Pharmacological Manipulation of Conventional Outflow Facility in Ex Vivo Mouse Eyes

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Support: A grant from National Glaucoma Research, a Program of the American Health Assistance Foundation (DRO), the National Eye Institute (EY17007, WDS; EY019696; DRO, WDS), and an unrestricted research gift from Allergan, Inc.

Prior Publications: A portion of this work has been previously published as an ARVO abstract.

Total word count: 5361 (not including abstract, references or figure legends)

Abstract word count: 250 (max 250)

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For Submission to IOVS

Abstract

Purpose: Mouse models are useful for glaucoma research, but it is unclear whether intraocular pressure (IOP) regulation in mice operates through mechanisms similar to those in humans. Our goal was to determine whether pharmacological compounds that affect conventional outflow facility in human eyes exert similar effects in C57BL/6 mice.

Methods: A computerized perfusion system was used to measure conventional outflow facility in enucleated mouse eyes ex vivo. Paired eyes were perfused sequentially, either immediately after enucleation or after 3 hrs storage at 4°C. Three groups of experiments examined: (i) sphingosine 1-phosphate (S1P); (ii) S1P with antagonists to $S1P_1$ and $S1P_2$ receptors; and (iii) the prostanoid EP_4 agonist 3,7-dithia PGE_1. We also examined whether a 24 hrs post-mortem delay affected the response to 3,7-dithia PGE_1.

Results: S1P decreased facility by 39%, and was almost completely blocked by an $S1P_2$, but not $S1P_1$, antagonist. $S1P_2$ antagonist alone increased facility nearly 2-fold. 3,7-dithia PGE_1 increased facility by 106% within 3 hrs post-mortem. By 24 hrs post-mortem, the facility increase caused by 3,7-dithia PGE_1 was reduced 3-fold, yet remained statistically detectable.

Conclusions: C57BL/6 mice show opposing effects of $S1P_2$ and EP_4 receptors on conventional outflow facility, as observed in human eyes. Pharmacological effects on facility are detectable up to 24 hrs post-mortem in enucleated mouse eyes. Mice are suitable models to examine the pharmacology of S1P and EP_4

receptor stimulation on IOP regulation as occurs within the conventional outflow pathway of human eyes, and are promising for studying other aspects of aqueous outflow dynamics.

Introduction

Mice provide important models for glaucoma research, due to their genetic malleability and the extensive catalog of molecular tools that may be exploited to investigate disease mechanisms[1]. While most glaucoma research involving mice has focused on the effect of elevated intraocular pressure (IOP) on the optic nerve, a small but growing community[2-13] has begun using mice to investigate the physiology of aqueous humor outflow, with the aim to better understand the mechanisms of IOP regulation. In fact, recent data show that the morphology and behavior of the murine conventional outflow pathway is more similar in some ways to humans than are non-human primates (e.g., like humans[14], mice do not appear to exhibit 'washout'[11], while 'washout' is observed in monkeys[14]). Notwithstanding the utility of mouse models, it remains an open question whether mice are appropriate models for IOP regulation at the level of the conventional outflow pathway as occurs within human eyes.

Compounds that affect IOP in humans tend to have similar effects in mice, however the response is not always through the same mechanisms, as noted previously[10]. For example, latanoprost lowers IOP[4,10,15-17] and increases conventional outflow facility[4,10] in mice without any detectible effects on unconventional outflow[4,10], unlike the response in human eyes where latanoprost increases both conventional[18] and unconventional outflow[19]. This suggests that the physiology and pharmacology of aqueous humor outflow may differ

substantially between mice and humans, and should be carefully examined before accepting the mouse as a reliable model for human IOP regulation.

The goal of this project was to determine whether pharmacological compounds that are known to affect conventional outflow facility in human eyes exert similar effects on conventional outflow facility in C57BL/6 mice. We specifically examined the facility response to two G-protein coupled receptor agonists, sphingosine 1-phosphate (S1P) and the prostanoid EP_4 agonist 3,7-dithia PGE_1, that respectively decrease[20] and increase[21] outflow facility in human eyes. By comparing the facility response measured in enucleated murine eyes against previous reports in enucleated human eyes[20,21], we aimed to determine whether C57BL/6 mice mimic aspects of human conventional outflow pathway pharmacology, which would identify this strain as a promising animal model for S1P and EP4-based regulation of IOP as occurs within human eyes. We also examined whether the pharmacological response is affected by prolonged post-mortem times, which is an important consideration for using the mouse model as a research tool when doing ex vivo perfusions.

Methods

All experiments were performed using ex vivo tissue and were done in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ex Vivo Mouse Eye Perfusion

C57BL/6 mice of either sex, aged 8-15 weeks, were killed by cervical dislocation. Eyes were enucleated within 10 minutes of death and perfused immediately or stored in phosphate buffered saline (PBS) at 4°C for 2-3 hours. For perfusion, each eye was mounted on a single well of a 96-well Stripwell plate (Corning) using cyanoacrylate glue to affix the extraocular muscles to the plastic sidewalls of a well. Special attention was given to maintain hydration throughout the experiment by covering the eye with tissue paper that was kept moist by regular drops of PBS. The perfusion solution was Dulbecco's PBS including divalent cations and 5.5 mM glucose (referred to as "DBG") filtered through a 0.22 μm filter before use. All perfusions were done at room temperature, with a post hoc correction to account for the viscosity difference between room and physiologic temperature[11,22].

Our perfusion method follows previously described techniques[11]. Briefly, a 33-gauge needle was used to cannulate the anterior chamber under a stereomicroscope using a micromanipulator. The needle was connected via rigid pressure tubing to a glass syringe (25 μL, Hamilton GasTight) placed on the rack of a motorized syringe pump (Pump33; Harvard Apparatus) under computer

control. A pressure transducer (142PC01G, Honeywell) monitored IOP through a three-way connector placed in the perfusion line. Custom written LabVIEW software[23] was used to automatically vary the flow rate from the syringe pump to maintain the eye at a user-defined IOP. Eyes were perfused at sequential pressure steps of 4, 8, 15, and 25 mmHg; we refer to this as our "standard perfusion regimen." Eyes were typically perfused for 20 minutes at each pressure step to obtain at least 10 minutes of stable perfusion data, and an average stable flow rate was calculated at each pressure step (Fig. 1). Data were considered acceptable if a stable flow rate was achieved in at least 3 of the 4 pressure steps. Based on this criterion, we rejected 12 eyes out of 88 valid perfusions. Selected eyes were fixed by removing the perfusion needle and immediately immersing the eye in 4% paraformaldehyde (PFA) in isotonic saline for 1 hour, followed by long-term storage in 0.1% PFA. For histology, eyes were processed for paraffin embedding, sectioned and stained using hematoxylin and eosin.

