Role of nitric oxide in murine conventional outflow physiology

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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 (68), identifying drugs that enhance conventional outflow may lead to new, more effective therapies for ocular hypertension and primary open-angle glaucoma. 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 regulation of aqueous humor outflow. NO is well known to mediate various physiological functions, including vasodilation, junctional complex assembly, and permeability, in vascular and lymphatic endothelial cells (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 NO 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.1 Our studies use ex vivo mouse eyes, because the conventional outflow pathway in mice is anatomically and functionally similar to that in 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 Association for Research in Vision and Ophthalmology 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 outflow facility, eyes were perfused with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) or its inactive analog N-acetyl-d-penicillamine (NAP). Because SNAP is light-sensitive, the NO release kinetics of SNAP and NAP were first characterized to identify an optimal lighting condition, which was controlled for all SNAP/NAP perfusion experiments. To examine whether endogenous NOS activity contributes to regulation of outflow facility, eyes were perfused with or without a broad-spectrum NOS inhibitor [N^ω-nitro-L-arginine methyl ester (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 previously reported that L-NAME decreases outflow 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 NO-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 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: 1) initial high-intensity light (700–1,000 lumens/m2) exposure of the stock solution (113 mM) for 10 min (measured with a luminometer; Mastech, GuangDong, China) followed by low-light (100–200 lumens/m2) conditions, where the NO donor was diluted to the working concentration (0.1–1 mM) and protected from surrounding laboratory light with aluminum foil, and 2) initial high-intensity light (700–1,000 lumens/m2) exposure of the stock solution for 10 min followed by continuous light exposure of the working solution at the same level (700–1,000 lumens/m2). NO release was measured over 60 min starting immediately after the initial 10-min light exposure for both conditions. Condition 1 was used for subsequent SNAP/NAP perfusions. Because 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 during continuous mixing with a magnetic stir bar.

Animal husbandry. This study used transgenic mice of the same C57BL/6 background strain as the WT controls. Perusions were performed with enucleated (ex vivo) eyes from mice of either sex. eNOS-GFPtg mice were 8–13 wk of age (for SNAP/NAP and cavtratin perfusions). WT mice were 6–12 wk of age (for SNAP/NAP perfusions) or 25–30 wk of age (for L-NAME and cavtratin perfusions). Mice were housed in individually ventilated cages, fed ad libitum, and maintained at 21°C with a 12:12-h light-dark cycle, with lights on from 6 AM to 6 PM.

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 twofold increase in plasma NO concentrations in large arteries (74), and to cause decreased aqueous humor outflow resistance and reduced IOP compared with 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 the manufacturer’s instructions (DNAeasy Blood & Tissue Kit, Qiagen, Hilden, Germany). Separate PCRs 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 AGGCGC-CAATGGTAAAACCTGGAAGAT and antisense primer AAGGCTC-TGGTGCGTGATGC 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 AGCTGACCGCTGAAAGTT and antisense primer TTGGTGATGC to yield a predicted product of 1133 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 of the NO donor (SNAP; Calbiochem) compared with 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 (DBG) passed through a 0.2-μm filter. 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 divided into aliquots and stored at −20°C, with care taken to protect 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 min followed by low ambient light exposure of the working solution (see above) immediately prior to initiation of the perfusion. Eyes were perfused at sequential pressure steps of 8, 15, 20, and 25 mmHg to determine outflow facility (see below).

In the second and third sets of experiments, we used a nonselective NOS inhibitor (L-NAME; second set) or an eNOS-selective inhibitor (cavtratin; third set) to examine the effect of NO inhibitors on conventional outflow facility. For L-NAME experiments, the treated eye was perfused with 10 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 previously 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, the treated eye was 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, which binds to and inhibits eNOS.
Cavitratin has been shown to enter murine vascular endothelial cells and inhibit eNOS activity and NO production for up to 20 h in vivo and in vitro (10, 29, 60, 65). Based on these data, we expect that cavitratin should inhibit eNOS within the trabecular outflow pathway of the murine eye. Cavitratin experiments were performed in WT and eNOS-GFPTg mice, with the contralateral control eye perfused with DBG alone. In a second set of cavitratin experiments, the contralateral eye was perfused with a scrambled cavitratin domain but an intact AP domain (cavitratin-X) at the same concentration (50 μM). Cavitratin was stored as a stock solution (8 mM in DMSO) at −20°C and diluted to a working concentration immediately prior to each experiment, with care taken to protect 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 those used in the first experimental set: because, in the first set of experiments, we observed nonlinear pressure-flow behavior at the highest (25 mmHg) pressure step, we therefore decreased the pressure range in the second and third sets of experiments to minimize this nonlinearity.

