The Role of Nitric Oxide in Murine Conventional Outflow Physiology

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Abstract
Elevated intraocular pressure (IOP) is the main risk factor for glaucoma. Exogenous nitric oxide (NO) decreases IOP by increasing outflow facility, but whether endogenous NO production contributes to the physiological regulation of outflow facility is unclear. Outflow facility was measured by pressure-controlled perfusion in ex vivo eyes from C57BL/6 wild-type (WT) or transgenic mice expressing human endothelial NO synthase (eNOS) fused to GFP superimposed on the endogenously expressed murine eNOS (eNOS-GFPtg). In WT mice, exogenous NO delivered by 100 µM S-nitroso-N-acetylpenicillamine (SNAP) increased outflow facility by 62±28% (mean±SD) relative to control eyes perfused with the inactive SNAP analogue N-acetyl-D-penicillamine (NAP; N=5; p=0.016). In contrast, in eyes from eNOS-GFPtg mice, SNAP had no effect on outflow facility relative to NAP (-9±4%, p=0.40). In WT mice, the non-selective NOS inhibitor L-NG-nitroarginine methyl ester (L-NAME, 10 µM) decreased outflow facility by 36±13% (N=5 each; p = 0.012), but 100 µM L-NAME had no detectible effect on outflow facility (-16±5%, p=0.22). An eNOS-selective inhibitor (cavtratin, 50 µM) decreased outflow facility by 19±12% in WT mice (p=0.011) and by 39±25% in eNOS-GFPtg mice (p=0.014). In the conventional outflow pathway of eNOS-GFPtg mice, eNOS-GFP expression was localized to endothelial cells lining Schlemm’s canal and the downstream vessels, with no apparent expression in the trabecular meshwork. These results suggest that endogenous NO production by eNOS within endothelial cells of Schlemm’s canal or downstream vessels contributes to the physiological regulation of aqueous humor outflow facility in mice, representing a viable strategy to more successfully lower IOP in glaucoma.

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Introduction

Elevated intraocular pressure (IOP) is a primary risk factor for glaucoma. All current glaucoma therapies aim to lower IOP, but no current treatments directly target the conventional outflow pathway that serves as the primary drainage route for aqueous humor. Because decreased outflow facility is the root cause of elevated IOP in primary open angle glaucoma (POAG) (68), identifying drugs that enhance conventional outflow may lead to new, more effective therapies for ocular hypertension and POAG.

Experimental evidence supports the idea that the bulk of aqueous humor outflow resistance is generated proximal to or at the endothelium of Schlemm’s canal (SC) (33); however, the mechanisms responsible for outflow regulation are not well understood. Recent data suggest that exogenous nitric oxide (NO) delivered via NO-donors decreases outflow resistance and IOP in several species (7, 21, 22, 32, 48, 61, 69) but it is unclear whether endogenous NO production by NO synthase (NOS) enzymes has a physiological role in aqueous humor outflow regulation. NO is well known to mediate various physiological functions in vascular and lymphatic endothelial cells, including vasodilation, junctional complex assembly and permeability (18, 55), and because SC is derived from vascular endothelia (30, 56) and expresses both vascular and lymphatic markers (3, 36, 52, 56), we hypothesize that NO release from SC cells contributes to physiological regulation of conventional outflow. Consistent with this notion, the presence of NADPH-diaphorase, an indicator of NOS activity, has been identified in the conventional outflow pathway of human donor eyes and suggests endogenous NO production by NOS enzymes (48), and decreased NADPH-diaphorase labeling has been reported in glaucomatous eyes (48). Recent studies have demonstrated that SC cells in culture secrete NO in response to increasing shear stress (2, 41), similar to vascular endothelial cells (13, 14), and the shear stress acting on SC cells in vivo during SC narrowing is predicted to approach the shear experienced by endothelial cells in arteries (24). Genome-wide association studies (34, 43, 54) have linked polymorphisms in the NOS3 gene encoding the endothelial-specific isoform of NOS (eNOS) to the pathogenesis of glaucoma, and overexpression of eNOS in a mouse model leads to decreased outflow resistance and lower IOP (69). Taken together, these data suggest that endogenous NO signaling within the conventional outflow pathway by NOS enzymes contributes to outflow regulation and that alterations in NO signaling in part mediate outflow dysfunction in glaucoma.
In this work, we test the hypothesis that endogenous NO production by eNOS influences conventional outflow facility in mice. Our studies use ex vivo mouse eyes because the conventional outflow pathway in mice is anatomically and functionally similar to humans (51, 67), and pharmacological modulation of outflow facility in mice resembles the responses previously reported in human eyes (8). To investigate the role of NO and NOS activity, we measured the effect of NO-donors and NOS inhibitors on outflow facility. Studies were performed in eyes from wild-type (WT) mice and from transgenic mice with elevated eNOS expression.
Materials and Methods

All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under UK Home Office Project License approval for research at Imperial College London and under Institutional Animal Care and Use Committee approval for research at Duke University.

