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The mechanism of increasing outflow facility by rho-kinase inhibition with Y-27632 in bovine eyes

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Abstract

Rho-kinase inhibitor Y-27632 (Y-27) affects actomyosin cytoskeletal networks and has been shown to significantly increase outflow facility (C) in enucleated porcine and rabbit eyes, as well as in vivo monkey eyes without obvious toxicity. The mechanisms underlying these responses remain largely unknown. In this study, we investigate how Y-27 affects aqueous humor C, the hydrodynamic patterns of outflow, and the morphology of the inner wall (IW) and juxtacanalicular connective tissue (JCT). 12 bovine eyes were perfused at 15 mmHg with Dulbecco's PBS containing 5.5 mM glucose (DPBS) to establish stable baseline C. The anterior chamber was exchanged and perfused with DPBS containing 50 μ M Y-27 in 7 eyes, while 5 eyes received DPBS alone. Eyes were then perfused with DPBS containing fluorescent microspheres (0.5 μ m; 0.002% v/v) at a fixed volume to deliver equivalent amounts of tracer to label the hydrodynamic filtration patterns. All eyes were perfusion-fixed with Karnovsky's fixative. Radial and frontal sections were prepared in all quadrants and confocal images were taken along the IW of the aqueous plexus (AP). The total length (TL) and filtration length (FL) of the IW were measured in ≥ 16 images/eye, and the average percent effective filtration length (PEFL=FL/TL) was calculated. Sections with AP were processed and examined by light and electron microscopy. The TL of the IW and length exhibiting JCT/IW separation (SL) were measured in ≥ 16 micrographs/eye, and the average percent separation length (PSL= SL/TL) was also calculated. After Y-27 treatment, C increased from 1.54 ± 0.34 ($\pm$ SEM) to 2.36 ± 0.54 μ L/min/mmHg ($58.2 \pm 18.9\%$) while control eyes changed from 1.67 ± 0.41 to 1.71 ± 0.39 μ L/min/mmHg ($6.0 \pm 9.3\%$) and the percent changes between the Y-27-treated and control eyes were significant ($p = 0.03$).

Control eyes showed segmental distribution of tracer in the trabecular meshwork tending to cluster near collector channel ostia, whereas Y-27-treated eyes showed a more uniform pattern and extensive labeling along the IW. In Y-27-treated eyes, PEFL was 3-fold larger ($57.6 \pm 3.7\%$) compared to control eyes ($18.2 \pm 4.5\%$; $p < 0.001$). Light microscopic examination revealed that, with Y-27, the IW and JCT were significantly distended compared to control eyes, with discernible separation between the IW and JCT. PSL was 2.8-fold larger in Y-27-treated eyes ($59.3 \pm 3.6\%$) than in controls ($20.8 \pm 2.0\%$; $p < 0.001$). A significant positive correlation was found between PEFL and PSL ($p = 0.003$) suggesting that as connectivity between the JCT and IW decreases the available area for aqueous humor drainage

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increases along the AP. Our study also demonstrates a significant positive correlation between C and the PSL ($p = 0.01$), a finding identical to what we reported to occur during the “washout” effect in bovine eyes. Our data suggests the structural correlate to the increase in C and PEFL after Y-27-treatment is physical separation between the JCT and IW. The increase in C after Y-27-treatment may share a similar mechanism comparable with the washout effect that occurs in bovine eyes. Overall, these findings support our hypothesis that JCT/IW connectivity influences local outflow hydrodynamics that regulate C, and suggest that strategies targeting JCT/IW connectivity are promising anti-glaucoma therapies to reduce IOP.