Outflow Facility Analysis

We calculated a pressure-dependent or "conventional outflow facility" (*C*) by fitting our pressure-flow rate data to the modified Goldmann equation[24]:

$$F = C\left(IOP\right) + F_u \qquad \text{(Eq. 1)}$$

where *F* represents the stable flow rate at each corresponding IOP. F_u in Equation 1 is usually taken as an estimate of the pressure-independent or "unconventional" outflow rate[11]. Equation 1 is valid only when (i) episcleral venous pressure is zero (appropriate for enucleated eyes)*;* (ii) *F* reaches

equilibrium at each value of IOP; and (iii) C and F_u are independent of IOP. The values of C and F_u are defined as the slope and intercept, respectively, of the best-fit linear regression to our measured F versus IOP data (Fig. 1B). In principle, C would be consistent with values obtained from a 2-level perfusion[3,4,6,7,25], except that the additional pressure steps give a much stronger confidence for estimating C[8,10]. Both C and F_u were multiplied by a factor of 1.38 to account for viscosity differences between physiologic and room temperatures, as previously described[11,22].

Experimental Design

We conducted three sets of perfusion experiments to measure how conventional outflow facility in the mouse eye responded to receptor-mediated compounds known to affect conventional outflow in human eyes. Experiments used paired eyes (treated vs. untreated contralateral controls), except for cases where data from one eye were rejected based on the stability criterion described above. Paired eyes were perfused sequentially (one eye immediately after enucleation, the contralateral eye 2-3 hrs after enucleation), where we randomised whether the control or experimental eye was perfused first. We also examined whether prolonged post-mortem time (24 hrs storage after enucleation at 4°C) affected the pharmacologic response between paired eyes.

In the first set of experiments (Group A), we examined the effect of sphingosine 1-phosphate (S1P), a bioactive lipid that decreases outflow facility by 31% in porcine eyes[26] and 36% in human eyes[20]. Experimental eyes were

perfused with 5 μM S1P in DBG containing fatty acid free bovine serum albumin (FAF-BSA; 2 mg/mL; Sigma-Aldrich A8806) and 17 μM sodium hydroxide (NaOH), while control eyes received DBG and FAF-BSA alone without S1P or NaOH. Independent studies demonstrated that 17 μM NaOH had a negligible effect on the pH of DBG (7.137±0.031 vs. 7.107±0.012 for Dulbecco's PBS with or without 17 μM NaOH, respectively, N = 3 independent trials each, p = 0.23; Student's t-test), and therefore NaOH was not included in the control solution. S1P (CAS 26993-30-6 from Sigma-Aldrich, UK) was dissolved from powder into 10 mM NaOH in water to give a 3 mM S1P stock solution that was stored at -20°C. Prior to cannulation, each needle was backfilled from the tip with 150 μL of the appropriate perfusion solution, a volume sufficient to last several hours even at the highest measured flow rates (~0.3 μL/min). The experimental eye was pre-treated with S1P-containing solution from a reservoir at 8 mmHg for 45 minutes to expose the outflow pathway to the drug prior to the start of the standard perfusion regimen. Control eyes were perfused from a reservoir for the same time with solution without S1P. Data from 14 individual eyes (8 S1P-treated and 6 controls, containing 6 pairs) passed the stability criterion and were included in Group A.

The aim of the second set of experiments (Group B) was to investigate the role of $S1P_1$ and $S1P_2$ receptors in mediating the S1P response. Following a previous study in human eyes[27], we used W146 (Avanti Polar Lipids) or JTE-013 (Cayman Chemical) that are selective antagonists to $S1P_1$ or $S1P_2$ receptors, respectively. W146 was dissolved in water as a 1 mM stock solution containing

10.6 mM cyclodextrin and 100 mM sodium carbonate (Na_2CO_3) as vehicle, and was stored at -20°C. JTE-013 was dissolved in DMSO as a 1 mM stock solution and stored at -20°C. Experimental eyes were pre-treated with antagonist and S1P-containing solution (45 minutes at 8 mmHg from a reservoir) followed by perfusion with the same solution over the standard perfusion regimen. For W146-treated experimental eyes, the perfusion solution contained 5 μM W146 in DBG + 5 μM S1P + 2 mg/mL FAF-BSA + 17 μM NaOH + 53 μM cyclodextrin + 500 μM Na_2CO_3. For JTE-treated experimental eyes, the perfusion solution contained 5 μM JTE-013 in DBG + 5 μM S1P + 2 mg/mL FAF-BSA + 17 μM NaOH + 70 mM DMSO. Antagonist concentrations (5 μM) were chosen to be consistent with concentrations used in prior perfusion studies with porcine and human eyes[27] and were several fold larger than reported IC_{50} values (0.83 μM for W146[28] and 1.0 μM for JTE-013[27]). Control eyes were perfused with 5 μM S1P in DBG + 2 mg/mL FAF-BSA + 17 μM NaOH, without antagonist, cyclodextrin, Na_2CO_3 or DMSO vehicle and without pre-treatment. Data from 8 eyes were included in the JTE study (4 JTE-treated and 4 controls, containing 4 pairs), and data from 7 eyes were included in the W146 study (3 W146-treated and 4 controls, containing 3 pairs).

To account for possible vehicle effects caused by cyclodextrin, Na_2CO_3 or DMSO, we repeated the W146 and JTE-013 antagonist studies using the same vehicle formulations in the control eyes. For these studies, the control eyes from the W146 study received 5 μM S1P in DBG + 2 mg/mL FAF-BSA + 17 μM NaOH + 53 μM cyclodextrin + 500 μM Na_2CO_3, while the experimental eyes from the

W146 study received the same solution with 5 μM W146. The control eyes from the JTE study received 5 μM S1P in DBG + 2 mg/mL FAF-BSA + 17 μM NaOH, + 70 mM DMSO, while the experimental eyes from the JTE study received the same solution with 5 μM JTE-013. Both control and experimental eyes were pretreated with perfusion solution for 45 minutes at 8 mmHg from a reservoir prior to starting the standard perfusion regimen. Data from 7 eyes were included in the JTE vehicle-controlled study (3 JTE-treated and 4 controls, containing 3 pairs), and data from 7 eyes were included in the W146 vehicle-controlled study (4 W146-treated and 3 controls, containing 3 pairs).

