eNOS, a Pressure-Dependent Regulator of Intraocular Pressure

W. Daniel Stamer,[1,2,3,4,5] *Yuan Lei,*[4,5,6] *Alexandra Boussommier-Calleja,*[4] *Darryl R. Overby,*[4] *and C. Ross Ethier*[4]

Purpose. Pathology in the primary drainage pathway for aqueous humor in the eye is responsible for ocular hypertension, the only treatable risk factor in patients with glaucoma. Unfortunately, the mechanisms that regulate pressure-dependent drainage of aqueous humor and thus intraocular pressure (IOP) are unknown. To better understand one possible underlying molecular factor that regulates IOP, nitric oxide (NO), pressure-dependent drainage in transgenic mice overexpressing endothelial NO synthase (eNOS) was studied.

Methods. IOP was measured by rebound tonometry in mice, and pressure versus flow data were measured by ex vivo perfusion at multiple pressures between 8 and 45 mm Hg, using mock AH ±100 μM L-NAME. A subset of eyes was examined histologically using standard techniques or was assayed for fusion protein expression by Western blot analysis.

Results. IOP was lower (9.6 ± 2.7 vs. 11.4 ± 2.5 mm Hg; mean ± SD; $P = 0.04$) and pressure-dependent drainage was higher (0.0154 ± 0.006 vs. 0.0066 ± 0.0009 μL/min/mm Hg; $P = 0.002$) in the transgenic mice than in the wild-type animals; however, pressure-independent drainage was unaffected. The NOS inhibitor L-NAME normalized pressure-dependent drainage in transgenic animals. For IOP >35 mm Hg, the slope of the pressure-flow curve in wild-type mice increased to match that seen in transgenic mice. Shear stress in the pressure-dependent pathway at elevated pressures was calculated to be in a range known to affect eNOS expression and activity in vascular endothelia.

Conclusions. Endothelial NOS overexpression lowers IOP by increasing pressure-dependent drainage in the mouse eye. Data are consistent with NO's having a mechanoregulatory role in aqueous humor dynamics, with eNOS induction at elevated IOPs leading to increased pressure-dependent outflow. (*Invest Ophthalmol Vis Sci.* 2011;52:9438–9444) DOI:10.1167/iovs.11-7839

From the Departments of [1]Ophthalmology and Vision Science, [2]Pharmacology, and [3]Physiology, The University of Arizona, Tucson, Arizona; and the [4]Department of Bioengineering, Imperial College London, London, United Kingdom.

[5]These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

[6]Present affiliation: Research Centre, Eye and ENT Hospital, Fudan University, Shanghai, China.

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Corresponding author: W. Daniel Stamer, Department of Ophthalmology, Duke University, Durham, NC 27710; dan.stamer@duke.edu.

Prolonged and substantial lowering of intraocular pressure (IOP) in those with primary open-angle glaucoma (POAG) slows or prevents vision loss.[1] IOP can be lowered by inhibiting the secretion of aqueous humor into, or enhancing drainage from, the eye. Although medical therapeutics available for daily use effectively reduce secretion, only one of the two outflow pathways is currently targeted to improve outflow. Sadly, there are no effective daily treatments that act primarily on the conventional pathway, which accounts for the majority (up to 90%) of total aqueous humor drainage in humans. The conventional outflow pathway is pressure-sensitive and is thus the main determinant of IOP[2]; drugs that act on this pathway to increase outflow would therefore be of great utility in treatment of ocular hypertension.

For most individuals, average IOP varies by <2 mm Hg of mercury throughout a lifetime.[3,4] However, in those who have ocular hypertension, the primary risk factor for POAG, elevated IOP, results from increased resistance to outflow in the conventional pathway.[5] Thus, it is likely that in some patients with primary ocular hypertension, the homeostatic mechanisms that regulate IOP are defective.

The mechanisms of IOP regulation have not been identified, but it seems likely that they are similar to those involved in the regulation of systemic blood pressure, including those that regulate vascular tone.[6] A key signaling molecule for local regulation of vascular tone is nitric oxide (NO), a free radical that is produced in vascular endothelia by the enzyme endothelial NO synthase (eNOS) via the conversion of L-arginine to L-citrulline. NO has several immediate consequences, including decreased platelet aggregation, decreased neutrophil adhesion, and smooth muscle relaxation.[7] NO also regulates assembly and disassembly of intercellular junctions, thereby affecting endothelial permeability.[8] Not surprisingly, some cardiovascular disorders, including systemic hypertension, are attributed in part to endothelial dysfunction and decreased availability of NO.[9] Endothelial NOS expression and localization in vascular endothelia has been shown to be shear-sensitive.[10,11] Interestingly, shear stress levels in human Schlemm's canal (SC) are comparable to those in the large arteries, particularly at elevated IOP when SC is narrow.[12] The shear levels acting on SC cells, in combination with the fact that SC cells are vascular in origin,[13,14] suggest that shear stress and NO may play a mechanoregulatory role in aqueous humor outflow.

