

Aqueous Humor Outflow Requires Active Cellular Metabolism in Mice

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PURPOSE. Conventional wisdom posits that aqueous humor leaves the eye by passive bulk flow without involving energy-dependent processes. However, recent studies have shown that active processes, such as cell contractility, contribute to outflow regulation. Here, we examine whether inhibiting cellular metabolism affects outflow facility in mice.

METHODS. We measured outflow facility in paired enucleated eyes from C57BL/6J mice using iPerfusion. We had three Experimental Sets: ES1, perfused at 35°C versus 22°C; ES2, perfused with metabolic inhibitors versus vehicle at 35°C; and ES3, perfused at 35°C versus 22°C in the presence of metabolic inhibitors. Inhibitors targeted glycolysis and oxidative phosphorylation (2-deoxy-D-glucose, 3PO and sodium azide). We also measured adenosine triphosphate (ATP) levels in separate murine anterior segments treated like ES1 and ES2.

RESULTS. Reducing temperature decreased facility by 63% [38%, 78%] (mean [95% confidence interval (CI)], $n = 10$ pairs; $P = 0.002$) in ES1 after correcting for changes in viscosity. Metabolic inhibitors reduced facility by 21% [9%, 31%] ($n = 9$, $P = 0.006$) in ES2. In the presence of inhibitors, temperature reduction decreased facility by 44% [29%, 56%] ($n = 8$, $P < 0.001$) in ES3. Metabolic inhibitors reduced anterior segment adenosine triphosphate (ATP) levels by 90% [83%, 97%] ($n = 5$, $P < 0.001$), but reducing temperature did not affect ATP.

CONCLUSIONS. Inhibiting cellular metabolism decreases outflow facility within minutes. This implies that outflow is not entirely passive, but depends partly on energy-dependent cellular processes, at least in mice. This study also suggests that there is a yet unidentified mechanism, which is strongly temperature-dependent but metabolism-independent, that is necessary for nearly half of normal outflow function in mice.

Keywords: aqueous flow, outflow resistance, metabolism, animal models, temperature

Aqueous humor drainage through the trabecular meshwork (TM) and into Schlemm's canal (SC) is thought to occur by passive bulk flow without active transport or energy-dependent cellular processes. Contributing to this view, Bárány¹ found that outflow facility was unaffected by several metabolic inhibitors in enucleated cattle eyes. VanBuskirk and Grant² found that outflow facility was insensitive to temperature change in enucleated human eyes. These classic studies suggest that TM or SC cells do not actively regulate outflow or contribute directly to outflow function. This led naturally to the view that the outflow pathway functions like a passive filter, without a direct role for the TM or SC cells themselves.^{3–11}

The notion that outflow functions like a passive filter is incongruent with recent findings showing that metabolically-dependent cellular processes, such as cell contractility,^{12–17} exocytosis,^{18,19} and signal transduction,^{20–24} affect conventional outflow facility. There are also two studies that, in contrast to VanBuskirk and Grant,² suggest that outflow facility is temperature dependent in enucleated mouse²⁵ and calf eyes.²⁶ Thus it remains an open

question as to what extent aqueous humor outflow requires active cellular metabolism. The goal of this study was to assess the effects of reduced temperature and metabolic inhibitors on outflow facility in freshly enucleated mouse eyes.

MATERIALS AND METHODS

Experimental Design

In this project, we examined the role of active cellular metabolism on outflow facility in ex vivo mouse eyes. We inhibited metabolic activity by reducing temperature or by perfusing with chemical inhibitors of cellular metabolism. We had three experimental sets. In Experimental Set 1 (ES1), we examined the effect of temperature reduction by perfusing paired eyes at 22°C versus 35°C, with both eyes receiving standard perfusate. In Experimental Set 2 (ES2), we perfused paired eyes with or without metabolic inhibitors, with both eyes at 35°C. In Experimental Set 3 (ES3), we examined the effect of temperature independently of metabolism by



perfusing paired eyes at 22°C versus 35°C, with both eyes receiving metabolic inhibitors. We also tested how temperature reduction and metabolic inhibitors affect intracellular ATP levels in the anterior segment.

To inhibit cellular metabolism, we used a combination of drugs that target two pathways involved in ATP production, glycolysis and oxidative phosphorylation. To inhibit glycolysis, we used 2-deoxy-D-glucose (2-DG) and (2E)-3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO). The 2-DG is a glucose analogue that is internalized by cells where it competitively inhibits the production of glucose-6-phosphate to prevent further glycolysis.^{27,28} The 3PO inhibits 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3),^{29,30} which is a rate-limiting enzyme in glycolysis. To inhibit oxidative phosphorylation, we used sodium azide (NaN₃) that blocks electron transport in mitochondria.^{31,32}

Animal Handling

This study used 40 male C57BL/6J mice (Charles River Laboratory, UK) aged between 10 and 13 weeks. Mice were fed ad libitum and kept in a 12-hour light/dark cycle. All mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under the authority of a UK Home Office Project License. Mice were humanely culled by cervical dislocation, and death was confirmed by scission of the femoral vein. Eyes were surgically enucleated within 10 minutes of death by cutting the extraocular muscles and the optic nerve with fine curved scissors. Once enucleated, eyes were stored in phosphate-buffered saline solution (PBS) at room temperature until perfusion (less than 20 minutes). All eyes were perfused within 30 minutes postmortem.

Measurement of Outflow Facility

We measured outflow facility using iPerfusion.³³ Briefly, iPerfusion consists of a thermal flow sensor (SLG150; Sensirion, Staefa, Switzerland) and a differential pressure transducer (PX409; Omegadyne, Sunbury, OH, USA) to measure the flow rate Q into the eye and the pressure P within the eye. An actuated reservoir is used to control the upstream applied pressure. The temperature of the eye is maintained in a bath of PBS with a computer-controlled heater that maintains the bath at the desired temperature, either 35° ± 0.5°C or 22° ± 0.2°C.

Within 10 minutes of enucleation, the eye was adhered to a support platform within the bath using tissue glue. The eye was then quickly submerged by filling the bath with PBS that was prewarmed to the desired temperature. We used a pulled glass micropipette to cannulate the eye via the anterior chamber for perfusion. The micropipette was beveled with a tip diameter of approximately 100 μm. Before cannulation, we measured the resistance of the micropipette, which would indicate whether there was a blockage or bubble that would require removal. The eye was then cannulated at an applied pressure of 8 mm Hg. Cannulation was done using a micromanipulator under a stereoscopic microscope. We measured outflow facility in two eyes simultaneously using two iPerfusion systems.

