Interaction Between Pilocarpine and Ripasudil on Intraocular Pressure, Pupil Diameter, and the Aqueous-Outflow Pathway

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PURPOSE. To explore interactions between pilocarpine and the ROCK inhibitor, ripasudil, on IOP and pupil diameter in human eyes, and morphological and functional changes in outflow tissues in vitro.

METHODS. IOP and pupil diameter were measured after pilocarpine and/or ripasudil, which were topically applied in healthy subjects. Human trabecular meshwork (HTM) cells were used in a gel contraction assay, for the evaluation of phosphorylation of myosin light chain and cofilin, and immunostaining for cytoskeletal proteins. Porcine ciliary muscle (CM) was used in a CM contraction assay. The permeability of human Schlemm’s canal endothelial (SCE) cells was evaluated by measuring transendothelial electrical resistance and fluorescein permeability.

RESULTS. Both pilocarpine and ripasudil significantly reduced IOP in human eyes, but pilocarpine interfered with ripasudil-induced IOP reduction when concomitantly introduced. Ripasudil significantly inhibited gel contraction, TGFβ2-induced stress fiber formation, smooth muscle actin expression, and phosphorylation of both myosin light chain and cofilin in HTM cells. Pilocarpine reduced these effects, significantly inhibited the ripasudil-induced HTM cell responses to TGFβ2 stimulation, and increased the permeability in SCE cells. In CM, ripasudil inhibited pilocarpine-stimulated contraction, but ripasudil did not have significant effects on pilocarpine-induced miosis.

CONCLUSIONS. Pilocarpine interfered with the direct effects of ROCK inhibitor on the conventional outflow pathway leading to IOP reduction and cytoskeletal changes in trabecular meshwork cells, but did not affect the relaxation effect of the ROCK inhibitor. It is therefore necessary to consider possible interference between these two drugs, which both affect the conventional outflow.

Keywords: ciliary muscle, conventional outflow, pilocarpine, ripasudil, trabecular meshwork

Intraocular pressure (IOP) reflects the balance between aqueous humor production and outflow through the conventional pathway via the trabecular meshwork (TM), and the uveoscleral pathway via the ciliary body to the suprachoroidal space. In glaucomatous eyes, IOP becomes elevated when outflow resistance increases, mainly in the TM. Pilocarpine, a parasympathetic M3 receptor agonist, rapidly increases conventional outflow attributable to indirect dilation of the flow space in the TM and Schlemm’s canal (SC).1–4 The longitudinal fibers of the ciliary muscle (CM) are anchored to the choroid, and pull on tendons extending into conventional outflow tissues, terminating in the TM and the inner walls of the SC.3,5 Thus, pilocarpine-induced contraction of the CM indirectly increases the space within the conventional pathway. The mechanism of pilocarpine-induced contraction involves drug binding to the CM via the M3 receptor followed by activation of rho-kinase (ROCK) and phospholipase C.6

The M3 receptor is also expressed by human TM (HTM) cells,7 wherein pilocarpine functions to activate Gq and the phospholipase C pathway.5 Thus, pilocarpine-induced morphological changes in TM cells, in turn, may alter the conventional outflow features.

Recently, the ROCK inhibitor, ripasudil (Glanatec; Kowa Company, Nagoya, Japan), has been launched in Japan; another ROCK inhibitor, Roclatan (Aerie, Durham, NC, USA) is now in clinical trials and Rhopressa (netarsudil 0.02%; Aerie) has been recently approved by the Food and Drug Administration as a new IOP-lowering drug for glaucoma patients. ROCK inhibitors were first reported to reduce rabbit IOP by relaxing the TM followed by expansion of the intertrabecular space via disruption of actin bundles,5,10 although other mechanisms, including degradation of the extracellular matrix and an increase in giant vacuole numbers within the SC, may also be in play.5,11,12
To date, ROCK inhibitors have been shown to exert additive effects when given together with prostaglandin analogs, beta-blockers, and carbonic anhydrase inhibitors, in efforts to increase uveoscleral outflow or decrease aqueous humor production.13–15 However, there have been no reports on the interaction between the ROCK inhibitor and pilocarpine, both of which increase the conventional outflow by different mechanisms. Pilocarpine triggers CM contraction and expands the intertrabecular space within the SC,7,8 whereas ROCK inhibitors relax TM cells and the CM.9,10 Thus, as M3 and ROCK are both present in the TM and the CM, the two drugs may exert opposite actions on these tissues, affecting the balance between contraction and relaxation, and in turn, modulating outflow and/or IOP reduction.

