPase [135–138].

Relaxation of TM and relaxation of blood vessels occur via similar mechanisms that involve the NO-cGMP pathway [139]. The cells of SC share properties with endothelial cells that line blood vessels, which may explain the mechanism of action for NO in IOP regulation. Exploration of the NO-dependent increase in outflow facility in porcine eyes demonstrated that this TM relaxation is GC-1 dependent and prevented by GC inhibitors, such as 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1–1 (ODQ) [70]. Additional studies link the NO-GC-1-cGMP pathway directly to AqH outflow capacity. Inhibition of NOS signaling in perfused donor human eyes with LNG-Nitroarginine methyl ester (L-NAME), a non-isoform specific NOS-inhibitor, decreases the rate of AqH outflow [140]. Conversely, a NO-donor compound increases AqH outflow rate [140]; the AqH outflow rate was directly proportional to increases in cGMP levels in perfused fluid, confirming a role for the NO-GC-1-cGMP pathway in regulation of AqH outflow in humans [140]. These findings were corroborated in animal models, where inhaled NO gas lowered IOP in both mice and sheep in a GC-1-dependent manner: in mice, lowering of IOP was attributed to increase in conventional aqueous outflow facility [141], and a small molecule GC-1 stimulator increased cGMP levels and increased AqH outflow in mouse eyes [142]. A long-term study on the effect on dietary nitrates on incidence of glaucoma was recently concluded, with the results strongly supporting a role for NO supplementation in the prevention of elevated IOP and thus POAG [143]. A greater intake of dietary nitrate was associated with a 20–30% lower risk of POAG; a particularly strong association was seen (40–50%) for those cases of glaucoma with early paracentral vision loss and vascular dysfunction [143]. This study further supports a role for impaired NO signaling in the development of glaucoma.

Although these studies strongly implicate the NO-GC-1-cGMP pathway as a regulator of IOP, the mechanism of action for downstream effectors is still poorly understood. However, cGMP-dependent changes in outflow are likely related to contraction and relaxation of the TM. In *ex vivo* preparations of TM and CM slices that were pre-contracted with carbachol, application of the NOS inhibitor L-nitroarginine led to an increased contraction of both TM and CM. In contrast, application of 8-Br-cGMP to pre-contracted CM and TM strips resulted in relaxation [144]. One possible mechanism for cGMP-mediated contraction is activation of protein kinase G (PKG) [70, 73, 125, 130]. Activated PKG can phosphorylate numerous targets with multiple downstream effects that relate to contractility, including ion channels and gap junctions [46, 71, 125, 145].

Contractile responses are linked to changes in membrane potential and ultimately, the activity of ion channels. For example, direct application of 8-Br-cGMP has a relaxing effect on bovine TM cells [146] and in CM [144] via activation of BKCa channels. The relaxation effect observed is much more pronounced in TM than in CM [144]. Activation of BKCa channels leads to K^+ efflux and cell hyperpolarization. This reduces cytosolic Ca^{2+} through the inhibition of voltage-operated (L-type) Ca^{2+} channels [147]. Similarly, Ca^{2+} channel blockers, such as topical verapamil, diltiazem, nifedipine, or flunarizine lower IOP in animal models and humans (reviewed in [148]). cGMP-dependent changes in Ca^{2+} dynamics may also involve increased uptake of calcium into the sarcoplasmic reticulum [149].

While the mechanism(s) underlying Ca^{2+}-dependent changes in TM relaxation are not well understood, evidence indicates a role for gap junctions [150]. One way in which gap junctions are modulated is through rho kinase inhibition. cGMP activates PK-G, which in turn phosphorylates Rho A, leading to its inhibition and subsequent inhibition of Rho Kinase [151]. Rho kinase inhibitors, such as netarsudil [152], activate myosin light chain phosphatase (Figure 3; reviewed in [153]). Subsequent dephosphorylation of the regulatory light chain of myosin prevents actin–myosin interaction, promoting cell relaxation [154–156]. This could lead to a widening of the intercellular spaces in the juxtacanalicular TM and SC, thus facilitate conventional AqH outflow to lower IOP [46, 71, 157, 158] (Figure 1).

It is clear that endogenous NO production and subsequent activation of the GC-1-cGMP pathway influence the cellular contractile mechanisms that mediate both AqH outflow and IOP. However, it is not only the TM that is important, the CM also plays a role in IOP regulation. Contraction of the CM causes relaxation of the TM, which increases intratrabecular spaces and increases outflow facility. The relaxation of the CM could possibly decrease conventional outflow. Thus, a functional antagonism between contractility of TM and CM exists and it is the balance between these modalities that may determine total AqH outflow through the conventional route (reviewed in [159]). Accordingly, pharmacological agents that preferentially relax the TM rather than the CM may have a beneficial impact on IOP.