In an additional 10 eyes (3 pairs and 4 unpaired eyes), we examined the influence of JTE antagonist alone on outflow facility. For these studies, experimental eyes were perfused with 5 μM JTE-013 + 70 mM DMSO in DBG without FAF-BSA (*N* = 5 eyes), while the control eyes were perfused with DBG alone without JTE, DMSO, or FAF-BSA (*N* = 5) using the standard perfusion regimen. Because 70 mM DMSO was found not to affect the facility response in JTE-treated eyes in the presence of S1P (see below), it was not included in the perfusion solution for the control eyes.

In the third set of experiments (Group C), we examined the influence of prostaglandin EP_4 receptor activation on conventional outflow in the mouse eye by perfusion with 3,7-dithia PGE_1, a highly selective PG-EP_4 receptor agonist[29] that increases conventional outflow facility in human[21] and monkey eyes[30] without affecting unconventional outflow. 3,7-dithia PGE_1 was dissolved in ethanol as a 10 mM stock solution and stored at -20°C. Experimental eyes were

pre-treated (45 min at 8 mmHg from a reservoir) and perfused with 10 nM 3,7-dithia PGE_1 in DBG + 17 μM ethanol without FAF-BSA. Control eyes were perfused with DBG alone without ethanol or pre-treatment. Ethanol was not included in the perfusion solution of the control eyes because 17 μM ethanol is approximately 1000-fold smaller than typical millimolar concentrations shown to have minimal effects on cultured cells[31,32]. Data examining the effects of 3,7-dithia PGE_1 included 13 eyes (7 treated and 6 untreated control eyes, containing 4 pairs).

In an additional 10 eyes (including 4 pairs and 2 unpaired eyes), we examined whether the response to 3,7-dithia PGE_1 was affected by post-mortem time. For these studies, eyes were enucleated and stored for 24 hrs at 4°C in Dulbecco's modified Eagle's medium (DMEM) and then perfused with 10 nM 3,7-dithia PGE_1 in DBG + 17 μM ethanol (N = 6) or with DBG + 17 μM ethanol (N = 4) using the standard perfusion regimen. We chose to examine the post-mortem facility response to 3,7-dithia PGE_1, rather than to S1P, for two reasons. First, 3,7-dithia PGE_1 causes a larger change in outflow facility compared to S1P (e.g., 69% increase following 3,7-dithia PGE_1[21] versus 36% decrease following S1P[20] in human eyes, respectively), and therefore 3,7-dithia PGE_1 would provide a more conservative test to detect smaller differences in facility that might occur with prolonged post-mortem times. Second, 3,7-dithia PGE_1 is an exogenous compound (unlike S1P) and is therefore more representative of potential candidate drugs that may affect conventional outflow. Therefore, by looking at how the facility response to 3,7-dithia PGE_1 changes with post-mortem time, we

can gain some insight into how post-mortem time may affect the interpretation of drug efficacy in perfusion experiments (that often incorporate eyes with post-mortem times up to 24 hours or use transgenic models where eyes are shipped overnight between laboratories).

Statistical Methods

All experiments included in this study contained some portion of unpaired eyes caused by one eye of a pair failing to pass the stability criterion. To analyze our data, we performed two statistical analyses: (i) a Welch's t-test that included the full set of eyes (accounting for unequal sample sizes); and (ii) a paired, 2-tailed Student's t-test that included only the subset of paired contralateral eyes. Wherever appropriate, we indicate whether a paired Student's t-test or a Welch's t-test was performed. The statistical significance threshold was taken to be a *p*-value of 0.05.

All facility values quoted in the text were temperature-corrected to account for viscosity differences, as described above. Figures showing flow rate data, however, were not corrected and represent the true flow rate output from the syringe pump.

Results

Group A, S1P Decreases Conventional Outflow Facility in Mice

Perfusion with 5 μM S1P caused a reduction in flow rate at each perfusion pressure (Fig. 1A). Compiling data from all eyes revealed a linear relationship between flow rate and IOP (Fig. 1B), consistent with the modified Goldmann equation (Eq. 1). In response to S1P, conventional outflow facility (*C*; slope of the linear regression) decreased by 38.9 ± 24.2% (mean ± SD; $p = 0.029$, paired Student's t-test, $N = 6$ pairs) compared to paired contralateral eyes perfused without S1P. After temperature correction, conventional outflow facility was 0.0125 ± 0.0037 and 0.0073 ± 0.0035 μL/min/mmHg for control and S1P-treated eyes ($p = 0.024$, Welch's t-test, $N = 8$ S1P-treated eyes and 6 controls), respectively. We observed no statistical difference in the intercept of the linear regression in response to S1P in either paired ($p = 0.71$) or unpaired analyses ($p = 0.56$, Welch's t-test). We observed no obvious differences in the morphology of the trabecular meshwork or Schlemm's canal following treatment with S1P (Fig. 2).

Group B, The $S1P_2$ Receptor Mediates the S1P Response

To determine which receptor mediates the S1P response measured in Group A, we perfused contralateral eyes with either S1P or S1P in combination with either an antagonist to the $S1P_2$ receptor (5 μM JTE-013; Fig. 3A) or an antagonist to the $S1P_1$ receptor (5 μM W146; Fig. 3B). In eyes treated with S1P without antagonist, the conventional outflow facility was 0.0074 ± 0.0034 μL/min/mmHg ($N = 15$ control eyes from the JTE and W146 experiments;

temperature-corrected), which was similar to the conventional facility measured for S1P-treated eyes in Group A. There was no difference in conventional outflow facility caused by vehicle, either for 70 mM DMSO (0.0072 ± 0.0052 vs. 0.0068 ± 0.0017 µL/min/mmHg in S1P-treated control eyes from the JTE studies with or without DMSO, respectively; temperature-corrected; p = 0.90, Welch's t-test, N = 4 for each group) or for 53 µM cyclodextrin and 500 µM Na_2CO_3 (0.0078 ± 0.0040 vs. 0.0079 ± 0.0035 µL/min/mmHg in S1P-treated control eyes from the W146 study with or without cyclodextrin + Na_2CO_3, respectively; temperature-corrected; p = 0.97, Welch's t-test, N = 3 or 4, respectively). For this reason, we compiled all data with and without vehicle control from the W146 or JTE perfusions with S1P. The compiled data shown in Figures 3A and 3B include 15 eyes for the JTE antagonist studies (8 controls + 7 experimentals, including 7 pairs) and 14 eyes for the W146 antagonist studies (7 controls + 7 experimentals, including 6 pairs).

