Sphingosine-1-Phosphate Enhancement of Cortical Actomyosin Organization in Cultured Human Schlemm's Canal Endothelial Cell Monolayers

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PURPOSE. Perfusion of sphingosine-1-phosphate (S1P) in whole eye organ culture models decreases outflow facility, whereas S1P promotes stress fiber formation and contractility in cultured trabecular meshwork (TM) cells. Because of S1P's known effect of increasing barrier function in endothelial cells, the authors hypothesized that Schlemm's canal (SC) cells in culture respond to S1P by increasing actomyosin organization at the cell cortex.

METHODS. Using primary cultures of human SC cells, the authors determined S1P activation of the GTP-binding proteins, RhoA and Rac (1,2,3). Time- and dose-dependent myosin light chain (MLC) phosphorylation in response to S1P and total expression of MLC were determined. Immunocytochemistry after S1P treatment was used to monitor filamentous actin (F-actin) and phospho-MLC organization and the localization of β -catenin, a component of adherens junctions. TM and human umbilical vein endothelial cell monolayers were used as controls.

RESULTS. S1P (1 μ M) activated RhoA and Rac after 5- and 30-minute treatments. S1P increased MLC phosphorylation with a similar time- and dose-dependent response in SC (EC_{50} = 0.83 μ M) compared with TM (EC_{50} = 1.33 μ M), though MLC expression was significantly greater in TM. In response to 1 μ M S1P treatment, phospho-MLC concentrated in the SC cell periphery, coincident with cortical actin assembly and recruitment of β -catenin to the cell periphery.

CONCLUSIONS. Results obtained in this study support the hypothesis that S1P increases actomyosin organization at the SC cell cortex and promotes intercellular junctions at the level of the inner wall of SC to increase transendothelial resistance and in part explains the S1P-induced decrease of outflow facility in organ culture. (*Invest Ophthalmol Vis Sci.* 2010;51:6633–6638) DOI: 10.1167/iovs.10-5391

Sphingosine-1-phosphate (S1P) is an endogenous bioactive lipid implicated in many systemic processes, such as bradycardia, vasoconstriction, angiogenesis, and lymphocyte egress.^{1,2} S1P binds with high affinity to five different G-protein-coupled receptor subtypes (S1P_{1–5}), formerly known as EDG receptors.³ Found in the nanomolar range in plasma and primarily bound to high-density lipoproteins,⁴

S1P has also been detected in aqueous humor.⁵ Perfusion of S1P in human and porcine whole eye organ culture models reduces outflow facility, thereby increasing resistance in the conventional outflow pathway.^{6,7} Most of the resistance to aqueous humor outflow is attributed to the juxtacanalicular region of the TM and the inner wall of SC.^{8–11} Histologic examination of S1P-treated and control porcine eyes revealed no differences in the structure of the TM.⁶ On the other hand, S1P-treated eyes displayed a dramatic increase in giant vacuoles along the endothelial lining of the aqueous plexus (analogous to SC in humans) compared with untreated control in eyes fixed at the same pressure. Giant vacuoles form in response to a pressure drop across the endothelial lining, indicating a potential enhancement of endothelial cell-cell junction complexity and an increase in resistance to outflow after the activation of S1P receptors along the endothelial lining. Activation of S1P receptors expressed by the inner wall of SC may also explain the dramatic effects of S1P on resistance to outflow observed in humans.⁷

In support of S1P affecting the junctions between SC cells lining the inner wall, vascular endothelial cells treated with S1P increase junctional proteins to sites of cell-cell contact.^{12–14} Additionally, S1P promotes cortical actin formation with an associated increase in MLC phosphorylation at the cell cortex.^{15,16} This actomyosin organization provides stability for the cell-cell and cell-matrix junctions and is essential in maintaining endothelial barrier function. On the other hand, MLC phosphorylation in S1P-treated contractile cells (like TM cells) occurs with focal adhesion and stress fiber formations.⁶ Although the contractile effects observed in TM cells involve the small GTPase Rho,⁶ the S1P effects observed in endothelial cells are conferred by the small GTPase Rac.¹⁷ Both Rac and Rho affect cytoskeleton dynamics and intercellular junctions and can either increase (Rac) or decrease (Rho) endothelial barrier function.¹⁸