6.2. Ocular blood flow

Vascular endothelial dysfunction and impaired blood flow have been associated with POAG, both in ocular hypertensive and normotensive subsets [80, 160–162]. The topic of vascular dysfunction and POAG has been recently reviewed (for further details see [115]). Briefly, the endothelial monolayer lies between the lumen of blood vessels and underlying smooth muscle tissue and is responsible for modulation of vascular tone, thrombus formation, cell adhesion, and sequestration of inflammatory mediators [115]. Impaired endothelial signaling in POAG is observed in tissues relevant for both outflow resistance and RGC support, including: 1) endothelial layers in the inner wall of SC, the ciliary body and the posterior longitudinal muscle (outflow resistance) and 2) vascular endothelial cells that underlie the luminal smooth muscle vessels that supply RGCs [115].

Several studies support vascular dysfunction as a key player in development of glaucoma. Acetylcholine-induced vasodilation, assessed non-invasively in forearm blood vessels, was impaired in NTG patients [80]. Several groups have studied flow-mediated vasodilation in

brachial arteries of both NTG and POAG patients with elevated IOP, and reported that these patient have abnormal responses compared with controls [160, 163, 164].

Although these studies could explain why glaucoma develops across a range of IOP levels, they do not elucidate the molecular mechanisms underlying vascular dysfunction in glaucoma. However, there is compelling evidence to suggest a role for NO-cGMP signaling in vascular dysfunction associated with glaucoma (see also section 5.1). Both vasoactive and vasoconstrictive factors, NO and endothelin-1 respectively, are produced by the vascular endothelium, and play a major role in the control of ocular blood flow [165–168]. In the retina and optic nerve head, endogenous NO helps to maintain basal blood flow [84, 169–171]. Blood flow through the optic nerve head is autoregulated. This means that local tissue blood flow (perfusion pressure) is kept constant despite physiological or metabolic changes. It is known that NO is involved in autoregulation at the optic nerve head under glaucoma-related conditions, i.e. ocular hypertension [172, 173]. Abnormalities in vascular autoregulation are implicated in glaucomatous optic neuropathy [174, 175], especially in patients with normal tension glaucoma (NTG)[176]. Several groups have investigated the role of NO in ocular blood flow autoregulation. These studies indicate that NO alters retinal, optic nerve head and choroidal autoregulation of blood flow in rabbits and pigs when IOP is elevated [172, 177]. Similarly, NO-donor molecules injected intravitreally enhance tissue oxygenation of the optic nerve head in preclinical animal models [178]. These data suggest that NO contributes to blood flow autoregulation in the retina and optic nerve.

Like IOP regulation, blood flow autoregulation is likely mediated by NO and downstream effectors such as cGMP. NO altered choroidal, retinal, and ONH autoregulation during experimental IOP elevations in rabbits and piglets [172, 177]. Sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, elevates cGMP levels and increases choroidal blood flow (reviewed in [179]) and optic neuropathy in GC-1$^{-/-}$ mice also presents with vascular dysfunction [64]. Impairment of the NO-GC-1-cGMP pathway may also impact ocular blood flow and autoregulation indirectly via changes in mean arterial pressure induced by systemic vascular dysfunction.

Blood flow autoregulation in the eye remains a complex phenomenon and the mechanism by which the NO-GC-1-cGMP pathway modulates retinal vascular autoregulation remains to be elucidated. A plausible mechanism may involve the downstream action of cGMP on calcium flux in cells: elevated cGMP leads to calcium efflux from smooth muscle cells, and therefore relaxation of the cell [180]. In arterioles (such as those in retinal vessels), this can lead to increased ocular blood flow. Although cGMP effects on calcium flux in cells have beneficial effects on ocular blood flow, a recent retrospective study suggests that use of calcium channel blockers confer a 30% increased risk of POAG progression. Previous studies have reported conflicting data. One study indicates that calcium channel blockers have no effect on the clinical course of glaucoma [181], while another indicates that calcium channel blockers may impede glaucoma progression [182]. Additionally, animal studies indicate that calcium channel inhibitors prevent ischemia-induced RGC degeneration by restoring impaired blood flow and directly inhibiting apoptosis pathways [183, 184].

Although there is no direct evidence that improved blood flow via modulation of NO-cGMP signaling leads to preservation of visual field, the studies aforementioned do highlight vascular dysfunction as a likely contributor in disease pathogenesis for both ocular hypertensive and NTG POAG patients. While it is clear that modulation NO-cGMP pathway can improve ocular blood flow, additional studies are required to evaluate any potential efficacy for this strategy in the treatment of glaucoma.

6.3. Neuroprotection

NO signaling is associated with both neuroprotective and neurotoxic outcomes depending on its concentration and the cell types involved. Elevated NO concentrations can lead to oxidative reduction reactions and the production of reactive nitrogen species (RNS) [185–188]. RNS, such as peroxynitrite, are implicated in the pathogenesis of many neurodegenerative disorders, such as Alzheimer's Disease [189]. Likewise, some studies indicate that NO may also have neurotoxic effects in retina. In an *in vitro* study, a NOS inhibitor significantly protected RGCs from anoxia-induced death [190]. In rat models of glaucoma, increased expression of NOS1 in retina was associated with RGC degeneration and inhibition of iNOS prevented RGC degeneration [191192, 193]. Finally, the δ-opiod receptor agonist (SNC-121) protects RGCs in a rat model of glaucoma via the suppression of iNOS activity [194].