Keywords

Aqueous outflow facility; Y-27632; inner wall; JCT; washout; bovine eye; trabecular meshwork; light microscopy; electron microscopy; confocal microscopy

1. Introduction

Drugs that disrupt the cytoskeleton or cytoskeletal contractility have been shown to reduce aqueous humor outflow resistance and intraocular pressure (IOP). In particular, such drugs include cytochalasin (Johnson, 1997; Kaufman and Erickson, 1982; Tian et al., 1999a), latrunculin (Ethier et al., 2006; Sabanay et al., 2006), H-7 (Bahler et al., 2004; Sabanay et al., 2004) and Y-27632 (Honjo et al., 2001; Rao et al., 2001; Tian and Kaufman, 2005; Waki, 2001), and are therefore important candidates for the next generation of glaucoma therapy. How these potential “glaucoma drugs” work to reduce outflow resistance, however, is much less understood, but determining their mechanism in the trabecular meshwork and their influence on aqueous outflow hydrodynamics is important for developing novel drugs that target specific sites of outflow limitation – namely, the inner wall endothelium of Schlemm’s canal and the underlying juxtacanalicular connective tissue (JCT) (Maepea and Bill, 1992). The overall goal of this project is to investigate how the resistance-decreasing effect of Rho-kinase inhibitor Y-27632 (“Y-27”) correlates with changes in the hydrodynamic filtration patterns and morphology within the JCT and inner wall.

Y-27 is a protein kinase inhibitor selective for Rho-associated kinase (ROCK) isoforms ROCK-I and ROCK-II (Davies et al., 2000; Ishizaki et al., 2000; Uehata et al., 1997), that regulate the phosphorylation of the regulatory myosin light chain (MLC) to promote actomyosin-driven cell contractility. Inhibiting ROCK with Y-27 decreases MLC phosphorylation by promoting MLC-phosphatase activity (Kaibuchi et al., 1999; Rosenthal et al., 2005), leading to cell relaxation and disassembly of actin stress fibers and focal adhesions in many cell types (Rao et al., 2001), including human trabecular meshwork and Schlemm’s canal endothelial cells in vitro (Honjo et al., 2001; Rao et al., 2001). In living rabbits, topical or intracameral delivery of Y-27 decreases IOP by 30–50% within 1–3 hrs (Honjo et al., 2001; Waki et al., 2001), which coincides with a 2-fold increase in trabecular outflow facility (Honjo et al., 2001). In living monkeys, perfusion with 10–100 μ M Y-27 dose-dependently increases outflow facility by 2–3 fold within 1–2 hrs (Tian and Kaufman, 2005). Similarly, Y-27-induced facility increase was reported in enucleated porcine eyes, but the magnitude of this effect was less (~80%) and saturated at 50 μ M (Rao et al., 2001).

Common morphologic features reported after drug-induced facility increase includes expansion or “ballooning” of the subendothelial extracellular matrix within the JCT, apparent separation between the JCT and the inner wall, and distension of the inner wall endothelium. These morphological changes were observed following Y-27 perfusion in porcine eyes (Rao et al., 2001) and H-7 (Sabanay et al., 2004) and latrunculin-B (Sabanay et al., 2006) in monkeys, and these changes are morphologically similar to the “matrix-matrix” type of inner wall/JCT

separation previously shown to correlate with increasing outflow facility during “washout” in bovine eyes (Overby et al., 2002; Scott et al., 2007). Conversely, enucleated human eyes show a smaller facility increase following H-7 (37%, 100 μ M) (Bahler et al., 2004) or latrunculin-B (64%, 1 μ M) (Ethier et al., 2006) and demonstrate a diminished inner wall/JCT separation compared to non-human primates that exhibit a 2 to 4-fold facility increase following perfusion with similar drug concentrations (Tian et al., 1999a; 1999b; Sabanay et al., 2000; 2004; 2006). While these differences between human and monkey eyes may be attributed to age or experimental perfusion methods, it is also likely that these differences may be related to the finding that human eyes do not exhibit inner wall/JCT separation or a facility increase after prolonged perfusion (Erickson-Lamy et al., 1990; Scott et al., 2007). These findings combined with previous reports that correlate inner wall/JCT separation with increasing outflow facility (Overby et al., 2002; Scott et al., 2007), support the notion that inner wall/JCT separation is a critical morphological mechanism for drug-induced increase in outflow facility.