The cells of the human conventional outflow pathway generate NO, as shown by the ability of these tissues to convert L-arginine to L-citrulline and positive staining for NADPH diaphorose.[15] Interestingly, glaucomatous eyes show decreased NADPH diaphorose labeling in the conventional pathway, compared with age-matched controls.[16] Although there is evidence of the expression of all three NOS isozymes in the conventional pathway, data are conflicting as to whether eNOS or inducible (i)NOS is primarily responsible for NO generation.[15,17] Regardless, exogenous compounds that liberate NO or affect the NO signaling pathway significantly increase conventional outflow

facility and lower IOP in rabbits, pigs, dogs, monkeys, and humans.[18–21] In a human anterior segment perfusion model, which isolates the conventional outflow pathway, one study has shown that perfusion with NOS inhibitors decreases outflow facility, whereas perfusion with NO donors has the opposite effect.[17] At the cellular level, NO has been shown to relax the trabecular meshwork (TM) and/or decrease cell volume, consistent with increased outflow facility.[19,22,23] NO expression in the TM also appears to be mechanoregulated, such that increasing IOP from 10 to 30 mm Hg increases the production of NO as well as the transcription of iNOS mRNA.[24]

We hypothesized that increased levels of NO production by eNOS in anterior segment endothelia would increase conventional outflow and thus decrease IOP. Using transgenic mice that overexpress a human eNOS-GFP fusion protein, we observed elevated outflow facility and reduced IOP compared with wild-type (WT) controls. Moreover, we found that inhibition of eNOS by L-NAME returned the pressure–flow relationship of transgenic mice to levels that were indistinguishable from those of WT animals.

Methods

eNOS-GFP Transgenic Mice

Transgenic mice were a gift from Rob Krams (Imperial College, London, UK) and Rini de Crom (Erasmus MC, Rotterdam, The Netherlands) and have been characterized previously.[25] The mice are hemizygous and express human eNOS fused to a GFP protein on a C57BL/6 background, driven by the human eNOS promoter. They therefore have elevated eNOS activity, with expression of the eNOS-GFP fusion protein superimposed on the endogenous murine eNOS. In these animals, eNOS-GFP expression has been shown to be restricted to endothelial tissues, leading to a two-fold increase in NO concentrations in large arteries.[25] WT C57BL/6 mice were used as controls and were obtained either from nontransgenic littermates or independent C57BL/6 mouse colonies culled for other experiments. All experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse Genotyping

Because homozygous transgenic mice are poor breeders, it was necessary to breed hemizygous animals with WT littermates, yielding only one or two hemizygous offspring per litter. It was therefore essential to identify transgene expression by using PCR to genotype each animal. Genotyping was performed on ear tissue samples obtained at weaning. Tissue lysis was performed according to the manufacturer's instructions (DirectPCRLysis Reagent; Viagen Biotech, Inc., Los Angeles, CA) with 0.3 mg/mL proteinase K (Sigma-Aldrich, St. Louis, MO). Normalized to weight, precleared ear lysate solution (2 μL) was added directly to the PCR. A hot-start mix (KAPA2G Robust HotStartReadyMix; Kapa-Biosystems, Cambridge, MA) was used in PCRs, run for 33 cycles at annealing temperatures of 62°C for primers directed against the sense (agctgaccctgaagttcatctg) and antisense (gacgttgtggctgttgtagttg) strands of GFP (predicted product size, 327 bp). PCR products were resolved by gel electrophoresis (2% 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).

Histology

Selected eyes were fixed at constant pressure (8 mm Hg) by infusion of 4% paraformaldehyde, stored in 4% paraformaldehyde (1–3 months), and then processed for paraffin embedding, sectioning (7 μm), and hematoxylin and eosin staining. Sections were viewed by standard light microscopy.

Western Blot Analysis

Methods for immunoblot analyses were similar to published methods.[26] Mouse eyes were snap frozen immediately after enucleation and then stored at −80°C until use. The eyes were pooled ($n = 4$), homogenized in 200 μL hypotonic lysis buffer (5 mM *N*-ethylmaleimide, 10 mM EDTA [pH 7.45], 200 μM phenylmethylsulfonylfloride, and EDTA-free protease inhibitor; Roche, Indianapolis, IN) supplemented with DNase (30 U/mL; Sigma-Aldrich). Laemmli sample buffer (2×) was then added to solution, followed by boiling for 10 minutes and centrifugation at 2000*g* for 1 minute. The supernatant (15 μL) was loaded onto either 7.5% or 10% polyacrylamide gel slabs and subjected to electrophoreses at 0.03 A for 65 minutes. Nitrocellulose membranes were blocked with 5% milk for 1 hour, then incubated overnight at 4°C with rabbit IgGs against GFP (1:3000 dilution[27]) or human eNOS (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used were horseradish-peroxidase–conjugated goat anti-rabbit IgGs (40 ng/mL; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. Nitrocellulose membranes were incubated with chemiluminescence reagent (HyGLO; Denville Scientific, Metuchen, NJ) and exposed to x-ray film (Genesee Scientific, San Diego, CA). To normalize for protein loading, membranes were reprobed with ascites fluid containing mouse monoclonal IgG against β-actin (1:10,000 dilution; Sigma-Aldrich), followed by secondary antibodies and chemiluminescence reagent. Protein bands were captured digitally and densitometry was performed (GeneSnap and GeneTools software; Syngene, Frederick, MD).