Conversely, NO can also act in a neuroprotective manner [195–199]. Activation of the NO-GC-1-cGMP pathway inhibits apoptotic cell death in a variety of primary neuronal cultures and neural-derived cell lines [200–202]. In traumatic brain injury, NO presence is associated with both detrimental secondary damage and neurological recovery [203]. The temporal importance of NO in neurological recovery was investigated using iNOS knockout mice. In these studies, oxidative stress markers were more pronounced in iNOS knockout mice than in wild type mice, suggesting a role for NO as an endogenous antioxidant and thus, neuroprotective agent [203]. In animal models of RGC degeneration, the neuroprotective effect of a novel NO-releasing beta-blocker (nipradilol) on RGC death is attributable to the released NO [204–208]. This neuroprotective effect is NO-dependent. Furthermore, nipradilol promotes regeneration of RGC axon in optic nerve, likely through S-nitrosylation of PTEN and subsequent activation of the mTOR/Akt pathway [209]. Further studies are needed to elucidate the neuroprotective capabilities of NO, in particular any involvement of cGMP, in the context of glaucoma.

The neuroprotective properties of NO in cell cultures appear to be mediated, at least in part, by the activation of the GC-cGMP pathway and its effect on downstream targets, such as protein kinases and Ca^{2+} channels [210, 211212]. As discussed in Section 6.2, cGMP may have an indirect neuroprotective effect via activation of BKCa channels and inhibition of L-type Ca^{2+} channels [183, 184]. In cerebellum, NO acts via the GC-1 pathway to prevent apoptosis through activation of protein kinase-G and Akt [213]. In Schwann cells, reduced expression of GC-1 leads to apoptosis of co-cultured neurons and glial cells [212]. Furthermore, in primary neuron cultures, direct application of NO or nitrite prevents endoplasmic reticulum-induced apoptosis in a GC-1-cGMP-dependent manner [214]. Although excess NO can be neurotoxic, the *in vitro* and *in vivo* evidenced outlined here supports a neuroprotective role for the NO-CG-1-cGMP pathway that should be further examined in glaucoma models.

7. The NO-GC-1-cGMP Pathway as a Target for Glaucoma Therapeutics

Given its established role in IOP regulation and ocular blood flow and its potential ability to serve as a neuroprotectant, NO signaling is a prime candidate for the development of novel multi-target therapeutics for glaucoma.

7.1. NO-Donor Compounds

For more than a century, NO-donating compounds, e.g. nitrovasodilators, have been used to successfully treat cardiovascular disease. Previous studies indicate that NO-donation via intravenous or oral administration of nitroglycerin or isosorbide dinitrate effectively lower IOP in both POAG patients and control subjects [215]. In animal studies, topical application of these same vasodilators also effectively lowers IOP [121, 122, 127, 216]. Accordingly, novel topical NO-releasing compounds for IOP management have recently been developed. For example, latanoprostene bunod (LBN) is a NO-donating prostaglandin F2α analog that is rapidly metabolized in situ (Table 1). Nipradilol is a beta-blocker ligated with a NO-donating moiety. Like their current counterparts, both LBN and nipradilol are effective at lowering IOP [124, 155, 217]. LBN is effective at lowering IOP in several animal models of glaucoma, where its parent compound latanoprost had minimal efficacy [158]. In Phase 2 and 3 clinical trials, LBN increased latanoprost IOP-lowering capacity by more than 1 mmHg, making it more effective than the preferred drug on the market for the treatment of POAG [218–222]. Nipradilol reduces IOP to a similar extent as timolol [223]. A recent review of non-clinical studies with LBN highlights that LBN has a dual action, enhancing AqH outflow via both the uveoscleral pathway (due to action of latanoprost) and the TM/SC of the conventional pathway (due to the effect of the NO moiety)[224].

Studies in animal models indicate that nipradilol also has neuroprotective qualities [204–206] (see section 6.3). There are conflicting data regarding translation of these neuroprotective qualities to humans; in comparative studies between nipradilol and timolol, no additional beneficial effects were observed in visual field performance of those taking nipradilol vs. timolol [225226, 227]. However, one study has shown that in addition to lowering IOP, nipradilol increases ocular blood flow in both control and NTG eyes [227], which suggests that nipradilol may have an advantage in prevention against glaucomatous damage by increasing ocular blood flow to the optic nerve head, over similar topical therapies. Further research is required to determine its neuroprotective efficacy and the mechanisms involved. There are several additional NO-donor compounds that exhibit pre-clinical efficacy for IOP reduction in animal models, including the prostaglandin analogue NCX 470 and two novel carbonic anhydrase inhibitors: NO-dorzolamide and NO-brinzolamide [228, 229230]. Together, these studies indicate that NO-donor compounds are viable and potentially potent therapeutic agents for IOP lowering in glaucoma patients as well as those with ocular hypertension.