A standard perfusion consisted of an initial pressurization step of 30 minutes at 8 mm Hg for acclimatization, followed by nine discrete pressure steps from 5 to 18.5 mm Hg and a final step at 8 mm Hg (Fig. 1A). The duration of each step

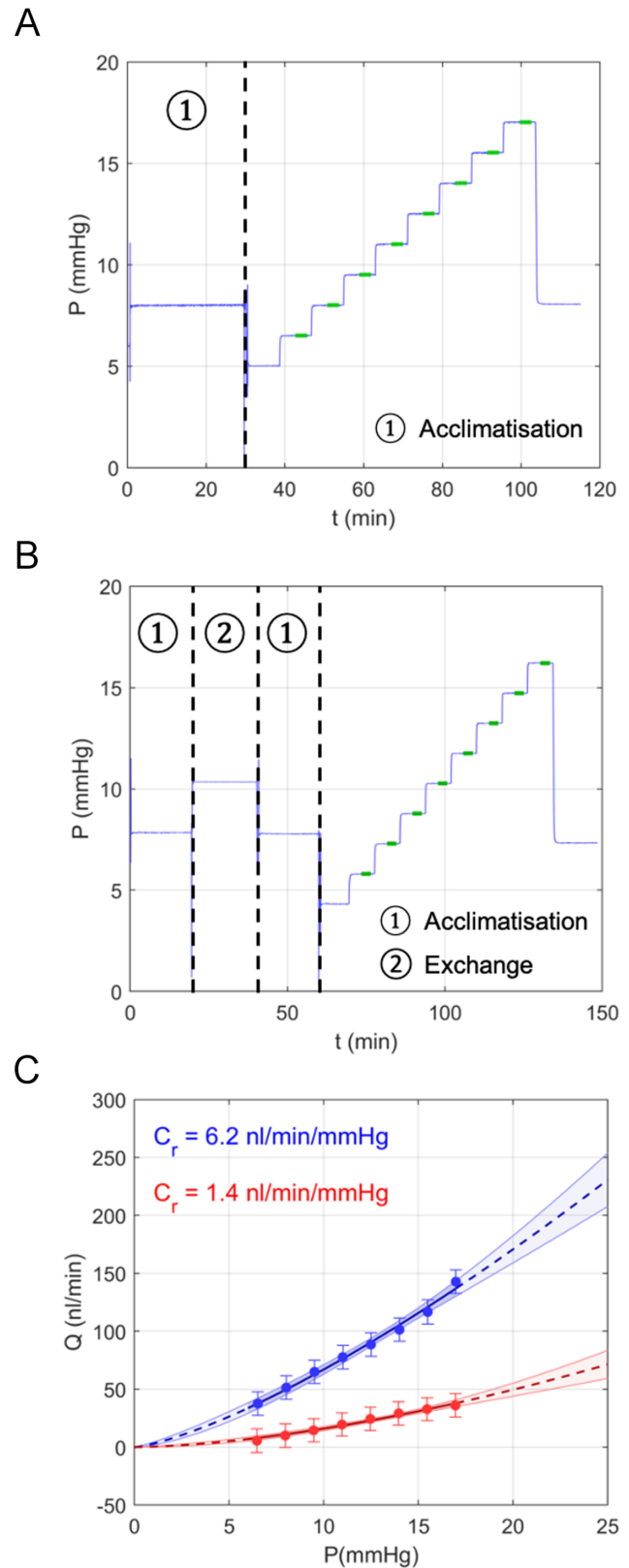


FIGURE 1. (A) The pressure stepping protocol used for Experimental Set (ES) 1 examining the effect of temperature. The green highlights show data used for the fitting by Equation 1. (B) The Pressure stepping protocol used for ES2 and ES3 that included an anterior chamber exchange to deliver metabolic inhibitors. (C) Flow (Q) and pressure (P) data for a pair of eyes from ES1 perfused at 22°C (red) or 35°C (blue). Data at 22°C are not viscosity corrected. Error bars

was typically eight to ten minutes and was controlled by the iPerfusion software based on a stability condition that advanced to the next step when Q changed by no more than 3 nL/min per minute over a six-minute moving window. Each step typically required two to four minutes to achieve stability.

Outflow facility was calculated as described by Sherwood et al.³³ Briefly, Q and P were measured over a sequence of nine increasing pressure steps, and the data were fit by the following power-law relationship:

$$Q = C_r \left(\frac{P}{P_r} \right)^\beta P \quad (1)$$

where C_r is the reference value of outflow facility that applies at a reference pressure P_r , defined to be 8 mm Hg to correspond to the physiological pressure drop across the outflow pathway (Fig. 1C). The β captures the nonlinearity of the Q - P relationship, which is perfectly linear when $\beta = 0$. Equation 1 requires that $Q = 0$ when $P = 0$, as has been confirmed in enucleated mouse eyes.³⁴ The Q and P values used for the fit were taken as the average over the last 300 seconds of each step that achieves our stability criterion. Any steps not reaching our stability criterion were excluded from the fit, along with any subsequent steps. All perfusions had at least six steps, and only increasing pressure steps were included in the fit (i.e., those shown in green highlights in Fig. 1).

The effect of a specific treatment on outflow facility was determined by calculating the average change in C_r between contralateral eyes. In this way, we account for the correlation in outflow facility between contralateral eyes,³³ and variations in baseline outflow facility between individual mice do not unduly influence the assessment of the relative difference in facility in response to the treatment. Statistical significance on the relative difference in outflow facility was determined using a weighted paired t -test as described previously.³³ Any change in C_r that exceeded 2.5 times the median absolute deviation from the median change was considered an outlier.³³

To examine the relationship between the relative difference in facility and the baseline outflow facility (defined as the value of $\ln C_r$ in the control eye), we used linear regression analysis for ES2 or analysis of covariance (ANCOVA) for ES1 and ES3. To consider the combined effects of temperature reduction in ES1 and ES3, we defined treatment with metabolic inhibitors within the ANCOVA as a categorical variable.

