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Sphingosine-1-Phosphate Effects on the Inner Wall of Schlemm's Canal and Outflow Facility in Perfused Human Eyes

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Abstract

Previous work has shown that sphingosine 1-phosphate (S1P) decreases outflow facility in perfused porcine eyes while dramatically increasing giant vacuole density in the inner wall of the aqueous plexus, with no obvious changes in the trabecular meshwork (TM). Due to known effects of S1P on cell-cell junction assembly in vascular endothelia, we hypothesized that S1P would decrease outflow facility in human eyes by increasing the complexity of cell-cell junctions in Schlemm's canal (SC) inner wall endothelia. Perfusion of enucleated post mortem human eyes at 8 mmHg constant pressure in the presence or absence of 5 μ M S1P showed that S1P decreased outflow facility by $36 \pm 20\%$ ($n = 10$ pairs; $p=0.0004$); an effect likely mediated by activation of S1P₁ and/or S1P₃ receptor subtypes, which were found to be the principal S1P receptors expressed by both TM and SC cells by RT-PCR, confocal immunofluorescence microscopy and western blot analyses. Examination of SC's inner wall using confocal microscopy revealed no consistent differences in VE-cadherin, β -catenin, phosphotyrosine or filamentous actin abundance/distribution between S1P-treated eyes and controls. Moreover, morphological inspection of conventional outflow tissues by light and scanning electron microscopy showed no significant differences between S1P-treated and control eyes, particularly in giant vacuole density. Thus, unlike the situation in porcine eyes, we did not observe changes in inner wall morphology in human eyes treated with S1P, despite a significant and immediate decrease in outflow facility in both species. Regardless, S1P receptor antagonists represent novel therapeutic prospects for ocular hypertension in humans.

Introduction

Lysophospholipids, such as sphingosine 1-phosphate (S1P), are membrane phospholipid metabolites that can function as autocrine/paracrine signaling molecules, influencing a broad range of cellular functions, such as cardiac development, immunity, platelet aggregation, cell

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movement and vascular permeability (Panetti 2002; Marsolais and Rosen 2009). S1P activity is mediated by binding to one or more of five G-protein coupled receptor subtypes (S1P receptors 1–5, formerly known as Edg1, 3, 5, 6, 8). The S1P receptor subtypes are differentially expressed in tissues, likely in alignment with specific functional tissue requirements. For example, S1P₁ and S1P₃ receptors are preferentially expressed by vascular endothelial cells, whereas smooth muscle cells express S1P₁, S1P₂ and S1P₃ (Donati and Bruni 2006).

Due to the fact that it is constantly bathed by secreted aqueous humor, the conventional outflow pathway has the potential to utilize S1P and/or other lysophospholipids as signaling molecules to modulate outflow resistance. In support of this idea, lysophospholipids are known to be constituents of aqueous humor (Liliom et al. 1998) and it has been shown that activation of S1P receptors in the conventional outflow tract dramatically and rapidly decreases outflow facility in porcine eyes. Specifically, outflow facility decreased by 31% in perfused porcine eyes after 5 hours of infusion of 5 μ M S1P (Mettu et al. 2004). Interestingly, while Mettu et al. observed no histological changes in the juxtacanalicular region of the trabecular meshwork of perfused porcine eyes, they did observe a dramatic increase in the density of giant vacuoles in the endothelial lining of the angular aqueous plexus (the porcine analogue of Schlemm's canal in human eyes). This observation is consistent with S1P affecting the pressure drop across the endothelial lining, perhaps by increasing the strength of cell-cell junctions between the endothelial cells lining the aqueous plexus. This premise is in turn consistent with well-described effects of S1P on cell-cell junction and circumferential actin assembly in endothelial cells via downstream effects on the small GTPase, Rac1 and subsequently decreased paracellular permeability (Garcia et al. 2001; Shikata et al. 2003; Dudek et al. 2004).

Despite obvious effects on cells lining the aqueous plexus, S1P receptor expression and activation has only been studied in TM cells in culture, where it was shown that TM cells express S1P₁ and S1P₃ receptor subtypes (Mettu et al. 2004). Further, Mettu et al. showed that S1P changed several measures of TM contractility, e.g. S1P promoted the phosphorylation of myosin light chain plus the formation of stress fibers and focal adhesions, which were shown to be mediated primarily through rho GTPase activation. Due to apparent rho-dominant signaling in TM cells, it was concluded that S1P₃ receptors mediate S1P effects on TM cell contractility and hence likely affect outflow facility in the porcine eye.

Motivated by this important work in porcine eyes, we sought to determine whether S1P affects outflow facility in human eyes, and if so, what role S1P receptor subtypes in the inner wall of SC might play in this process. We hypothesized that S1P increases outflow resistance in human eyes by activating receptors in the inner wall of SC, driving circumferential actin and associated cell-cell junction assembly. In the present study, we observed that, similar to porcine eyes, S1P dramatically and rapidly decreases outflow facility in enucleated human eyes. However, unlike the situation in porcine eyes, the inner wall of SC in treated human eyes was not morphologically different from untreated eyes. At the molecular level, the inner wall of SC expressed S1P₁ and S1P₃ receptor subtypes, but activation of these receptors did not result in detectable changes in cortical actin, VE-cadherin, phosphotyrosine or β -catenin distribution/abundance.