The eyes were enucleated within 10 min of death by cervical dislocation and stored in PBS (Invitrogen) at 4°C until perfusion, typically within 1–3 h. Experiments used paired contralateral eyes, except in the cases where data from one eye were rejected on the basis of the stability criterion described below. Paired eyes were perfused sequentially (one eye immediately after enucleation, the second eye 2–3 h after enucleation), with the treatment assigned randomly to the first or second eye to control for any time-dependent postmortem changes. No differences in outflow facility were observed between untreated controls perfused immediately and those perfused 2–3 h 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, Hitchin, UK), with the tip positioned in the anterior chamber using a micromanipulator (Fig. 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 (25 μl; Hamilton GasTight, Reno, NV) mounted on a motorized syringe pump (PHD Ultra, Harvard Apparatus, Cambridge, MA). Custom-written LabVIEW software (National Instruments, Austin, TX) was used to control the syringe pump flow rate to maintain the eye at a user-defined perfusion pressure (50), which was monitored by a pressure transducer (model 142PC01G, Honeywell, Columbus, OH) located upstream of the cannulation needle. Eye temperature was maintained at 34–37°C, with the eye kept hydrated by submersion to the limbus in PBS and the cornea covered with moist tissue paper. Both the experimental and control eyes were pressurized from a reservoir at 8 mmHg for 45 min before initiation of the perfusion to allow sufficient time for the pressure to stabilize within the eye.

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

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F = C \cdot P_p + F_u
\]

where \( F \) represents the average stable flow rate at each perfusion pressure \( P_p \), and \( F_u \) 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 \( F \) vs. \( P_p \) data. At the end of the perfusion, eyes were fixed by immersion in 4% paraformaldehyde for 1 h followed by long-term storage in 0.1% paraformaldehyde.

**Immunofluorescence microscopy.** The expression and localization of eNOS-GFP in the conventional outflow pathway were 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 and then with 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 1 h at room temperature, rehydrated in PBS, permeabilized with 10% Triton X-100, and blocked with 10% nonfat milk. The sections were stained with a primary antibody against eNOS (a gift from Dr. Guglielmo Gotti, University of Pisa, Pisa, Italy; 1:200), secondary antibody against rabbit IgG (1:200; Invitrogen), and 4′,6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining. Slides were imaged with a confocal microscope (Olympus FV1000, Olympus America, Center Valley, PA) equipped with a 20× objective. Confocal images were analyzed with Fiji software (National Institutes of Health). Immunofluorescence images were pseudocolored and merged with DAPI images to create the final images shown.
in 0.2% Triton X-100 in PBS for 5 min, 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 h at room temperature and then with rat anti-mouse CD31 antibodies (clone mec13.3, BD Pharmingen, Mississauga, ON, Canada) at a dilution of 1:100 for 2 h 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, Burlington, ON, Canada) diluted 1:200 in PBS at room temperature for 2 h at room temperature and then with rat anti-mouse CD31 antibodies raised in rabbit against GFP (clone mec13.3, BD Pharmingen, Mississauga, ON, Canada) at a dilution of 1:100 for 2 h at room temperature to label nuclei, mounted in Prolong Gold fluorescent mounting medium (Life Technologies), and examined with a confocal microscope (LSM 510, Zeiss, Jena, Germany).

Statistical analysis. Differences in outflow facility between treated and untreated eyes were examined with the linear mixed model using commercial statistical software (SPSS, IBM) following previous methods (9). The linear mixed model 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 <0.05.

RESULTS

Effect of light on NO donors. The initial 10-min exposure of the stock solution at 700–1,000 lumens/m² triggered NO release from SNAP that increased over 1 h under ambient light conditions (Fig. 2A). Continuous light exposure at 700–1,000 lumens/m², however, led to an initial rise in NO followed by a decline to zero within 1 h (Fig. 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 exposed to 700–1,000 lumens/m² for 10 min prior to the perfusion, which was performed under low ambient light. After this protocol, the concentration of NO released from 100 µM SNAP reached 140 ± 20 (SD) nM (n = 3) at 1 h (Fig. 2B). The NO concentration released from 100 µM SNAP far exceeds the Michaelis concentration (Kₘ = 23 nM) (15) for the reaction between NO and soluble guanylate cyclase (sGC) and, thus, likely yields a saturating dose of NO. In contrast, 100 µM NAP released no detectable NO (0 ± 4 nM, n = 3) under otherwise identical conditions (Fig. 2B). We also examined an alternative NO donor (100 µM diethylenetriamine-NO; DETA-NO), but we found that NO release by DETA-NO was nearly threefold less than NO release by the same concentration of SNAP (140 ± 20 vs. 49 ± 9 nM; data not shown) over 60 min.