Experimental Design

This study investigated the influence of exogenous NO and endogenous NOS activity on conventional outflow facility in enucleated mouse eyes. Eyes were obtained from C57BL/6 WT or transgenic mice that express the human variant of eNOS fused to GFP (eNOS-GFPtg) superimposed on the C57BL/6 background expression of eNOS (69, 74). To examine whether exogenous NO influences facility, eyes were perfused with the NO-donor S-nitroso-N-acetylpenicillamine (SNAP) or its inactive analog N-acetyl-Dpenicillamine (NAP). Because SNAP is light sensitive, the NO release kinetics of SNAP and NAP were first characterized to identify an optimal lighting condition that was controlled for all SNAP/NAP perfusion experiments. To examine whether endogenous NOS activity contributes to facility regulation, eyes were perfused with or without a broad spectrum NOS-inhibitor (L-NAME) or an eNOS-selective inhibitor (Cavtratin; caveolin-1 scaffolding domain peptide). All experiments were performed using both WT and eNOS-GFPtg eyes, with the exception of L-NAME perfusions, since we have previously reported that L-NAME decreases facility in eNOS-GFPtg eyes to levels consistent with WT eyes (69).

Calibration of NO-donor

Because SNAP and NAP are light sensitive, NO release from SNAP and NAP was first characterized using a nitric oxide sensitive electrode (ISO-NOS II; WPI, Hertfordshire, UK), calibrated following the manufacturer’s instructions. Briefly, the NO probe was placed in a solution containing 0.1 M H₂SO₄ and 0.1 M KI to polarize the sensor and to obtain a baseline current. To determine the standard curve, changes in current were measured in response to incremental volumes of 50 µM KNO₂ added to the H₂SO₄/KI solution within a glass vial. Conversion of KNO₂ to NO occurs rapidly and
follows 1:1 stoichiometry such that the NO concentration is dictated by the added concentration of KNO₂.

Once the standard curve was generated, we examined NO release from SNAP and NAP. Preliminary data indicated that NO release from SNAP depended on the intensity and duration of light exposure. Thus, we tested NO release under two lighting conditions that could be controlled during the perfusion: (i) initial high intensity light exposure of the stock solution (113 mM) for 10 minutes at 700-1000 lumens/m² (measured with a luminometer; Mastech, GuangDong, China) followed by low light conditions (100-200 lumens/m²) where the NO-donor was diluted to the working concentration (0.1 – 1 mM) and protected from surrounding laboratory light with aluminum foil, and (ii) initial high intensity light exposure of the stock solution for 10 minutes at 700-1000 lumens/m² followed by continuous light exposure of the working solution at the same level (700-1000 lumens/m²). NO release was measured over 60 minutes starting immediately after the initial 10 minute light exposure for both conditions. Condition (i) was used for subsequent SNAP/NAP perfusions. Because the conversion of SNAP to NO is catalyzed by metal ions and is temperature sensitive, all NO measurements were performed in perfusion solution (containing divalent cations) at 37°C. The probe tip was immersed in 10 mL of solution within a glass vial while being continuously mixed with a magnetic stir-bar.

Animal Husbandry

This study used transgenic mice having the same C57BL/6 background strain as the WT controls. Perfusion were performed with enucleated (ex vivo) eyes from mice of either gender. eNOS-GFPtg mice were aged 8 to 13 weeks (for SNAP/NAP and cavtratin perfusions). WT mice were aged 6 to 12 weeks (for SNAP/NAP perfusions) or 25 to 30 weeks (for L-NAME and cavtratin perfusions). Mice were housed in individually ventilated cages, fed ad libitum and maintained at 21°C with a 12-hour light (6 AM to 6 PM) and 12-hour dark cycle.

Transgenic mice express the human gene variant of eNOS fused to green fluorescent protein (GFP) on a C57BL/6 background. The transgene contains the full-length human eNOS promoter such that transgene expression is regulated by endogenous transcriptional activity and is superimposed on the background expression
of murine eNOS (74). In these animals, eNOS-GFP expression has been shown to be specific to endothelial tissues, to lead to a two-fold increase in plasma NO concentrations in large arteries (74), and to cause decreased aqueous humor outflow resistance and reduced IOP compared to age-matched controls (69). eNOS-GFPtg mice also have reduced mean aortic pressure, reduced heart rate and lower systemic vascular resistance (74). Transgenic mice were a kind gift from Prof. Rob Krams (Imperial College London, UK) and Prof. Rini de Crom (Erasmus MC, Rotterdam, The Netherlands).

Genotyping of transgenic mice was performed to detect sequences of GFP and the human variant of eNOS present in the genome of eNOS-GFPtg mice but absent from the genome of WT mice. Genotyping was performed using ear tissue samples obtained at weaning, with tissue lysis and DNA purification performed according to manufacturer’s instructions (DNeasy Blood & Tissue kit, Qiagen Hilden, Germany). Separate PCR reactions were performed using a hot-start mix (KAPA2G Robust HotStartReadyMix; Kapa- Biosystems, Cambridge, MA). For human eNOS, 29 cycles were performed with an annealing temperature of 63°C using sense primer AGGGCGCAATGGTAACCTGAAGAT and antisense primer AAAGCTCTGGGTGCGTATGCGG to yield a predicted product of 699 bp. These sequences are specific for the human eNOS and are not predicted to react with C57BL/6 murine eNOS. For GFP, 29 cycles were performed with an annealing temperature of 59.9°C using sense primer AGCTGACCCTGAAGTTCATCTG and antisense primer GACGTTGTGGCTGTTGTAGTTG to yield a predicted product of 327 bp. PCR products were resolved by gel electrophoresis (1% agarose) in the presence of DNA gel stain (SYBR Safe; Invitrogen, Carlsbad, CA). Bands were visualized on an imaging station (Biospectrum 500; UVP, Upland, CA).