Materials and Methods

Cell Culture

Human donor eyes were obtained from Life Legacy Foundation (Tucson, AZ), National Disease Research Interchange (Philadelphia, PA) and Sun Health Research Institute (Sun City, AZ). Schlemm's canal (SC) cells were isolated from conventional outflow tissues of human eyes using a cannulation technique and then were characterized and cultured as previously described (Stamer et al. 1998). Using a blunt dissection procedure followed by extracellular

matrix digestion, trabecular meshwork (TM) cells were isolated from human eyes and were characterized and cultured as previously described (Stamer et al. 1995). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, low glucose), supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and glutamine (0.29 mg/ml). Six different SC cell strains (SC42, SC44, SC45, SC51, SC55, and SC 56) and four TM cell strains (TM26, TM86, TM87, and TM90) were used in the present study and chosen based on strain availability at the time of experiments.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from cell strains using the TRIzol reagent (GIBCO). RNAs were used as templates for reverse transcription synthesis of cDNAs using the ThermoScript RT-PCR kit (Invitrogen). Amplification of DNA by the polymerase chain reaction (PCR) was performed using *Taq* DNA polymerase (Invitrogen) for 30 cycles (denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 45s). Oligonucleotide primers used for subtype-specific amplification to detect the S1P receptors were previously characterized (Mettu et al. 2004): *edg1* (5'-ACGTCAACTATGATATCATCGTCCG, 3'-CATTTTCAGCATTGTGATATAGCGC); *edg5* (5'-ACTGTCCTGCCTCTCTACGCC, 3'-GTCTTGAGCAGGGCTAGCGTC); and *edg3* (5'-ACCATCGTGATCCTCTACGCAC, 3'-CTTGATTACTTCTGCTTGGGTCTG). Positive control primers were directed against *gapdh* (GAPDH6- GAAGGTGAAGGTCGGAGTC, 3'GAPDH 212- GAAGATGGTGATGGGATTTC). PCR products were loaded into 1% agarose gel slabs, resolved by electrophoresis and visualized using ethidium bromide and ultraviolet light.

Western Blot Analyses

Mature and confluent SC and TM cell monolayers were scraped from culture plates and solubilized in Laemmli sample buffer containing 10% β -mercaptoethanol. The whole cell lysates were boiled for 10 minutes, loaded onto a 10% polyacrylamide gel, and proteins were fractionated by SDS-PAGE. Proteins were electrophoretically transferred from gel slabs to nitrocellulose membranes for 90 min at 100 V. Membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline (137 mM NaCl, 25 mM Tris, 2.7 mM KCl, pH 7.4) containing 2% Tween-20 (TBS-T) for 60 min, then incubated with rabbit polyclonal IgGs that specifically recognize S1P₁ (0.1 μ g/ml, Affinity Bioreagents), S1P₂ (EDG5, 0.2 μ g/ml, Santa Cruz), or mouse monoclonal IgGs against S1P₃ (EDG3, 0.1 μ g/ml, Exalpha Biologicals) receptor subtypes. Following overnight incubation at 4°C, membranes were washed (3 $\times$ 10 min) with TBS-T, incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgGs (40 ng/ml, Jackson ImmunoResearch Laboratories) for 60 min at room temperature, and washed again with TBS-T (3 $\times$ 10 min). To visualize proteins, membranes were incubated with either ECL Advance (Amersham) or HyGLO (Denville Scientific) chemiluminescence reagents and exposed to X-ray film (Genesee Scientific). All membranes were re-probed with ascites fluid containing a mouse monoclonal IgG against β -actin (1:10,000 dilution, Sigma-Aldrich) and subsequent HRP-conjugated goat anti-mouse IgG (40 ng/ml, Jackson ImmunoResearch Laboratories) for loading control. S1P₁-specific bands were determined with the inclusion of EDG1 transfected cell lysates (Exalpha Biologicals).

Immunofluorescence Microscopy of Frozen Ocular Sections

Human cadaveric eyes (donor ages= 79, 88 and 98) were received within 36 hours of death and their anterior portions were dissected into radially oriented wedges. Tissue wedges were immersed in OCT compound, frozen at -80°C and then sagittally cryosectioned (8 μ m). The tissue sections on slides were fixed in 4% paraformaldehyde, then blocked for 30 min with 10% goat serum in 100 mM Tris-HCl containing 0.05% Tween-20, pH 7.4. The sections were then incubated overnight in a moist chamber at 4°C with rabbit polyclonal IgGs that recognize