In the present study, we explored the interactions between pilocarpine and a ROCK inhibitor in terms of the outflow pathway. We first evaluated the IOP-lowering effect and the pupil diameter of a ROCK inhibitor, ripasudil, and the additional effect of pilocarpine in healthy human subjects. We then studied the morphological and functional changes in human TM cells, human SC endothelial (SCE) cells, and the porcine CM in vitro.

METHODS

Subjects

This study was approved by the ethics committee of the University of Tokyo and Miyata Eye Hospital, and adhered to the tenets of the Declaration of Helsinki. All subjects provided written informed consent before participation in the study.

A total of 20 healthy volunteers were recruited, and all subjects underwent a comprehensive ophthalmic examination. The criteria for inclusion of healthy subjects were as follows: at least 18 years of age; an IOP between 10 and 21 mm Hg; normal anterior chamber depth with an open angle; and no family history of glaucoma, ophthalmic diseases, surgery, or systemic diseases. The right eye of each subject was used for analyses of the IOP and pupil diameter.

IOP and Pupil Diameter Measurements After Administration of Pilocarpine and the ROCK Inhibitor, Ripasudil, in Healthy Volunteers

The study design for IOP reduction was a single blind comparative study of four regimens for 20 subjects with a single drop for one eye. The study was performed over four visits with a minimum interval of 6 days between each visit. IOP and pupil diameter were measured before (9 AM) and at 2 (11 AM), 4 (1 PM), and 8 hours (5 PM) after drug instillation. The IOP and pupil diameter were measured using a Goldmann tonometer after instillation of topical anesthesia and an autorefractometer (Tomey Corporation, Nagoya, Japan), respectively. Three measurements were obtained, and the average IOP was recorded. The pupil diameter was measured in a dark room. The subjects were divided into four groups and one of four regimens was randomly allocated for each group, with four regimens completed during the four visits. The four regimens included the baseline IOP variation without any eye drops as a control group. The study subjects were treated with 0.4% ripasudil ophthalmic solution (R-group: Sanpilo), 2% pilocarpine ophthalmic solution (P-group: Sanpilo; Santen Pharmaceutical, Osaka, Japan), or a combination of 0.4% ripasudil and 2% pilocarpine ophthalmic solution (R/P-group). One of the researchers (TK) allocated four regimens for each group. The examiners were all blinded to the allocated regimens.

Preparation of Human TM and Monkey SCE Cells, and Drug Concentrations

Primary human TM (HTM) cells and primary monkey SCE cells were isolated from human donor eyes or monkey eyes and characterized as described previously.16–18 Only well-characterized normal HTM cells from passages 3 to 8 and SCE cells from passages 3 to 5 were used in subsequent studies.

The ripasudil concentrations in aqueous humor 30 to 60 minutes after a single instillation of ripasudil ophthalmic solution (1.0%, wt/vol) were approximately 10 μg/mL in the rabbit and 1 μg/mL in the monkey, corresponding to approximately 25 μM and 2.5 μM, respectively.19 A previous report showed that the pilocarpine level in aqueous humor of the rabbit eye 30 to 60 minutes after 2% (wt/vol) pilocarpine instillation was 2.53 to 3.72 μg/mL, or approximately 92 μM.20 In addition, we have preliminarily evaluated the concentrations of 1, 10, 50, and 100 μM for both ripasudil and pilocarpine in vitro studies and investigated the dose-response and maximal effects of each drug, and then we determined the appropriate concentration of each drug. Although among species differences in aqueous humor concentrations may be in play, we chose final ripasudil concentrations between 10 and 50 μM, and a pilocarpine concentration of 100 μM for in vitro studies with cells, unless otherwise specified.