Ex Vivo Mouse Eye Perfusion

The first set of experiments examined the effect the NO-donor (SNAP; Calbiochem) compared to its inactive analog (NAP; Sigma) on outflow facility. Treated eyes were perfused with 100 µM SNAP in Dulbecco’s phosphate buffered saline (PBS) including divalent cations and 5.5 mM D-glucose passed through a 0.2 µm filter (referred to as “DBG”). Contralateral control eyes were perfused with 100 µM NAP in DBG. Stock solutions of SNAP or NAP at 113 mM in dimethyl sulfoxide (DMSO) were aliquoted and
stored at –20°C, protecting the solutions from light at all times. Working solutions were prepared immediately prior to each experiment, with high intensity light exposure of the stock solution for 10 minutes followed by low ambient light exposure of the working solution (see above) immediately prior to starting the perfusion. Eyes were perfused at sequential pressure steps of 8, 15, 20 and 25 mmHg to determine outflow facility as described below.

In the second and third sets of experiments, we examined the effect of NOS-inhibitors on conventional outflow facility, using either a non-selective NOS inhibitor (L-NAME; second set) or an eNOS-selective inhibitor (cavtratin; third set). For L-NAME experiments, treated eyes were perfused with either 10 µM or 100 µM L-NAME in DBG, while the contralateral control eye was perfused with DBG alone. L-NAME perfusions were performed only in WT mice, because we have already reported the effects of L-NAME in eNOS-GFPtg mice (69). L-NAME solution was prepared from powder immediately prior to each experiment. For cavtratin experiments, treated eyes were perfused with 50 µM cavtratin in DBG containing 0.6% DMSO, while the contralateral vehicle control eye was perfused with DBG containing 0.6% DMSO without cavtratin. Cavtratin is a chimeric peptide that contains a cellular internalization sequence antennapedia (AP) domain fused to the caveolin-1 scaffolding domain that binds to and inhibits eNOS. Cavtratin has been shown to enter murine vascular endothelial cells and inhibit eNOS activity and NO production for up to 20 hrs in vivo and in vitro (10, 29, 60, 65). Based on these data, we expect that cavtratin should inhibit eNOS within the trabecular outflow pathway of the murine eye. Cavtratin experiments were performed in both WT and eNOS-GFPtg mice, with the contralateral control eyes perfused with DBG alone. A second set of cavtratin experiments perfused the contralateral eye with a scrambled cavtratin domain but an intact AP domain (cavtratin-X) at the same concentration (50 µM). Cavtratin was stored as a stock solution (8 mM in DMSO) at –20°C and diluted to a working concentration immediately prior to each experiment, protecting the solutions from light at all times. Eyes were perfused at sequential pressure steps of 4, 8, 15 and 20 mmHg. These pressure steps differ from the first experimental set because in the first set we observed nonlinear pressure-flow behavior at the highest pressure step (25 mmHg), and we therefore decreased the pressure range in the later sets to minimize this nonlinearity.
The eyes were enucleated within 10 minutes of death by cervical dislocation and stored in PBS (Invitrogen) at 4°C until perfusion, typically within 1-3 hours. Experiments used paired contralateral eyes, except in the cases where data from one eye were rejected based on the stability criterion described below. Paired eyes were perfused sequentially (one eye immediately after enucleation, the second eye 2-3 hours after enucleation), with the treatment assigned randomly to the first or second eye so as to control for any time-dependent post-mortem changes. No differences in facility were observed between untreated controls perfused immediately versus those perfused 2-3 hours later (not shown).

Our perfusion method follows previously described techniques (8, 9, 40, 69). The eye was affixed to a support using cyanoacrylate glue to stabilize the eye for cannulation by a 33-gauge beveled needle (Nanofil; World Precision Instruments, Europe; Hitchin, UK) with the tip positioned in the anterior chamber using a micromanipulator (Figure 1A). Before cannulation, each needle was backfilled from the tip with 200 µL of the perfusate, a volume sufficient to last several hours during perfusion. The needle was connected via pressure tubing to a glass syringe (25µL; Hamilton GasTight, Reno, NV) mounted on a motorized syringe pump (PHD Ultra; Harvard Apparatus, MA). Custom written LabVIEW software (National Instruments Corp., Austin, TX) was used to control the syringe pump flow rate to maintain the eye at a user-defined perfusion pressure (50) that was monitored by a pressure transducer (142PC01G; Honeywell, Columbus, OH) located upstream of the cannulation needle. Eye temperature was maintained at between 34°C and 37°C, with the eye kept hydrated by submerging it to the limbus in PBS and by covering the cornea with moist tissue paper. Both the experimental and control eyes were pressurized from a reservoir at 8 mmHg for 45 minutes before starting the perfusion to allow sufficient time for the pressure to stabilize within the eye.