S1P₁ (EDG1, 4 µg/ml, Santa Cruz), S1P₂ (EDG5, 4 µg/ml, Santa Cruz), or S1P₃ (EDG3, 4 µg/ml, Santa Cruz) receptor subtypes. Following antibody incubations, sections were washed extensively with 100 mM Tris-HCl containing 0.05% Tween-20 (4 × 10 min). Antigen binding was detected by a 1 hr incubation with CY3-conjugated goat anti-rabbit IgG (0.75 µg/ml, Jackson ImmunoResearch Laboratories), counterstained with SYTOX green nucleic acid stain (100 nM, Invitrogen) for 1 minute, and washed extensively (4 × 10 min) before visualization. Background and auto-fluorescence was monitored by incubating tissues with CY3-conjugated goat anti-rabbit IgG in the absence of primary antibodies. Labeled tissue sections were visualized and captured digitally using a Nikon PCM 2000 confocal microscope (Melville, NY).

Whole Eye Perfusion and Facility Measurements

Dulbecco's phosphate buffered saline containing 5.5 mM glucose (DBG) and prefiltered through a 0.22 µm Millex-GS filter (Millipore, Bedford, MA) served as mock aqueous humor for the perfusion studies. Sphingosine-1-phosphate (S1P), acquired from Biomol International (Plymouth Meeting, PA), was dissolved in 65°C boiling methanol (0.5mg/mL) and aliquotted into glass tubes. The solvent was evaporated with streaming nitrogen gas to deposit a thin film inside the tube and the aliquots stored at -20°C. Working solutions of 5µM S1P were obtained by dissolving the stock aliquot into DBG containing 0.2% fatty-acid free BSA (Roche, Penzberg, Germany) on a rotator overnight at 37°C. Control solutions also contained 0.2% fatty-acid free BSA.

Ostensibly normal human eyes were obtained from the Eye Bank of Canada (Ontario Division, Toronto, Ontario) and NDRI (Philadelphia, PA). After excluding six pairs of eyes with unstable or asymmetric baseline facility traces, 10 pairs remained in the study. Mean donor age was 79.8 years (range 74–92 years) and mean post mortem time to start of perfusion was 30.8 hours (range 15.5–39.0 hours) (table 1). DBG was introduced into eyes at a constant pressure of 8 mmHg (corresponding to 15 mmHg in vivo) via a needle inserted through the cornea to the posterior chamber as described previously (Ethier et al. 1993). Baseline facility was measured for 60–90 min and then one eye of each pair received anterior chamber exchange with 5 µM S1P, while the contralateral eye was exchanged with DBG containing only vehicle. During the anterior chamber exchange a constant IOP of 8 mmHg was maintained after which the perfusion was allowed to continue for approximately 180 min at 8 mmHg. The percent increase in outflow facility (C) is $100 \times (C_{\text{final}}/C_{\text{Baseline}} - 1)$ and net change in C is percent increase in experimental eye minus percent increase in control eye. Average net change values were analyzed by a two-tailed, paired Student's t-test assuming unequal variance and differences were considered significant at $p < 0.05$.

Following S1P perfusion, eyes were fixed by anterior chamber exchange and perfusion with 3% formalin at 8 mmHg, then hemisected, and the outflow tissue cut into wedges. Half from each eye, including tissue wedges from every quadrant, were immersed overnight in universal fixative (UF, 2.5% formalin, 2.5% glutaraldehyde in Sorensen's buffer) for use in scanning electron microscopy (SEM) and semi-thin sectioning. The rest, to be used for confocal microscopy, were fixed in 3% formalin, the exception being eyes 07-025/026 and 08-005-006, which were perfusion fixed only, then stored in 15% glycerol in DBG at -20°C.

Labelling and Confocal Microscopy of the Inner Wall of SC

Radial segments of the limbal area were microdissected and labeled as previously described (Ethier et al., 2004). Briefly, Schlemm's canal was opened by an incision along its posterior margin, and the trabecular meshwork and adherent inner wall reflected anteriorly. The inner wall and underlying trabecular meshwork were fluorescently labeled to visualize F-actin, nuclei, and either VE-cadherin, β -catenin or phosphotyrosine. Tissue was permeabilized with

0.2% Triton X-100 in DPBS for 5 min at room temperature (RT) and blocked with 5% goat serum (Sigma Corp., St. Louis, MO) in DPBS for 45 min at 37°C. VE-cadherin was labeled with mouse anti-VE-cadherin IgG (clone 9H7, provided by Dr. Ron Heimerl) diluted in calcium- and magnesium-free DPBS, and incubated overnight at RT, followed by incubation in Alexa-647 goat anti-mouse IgG (Invitrogen, Austin, TX) diluted 1:150 in DPBS, for 75 min at 37°C. β -catenin was labeled with rabbit anti- β -catenin IgG (Abcam, Cambridge, MA), diluted 1:200 in DPBS, and incubated for 2h at RT, followed by incubation in Alexa-647 goat anti-rabbit IgG (Invitrogen, Austin, TX) diluted 1:150 for 75 minutes at 37°C. Phosphotyrosine was labeled with mouse anti-phosphotyrosine IgG (clone 4G10; Upstate, Lake Placid, NY), diluted 1:200 and incubated at 37°C for 60 min followed by incubation in Alexa-647 goat anti-mouse IgG (Invitrogen, Austin, TX). For negative controls, tissue was treated as above while excluding the primary antibodies. F-actin was labeled by incubating for 30 min at RT in rhodamine-phalloidin (Invitrogen, Austin, TX), diluted 1:40 in DPBS. Nuclei were marked by incubating for 5 min at RT in SYTOX (Invitrogen, Austin, TX), diluted 1:2400 in Tris-buffered saline or DAPI (Invitrogen, Austin, TX) diluted 2 μ g/mL in DPBS.