Effect of NO donors on conventional outflow facility. To determine whether NO donors affect conventional outflow facility, we perfused paired eyes from WT or eNOS-GFpGt mice with 100 µM SNAP or 100 µM NAP. In WT mice, SNAP increased the flow rate at each perfusion pressure and increased conventional outflow facility by 62 ± 28% compared with NAP [0.051 ± 0.015 (n = 6) vs. 0.031 ± 0.010 (n = 5) µl/min⁻¹·mmHg⁻¹, P = 0.016; Fig. 3A]. In eNOS-GFpGt mice, SNAP had no detectable effect on the flow rate (Fig. 3B) and there was no difference in conventional outflow facility between SNAP- and NAP-treated eyes [0.019 ± 0.006 (n = 5) vs. 0.021 ± 0.005 (n = 4) µl/min⁻¹·mmHg⁻¹, P = 0.40]. Taken together, these data reveal that a NO donor increases outflow facility in WT mice but has no detectable effect in eNOS-GFpGt 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 nonselective inhibitor of all NOS isoforms. At 10 µM, l-NAME reduced the flow rate at each perfusion pressure relative to vehicle-treated eyes (Fig. 4A) and decreased conventional outflow facility by 36 ± 13% (0.014 ± 0.002 vs. 0.021 ± 0.007 µl/min⁻¹·mmHg⁻¹, n = 5 each, P = 0.012). However, 100 µM l-NAME had no apparent effect on
the flow rate or conventional outflow facility $[0.028 \pm 0.007 (n = 9) \text{ vs. } 0.024 \pm 0.005 (n = 8) \mu \text{l min}^{-1} \text{mmHg}^{-1}, P = 0.22]$. These data reveal that L-NAME has a biphasic effect on conventional outflow facility 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 conventional outflow facility in WT eyes by 19 ± 2% relative to vehicle-treated contralateral eyes $[0.021 \pm 0.009 (n = 7) \text{ vs. } 0.025 \pm 0.012 (n = 8) \mu \text{l min}^{-1} \text{mmHg}^{-1}, P = 0.011]$ (Fig. 4C). In a separate set of experiments, we compared the effects of cavtratin with the effects of a control peptide with a scrambled cavtratin domain and an intact AP domain (cavtratin-X) and found a similar 25 ± 17% reduction in conventional outflow facility (0.019 ± 0.008 vs. 0.014 ± 0.007 $\mu \text{l min}^{-1} \text{mmHg}^{-1}, n = 9$ each, $P = 0.026$; not shown). However, in eNOS-GFPtg mice, 50 µM cavtratin decreased conventional outflow facility by 39 ± 25% relative to vehicle-treated contralateral eyes $[0.008 \pm 0.004 (n = 7) \text{ vs. } 0.014 \pm 0.006 (n = 5) \mu \text{l min}^{-1} \text{mmHg}^{-1}, P = 0.014]$ (Fig. 4D). These data suggest that eNOS may be the predominant isoform involved in aqueous humor outflow regulation, decreasing conventional outflow facility 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 colocalized 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 (Fig. 5), GFP immunofluorescence was restricted to the endothelium of SC, collector channels, and episcleral vessels, which also expressed CD31 (31). Interestingly, there was no apparent GFP staining of the trabecular meshwork (TM), and GFP labeling was entirely absent from WT mice, which 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 outflow facility-increasing effect of various NO donors was first demonstrated in living monkeys (32, 63) and, subsequently, in living rabbits (38) and in eyes obtained postmortem 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 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.

Precisely how NO affects outflow facility and IOP remains unclear but likely involves 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. For instance, sGC mediates the vasodilatory effect of NO on smooth muscle cells in the cardiovascular system, and sGC is necessary for the increase in outflow facility 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 (23) cell volume 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 per-
meability of vascular endothelia (13, 18) and, thereby, may disrupt the adherens junctions along the inner wall of SC (31) to increase outflow facility. Downstream of SC, NO may induce relaxation of smooth muscle cells that surround the collector channels (17) to increase intrascleral vessel diameter and, therefore, may influence posttrabecular 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 increase in outflow facility putatively caused by TM/SC relaxation. This effect should be more pronounced in primates (57, 58, 72) and mice (51), which have a ciliary muscle with more extensive tendinous connections to the juxtacanalicular tissue and inner wall endothelium of SC than 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 three genes and their splice variants, including the calcium-dependent eNOS and nNOS, which are constitutively expressed in endothelial cells and nitrergic neurons, respectively, and the calcium-independent iNOS, which is expressed predominantly in macrophages. While Nathanson and McKee (48) argue, based on immunohistochemistry, that eNOS is the primary NOS isoform in the human outflow pathway, the TM is innervated by nitrergic nerve fibers, which