Although the effects of S1P on human TM have been investigated, the mechanism by which S1P works through SC cells has not. The present study was performed to test the hypothesis that SC cells respond to S1P in culture by activating Rac and increasing actomyosin reorganization at the cell cortex. To accomplish this objective, we monitored the extent of RhoA compared with Rac activation and MLC phosphorylation in S1P-treated human SC cells in culture compared with control. Additionally, we examined phospho-MLC, filamentous (F)-actin, and β -catenin localization to determine S1P involvement in cytoskeletal organization and mobilization of cell junction proteins in SC cells.

MATERIALS AND METHODS

Cell Culture

Human SC and TM cells were isolated from donor eyes using methods previously described.^{19,20} Both cell types were maintained in Dulbecco's modified Eagle's medium (DMEM, low glucose; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and glutamine (0.29 mg/mL). At

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least two different cell strains isolated from the eyes of two individual donors were tested in each type of experiment. The cell strains chosen for each experiment were based on availability at the time of the experiments. Human umbilical vein endothelial cells (HUVEC-2; BD Biosciences, Bedford, MA) were maintained in medium (Medium 199; Invitrogen) supplemented with 15% fetal bovine serum, heparin sodium salt (90 $\mu\text{g/mL}$; Sigma-Aldrich, St. Louis, MO), endothelial mitogen (0.1 mg/mL; Biomedical Technologies Inc., Stoughton, MA), penicillin (100 U/mL), streptomycin (0.1 mg/mL), and glutamine (0.29 mg/mL).

Immunoblot Analyses

Cells, after 2-hour serum starvation and appropriate treatments, were washed twice with ice-cold PBS, lysed with ice-cold Laemmli buffer, and boiled for 5 minutes. The samples were then separated on a 12% polyacrylamide gel by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes for 90 minutes at 100 V. The membranes were blocked with 5% milk in Tris-buffered saline containing 0.2% Tween-20 (TBS-T) for 1 hour, then incubated overnight at 4°C with rabbit IgG against myosin light chain or phospho (Thr18/Ser19)-myosin light chain (1:1000 dilution; Cell Signaling, Beverly, MA). After exposure to primary antibody, membranes were washed with TBS-T, incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (40 ng/mL; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature, then washed again with TBS-T (3 $\times$ 10 minutes). Membranes were incubated with chemiluminescence reagents (Amersham ECL Advance [GE Healthcare, Piscataway, NJ] or HyGLO [Denville Scientific, Metuchen, NJ]) and exposed to X-ray film (Genesee Scientific, San Diego, CA). Membranes were re-probed with ascites fluid containing mouse monoclonal IgG against β -actin (1:10,000 dilution; Sigma-Aldrich) for loading control. Signals in the linear range of the X-ray film were captured digitally, and densitometry was performed with a gel documentation and analysis system (InGenius analysis system with GeneSnap and GeneTools software; Syngene, Frederick, MD).

RhoA Activation Assay

Active RhoA was measured using a RhoA activation assay kit (Millipore, Billerica, MA). The assay is based on affinity precipitations previously described to pull down GTP-bound Rho.²¹ In brief, cells in a six-well plate at confluence for at least 1 week were serum starved for 2 hours before treatment. After treatment, cells were washed twice with ice-cold PBS, lysed with ice-cold magnesium lysis buffer (MLB), and clarified by centrifugation at 14,000g at 4°C for 5 minutes. Cell lysates were then incubated with 7.5 μg Rho assay reagent slurry (glutathione-agarose with GST-tagged Rhotekin Rho binding domain) for 45 minutes at 4°C. Additional cell lysates were loaded with GDP or GTP γ S before pull-down to serve as negative and positive controls, respectively. After pull-downs, beads were washed three times with MLB. Laemmli sample buffer was then added to the samples and boiled for 5 minutes. GTP-bound RhoA was detected by immunoblot analysis using a mouse monoclonal antibody against RhoA (26C4, 1 $\mu\text{g/mL}$; Santa Cruz Biotechnology, Santa Cruz, CA). A sample from each cell lysate was used to probe for total RhoA.