The thin, 2 mm circumferential section of tissue comprising the inner wall of Schlemm's canal, JCT and trabecular meshwork (TM) was separated from the ciliary body and root of the iris by an oblique cut at the posterior margin of Schlemm's Canal, then mounted with DAKO® fluorescent mounting medium (DAKO, Glostrup, Denmark) and a No. "0" cover slip. Samples were examined using a Zeiss LSM 510 meta confocal microscope (Carl Zeiss Inc., Jena, Germany). Six pairs of perfused eyes (7025/6, 8005/6, 683/4, 695/6, 697/8, 6109/10) were examined in a masked fashion by three observers, and the average number of preparations examined was eight per eye. Z-series were collected using a $\times 63$ oil or water immersion lens, with the focal plane beginning at the apex and advancing deeper into the tissue. Images were viewed using Zeiss Image Browser software, version 5.

SEM and Semi-thin Sectioning

Outflow tissue from eyes that had been fixed in UF was dissected out, post-fixed in 1% osmium tetroxide (EMS, Hatfield, PA), dehydrated, infiltrated, and embedded in Epon-Araldite (EMS, Hatfield, PA). Half-micron thick radial sections were stained with toluidine blue, examined and photographed with a Zeiss Axiovert microscope. Samples from all four quadrants of four pairs of eyes were examined in detail (6105/6, 683/4, 813/4, 697/8).

For SEM, inner wall samples from all four quadrants in two pairs of eyes were examined in detail (6105/6 and 813/4). Eyes were microdissected to expose the inner wall of Schlemm's canal, as described above, fixed in UF, incubated in a 2% solution of guanidine-HCl/tannic acid (Sigma Corp., St. Louis, MO) for 2h, post-fixed in 1% osmium tetroxide for 1h, washed then dehydrated through an ethanol series, incubated in hexamethyldisilazane (Sigma Corp., St. Louis, MO) and air dried in a fume hood. They were mounted on SEM stubs, gold-coated and examined with a Hitachi S-3400N VP SEM (Pleasanton, CA).

Results

We used three complementary approaches to test the hypothesis that S1P effects on conventional outflow in human are mediated at the level of cell-cell junctions between cells of the inner wall of Schlemm's canal (SC). Using primary cultures of human SC endothelial cells, we first examined the expression of three S1P receptor subtypes (S1P₁₋₃) via RT-PCR and Western blot analyses. Similar to porcine TM cells (Mettu et al. 2004), we observed that SC cells express messenger RNA for all three receptor subtypes tested (not shown), but only protein for S1P₁ and S1P₃ receptors (Figure 1).

These results are consistent with data gathered from confocal indirect immunofluorescence studies using subtype-specific antibodies to label S1P receptors in human cadaveric eyes (Figure 2). We observed the presence of S1P₁ and S1P₃ receptor subtypes in the SC endothelia. These studies also confirmed that both S1P₁ and S1P₃ receptor subtypes were expressed by cells on the trabecular beams and in the juxtacanalicular tissue. Interestingly, labeling of S1P₁ and S1P₃ receptors appeared stronger on SC endothelia than on TM cells in all donor eyes examined. In two of three eyes analyzed, we did notice some non-specific labeling of S1P₃ antibodies to non-cellular material of sclera. Additionally, some modest SC and TM labeling cells was observed using antibodies that recognize S1P₂ receptors (Figure 2).

Next, we examined whether S1P affects outflow facility in human eyes in a fashion similar to that reported for porcine eyes. We tested 10 pairs of enucleated human donor eyes (table 1) in which stable baseline outflow facilities were found to be similar between control (0.235 ± 0.07 $\mu\text{l}/\text{min}/\text{mmHg}$) and their experimental counterparts (0.257 ± 0.04 $\mu\text{l}/\text{min}/\text{mmHg}$) and within a physiological range (table 1). Our results showed that S1P significantly decreased outflow facility by $36 \pm 20\%$ (figure 3; $p=0.0004$), which was similar in magnitude to that reported for porcine eyes. Impressively, S1P effects were observed very rapidly, within 20 minutes of anterior chamber exchange.