ES1: Investigating the Effects of Temperature Reduction

To investigate the effect of temperature reduction on outflow facility, one eye of a pair was perfused at a physiological temperature of 35°C while the contralateral eye was perfused at 22°C. Both eyes were perfused with Dulbecco's PBS containing divalent cations and 5.5 mmol/L glucose that was passed through a 0.22 μ m filter before use. ES1 used

10 mice, which were perfused using the pressure-stepping protocol summarized in Figure 1A.

Measurements of outflow facility at different temperatures require correction to account for changes in perfusate viscosity. Assuming that the viscosity of the perfusate behaves like that of water, the value of C_r measured at 22°C must be multiplied by the ratio $\mu_{22^\circ\text{C}}/\mu_{35^\circ\text{C}}$, where $\mu_{22^\circ\text{C}}$ is the dynamic viscosity of water at 22°C (954 Pa.s) and $\mu_{35^\circ\text{C}}$ is the dynamic viscosity of water at 35°C (719 Pa.s).³⁵ Note that this ratio is greater than unity, meaning that the corrected facility is larger than the measured facility (i.e., the correction reduces, not increases, the apparent effect of temperature on facility). The corrected outflow facility represents the facility that would have been measured if perfusate viscosity were unaffected by temperature. Thus, by comparing the corrected C_r at 22°C versus the measured C_r at 35°C, we can isolate the effects of temperature on outflow facility, independent of changes in viscosity.

ES2: Investigating the Effects of Metabolic Inhibitors

To test the effect of the metabolic inhibitors, paired eyes were perfused with or without metabolic inhibitors at a physiological temperature of 35°C. The inhibitors targeted glycolysis and oxidative phosphorylation and included 11 mmol/L 2-DG, 4 mmol/L NaN₃, and 100 μ mol/L 3PO (Calbiochem, Merck, Darmstadt, Germany) in Dulbecco's PBS without additional glucose. The contralateral eye not receiving metabolic inhibitors was perfused with isosmotic vehicle, which was Dulbecco's PBS containing 11 mmol/L glucose, 4 mmol/L NaCl and 0.2% DMSO. All solutions were filtered through a 0.22 μ m filter before use. ES2 used 10 mice.

We used anterior chamber exchange to deliver a known concentration of metabolic inhibitors that is maintained throughout the perfusion. A sham exchange was performed for the eye receiving isosmotic vehicle. To perform an exchange, the eye was cannulated with two micropipettes held in a custom device mounted on a micromanipulator to allow for simultaneous dual cannulation. Both micropipette tips were positioned in the anterior chamber. One micropipette was connected to the iPerfusion system, whereas the other was connected to a 1 mL glass syringe (Gastight 1700 Series, Hamilton Company, Reno, NV, USA) mounted on a syringe pump (PHD ULTRA; Harvard Apparatus, Holliston, MA, USA). The micropipette and upstream tubing connecting to iPerfusion were filled with perfusion fluid containing metabolic inhibitors (or isosmotic vehicle). To perform the exchange, the syringe pump was programmed to withdraw fluid from the anterior chamber at a flow rate of 5 μ L/min for 20 minutes. This draws fluid into the eye through the micropipette connected to iPerfusion, thereby filling the anterior chamber with the desired concentration of drug or vehicle. The eye pressure during the exchange was controlled using an actuated reservoir set to give an applied upstream pressure P_a of:

$$P_a = P_e + Q_e R_n \quad (2)$$

where P_e is the desired value of IOP during the exchange (defined to be 8 mm Hg), Q_e is the exchange flow rate (5 μ L/min) and R_n is the resistance of the micropipette (typical range of 0.1–1.0 mm Hg/ μ L/min). We measured R_n before

represent 95% CI on each measurement. The curves represent the best fit by Equation 1, with C_r values shown for each case. The shading around the curve depicts the 95% CI on the fit.

each experiment by placing the pipette tip in the eye bath and using iPerfusion to measure its resistance. To ensure a correct exchange, both micropipettes should have a similar resistance. Equation 2 accounts for the pressure drop across the micropipette, which is normally negligible when flow rates are small (< 200 nL/min), as occurs during a perfusion, but must be accounted for when flow rates are large, as occurs during the exchange.

ES2 used the following pressure stepping protocol. After cannulation, the eye was acclimated to the perfusion system for 20 minutes at an applied pressure of 8 mm Hg. The anterior chamber was then exchanged with either metabolic inhibitors or vehicle at 8 mm Hg for 20 minutes, as described above. The eye was then allowed to further acclimate after the exchange for 20 minutes at 8 mm Hg. The eye was then perfused over nine increasing pressure steps as for ES1 to determine C_r (Fig. 1B).

ES3: Investigating the Effect of Temperature, Independent of Metabolic Inhibitors

Temperature reduction examined in ES1 will tend to suppress enzyme activity, and thereby reduce metabolic processes. This should mimic the effects of the metabolic inhibitors examined in ES2. However, it is possible that temperature reduction has additional effects on outflow, for example by affecting the mechanical properties of cells, such as cell stiffness³⁶ and contractility.^{37,38} We thus investigated the effects of temperature independent of metabolic inhibitors. To do this, we put metabolic inhibitors into both eyes, but perfused one eye at 35°C while the contralateral was perfused at 22°C. The cocktail of metabolic inhibitors was the same as that for ES2, delivered into both eyes using an anterior chamber exchange, as described above. Outflow facility at 22°C was corrected to account for changes in viscosity, as described for ES1. The pressure stepping protocol was identical to that used for ES2 (Fig. 1B). ES3 used 10 mice.

Measurement of ATP in the Anterior Segment

Our aim was to assess how temperature reduction and the metabolic inhibitors affect ATP. Because of the difficulty of manually dissecting the tiny outflow pathway of mice, we resorted to measuring ATP levels in the anterior segment, which includes the cornea, corneoscleral rim, and conventional outflow pathway. These measurements used 10 mice.

The mice were handled as described above for perfusions. After enucleation, eyes were stored in PBS at room temperature and, within 20 minutes, eyes were hemisected posteriorly to the limbus. The posterior segment and lens were discarded, and the iris was carefully removed. This was necessary because the heavy iris pigmentation interfered with the luminescence measurements described below. Anterior segments were then incubated with metabolic inhibitors versus isosmotic controls at 35°C ($n = 5$ pairs) or incubated in standard perfusion solution at 35°C versus 22°C ($n = 5$ pairs) for two hours. These two conditions mimic what the eyes experience during perfusion for ES1 and ES2.

