Pharmacologic Manipulation of Conventional Outflow Facility in Ex Vivo Mouse Eyes

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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 pharmacologic 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 hours storage at 4°C. Three groups of experiments examined sphingosine 1-phosphate (S1P), S1P with antagonists to S1P₁ and S1P₂ receptors, and the prostanoid EP₄ receptor agonist 3,7-dithia PGE₁. We also examined whether a 24-hour postmortem delay affected the response to 3,7-dithia prostaglandin E₁ (PGE₁).

RESULTS. S1P decreased facility by 39%, and was blocked almost completely by an S1P₂, but not S1P₁, receptor antagonist. The S1P₂ receptor antagonist alone increased facility nearly 2-fold. 3,7-dithia PGE₁ increased facility by 106% within 3 hours postmortem. By 24 hours postmortem, the facility increase caused by 3,7-dithia PGE₁ was reduced 3-fold, yet remained statistically detectable.

CONCLUSIONS. C57BL/6 mice showed opposing effects of S1P₂ and EP₄ receptor activation on conventional outflow facility, as observed in human eyes. Pharmacologic effects on facility were detectable up to 24 hours postmortem in enucleated mouse eyes. Mice are suitable models to examine the pharmacology of S1P and EP₄ 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. (*Invest Ophthalmol Vis Sci.* 2012;53:5838–5845) DOI:10.1167/iov.12-9923

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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 understand better the mechanisms of IOP regulation. In fact, recent data show that the morphology and behavior of the murine conventional outflow pathway are more similar in some ways to humans than are nonhuman primates (e.g., like humans,¹⁴ mice do not appear to exhibit “washout,”¹¹ while “washout” is observed in monkeys¹⁴). 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 detectable effects on unconventional outflow,^{4,10} unlike the response in human eyes where latanoprost increases conventional¹⁸ 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 examined carefully before accepting the mouse as a reliable model for human IOP regulation.

The goal of our project was to determine whether pharmacologic 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 EP₄ agonist 3,7-dithia prostaglandin E₁ (PGE₁), which respectively decrease²⁰ and increase²¹ 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 pharmacologic response is affected by prolonged postmortem 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 to 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 to 3 hours. For perfusion, each eye was mounted on a single well of a 96-well Stripwell plate (Corning, Leicestershire, UK) 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 μ m 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 μ L; Hamilton GasTight, Reno, NV) placed on the rack of a motorized syringe pump (Pump33; Harvard Apparatus, Holliston, MA) under computer control. A pressure transducer (142PC01G; Honeywell, Columbus, OH) monitored IOP through a three-way connector placed in the perfusion line. Custom written LabVIEW software²³ (National Instruments Corp., Austin, TX) was used to vary the flow rate automatically 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 mm Hg; we refer to this as our "standard perfusion regimen." Eyes typically were 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

$$F = C(IOP) + F_u \quad (1)$$

where F represents the stable flow rate at each corresponding IOP. F_u in equation 1 usually is taken as an estimate of the pressure-independent or "unconventional" outflow rate.¹¹ Equation 1 is valid only when episcleral venous pressure is zero (appropriate for enucleated eyes), F reaches equilibrium at each value of IOP, and 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 described previously.^{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 versus untreated contralateral controls), except for cases where data from one eye

FIGURE 1. Data from Group A. (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 mm Hg). To enable flow rate traces from 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. (B) the average flow rate at each pressure level for all control (filled circles) and S1P-treated eyes (open circles) from Group A. Bars: SD. Lines: the best-fit linear regressions to average data. FAF-BSA was included in the perfusion medium for all eyes represented in (B). Flow rate data are not temperature corrected.