Collagen Gel Contraction Assay Using TM Cells

Collagen gel contraction assays were performed using a Cell Contraction Assay Kit (Nitta Gelatin, Inc., Osaka, Japan) as described previously.21 Ripasudil to final concentrations of 1, 10, and 100 μM, or/and pilocarpine to final concentrations of 1, 10, and 100 μM, were added to the tops of the collagen gel lattices, and gel areas were photographically recorded. The dose-dependent effects of ripasudil and pilocarpine were recorded at 1, 2, 3, 18, 24, and 48 hours, and the additive effect of 10 μM ripasudil and 100 μM pilocarpine was also investigated at 24, 48, 72, and 120 hours, and analyzed with the aid of ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The -fold area changes in the different treatment groups were recorded in bar graphs.

Immunostaining of Cultured HTM Cells

Immunostaining of cultured HTM cells was performed as described previously.19,21 After serum starvation for 24 hours, drugs were added to final concentrations of 50 μM ripasudil, 100 μM pilocarpine, and 50 μM ripasudil + 100 μM pilocarpine, and the cells were washed with PBS, and then stimulated with 5 ng/mL TGF-β2 (mimicking the pathogenic development of primary open-angle glaucoma). To identify actin bundles and cytoskeletal changes, the slides were incubated with anti-alpha smooth muscle actin (αSMA) antibody (DAKO, Tokyo, Japan), phalloidin-rhodamine (Sigma-Aldrich Corp., St. Louis, MO, USA), and anti-vinculin antibody (Sigma-Aldrich Corp.). After washing and incubation with the secondary antibody Alexa Fluor 488 (Invitrogen, Waltham, MA, USA), the slides were imaged under a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan).

Western Blotting

The expression of αSMA, and phosphorylation status of the myosin light chain (MLC) and cofilin were determined by Western blotting, using mouse monoclonal antibody against αSMA, rabbit polyclonal antibodies against phospho-MLC (Cell Signaling Technology, Danvers, MA, USA) or phosphor-cofilin...
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(Cell Signaling Technology), as previously described.\(^9\)\(^,\)\(^24\) HTM cells were cultured in six-well dishes and, after serum starvation for 24 hours, drugs were added to final concentrations of 10 or 50 \(\mu\)M ripasudil; 100 \(\mu\)M pilocarpine; and 10, 30, or 50 \(\mu\)M ripasudil \(+\) 100 \(\mu\)M pilocarpine; the cells were washed with PBS and then stimulated with 5 \(\mu\)g/mL TGF\(\beta\)2 for 24 hours. \(\beta\)-tubulin served as the loading control. All membranes were stripped of antibodies using Western blot stripping solution and incubated with mouse monoclonal antibody \(\beta\)-actin (1:1000), and subsequently with H goat anti-mouse IgG antibody (1:2000) as a loading control. Densitometry of scanned films was performed using ImageJ 1.49, and results are expressed relative to the loading control (\(\beta\)-tubulin).

**CM Contraction Assay**

CM contraction was measured as described previously.\(^24\) We used fresh porcine eyes within 2 hours after enucleation, and ciliary muscle strips were excised according to the methods described previously.\(^9\)\(^,\)\(^24\) The dissected ciliary muscle strips (approximately 8 \(\times\) 2 \(\times\) 2 mm) were placed in 10-mL tissue baths, and filled with pre-aerated Krebs’ bicarbonate solution at 37°C. The upper end of the preparation was tied to an isometric transducer and preloaded with 100 mg. The strips in tissue baths were allowed to equilibrate for at least 1 hour. After equilibration, we confirmed the contractile response of CM by 10\(^{-6}\) M carbachol. After that, the tissue bath was rinsed twice with Krebs-Henseleit physiologic solution and equilibrated again for 1 hour. The strips that sowed stable tone for after stimulation, and FITC-dextran permeability was measured in a cumulative manner in CM strips. Relaxation responses were expressed as percentages of the maximum effect (100%) elicited by pilocarpine in each strip.