After incubation, the tissues were washed in ice-cold PBS and homogenized in 500 μ L of cold lysis buffer (10 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 1 mmol/L ethylenediamine tetra-acetic acid and 0.01% Triton X-100 in dH₂O)

using a tissue homogenizer (T10 basic Ultra-Turrax, IKA, Staufen, Germany). The homogenization was done in three cycles of 15 seconds leaving the tubes in ice for 10 seconds in between each cycle. Tissue homogenates were spun in a centrifuge at 17×10^3g for five minutes at 4°C, and the supernatant was kept in ice for ATP and protein quantification. ATP levels were measured by luminescence using an ATP determination kit (A22066; ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's instructions. To normalize for the quantity of tissue, protein concentration was measured using the Pierce BCA Protein Assay Kit (no. 23227, ThermoFisher Scientific) following manufacturer's instructions. We calculated the quantity of ATP (in picomoles) per unit mass of protein (in milligrams) and determined the relative change in normalized ATP levels between contralateral eyes. Statistical significance was evaluated using a *t*-test on the average percentage change in ATP between contralateral eyes.

RESULTS

Effect of Temperature and Metabolic Inhibitors on Outflow Facility

ES1 examined the effect of temperature reduction on outflow facility (Fig. 2A). In eyes perfused at 35°C, C_r was 4.8 [3.7, 6.3] nL/min/mm Hg (geometric mean [95% CI]). In eyes perfused at 22°C, C_r before viscosity correction was 1.3 [0.9, 2.0] nL/min/mm Hg, but C_r increased to 1.8 [1.2, 2.7] nL/min/mm Hg after viscosity correction. Between contralateral pairs, reducing temperature from 35°C to 22°C decreased C_r by 63% [38%, 78%] after viscosity correction ($P = 0.002$, $n = 10$ pairs, Fig. 3 left panel).

ES2 examined the effect of metabolic inhibitors on outflow facility at physiological temperature (35°C; Fig. 2B). In eyes perfused with isosmotic vehicle, C_r was 4.8 [2.4, 9.9] nL/min/mm Hg. In eyes perfused with metabolic inhibitors, C_r was 3.8 [1.8, 7.8] nL/min/mm Hg. Between contralateral pairs, metabolic inhibitors reduced C_r by 21% [9%, 31%] ($P = 0.006$, $n = 9$ pairs, Fig. 3, central panel). Data from one of the 10 pairs perfused was identified as an outlier and was not included in the statistical analysis.

ES3 examined the effect of temperature reduction in the presence of metabolic inhibitors (Fig. 2C). In eyes perfused at 35°C with metabolic inhibitors, C_r was 7.2 [6.0, 8.6] nL/min/mm Hg. In eyes perfused at 22°C with metabolic inhibitors, C_r before viscosity correction was 3.1 [2.6, 3.6] nL/min/mm Hg, which increased to 4.1 [3.4, 4.8] nL/min/mm Hg after viscosity correction. Between contralateral pairs both receiving metabolic inhibitors, reducing temperature from 35°C to 22°C decreased C_r by 44% [56%, 29%] ($P < 0.001$, $n = 8$ pairs, Fig. 3 right panel). Two eyes of the 10 pairs perfused did not achieve the stability criteria described above, and data from both pairs were not included in the statistical analysis.

We also examined whether there was a relationship between the relative decrease in outflow facility and the baseline outflow facility, defined as the log-transformed value of C_r in the control eye. For eyes treated with metabolic poisons in ES2, there was no detectible relationship between the relative change in facility and the value of $\ln C_r$ in the control eye ($P = 0.83$, $n = 9$). However, for eyes experiencing temperature reduction in ES1 and ES3, there was a significant relationship, with a larger relative decrease in facility