Finally, we studied whether S1P treatment induced morphological changes in outflow tissues (including SC) of perfused human eyes, and whether such changes were similar to those reported in porcine eyes (Mettu et al. 2004). We first examined sagittal sections in each of four quadrants of perfused conventional outflow tissues by light microscopy. In all cases, no significant differences in inner wall morphology between S1P-treated eyes and their paired controls were observed (Figure 4). Overall, outflow structures were populated by appropriate numbers of cells for the age of the donors, the cells appeared healthy and the inner wall was intact in both control and treated eyes. Moreover, giant vacuole density was examined qualitatively and found not to be different between treated and control eyes. We also studied the morphology of the inner wall *en face* using scanning electron microscopy. Although we did not quantify giant vacuole or pore density in the inner wall, no notable differences between S1P-perfused and control eyes were observed, with each displaying similar vacuole densities, vacuole sizes/shapes, inner wall pore appearances and cell-cell junction appearances (Figure 4).

To explore whether the decreased outflow facility in S1P-treated eyes could be linked with changes at the molecular level, we used indirect immunofluorescence confocal microscopy to study the expression and cellular distribution of several components known to influence endothelial permeability: F-actin, VE-cadherin, β -catenin and phosphotyrosine. While we did notice circumferential reorganization of F-actin in some S1P-treated eyes compared to their contralateral controls, these observations were not consistent amongst all treated eyes (Figure 5 and Figure 6). Similarly, VE-cadherin labeling (Figure 5), β -catenin labeling (Figure 6) and phosphotyrosine labeling (data not shown) appeared unaltered by S1P treatment. In fact, extensive masked analysis of labeled tissues failed to identify any constant effect of S1P on the distribution or amount of the above factors.

Discussion

This study demonstrates that S1P causes rapid and substantial decreases in outflow facility in human eyes. Because receptors for S1P were found on both TM and SC cells in the conventional outflow pathway, the involvement of both cell types in the S1P response is possible. Interestingly, even though S1P receptors appeared more abundant in SC endothelia, we did not find morphologic alterations of inner wall cells in human eyes similar to the dramatic ballooning of aqueous plexus endothelial cells observed in porcine eyes treated with the same

dose and time course of S1P (Mettu et al., 2004). In fact, we could observe no consistent morphological or molecular changes to explain the facility decrease due to S1P perfusion in human eyes. This suggests that a number of subtle synergistic changes in outflow tissues are responsible for S1P's rapid and substantial effect on facility.

While the time course and magnitude of facility changes in response to S1P were remarkably similar between porcine (Mettu et al., 2004) and human eyes, the reason(s) for the differences in giant vacuole density between these two species is unclear. Differences in physiology between the porcine and human outflow tracts (e.g. greater connectivity between the JCT-TM cells and inner wall of SC is observed in human eyes compared to other species (Overby et al. 2002; Scott et al. 2007) may underlie dissimilarities. Alternatively, there may be a species difference in the distribution and/or abundance of S1P receptors between TM and SC cells.

A limitation of this study was that we did not quantify giant vacuole density or expression levels of VE-Cadherin and β -catenin. This was primarily due to technical issues; for example, quantification of giant vacuoles from SEM is problematic due to issues with differentiating between nuclear bulges and giant vacuoles, necessitating time-consuming analysis of serial micrographs. Moreover, for analyses of VE-cadherin and β -catenin, the small volume of TM/SC tissues requires tissue pooling for quantitative analysis of protein levels. Thus, we cannot exclude the possibility that there were subtle changes in these endpoints that were not evident by qualitative inspection, although considering the degree of heterogeneity around the circumference of the TM; it is our experience that changes typically need to be qualitatively evident to be statistically robust.

Activation of S1P receptors in a contractile cell increases cellular tone; S1P would therefore be expected to increase TM cell tone, which is predicted to decrease outflow facility (Tian et al. 1998; Ethier and Chan 2001). Further, S1P increases barrier function in endothelia, and therefore would be expected to decrease the permeability of SC cells. Since activation of S1P receptors in both cell types is predicted to decrease outflow facility, it is possible that both TM cells and SC cells participate in the S1P-mediated effects observed in human whole eye perfusions. At this time the relative contribution of each cell type to the total facility change is uncertain.

Activation of S1P1 and S1P3 receptors in vascular endothelia, often due to release of S1P from activated platelets, decreases paracellular permeability due to assembly of the circumferential actin cytoskeleton and adherens junctions (Sanchez et al. 2003; Mehta et al. 2005; Sun et al. 2009). Thus, in the present study we examined filamentous actin, VE-cadherin, β -catenin and phosphotyrosine at cell-cell borders of the inner wall of SC following S1P treatment. We chose to study adherens junction proteins because they appear integral to the development and maintenance of endothelial barrier properties (Vittet et al. 1997; Carmeliet et al. 1999; Corada et al. 1999; Gumbiner 2000). While cadherins directly mediate cell-cell interactions, β -catenin regulates the association of the cadherin junction complex with the circumferential actin cytoskeleton. For example, phosphorylation of catenins modulate the adhesive function of the cadherin junction complex by decreasing the affinity of a cadherin for an adjacent cadherin or the affinity of the cadherin/catenin complex for the circumferential actin cytoskeleton (Rajasekaran et al. 1996; Roura et al. 1999; Wong et al. 2000; Xia et al. 2003). In vascular endothelia, PECAM-1 (CD31) localizes at lateral cell borders and associates with adherens junctions. We examined phosphotyrosine staining patterns due to two recent reports showing modulation of PECAM-1 phosphorylation by S1P and mechanical stress, which correlated with decreased endothelial monolayer permeability (Chiu et al. 2008; Huang et al. 2008).

