Shear Stress-Triggered Nitric Oxide Release From Schlemm's Canal Cells

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PURPOSE. Endothelial nitric oxide (NO) synthase is regulated by shear stress. At elevated intraocular pressures when the Schlemm's canal (SC) begins to collapse, shear stress is comparable with that in large arteries. We investigated the relationship between NO production and shear stress in cultured human SC cells.

METHODS. Schlemm's canal endothelial cells isolated from three normal and two glaucomatous human donors were seeded into Ibidi flow chambers at confluence, cultured for 7 days, and subjected to steady shear stress (0.1 or 10 dynes/cm²) for 6, 24, or 168 hours. Cell alignment with flow direction was monitored, and NO production was measured using 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) and Griess reagents. Human trabecular meshwork (TM) and umbilical vein endothelial cells (HUVECs) were used as controls.

RESULTS. Normal SC strains aligned with the direction of flow by 7 days. Comparing 0.1 vs. 10 dynes/cm², NO levels increased by 82% at 24 hours and 8-fold after 7 days by DAF-FM, and similar results were obtained with Griess reagent. Shear responses by SC cells at 24 hours were comparable with HUVECs, and greater than TM cells, which appeared shear-insensitive. Nitric oxide production by SC cells was detectable as early as 6 hours and was inhibited by 100 μ M nitro-L-arginine methyl ester. Two glaucomatous SC cell strains were either unresponsive or lifted from the plate in the face of shear.

CONCLUSIONS. Shear stress triggers NO production in human SC cells, similar to other vascular endothelia. Increased shear stress and NO production during SC collapse at elevated intraocular pressures may in part mediate IOP homeostasis.

Keywords: eNOS, glaucoma, aqueous humor, conventional outflow, intraocular pressure

Nitric Oxide (NO) is a labile gas that is produced in endothelia by the enzyme endothelial NO synthase (eNOS) through the conversion of L-arginine to L-citrulline. Once produced, NO performs a host of functions including regulating the assembly and disassembly of intracellular junctions, affecting endothelial permeability,¹ and smooth muscle relaxation resulting in vasodilation.²

Abnormal NO homeostasis has been associated with several diseases, such as systemic hypertension, heart failure,^{3,4} and glaucoma.^{5,6} Primary open-angle glaucoma, the most common type, is a leading cause of blindness and is often characterized by elevated intraocular pressure (IOP). Elevated IOP (ocular hypertension) is caused by dysfunction of the conventional outflow pathway of the eye. Three different genetic association studies have suggested that NO plays a role in the regulation of IOP.^{7–9} Further, patients with glaucoma have diminished NO levels in their aqueous humor and compromised ocular hemodynamics compared with age-matched control patients.^{10,11}

Consistent with these observations, NO donating compounds effectively increase conventional outflow facility leading to a decrease in IOP in mice, rabbits, pigs, dogs, monkeys, and humans.^{6,12,13} In contrast, perfusion of human eyes with NOS inhibitors decreases outflow facility.¹⁴ The site

of NO activity is unknown, but likely to involve the two cell types that populate the conventional outflow pathway, namely trabecular meshwork (TM)¹⁵ and Schlemm's canal (SC) cells.^{13,16}

Schlemm's canal is a circular microvessel into which the aqueous humor enters, to exit the eye and join the general circulation. The endothelial NO synthase, eNOS, is expressed by SC cells and overexpression of eNOS in transgenic mice results in decreased IOP¹³ and decreased outflow resistance. Thus, elements that regulate eNOS activity and expression, such as shear stress,^{17–19} may impact intraocular pressure.

Wall shear stress levels in SC are calculated to be comparable with the levels found in large arteries (2–20 dynes/cm^{2,20}), particularly at elevated IOP on account of IOP-induced narrowing of SC.²¹ Data in several species, including living mice²¹ and humans²² indicate that when IOP increases, SC lumen narrows, experiencing full collapse in some regions. However, effects of shear stress on NO production by SC cells have never been tested. In the present study, we investigated if NO production by cultured human SC cells increases with shear stress, as part of our overall hypothesis that shear stress functions as a modulator in an endogenous feedback loop that regulates IOP. We predict that as SC narrows at elevated IOPs,

the shear stress acting on SC cells increases; leading to elevated NO production.