In this study, we examine the hypothesis that loss of adhesion and physical separation between the inner wall and JCT induced by Y-27-treatment is a salient feature responsible for an increase in the effective filtration area of inner wall and JCT and therefore increased outflow facility. This hypothesis is based on the idea that outflow facility is proportional to the effective filtration area (EFA), such that facility increases when outflow hydrodynamics transition from a “segmental” or non-uniform pattern (corresponding to the normal state (Hann et al., 2005) to a less confined or uniform pattern that allows for wider drainage areas through the resistive tissues of the JCT and inner wall. To test this hypothesis, we introduce a newly developed method using fluorescent microspheres to visualize changing hydrodynamic patterns of outflow through the trabecular meshwork. We also identify morphologic changes that bear consistent relationships with outflow facility measurements within the same eyes to more closely investigate the underlying mechanism of how Y-27632 increases outflow facility.

2. Materials and Methods

Twelve bovine eyes were obtained from a local abattoir (Arena and Sons, Hopkinton, MA) and delivered on ice within 6 h post-mortem. Eyes with any discernible damage or accumulated blood in the angle of the anterior chamber were excluded. Each eye was confirmed to be grossly normal by examination under a dissecting microscope.

2.1. Experimental ocular perfusion

All eyes were perfused at 34°C with Dulbecco’s phosphate-buffered saline (Life Technologies, Grand Island, NY) containing 5.5 mM glucose (collectively referred to as DPBS). Y-27632 was obtained from Calbiochem, CA (Lot#: B64617). Red Fluorescent Microspheres (FluoSphere, 0.5 μ m diameter), Lot#: 41434A, from Molecular Probes, Inc., Eugene, Oregon, USA) at a concentration of 0.002% v/v (3×10^{10} #/mL) were used to trace the hydrodynamic filtration patterns. Constant pressure perfusion of bovine eyes was performed using a previously described electronic perfusion system (Erickson-Lamy et al., 1990). Briefly, intraocular pressure was maintained by a “perfusion” reservoir placed at a known height above the eye (corresponding to 15 mmHg), while the weight of the reservoir was measured using an isometric force transducer and a data acquisition computer programmed using LabVIEW (version 7; National Instruments). Perfusion flow rate (Q) was calculated from the changing weight of the perfusion reservoir, and outflow facility ($C=Q/IOP$) was continually measured at 10 Hz, ensemble averaged over a 10-second window, and electronically recorded every 10 seconds throughout the entire duration of the perfusion.

All bovine eyes were cleaned of extraocular tissue, submerged to the limbus in phosphate-buffered saline (PBS) at 34°C and draped with moist gauze. A 21-gauge infusion needle (connected to the perfusion reservoir) was inserted through the peripheral cornea with the

needle tip positioned in the posterior chamber to prevent artificial deepening of the anterior chamber (Ellingsen and Grant, 1971; Francois et al., 1955). Another needle was inserted intracamerally and connected to a second “collection” reservoir that was clamped except during anterior chamber exchange. In all perfusions we attempted to use paired bovine eyes; however, technical complications with the perfusion system, such as air bubble occlusion in perfusion lines, forced some data to be excluded. All eyes were perfused at 15 mmHg with DPBS for at least 30 minutes or until a stable baseline outflow facility was achieved. Anterior chamber contents were then exchanged (7 mL exchange volume) with either DPBS containing 50 μ M Y27 (N= 7 eyes) or DPBS alone (N = 5 eyes), followed by 0.5 mL perfusion with the same solution to hold the volume of fluid perfused constant in all eyes. To label the hydrodynamic patterns of outflow, anterior chamber contents of all eyes were then exchanged (7 mL) with DPBS containing red fluorescent microspheres (0.5 μ m, 0.002% v/v) followed by 0.5 mL perfusion of this same solution to deliver an equivalent volume of tracer to the trabecular meshwork of each eye. Eyes were then exchanged (7 mL) with DPBS to remove tracer, followed by exchange (7 mL) and perfusion (0.5 mL) with Karnovsky’s fixative (2.5% glutaraldehyde and 2% paraformaldehyde, pH 7.3). Eyes were hemisected along the equator and immersed overnight in Karnovsky’s fixative in PBS.