7.2. Alternative NO-GC-1-cGMP Targeting

Although NO-donor compounds are promising as therapeutics for IOP regulation, they may not be ideal neuroprotective agents. As described in this review, the neuroprotective activities of NO can be linked specifically to its activation of GC and subsequent modulation of cGMP. While NO-donation can facilitate activation of GC-1, it also has the potential to

induce the production of RNS and promote oxidative stress outcomes. An alternative method of harnessing the neuroprotective properties of NO-GC-1-cGMP, while avoiding the possible GC-independent neurotoxic effects of NO, is the direct targeting of GC or cGMP. As discussed above, NO modulation of both IOP and ocular blood flow is linked to activation of GC-1 and subsequent induction of cGMP-mediated pathways. Targeting GC-1 or cGMP would not only promote the activation of neuroprotection pathways associated with NO, but would likely maintain equivalent IOP lowering efficacy. There are several opportunities for therapeutic activation of either GC-1 or cGMP, including pharmacological activation of GC-1 and inhibition of cGMP degradation. Recent evidence, described below, supports the neuroprotective benefit of these strategies in the CNS.

7.2.a PDE Inhibitors—cGMP is degraded by PDEs (Figure 2). PDE inhibitors prevent the breakdown of cGMP, thereby increasing cGMP bioavailability and prolonging cGMP-mediated activation of downstream pathways (Figure 2). In a rat model of hypoxic ischemia, elevation of cGMP levels by sildenafil, a PDE5 inhibitor, reduces apoptosis, astrocytosis, and microgliosis in the brain [231]. Similarly, tadalafil, another CNS penetrant PDE5 inhibitor, is neuroprotective both in spinal cord injury [232] and in ischemia/reperfusion injury [233]. In the eye, PDE6 inhibition prevents hypoxia-induced cell death throughout the whole retina in porcine retinal explants via a cGMP-dependent mechanism [234].

As highlighted in this review, cGMP is involved in AqH dynamics and thus, PDE5 inhibitors have the potential to affect IOP [131, 235]. However, findings from several clinical studies indicate that the use of PDE5 inhibitors do not alter IOP. In a Phase I clinical trial, a single dose of PDE5 inhibitor sildenafil does not alter IOP in healthy volunteers either 1 hour or 48 hours post-administration [236]. A second study with a larger sample size yielded the same results [237]. A similar study also determined that a single dose of sildenafil does not alter IOP either 1 hour or 5 hours post-administration in glaucoma patients [238]. Although these studies suggest that PDE5 inhibition does not impact IOP, the findings pertain to only a single administration. To date, there is only one study that examined the long-term effects of sildenafil treatment on IOP. In this study, a small cohort of patients with erectile dysfunction (n=10) received 50mg of sildenafil citrate one or more times a week for a minimum of 3 months and displayed no change in IOP [239]. Together, these studies suggest that PDE5 inhibitors likely do not impact IOP. However, a study of long-term use in both healthy volunteers and glaucoma patients would be beneficial..

Although PDE5 inhibitors appear to not influence IOP, visual disturbances have been reported [240, 241]. The most common visual disturbances are increased blue tinge in the visual image and an increased sensitivity to light [242–244]. The rate of occurrence for these symptoms is low; in 3–11% of men taking sildenafil 25–100 mg [241], 0.3–2% of vardenafil [245, 246], and 0.1% of tadalafil users [247]. The symptoms tend to be mild, transient, dose-dependent, and completely reversible. These symptoms likely arise from off-target inhibition of PDE6 in the retina. PDE6 expression in retina is restricted to rod and cone outer segments, where it contributes to phototransduction [248]. PDE5 inhibitors currently prescribed having varying selectivity for PDE5 over PDE6: 10-fold for sildenafil [249], 15-fold for vardenafil [250], and 700-fold for tadalafil [251]. While these visual disturbances are

manageable for intermittent use, they may have greater implications for the use of PDE5 inhibitors in a chronic treatment paradigm, as would be necessary for treatment of glaucoma.

In addition to visual disturbances, a few more serious ocular events have also been noted in male patients prescribed PDE5 inhibitors, including: non-arteritic anterior ischemic optic neuropathy (NAAION) with attendant vision loss, cilio-retinal artery occlusion, central retinal vein occlusion (CRVO), and pupil sparing third nerve palsy (reviewed in [252]). However, a direct cause and effect relationship between these conditions and the use of PDE5 inhibitors has not been established and the rate of incidence does not appear higher than that in male populations generally.[252]

7.2.b. GC Stimulators—Given the potential off-target effects associated with PDE5 inhibition (e.g. PDE6 inhibition in photoreceptors), direct targeting of cGMP production by GC has emerged as a novel therapeutic strategy to lower IOP, and potentially provide neuroprotection in glaucoma [142]. GC stimulators, small molecule drugs that synergistically increase GC enzyme activity with NO, are already clinically available or are in clinical trials for a variety of diseases. Riociguat is approved for treatment of pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension [253, 254].