METHODS

Cell Culture

Three cell types were used in experiments: human umbilical vein endothelial cells (HUVECs; Becton, Dickinson & Company, Franklin Lakes, NJ, USA); human SC cells (isolated, cultured, and characterized as previously described²³); and human trabecular meshwork (TM) cells (isolated, cultured, and characterized as before²⁴). Both the human SC and TM cells were used at passages two through five. HUVECs were isolated from a single human donor and used between passages three through six. Individual experiments for all cell types were run on different days/times.²⁵ Five SC cell strains isolated from three nonglaucomatous donor eyes (SC60, SC65, and SC78, aged 58, 68, and 77 years, respectively) and two glaucomatous donor eyes (SC57g and SC63g, both aged 78 years at time of death with a history of primary open-angle glaucoma recorded in their medical records) were used in experiments. Two trabecular meshwork cell strains isolated from two non-glaucomatous donor eyes (TM122 and TM126, aged 54 and 88 years, respectively) were evaluated.

We cultured HUVECs in medium (Medium 199, Gibco; Life Technologies, Grand Island, NY, USA) supplemented with 15% Hyclone fetal bovine serum (FBS; Thermo Fisher Scientific, South Logan, UT, USA); penicillin-streptomycin-glutamine (PSG, 100 U/mL; Life Technologies); heparin sodium salt (90 µg/mL, Sigma-Aldrich Corp., St. Louis, MO, USA); and endothelial mitogen (0.1 mg/mL; Biomedical Technology, Inc., Stoughton, MA, USA). Schlemm's canal cells were cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose ×1 medium (Life Technologies) supplemented with 10% FBS and PSG (100 U/mL). Trabecular meshwork cells were cultured in DMEM low glucose ×1 medium (Life Technologies) supplemented with 1% FBS and PSG (100 U/mL). In inhibition experiments with NOS, medium was supplemented with 100 µM Nw-Nitro-L-arginine methyl ester chloride (L-NAME; Sigma-Aldrich Corp.).

Shear Stress Experiments

Shear stress was applied to confluent HUVECs and SC cells using an Ibidi pump system (Ibidi, Munich, Germany). Cells (3.3×10^5) were loaded onto µ-slides I^{0.6} (Ibidi) and placed in an incubator at 37°C with 5.0% CO₂. Slide surfaces treated with a physical modification used to improve cell adhesion (ibiTreat; Ibidi). These µ-slides hold a volume of 150 µL, with a channel height of 600 µm and a cell culture surface area of 2.5 cm² (5 mm width × 50 mm length).

We loaded HUVECs onto µ-slides and, other than a daily change of media, were allowed to settle for 3 days before induction of shear. In contrast, SC cells were loaded into µ-slides and allowed to settle for 1 to 2 weeks before a constant shear was applied. Preliminary experiments indicated that exposing SC cells to shear prior to this time resulted in cells detaching from the µ-slide (data not shown).

The Ibidi pump system was set up per the company's instructions and proprietary software was used to control the level of shear applied to cells by controlling total media flow rate through the channels of known dimensions. We used the yellow/green type perfusion tubing set that connects to the µ-slide and is able to maintain a flow rate from 1.98 to 27.44 mL/min. Sterile filters (Sartorius Minisart, 0.2-µm pore size, Teflon; Sigma-Aldrich Corp.) were used to filter the air entering

the tubing system, via an air pressure pump, which applied the force required to maintain the flow rate of the media through the tubing and across the µ-slide. The air pressure pump responds to the company's proprietary software and provides air to the perfusion sets at the designated pressure with a range of −100 mbar to +100 mbar. The air pumped into the system came from the incubator itself and thus contained 5% CO₂.

Cell Alignment

A minimum of three phase-contrast images of cells per µ-slide exposed to shear were collected from each individual experiment with a microscope (Zeiss AXIO Observer.D1, ×100 total magnification; PH1, Thornwood, NY, USA). Cell alignment was measured manually using ImageJ (Supplementary Fig. S1, <http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health (NIH), Bethesda, MD, USA). The 0° line was defined as the direction of flow. Another line was then drawn parallel to the major axis of individual cells (typically 130 cells per field of view), and the angle with respect to the direction of flow was measured. The measured cell alignment angles were then sorted into 15° bins (0–15°, 15–30°, 30–45°, 45–60°, 60–75°, and 75–90°), and the binned data from each individual µ-slide were pooled together to obtain a histogram describing the distribution of cell alignment for each experimental condition.