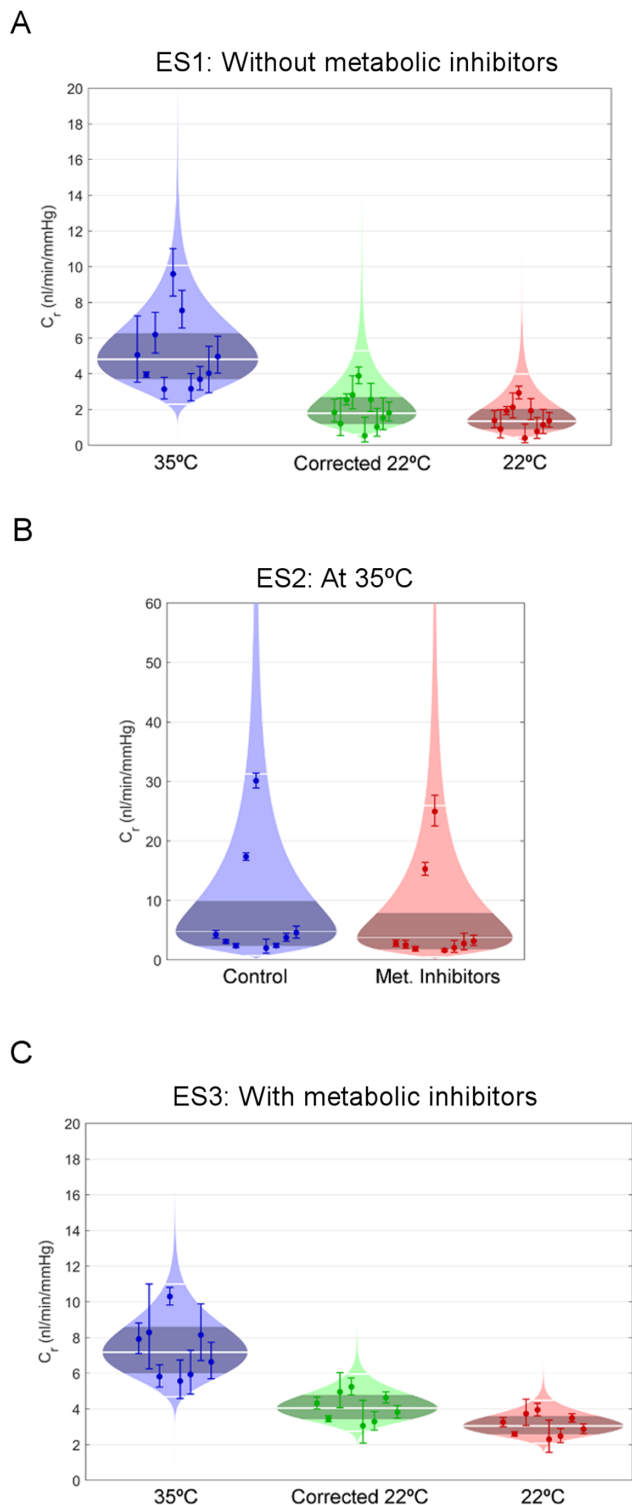


FIGURE 2. Cello plots of outflow facility (C_r) data from ES1 (A), ES2 (B) and ES3 (C). **(A)** ES1: The *left plot* represents eyes perfused at 35°C, whereas the *right plot* represents eyes perfused at 22°C (uncorrected value of C_r). The *central plot* represents the value of C_r for eyes perfused at 22°C after viscosity correction. The cello plots show all measured values of C_r (data points) with the *error bars* representing the corresponding 95% CI on each measurement. The light shaded region shows the log-normal distribution that best fits the data. The central *white line* represents the geometric mean, and the outer white lines represent the range that encompasses 95% of the data. The central dark shaded region represents the 95% CI of the mean. **(B)** ES2: eyes perfused with metabolic inhibitors

observed in eyes with a larger value of $\ln C_r$ in the control eye ($P = 0.013$, $\eta^2 = 0.31$, $n = 18$; ANCOVA).

Effect of Temperature and Metabolic Inhibitors on ATP Levels in the Anterior Segment

We measured how ATP levels were affected by temperature reduction and metabolic inhibitors (Fig. 4). In anterior segments at 35°C, the ATP level was 5.1 [3.9, 6.2] pmol/mg protein (mean [95% CI]), whereas in anterior segments at 22°C, the ATP level was 6.1 [3.4, 8.8] pmol/mg protein. These differences were not statistically significant between contralateral pairs ($P = 0.36$, $n = 5$ pairs). In anterior segments treated with metabolic inhibitors, the ATP level was 0.4 [0.1, 0.8] pmol/mg protein, whereas for eyes treated with isosmotic vehicle the ATP level was 4.3 [3.7, 4.9] pmol/mg protein. Between contralateral pairs, metabolic inhibitors reduced ATP levels by 90% [83%, 97%] ($P \ll 0.001$, $n = 5$ pairs).

DISCUSSION

Our results demonstrate that outflow facility in mice is sensitive to metabolic inhibitors and exquisitely sensitive to temperature. Reducing temperature from 35°C to 22°C reduced outflow facility by 63% on average in ES1, whereas metabolic inhibitors reduced outflow facility by 21% in ES2. We initially surmised that temperature reduction should effectively inhibit most enzymatic processes involved in metabolism, and thus we reasoned that temperature reduction should have a similar effect as metabolic inhibitors on outflow facility. However, to our surprise, we found that the effect of temperature reduction on outflow facility was three-fold greater than metabolic inhibitors alone. Consistent with this notion, when controlling for the presence of metabolic inhibitors in ES3, temperature reduction from 35°C to 22°C still reduced outflow facility by 44%.

Interestingly, the magnitude of these effects appeared to be additive. Specifically, the 63% facility decrease in response to temperature reduction was roughly equal to the sum of the effect of metabolic inhibitors alone (21%) and the effect of temperature reduction in the presence of metabolic inhibitors (44%). This suggests that temperature reduction suppresses both *metabolism-dependent* and *metabolism-independent* mechanisms to affect outflow facility. The metabolism-dependent mechanisms appear to account for roughly one fifth of normal outflow function in normal healthy adult mice, whereas the metabolism-independent and temperature-dependent mechanisms appear to account for just under one half of normal outflow function.

Before proceeding, it is worth benchmarking the effects of temperature reduction and metabolic inhibitors against other compounds that are known to reduce outflow facility in mice. Sphingosine 1-phosphate (S1P) decreases outflow facility by roughly 39% in enucleated mouse eyes,¹² approximating the response observed in human

versus isosmotic vehicle (control). Both eyes are perfused at 35°C. Symbols are as defined in panel A. **(C)** ES3: The *left plot* represents eyes perfused at 35°C, whereas the *right plot* represents eyes perfused at 22°C (uncorrected value of C_r), all eyes receiving with metabolic inhibitors. The *central plot* represents the value of C_r for eyes perfused at 22°C after viscosity correction. Symbols are as defined in panel A.

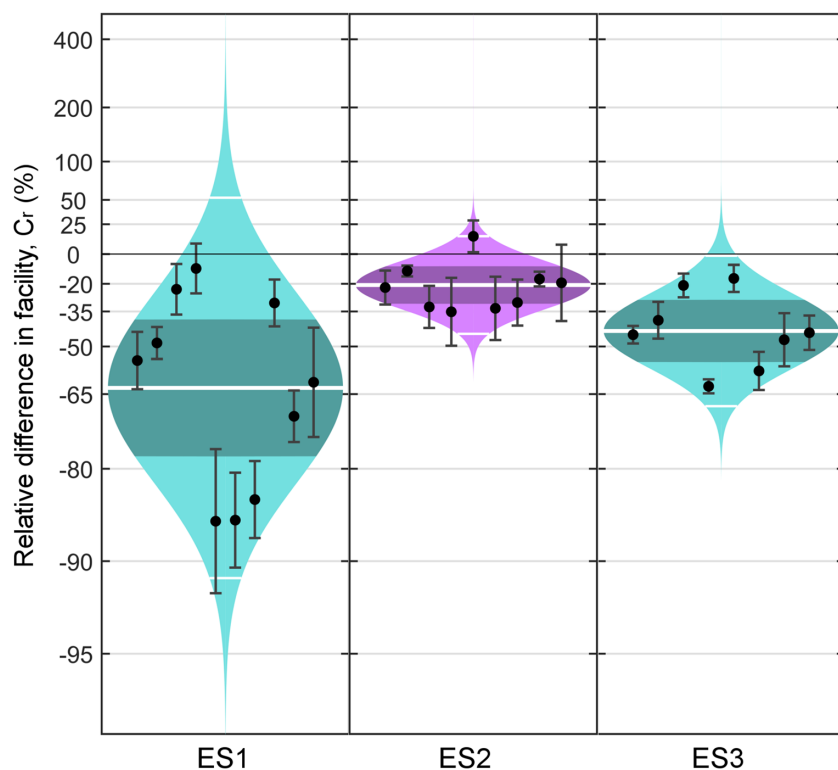


FIGURE 3. Cello plots showing the relative difference in outflow facility (C_r) between contralateral eyes. *Left panel*, Outflow facility of eyes perfused at 22°C relative to 35°C with viscosity correction (ES1), all eyes perfused with vehicle. *Central panel*, Outflow facility of eyes perfused with metabolic inhibitors relative to isosmotic controls, all eyes perfused at 35°C (ES2). *Right panel*, Outflow facility of eyes perfused at 22°C relative to 35°C with viscosity correction, all eyes perfused with metabolic inhibitors (ES3). The symbols are as described in [Figure 2](#).