In eyes perfused with both S1P and JTE, the temperature-corrected conventional facility was 0.0133 ± 0.0019 µL/min/mmHg, nearly 2-fold larger than eyes treated with S1P without antagonist (0.0070 ± 0.0036 µL/min/mmHg; p = 0.0012, Welch's t-test , N = 8 S1P vs. 7 S1P + JTE,) and similar to the conventional facility of control eyes from Group A. In contrast, conventional facility was unchanged between eyes perfused with S1P or S1P and W146 (0.0079 ± 0.0034 vs. 0.0094 ± 0.0032 µL/min/mmHg; temperature-corrected; p = 0.41, Welch's t-test, N = 7 S1P vs. 7 S1P + W146; β = 0.145, α = 0.05, assuming the facility values for S1P-treated and untreated eyes from Group A with N = 7 for

each group). These data demonstrate that JTE largely blocks the facility-reducing effect of S1P, while W146 has little effect, suggesting that the $S1P_2$ receptor, and not the $S1P_1$ receptor, is principally responsible for mediating the S1P response in C57BL/6 mice. We did not observe significant effects of JTE or W146 in the presence of S1P on the intercept of the pressure-flow relationship ($p \geq 0.27$).

In eyes perfused with 5 μM JTE without S1P, temperature-corrected conventional facility was nearly 2-fold greater than in untreated control eyes (Fig. 3C), increasing from 0.0096 ± 0.0026 to 0.0194 ± 0.0056 μL/min/mmHg ($p = 0.017$; $N = 5$ control and $N = 5$ JTE-treated eyes, Welch's t-test). There was no significant difference in the intercept of the pressure-flow relationship between JTE-treated and untreated eyes ($p = 0.19$). These data suggest that endogenous S1P signaling may be regulating conventional outflow facility in the mouse trabecular meshwork, which can be blocked by $S1P_2$ receptor antagonist JTE-013.

Group C, EP_4 Receptor Agonist Increases Conventional Outflow Facility in Mice

We measured a two-fold increase in conventional outflow facility following perfusion with 10 nM 3,7-dithia PGE_1 (Fig. 4A), with the temperature-corrected facility increasing from 0.0062 ± 0.0005 to 0.0131 ± 0.0024 μL/min/mmHg ($p = 0.0003$; Welch's t-test; $N = 6$ or 7 for untreated eyes or eyes treated with 3,7-dithia PGE_1, respectively). Considering only paired eyes, the conventional facility increased by 105.8 ± 48.4% following 3,7-dithia PGE_1 treatment compared to untreated contralateral eyes ($p = 0.02$; paired Student's t-test, $N = 4$ pairs). In

contrast, 3,7-dithia PGE_1 inconsistently affected the intercept of the pressure-flow relationship. More specifically, while we observed a statistically detectable decrease in the intercept between treated and untreated groups using unpaired analysis ($p = 0.04$), we observed no difference using paired analysis ($p = 0.31$).

After 24 hrs post-enucleation and storage at 4°C in DMEM, eyes appeared well preserved with clear corneas and constricted pupils. In contrast, eyes stored for the same period in PBS at 4°C appeared “mushy” with cloudy corneas and dilated pupils. Following 24 hr storage in DMEM, conventional outflow facility in eyes perfused with 3,7-dithia PGE_1 was 0.0128 ± 0.0034 µL/min/mmHg ($N = 6$ eyes; Fig. 4B), which was significantly larger ($p = 0.037$, Welch’s t-test) than the baseline facility of untreated eyes (0.0087 ± 0.0014 µL/min/mmHg, $N = 4$ eyes) by unpaired analysis. When considering only paired eyes, the relative facility increase following 3,7-dithia PGE_1 was nearly 3-fold smaller after 24 hrs (38.1 ± 34.5%; $N = 4$ pairs) versus after 3 hrs (see above) and failed to achieve statistical significance ($p = 0.126$, paired Student’s t-test). Similarly, after 24 hrs for pressures of 8 mmHg and above, there was a tendency for 3,7-dithia PGE_1 to increase the flow rate, but even at 25 mmHg the flow rate increase failed to achieve statistical significance ($p = 0.10$). This is in contrast to eyes perfused within 3 hrs post-mortem, when 3,7-dithia PGE_1 caused a statistically significant increase in flow rate for all pressures of 8 mmHg or larger ($p < 0.05$). This suggests that while the facility-increasing effect of 3,7-dithia PGE_1 was present 24 hrs after enucleation, the effect was subtle and detectable only when flow

rates were measured over several perfusion pressures and linear regression analysis was used to calculate the slope of the flow rate-pressure relationship.

Discussion

This study demonstrates that C57BL/6 mouse eyes respond to S1P and EP_4 agonist in a similar manner as that previously reported for human eyes. Specifically, S1P decreased murine outflow facility by 39%, which was nearly identical to the 36% decrease previously reported in human eyes[20]. The prostanoid EP_4 agonist, 3,7-dithia PGE_1, caused a facility increase of 106% in mice, which was somewhat larger than the 69% increase observed in human eyes[21]. Quantitative differences aside, the similarity in qualitative response between species suggests that similar pharmacological signaling mechanisms underlie the facility response to S1P and 3,7-dithia PGE_1 between C57BL/6 mice and human eyes.