2.2. Confocal microscopy

Fixed anterior segments were quadrisectioned and cut into radial and frontal histological sections approximately 1–1.5 mm thick. Frontal sections were oriented tangential to the corneo-scleral limbus and perpendicular to the ocular surface (Fig. 1), as previously described (Parc et al., 2000). Sections were counter-stained with TOPRO-3 iodide (Lot#: 18C1-5, Molecular Probes, Inc., Eugene, Oregon, USA) for 30 minutes to label cell nuclei, washed 3-times in PBS, mounted and examined with a confocal microscope (Carl Zeiss 510, Axiovert 100M Laser Scanning Microscope, Heidelberg, Germany) using a 20X objective. Fluorescent images of cell nuclei and tracer patterns were taken at random points along the inner wall of the aqueous plexus (the bovine equivalent of Schlemm’s canal; Tripathi, 1971), independent of local tracer accumulation.

To examine the hydrodynamic patterns of outflow, a masked analysis of the total length of the inner wall (TL) and the “filtration length” of the inner wall (FL) – identified based upon local tracer accumulation – were measured in at least 16 images per eye from all 4 quadrants (Figure 2A). The percent effective filtration *length* (PEFL; the 1-dimensional equivalent to effective filtration *area*) was then calculated for each eye according to $PEFL = \Sigma FL / \Sigma TL$, where Σ represents the summation over N, the number of images per eye. Alternatively, PEFL could be calculated as $\frac{1}{N} \Sigma (FL/TL)$, but this definition yields results that are statistically indistinguishable from the former definition, and the former definition was used for all analysis. Inter-observer variability in PEFL was always less than 6% and typically within 2%, measured in a subset of images (N=20).

2.3. Light and electron microscopy

Tissue sections found to contain aqueous plexus and/or collector channel ostia by confocal microscopy were then processed for light and electron microscopy to more closely examine the morphology of the inner wall and JCT and the degree of inner wall/JCT separation. These sections were post-fixed in 2% osmium tetroxide and 1.5% potassium ferrocyanide for 2 hrs, dehydrated in a graded series of ethanols, and embedded in Epon-Araldite (Electron Microscopy Sciences, Hatfield, PA, USA). For light microscopy, semi-thin sections (3 μ m) were cut and stained with 1% Toluidine Blue (Fisher Scientific, Pittsburgh, PA, USA), and examined using a 20X objective. For electron microscopy, ultrathin sections (90 nm) were cut using an ultramicrotome, counterstained with uranyl acetate and lead citrate, and examined

using transmission electron microscopy (Model 300; Philips, Eindhoven, The Netherlands) at an original magnification of 3300X. Light micrographs of the inner wall were taken from all four quadrants per eye and electron micrographs were taken from two to four quadrants per eye.

2.3.1. Morphological assessment of inner wall/JCT separation—Inner wall/JCT separation was examined using both light and electron microscopy. As previously described (Overby et al., 2002; Scott et al., 2007), inner wall/JCT separation can take two distinct forms: “cell-matrix” separation between the inner wall cells and their underlying basal lamina, and “matrix-matrix” separation between the basal lamina and the extracellular matrix within the JCT (Overby et al., 2002). Using light microscopy, however, the basal lamina is not clearly resolvable, and inner wall/JCT separation was identified based upon apparent expansion of the JCT and “ballooning” of the inner wall that protrudes into the lumen of the aqueous plexus, as demonstrated in Fig. 2B and consistent with previous definitions (Scott et al., 2007). To semi-quantitatively examine changes in inner wall/JCT separation between control and Y-27-treated eyes, a trained masked observer measured the total length of inner wall (TL) and the apparent separated length of the inner wall (SL) – identified based upon local JCT expansion and inner wall “ballooning” – was measured (ImageJ 1.38X, National Institutes of Health, USA) in at least 16 light micrographs per eye from all 4 quadrants (Fig. 2B). The average percent separation length (PSL) was calculated for each eye according to $PSL = \Sigma SL / \Sigma TL$. Intra-observer variability in measured PSL was always less than 6% and typically within 2% (N=20). Separated and non-separated regions were then examined more closely to count the density of giant vacuoles along the inner wall and to distinguish between “cell-matrix” and “matrix-matrix” types of separation using electron microscopy.

2.4. Statistical methods

Two-tailed Student's *t*-test and linear regression analysis were applied with a required significance level of 0.05.