Each pressure step during perfusion was maintained for 20-30 minutes, and the average stable flow rate was calculated over approximately 10 minutes near the end of each pressure step (Figure 1B). Data were considered acceptable if a stable flow rate was achieved in at least 3 of the 4 pressure steps. Conventional outflow facility (C) was estimated by fitting our pressure-flow rate data to the modified Goldmann equation (40):

\[ F = C \times P_p + F_u \]  

(1)
where $F$ represents the average stable flow rate at each perfusion pressure ($P_p$). $F_u$ represents the apparent flow rate at zero pressure. Equation 1 is only valid when aqueous humor production and episcleral venous pressure are zero (appropriate for enucleated eyes), when $F$ reaches equilibrium at each value of $P_p$, and when $C$ and $F_u$ are independent of $P_p$. The value of $C$ is estimated as the slope of the best-fit linear regression to the measured $F$ versus $P_p$ data. At the end of the perfusion, eyes were fixed by immersion in 4% paraformaldehyde (PFA) for 1 hour, followed by long-term storage in 0.1% PFA.

**Immunofluorescence Microscopy**

The expression and localization of eNOS-GFP in the conventional outflow pathway was examined by immunofluorescence microscopy of eyes from eNOS-GFPtg and WT mice (N = 2 each). After immersion fixation, eyes were opened at the cornea and infiltrated with 30% sucrose in PBS, followed by a 1:1 mixture of 30% sucrose solution and cryostat embedding compound (Tissue-Tek OCT; Sakura-Finetek, Torrance, CA). The eyes were embedded in cryomolds (Sakura-Finetek), frozen on dry ice, mounted in a cryostat (Microm HM 525; ThermoFisher Scientific, Waltham, MA), and sagittally sectioned in steps of 16 µm. Cryosections were dried for 1h at room temperature, rehydrated in PBS, permeabilized in 0.2% Triton X-100 in PBS for 5 minutes and blocked in 10% normal goat serum in PBS for 30 min. The sections were incubated with antibodies raised in rabbit against GFP (70) at a dilution of 1:500 in PBS for 2 hours at room temperature, followed by rat anti-mouse CD31 antibodies (clone mec13.3; BD Pharmingen, Mississauga, ON) at a dilution of 1:100 for 2 hours at room temperature to label the endothelium of SC. The sections were then incubated with Alexa 555 goat anti-rat and Alexa 647 goat anti-rabbit secondary antibodies (Life Technologies Inc., Burlington, ON) diluted 1:200 in PBS for room temperature for 2 hours. The sections were incubated with 5 µM Sytox (Life Technologies Inc., Burlington, ON) for 10 minutes at room temperature to label nuclei, mounted in Prolong Gold Fluorescent mounting media (Life Technologies Inc., Burlington, ON) and examined with a Zeiss LSM 510 Confocal microscope (Zeiss, Jena, Germany).

**Statistical Analysis**
Differences in outflow facility between treated and untreated eyes were examined with the linear mixed model (LMM) using commercial statistical software (SPSS, IBM), following previous methods (9). The LMM accounts for correlated errors between paired eyes from individual mice, while allowing for unpaired eyes that arise when the contralateral eye fails to meet the stability criteria. Differences were considered statistically significant when the $P$ value (estimating the probability that the null hypothesis was true) was less than 0.05.

Results

Effect of Light on NO-Donors

The initial 10 minute exposure of the stock solution at 700-1000 lumens/m$^2$ triggered NO-release from SNAP that increased over one hour under ambient light conditions (Figure 2A). Continuous light exposure at 700-1000 lumens/m$^2$, however, led to an initial rise in NO followed by a decline in NO concentration to zero within one hour (Figure 2A). These data reveal that NO release by SNAP is light dependent, but that prolonged light exposure can disrupt NO release kinetics. Importantly, this suggests that lighting conditions must be carefully controlled during SNAP perfusion experiments. Therefore, for all subsequent SNAP/NAP experiments, the SNAP-containing solutions were pre-treated with 700-1000 lumens/m$^2$ for 10 minutes prior to the perfusion, with the perfusion performed under low ambient light. Following this protocol, the concentration of NO released from 100 µM SNAP reached 140 ± 20 nM (mean ± SD; N = 3) at one hour (Figure 2B). The NO concentration released from 100 µM SNAP far exceeds the Michaelis concentration ($K_M = 23$ nM) (15) for the reaction between NO and soluble guanylate cyclase, and thus likely yields a saturating dosage of NO. 100 µM NAP, in contrast, released no detectible NO (0±4 nM; N = 3) under otherwise identical conditions (Figure 2B). We also examined an alternative NO-donor (100 µM DETA-NO), but found that NO release by DETA-NO was nearly 3-fold less than by the same concentration of SNAP (140 ± 20 nM vs. 49 ± 9 nM; data not shown) over 60 minutes.

Effect of NO-Donors on Conventional Outflow Facility

To determine whether NO-donors affect conventional outflow facility, we perfused paired eyes from either WT or eNOS-GFPtg mice with 100 µM SNAP or 100 µM NAP. In WT mice, SNAP increased the flow rate at each perfusion pressure and increased $C$ by 62 ± 28% compared to NAP (0.051 ± 0.015 vs. 0.031 ± 0.010
µL/min/mmHg; N = 6 vs. 5; P=0.016; Figure 3A). In eNOS-GFPtg mice, SNAP had no
detectible effect on the flow rate (Figure 3B) and there was no difference in C between
SNAP and NAP-treated eyes (0.019 ± 0.006 vs. 0.021 ± 0.005 µL/min/mmHg; N = 5 vs.
4; P = 0.40). Taken together, these data reveal that a NO donor increases outflow facility
in WT mice, but has no detectible effect in eNOS-GFPtg mice.