**Measurement of Monolayer Transendothelial Electrical Resistance (TEER) and Permeability**

Measurement of monolayer TEER and permeability of FITC-dextran (average molecular weight, 4000; Sigma-Aldrich Corp.) of SCE cells were performed according to a previously described method.\(^12\)\(^,\)\(^16\) After serum starvation overnight, SCE cells were treated with 50 \(\mu\)M ripasudil, 100 \(\mu\)M pilocarpine, or a mixture of both. TEER was measured at 1, 4, 6, and 24 hours after stimulation, and FITC-dextran permeability was measured at 24 hours. Each experiment was performed at least four times.

**Statistics**

The summary statistics of the continuous variables of the healthy volunteers and experimental results are presented as means \(\pm\) SDs. The estimates of the mean using a mixed-effect model were presented as means estimate with 95% confidence intervals. Statistical analysis was performed with the aid of SAS Ver9.4 and JMP Pro 11 software (SAS Institute, Inc., Cary, NC, USA). The changes from baseline of the IOPs and pupil diameters in each group were evaluated using the mixed-effects model. The significance level of alpha = 0.05 was used in all statistical tests. In experimental results, Student’s \(t\)-test in combination with a Bonferroni post hoc test was used for between-group comparisons. Dunnet’s multiple comparisons test was used as a post hoc test following ANOVA to compare more than two groups. All data represent the means of at least three independent experiments. A difference was considered statistically significant at a \(P < 0.05\).

**RESULTS**

**IOP Reduction by Ripasudil and Pilocarpine in Healthy Volunteers**

Twenty healthy volunteers (3 males and 17 females) were included in the study. The mean age was 40.8 \(\pm\) 11.6 years (mean \(\pm\) SD). Baseline IOP was 14.9 \(\pm\) 2.28 mm Hg (mean \(\pm\) SD). No eyes showed adverse effects to the treatments. Time course of IOP reduction from the baseline IOP at 0 hour without any eye drops (control group) of control and three groups is shown in Figure 1A. After instillation, among three groups of R, P, and R/P, IOP reduction from the control group was significant in R at 2 hours (\(P = 0.0033\)) and in R/P at 4 hours (\(P = 0.0288\)). At 2 hours after instillation, which was the time point of the peak IOP reduction by ripasudil, a significant difference of IOP reduction was observed only between R- and P-groups (\(P = 0.0174\)), but not between R- and R/P-groups, and also not between P- and R/P-groups (\(P = 0.1017\) and \(P = 0.4553\), respectively). Comparison between groups at 2 hours showed the significant difference between control and R- (\(P < 0.0001\)), control and R/P (\(P < 0.0019\)), and R- and P-groups (\(P = 0.0005\)). At 4 hours after instillation, which was the time point of the peak IOP reduction by pilocarpine, no significant IOP reduction was observed among R-, P-, and R/P-groups. Comparison between groups at 4 hours showed the significant difference between control and R- (\(P = 0.0093\)), control and P (\(P = 0.0208\)), and control and R/P- groups (\(P < 0.0001\)). Collectively, additional instillation of pilocarpine on ripasudil did not cause a significant additional IOP reduction; on the contrary, pilocarpine compromised the ripasudil-induced IOP reduction at 2 hours after instillation in healthy human volunteers.