The fact that we were unable to discern consistent changes in abundance or localization of junctional proteins suggests that junctional complexity is already high under basal conditions,

and/or that additional increases in junctional assembly were not resolvable using the confocal microscopy techniques used in the present study. It may be easier to notice disassembly of junctions between SC cells due to appearance of pores or gaps in staining. Alternatively, it may be that consistent changes were not observed because it is estimated that in older eyes only a fraction of the total circumference of the conventional outflow pathway typically conducts flow (Overby et al. 2009). Since flow pathways were not “marked” with tracer in the present study, only a subset of the eye regions that were examined by microscopy will have been exposed to S1P; this may partially explain the inconsistent changes we observed with filamentous actin staining.

We and Mettu et al. did not notice a change in the morphology of the TM following S1P treatment. While the TM probably participates in the S1P effects, morphological alterations in the TM were not discernable due to the limitation of the methods used in our study and the previous one. If cellular contraction occurs in the TM following exposure to S1P, then other endpoints, such as myosin light chain phosphorylation status, or filamentous to globular actin ratio in the TM may represent better ways to monitor change in TM contractility.

As a constituent of aqueous humor and released factor from activated platelets in the SC lumen, S1P is expected to contribute to a tonal level of signaling and tissue homeostasis in the conventional outflow pathway (Wan et al. 2008). Thus, a potential therapeutic strategy to treat those with ocular hypertension is the development of S1P receptor antagonists, which should decrease S1P-mediated tone and hence increase outflow facility. The relative contribution of S1P receptor subtypes expressed by outflow cells will dictate whether specific- or pan-antagonists to the S1P₁ and S1P₃ receptors would be more efficacious.

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References

- Carmeliet P, Lampugnani M, Moons L, Breviario F, Compernelle V, Bono F, Balconi G, Spagnuolo R, Oosthuysen B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter M, Gittenberger-de-Groot A, Poelmann R, Lupu F, Herbert J-M, Collen D, Dejana E. Targeted deficiency of cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999;98:147–157. [PubMed: 10428027]
- Chiu YJ, McBeath E, Fujiwara K. Mechanotransduction in an extracted cell model: Fyn drives stretch- and flow-elicited PECAM-1 phosphorylation. *J Cell Biol* 2008;182:753–763. [PubMed: 18710921]
- Corada M, Mariotti M, Thurston G, Smith K, Kunkel R, Brockhaus M, Lampugnani M, Martin-Padura I, Stoppacciaro A, Ruco L, McDonald D, Ward P, Dejana E. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc. Natl. Acad. Sci. USA* 1999;96:9815–9820. [PubMed: 10449777]
- Donati C, Bruni P. Sphingosine 1-phosphate regulates cytoskeleton dynamics: implications in its biological response. *Biochim Biophys Acta* 2006;1758:2037–2048. [PubMed: 16890187]
- Dudek SM, Jacobson JR, Chiang ET, Birukov KG, Wang P, Zhan X, Garcia JG. Pulmonary endothelial cell barrier enhancement by sphingosine 1-phosphate: roles for cortactin and myosin light chain kinase. *J Biol Chem* 2004;279:24692–24700. [PubMed: 15056655]
- Ethier C, Ajerssch P, Pirog R. An improved ocular perfusion system. *Curr Eye Res* 1993;12:765–770. [PubMed: 8222738]