Intraocular Pressure Measurements in Mice

To validate a commercially available rebound tonometer (TonoLab; ICare Espoo, Finland;[28]), we measured IOP in one eye of each of seven cadaveric mice. Each eye was cannulated near the limbus, and IOP was varied manometrically between 8 and 35 mm Hg by means of an open reservoir while tonometry was performed. According to the manufacturer's recommendations, the final IOP reading was calculated after six consecutive rebound measurements were made, with the final value reported as the average of four readings after the highest and lowest readings were discarded by the instrument. In three additional cadaveric mice, we measured IOP while the eyes were perfused at constant pressures between 8 and 35 mm Hg, thus avoiding the potential influence of the open reservoir on the rebound tonometer response that could lead to an underestimation of tonometric IOP.[28,29] Both methods showed similar results. A calibration curve for the tonometer was generated from data obtained in experiments using the open reservoir, and all reported values have been corrected according to this calibration curve.

To compare the transgenic to the WT mice, we measured IOP in both eyes (134 eyes total) in mice aged 6 to 46 weeks. IOP was measured immediately after the mice lost consciousness due to an intraperitoneal injection of pentobarbitone sodium (20 mg; Centaur Pharmaceuticals, Mumbai, India), and death was confirmed by cervical dislocation. Five of the 134 eyes exhibited cataracts and artificially elevated IOP readings (all >20 mm Hg) and were excluded from the study.

Mouse Eye Perfusion

Perfusion was performed in mice of both genders aged 12 to 24 weeks at the time of death (by cervical dislocation). The eyes were enucleated within 10 minutes of death and were kept in Dulbecco's modified Eagle's medium (ATCC, Manassas, VA) at room temperature until perfusion. The elapsed time from enucleation to perfusion was 1 to 4 hours.

The experimental setup was developed by our laboratory and is described in detail elsewhere.[30] Briefly, enucleated mouse eyes were cannulated with a 33-gauge bevelled-tip needle (Nanofil; World Precision Instruments, Europe; Hitchin, UK). The cannula was connected to a glass syringe (50 μL; Hamilton, Reno, NV) and a pressure transducer (model 142PC01G; Honeywell, Morristown, NJ). A computer-con-

trolled syringe pump (model 33; Harvard Apparatus, Holliston, UK) delivered a variable flow rate to the anterior chamber (AC) so as to maintain a desired IOP, as monitored by the pressure transducer connected to a computer control system (Labview Software; National Instruments, Austin, TX).

Eyes of the eNOS-GFP mice were perfused with Dulbecco's phosphate-buffered saline (PBS; Invitrogen) at sequential pressures of 8, 15, 22, 30, 35, 40, and 45 mm Hg, with a final measurement at 15 mm Hg, and compared to eyes from the WT animals that were perfused at the same pressures.[30] The perfusion duration was at least 10 minutes at each pressure level. In some experiments, one eye was perfused with PBS at sequential pressures of 8, 15, 22, 30 mm Hg, with a final measurement at 15 mm Hg, whereas the contralateral eye was perfused with PBS containing 100 μM L-NAME (Sigma-Aldrich) at the same sequence of pressures. For all eyes, total outflow facility (C_{total}) was calculated as C_{total} = total inflow rate (F)/intraocular pressure (IOP), where we assumed that at equilibrium, the total inflow rate equals the total outflow rate and episcleral pressure is 0 for the enucleated eyes. Conventional (pressure-dependent) outflow facility was estimated as the slope of the linear regression of the relationship between flow and pressure over five different pressures (8–35 mm Hg). Unconventional (pressure-independent) outflow was estimated by extrapolation of the linear regression to 0 mm Hg (y-intercept).

Statistical Analyses

For IOP and flow rate measurements, data were compared by the Mann-Whitney U test (SPSS 17 for Windows; IBM- SPSS, Chicago, IL). The SE of the slope and intercept of the pressure–flow graph were analyzed (LINEST function in Excel; Microsoft, Redmond WA). In all cases, differences were considered significant at $P < 0.05$.