Stimulation of GC may have therapeutic benefits outside the cardiovascular system, and particularly in the eye. For example, GC-1 stimulation by IWP-953 increased AqH outflow in enucleated mouse eyes, highlighting the therapeutic potential for GC stimulators as novel ocular hypotensive drugs [142]. Safety, tolerability, and efficacy of the GC activator, MGV354, has shown promising IOP-lowering effects in pigmented rabbits and in a cynomolgus monkey model of glaucoma [255]. A single topical ocular dose caused a significant dose-dependent IOP reduction of 20% to 40% (versus vehicle), lasting up to 6 hours in pigmented rabbits. The MGV354-induced IOP lowering was sustained for up to 7 days following once-daily dosing in a monkey model of glaucoma and was greater in magnitude compared to travoprost-induced IOP reduction [255]. It is not yet clear whether this approach also provides neuroprotection to RGCs beyond that afforded by IOP reduction. Together, these data indicate that pharmacological targeting of GC and cGMP may be a fruitful and beneficial alternative to NO-releasing compounds for glaucoma therapy. However, further studies to evaluate this compound *in vivo* are necessary.

8. Conclusions

Glaucoma incidence is on the rise, with many more cases expected to surface in the few decades. Despite effective treatments to lower IOP, glaucoma is still a major cause of blindness worldwide. Advances in treatment are impeded by the complex etiology of the disease and lack of understanding of IOP-independent facets of the disease, i.e. neurodegenerative mechanisms. Ideally, novel glaucoma therapeutics would target both IOP-dependent and -independent mechanisms of the disease.

Recent evidence, reviewed here, indicates that impairment in the NO-GC-cGMP pathway is implicated in glaucoma onset and progression. Involvement of the NO-GC-cGMP pathway with both IOP regulation and ocular blood flow and its potential to elicit neuroprotective

responses in neural retina make this pathway a strong candidate for therapeutic targeting of multiple pathogenic mechanisms in glaucoma.

The potential of the NO-GC-cGMP pathway to prevent and/or treat glaucoma underlies the development of NO-donor compounds as IOP lowering therapeutics. There are many benefits of targeting the NO-GC-cGMP pathway when developing novel therapeutics for glaucoma. NO increases AqH humor outflow through the conventional pathway, which aids in IOP reduction, whilst also increasing retinal perfusion and having putative neuroprotective effects (Figure 4). The potential benefit in NO-releasing treatments is however offset by the delicate balance necessary to promote beneficial outcomes and avoid negative consequences due, for example, to induction of RNS, and other oxidative stress leading to nitrate tolerance which can ultimately promote insensitivity to long-term NO exposure and inhibition on GC [256]. This risk is not alleviated by available NO donor compounds, which activate GC to produce cGMP.

Here, we advocate for the development and further study of compounds that activate the NO-GC-cGMP pathway by targeting GC and cGMP directly. IOP and blood flow regulation by NO is attributable to GC-mediated elevation of cGMP. Likewise, neuroprotective outcomes associated with NO, i.e. anti-apoptotic signaling, are also dependent on GC activation and cGMP modulation. Thus, direct targeting of GC activity or cGMP levels has the potential to promote IOP reduction, increase ocular blood flow and activate neuroprotective mechanisms without the generation of RNS and nitrate (and NO) tolerance. While a few studies suggest that compounds targeting GC activation and cGMP levels are acceptable alternatives to NO-releasing compounds for the treatment of glaucoma, additional research is needed to systematically evaluate the therapeutic efficacy of this approach.

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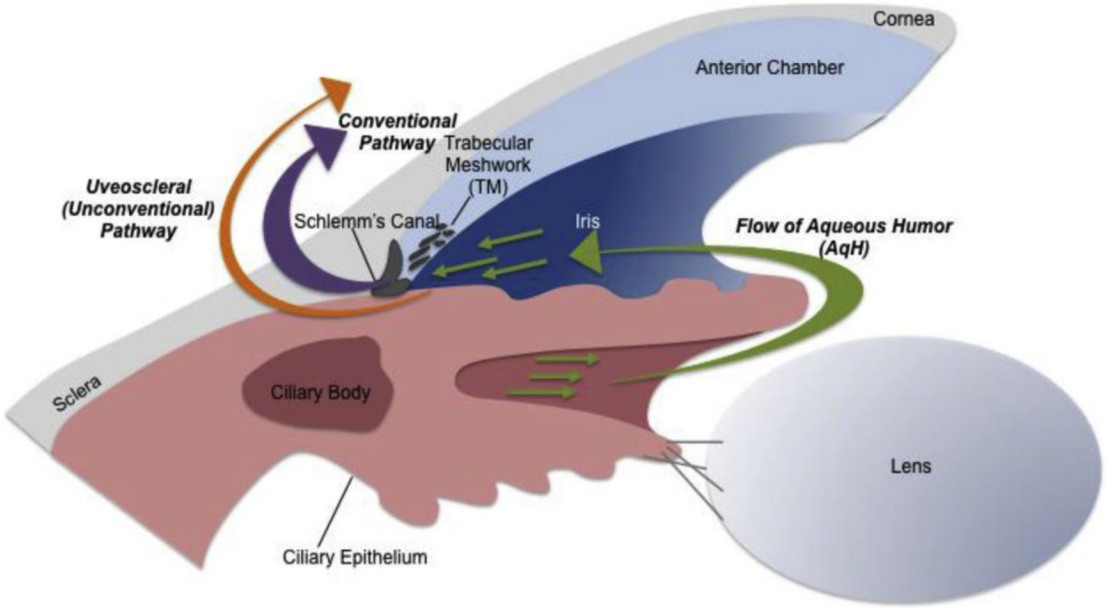

Fig. 1.