Rac Activation Assay

A colorimetric assay kit (Rac G-Lisa; Cytoskeleton, Denver, CO) was used to measure Rac (1,2,3) activation. On reaching confluence, cells were serum starved for 2 hours before treatments. After treatments, cells were washed once with ice-cold PBS, lysed, and clarified by centrifugation at 14,000 rpm at 4°C for 2 minutes. An aliquot from each sample was used to measure and normalize protein concentrations between the samples. Fifty microliters of each lysate was added to separate wells of the plate coated with the Rac-GTP binding domain of p21 activated kinase. Additional wells were filled with lysis buffer or nonhydrolyzable Rac to serve as a negative and a positive control, respectively. The plate was placed immediately on an orbital shaker set

at 200 rpm for 30 minutes at 4°C and was then washed three times with wash buffer at room temperature, incubated with 200 μL antigen-presenting buffer for 2 minutes, and washed three more times. Mouse monoclonal IgG against Rac (1:200 dilution) was added to each well and incubated for 45 minutes on the orbital microplate shaker at room temperature. After three more washes, secondary anti-horseradish peroxidase (HRP)-labeled antibody (1:100 dilution) was added to each well and incubated on the orbital microplate shaker for an additional 45 minutes. The plate was washed three times, after which 50 μL HRP detection reagent was added and incubated at 37°C for 5 minutes. HRP stop buffer (50 μL) was added, and the absorbance readings at 490 nm were measured with a plate reader (Flexstation 3; Molecular Devices, Sunnyvale, CA). The mean Abs₄₉₀ values acquired for each treatment were compared with the control and analyzed by a two-tailed, unpaired Student's *t*-test. Differences were considered significant at *P* < 0.05.

Immunofluorescence Microscopy

Cells plated on glass coverslips were maintained at confluence for at least 1 week and then serum starved for 2 hours before treatments. After treatments, cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde for 10 minutes, permeabilized with PBS containing 0.2% Triton X-100 (PBS-T) for 5 minutes, and blocked with 10% goat serum in PBS-T for 30 minutes. After blocking, the cells were incubated for 1 hour at room temperature with rabbit IgG against either phosphospecific (Ser19) myosin light chain (2.5 $\mu\text{g/mL}$; Millipore), β -catenin (1 $\mu\text{g/mL}$; Santa Cruz Biotechnology), or cortactin (1:100 dilution; Cell Signaling Biotechnology). The cells then underwent three washes with PBS, 1-hour incubation with CY3-conjugated goat anti-rabbit IgG (0.75 $\mu\text{g/mL}$; Jackson ImmunoResearch Laboratories), one wash with PBS, counterstain with green nucleic acid stain (SYTOX, 100 nM; Invitrogen) for 1 minute, and three more washes with PBS before visualization. Background and autofluorescence was detected by incu-

FIGURE 1. S1P-induced RhoA activation and MLC phosphorylation in SC cell monolayers. (A) RhoA activation (RhoA-GTP) in SC cells treated with 1 μM S1P for 5 and 30 minutes were compared with control (C). Cell lysates were also probed for total RhoA and used for comparison between the treatment groups. Blots are representative of four different experiments using two SC cell strains. (B) TM and SC cells were treated with 1 μM S1P or left untreated (C) and were probed for phospho-MLC over a time course (1, 3, 5, 30, and 60 minutes) that reflected the S1P effect in organ culture. β -Actin was used as a loading control. The TM blot is representative of seven experiments using three cell strains, whereas the SC blot is representative of eight experiments using three cell strains.