3. Results

3.1. Outflow facility

Eyes treated with Y-27 demonstrated a significant increase in outflow facility of $58 \pm 19\%$ from baseline 1.54 ± 0.34 to 2.36 ± 0.54 $\mu\text{L}/\text{min}/\text{mmHg}$ (mean $\pm$ SEM) just prior to fixation as compared to the $6 \pm 9\%$ ($p = 0.037$; 2-tailed Student's *t*-test) increase observed in control eyes (Fig. 3). The magnitude of this facility increase is comparable to the 80% facility increase observed in porcine eyes after 3 hrs perfusion (Rao et al., 2001), but was less than the 2- and 3-fold increase observed after 3 hrs in rabbits (Honjo et al., 2001) and after 60–90 min in monkeys (Tian et al., 2005), respectively. Perfusion with tracer microspheres did not appear to influence outflow facility compared to the value immediately prior to tracer perfusion ($8 \pm 7\%$, $p = 0.31$).

3.2. Tracer patterns and effective filtration length

In control eyes, confocal microscopy revealed a segmental distribution of tracer in the trabecular meshwork, with punctate labeling along the inner wall (Fig. 4A). In contrast, the tracer distribution was more uniform in Y-27-treated eyes, with extensive inner wall labeling (Fig. 4B), despite both eyes receiving an equivalent volume of tracer. Tracer accumulation tended to coincide with the presence of a collector channel ostium observed in the same or in neighboring sections (Fig. 4C & Fig 4D). Analysis of tracer patterns along the inner wall revealed a 3-fold increase in PEFL in Y-27-treated eyes (PEFL = $58 \pm 7\%$ vs. $18 \pm 5\%$; $p = 6 \times 10^{-4}$) compared to controls (Fig. 4E). There was no apparent correlation between PEFL and *absolute value* of outflow facility, measured either before or during tracer perfusion ($p > 0.38$;

Fig. 5A), but there was a positive correlation between PEFL and the *relative* facility increase (with respect to baseline) after Y-27 or sham treatment just prior to tracer perfusion ($p = 0.03$; Fig. 5B). The significance of this correlation increases when a potential outlier (indicated by arrow in Fig. 5B) is removed from the regression analysis ($p = 0.006$).

3.3. Inner wall/JCT separation and effective separation length

Light microscopy examination revealed regions of JCT expansion and inner wall “ballooning” indicative of inner wall/JCT separation that appeared to be more prevalent and more exaggerated in Y-27-treated eyes compared to controls (Fig. 6A,B). In several Y-27-treated sections, separation was sufficient for the inner wall to physically contact the outer wall, with near complete collapse of the aqueous plexus lumen. Analysis revealed that PSL was nearly 3-fold larger in Y-27-treated eyes ($59 \pm 4\%$) compared to controls ($21 \pm 2\%$; $p = 7 \times 10^{-6}$; Fig 6C), and interestingly these PSL values are similar in magnitude to the values of PEFL reported above.

Plotting PSL versus PEFL for each eye (Fig. 7) revealed two populations corresponding to the control and Y-27-treated eyes, and both populations fall approximately along the line of unit slope (actual slope = 0.90, $R^2 = 0.60$) that would indicate a one-to-one correspondence between the two variables. In sections containing a collector channel ostium, inner wall/JCT separation tended to be greater than sections lacking an ostium (PSL = $70.8 \pm 5.7\%$ vs. 56.7 ± 3.6 , $N = 112$), but this difference did not achieve statistical significance ($p = 0.06$). There was a trend that higher PSL coincided with larger *absolute values* of outflow facility measured before tracer perfusion ($p = 0.14$, Fig. 8A), and there was a significant correlation between PSL and the *relative* increase in facility (with respect to baseline) prior to tracer perfusion ($p = 0.008$; Fig. 8B).

When examined using electron microscopy, similar findings emerged. Regions of inner wall/JCT separation were identified based upon the appearance of loose or disorganized extracellular matrix within the JCT and a distended inner wall that protruded into the lumen of the aqueous plexus. Consistent with light microscopy observations, regions of inner wall/JCT separation were more frequent in Y-27-treated eyes compared to controls (Figure 9A and 9B). The morphology of the separated regions was consistent with the previously described “matrix-matrix” type of separation (Overby et al., 2002) that occurred between the basal lamina of the inner wall and the extracellular matrix of the JCT. The JCT cells in separated regions tended to remain nearer to the trabecular beams as the JCT and inner wall expanded outwards into the lumen (Figure 9C and 9D). Tracer microspheres were occasionally found underneath pore-like openings or gaps between neighboring endothelial cells along the inner wall (Figure 9E).