Nitric Oxide Detection

Two methods were used to monitor NO levels. The first method was direct and utilized a DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) probe (Life Technologies); DAF-FM is essentially nonfluorescent until it reacts with NO to form benzotriazole, which is fluorescent (excitation/emission = 495/515 nm). We incubated DAF-FM diacetate (solubilized in high-quality anhydrous DMSO, 1% final concentration in media) with cells immediately at the end of the shear exposure time points. Cells were imaged with a microscope (Nikon Eclipse 90i with a Nikon D-Eclipse C1 Si Laser; Melville, NY, USA). Identical gain and exposure settings were used to capture all images during a single viewing session. Five images were captured along the approximate centerline of the µ-slide roughly equidistant from each other down the length of the slide. Each image was analyzed with ImageJ (NIH) to calculate the average fluorescence intensity across the field of view. Due to artifactual darkening near the edges of each image (likely due to µ-slide contours or nonuniform illumination), only the center portion of each image (1173.5 µm × 1128.8 µm, containing approximately 100 cells) was analyzed (Supplementary Fig. S2). A single mean fluorescence intensity value was calculated for each µ-slide by averaging over the five images. Using HUVECs as our positive control, we tested several concentrations of the DAF-FM diacetate probe (1–50 µM) in preliminary experiments over a range of shear levels (0.1–15 dynes/cm²); the fluorescence signal at 50 µM DAF-FM was linear between 0.1 and 10 dynes/cm² (data not shown).

In the second method, NO concentration was indirectly assessed by measuring the concentration of nitrite, a by-product of NO degradation, with a Griess reagent analysis kit (Invitrogen, Carlsbad, CA, USA). Equal volumes of N-(1-naphthyl) ethylenediamine and sulfanilic acid were mixed together to form the Griess reagent. The Griess reagent (20 µL), ultrapure water (Milli-Q 130 µL; Millipore Corp., Billerica, MA, USA) and conditioned media (150 µL) from the shear experiments (collected immediately following the experiment and kept at −80°C until time to run the Griess reagent assay) were combined and added to a 96-well plate. Controls included

FIGURE 1. Alignment of normal SC and HUVECs induced by shear stress. Phase-contrast images of HUVECs exposed to 0.1 dynes/cm² or 10 dynes/cm² for 24 hours show alignment at the higher value of shear stress. The direction of flow/shear is indicated by arrows. Similarly, SC cells were exposed to shear stress for 1 week and cell alignment was assessed. Images are representative, and show data from one experiment of five total for HUVECs and of eight total for SCs, using two SC cell strains.

a photometric reference sample (20 μ L Griess reagent and 280 μ L Milli-Q water) and serial dilutions of a nitrite standard solution in ultrapure water (to generate a standard calibration curve; Millipore Corp.). The samples and standard mixtures were incubated for 30 minutes at room temperature and the absorbance of the nitrite-containing samples were measured at 570 nm using a plate reader coupled with data acquisition and analysis software (SpectraMax M3 and SoftMax Pro 5; Molecular Devices, Sunnyvale, CA, USA). Linear regression analysis using data from the standard curve was used to estimate the nitrite concentrations of the samples (Supplementary Fig. S3).

In preliminary experiments with HUVECs, we also attempted to measure NO directly using a NO probe (inNO-TII NO measuring system coupled with an amino-700 model NO sensor with inoII software; Innovative Instruments, Inc., Tampa, FL, USA); however, we found that this equipment was incompatible with the Ibidi system, despite several system modifications and trials.

Cell Viability and LDH Release

After exposure to shear stress, cells were visually monitored via phase-contrast microscopy to verify attachment to the μ -slide. To assess the health of the cells after they were exposed to shear stress, lactate dehydrogenase (LDH) content in conditioned media was measured with an LDH assay kit (Sigma-Aldrich Corp.). A lactate dehydrogenase assay buffer (48 μ L), LDH substrate mix (2 μ L) and conditioned media (50 μ L) from the shear experiments were combined and added to a 96-well plate. Controls included a photometric reference sample (50 μ L of LDH assay buffer) and parallel dilutions of a 1.25 mM nicotinamide adenine dinucleotide-hydrogen (NADH) standard solution in LDH assay buffer (to generate a standard calibration curve). The samples and standard mixtures were incubated for