were rejected based on the stability criterion described above. Paired eyes were perfused sequentially (one eye immediately after enucleation, the contralateral eye 2–3 hours after enucleation), where we randomized whether the control or experimental eye was perfused first. We also examined whether prolonged postmortem time (24 hours 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 S1P, a bioactive lipid that decreases outflow facility by 31% in porcine eyes²⁶ and 36% in human eyes.²⁰ Experimental eyes were perfused with 5 μ M S1P in DBG containing fatty acid-free bovine serum albumin (BSA) (FAF-BSA; 2 mg/mL; Sigma-Aldrich A8806, Dorset, UK) and 17 μ M sodium hydroxide (NaOH), while control eyes received DBG and FAF-BSA alone without S1P or NaOH. Independent studies demonstrated that 17 μ M 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 μ M 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 . Before cannulation, each needle was backfilled from the tip with 150 μ L of the appropriate perfusion solution, a volume sufficient to last several hours even at the highest measured flow rates (~ 0.3 μ L/min). The experimental eye was pretreated with S1P-

containing solution from a reservoir at 8 mm Hg for 45 minutes to expose the outflow pathway to the drug before 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 S1P₁ and S1P₂ receptors in mediating the S1P response. Following a previous study in human eyes,²⁷ we used W146 (Avanti Polar Lipids, Alabaster, AL) or JTE-013 (Cayman Chemical, Ann Arbor, MI), which are selective antagonists to S1P₁ or S1P₂ receptors, respectively. W146 was dissolved in water as a 1 mM stock solution containing 10.6 mM cyclodextrin and 100 mM sodium carbonate (Na₂CO₃) as vehicle, and was stored at -20°C . JTE-013 was dissolved in dimethyl sulfoxide (DMSO) as a 1 mM stock solution and stored at -20°C . Experimental eyes were pretreated with antagonist and S1P-containing solution (45 minutes at 8 mm Hg 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 Na₂CO₃. 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 IC₅₀ values (0.83 μM for W146²⁸ and 1.0 μM for JTE-013²⁷). Control eyes were perfused with 5 μM S1P in DBG + 2 mg/mL FAF-BSA + 17 μM NaOH, without antagonist, cyclodextrin, Na₂CO₃, or DMSO vehicle and without pretreatment. 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, Na₂CO₃, 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 Na₂CO₃, 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. Control and experimental eyes were pretreated with perfusion solution for 45 minutes at 8 mm Hg from a reservoir before 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 EP₄ receptor activation on conventional outflow in the mouse eye by perfusion with 3,7-dithia PGE₁, a highly selective PG-EP₄ receptor agonist²⁹ that increases conventional outflow facility in human²¹ and monkey eyes³⁰ without affecting unconventional outflow. 3,7-dithia PGE₁ was dissolved in ethanol as a 10 mM stock solution and stored at -20°C . Experimental eyes were pretreated (45 minutes at 8 mm Hg from a reservoir) and perfused with 10 nM 3,7-dithia PGE₁ in DBG + 17 μM ethanol without FAF-BSA. Control eyes were perfused with DBG alone without ethanol or pretreatment.

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 PGE₁ 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 PGE₁ was affected by postmortem time. For these studies, eyes were enucleated and stored for 24 hours at 4°C in Dulbecco's modified Eagle's medium (DMEM) and then perfused with 10 nM 3,7-dithia PGE₁ 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 postmortem facility response to 3,7-dithia PGE₁, rather than to S1P, for two reasons. First, 3,7-dithia PGE₁ causes a larger change in outflow facility compared to S1P (e.g., 69% increase following 3,7-dithia PGE₁²¹ versus 36% decrease following S1P²⁰ in human eyes, respectively), and therefore 3,7-dithia PGE₁ would provide a more conservative test to detect smaller differences in facility that might occur with prolonged postmortem times. Second, 3,7-dithia PGE₁ is an exogenous compound (unlike S1P) and, therefore, is more representative of potential candidate drugs that may affect conventional outflow. Therefore, by looking at how the facility response to 3,7-dithia PGE₁ changes with postmortem time, we can gain some insight into how postmortem time may affect the interpretation of drug efficacy in perfusion experiments (which often incorporate eyes with postmortem 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: a Welch's *t*-test that included the full set of eyes (accounting for unequal sample sizes) and 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 (equation 1). In response to S1P, conventional outflow facility (*C*, slope of the linear regression) decreased by $38.9 \pm 24.2\%$ (mean $\pm$ 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 $\mu\text{L}/\text{min}/\text{mm Hg}$ 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).