Effect of NOS-Inhibitors on Conventional Outflow Facility

To determine whether endogenous NOS activity contributes to the regulation of
conventional outflow facility, we perfused WT mice with L-NAME, a non-selective
inhibitor of all NOS isoforms. At low dosage, 10 µM L-NAME reduced the flow rate at
each perfusion pressure relative to vehicle-treated eyes (Figure 4A) and decreased C by
36 ± 13 % (0.014 ± 0.002 vs. 0.021 ± 0.007 µL/min/mmHg; N = 5 each, P=0.012).
However, at a higher dosage, 100 µM L-NAME had no apparent effect on the flow rate
nor on C (0.028±0.007 vs. 0.024±0.005 µL/min/mmHg; N = 9 vs. 8; P=0.22; Figure 4B).
These data reveal that L-NAME has a biphasic effect on outflow facility in WT mice,
decreasing C at lower concentrations but having little effect on C at higher
concentrations.

To investigate specifically the influence of eNOS, as opposed to other NOS
isoforms, we perfused eyes with cavtratin, an eNOS-selective inhibitor. Cavtratin at
50 µM decreased C in WT eyes by 19 ± 12% relative to vehicle-treated contralateral
eyes (0.021 ± 0.009 vs. 0.025 ± 0.012 µL/min/mmHg; N = 7 vs. 8; P=0.011; Figure 4C).
In a separate set of experiments we compared cavtratin effects to a control peptide with a
scrambled cavtratin domain and an intact AP domain (cavtratin-X ), and found a similar
25 ± 17% reduction in C (0.019 ± 0.008 vs. 0.014 ± 0.007 µL/min/mmHg; N = 9 each;
P=0.026; not shown). However, in eNOS-GFPtg mice, 50 µM cavtratin decreased C by
39 ± 25% relative to vehicle-treated contralateral eyes (0.008 ± 0.004 vs. 0.014 ± 0.006
µL/min/mmHg; N = 7 vs. 5 each; P=0.014; Figure 4D). These data suggest that eNOS
may be the predominant isoform involved in aqueous outflow regulation, decreasing C in
both WT and eNOS-GFPtg mice.

Localization of eNOS Transgene Expression within the Conventional Outflow Pathway

To confirm and localize expression of the eNOS transgene within the
conventional outflow pathway, eyes from eNOS-GFPtg mice were enucleated and
processed for immunofluorescence microscopy. The immunofluorescence signal from
antibody complexes against GFP in eNOS-GFPtg mice co-localized with the endothelial marker CD31 within blood vessels of the iris and ciliary body, confirming the endothelial-specific expression of the eNOS-GFP transgene and its promoter (74). In the conventional outflow pathway (Figure 5), GFP immunofluorescence was restricted to the endothelium of SC, collector channels and episcleral vessels that also expressed CD31 (31). Interestingly, there was no apparent GFP staining of the trabecular meshwork (TM), and GFP labelling was entirely absent from WT mice that lacked the eNOS-GFP transgene. No obvious differences were observed in the morphology of SC and TM between eNOS-GFPtg and WT mice.
Discussion

Exogenously delivered NO typically decreases IOP by increasing outflow facility. The facility-increasing effect of various NO donors was first demonstrated in living monkeys (32, 63), and subsequently in living rabbits (38) and in post-mortem eyes from pigs (21, 22) and humans (61). The current study demonstrates that exogenous NO, delivered via SNAP, also increases outflow facility in enucleated eyes from C57BL/6 mice. This suggests that there exists a common outflow facility response to exogenous NO across several species that can be examined in mice as a model system that resembles the functional anatomy (51) and pharmacology (8) of the human outflow pathway.

The precise details of how NO affects outflow facility and IOP remain unclear, but likely involve mechanisms related to cellular contractility or endothelial permeability. NO is a free radical gas that can rapidly diffuse across cell membranes to affect the biological activity of several proteins and enzymes. Soluble guanylate cyclase (sGC), for instance, mediates the vasodilatory effect of NO on smooth muscle cells in the cardiovascular system, and sGC is necessary for the facility increase following exogenous NO in ex vivo pig eyes (22). Deficiencies in sGC signaling contribute to ocular hypertension and glaucomatous optic neuropathy in mice (12). Similarly, NO induces relaxation of TM cells (20) and decreases TM (19) and SC cell volume (23) to potentially affect the dimensions of inner wall pores or flow pathways in the juxtacanalicular tissue. Alternatively, NO mediates the disruption of adherens junctions to increase permeability of vascular endothelia (13, 18), and NO may thereby disrupt the adherens junctions along the inner wall of SC (31) to increase facility. Downstream of SC, NO may induce relaxation of smooth muscle cells that surround the collector channels (17) to increase intrascleral vessel diameter and so may influence post-trabecular or distal outflow resistance downstream of SC (59).

NO also induces relaxation of the ciliary muscle (6, 26, 28, 75). However, as pointed out previously (63, 75), ciliary muscle relaxation tends to decrease outflow facility and oppose the facility increase putatively caused by TM/SC relaxation. This effect should be more pronounced in primates (57, 58, 72) and mice (51) that have a ciliary muscle with more extensive tendinous connections to the juxtacanalicular tissue and inner wall endothelium of SC relative to other species such as pigs (4, 45). NO may
also affect other aspects of aqueous humor dynamics; for example, NO-donors may decrease aqueous humor inflow (66), increase episcleral venous pressure (77) and affect choroidal blood volume (35). Thus, there are numerous potential mechanisms by which NO may affect aqueous humor dynamics, and, depending on the site of NO activity and the species being examined, NO may either increase or decrease IOP. While the downstream mechanisms by which NO regulates IOP are certainly important, the current study focused upstream on the role of NOS enzymes and how production of endogenous NO may be involved in the physiological regulation of outflow facility.