3.4. Giant vacuoles

Consistent with observations in porcine eyes (Rao et al., 2001), more giant vacuoles were observed per 100 μm of inner wall in Y-27-treated eyes (3.1 ± 0.2 ; $N = 112$, total # of 100 μm lengths counted) compared to control eyes (1.4 ± 0.2 ; $N = 80$, total # of 100 μm lengths counted; $p = 0.0004$). Consistent with a previous study (Parc et al., 2000), giant vacuoles were more prevalent in sections containing a collector channel ostium (3.7 ± 0.2 per 100 μm ; $N = 24$) compared to sections lacking an ostium (2.9 ± 0.3 per 100 μm ; $N = 88$, $p = 0.04$; Y-27-treated eyes only).

4. Discussion

Drugs that disrupt the cytoskeleton or actomyosin-driven cell contractility often increase aqueous humor outflow facility, but the mechanism for the increase in outflow facility increase

is largely unknown. In this study, we examined the effects of Rhokinase inhibitor Y-27632 (Y-27) on outflow facility, hydrodynamic patterns of outflow, and outflow pathway morphology in bovine eyes. There were three important observations after bovine eyes were perfused with 50 μ M Y-27: (i) a significant increase in outflow facility (~60%), (ii) a 3-fold increase in the PEFL that transitioned from a punctate to a more uniform distribution along the inner wall, and (iii) a 3-fold increase in the PSL that was characterized by increased “ballooning” of the JCT and expansion of the inner wall.

The hypothesis driving this research is that outflow facility is proportional to the effective filtration area of inner wall and JCT, such that increasing filtration area would predictably coincide with an increase in outflow facility. Furthermore, loss of adhesion and physical separation between the inner wall and JCT is hypothesized to be the salient feature responsible for the hydrodynamic redistribution of outflow patterns and increased facility. Thus, according to our hypothesis, outflow facility, outflow pathway morphology and outflow hydrodynamics are highly coupled, inter-dependent phenomena that together provide a mechanism explaining how cytoskeletal drugs influence outflow facility by concurrently affecting outflow pathway morphology and outflow hydrodynamics.

To test our hypothesis and examine outflow hydrodynamics and effective filtration area, we developed a new tracer technique using fluorescent microspheres (0.5 μ m). This technique is advantageous compared to previous tracers (e.g., cationic ferritin, colloidal gold) because outflow patterns can be visualized with minimal processing using confocal microscopy and patterns can be imaged over tissue-level dimensions that are much larger than the tracer particle size and may be more relevant for outflow hydrodynamics. In contrast, conventional tracers, such as cationic ferritin and colloidal gold (Melamed et al., 1986) require electron microscopy to resolve individual particles, are typically limited to cellular-level dimensions and thereby challenged to achieve a global appreciation of tissue-level hydrodynamics. To avoid the tendency for greater accumulation of tracer in eyes having larger facilities (and thus larger perfusion flow rates at constant IOP), the volume of tracer perfusion was held constant (0.5 ml) between all eyes (as opposed to holding constant the time duration of tracer perfusion). Like all tracer techniques, our approach implicitly assumes that tracer patterns are reliable representations of outflow hydrodynamics and that experimental treatments such as Y-27 do not significantly influence tracer properties such as binding affinity or diffusivity within tissues that would influence observed tracer patterns.

Perfusion with fluorescent tracer microspheres revealed non-uniform or “segmental” outflow patterns in the normal state of bovine eyes (without Y-27) that were generally consistent with previous descriptions of segmental outflow in human eyes reported using cationic ferritin (Ethier et al., 2001; Hann et al., 2005). These data support the idea that only a portion of the outflow pathway is actively involved in hydrodynamic filtration at any given instant, and the images generated from this study indicate that regions of tracer decoration tend to coincide with the presence of collector channel ostia (Fig. 4). Furthermore, the density of giant vacuoles – believed to be functional markers for sites of active filtration along the inner wall (Parc et al., 2000) – increased 2-fold following perfusion with Y-27, and were more prevalent nearer to collector channel ostia. Taken together, these data suggest that collector channel ostia may have an important contribution to outflow resistance by influencing the patterns of segmental outflow (Campbell et al., 1984; Tanchell et al., 1984), possibly by affecting the pressure distribution within Schlemm’s canal (Johnson and Kamm, 1983).