3 minutes at 37°C and the absorbances of the samples were measured at 450 nm using a plate reader coupled with software (SpectraMax M3 with SoftMax Pro 5; Molecular Devices) for an initial measurement. Absorbance of the samples were then measured every 5 minutes following this initial time-point until the absorbance of the most active sample exceeded the absorbance of the highest standard concentration. Linear regression analysis using data from the standard curve was used to estimate the NADH concentrations of the samples (Supplementary Fig. S4). The activity of LDH was then calculated by multiplying the concentration by the sample dilution factor (1) and dividing it by the product of the time of the reaction (40 minutes) and the sample volume of the well (50 μ L). All four SC cell strains used for the LDH assay were exposed to 10 dynes/cm² for 24 hours.

Statistical Analyses

For cell alignment analyses at 0.1 dynes/cm² and 10 dynes/cm², a Wilcoxon rank sum test was performed to compare the sample cell numbers in each individual bin to a uniform distribution where the expected percentages in each of the six bins would be 16.7%. For all DAF-FM fluorescence and Griess reagent analyses, a Wilcoxon rank-sum test was used to compare the samples, due to small sample sizes and thus not being able to confirm a normal distribution of the samples. For the experiments lasting 1 week or for 24 hours, measurements from cells exposed to 0.1 dynes/cm² were compared with those from cells exposed to 10 dynes/cm² to determine significance. For the 6-hour experiments, 10 dynes/cm² results were compared with those from 10 dynes/cm² with media supplemented with L-NAME.

For the normal SC cell strains, the three strains were compared using a Kruskal-Wallis one-way ANOVA analysis, at each shear condition, to ensure that there was no significant

FIGURE 2. Histograms showing cultured endothelial cell alignment relative to the direction of flow/shear (defined as 0°) when exposed to shear stresses of 0.1 or 10 dynes/cm². (A) Displays pooled cell orientation data obtained from images of HUVECs (mean $\pm$ SD, $n = 5$) exposed to shear stress for 24 hours. The dashed line indicates the expected frequency of 16.7% for each 15° bin, corresponding to the case of uniform distribution between bins (random cell orientation). (B) Shows distribution of Schlemm's canal cells across bins (combined data from two normal strains, mean $\pm$ SD, $n = 8$) exposed to shear stresses for 1 week. Significant differences were determined by comparing the sample numbers in each individual bin at each shear stress level to the expected frequency. * $P < 0.05$.

difference between the three normal SC cell strains. The absence of such a difference allowed us to combine results into one group across these strains, for each of the fluorescence and Griess reagent measurements. However, if a significant difference existed, the strains were compared with a Wilcoxon rank sum test to determine which cell strains could be combined for each shear condition and each NO measurement, and which strains must be analyzed separately. The threshold for significance was $P < 0.05$.

RESULTS

Cell Alignment

We exposed HUVECs and human SC cells to shear stress of 10 dynes/cm² and cell alignment relative to the direction of shear was assessed at 24 hours and 1 week, respectively (Fig. 1). While HUVECs aligned with the direction of flow/shear by 24 hours, SC cells required a full week to become aligned, with no obvious alignment at earlier time points (24, 48, or 120 hours).

In contrast, both cell types exposed to a shear stress of 0.1 dynes/cm² (used as a low shear control, providing sufficient media turnover for cell culture within the Ibidi chamber, but delivering nearly no mechanostimulation to cells) did not appear to align with flow (Fig. 1). Quantitative assessment of cell alignment revealed that more than 60% of HUVECs were oriented within 15° of the direction of flow after 24 hours of exposure to 10 dynes/cm² (Fig. 2A). Another 30% of the cells were aligned within 15 to 30° of the flow direction, demonstrating that HUVECs align preferentially in the direction of 10 dynes/cm² shear stress within 24 hours. In contrast, HUVECs exposed to 0.1 dynes/cm² did not exhibit alignment, and the distribution of cell alignment angles was not significantly different from the uniform distribution. Similarly, after 1 week of exposure to 10 dynes/cm², 67% of SC cells were aligned within 15° of the direction of flow and another 17% were within 15 to 30°; showing a strong distribution favoring alignment with the direction of flow. When exposed to 0.1 dynes/cm² for 1 week, there was a relatively uniform distribution of cell orientations.