FIGURE 2. Dose-dependent S1P-mediated MLC phosphorylation in both TM and SC cell monolayers. SC and TM cells were treated with a range (10^{-4} to 10^{-8} M) of S1P concentrations for 5 minutes. Graphs represent the percentage of MLC phosphorylation observed (phospho-MLC/ β -actin) compared with the maximum response of each experiment (mean $\pm$ SEM). (A) SC cells displayed an EC_{50} value of $0.83 \mu\text{M}$ ($n = 7$), whereas (B) TM cells displayed a value of $1.33 \mu\text{M}$ ($n = 5$).

bation of CY3-conjugated goat anti-rabbit IgG without previous primary antibody incubations. To detect F-actin, confluent cultures of cells were stained with AlexaFluor 568 phalloidin (Invitrogen) for 20 minutes. Labeled cells were visualized and captured digitally using a confocal microscope (PCM 2000; Nikon, Melville, NY).

RESULTS

To study the effects of S1P on cultured SC cell monolayers, we first investigated the Rho/Rho-kinase/MLC phosphorylation pathway activated by S1P in TM cells.⁶ Stimulation of this pathway alters the contractile state of cells by modifying actomyosin architecture. Using a pull-down assay to detect GTP-bound (active) RhoA, $1 \mu\text{M}$ S1P treatments increased activated RhoA in a time-dependent manner compared with untreated control (Fig. 1A).

Downstream of activated Rho, Rho-kinase phosphorylates and deactivates MLC phosphatase, thus allowing an increase in phospho-MLC species.²² Corresponding to the acute effect of S1P in organ culture,^{6,7} we examined the effects of $1 \mu\text{M}$ S1P on MLC phosphorylation in TM and SC cell monolayers between 1 and 60 minutes. For both TM and SC cells, maximal MLC phosphorylation levels occurred within 5 to 30 minutes (Fig. 1B). No significant differences in β -actin levels were observed between the different time treatments. With similar time-dependent increases in phospho-MLC, we next investigated S1P dose-related MLC phosphorylation in both SC and TM cells over a range of S1P concentrations (10^{-4} to 10^{-8} M) at 5 minutes. We observed that SC (Fig. 2A) and TM (Fig. 2B) cells dose dependently increased phospho-MLC with increas-

ing S1P concentrations. Maximal phospho-MLC levels in both cell types were approached with 10 to $100 \mu\text{M}$ S1P with EC_{50} values of $0.83 \mu\text{M}$ and $1.33 \mu\text{M}$ for SC and TM cells, respectively. Thirty-minute preincubation with the Rho-kinase inhibitor Y-27632 blocked S1P-induced MLC phosphorylation in SC cells similarly to what is observed in TM cells, indicative of S1P activation of the RhoA/Rho-kinase/MLC phosphorylation pathway (Fig. 3). Y-27632 also inhibited MLC phosphorylation by thrombin, a known activator of the same pathway.

On examining these two cell types more closely, we found that TM cells contained a greater abundance of phospho-MLC species compared with SC cells after S1P treatment (Fig. 3). Comparisons were made in experiments in which attention was paid to equal protein loading of SC and TM cell lysates and differences were revealed after analysis of two different time exposures of X-ray film exposed to a single blot (Fig. 3). Similarly, a greater expression level of MLC was observed in TM than in SC cell strains (Fig. 4) in all three cell strains of each cell type tested.

S1P-mediated Rac activation in endothelial cells promotes barrier integrity and decreased paracellular permeability.¹⁸ Given that S1P receptors in endothelia often preferentially use the Rac pathway, we next investigated whether S1P receptors in SC endothelial cells signal through the Rac pathway. In fact, compared with untreated control (0.20 ± 0.04), $1 \mu\text{M}$ S1P treatment for 5 minutes (0.36 ± 0.07) and 30 minutes (0.29 ± 0.03) significantly increased Rac (1,2,3) activation (mean $Abs_{490} \pm$ SD; $P < 0.01$) (Fig. 5) in mature SC monolayers.