Flow of aqueous humor (AqH) in the eye. AqH is produced at the ciliary body and flows (green arrows) through one of two independent pathways that regulate AqH dynamics: the conventional pathway through the trabecular meshwork (TM) and Schlemm's canal (purple arrow) and the non-conventional pathway via the uveoscleral tract (orange arrow). Intraocular pressure (IOP) in the eye is established by the balance of (AqH) production and elimination in the anterior chamber.

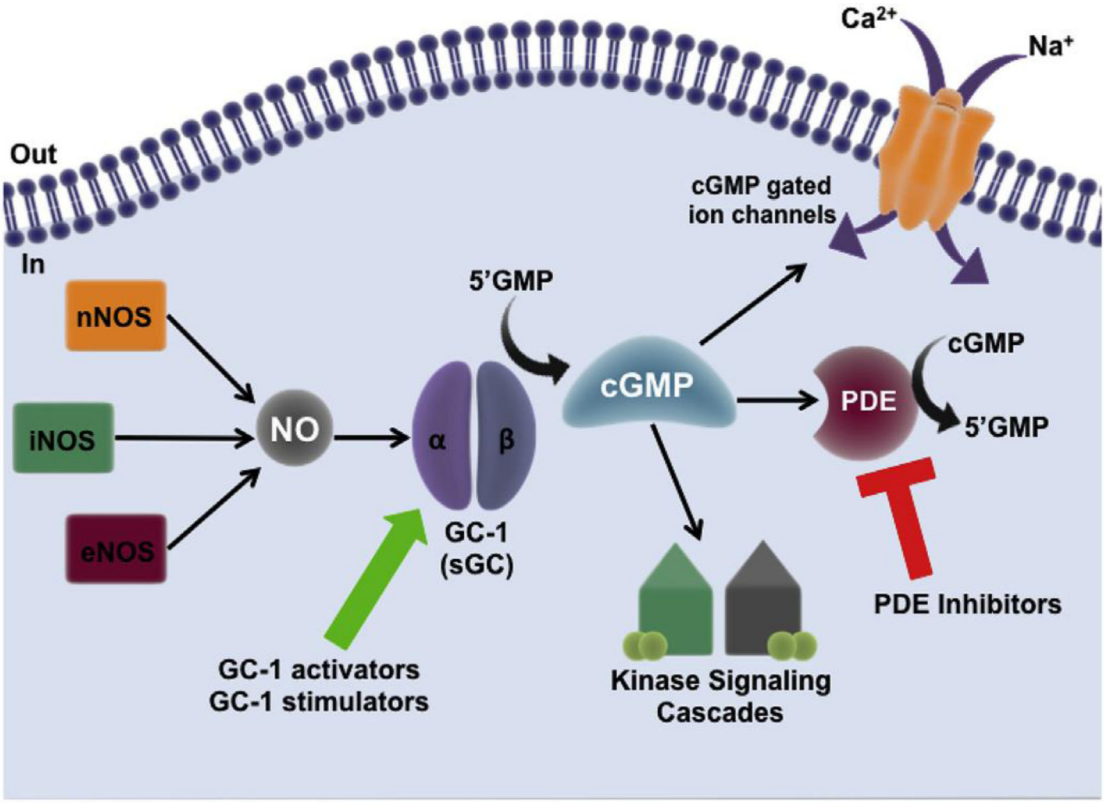

Fig. 2. The NO-GC-1-cGMP pathway.

NO is produced from L-arginine by nitric oxide synthase (NOS) of which there are three isoforms: neuronal NOS1 (nNOS), endothelial NOS3 (eNOS) and inducible NOS2 (iNOS). NO targets guanylate cyclase-1 (GC-1), a heterodimeric protein capable of converting GMP to cGMP. cGMP produced by GC-1 can target cGMP-gated ion channels, and activate downstream kinase signaling cascades. Phosphodiesterase enzymes (PDE) bind to cGMP and catalyse its breakdown into GMP – PDEs act as important regulators of signal transduction mediated by cGMP. cGMP bioavailability in the cell can be modulated in two ways: 1) through the use of GC-1 stimulators and activators, which increase production of cGMP, or, 2) through the use of PDE inhibitors which prevent the breakdown of cGMP in the cell.

