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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 μM S1P showed that S1P decreased outflow facility by 36 ± 20% ($n = 10$ pairs; $p = 0.0004$); an effect likely mediated by activation of $S1P_1$ and/or $S1P_3$ 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.

1. 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 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 know 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, $S1P_1$ and $S1P_3$ receptors are preferentially expressed by vascular endothelial cells, whereas smooth muscle cells express $S1P_1$, $S1P_2$ and $S1P_3$ (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 h of infusion of 5 μM 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

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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 $S1P_1$ and $S1P_3$ 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 $S1P_3$ 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 $S1P_1$ and $S1P_3$ receptor subtypes, but activation of these receptors did not result in detectible changes in cortical actin, VE-cadherin, phosphotyrosine or β-catenin distribution/abundance.

2. Materials and methods

2.1. 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.

2.2. 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 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s). Oligonucleotide primers used for subtype-specific amplification to detect the S1P receptors were previously characterized (Mettu et al., 2004): *edg*1 (5′-ACGTCAACTATGATATCATCGTCCG, 3′-CATTTTCAGCATTGTGATATAGCGC); *edg*5 (5′-ACTGTCCTGCCTCTCTACGCC, 3′-GTCTTGAGCAGGGCTAGC GTC); and *edg*3 (5′-ACCATCGTGATCCTCTACGCAC, 3′-CTTGATTTAC TTCTGCTTGGGTCG). 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.

2.3. 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 min, 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 $S1P_1$ (0.1 μg/ml, Affinity Bioreagents), $S1P_2$ (EDG5, 0.2 μg/ml, Santa Cruz), or mouse monoclonal IgGs against $S1P_3$ (EDG3, 0.1 μg/ml, Exalpha Biologicals) receptor subtypes. Following overnight incubation at 4 °C, membranes were washed (3 × 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 × 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 reprobed 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. $S1P_1$-specific bands were determined with the inclusion of EDG1 transfected cell lysates (Exalpha Biologicals).

2.4. Immunofluorescence microscopy of frozen ocular sections

Human cadaveric eyes (donor ages = 79, 88 and 98) were received within 36 h 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 saggitally cryosectioned (8 μm). 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 $S1P_1$ (EDG1, 4 μg/ml, Santa Cruz), $S1P_2$ (EDG5, 4 μg/ml, Santa Cruz), or $S1P_3$ (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 h 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 min, and washed extensively (4 × 10 min) before visualization. Background and auto-fluorescence was monitored by incubating tissues with CY3-conjugated goat anti-rabbit