With S1P-induced Rho and Rac activation known to affect cytoskeletal organization and endothelial permeability,^{18,23} we next examined the effect of S1P on F-actin arrangement and

FIGURE 3. Rho-kinase dependent MLC phosphorylation following S1P treatment of SC cell monolayers. TM and SC cells were treated with 1 U/mL thrombin (T) or $1 \mu\text{M}$ S1P (S) or were left untreated (C) for 5 minutes. $10 \mu\text{M}$ Y-27632 (Y) preincubated for 30 minutes before thrombin or S1P treatment. Two exposure time points (exposure 1 and exposure 2) of blots on film are displayed to compare phospho-MLC levels between TM and SC, with β -actin used as a loading control. Blots are representative of at least six experiments.

FIGURE 4. MLC expression levels in TM and SC cell monolayers. Whole cell lysates from three TM (86, 90, 93) and three SC (51, 53, 56) cell strains were probed for MLC. Two exposure time points (exposure 1 and exposure 2) of blots on film are displayed to distinguish MLC levels between and within each cell type, with β -actin used as a loading control.

FIGURE 5. S1P-induced Rac activation in SC cell monolayers. Rac (1,2,3) activation in SC cells treated with 1 μ M S1P for 5 and 30 minutes were compared with control. The graph represents the mean $\text{Abs}_{490} \pm \text{SD}$ observed in three different cell strains ($n = 6$; $*P < 0.01$).

phospho-MLC localization. In SC cells, S1P decreased stress fiber polymerization and increased cortical actin assembly compared with control after 5- and 30-minute treatments (Fig. 6A). Similarly, phospho-MLC staining was detected in a cortical arrangement after S1P treatment (Fig. 6B). The cortical staining pattern was similar to what was observed in HUVECs (Supplementary Fig. S1, <http://www.iovs.org/content/51/12/6633/suppl/DC1>). An increase in global phospho-MLC staining in S1P-treated over control cells was consistent with the increase in phospho-MLC observed through immunoblot analysis (Fig. 2A). Interestingly, we did not detect a significant difference in the localization of cortactin, a protein that associates with sites of actin polymerization, between S1P-treated and control cells (not shown).

After the observation of increased cortical actin and phospho-MLC following S1P treatment, we next investigated whether β -catenin is also recruited to the cell periphery. β -Catenin is an adaptor protein associated with adherens junctions and has been shown to increase localization to sites of cell-cell contact in vascular endothelial cells.¹² Indeed, S1P treatment increased β -catenin recruitment to the cell periphery of SC cell monolayers (Fig. 7).

FIGURE 7. S1P increases β -catenin recruitment to SC cell periphery. Monolayers of SC cells were treated with 1 μ M S1P for 30 minutes or were left untreated. Cells were immunolabeled for β -catenin (red) and counterstained to identify nuclei (green). Arrows: β -Catenin at the cell periphery. Inset: secondary antibody staining alone (2°Ab). Results shown are representative of five experiments with three different cell strains.

DISCUSSION

The major findings of this study are that human SC cell monolayers in culture respond to S1P by activating both RhoA and Rac. Stimulation of these small GTPases results in a coordinated increase in MLC phosphorylation, F-actin, and β -catenin at the cell cortex. These results suggest that the endothelial cells lining the inner wall of SC contribute to the S1P effects on outflow facility through actomyosin organization at the cell cortex and enhancement of the cell-cell junction assembly, thereby increasing transendothelial resistance.

We observed that S1P-treated SC cells respond in part through RhoA activation and Rho kinase-dependent MLC phosphorylation. In addition, SC cells increase MLC phosphorylation in a time- and dose-dependent manner similar to that for TM cells. Although S1P activates the RhoA-MLC signaling pathway in both cell types, the greater MLC expression levels and the larger extent of MLC phosphorylation in S1P-treated TM cells indicate that the Rho pathway is likely more dominant in TM than in SC. These data are in agreement with S1P increasing stress fiber formation in TM cells⁶ and TM behavior as a contractile tissue.