**Pupil Diameter Changes Induced by Ripasudil and Pilocarpine in Healthy Volunteers**

The baseline pupil diameter was 5.59 \(\pm\) 0.66 mm. Ripasudil did not affect the pupil diameter when compared with baseline. Pilocarpine caused significant miosis from 2 hours after instillation, and addition of ripasudil did not abrogate the miosis induced by pilocarpine (Fig. 1B).

**Human TM-mediated Collagen Gel Contraction**

We used the collagen gel contraction assay to explore the dose-dependence of ripasudil and pilocarpine on HTM contraction. Ripasudil at 10 and 100 \(\mu\)M significantly suppressed contraction of the collagen gel in a dose-dependent manner, especially between 24 and 48 hours (Fig. 2A). However, pilocarpine did not show any significant effect up to 48 hours (Fig. 2B). Therefore, we next explored the interaction and additional prolonged effects of 10 \(\mu\)M ripasudil and 100 \(\mu\)M pilocarpine up to 120 hours. Pilocarpine did not abrogate the relaxation by ripasudil on TM-mediated collagen gel contraction (Fig. 2C).

**Effects of Ripasudil and Pilocarpine on TGF\(\beta\)2-induced Cytoskeletal Rearrangements and Colocalization of \(\alpha\)SMA to Stress Fibers in HTM Cells**

HTM cells were stained with an anti-\(\alpha\)SMA antibody to evaluate the extent of the epithelial-mesenchymal-transition-like phenomenon (indicated by a green color). Phalloidin-rhodamine...
was used to highlight the F-actin cytoskeleton (red), an anti-vinculin antibody (green) to reveal focal adhesions, and 4',6-diamidino-2-phenylindole (DAPI) to identify the nucleus (blue).

In line with a previous report, and our HTM collagen gel contraction assay results, ripasudil inhibited TGFβ2-dependent actin stress fiber formation without compromising cell viability (data not shown).25 Pilocarpine did not affect TGFβ2-mediated actin bundle polymerization. As previously observed,26,27 in the context of the TGFβ2-dependent myofibroblast-like transition of HTM cells, TGFβ2 induced significant increases in αSMA and vinculin accumulation, and in the numbers of actin stress fibers, compared with the control values (Figs. 3A, 4). Ripasudil completely blocked the increases in αSMA and vinculin accumulation; however, pilocarpine did not significantly affect TGFβ2-induced cytoskeletal changes. Concomitant addition of ripasudil and pilocarpine slightly reduced the TGFβ2-induced myofibroblast-like transition, and the cytoskeletal changes, compared with those observed after addition of ripasudil alone.

Western blotting was used to detect αSMA in efforts to explore this interaction between ripasudil and pilocarpine in terms of the TGFβ2-dependent myofibroblast-like transition of HTM cells (Fig. 3B). TGFβ2 significantly induced αSMA expression (an increase of approximately 340%; P < 0.05), whereas 50 μM ripasudil significantly suppressed such expression (P < 0.05), but concomitant addition of ripasudil and pilocarpine mitigated the reduction in TGFβ2-induced αSMA expression induced by ripasudil alone (Fig. 3B, lower panel). Ripasudil completely inhibited the TGFβ2-induced recruitment of vinculin to the ends of actin stress fibers, but concomitant addition of ripasudil and pilocarpine mitigated the reduction in TGFβ2-induced vinculin accumulation induced by ripasudil alone (Fig. 4, arrowheads). Together, the data suggest that pilocarpine compromises the effects of
FIGURE 2. Human TM-mediated collagen gel contraction. The dose-dependent effects of ripasudil (A) and pilocarpine (B) on TM-mediated contraction were investigated using a collagen gel contraction assay. The additive effect of 10 μM ripasudil and 100 μM pilocarpine was also investigated (C). Affected gel areas were photographed at 1, 2, 3, 18, 24, and 48 hours for (A) and (B) and at 24, 48, 72, and 120 hours for (C). Data are expressed as means ± SD (n = 4 per time point). *P < 0.05 or **P < 0.01; control versus each group; Dunnett’s test.
Effect of Ripasudil and Pilocarpine on MLC and Cofilin Phosphorylation