Endogenous NO is produced in vivo by a family of NOS enzymes encoded by 3 genes and their splice variants, including the calcium-dependent eNOS and nNOS that are constitutively expressed in endothelial cells and nitrergic neurons, respectively, and the calcium-independent iNOS that is expressed predominately in macrophages. While Nathansan and McKee argue, based on immunohistochemistry, that eNOS is the primary NOS isoform in the human outflow pathway (48), the TM is innervated by nitrergic nerve fibers that contain nNOS in primate and porcine eyes (44, 64). Macrophages are commonly found within the TM, particularly after laser trabeculoplasty (1), and express iNOS (76) that when activated yields greater quantities of NO relative to eNOS or nNOS (48, 61, 62). Within the TM, the NOS expression profile, particularly for iNOS, changes in response to IOP (62). Downstream of the TM, nNOS-labeled nerve fibers and terminals surround episcleral veins in mice (51) and arteriovenous anastomoses within the intrasceral vessel network in primates (25). eNOS itself, while expressed by SC inner wall cells, is also expressed by the endothelium of the intrasceral collector channels and aqueous veins (69). In primates, the longitudinal ciliary muscle fibers are enriched in eNOS (48), and insert directly into the juxtacanalicular region (57, 58). Thus, all three NOS isoforms, and likely their splice variants, are present throughout the trabecular, post-trabecular and ciliary muscle regions, suggesting multiple locations where NOS activity may influence outflow physiology.

To examine the physiological role of endogenous NO in aqueous humor dynamics, we perfused enucleated mouse eyes with inhibitors of NOS enzymes and measured conventional outflow facility. By examining enucleated eyes, one can measure outflow facility independently of NO-mediated effects on inflow, episcleral venous pressure (EVP) or unconventional outflow, since such effects are very likely eliminated in...
enucleated eyes and would otherwise confound measurements in vivo. This assumes
that the facility is unaffected by post-mortem changes, and that the NO signaling
pathways are preserved ex vivo. In support of the ex vivo model, Millar and colleagues
have shown that the facility measured in vivo is not significantly different than the facility
measured immediately after death in the same mice prior to enucleation (46).
Furthermore, the pharmacological facility response measured in enucleated mouse eyes
mimics the response previously observed in enucleated human eyes to several receptor-
mediated compounds including sphingosine 1-phosphate (8, 71), prostaglandin EP4
agonist (8, 47) and pilocarpine (5, 51).

In wild-type mice, non-selective inhibition of NOS enzymes by L-NAME
decreased outflow facility at 10 µM, suggesting that there is a basal tone of endogenous
NO involved in facility regulation. However, there was no detectible facility decrease
observed in response to L-NAME at 100 µM. It is puzzling why higher concentrations of
L-NAME had no detectible effect on outflow facility; one possibility is that there is
differential NOS inhibition between different tissues of the outflow pathway that may, in
some cases, exert opposing effects on outflow facility. For example, one may speculate
that the higher concentration of L-NAME is more assessable to the ciliary muscle where
NO-mediated ciliary muscle relaxation may tend to oppose the facility increase caused
NO action within the TM (see above). Alternatively, the data may reflect differences in
the inhibition of different NOS isoforms by L-NAME that tends to be more selective for
nNOS (IC50 = 0.15 µM) relative to eNOS (IC50 = 2.7 µM) or iNOS (IC50 = 14 µM) (73),
and perhaps the locations or actions of the different isoforms have opposing effects on
outflow facility. L-NAME must also be converted into a free acid to become bioactive
(53), and the kinetics of this reaction may vary between the TM and ciliary muscle that
exert opposing effects on facility. Alternatively, L-NAME may act as a muscarinic
antagonist (11), affecting the M2 and M3 receptors present within the ciliary body and iris
sphincter of humans (27, 32). If a similar receptor profile is present in mice, then L-
NAME could presumably act directly on muscarinic receptors to modulate ciliary muscle
contracility that affects outflow facility in mice (42, 51). Apart from NOS, NO itself
exhibits a biphasic effect on TM cell contractility (20) and IOP (39, 49), and thus the
response to L-NAME that we observed may reflect the underlying biphasic nature of how
NO affects outflow facility. Regardless of the underlying mechanism, the literature is
inconsistent on how L-NAME affects aqueous humor dynamics. For example, in living
monkeys L-NAME does not appear to affect IOP (32), yet in monkey anterior segment organ culture perfusion L-NAME lowers IOP by increasing outflow facility (61). More studies are necessary to better understand how NOS inhibition by L-NAME influences aqueous humor dynamics and outflow facility in particular.