Despite the activation of RhoA and the subsequent phosphorylation of MLC in S1P-treated SC cells, the peripheral

FIGURE 6. S1P promotes cortical actin assembly and peripheral localization of phospho-MLC localization in SC monolayers. SC cells were treated with 1 μ M S1P for 5 and 30 minutes or left untreated (control). (A) Cells were stained with AlexaFluor phalloidin 568. Arrows: cortical actin. Arrowheads: stress fiber formations. Results shown are representative of seven experiments with two different cell strains. (B) In parallel experiments, cells were also immunolabeled for phospho-MLC (red) and were counterstained to identify nuclei (green). (Arrows: cortical arrangement of phospho-MLC. Left inset: secondary antibody staining alone (2°Ab). Right inset: enlargement focusing on cortical phospho-MLC staining. Results shown are representative of four experiments with three different cell strains.

localization of phospho-MLC and the concurrent cortical actin formation suggest the strengthening of the cytoskeletal network at the cell cortex. Hence, actomyosin reorganization in S1P-stimulated endothelial cells is linked to an increase in transendothelial electrical resistance and cell barrier integrity,¹⁵ two parameters that are dependent on Rac activation and adherens junction assembly.^{12,18} When SC cells were tested in the present study, S1P robustly increased Rac activity and mobilization of the adherens adaptor protein β -catenin. Taken together, SC cells appear unique in that S1P signals effectively through both Rac and RhoA compared with their fellow endothelia, likely because of the unusual requirement of SC to withstand fluid flow in the basal-to-apical direction (in contrast to the apical-to-basal direction for nearly all other endothelia). Thus for tissue homeostasis, the inner wall of SC must rely not only on cell-cell attachments to maintain a patent monolayer but also on unusually strong cell-matrix attachments through focal adhesions, secured intracellularly by actin stress fibers.

Although S1P effects on the actomyosin architecture in SC and TM may differ, a combination of those responses may be essential for decreasing outflow facility in situ. In TM cells, a Rho-dominant response to S1P leads to elevated MLC phosphorylation with the assembly of stress fibers. In SC cells, a moderate increase in Rho activity and MLC phosphorylation leads to stress fiber assembly. In parallel, an increase in Rac activity leads to cortical actomyosin arrangement (Fig. 8). Thus, simultaneous TM cell contraction and an increase in SC endothelial barrier integrity with a decrease in aqueous humor flow across the inner wall may underlie S1P's dramatic and immediate effect on outflow facility.

Our observed change in the distribution of β -catenin after S1P treatment of cultured SC cells in this study is similar to findings in some, but not all, areas of the inner wall examined in a recent in situ study⁷ by our group. One possible explanation for this inconsistency is segmental flow of aqueous humor through the conventional outflow pathway.²⁴ With segmental flow, only a fraction of the conventional outflow tissues (including the inner wall) encounter flow (and thus S1P), making the interpretation of data obtained from areas of the inner wall, where exposure to segmental flow is unknown, difficult.

With the establishment of S1P's effects in both organ culture and cell-based assays, we can now use subtype-specific

antagonists to identify the receptors responsible for each response. Binding of an antagonist to the S1P receptor subtype responsible for increasing outflow resistance may inhibit endogenous tone, decrease outflow resistance, and thus provide a novel drug target for primary open-angle glaucoma to reduce ocular hypertension. Interestingly, intravenous injection of an S1P₁-specific antagonist in mice increases lung capillary leakage.²⁵ Here, the S1P₁ receptor is linked to maintaining endothelial barrier integrity through mechanisms involving Rac activation, cytoskeletal rearrangement, and junctional organization.¹⁷ Thus, we predict that an S1P₁ antagonist-induced decrease in basal S1P₁ receptor activity in SC cells may decrease outflow resistance, thereby increasing aqueous humor outflow.

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FIGURE 8. Model for differential S1P receptor coupling to Rac/Rho pathways in outflow cells. The Rho pathway is preferentially activated by S1P in TM cells and promotes stress fiber formation and cell contractility, whereas the Rac pathway is preferentially activated in vascular endothelial cells (VEC) and promotes cortical actin formation with an increase in endothelial barrier function. In SC cells, both Rho and Rac are activated by S1P, with a transition from stress fibers to cortical actin formation.

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