![Fig. 4. Effect of NOS inhibitors on conventional outflow facility in enucleated eyes from WT and eNOS-GFPtg mice. A and B: perfusion flow rate measured as a function of pressure in WT eyes perfused with L-NAME (nonselective NOS inhibitor) at 10 µM or 100 µM in DBG vs. DBG vehicle. C and D: perfusion flow rate measured as a function of pressure in eyes perfused with 50 µM cavatrain (eNOS-selective inhibitor) vs. vehicle (DBG + 0.6% DMSO) from WT or eNOS-GFPtg mice. Outflow facility is estimated on the basis of the slope on the linear regression through the data points, as described by Eq. 1. Error bars represent SD.](image-url)
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), which, when activated, yields greater quantities of NO than either 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 intrascleral vessel network in primates (25). eNOS itself, while expressed by SC inner wall cells, is also expressed by the endothelium of the intrascleral collector channels and aqueous humor veins (69). In primates, the longitudinal ciliary muscle fibers are enriched in eNOS (48) and insert directly into the juxtaocular 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, or unconventional outflow, since such effects are very likely eliminated in enucleated eyes and would otherwise confound measurements in vivo. This assumes that the outflow facility is unaffected by postmortem changes and that the NO signaling pathways are preserved ex vivo. In support of the ex vivo model, Millar and colleagues (46) showed that the outflow facility measured in vivo is not significantly different from that measured immediately after death in the same mice prior to enucleation. Furthermore, the pharmacological outflow 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 WT mice, nonselective inhibition of NOS enzymes by 10 μM L-NAME decreased outflow facility, suggesting that there is a basal tone of endogenous NO involved in regulation of outflow facility. However, no detectable facility decrease was observed in response to 100 μM L-NAME. It is puzzling why higher concentrations of L-NAME had no detectable effect on outflow facility; one possibility is that differential NOS inhibition between different tissues of the outflow pathway may, in some cases, exert opposing effects on outflow facility. For example, one may speculate that the higher concentration of L-NAME is more available to the ciliary muscle, where NO-mediated ciliary muscle relaxation may tend to oppose the increase in outflow facility caused by NO action within the TM (see above). Alternatively, the data may reflect differences in the inhibition of different NOS isoforms by L-NAME, which tends to be more selective for nNOS (IC50 = 0.15 μM) than 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 to a free acid to become bioactive (53), and the kinetics of this reaction may vary between the TM and ciliary muscle, which exert opposing effects on outflow facility. Alternatively, L-NAME may act as a muscarinic antagonist (11), affecting the M2 and M3 receptors 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 contractility, which affects outflow facility in mice (42, 51). Apart from NOS, NO itself exhibits a biphasic effect on TM cell contracility (20) and IOP (39, 49); thus the response to L-NAME that we observed may reflect the underlying biphasic nature of the effect of NO on 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, in particular, outflow facility.

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) showed 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, although it significantly increases outflow facility in WT mice. This demonstrates that the outflow facility-increasing effect of NO becomes saturated under conditions of elevated eNOS expression. When eNOS expression was selectively inhibited by cavitatin, outflow facility was reduced in WT and eNOS-GFPtg mice, with a twofold greater reduction in the transgenic mice, which have elevated eNOS activity. Similarly, Stamer et al. reported a similar reduction in outflow facility 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 physiological 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 was consistently larger in WT than eNOS-GFPtg mice. In contrast, Stamer et al. (69) reported a twofold-larger outflow facility in eNOS-GFPtg mice than in their WT littermates. Two important differences may contribute to this discrepancy between the present study and that of Stamer et al. 1) Perfusions in the current study were done at physiological temperature, with the eyes submerged under isotonic saline, whereas perfusions in the prior study were conducted with the eyes at room temperature and 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 differences in baseline outflow facility between the current study and prior studies. 2) The current study did not directly compare 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, which may have exhibited a baseline outflow facility different from that of the transgenic colony. For these reasons, the current experiments were not designed to detect differences in baseline outflow facility between WT and eNOS-GFPtg mice but, rather, 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, 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 porcine angular aqueous plexus cells (the porcine equivalent of SC cells) (41). This presents the possibility that eNOS may act as part of an endogenous feedback loop to regulate IOP and 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, which increases the shear stress acting on the inner wall, because the same volumetric flow rate of the aqueous humor 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 outflow 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, which would tend to increase IOP. Such a mechanism may underlie IOP homeostasis and contribute to the relative consistency of IOP, which lies within a surprisingly narrow range, despite decades of life, in healthy individuals (16, 37). This homeostatic mechanism may become impaired in glaucomatous eyes, in which NOS activity in the conventional outflow pathway appears to be reduced (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 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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