Results

The goal of this project was to determine the impact of overexpression of eNOS on IOP and conventional outflow function. To accomplish our goal, we used a transgenic mouse model that expresses a human eNOS-GFP fusion protein in addition to endogenous murine eNOS.[25] Genotyping confirmed transgene expression in some mice (positive transgenic animals), revealing a PCR product of the predicted 327-bp size (Fig. 1A). Enucleated eyes from transgenic mice expressed human eNOS and GFP protein in ocular tissues, whereas WT mice did not, as confirmed by Western blot (Fig. 1B). Interestingly, immunofluorescence staining of GFP as a reporter for transgene expression revealed inconsistent labeling of ocular vascular endothelia, including SC. In some sections, we observed a clear but faint and often discontinuous labeling about SC, whereas other sections exhibited no detectable labeling above background. These data (not shown) suggest regional variations in transgene expression, which is consistent with a prior report suggesting nonuniform NOS activity in SC endothelium.[15] To determine whether transgene expression affected the anatomic structure of the conventional outflow pathway and nearby tissues, enucleated mice eyes were inspected by standard histology, and no differences were observed between WT and transgenic animals (compare Figs. 1C and 1D).

We next examined IOP in the eNOS-GFP mice using rebound tonometry. Calibration revealed that the tonometer (Tonolab; ICare) produced a linear output as a function of manometric pressure (R^2 = 0.992) that tended to slightly underestimate the set manometric pressure (e.g., by 0.4 mm Hg at a manometric IOP of 10 mm Hg; Fig. 2A). Hence, we used this calibration curve to correct all tonometric measurements from all the mice. We observed that there was no significant difference in IOP between the WT littermates and the C57BL/6 animals from an independent colony (11.4 ± 2.5 vs. 11.5 ± 2.7 mm Hg; Fig. 2B). However, IOP was significantly reduced in eNOS-GFP mice (9.6 ± 2.7 mm Hg) compared with either control group ($P < 0.05$).

To determine whether changes in conventional outflow facility were responsible for the observed differences in IOP, we perfused enucleated mouse eyes using a technique developed by our laboratory.[30] Figure 3A shows a tracing from a typical perfusion experiment, where we recorded the flow rate necessary to maintain IOP at each pressure level in a series of increasing IOP steps (8–45 mm Hg). The corresponding total outflow facility is shown in Figure 3B for the same eye. For all the eyes, we then plotted the flow rate as a function of IOP, comparing data from the transgenic versus the WT littermates, and we performed a linear regression on these data (Fig. 4). The slope of the flow rate versus pressure relationship for the transgenic mice was more than two times larger than for WT

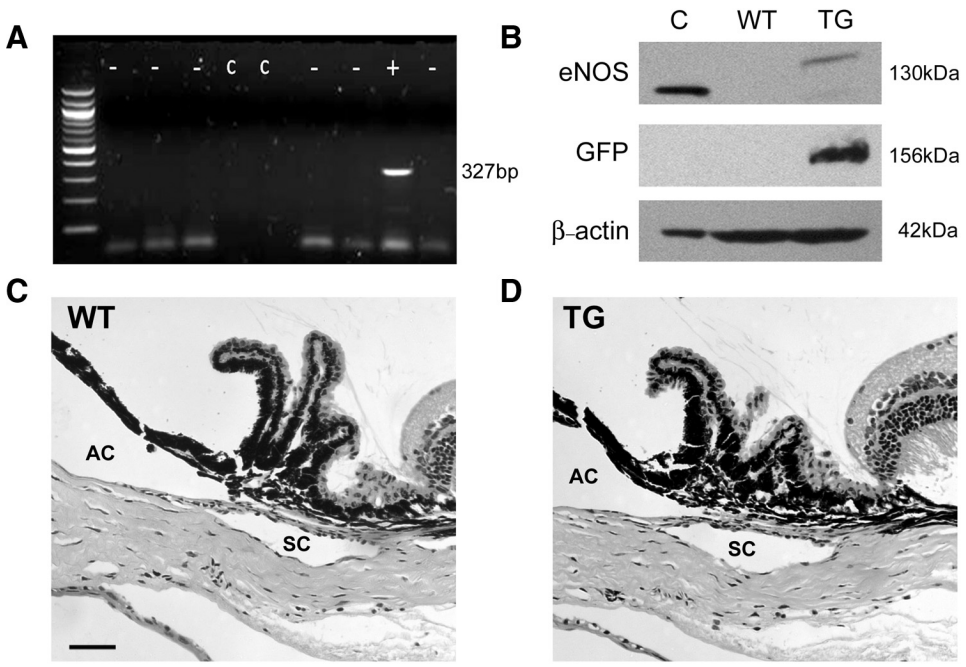

Figure 1. Transgenic mice expressing human eNOS-GFP. (**A**) Genotyping results of a typical litter of pups by PCR analyzed using electrophoresis of a stained agarose gel. In this case, one animal was found to have the transgene (+), whereas six littermates did not (−). Base pair markers are shown on left and controls for the PCR are indicated by C above two lanes. (**B**) Western blot analysis of ocular tissues from transgenic mice expressing human eNOS-GFP (TG) or WT littermates. Blots were probed with antibodies raised against human eNOS, GFP, or β-actin. Human umbilical vascular endothelial cells (HUVECs) were used as positive control (C) for human eNOS expression. The higher eNOS band in the TG lane corresponds to eNOS-GFP fusion protein expression. (**C**, **D**) show images of paraffin sections taken from anterior angle of eyes from WT and transgenic mice (eNOS-GFP) that were perfusion fixed and then inspected after hematoxylin and eosin staining. Bar, 100 μm. AC, anterior chamber; SC, Schlemm's canal.