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: 50 μ m. The absence of giant vacuoles in the inner wall of Schlemm's canal may reflect the fact that the eyes were fixed by immersion.

Group B, The S1P₂ Receptor Mediates the S1P Response

To determine which receptor mediates the S1P response measured in Group A, we perfused contralateral eyes with S1P alone or in combination with either an antagonist to the S1P₂ receptor (5 μ M JTE-013, Fig. 3A) or an antagonist to the S1P₁ receptor (5 μ M W146, Fig. 3B). In eyes treated with S1P without antagonist, the conventional outflow facility was 0.0074 ± 0.0034 μ L/min/mm Hg ($N = 15$ 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 μ L/min/mm Hg 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 μ M cyclodextrin and 500 μ M Na₂CO₃ (0.0078 ± 0.0040 vs. 0.0079 ± 0.0035 μ L/min/mm Hg in S1P-treated control eyes from the W146 study with or without cyclodextrin + Na₂CO₃, 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 (Figs. 3A, 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 S1P and JTE, the temperature-corrected conventional facility was 0.0133 ± 0.0019 μ L/min/mm Hg, nearly 2-fold larger than eyes treated with S1P without antagonist (0.0070 ± 0.0036 μ L/min/mm Hg; $P = 0.0012$, 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 μ L/min/mm Hg; temperature-corrected; $P = 0.41$, Welch's t -test, $N = 7$ S1P vs. 7 S1P + W146; $\beta = 0.145$, $\alpha = 0.05$, assuming the facility values for S1P-treated and untreated eyes from Group A with $N = 7$ for each group). These data demonstrated that JTE largely blocks the facility-reducing effect of S1P, while W146 has little effect, suggesting that the S1P₂ receptor, and not the S1P₁ receptor, is responsible principally

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 μ M 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 μ L/min/mm Hg ($P = 0.017$; $N = 5$ 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 suggested that endogenous S1P signaling may be regulating conventional outflow facility in the mouse trabecular meshwork, which can be blocked by S1P₂ receptor antagonist JTE-013.

Group C, EP₄ 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 PGE₁ (Fig. 4A), with the temperature-corrected facility increasing from 0.0062 ± 0.0005 to 0.0131 ± 0.0024 μ L/min/mm Hg ($P = 0.0003$, Welch's t -test, $N = 6$ or 7 for untreated eyes or eyes treated with 3,7-dithia PGE₁, respectively). Considering only paired eyes, the conventional facility increased by $105.8 \pm 48.4\%$ following 3,7-dithia PGE₁ treatment compared to untreated contralateral eyes ($P = 0.02$, paired Student's t -test, $N = 4$ pairs). In contrast, 3,7-dithia PGE₁ 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 hours 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-hour storage in DMEM, conventional outflow facility in eyes perfused with 3,7-dithia PGE₁ was 0.0128 ± 0.0034 μ L/min/mm Hg ($N = 6$ 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 μ L/

FIGURE 3. Data from Group B. (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. (B) Average flow rate at each pressure level for S1P- (open circles) and S1P + W146-treated eyes (filled squares). (C) Average flow rate at each pressure level for control (open diamonds) and JTE-treated eyes without S1P (filled circles). Bars: SD 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.

min/mm Hg, $N = 4$ eyes) by unpaired analysis. When considering only paired eyes, the relative facility increase following 3,7-dithia PGE₁ was nearly 3-fold smaller after 24 hours ($38.1 \pm 34.5\%$, $N = 4$ pairs) versus after 3 hours (see above) and failed to achieve statistical significance ($P = 0.126$, paired Student's *t*-test). Similarly, after 24 hours for pressures of 8 mm Hg and above, there was a tendency for 3,7-dithia PGE₁ to increase the flow rate, but even at 25 mm Hg the flow rate increase failed to achieve statistical significance ($P = 0.10$). This is in contrast to eyes perfused within 3 hours postmortem, when 3,7-dithia PGE₁ caused a statistically significant increase in flow rate for all pressures of 8 mm Hg or larger ($P < 0.05$).