Despite the potential role of other NOS isoforms, eNOS appears to be particularly important for the physiological regulation of outflow facility. Stamer et al. (69) have shown that elevated eNOS expression in eNOS-GFPtg mice leads to reduced IOP and increased outflow facility. However, our data suggest that exogenous NO does not increase outflow facility in eNOS-GFPtg mice, despite significantly increasing facility in wild-type. This demonstrates that the facility-increasing effect of NO becomes saturated under conditions of elevated eNOS expression. When eNOS expression was selectively inhibited by cavtratin, facility was reduced in both WT and eNOS-GFPtg mice, with a 2-fold greater reduction in the transgenic mice that have elevated eNOS activity. Similarly, Stamer et al. (69) reported a similar facility reduction in the eNOS-GFPtg mice in response to L-NAME. This is consistent with the hypothesis that NO production by eNOS contributes to the normal physiologic regulation of outflow facility in mice. Disruption of normal eNOS activity, as may occur in some cases of glaucoma (34, 43, 54) may therefore contribute to the pathogenesis of ocular hypertension associated with the disease.

One limitation of this study was that the baseline outflow facility in WT mice was consistently larger than the baseline facility of eNOS-GFPtg mice. This contrasts with the prior study of Stamer et al. (69) who reported a two-fold larger facility in eNOS-GFPtg mice compared to WT littermates. There are two important differences that may contribute to this discrepancy between studies. Firstly, the perfusions in the current study were done at physiological temperature with the eyes submerged under isotonic saline, where perfusions in the prior study were conducted with the eyes at room temperature exposed to room air. Environmental factors such as temperature and hydration likely influence the measurement of outflow facility (9), and thus may have contributed to baseline facility differences between the current and prior studies. Secondly, the current study did not directly compare between transgenic and WT mice from the same litter. Instead, WT mice used in the current study, despite being from the same background strain, were obtained from a separate colony that may have exhibited a different baseline facility relative to the transgenic colony. For these reasons, the
current experiments were not designed to detect baseline facility differences between WT and eNOS-GFPtg mice, but rather were designed to examine the effect of NO-donors or NOS inhibitors between paired eyes of a given cohort.

The mechanism by which eNOS regulates outflow facility remains to be determined. However, the expression of the eNOS-GFP transgene in the conventional outflow pathway was localized to the endothelium of SC and the intrascleral vessels, with no apparent expression within the TM. This strongly suggests that NO release by endothelial cells within SC or intrascleral vessels influences outflow facility. Furthermore, because NO production by eNOS is shear sensitive (13, 14) and because the shear stress acting on the inner wall reaches levels known to activate eNOS in vascular endothelia (24), it is possible that SC cells produce NO in response to circumferential flow within SC. In fact, recent studies have demonstrated shear-induced NO production by human SC cells (2) and by porcine angular aqueous plexus cells (41) (the porcine equivalent of SC cells). This presents the possibility that eNOS may act as part of an endogenous feedback loop to regulate IOP and to offset any potential changes in outflow resistance within the TM. According to this hypothesis, originally outlined by Stamer et al. (69), IOP elevation causes collapse of SC that increases the shear stress acting on the inner wall because the same volumetric flow rate of aqueous must then flow through a smaller SC lumen. Shear-induced NO release by SC cells then increases outflow facility to oppose the IOP elevation. One should note that this scenario requires that both the original outflow obstruction and the action of NO on facility occur within the TM, and not downstream of the inner wall of SC. The same mechanism could respond to reductions in IOP, reducing basal NO production that would tend to increase IOP. Such a mechanism may underlie IOP homeostasis and contribute to the relative consistency of IOP that lies within a surprisingly narrow range despite decades of life in healthy individuals (16, 37). This homeostatic mechanism may become impaired in glaucomatous eyes that appear to have reduced NOS activity in the conventional outflow pathway (48). Our findings predict that in order for NO-based therapeutics to achieve optimal IOP reduction, NO must be released in close proximity to the juxtacanalicular region and inner wall of SC, where endogenous eNOS regulation of outflow normally occurs. NO-donors that release NO in the anterior chamber, for instance, may not achieve full therapeutic benefit, and NO release in other tissues such as in the ciliary muscle or episcleral veins may be counterproductive and potentially lead to elevations in
IOP. Thus, targeting the NO-regulatory machinery within the conventional outflow pathway may provide a promising therapeutic target for treating glaucoma.
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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).
Figure Legends:

**Figure 1:** The perfusion system to measure outflow facility in enucleated ex vivo mouse eyes. (A) A computer-controlled syringe pump controls the perfusion flow rate ($F$) into the mouse eye to maintain a user-defined perfusion pressure that is measured by a pressure transducer ($P_p$; see Equation 1). The mouse eye is held within an isotonic bath at 34-37°C and is cannulated by a 33 gauge needle that is positioned into the anterior chamber using a micromanipulator while viewing under a stereomicroscope. The perfusion is open to a reservoir set to 8 mmHg above the eye during needle insertion and during the initial pressurization period, but the reservoir is closed during the perfusion. (B) Representative perfusion tracings showing the perfusion pressure (blue line) and flow rate data from paired eyes perfused with 100 µM N-acetyl-D-penicillamine (NAP; grey line) or 100 µM S-Nitroso-N-acetylpenicillamine (SNAP; black line). Red highlighted regions represent data used to calculate the average flow rate at each corresponding pressure step. Spikes represent rapid increases in flow rate to maintain the user-defined perfusion pressure. Only one perfusion pressure tracing is shown, but pressure tracings are generally similar for both eyes.