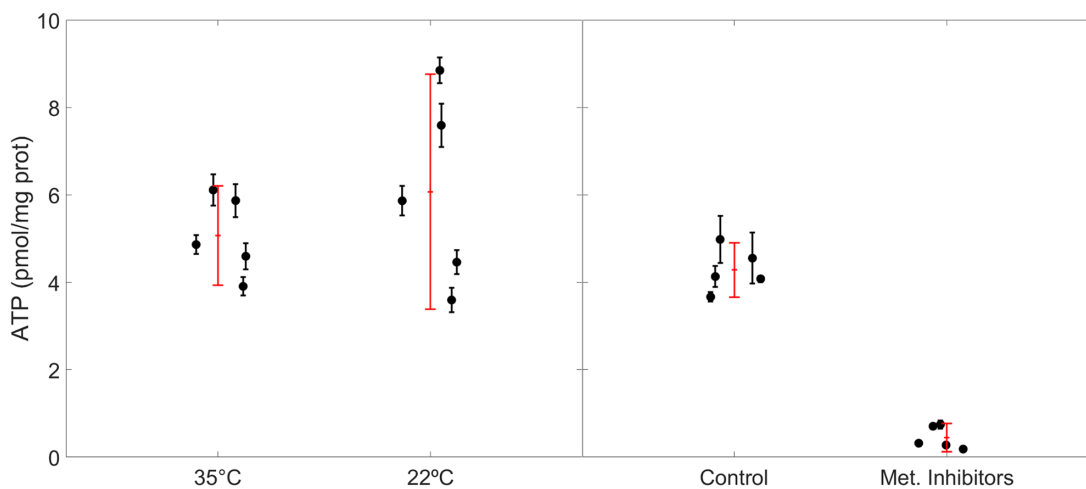


FIGURE 4. ATP levels in anterior segments of mouse eyes treated with temperature reduction (*left panel*) or metabolic inhibitors (*right panel*). Each data point represents the normalized ATP level (pmol ATP per mg of protein) obtained from one anterior segment with three technical replicates (mean \pm SD). *Red symbols* represent the overall mean and 95% CI.

eyes.¹³ S1P is thought to act by affecting cell contractility and stiffness via phosphorylation of myosin light chain.³⁹ Dexamethasone (DEX) decreases outflow facility by approximately 52% in mice after three to four weeks of exposure, with effects attributed to increased extracellular matrix and basement membrane deposition underlying the inner wall of SC⁴⁰ and increased TM stiffness.⁴¹

Blockade of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR-2) with the Ki8751 or VEGF-A₁₆₅b decreases outflow facility by 34% and 44%, respectively.²² Martenoxin (MarTX), which blocks the large-conductance potassium ion channels K_{Ca}1.1 (or BK, maxi-K) that contain the β_4 subunit, decreases outflow facility by roughly 35%, whereas iberiotoxin, which blocks K_{Ca}1.1 channels that lack

β_4 , decreases outflow facility by roughly 16%.⁴² Thus the effect of metabolic inhibitors on outflow facility is somewhat smaller than previously reported effects of S1P, DEX, MarTX, and VEGFR-2 antagonists. However, temperature reduction has the largest effect of any previously reported compound or treatment that has been shown to decrease outflow facility, at least in mice. Furthermore, it may be important that eyes with a higher baseline outflow facility exhibited a larger facility decrease in response to temperature reduction, suggesting that temperature-dependent mechanisms may contribute to differences in baseline outflow facility between individuals. Future studies should thus investigate how temperature reduction affects outflow function because this mechanism appears to have a critical role in outflow function.

Comparison Against Prior Studies: Effects of Temperature

Our temperature studies in ES1 contrast with classic studies by VanBuskirk and Grant² and Pollack et al.⁴³ who did not find any significant change in outflow facility in response to temperature reduction in human and rabbit eyes respectively. Our results, however, are consistent with prior studies in mice from our own laboratory,²⁵ as well as with data reported by Suzuki and Anderson²⁶ in enucleated calf eyes.

VanBuskirk and Grant² examined the effect of temperature reduction on outflow facility in 29 ostensibly normal enucleated human eyes. After correcting for changes in viscosity, they reported that “the reduction in temperature appeared to have little if any functional influence on the aqueous outflow itself.” Pollack et al.⁴³ reported similar findings in live rabbits. Perhaps the outflow system in mice is more sensitive to temperature than other species. Alternatively, the effects of prolonged enucleation or post-mortem time, which were less than six hours and <48 hours, respectively, in the human study,² or advanced age of the donors could have suppressed the effects of temperature. In our study, young adult mouse eyes were perfused within 30 minutes of death, which may have better preserved the physiological function of the outflow pathway, but this does not explain the lack of the temperature effect observed in rabbits.⁴³

Boussommier-Calleja et al.²⁵ measured a 44% reduction in outflow facility in enucleated mouse eyes perfused at 20°C relative to 35°C after viscosity-correction. The mice in that study were of a similar genetic background and age as in our current study, but the perfusion in that study was done using an older pump-based perfusion system,^{44,45} rather than the reservoir-based iPerfusion system,³³ which we believe to be more accurate. Additionally, because the contralateral eyes of that study²⁵ were perfused consecutively, the postmortem time ranged up to three hours, with eyes stored at 4°C in PBS after enucleation, which occurred within 15 minutes of death. It is thus possible that the longer postmortem times or less-accurate techniques used in our previous study²⁵ may have reduced the apparent effect of temperature on outflow facility relative to our current work.