- Ethier C, Chan D. Cationic ferritin changes outflow facility whereas anionic ferritin does not. *Invest. Ophthalmol. Vis. Sci* 2001;42:1795–1802. [PubMed: 11431444]
- Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest* 2001;108:689–701. [PubMed: 11544274]
- Gumbiner B. Regulation of cadherin adhesive activity. *J. Cell Biol* 2000;148:399–403. [PubMed: 10662767]
- Huang YT, Chen SU, Chou CH, Lee H. Sphingosine 1-phosphate induces platelet/endothelial cell adhesion molecule-1 phosphorylation in human endothelial cells through cSrc and Fyn. *Cell Signal* 2008;20:1521–1527. [PubMed: 18502612]
- Liliom K, Guan Z, Tseng JL, Desiderio DM, Tigyi G, Watsky MA. Growth factor-like phospholipids generated after corneal injury. *Am J Physiol* 1998;274:C1065–C1074. [PubMed: 9575804]
- Marsolaïs D, Rosen H. Chemical modulators of sphingosine-1-phosphate receptors as barrier-oriented therapeutic molecules. *Nat Rev Drug Discov* 2009;8:297–307. [PubMed: 19300460]
- Mehta D, Konstantoulaki M, Ahmmed GU, Malik AB. Sphingosine 1-phosphate-induced mobilization of intracellular Ca²⁺ mediates rac activation and adherens junction assembly in endothelial cells. *J Biol Chem* 2005;280:17320–17328. [PubMed: 15728185]
- Mettu P, Deng P, Misra U, Gawdi G, Epstein D, Rao P. Role of lysophospholipid growth factors in the modulation of aqueous humor outflow facility. *Invest. Ophthalmol. Vis. Sci* 2004;45:2263–2271. [PubMed: 15223804]
- Overby D, Gong H, Qiu G, Fredde TF, Johnson M. The mechanism of increasing outflow facility during washout in the bovine eye. *Invest Ophthalmol Vis Sci* 2002;43:3455–3464. [PubMed: 12407156]
- Overby DR, Stamer WD, Johnson M. The changing paradigm of outflow resistance generation: towards synergistic models of the JCT and inner wall endothelium. *Exp Eye Res* 2009;88:656–670. [PubMed: 19103197]
- Panetti TS. Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. *Biochim Biophys Acta* 2002;1582:190–196. [PubMed: 12069828]
- Rajasekaran A, Hojo M, Huima T, Rodriguez-Boulán E. Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *J. Cell Biol* 1996;132:451–463. [PubMed: 8636221]
- Roura S, Miravet S, Piedra J, Garcia D, Dunach M. Regulation of E-cadherin/Catenin association by tyrosine phosphorylation. *J. Cell Biol* 1999;274:36734–36740.
- Sanchez T, Estrada-Hernandez T, Paik JH, Wu MT, Venkataraman K, Brinkmann V, Claffey K, Hla T. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J Biol Chem* 2003;278:47281–47290. [PubMed: 12954648]
- Scott PA, Overby DR, Fredde TF, Gong H. Comparative studies between species that do and do not exhibit the washout effect. *Exp Eye Res* 2007;84:435–443. [PubMed: 17173894]
- Shikata Y, Birukov KG, Garcia JG. S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. *J Appl Physiol* 2003;94:1193–1203. [PubMed: 12482769]
- Stamer WD, Roberts BC, Howell DN, Epstein DL. Isolation, culture and characterization of endothelial cells from Schlemm's canal. *Invest Ophthalmol Vis Sci* 1998;39:1804–1812. [PubMed: 9727403]
- Stamer WD, Seftor RE, Williams SK, Samaha HA, Snyder RW. Isolation and culture of human trabecular meshwork cells by extracellular matrix digestion. *Curr Eye Res* 1995;14:611–617. [PubMed: 7587308]
- Sun X, Shikata Y, Wang L, Ohmori K, Watanabe N, Wada J, Shikata K, Birukov KG, Makino H, Jacobson JR, Dudek SM, Garcia JG. Enhanced interaction between focal adhesion and adherens junction proteins: involvement in sphingosine 1-phosphate-induced endothelial barrier enhancement. *Microvasc Res* 2009;77:304–313. [PubMed: 19323978]
- Tian B, Kaufman PL, Volberg T, Gabelt BT, Geiger B. H-7 disrupts the actin cytoskeleton and increases outflow facility. *Arch Ophthalmol* 1998;116:633–643. [PubMed: 9596500]
- Vittet D, Buchou T, Schweitzer A, Dejana E, Huber P. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc. Natl. Acad. Sci. USA* 1997;94:6273–6278. [PubMed: 9177207]

- Wan Z, Woodward DF, Stamer WD. Endogenous Bioactive Lipids and the Regulation of Conventional Outflow Facility. *Expert Rev Ophthalmol* 2008;3:457–470. [PubMed: 19381354]
- Wong E, Morgan L, Smales C, Lang P, Gubby S, Staddon J. Vascular endothelial growth factor stimulates dephosphorylation of the catenins p120 and p100 in endothelial cells. *Biochem J* 2000;346:209–216. [PubMed: 10657259]
- Xia X, Mariner D, Reynolds A. Adhesion-associated and PKC-modulated changes in serine/threonine phosphorylation of p120-catenin. *Biochem* 2003;42:9195–9204. [PubMed: 12885254]

Figure 1. Western blot analyses of S1P receptor subtype expression in monolayers of Schlemm's canal (SC) endothelial cells

Whole cell lysates derived from three mature SC cell monolayers (strains SC44, SC55, and SC56) were probed with antibodies specific for S1P₁, S1P₂ and S1P₃ receptor subtypes. Whole cell lysates prepared from human microvascular endothelia (HM), human umbilical vein endothelia (HV) and human trabecular meshwork (TM26) cells were also analyzed and used as controls. Blots were reprobbed with antibodies specific for β-actin to control for equivalent loading between samples.