S1P is known to bind to one of five G protein-coupled receptors ($S1P_{1-5}$) each of which exhibit different downstream signaling events and are differentially regulated in different tissues[33]. S1P rapidly decreases outflow facility[20,26], and lysophospholipids similar to S1P are found in aqueous humor[34], possibly acting as endogenous regulators of outflow facility[35]. In C57BL/6 mice, we detected expression of $S1P_1$ and negligible levels of $S1P_2$ and $S1P_3$ in Schlemm's canal endothelium by in situ confocal immunofluorescence (Supplemental Figure 1). The relative absence of $S1P_2$ and $S1P_3$ labeling could be attributed to reduced antibody sensitivity compared to prior studies by our group[20] that reported relatively low levels of $S1P_2$ and $S1P_3$ expression, compared to $S1P_1$, by human

Schlemm's canal cells in situ. Despite the seemingly low levels of $S1P_2$ expression, the facility-decreasing effect of S1P in mice was almost completely abolished by JTE-013, an antagonist to the $S1P_2$ receptor, but not by W146, an antagonist to the $S1P_1$ receptor. Similar effects were observed in human eyes[27], where JTE-013 blocked facility reduction as well as phosphorylated myosin light chain (pMLC) in response to S1P, while no blocking effect on pMLC was observed in the presence of either W146 or VPC23019 (a dual antagonist to $S1P_1$ and $S1P_3$). Perfusion with JTE-013 alone increased conventional outflow by two-fold in C57BL/6 mice, yielding a conventional outflow facility (0.0194 ± 0.0056 µL/min/mmHg, N = 5) that was larger than that measured in either untreated eyes (0.0125 ± 0.0037 µL/min/mmHg, N = 6, from Group A; p = 0.056, Welch's t-test) or eyes perfused with 3,7-dithia PGE_1 (0.0131 ± 0.0024 µL/min/mmHg, N = 7, from Group C; p = 0.063, Welch's t test). These data strongly implicate the $S1P_2$ receptor as a key mediator of the facility-regulating effect of S1P and suggest an endogenous concentration of S1P within the trabecular meshwork. It should be noted however, that the selectivity of JTE-013 to the $S1P_2$ receptor has been recently called into question[36,37] based on data reporting an effect of JTE-013 in $S1P_2$ knock-out mice[38]. Therefore, future studies should account for potential off-target effects, possibly by incorporating genetically altered mice to better understand the underlying mechanisms by which S1P regulates outflow facility.

3,7-dithia PGE_1 was the first selective agonist developed against the EP_4 receptor[29] and has been shown to lower IOP by nearly 40% in cynomolgus

monkeys by increasing trabecular outflow facility without affecting uveoscleral outflow[30]. Similarly, 3,7-dithia PGE_1 increases conventional outflow facility in post-mortem human eyes by 69%[21], consistent with the expression of PG-EP_4 in human trabecular meshwork and Schlemm's canal in situ[21,39]. At 10 nM concentration, however, the effect of 3,7-dithia PGE_1 appears to be selective for Schlemm's canal cells, with no observed effect on trabecular meshwork cells, based on cell culture assays of cAMP accumulation following PG-EP_4 receptor activation[21]. Compared to humans, our data show that 3,7-dithia PGE_1 had a nearly two-fold larger effect in C57BL/6 mice (106% vs. 69%[21]), and expression of PG-EP_4 has already been demonstrated within the trabecular meshwork and Schlemm's canal in other strains of mice[40]. The larger facility increase in mice may reflect differences in EP_4 sensitivity between species or with age, or it may be due to improved preservation of mouse tissue due to shorter post-mortem times. Alternatively, because mice have a more prominent Schlemm's canal with only 2-4 trabecular beams compared to 12-20 in humans[41], mice may be predisposed to exhibit a more robust facility response to compounds such as 3,7-dithia PGE_1 that preferentially affect Schlemm's canal (see above), as opposed to trabecular meshwork, cells[21]. Regardless, the robust facility-increasing response following 3,7-dithia PGE_1 strongly suggests that C57BL/6 mice are a good pharmacological model for investigating the contribution of EP_4, and possibly other prostanoid receptors, in the regulation of conventional outflow. This may be particularly interesting given that prostaglandin PGE_2, a natural ligand for EP_4 receptors[42], is present within aqueous humor at reduced

concentrations in eyes from patients with primary open-angle and steroid-induced glaucoma[43].

Post hoc comparison of facility data revealed that conventional outflow facility was, rather surprisingly, nearly 60% larger in eyes perfused with 0.2% FAF-BSA in DBG compared to eyes perfused with DBG alone. More specifically, there was a statistical difference (p = 0.028, Welsh's t-test) between eyes perfused with 0.2% FAF-BSA from Group A (0.0125 ± 0.0037 µL/min/mmHg, N = 6) compared to eyes perfused without FAF-BSA aggregated from Groups B and C (0.0078 ± 0.0025 µL/min/mmHg, N = 11). FAF-BSA was included as a carrier for S1P in the perfusion medium, following prior studies in human eyes[20], and therefore FAF-BSA was excluded from experiments that did not contain S1P (i.e., all experiments from Group C and experiments from Group B examining JTE alone). We are not certain as to the cause of this difference, nor were there any obvious differences in the gender, age or genetic background of the mice that could explain this difference. Because elevated FAF-BSA would be expected to decrease (due to possible obstruction), rather than increase, outflow facility[44], these data may indicate a potential trace contaminant carried from the FAF-BSA source (e.g., lipoprotein, phospholipid or lipopolysaccharide) that may itself increase facility. We observed that the effect of FAF-BSA on facility persisted through two different batches from the supplier (lot numbers 040M7715V and 108k7425), suggesting a potential widespread, rather than batch-dependent, contaminant. Nevertheless, because our experiments used a paired perfusion approach, in which we compared the relative effects of selective receptor

agonists or antagonists diluted in otherwise identical perfusion solutions (both control and experimental), we conclude that the facility effects we observed were in fact due to drug treatment, and not to the presence or absence of FAF-BSA in the perfusion media.