**Figure 2:** Characterization of NO concentration following release by SNAP or NAP under varying lighting conditions. (A) NO release following initial high intensity light exposure of the stock solution (113 mM) (700-1000 lumens/m²) for 10 minutes, followed by dilution to the working solution (1 mM in DBG) and either low intensity light (100-200 lumens/m²; filled circles) or prolonged high intensity light (700-1000 lumens/m²; open circles). Data is from one experiment, but is representative of three individual experiments. Time zero corresponds to the end of the initial 10 minute period, during which time the SNAP stock was exposed to high intensity light and after which time the NO release was measured in the working solution by the probe. (B) NO release from 100 µM SNAP (filled circle) or 100 µM NAP (open circles) in DBG (N = 3 each; bars are SD). Each stock solution was treated with an initial 10 minute high intensity light exposure (700-1000 lumens/m²) followed by low intensity light (100-200 lumens/m²) of the working solution starting at time zero. NO was measured using an NO-sensitive probe (ISO-NOS II). The data shown in panel B are representative of the NO release during the 100 µM SNAP/NAP perfusions.
Figure 3: The effects of NO-donor on conventional outflow facility in eyes from wild-type (WT) and eNOS-GFPtg mice. (A) Perfusion flow rate measured as a function of pressure in enucleated eyes from WT mice perfused with 100 µM SNAP (NO-donor; filled circles) or 100 µM NAP (inactive analog; open circles). Outflow facility was estimated based on the slope on the linear regression through the data points, as described by Equation 1. (B) Perfusion flow rate measured as a function of pressure in enucleated eyes from eNOS-GFPtg mice perfused with 100 µM SNAP (filled circles) or 100 µM NAP (open circles). Bars represent SD. Note different vertical axis scales in panels A and B.

Figure 4: The effect of NOS-inhibitors on conventional outflow facility in enucleated eyes from wild-type (WT) and eNOS-GFPtg mice. (A,B) Perfusion flow rate measured as a function of pressure in WT eyes perfused with L-NAME (non-selective NOS inhibitor; filled circles) at 10 µM (A) or 100 µM (B) in DBG versus DBG vehicle alone (open circles). (C,D) Perfusion flow rate measured as a function of pressure in eyes perfused with 50 µM cavatrin (eNOS-selective inhibitor; filled circles) versus vehicle control (DBG + 0.6% DMSO; open circles) from WT (C) or eNOS-GFPtg (D) mice. Outflow facility is estimated based on the slope on the linear regression through the data points, as described by Equation 1. Error bars represent SD.

Figure 5: Localization of transgene expression in the conventional outflow pathway of eNOS-GFPtg mice. (A) Sagittal section through the trabecular meshwork (TM) and Schlemm’s canal (asterisk) labeled with the endothelial marker CD31, showing labeling of SC endothelium as well as blood vessels (arrowheads) in the ciliary body (CB) and sclera. (B) Indirect immunofluorescence against GFP from the same section shown in panel A, revealing co-localization of the eNOS-GFP transgene along the endothelium of SC and along blood vessels in the CB. (C) Merged color image showing labeling for CD31 and GFP superimposed on the brightfield image to show the location of SC and TM with respect to the CB, iris and sclera. Insert: lower magnification of the brightfield sagittal section showing the location of the outflow pathway with respect to the iris and lens. Data are representative images from two eNOS-GFPtg mouse eyes.


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**A**

- Enucleated mouse eye
- 33G Needle
- Micro-manipulator
- Temperature-controlled Waterbath
- Microscope
- Reservoir
- Syringe Pump
- Pressure Transducer
- Computer Controller

**B**

- **Flow Rate, SNAP**
- **Flow Rate, NAP**
- **Perfusion Pressure**

**Graph**

- Y-axis: Perfusion Pressure (mmHg)
- X-axis: Flow Rate (μL/min)
- Time: 15 min
A

- 1mM SNAP (high intensity light exposure)
- 1mM SNAP (low intensity light exposure)

Addition of NO-donor

Time (minutes)

NO concentration (nM)

B

- 100μM SNAP (n=3)
- 100μM NAP (n=3)

Addition of NO-donor

Time (minutes)

NO concentration (nM)
A  WT-mice + NO-Donor

B  eNOS-GFPtg + NO-Donor

Flow Rate (μL/min) vs. Perfusion Pressure (mmHg)

For WT-mice + NO-Donor:
- SNAP, 100μM (N=6)
- NAP, 100μM (N=5)

Regression equation:
- $F = 0.051 P_P - 0.309$
- $R^2 = 0.956$

For eNOS-GFPtg + NO-Donor:
- SNAP, 100μM (N=5)
- NAP, 100μM (N=4)

Regression equation:
- $F = 0.019 P_P - 0.077$
- $R^2 = 0.973$

Regression equation:
- $F = 0.021 P_P - 0.123$
- $R^2 = 0.970$
A  WT-mice + NOS inhibitors

F = 0.021 \( P_p \) - 0.011
\( R^2 = 0.982 \)

B  WT-mice + NOS inhibitors

F = 0.028 \( P_p \) - 0.040
\( R^2 = 0.990 \)

C  WT-mice + NOS inhibitors

F = 0.025 \( P_p \) - 0.064
\( R^2 = 0.985 \)

D  eNOS-GFPtg + NOS inhibitors

F = 0.014 \( P_p \) - 0.042
\( R^2 = 0.988 \)

F = 0.008 \( P_p \) - 0.029
\( R^2 = 0.992 \)