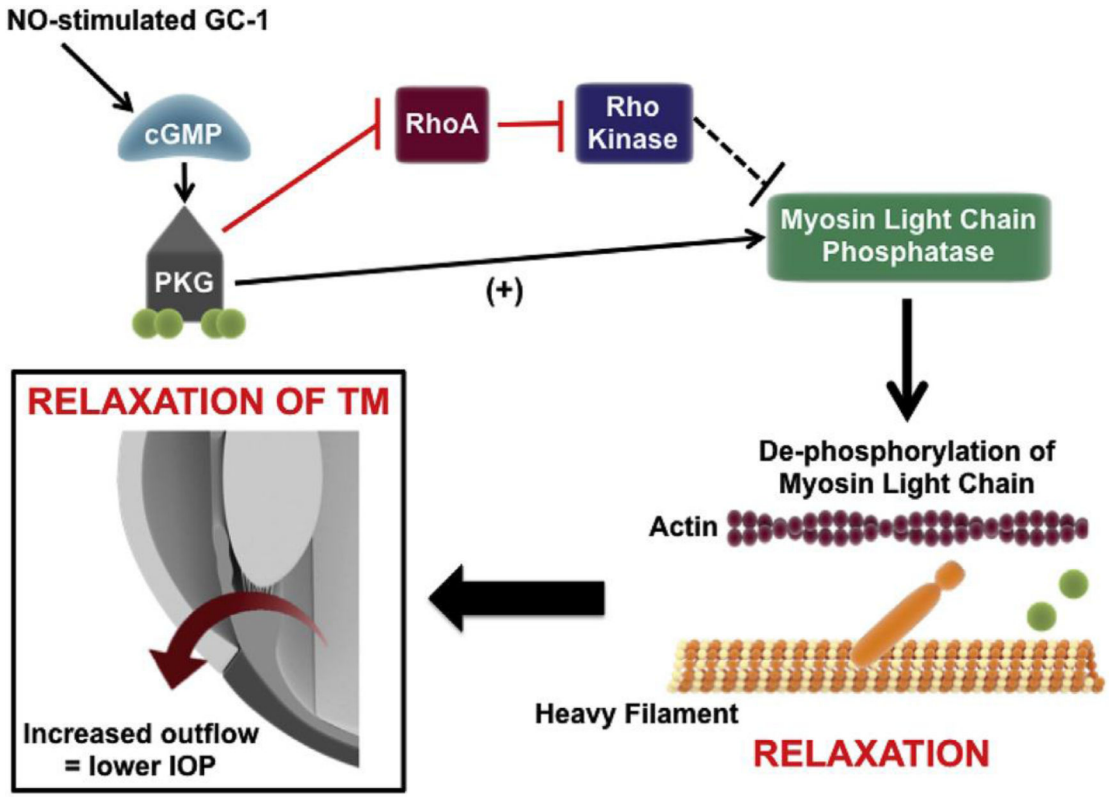

Fig. 3. cGMP-mediated modulation of IOP through increase in AqH outflow.

NO triggers production of cGMP by GC-1. cGMP activates protein kinase G (PKG). Activated PKG can phosphorylate numerous targets with multiple downstream effects, including inhibition of Rho A, thus preventing inhibition of myosin phosphatase by Rho Kinase. In addition to inhibition of Rho A, activated PKG can directly activate myosin light chain phosphatase (MLCP). Subsequent dephosphorylation of the regulatory light chain of myosin by MLCP prevents actin–myosin interaction, promoting cell relaxation. This in turn leads to a widening of the intercellular spaces in the juxtacanalicular TM and Schlemm's canal, thus facilitating conventional AqH outflow and relieving IOP.