Data from the current study suggest that the fraction of unconventional outflow in mice may be significantly smaller than previously estimated[3,4,6,11]. We calculate the unconventional fraction of total outflow as

$$\frac{F_U}{C(IOP)+F_U}$$

Using the regression parameters from control eyes from Group C (perfused within 3 hrs post-enucleation without FAF-BSA; Fig. 4A) yields a relative contribution of 37.9 ± 15.6% unconventional to total outflow at 8 mmHg. In contrast, our previous work estimated that unconventional outflow represents 66% of total outflow in C57BL/6 mice[11]. This difference is largely attributable to the 5-fold difference in F_U between our current study (0.035 ± 0.025 µL/min; temperature-corrected; control eyes of Group C at 3 hrs) and our previous study (0.157 ± 0.026 µL/min)[11], with a more modest difference observed in conventional outflow facility (0.0062 ± 0.0005 vs. 0.0091 ± 0.0012 µL/min/mmHg[11]). We do not understand the reasons for such a large discrepancy in F_U, but it may be related to differences in experimental techniques, and in particular the hydration of the eye, between the two studies. In the current study, the eye was covered with tissue paper that was kept moist by

regular drops of saline, while our previous study[11] used regular drops of saline without tissue paper. It is thus possible that evaporation from the eye contributed to overestimation of F_U in our previous study, and ongoing experiments are closely examining this hypothesis. Along these lines, a recent study[10] has reported that the fraction of unconventional outflow in BALB/cJ mice (20.5%) is more consistent with the lower range of unconventional outflow estimated in human eyes (4 – 14%[45]), while unconventional outflow may be larger in other strains of mice (e.g., ~80% in NIH Swiss White mice[3,4]) and more consistent with the upper range of unconventional outflow estimated in human eyes (46-54%)[46]. This suggests that particular strains of mice (e.g., C57BL/6 or other strains that exhibit similar pharmacological behavior) may serve as better models for the physiology or pharmacology of IOP regulation as occurs within the conventional outflow pathway of human eyes.

The conventional outflow pathway is sensitive to post-mortem degradation[47], and enucleated whole human globes are routinely accepted for perfusion studies up to 24 hrs or longer after death. After 24 hrs post-enucleation and storage in DMEM at 4°C, the facility-increasing effect of 3,7-dithia PGE_1 remained statistically detectable in C57BL/6 mouse eyes, but only in the larger data set (unpaired set with 10 eyes) when conventional facility was measured as the slope of the flow rate-pressure relationship. When considering only perfusion data at individual pressures, the flow rate increase caused by 3,7-dithia PGE_1 failed to achieve statistical significance at 24 hrs ($p \geq 0.10$). This suggests that a 24 hr post-mortem delay likely represents an upper limit for detection of

pharmacological effects in ex vivo mouse eyes. More to this point, post-hoc analysis of conventional facility in DBG-perfused mouse eyes after 24 hrs (0.0087 µL/min/mmHg, temperature-corrected, from Group C) was approximately 40% greater ($p = 0.043$; Welch's t-test) than the facility measured in eyes within 3 hrs after enucleation (0.0062 µL/min/mmHg, temperature-corrected, from Group C). This change in baseline facility explains why the relative facility increase following 3,7-dithia PGE_1 after 24 hrs (38%) was nearly 3-fold less than that measured within 3 hrs (106%). Taken together, these data demonstrate that post-mortem changes occurring within 24 hrs can affect both conventional facility and the relative facility response to pharmacological compounds. We are not aware of any studies that have examined outflow facility as a function of post-mortem time in human eyes, but if the post-mortem response of human and mouse eyes are similar, then these data suggest that there may be a considerable loss of sensitivity for detecting pharmacologically-induced changes in outflow facility using human eyes even at 24 hrs post-mortem.

In conclusion, we demonstrate that conventional outflow facility in C57BL/6 mice mimics the pharmacological response of human eyes to PG-EP_4 and S1P receptor agonists that respectively increase and decrease outflow facility. These data strongly support the mouse eye (and possibly C57BL/6 or other strains) as a promising and robust model for the pharmacology of PG-EP_4 and S1P receptor activity on IOP regulation as occurs within the conventional outflow tract of human eyes, as well as for investigating the basic mechanisms of outflow resistance generation as relevant for glaucoma.

Acknowledgements

Dr. Grant Sumida (University of Arizona) provided assistance for the preparation of S1P solution. Dr. Christina Abbot and Ms. Kyasha Sri Ranjan (Imperial College London) provided assistance in the preparation of S1P antagonist solutions. Ms. Lorraine Lawrence (Imperial College London) and Ms. Kristin Perkumas (University of Arizona) provided histology support. The donors of National Glaucoma Research, a Program of the American Health Assistance Foundation, provided funding for this work. Alexandra Boussommier-Calleja is a recipient of an Entente Cordiale studentship managed by the British Council.

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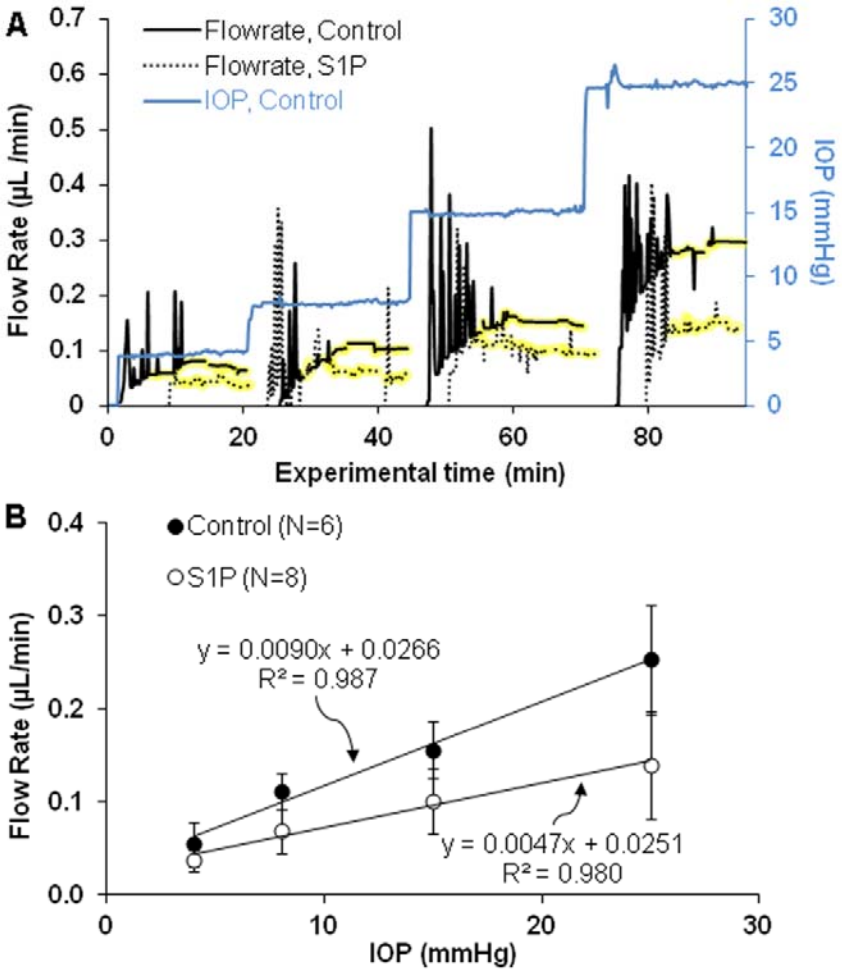
Figure Legends