The MLC and cofilin play crucial roles in regulating dynamic rearrangements of the cytoskeleton. Phosphorylation of these proteins stabilizes actin filaments and triggers the formation of stress fibers. To explore the details of how pilocarpine compromises the effects of ripasudil on the cytoskeletal changes in HM cells observed by immunostaining, we used Western blotting to detect phosphorylated MLC (p-MLC) and phosphorylated cofilin (p-cofilin). As shown in Figure 5, TGFβ2 induced significant phosphorylation of MLC (an increase of approximately 250% \( P < 0.01 \)) compared with the control, and 10 \( \mu M \) and 30 \( \mu M \) ripasudil significantly inhibited this induction \( P < 0.05, P < 0.01 \), respectively). However, pilocarpine had no effect on MLC phosphorylation, and concomitant ripasudil (10 \( \mu M \)) and pilocarpine (100 \( \mu M \))

**Figure 3.** Effect of ripasudil and pilocarpine on TGFβ2-induced αSMA expression and colocalization of αSMA and stress fibers in HTM cells. After serum starvation for 24 hours, cultured TM cells were pretreated with 50 \( \mu M \) ripasudil, 100 \( \mu M \) pilocarpine, or 50 \( \mu M \) ripasudil + 100 \( \mu M \) pilocarpine for 1 hour and then stimulated with 5 ng/mL TGFβ2 for 24 hours before immunostaining for αSMA (green) and F-actin (red) (A). Cell nuclei were counterstained with DAPI (blue). Scale bar: 100 \( \mu M \). The upper panel shows representative Western blots of αSMA in (B). Lower panel in (B) is a relative αSMA level, in which the data are shown as means ± SE, \( n = 3 \). * \( P < 0.05 \), compared with control; Student’s t-test.

**Figure 4.** Effect of ripasudil and pilocarpine on TGFβ2-induced cytoskeletal rearrangements in HTM cells. After serum starvation for 24 hours, cultured TM cells were pretreated with 50 \( \mu M \) ripasudil, 100 \( \mu M \) pilocarpine, or 50 \( \mu M \) ripasudil + 100 \( \mu M \) pilocarpine for 1 hour, and then stimulated with 5 ng/mL TGFβ2 for 24 hours before immunostaining for vinculin (green) and F-actin. White arrowheads: vinculin staining. Cell nuclei were counterstained with DAPI (blue). Scale bar: 60 \( \mu M \).

**Effects of Ripasudil and Pilocarpine on MLC and Cofilin Phosphorylation**

The MLC and cofilin play crucial roles in regulating dynamic rearrangements of the cytoskeleton. Phosphorylation of these proteins stabilizes actin filaments and triggers the formation of stress fibers. To explore the details of how pilocarpine compromises the effects of ripasudil on the cytoskeletal changes in HM cells observed by immunostaining, we used Western blotting to detect phosphorylated MLC (p-MLC) and phosphorylated cofilin (p-cofilin). As shown in Figure 5, TGFβ2 induced significant phosphorylation of MLC (an increase of approximately 250%; \( P < 0.01 \)) compared with the control, and 10 \( \mu M \) and 30 \( \mu M \) ripasudil significantly inhibited this induction \( P < 0.05, P < 0.01 \), respectively). However, pilocarpine had no effect on MLC phosphorylation, and concomitant ripasudil (10 \( \mu M \)) and pilocarpine (100 \( \mu M \))
downregulated p-MLC production. Thus, pilocarpine compromised the ripasudil-induced effect (Fig. 5A). Similar results were observed when phosphocofilin levels were assayed, but the differences were not significant (Fig. 5B).

Effect of Ripasudil on Pilocarpine-induced Contraction of CM

It is well known that pilocarpine induces CM contraction. When ripasudil was added to muscle strips precontracted with pilocarpine (10⁻⁵ M), muscle contraction was inhibited in a concentration-dependent manner (Fig. 6). Ripasudil at both 10⁻⁵ and 10⁻⁴ M significantly inhibited contraction compared with the control value (P < 0.01, t-test).