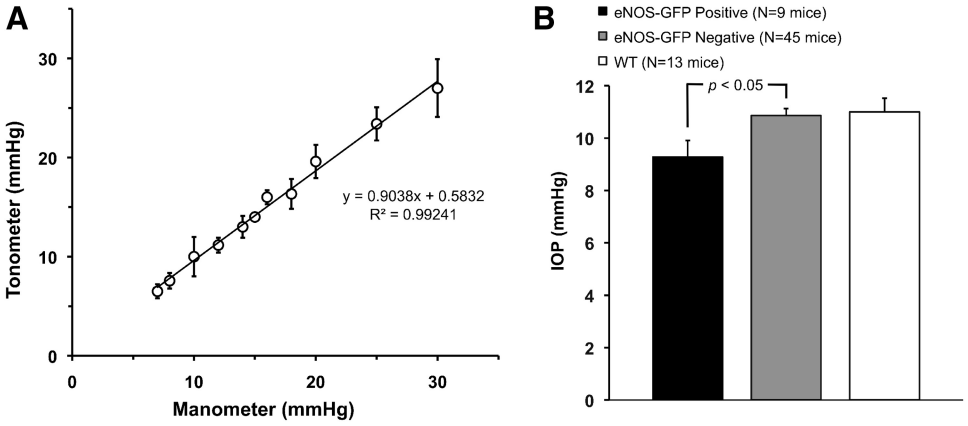

Figure 2. IOP measured by rebound tonometry in transgenic mice expressing human eNOS-GFP. (**A**) Calibration curve of the tonometer in postmortem mouse eyes in situ. (**B**) Corrected IOP measurements taken from transgenic mice expressing eNOS-GFP (■), WT littermates (▨), and WT mice obtained from an independent C57BL/6 colony (□). A significant difference in IOP was observed between eNOS-GFP-positive and -negative mice ($P = 0.04$). The mice were under deep anesthesia (sodium pentobarbitone) during IOP measurements, as described in the text.

littermates (0.0154 ± 0.006 vs. 0.0068 ± 0.0009 μL/min/mm Hg; $P = 0.002$), consistent with greater outflow through the conventional outflow pathway. In contrast, the zero pressure intercept of the pressure–flow relationship was not significantly different between the two groups (0.128 ± 0.13 vs. 0.108 ± 0.02 μL/min; $P = 0.10$), consistent with no change in the pressure-independent (unconventional) outflow.

To determine whether NOS plays a role in the increased outflow facility in eNOS-GFP mice eyes, we used a well-characterized NOS inhibitor, L-NAME. Paired eNOS-GFP mouse eyes were tested, with one eye perfused with L-NAME and the contralateral eye perfused with vehicle. Pressure–flow relationships in Figure 5A demonstrated that, at every pressure in every pair of eyes tested, total flow was less in the eyes perfused with L-NAME than in the contralateral control eye. Summarized data (Fig. 5B) showed that treatment of eNOS-GFP eyes with L-NAME significantly decreased the flow rate at every pressure tested ($P < 0.05$), with the exception of 15 mm Hg ($P = 0.055$). Interestingly, flow rates in the eNOS-GFP eyes treated with L-NAME were not significantly different from those in the untreated WT littermate eyes at all pressures tested ($P > 0.05$).