Figure 1: Data from Group A. Panel A) Typical perfusion tracings showing IOP (blue) and flow rate data for a control (solid black) and an S1P treated (dashed black) eye as a function of time (data taken from unpaired eyes). Yellow highlighted regions represent data used to calculate the average flow rate at each pressure level (4, 8, 15, 25, 8 mmHg). To enable flow traces from both control and experimental eyes to be seen on the same graph, flow rate tracings for the S1P treated eye were shifted by several minutes, and the pressure curve for the S1P-treated eye was omitted. Panel B) the average flow rate at each pressure level for all control (filled circles) and S1P treated eyes (open circles) from Group A. Bars are S.D. and lines represent the best-fit linear regressions to average data. FAF-BSA was included in the perfusion medium for all eyes represented in Panel B. Flow rate data are not temperature corrected.

Figure 2: No obvious differences are observed in the histology of the iridocorneal angle from control (A) and S1P-treated (B) mouse eyes. AC, anterior chamber; TM, trabecular meshwork; SC, Schlemm's canal; I, iris; C, cornea; S, sclera. Bars are 50 μm. The absence of giant vacuoles in the inner wall of Schlemm's canal may reflect the fact that the eyes were fixed by immersion.

Figure 3: Data from Group B. Panel A) Average flow rate at each pressure level for S1P (open circles) and S1P+JTE-013-treated eyes (filled circles). Controls for Panels A and B include eyes with and without vehicle formulations, as described in the text. Panel B) Average flow rate at each pressure level for S1P (open circles) and S1P+W146-treated eyes (filled squares). Panel C) Average flow rate at each pressure level for control (open diamonds) and JTE-treated eyes without S1P (filled circles). Bars are S.D. and lines represent the best-fit linear regression to average data. FAF-BSA was included in the perfusion medium for all eyes represented in Panels A and B, but no FAF-BSA was included in the perfusion medium for Panel C. Flow rate data are not temperature corrected.

Figure 4: Data from Group C. Average flow rate at each pressure level for control (filled circles) and 3,7-dithia PGE_1 (open squares) treated eyes, within 3 hrs (Panel A) or after 24 hrs post-mortem storage at 4°C (Panel B). Bars are S.D. and lines represent the best-fit linear regressions to average data. For Panels A and B, there was no FAF-BSA in the perfusion medium. Flow rate data are not temperature corrected.



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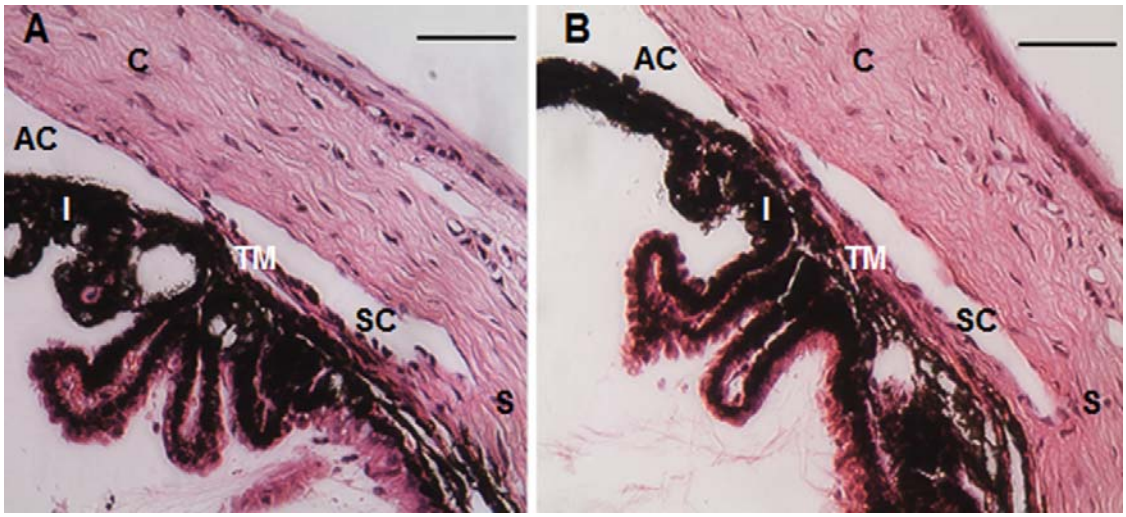

Figure 2: No obvious differences are observed in the histology of the iridocorneal angle from control (A) and S1P-treated (B) mouse eyes. AC, anterior chamber; TM, trabecular meshwork; SC, Schlemm's canal; I, iris; C, cornea; S, sclera. Bars are 50 μm. The absence of giant vacuoles in the inner wall of Schlemm's canal may reflect the fact that the eyes were fixed by immersion.