FIGURE 4. Data from Group C. Average flow rate at each pressure level for control (filled circles) and 3,7-dithia PGE₁ (open squares) treated eyes, within 3 hours (A) or after 24 hours postmortem storage at 4°C (B). Bars: SD. 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.

This suggests that while the facility-increasing effect of 3,7-dithia PGE₁ was present 24 hours 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

Our study demonstrated that C57BL/6 mouse eyes respond to S1P and PG-EP₄ receptor agonist in a similar manner as that reported previously for human eyes. Specifically, S1P decreased murine outflow facility by 39%, which was nearly identical to the 36% decrease reported previously in human eyes.²⁰ The prostanoid EP₄ receptor agonist, 3,7-dithia PGE₁, 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 pharmacologic signaling mechanisms underlie the facility response to S1P and 3,7-dithia PGE₁ between C57BL/6 mice and human eyes.

S1P is known to bind to one of five G protein-coupled receptors (S1P₁₋₅) each of which exhibits different downstream signaling events and are regulated differentially in different tissues.³³ S1P decreases outflow facility rapidly,^{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 S1P₁ and negligible levels of S1P₂ and S1P₃ in Schlemm's canal endothelium by in situ confocal immunofluorescence (Supple-

mentary Figure S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9923/-DCSupplemental>). The relative absence of S1P₂ and S1P₃ labeling could be attributed to reduced antibody sensitivity compared to prior studies by our group²⁰ that reported relatively low levels of S1P₂ and S1P₃ expression, compared to S1P₁, by human Schlemm's canal cells *in situ*. Despite the seemingly low levels of S1P₂ expression, the facility-decreasing effect of S1P in mice was abolished almost completely by JTE-013, an antagonist of the S1P₂ receptor, but not by W146, an antagonist of the S1P₁ 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 S1P₁ and S1P₃). Perfusion with JTE-013 alone increased conventional outflow by two-fold in C57BL/6 mice, yielding a conventional outflow facility ($0.0194 \pm 0.0056 \mu\text{L}/\text{min}/\text{mm Hg}$, $N = 5$) that was larger than that measured in either untreated eyes ($0.0125 \pm 0.0037 \mu\text{L}/\text{min}/\text{mm Hg}$, $N = 6$, from Group A; $P = 0.056$, Welch's *t*-test) or eyes perfused with 3,7-dithia PGE₁ ($0.0131 \pm 0.0024 \mu\text{L}/\text{min}/\text{mm Hg}$, $N = 7$, from Group C; $P = 0.063$, Welch's *t*-test). These data strongly implicate the S1P₂ 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 S1P₂ receptor recently has been called into question^{36,37} based on data reporting an effect of JTE-013 in S1P₂ knock-out mice.³⁸ Therefore, future studies should account for potential off-target effects, possibly by incorporating genetically altered mice to understand better the underlying mechanisms by which S1P regulates outflow facility.