Figure 2. Expression of S1P receptor subtypes by human Schlemm's canal (SC) endothelia *in situ* Confocal immunofluorescence microscopy of frozen serial saggital sections of human anterior segments shows labeling with antibodies specific for S1P₁, S1P₂ and S1P₃ receptor subtypes (as indicated, red) and cell nuclei (SYTOX, green). Insets indicate internal positive control staining for S1P₁ and S1P₃ receptors located on scleral vessels (V) in same tissue section. The lower right panel is provided to show background staining of tissues labeled only with rhodamine-conjugated secondary antibodies and nuclei stain (control). Arrows point to labeling in trabecular meshwork (TM). Results shown are all from one human donor eye and are representative of three different human eyes analyzed.

Effects of S1P on Facility

Figure 3. Effect of S1P on outflow facility in paired whole human eye perfusions

After one hour of relatively stable facility, anterior chamber contents in one eye were exchanged with medium (DPBS + 5.5 mM glucose + 0.2% fatty-acid free BSA) containing 5 μ M S1P while the contralateral eye was exchanged with medium only, and then perfused for an additional 3.5 hours (post-exchange). The plotted quantity is measured outflow facility normalized by each eye's stable facility value just prior to exchange (mean $\pm$ SEM for ten pairs of eyes). Normalized facility values less than one therefore indicate a decrease in facility compared to baseline.

Figure 4. Morphological analyses of the inner wall of Schlemm's canal and associated conventional outflow structures in human eyes perfused with S1P

Shown are semi-thin (plastic) sections of outflow tissues from a control (panel A) and S1P-perfused (panel B) eye from a pair, fixed at 8 mmHg, sagittally sectioned (0.5 μ m) and stained with toluidine blue (bar= 100 μ m). Micrographs are from a representative eye pair (6105/6) of four that were examined in detail. The inner wall of SC (viewed from the luminal side) in perfused eyes was examined by scanning electron microscopy. Displayed in panel C is a micrograph taken from a control eye, while panel D was taken from the contralateral, S1P-treated eye (6105/6). Results shown are representative of 2 eye pairs that were examined by SEM in all four quadrants (bar=25 μ m). Representative giant vacuoles are indicated by arrowheads.

Figure 5. Actin architecture and Vascular Endothelial (VE)-cadherin expression in Schlemm's canal inner wall of eyes perfused with S1P

Post perfusion, outflow tissues were probed with rhodamine phalloidin to label filamentous actin (red) and monoclonal antibodies specific for VE-cadherin (green). Shown are confocal images taken of a single area of human SC inner (viewed from luminal side) wall in paired eyes. Merged images are shown in right panels. Shown are micrographs from a single eye pair (7025/026) to indicate variability that was observed in staining patterns between S1P-treated and control eyes. Data shown are representative of six pairs of eyes that were examined.

Figure 6. Actin architecture and β -catenin expression in Schlemm's canal inner wall of eyes perfused with S1P

Post perfusion, outflow tissues were probed with rhodamine phalloidin to label filamentous actin (red) and monoclonal antibodies specific for β -catenin (green). Shown are confocal images taken of a single area of human SC inner (viewed from luminal side) wall in paired eyes perfused with S1P or vehicle control. Merged images are shown in right panels. Shown are micrographs from a representative eye pair (8005/6) of two pairs of eyes that were examined in total.

Table 1

Summary of Eye Donor Characteristics and Perfusion Results with S1P

ID	Donor Age (yrs)	Donor Gender	Time to Enucleation (hrs)	Time to Perfusion (hrs)	Baseline C (μl/min/mmHg)	Ending C (μl/min/mmHg)	Increase in C (%)	Net Change in C (%)
797-e	79	F	0.5	20	0.21	0.1	-52.4	-32.4
798-v					0.15	0.12	-20.0	
811-e	75	M	2.3	35.8	0.2	0.14	-30.0	-87.9
812-v					0.19	0.3	57.9	
813-e	74	F	2.3	33	0.3	0.19	-36.7	-40.2
814-v					0.29	0.3	3.5	
683-e	92	F	3.2	27	0.23	0.18	-21.7	-21.7
684-v					0.19	0.19	0	
695-e	75	F	7.2	38.3	0.3	0.13	-56.7	-36.7
696-v					0.15	0.12	-20.0	
697-v	83	M	2.0	28.5	0.22	0.19	-13.6	-40.6
698-e					0.24	0.11	-54.2	
6101-v	76	M	4.0	32	0.28	0.25	-10.7	-16.6
6102-e					0.33	0.24	-27.3	
6105-e	78	F	1.5	38.5	0.26	0.22	-15.4	-23.3
6106-v					0.38	0.41	7.9	
7025-v	76	M	NA	39	0.26	0.24	-7.7	-42.3
7026-e					0.24	0.12	-50.0	
8005-e	90	F	NA	15.5	0.26	0.14	-46.2	-21.2
8006-v					0.24	0.18	-25.0	
	79.8	6F/4M	2.9	30.8				
					Control mean		-2.8	
					S1P-treated mean	0.16	-39.1	

e=experimental

v=vehicle

ID=eye donor identification number

C=outflow facility