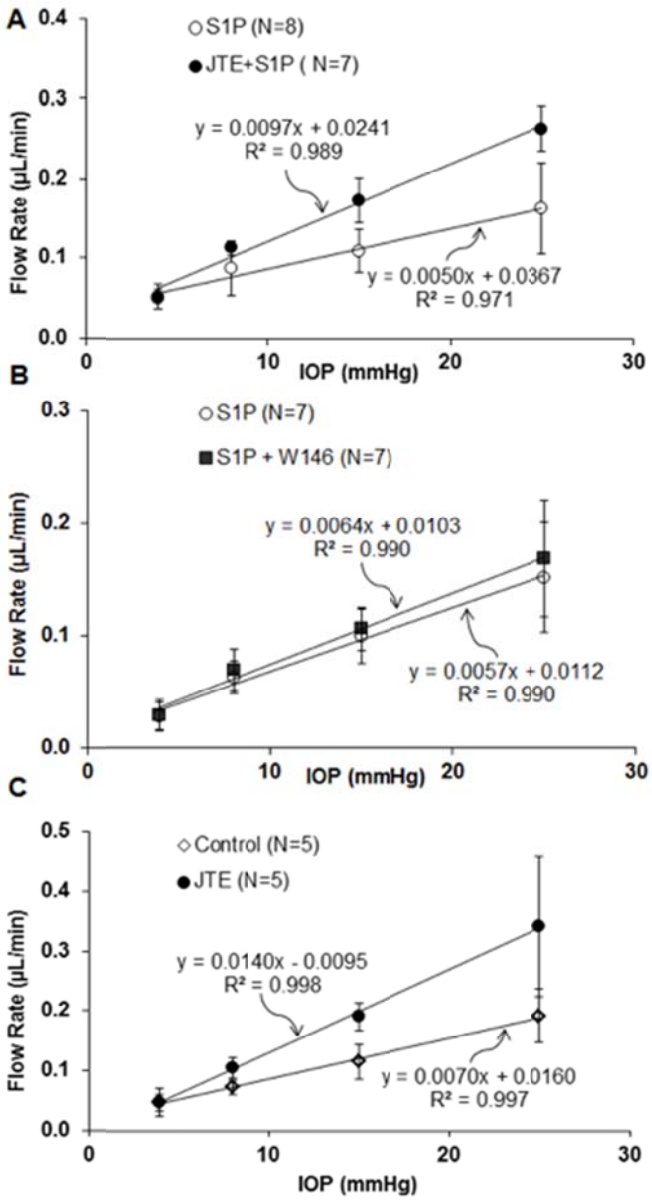

Figure 3: Data from Group B. Panel A) Average flow rate at each pressure level for S1P (open circles) and S1P+JTE-013-treated eyes (filled circles). Controls for Panels A and B include eyes with and without vehicle formulations, as described in the text. Panel B) Average flow rate at each pressure level for S1P (open circles) and S1P+W146-treated eyes (filled squares). Panel C) Average flow rate at each pressure level for control (open diamonds) and JTE-treated eyes without S1P (filled circles). Bars are S.D. and lines represent the best-fit linear regression to average data. FAF-BSA was included in the perfusion medium for all eyes represented in Panels A and B, but no FAF-BSA was included in the perfusion medium for Panel C. Flow rate data are not temperature corrected.

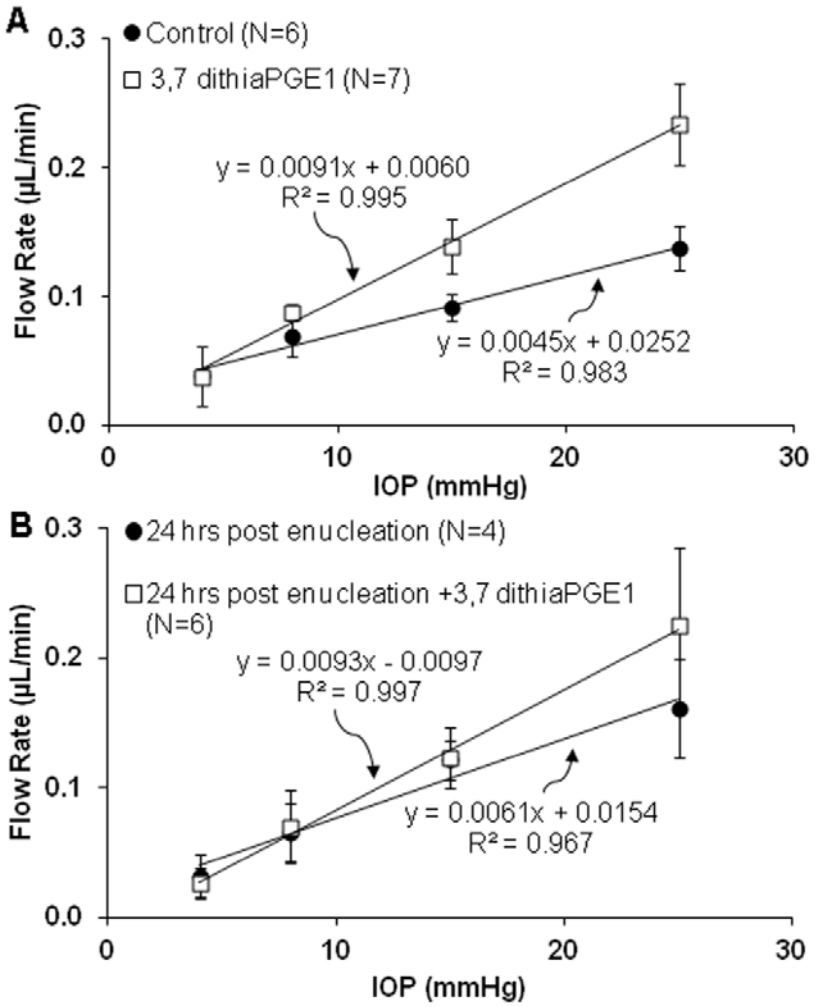

Figure 4: Data from Group C. Average flow rate at each pressure level for control (filled circles) and 3,7-dithia PGE_1 (open squares) treated eyes, within 3 hrs (Panel A) or after 24 hrs post-mortem storage at 4°C (Panel B). Bars are S.D. and lines represent the best-fit linear regressions to average data. For Panels A and B, there was no FAF-BSA in the perfusion medium. Flow rate data are not temperature corrected.

Supplemental Figures

Supplemental Figure 1: Confocal immunofluorescence microscopy of S1P receptor expression in angle tissues of the C57BL/6 mouse eye. Slides containing frozen sections of mouse eyes (10 µm; embedded in OCT) were probed with polyclonal antibodies raised against peptides that correspond to carboxyl terminus of *human* S1P receptors 1 (sc-25489), 2 (sc-30024) and 3 (sc-25491), but cross react with mouse. Binding of primary antibodies to mouse tissues was visualized using goat anti-rabbit antibodies conjugated to Dylight that were excited and fluorescence signals digitally captured by a Leica SP5 confocal microscope. Images from all sections were recorded during the same session using identical confocal settings. As a positive control for tissue quality and localization of Schlemm's canal endothelia (asterisks), slides containing mouse eye sections were probed with monoclonal antibodies that specifically recognize PECAM-1, followed by goat anti-mouse secondary antibodies conjugated to Dylight. As a negative control, some slides containing mouse sections were probed only with goat anti-rabbit antibodies conjugated to Dylight (2).