Nitric Oxide Production

To evaluate NO production after alignment, HUVECs were exposed to shear stresses of 0.1 and 10 dynes/cm² for 24 hours and evaluated with a DAF-FM fluorescent probe (Fig. 3). The mean DAF-FM fluorescence measured at 24 hours increased significantly by 82% from low shear (0.1 dynes/cm²) to high shear (10 dynes/cm², Fig. 4A, $n = 5$, $P = 0.01$). This result was consistent with data obtained with the Griess reagent that demonstrated a significant 48% increase in nitrite concentration (Fig. 4B, $P = 0.05$).

Since alignment of SC cells took longer (1 week), we first tested the influence of shear stress on NO production at the 1-week time point. We observed an 8-fold increase in NO production with shear stress, either as measured using DAF-FM fluorescence (Fig. 5A, $n = 8$, $P = 0.00008$) or the Griess reagent assay (2-fold, Fig. 5B, $P = 0.004$). There was no significant difference between two of the normal cells strains (SC60 and 65) for either shear level, for either fluorescence ($P = 1$ for 0.1 dynes/cm² and $P = 0.47$ for 10 dynes/cm²) or Griess reagent analysis ($P = 0.31$ for 0.1 dynes/cm² and $P = 0.06$ for 10 dynes/cm²).

For direct comparisons to HUVECs, we also measured NO release from SC cells at earlier time points than the 7 days needed for cell alignment. Interestingly, after only 24 hours of shear stress exposure, DAF-FM fluorescence from SC cells increased significantly from 2- to 8-fold, depending upon the cell strain (Fig. 6A, $P < 0.05$). These results were consistent with approximately a 2-fold elevation of NO concentrations across all SC cell strains measured using the Griess reagent (Fig. 6B, $P < 0.05$).

To evaluate a possible role of NO in glaucoma, we next measured the responses to shear of two SC cell strains isolated from two patients with a confirmed diagnosis of open-angle glaucoma. While the first cell strain (SC63g) behaved like other cells tested at 0.1 dyne/cm², at higher shear levels patches of cells lifted off of the substrate in the shear chamber at 5 and 15 dynes/cm² (data not shown). Thus, accurate DAF-FM or Griess reagent measurements were not possible with the SC63g cell strain. In contrast, a second glaucomatous SC cell strain (SC57g) stayed attached at all shear levels tested, including 10 dynes/cm² (Supplementary Fig. S5). Interestingly, no significant change in NO production with shear stress was seen in this SC cell strain (SC57g), both as measured by DAF-FM fluorescence (Fig. 6A, $n = 4$, $P = 0.9$) or nitrite concentration (Fig. 6B, $n = 4$, $P = 0.8$).

FIGURE 3. Nitric oxide levels assessed by DAF-FM fluorescence in HUVECs and Schlemm's canal (SC) cells exposed to shear stress. We exposed HUVECs and SC cells to 0.1 dynes/cm² or 10 dynes/cm² and DAF-FM fluorescence was evaluated using fluorescence microscopy. We exposed HUVECs to shear for 24 hours while SC cells were exposed to shear for 1 week. At the end of each experiment, a DAF-FM probe was applied. Shown are images of a single field of view (five total taken) from a single condition, representative of five total experiments for HUVECs and eight total experiments using two SC cell strains.

Due to the above-mentioned behavior of SC63g in response to shear stress, we sampled the media of all five cell strains exposed to 10 dynes/cm² for 24 hours, analyzing for LDH content (Fig. 6C). There was a significant difference in the LDH released into the media between the normal cell strains and one glaucoma cell strain (SC63g). In fact, we noticed more than a 2-fold increase in the amount of LDH content in conditioned media taken from SC63g compared with the other normal cell strains, likely due to cell damage and/or cells lifting off of the shear chamber. In contrast, LDH release by the other glaucoma strain (SC57g) was not different than the three normal strains or SC63g.

To confirm involvement of NO synthase in the observed shear stress responses, media used in selected experiments was supplemented with 100 μ M L-NAME.²⁶ In the presence of L-NAME at 10 dynes/cm², two of the three normal SC cell strains showed a decrease in DAF-FM fluorescence (Fig. 7A, $n = 5$, $P = 0.01$ for SC65, $P = 0.01$ for SC60). This decrease in fluorescence was also seen in a glaucomatous SC strain ($n = 4$, $P = 0.04$); and HUVECs ($n = 4$, $P = 0.01$). Results with DAF-FM were not corroborated with the nitrite concentration in the normal SC strains. However, L-NAME prevented shear-induced increases in nitrite levels in HUVECs and lowered basal nitrite levels in a glaucomatous SC cell strain (Fig. 7B, $P = 0.05$ for SC57g; and $P = 0.014$ for HUVECs).