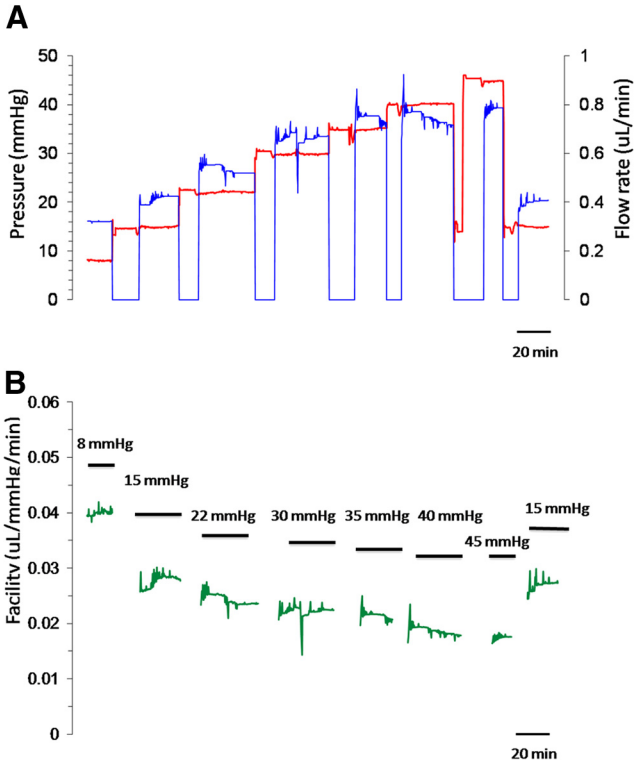

Figure 3. Representative tracings from a perfused eNOS-GFP mouse eye. Shown is a typical example of a perfusion experiment from a single enucleated eNOS-GFP mouse eye at constant pressures of 8, 15, 22, 30, 35, 40, and 45 mm Hg for 10 to 20 minutes at each pressure. Our experimental design included a final measurement at 15 mm Hg to verify that high pressures did not damage eyes. (**A**) Pressure in *red* and flow rate in *blue*, and (**B**) shows the corresponding total outflow facility in *green* (flow rate divided by pressure) derived from the data in (**A**). Intervals where facility data are absent indicate when the syringe pump was turned off (~5 minutes, refer to *blue trace* in **A**), and the eye was pressurized by an elevated open reservoir, to rapidly establish the new pressure value. Outflow facility was not computed during such intervals.

Discussion

By in situ IOP measurements and perfusion of enucleated mouse eyes, we show that overexpression of a single gene product, eNOS, dramatically increased pressure-dependent outflow and decreased IOP in the mouse eye, while pressure-independent outflow remained unchanged. We further showed that this effect was NO-dependent. To our knowledge this is the first demonstration of a transgenic mouse model

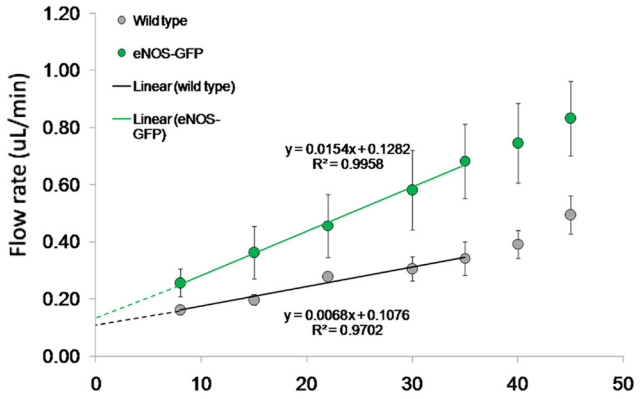

Figure 4. Summary of pressure–flow relationships for transgenic mice eyes expressing human eNOS-GFP. Shown are mean flow rates and IOPs averaged over all perfused transgenic mice eyes (*green*; $n = 11$). The slope of the regression line (*solid green*) is the conventional (pressure-dependent) outflow facility (C_{conv}) for transgenic mice, which is 0.0154 μL/min/mm Hg. Compare the slope for the transgenic mice with that of WT littermates (*solid black line*) which is significantly less, having an outflow facility of 0.0068 μL/min/mm Hg.[30] The intercepts of the regression lines (*dotted lines*) are the pressure-independent outflows (F_u). Values obtained for transgenic and WT mice were not significantly different (0.128 μL/min versus 0.108 μL/min; $P = 0.10$).