3,7-dithia PGE₁ was the first selective agonist developed against the EP₄ receptor²⁹ 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 PGE₁ increases conventional outflow facility in postmortem human eyes by 69%,²¹ consistent with the expression of PG-EP₄ receptor in human trabecular meshwork and Schlemm's canal *in situ*.^{21,39} At 10 nM concentration, however, the effect of 3,7-dithia PGE₁ 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-EP₄ receptor activation.²¹ Compared to humans, our data show that 3,7-dithia PGE₁ had a nearly 2-fold larger effect in C57BL/6 mice (106% vs. 69%²¹), and expression of PG-EP₄ receptors already has 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 EP₄ receptor abundance or sensitivity between species or with age, or it may be due to improved preservation of mouse tissue due to shorter postmortem times. Alternatively, because mice have a more prominent Schlemm's canal with only 2 to 4 trabecular beams compared to 12 to 20 in humans,⁴¹ mice may be predisposed to exhibit a more robust facility response to compounds, such as 3,7-dithia PGE₁, 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 PGE₁ suggests strongly that C57BL/6 mice are a good pharmacologic model for investigating the contribution of EP₄ receptors, and possibly other prostanoid receptors, in the regulation of conventional outflow. This may be interesting particularly given that prostaglandin PGE₂, a natural ligand for EP₄ 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 ($P = 0.028$, Welch's *t*-test) between eyes perfused with 0.2% FAF-BSA from Group A ($0.0125 \pm 0.0037 \mu\text{L}/\text{min}/\text{mm Hg}$, $N = 6$) compared to eyes perfused without FAF-BSA aggregated from Groups B and C ($0.0078 \pm 0.0025 \mu\text{L}/\text{min}/\text{mm Hg}$, $N = 11$). 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 sex, 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 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 (control and experimental), we concluded 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 our current study suggested that the fraction of unconventional outflow in mice may be significantly smaller than estimated previously.^{3,4,6,11} We calculated 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 hours post-enucleation without FAF-BSA, Fig. 4A) yields a relative contribution of $37.9 \pm 15.6\%$ unconventional to total outflow at 8 mm Hg. In contrast, our previous work estimated that unconventional outflow represents 66% of total outflow in C57BL/6 mice.¹¹ This difference is attributable largely to the 5-fold difference in F_U between our current study ($0.035 \pm 0.025 \mu\text{L}/\text{min}$, temperature-corrected, control eyes of Group C at 3 hours) and our previous study ($0.157 \pm 0.026 \mu\text{L}/\text{min}$),¹¹ with a more modest difference observed in conventional outflow facility (0.0062 ± 0.0005 vs. $0.0091 \pm 0.0012 \mu\text{L}/\text{min}/\text{mm Hg}$ 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 our current study, the eye was covered with tissue paper that was kept moist by regular drops of saline, while our previous study¹¹ used regular drops of saline *without* tissue paper. Thus, it is possible that evaporation from the eye contributed to overestimation of F_U in our previous study, and ongoing experiments are examining this hypothesis closely. Along these lines, a recent study¹⁰ 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 pharmacologic 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 postmortem degradation,⁴⁷ and enucleated whole human globes are accepted routinely for perfusion studies up to 24 hours or longer after death. After 24 hours post-enucleation and storage in DMEM at 4°C, the facility-increasing effect of 3,7-dithia PGE₁ 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 PGE₁ failed to achieve statistical significance at 24 hours ($P \geq 0.10$). This suggested that a 24-hour postmortem delay likely represents an upper limit for detection of pharmacologic effects in ex vivo mouse eyes. More to this point, post-hoc analysis of conventional facility in DBG-perfused mouse eyes after 24 hours (0.0087 $\mu\text{L}/\text{min}/\text{mm Hg}$, temperature-corrected, from Group C) was approximately 40% greater ($P = 0.043$, Welch's t -test) than the facility measured in eyes within 3 hours after enucleation (0.0062 $\mu\text{L}/\text{min}/\text{mm Hg}$, temperature-corrected, from Group C). This change in baseline facility explains why the relative facility increase following 3,7-dithia PGE₁ after 24 hours (38%) was nearly 3-fold less than that measured within 3 hours (106%). Taken together, these data demonstrated that postmortem changes occurring within 24 hours can affect conventional facility and the relative facility response to pharmacologic compounds. We are not aware of any studies that have examined outflow facility as a function of postmortem time in human eyes, but if the postmortem response of human and mouse eyes are similar, then these data suggest that there is a considerable loss of sensitivity for detecting pharmacologically-induced changes in outflow facility using human eyes even at 24 hours postmortem.

In conclusion, we demonstrated that conventional outflow facility in C57BL/6 mice mimics the pharmacologic response of human eyes to PG-EP₄ and S1P receptor agonists that respectively increase and decrease outflow facility. These data strongly advocate for the mouse eye (and possibly C57BL/6 or other strains) as a promising and robust model for the pharmacology of PG-EP₄ 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.

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