Following perfusion with 50 μ M Y-27, outflow facility increases by nearly 60% in bovine eyes, while at the same time tracer patterns transition from the normal “segmental” pattern to a more uniform pattern along the inner wall, coincident with a 3-fold increase in PEFL. These data show that increasing outflow facility coincides with an increase in PEFL when analyzed

over the sample population, suggestive of a positive relationship between these two parameters that is generally consistent with our hypothesis. Correlation analysis applied to individual eyes revealed a statistically significant positive correlation between PEFL and the *relative increase* in outflow facility between baseline and immediately after Y-27 or sham treatment (Fig. 5B). These data show that individual eyes having a larger facility *increase* in response to Y-27 tend to have a larger PEFL, but there was no observed relationship between PEFL and the *absolute value* of outflow facility as would be expected if facility were directly proportional to PEFL. This latter result could be due to large intrinsic variability in outflow properties between eyes or due to significant portion of outflow resistance being attributable to factors independent of PEFL. Regardless, the observed relationship between PEFL and relative facility increase in Fig. 5B is noteworthy and largely consistent with our hypothesis.

The outflow pathway morphology following Y-27-treatment in bovine eyes was similar to that induced by other cytoskeletal drugs in various species that exhibit washout effect. Following H-7 (Sabanay et al., 2000; 2004), latrunculin-B (Sabanay et al., 2006) or Y-27 in monkey (Tian and Kaufman, 2005) or porcine (Rao et al., 2001) there were fairly consistent reports of JCT expansion or inner wall “ballooning” that were morphologically similar to inner wall/JCT separation described in our current and previous studies (Overby et al., 2002; Scott et al., 2007). Closer inspection with electron microscopy revealed that the Y27-induced separation is of the matrix-matrix type previously shown to correlate with increasing outflow facility during washout in bovine eyes (Overby et al., 2002; Scott et al., 2007). Likewise, in the current study, we observed a significant correlation between PSL and the *relative* facility increase following Y-27 or sham treatment with respect to baseline, which was similar to the correlation for PEFL discussed above. Furthermore, correlation analysis of data from individual eyes revealed that PEFL is highly correlated to PSL with nearly a one-to-one relationship (Fig. 7), meaning that changes in PEFL induced by Y-27 are mirrored with nearly identical changes in PSL. This suggests that these variables are either highly dependent upon one another or depend similarly upon an unidentified third variable. Regardless, these data support the hypothesis that outflow pathway morphology, specifically inner wall/JCT separation, is tightly coupled to outflow hydrodynamics and outflow facility, but further studies are needed to determine whether this is a *causal* relationship.

Our current and previous data (Overby et al., 2002) suggest that the facility changes after Y-27-treatment and during washout in bovine eyes correspond with matrix-matrix connectivity between the inner wall and JCT. Similar changes were also found in monkey eyes but not in human eyes after short-term Y-27 perfusion (0.5ml) (Lu et al, 2007). These results suggest that the increase in outflow facility induced by the Rhokinase inhibitor Y-27 may share a similar mechanism with eyes exhibiting increased outflow facility due to “washout”. Given that the human eye does not wash-out, why should we continue to study bovine eyes, which are so different structurally from those of humans? It is important to note that, despite its striking morphological similarities to the human eye, the monkey eye behaves more like the bovine than the human in its outflow characteristics. If we fully understood the mechanisms underlying washout we could bring this knowledge to bear inducing washout in human eyes to reduce intraocular pressure in glaucoma.