Suzuki and Anderson²⁶ examined how temperature reduction influenced the action of metabolic inhibitors on outflow facility in enucleated calf eyes. Although the effect of temperature alone was not the main focus of their

study, their data suggest a 47% reduction in the measured outflow facility at 22°C relative to 36°C in the absence of metabolic inhibitors. There was no mention of whether these differences achieved statistical significance or whether the facility values were corrected for changes in viscosity. If viscosity changes were not accounted for in their reported data, then applying the correction yields a temperature-dependent facility decrease of 28%. The postmortem time was not reported, but as the eyes were obtained from a local abattoir, it was likely at least a few hours, with the eyes kept cold until use.

The discrepancy between these studies,^{2,25,26,43} requires further investigation to determine whether, as previously suggested, postmortem time,^{46,47} in addition to age, species, or methodologic differences (e.g., enucleation) may explain the temperature sensitivity to outflow observed in mouse (and possibly calf) eyes versus in human and rabbit eyes. If species differences are found to be responsible and the ex vivo mouse eye is indeed unique in regard to temperature-sensitive outflow, then this would raise questions about the validity of the ex vivo mouse eyes (and potentially mouse models in general) for investigating outflow physiology, particularly if such temperature sensitivity is not observed in primates.

Comparison Against Prior Studies: Effects of Metabolic Inhibitors

Bárány¹ studied the effect of several metabolic inhibitors on outflow facility in enucleated eyes from cattle. Figure 5 summarizes the inhibitors used by Bárány, which target glycolysis (iodoacetic acid [IAA]; sodium arsenite [NaAsO₂]; sodium fluoride [NaF]), production of acetyl-coenzyme A (NaAsO₂), and oxidative phosphorylation (NaF; NaN₃; sodium cyanide [NaCN]; 2-4-dinitrophenol [DNP]). None of these compounds perfused on their own were found to have any effect on outflow, except for IAA, which increased outflow facility, likely via its sulfhydryl effects rather than via its metabolic effects.^{26,48,49}

In Bárány's study¹ each inhibitor was examined in only three pairs of eyes, and thus it is possible that any effects could have been simply missed due to small sample sizes. Furthermore, Bárány's perfusions were performed at room temperature,¹ where effects of metabolic inhibitors may be suppressed. For example, Suzuki and Anderson²⁶ showed that NaF had a negligible effect on outflow facility at room temperature, but NaF decreased facility by as much as 50% (average of 30%) at physiological temperature. This is consistent with our finding that temperature reduction has a larger effect on outflow than metabolic inhibitors.

Suzuki and Anderson²⁶ examined the effect of DNP, 2-DG, IAA, iodoacetamide (IA) and potassium cyanide (KCN) on outflow facility in enucleated calf eyes. IAA and IA increased facility, consistent with findings by Bárány,¹ but with the exception of NaF, no other inhibitor was found to have any effect on outflow facility. Importantly, NaF is an inhibitor of both glycolysis (via enolase⁵⁰) and oxidative phosphorylation^{51,52} (Fig. 5), and, hence, NaF is similar to our inhibitor cocktail by targeting multiple metabolic pathways simultaneously. The metabolism of the trabecular meshwork is thought to be largely anaerobic and driven by glycolysis.⁵³ However, inhibition of one metabolic pathway may lead to compensation by other pathways that may make use of alternative energy sources. Thus a single inhibitor that targets

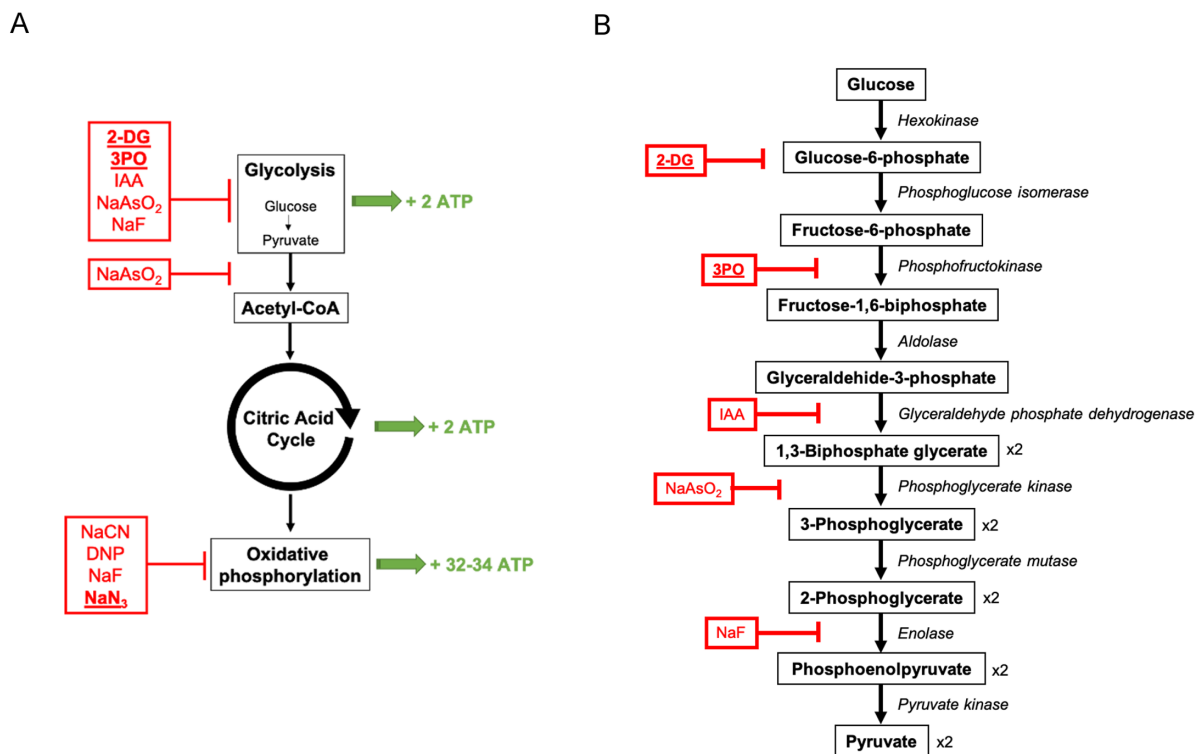


FIGURE 5. (A) A summary of the main metabolic pathway for ATP production and where metabolic inhibitors affect this pathway. Inhibitors that are underlined are those used in this study, whereas those that are not underlined were used by Bányai.¹ The net ATP production per molecule of glucose is shown in green. (B) Schematic representation of glycolysis, with metabolic inhibitors shown in red. 2-DG: 2-Deoxy-D-glucose; 3PO: (2E)-3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; IAA: iodoacetic acid; NaAsO₂: sodium arsenite; NaF: sodium fluoride; NaCN: sodium cyanide; NaN₃: sodium azide; DNP: 2,4-Dinitrophenol.

multiple pathways, such as NaF, or an inhibitor cocktail may be more effective at examining the role of active cellular metabolism in outflow function.