To compare SC cell results with the other cell type in the conventional outflow tract, two different TM cell strains were tested for shear-induced NO production (Fig. 8). Though basal NO production (at 0.1 dynes/cm²) measured by DAF-FM and Griess reagent was similar to that found in SC cells and HUVECs, there was no significant shear-mediated increase in NO production at 10 dynes/cm². There was also no significant difference in NO production between the two cell strains at either level of shear stress tested.

DISCUSSION

The present study establishes that human Schlemm's canal endothelial cells respond to shear stress similar to other vascular endothelia. Specifically, we observed that cultured human SC cells react to physiological levels of shear stress by aligning with the direction of flow and by increasing production of NO. Consistent with a role for NO synthase in this process, treatment of cells with L-NAME significantly reduced shear-dependent NO production by SC cells. Interestingly, we found that SC cells isolated from glaucomatous eyes were either shear-unresponsive or lifted from their substrate in the presence of shear stress. Taken together, these data are consistent with the hypothesis that NO production by SC cells has a homeostatic signaling function during times of elevated IOP, when SC narrows and shear stress on SC cells increases. Shear-stimulated production of NO by SC cells would then increase outflow facility, normalizing IOP.^{6,12,13,27,28} Further, although our data with glaucomatous cells is limited, there are preliminary indications that this process may be compromised in glaucoma.

Shear Stress and NO Production

Our positive control for shear-responsiveness was HUVECs, which in our hands showed a similar trend to previous reports.^{29,30} Specifically, for physiological levels of shear stress, HUVECs aligned with the direction of flow within 24 hours, and increased production of NO as measured with two assays. Ideally we would have also measured changes in eNOS protein levels in cultured cells. However, due to the small number of cells in the chamber slides (2.5 cm² area) of the Ibidi system this was not possible. Hence, initial attempts using immunoblot analyses and two different antibodies revealed that eNOS protein levels were below the level of

FIGURE 4. Nitric oxide production by HUVECs exposed to shear stress for 24 hours. Cells were exposed to shears of 0.1 or 10 dynes/cm². (A) Shows the quantification of NO content from DAF-FM fluorescence and (B) nitrite concentration from Griess reagent analysis (mean ± SD, $n = 5$).

detection (data not shown). Previous studies have shown that shear stress leads to an increase in eNOS protein expression by HUVECs.^{31,32}

While HUVECs aligned with the direction of flow within 24 hours, nonglaucomatous SC cell strains took a week to align. In situ, SC cell alignment is seen in regions of highest flow or shear; for example, commonly occurring near the ostia of collector channels, whereas in locations distal from ostia there is less alignment.²⁰ Interestingly, we were able to detect shear-dependent increases in the production of NO at time points (6 and 24 hours) prior to cell alignment in all three normal SC cell strains tested, suggesting that cell alignment and NO production are independent events. This type of effect, in which there is an increase in NO production before cell alignment, has been seen by others in HUVECs exposed to shear for 6 hours.^{31,33} At the 6-hour time point, shear-dependent NO production in HUVECs was inhibited by the NOS inhibitor, L-NAME, confirming shear-regulated NOS activity. Similar results were seen in two of the three SC cell strains (SC60 and SC65) using DAF-FM; however, significance was not reached in assays using the Griess reagent. More research is needed to better understand mechanistically shear-dependent changes in SC cells as shown before in HUVECs.^{31,32}

FIGURE 5. Nitric oxide production by normal Schlemm's canal cells exposed to shear stress for 1 week. Cells were exposed to 0.1 or 10 dynes/cm² for 1 week. (A) Shows the quantification of NO content derived from DAF-FM fluorescence and (B) quantification of nitrite concentration from Griess reagent analysis for normal SC cell strains. Shown are combined data from two normal cell strains (SC60 and 65, mean ± SD, $n = 8$).