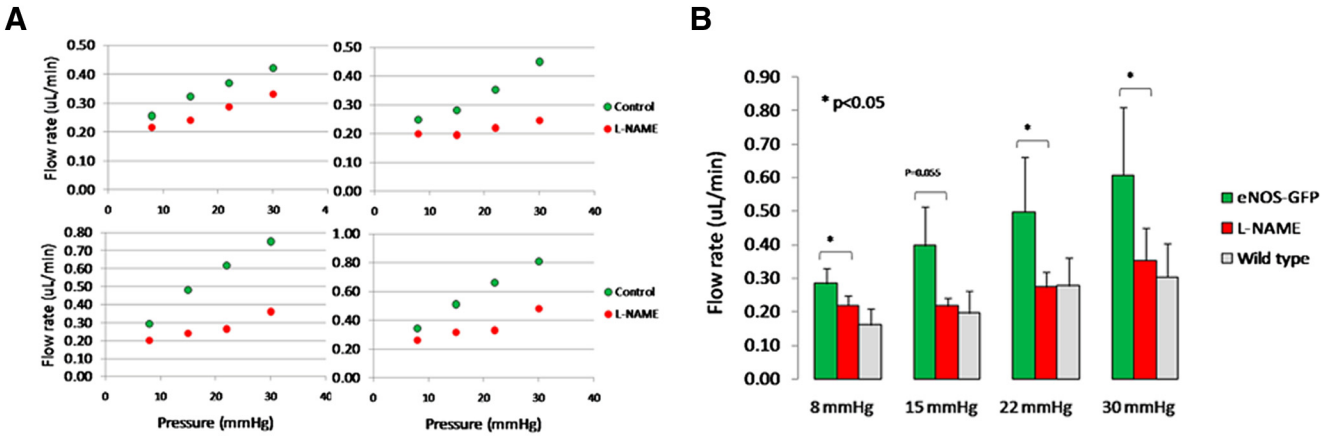

Figure 5. Effect of L-NAME on pressure–flow relationship in transgenic mice eyes expressing human eNOS-GFP. (**A**) Results from experiments with paired eyes from transgenic mice. At constant pressure (10, 15, 22, and 30 mm Hg), one eye was perfused with mock aqueous humor and the contralateral eye was perfused with mock aqueous humor containing 100 μM L-NAME. Each panel shows data obtained from one pair of eyes ($n = 4$ pairs). (**B**) A summary of data (mean ± SEM) with transgenic mice perfused with mock aqueous humor, mock aqueous humor plus L-NAME or WT littermate eyes at the same IOPs.[30] Flow rates obtained from transgenic eyes treated with L-NAME were significantly lower than those in untreated eyes ($P < 0.05$), except at 15 mm Hg ($P = 0.055$). Flow rates of L-NAME treated transgenic eyes were not significantly different from flow rates in WT eyes at all pressures tested ($P > 0.05$).

with depressed IOP, elevated conventional outflow facility plus a morphologically normal and open angle.

An interesting feature of the pressure–flow curves in the WT mouse eyes was that the pressure-dependent outflow (the slope of the pressure/flow relationship) increased at pressures higher than 35 mm Hg. Although we had only three IOP levels at elevated pressures (> 35 mm Hg), the slope of the pressure-flow curve in the WT mice at these higher pressures was indistinguishable from the slope seen in the eNOS transgenic mice at much lower pressures. These data suggest the induction of a mechanism at high pressures in the WT animals that is continually present at lower pressures in transgenic animals. We speculate that this mechanism is eNOS dependent, which is consistent with the known mechanosensitivity of eNOS activity in the systemic vasculature.[25,31,32] Likewise, analysis of SC dimensions predicts that the shear stresses in the SC of the mouse are in a range known to induce mechanical effects in cells at elevated pressures when the SC begins to narrow (Fig. 6), confirming a potential mechanoregulatory role of shear stress acting in the SC of the mouse.

We hypothesize that shear stress acting on the endothelial cells of SC functions as a "barostat" within an endogenous feedback loop that detects changes in IOP and regulates outflow facility so as to restore IOP homeostasis, partly through NO signaling. In this scenario, NO production by SC cells increases as IOP increases on account of SC collapse, which elevates shear stress as outflow is forced through a smaller canal lumen. Transport of NO, either by retrograde diffusion through the TM and/or advection along the circumference of the canal, may induce resistance changes, both at the level of the inner wall and the juxtacanalicular tissue (JCT). Because SC is largely free of blood constituents and thereby is low in heme and thiol compounds, NO decay is most likely dominated by its reaction with dissolved oxygen, suggesting that the NO half-life