In summary, we investigated hydrodynamic and morphological changes, and their relationship to aqueous outflow as a presumed mechanism for the increase in outflow facility after Y-27 treatment and compared it with our previous study that examined increasing outflow facility during washout in bovine eyes. Our study demonstrates that Y-27 significantly increases outflow facility, the percent effective filtration length, and the percent separation length along the inner wall of the aqueous plexus in bovine eyes. Significant correlations between the effective filtration length and separation of inner wall and JCT as well as between percent separated length and facility change were found. The changes in outflow facility and percentage

separation length are consistent with previous findings that correlate with increasing outflow facility during “washout” (Overby et al, 2002). Therefore, Y-27 and washout may likely act through similar mechanisms that increase outflow facility by redistributing aqueous outflow through a larger area of the inner wall and JCT. These findings support the hypothesis that inner wall/JCT connectivity influences local outflow hydrodynamics to control outflow facility. Therefore, the molecular constituents tethering the inner wall to the underlying JCT represent promising targets for new pharmacological strategies that disrupt the connectivity between the inner wall and the JCT to induce a washout-like facility increase in glaucomatous human eyes.

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Figure 1. Schematic drawing of frontal Section

The plane of the section is tangential to the corneo-scleral limbus and perpendicular to the ocular surface.

Figure 2. Measurement of percent effective filtration length (PEFL) and percent separation length of inner wall and JCT (PSL)

A. $PEFL = FL/TL$. Green line represents tracer-decorated filtration length (FL) and white line represents total length of aqueous plexus (TL). The tracer distribution was shown by pink color.
 B. $PSL = SL/TL$. Red line represents separation length (SL) and yellow line represents total length of aqueous plexus (TL).

TM: trabecular meshwork. AP: aqueous plexus.

Figure 3. Outflow facility

A: Changes in outflow facility in Y27- treated and control eyes

B: Percentage increases of outflow facility in Y27- treated and control eyes

Figure 4. Confocal microscopic analysis

- A. In a control eye, a segmental distribution of tracers (pink color) was seen along the inner wall of aqueous plexus (AP).
- B. In a Y27- treated eye, the tracer distribution (pink color) was more uniform along the inner wall of AP.
- C. In a control eye, when collector channel (CC) was seen, the segmental tracer accumulation tended to concentrate near the ostium of the CC.
- D. In a Y27- treated eye, a more uniform tracer distribution near the collector channel ostia region was observed as compared to control eyes.
- E. Percentage of Effective Filtration length in Y27- treated and control eyes.

TM: trabecular meshwork

Figure 5. Correlation between PEFL and outflow facility

A. No apparent correlation between PEFL and *absolute value* of outflow facility either before or during tracer perfusion.

B There was a positive correlation between PEFL and the *relative* facility increase (with respect to baseline) prior to tracer perfusion. The significance of this correlation increases when a potential outlier (indicated by arrow) is removed from the regression analysis

Figure 6. Light microscopic analysis

A. No separation was found between the inner wall of aqueous plexus (AP) and juxtacanalicular connective tissue (JCT) in control eyes.

B. Separation was found between the inner wall of AP and JCT (double arrow) in Y27- treated eyes.

C. Percentage of separation length in Y27- treated and control eyes

TM = trabecular meshwork

Figure 7. Correlation between PEFL and PSL

Plotting PSL versus PEFL for each eye revealed two populations corresponding to the control and Y27-treated eyes, and both populations fall approximately along the line of unit slope indicating a one-to-one correspondence between two variables.

Figure 8. Correlation between PSL and outflow facility

A. There was borderline significant correlation between PSL and the *absolute* value of outflow facility measured before tracer perfusion.

B: There was a significant correlation between PSL and the *relative* increase in facility (with respect to baseline) prior to tracer perfusion

Figure 9. Electron microscopic analysis

- A. In a control eye, the inner wall and JCT were more in contact with underlying structures, the JCT appeared compact and without separation between the inner wall and JCT.
- B. In a Y27-treated-eye, the inner wall appeared distended, the JCT was loose with significant separation between the inner wall and JCT (double arrow).
- C. In areas without separation, the connection between the inner wall and JCT cells and between the inner wall and JCT matrix were maintained.
- D. In separated areas, the connection between the inner wall and JCT cells and between the inner wall and JCT matrix were lost (double arrow).
- E. Microspheres (arrow) were often found underneath border pores or gaps in the inner wall.