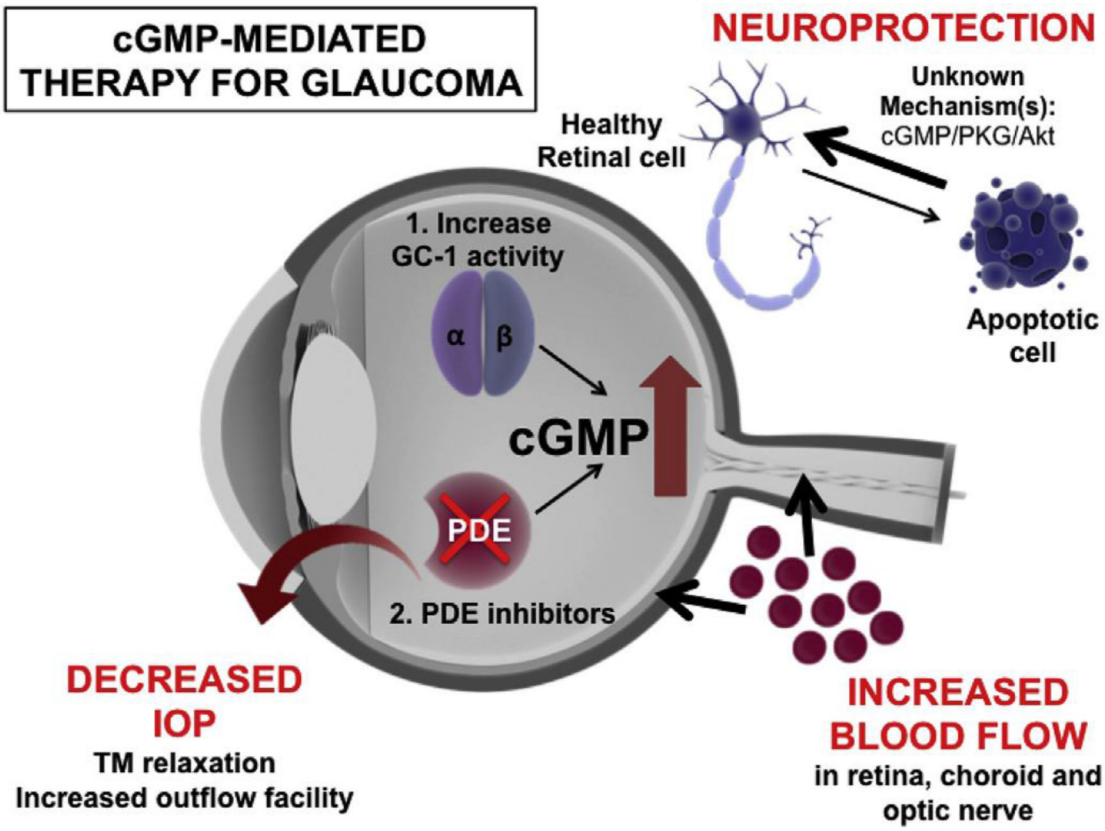

Fig. 4. –. GC-1-directed therapy for glaucoma is pleiotrophic in its action.

Increased levels of cGMP have been shown to have pleiotrophic targets that are beneficial in the treatment of glaucoma, including: relaxation of the TM to increase outflow facility which leads to decreases in IOP; increasing blood flow to the retina, choroid and optic nerve head; prevention of degeneration of retinal ganglion cells through mechanisms that may involve downstream kinase pathways. cGMP levels in the eye can be increased in two ways: 1) through the use of GC-1 stimulators and activators, which aim to increase production of cGMP; or 2) through the use of PDE inhibitors which prevent the breakdown of cGMP in the cell to increase bioavailability.

Table 1.

Current IOP-lowering medications for the treatment of Glaucoma.

Type of Medication	Examples	Mechanism of Action	Adverse Effects
Prostaglandin analogues (PGAs)	Latanoprost Travoprost Tafluprost Bimatoprost Timolol	Enhanced outflow of AqH through the uveoscleral pathway	Conjunctival hyperemia, lengthening and darkening of eyelashes, uveitis, macular edema, periocular hyperpigmentation, increased iris pigmentation
β-Adrenergic blockers	Levobunolol Carteolol Betaxolol Brimonidine	Reduction of AqH production	Ocular irritation, dry eyes, bronchoconstriction, bradycardia
α-Adrenergic agonists	Apraclonidine Dorzolamide	Decreased AqH production, Enhanced uveoscleral outflow of AqH	Allergic conjunctivitis, dry eyes, contact dermatitis, CNS effects, renal failure
Carbonic anhydrase inhibitors	Brinzolamide Acetazolamide (oral) Pilcocaprine	Decreased AqH production	Ocular irritation, dry eyes, metallic taste, nausea, renal stones
Cholinergic agonists		Increased outflow of AqH through conventional pathway	Itching/burning/stinging of the eye, poor vision in dim light, temporary vision loss, headache, brow ache
Rho Kinase Inhibitors Modified PGAs	Netarsudil Latanoprost bunod (LBN)	Increased outflow of AqH through conventional pathway Dual mechanism: increased AqH outflow through conventional pathway (via latanoprost acid), and increased outflow of AqH through conventional pathway via nitric oxide release	Conjunctival hyperemia, corneal verticullata Conjunctival hyperemia, punctata keratitis, eye pain, vision loss

Table 2.

Ocular localization of nitric oxide synthase (NOS) and soluble guanylate cyclase (GC-1).

Gene/isoform	Species	Site of expression (cell or tissue)	Reference
NOS1 (nNOS)	Human	Ciliary non-pigmented epithelium	[51]
	Human	ONH astrocytes, lamina cribrose	[44]
	Monkey	Amacrine cells, rod and cone photoreceptors, RGC	[56]
	Canine	RGC	[57]
	Rabbit	Amacrine cells, rod and cone photoreceptors, RGC	[56]
	Rat	Ciliary process epithelium, INL, IPL, RGC layer, photoreceptors	[52,63]
	Murine	Retinal amacrine cells	[58]
	Murine	Retinal amacrine cells, RGC layer somata; IPL puncta	[59]
	Murine	Müller cells	[60]
	Guinea pig	RGC layer, INL, IPL	[61]
	Chicken	RGC layer, INP, IPL	[62]
NOS2 (iNOS)	Human	Macrophages in stroma and ciliary processes	[46]
	Human	Astrocytes	[64]
	Chicken	RPE	[62]
NOS3 (eNOS)	Human	Longitudinal CM fibers, TM, SC	[46]
	Human	Retinal vasculature	[44]
	Human	TM	[47]
GC-1	Human	RGC, IPL, ONL	[66]
	Human	TM cells	[67]
	Rabbit	Amacrine cells, bipolar cells, cone photoreceptors, RGC	[56]
	Murine	Somata in the INL, ONL, IPL, and OPL	[59]
	Murine	RGC, IPL, ONL	[66]
	Turtle	Amacrine cells, bipolar cells, RGC layer, IPL	[69]

ONH, optic nerve head; RGC, retinal ganglion cell; IPL, inner plexiform layer; CM, ciliary muscle; TM, trabecular meshwork; SC, Schlemm's canal; ONL, outer nuclear layer; INL, inner nuclear layer; OPL, outer plexiform layer.