Shear Stress Responses From Glaucomatous SC Cells

Using two different methods of NO detection, no significant difference in NO production in a single glaucomatous SC cell strain was seen in response to increasing shear stress from 0.1 to 10 dynes/cm². A second glaucomatous SC cell strain (SC63g) was also tested; however, cells did not remain attached to the flow chamber slide when subjected to shears above 0.1 dynes/cm². Both glaucomatous SC cell strains appeared to behave differently from the three normal SC cell strains in regards to shear-induced NO release. However, due to the limited number of glaucomatous SC cell strains examined it is impossible to make definite conclusions on this point. One could speculate that differences in cell attachment or response to shear may contribute to the development of ocular hypertension in glaucoma. Perhaps some glaucoma cell strains require being on the μ -slide for longer duration than "normal" SC lines, which required 1 to 2 weeks (compared with 2 days for HUVECs) to adhere before the adhesive forces could withstand the shear stress without cells detaching (data not shown). Interestingly, we observed a significant decrease in NO production by a glaucomatous SC cell strain when treated with L-NAME, a NOS

FIGURE 6. Nitric oxide production and LDH release by Schlemm's canal cells exposed to shear stress for 24 hours. Cells were exposed to 0.1 or 10 dynes/cm² for 24 hours and NO content was measured by (A) DAF-FM fluorescence and (B) Griess reagent for normal SC cell strains (combined data from three normal strains) and one glaucomatous cell strain (SC57g). (C) LDH activity assay to estimate cell viability in cells exposed to 10 dynes/cm² of shear for 24 hours. All data shown are expressed as mean values ± SD.

FIGURE 7. Nitric oxide production in Schlemm's canal cells and HUVECs exposed to 10 dynes/cm² shear stress $\pm$ L-NAME for 6 hours. (A) Shows the quantification of NO content from DAF-FM fluorescence and (B) nitrite concentration from Griess reagent analysis (mean $\pm$ SD).

inhibitor, like in the two normal SC cell strains. Thus, the glaucoma SC cells still have constitutive production of NO that can be inhibited, but appear unresponsive to an increase in shear.

Shear Stress Responses From TM Cells

Similar to previous studies in vitro and in situ^{34–38} we observed that TM cells produce NO at levels comparable with SC and HUVECs (Fig. 8). In contrast to normal SC cells and HUVECs, however, we observed no significant increase in NO

production in response to 10 dynes/cm² of shear for 24 hours, suggesting differences in mechanotransduction or NOS isotype expression in TM cells. These data are consistent with recent studies in our laboratory showing that eNOS is not expressed by TM, but instead localizes specifically to SC and distal venous endothelia of the conventional outflow tract (unpublished data). Taken together, it appears that NO signaling in the conventional outflow tract is complicated, involving multiple cell types and potentially different isoforms of NOS.

FIGURE 8. Nitric oxide production by trabecular meshwork cells exposed to shear stress for 24 hours. Shown are combined data (mean $\pm$ SD) from two cell strains exposed to 0.1 or 10 dynes/cm² for 24 hours. NO content was measured by (A) DAF-FM fluorescence and (B) Griess reagent analysis.

Physiological Relevance of Findings

In the healthy eye, we hypothesize that shear stress and NO production plays a role in the regulation of IOP by increasing the permeability of the SC inner wall and decreasing contractility of the juxtacanalicular TM. Specifically, during elevated IOP, we know that SC narrows,^{21,39,40} thus increasing average shear stress acting on SC cells. In a simplified model, shear stress has been calculated to range from 2 to 20 dynes/cm²,²⁰ similar to that occurring in large vessels, and to increase strongly as SC collapses. The present study shows that SC cells respond to physiological shear stress by aligning with the direction of shear (10 dynes/cm²) as observed in vivo.²⁰ Schlemm's canal cells also respond by increasing NO production, consistent with previous results showing NO production in several cells of the outflow pathway including the SC.³⁸ Interestingly, we did not see any additional production of NO by SC at shear levels above 10 dynes/cm² (data not shown). Because NO is a gas with a relatively high diffusivity in aqueous, it can diffuse "upstream" of the flow direction when released from SC to reach the TM, where it has the potential to relax the TM via paracrine signaling or to increase SC permeability, similar to the situation in the systemic vasculature.^{1,2}

In summary, data presented are consistent with the idea that NO production by SC cells increases upon SC collapse at

elevated IOPs and may be part of a homeostatic feedback loop that normalizes IOP. Data from two glaucoma cell strains suggested a depressed responsiveness to shear. If these data hold true for a larger sample number of glaucoma strains, then this aberrant regulation of NO production may prevent proper normalization of IOP and play a role in ocular hypertension in glaucoma.

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