Effects on ATP Levels

Our data show that simultaneous inhibition of glycolysis and oxidative phosphorylation reduces ATP levels by nearly 90% after two hours. These measurements provide biochemical validation that our inhibitory cocktail was functional within the mouse eye. These ATP measurements were made of the anterior corneoscleral shell, but we may assume with reasonable confidence that ATP levels in the TM would follow similar trends. Although we point out that measurements of ATP in the anterior segment as a whole may not be entirely reflective of changes within the TM and may be strongly influenced by more metabolically active tissues such as the cornea. For instance, the integrity of the cornea has been used as indicator of the health of the trabecular meshwork.⁵⁴ Presumably, the metabolic inhibitors effectively suppressed ATP production without affecting consumption, such that ATP levels declined after exposure to the inhibitors. Because we only measured ATP levels after two hours of exposure, at a time point corresponding to the approximate end of the perfusion, we cannot comment on the time course of ATP depletion. Thus, it could have been possible that ATP levels were not yet fully exhausted at the earlier stages of the perfusion. If that were the case, however, we should expect the true effect of metabolic inhibitors on outflow facility to be even larger than the 21% effect measured in this study.

Regardless, these results demonstrate that inhibiting glycolysis or oxidative phosphorylation coincides with a reduction in outflow facility, suggesting that metabolic production of ATP is necessary to maintain roughly one fifth of normal outflow function.

ATP levels were not significantly affected by temperature reduction. This is likely because temperature reduction, in contrast to the metabolic inhibitors, affects both the consumption and production of ATP. Both processes involve enzymatic activity, and all enzymes have an optimal temperature where the rate of reaction is greatest. As a general rule, a temperature change of 1° or 2°C may introduce 10% to 20% change in enzyme reaction rate.⁵⁵ If the rates of production and consumption of ATP are decreased similarly by temperature reduction, then we would expect ATP levels to remain fairly constant, as we had observed. Otherwise, any increase in the consumption rate must have been offset by an increase in the production rate, which seems unlikely. It thereby seems reasonable to assume that temperature reduction decreases the rate of ATP consumption in the anterior segment and, by extension, the conventional outflow pathway.

Thus, despite ATP levels being maintained, the decrease in ATP consumption imposed by temperature reduction should have had a similar metabolic effect as ATP depletion, because both processes effectively inhibit the downstream effects of ATP. To our surprise, temperature reduction had a threefold larger effect on outflow facility than metabolic inhibitors. A portion of the total effect of temperature reduction can be attributed to suppression of metabolic

activity, but even in the presence of metabolic inhibitors, temperature reduction decreased outflow facility by a further 44%. This reveals that temperature-reduction affects both metabolism (or ATP)-dependent mechanisms and ATP-independent mechanisms to impair outflow function. Understanding these separate mechanisms is important for understanding the basis for outflow resistance generation.

Implications for Outflow Resistance Generation

The current consensus attributes the majority of outflow resistance generation to flow through extracellular matrix material within the juxtacanalicular connective tissue (JCT) immediately underlying the inner wall endothelium of SC.^{56,57} Resistance generated via this mechanism should be almost entirely passive (ATP-independent) and temperature-insensitive (apart from the effects on aqueous humor viscosity). Our results are thus at odds with the view that extracellular matrix in the JCT is *solely* responsible for resistance generation. However, it is now fairly well accepted that outflow resistance generation arises because of a synergistic interaction between the inner wall and JCT.⁵⁸ Regardless of the details of any particular synergistic mechanism (e.g., funnelling^{45,59}), our results demonstrate that TM or SC cells must be directly involved in outflow resistance generation, at least in mice. In other words, flow through extracellular matrix cannot be the sole mechanism of outflow resistance generation.

TM and SC cells contribute to development and maintenance of the outflow pathway. It is thus not entirely surprising that inhibiting the metabolism of these cells would disrupt outflow function. What is surprising, however, is the timescale of the observed effects. Our results reveal that inhibiting metabolism or reducing temperature has practically an immediate inhibitory effect on outflow function. Such a fast response is inconsistent with the slower timescale of extracellular matrix remodeling and turnover, which typically operates over several hours to days.⁶⁰ This suggests that TM and SC cells may function over a fast timescale to modulate outflow resistance, with extracellular matrix providing a longer-lasting, more stable structure that provides the basis for resistance generation.^{61–63}

There are ample means by which TM and SC cells may quickly modulate outflow resistance without requiring active transport of aqueous humor per se. Focal extracellular matrix remodeling by podosome or invadopodia-like structures⁶⁴ occurs over the timescale of several minutes and would be inhibited by ATP-depletion or temperature reduction. Signaling pathways involving NO,^{21,65–67} VEGF,²² pigment epithelium-derived factor (PEDF),²³ transforming growth factor- β ^{24,68} and prostaglandins^{20,69,70} have all been shown to quickly modulate outflow resistance and require metabolic energy. Modulation of cell junctions,^{71–76} cytoskeletal contractility,^{15,77–82} cell remodelling,⁸³ and even ATP-sensitive^{42,84} or temperature-dependent^{85,86} ion channels may contribute as well. ATP depletion or temperature reduction could also affect the mechanical properties of TM and SC cells, as in other cell types,⁸⁷ to affect outflow resistance via cell or tissue stiffness or rheology.^{58,88,89} Future work should examine the basis for how inhibition of metabolism and temperature reduction in particular affects outflow resistance generation.

In conclusion, this study reveals that cells in the outflow pathway actively modulate outflow function over fast timescales through both metabolism-dependent

and metabolism-independent mechanisms. Metabolism-dependent mechanisms account for roughly one fifth of total outflow function in normal healthy adult mice, whereas metabolism-independent cellular mechanisms account for just under one half of outflow function. This challenges the prevailing view that aqueous humor outflow is largely passive, at least in mice, and further emphasizes the need to understand the mechanisms by which TM and SC cells actively modulate outflow resistance generation. This study also reveals a yet unidentified mechanism that is exquisitely temperature-sensitive but occurs independently of ATP that contributes to roughly half of outflow function.

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