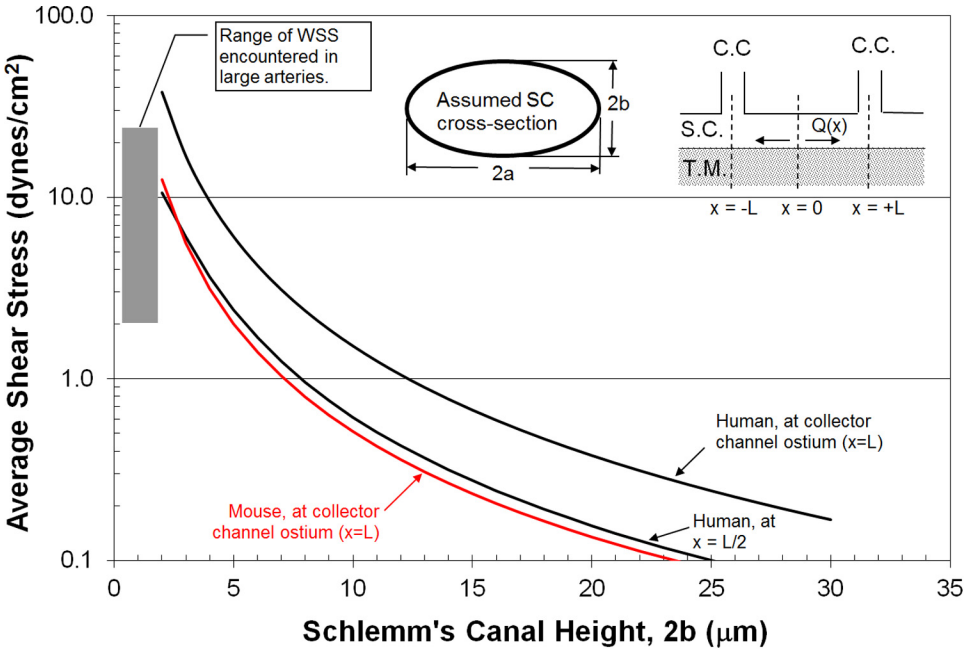

Figure 6. Theoretical shear stress in SC of mouse compared to human. The *y*-axis is the circumferentially averaged shear stress computed from a simple model in which SC is elliptical in cross section.[12] Flow rates for mouse eyes were taken from Aihara et al.,[48] and the number of CCs and canal size were taken from Tables 1 and 2 in van der Merwe E,[47] for 6- to 8-month WT C57 mice. The *x*-axis is the minor axis length of the elliptical canal (i.e., the separation between inner and outer SC walls). The *red line* indicates predicted values of shear stress in Schlemm's canal over a range of canal heights for the mouse eye, while *black lines* show predicted values of shear stress for human Schlemm's canal over a range of canal heights at CC ostium (*top line*) or at midpoint between ostia (*bottom line*). *Inset* shows the assumed cross-sectional shape of the canal and the terminology for computing the flow rate as a function of position in the canal, $Q(x)$.

in SC is on the order of several minutes.[33,34] This scenario predicts elevated NO concentrations near collector channel (CC) ostia, as NO is convected within SC, and may explain the segmental outflow that tends to concentrate in these regions.[35–37] On the other hand, if large regions of the TM were starved of flow (e.g., either by diverted flow or decreased aqueous humor production, such as occurs with aqueous suppressant drugs) then local shear stresses and NO levels would fall, contributing to a vicious cycle that would further reduce local outflow conductance.[38] Future studies need to determine whether defective NO signaling contributes to the glaucomatous decrease in outflow facility.

Our findings in mice are consistent with those in previous studies in humans that show that conventional outflow tissues generate NO.[15,17] Not as clear, though, is the NOS isotype responsible for NO production. In a study in which monoclonal antibodies directed against unique regions of the three major NOS isotypes (eNOS, iNOS, and nNOS) were used, results from one study showed that eNOS is the only isozyme expressed in both TM and SC cells.[39] However, the expression data are difficult to interpret, because background labeling was high for eNOS IgGs in immunohistochemistry, whereas Western blots were not performed to demonstrate antibody specificity in human conventional tissues. In contrast, another study showed that iNOS was the predominant isozyme in the TM.[17] Unfortunately, this study did not specifically examine NOS expression in SC.

Understanding the role of eNOS in conventional outflow regulation is relevant to human disease where polymorphisms in the *NOS3* gene have been shown to associate with POAG in three studies to date. All the studies demonstrated that ocular hypertension was a component of POAG that positively associated with variants in the *NOS3* gene. One study found amino acid changes in the coding region of eNOS.[40] These subjects with POAG and eNOS mutations also had migraine, consistent with in eNOS mutations contributing to multiple vascular-related ailments. In this context, it is interesting that NOS inhibition in POAG patients leads to altered hemodynamics compared to age-matched controls.[41] In the other two genomic studies implicating the *NOS3* gene in POAG, the promoter region was the location of polymorphisms, suggesting that regulation of eNOS expression is important in the development of POAG.[42,43] These findings are consistent with data from a previous study showing decreased NO production (NADPH diaphorase staining) in outflow tissues from POAG patients compared with age-matched controls.[16]

When mice were clamped in the physiological pressure range (8–15 mm Hg), we found that those overexpressing eNOS had approximately a twofold increase in outflow facility compared with the WT animals. Using NO-donating agents, others have found similar results, with increases in outflow facility of approximately twofold in enucleated porcine eyes,[19] of 30% to 90% in living monkey eyes,[21] and of 60% to 80% in living rabbit eyes.[44] However, the reported increase in human anterior segment preparations was only ~10%.[17] The lower response level in human eyes may be due to the nature of the cadaveric tissue tested (i.e., older donors, extended time between death, and enucleation–perfusion plus low baseline outflow facilities).

An NO-donating drug conjugated to latanoprost (PF-207) has recently been developed and tested for clinical use. In animal models, PF-207 lowered IOP better than latanoprost alone, suggesting activity in the unconventional and conventional outflow pathways.[18,45,46] However, in phase I and II trials, PF-207 did not show significant improvement over latanoprost alone in POAG and ocular hypertensive (OHT) patients. Based on our findings in mouse eyes, we hypothesize that, for two possible reasons, PF-207 failed to access the JCT region of the conventional outflow pathway where the NO-regulatory circuit is functionally active: (1) NO was released in the cornea, aqueous humor, or inner TM, before reaching the JCT, or (2) flow through the conventional outflow pathway was diminished in OHT patients, particularly in the presence of latanoprost, which acts to shunt flow away from conventional tissues. To address these potential shortcomings, future studies should focus on modification of NOS regulation at the level of SC endothelia, where NO is produced and resistance is generated. Further, we are confident that intervening in the regulation of endogenous production of NO is a better strategy than exogenous delivery of NO, as there will be more opportunities to target this key regulatory feedback loop once it is better understood.

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