Effects of Ripasudil and Pilocarpine on Barrier Function and Permeability of SCE Cell Monolayers

To evaluate the barrier function and permeability of the SCE cell monolayer, we measured TEER. Following treatment with 50 µM ripasudil, FITC-dextran permeability was significantly increased at 24 hours after treatment. Addition of 100 µM pilocarpine slightly attenuated this increased FITC-dextran permeability induced by ripasudil treatment (Fig. 7A). We then evaluated the effects of ripasudil and pilocarpine on TEER, and found that TEER was significantly decreased by 50 µM ripasudil treatment in a time-dependent manner until 4 hours after drug treatment (Fig. 7B). Although treatment with pilocarpine alone induced no significant difference in TEER when compared with the control, the decrease of TEER by ripasudil was significantly attenuated by concomitant treatment of ripasudil and pilocarpine at 4 hours after treatment (Fig. 7B). These observations suggest that simultaneous administration of pilocarpine inhibited the ripasudil-induced decrease in barrier function of the cultured SCE cell monolayers. The Table summarizes the interactions between these two drugs.

DISCUSSION

We first explored the interactions between two antiglaucoma drugs with different mechanisms of action, but the same target, which are used to increase conventional outflow to reduce IOP, and that cause morphological changes in TM, SC, and CM. Pilocarpine induces CM contraction via the muscarinic M3 receptor.
Effects of ripasudil, pilocarpine, and ripasudil/pilocarpine on FITC-dextran permeability and transendothelial electrical resistance in SCE cell monolayers. (A) Ripasudil increased the permeability of FITC-dextran in SCE cell monolayers. The increase of permeability by ripasudil was slightly attenuated \((P = 0.192\) ripasudil versus ripasudil-pilocarpine with Student’s \(t\)-test) by concomitant treatment of ripasudil and pilocarpine. Data are expressed as mean ± SD \((n = 4–5/\text{column})\). *Indicates \(P < 0.05\); control versus the ripasudil-treated group using Student’s \(t\)-test. (B) Ripasudil decreased TEER significantly, and the decrease of TEER by ripasudil was significantly attenuated by concomitant treatment of ripasudil and pilocarpine at 4 hours \((P = 0.044\). Data are expressed as mean ± SD \((n = 4–5/\text{column})\). * and ** indicate \(P < 0.05\) or \(P < 0.01\) when comparing the control versus the ripasudil group using Student’s \(t\)-test. # indicates \(P < 0.05\) when comparing ripasudil only versus ripasudil + pilocarpine.

**Figure 7.** Effects of ripasudil, pilocarpine, and ripasudil/pilocarpine on FITC-dextran permeability and transendothelial electrical resistance in SCE cell monolayers. (A) Ripasudil increased the permeability of FITC-dextran in SCE cell monolayers. The increase of permeability by ripasudil was slightly attenuated \((P = 0.192\) ripasudil versus ripasudil-pilocarpine with Student’s \(t\)-test) by concomitant treatment of ripasudil and pilocarpine. Data are expressed as mean ± SD \((n = 4–5/\text{column})\). *Indicates \(P < 0.05\); control versus the ripasudil-treated group using Student’s \(t\)-test. (B) Ripasudil decreased TEER significantly, and the decrease of TEER by ripasudil was significantly attenuated by concomitant treatment of ripasudil and pilocarpine at 4 hours \((P = 0.044\). Data are expressed as mean ± SD \((n = 4–5/\text{column})\). * and ** indicate \(P < 0.05\) or \(P < 0.01\) when comparing the control versus the ripasudil group using Student’s \(t\)-test. # indicates \(P < 0.05\) when comparing ripasudil only versus ripasudil + pilocarpine.

**Table. Summary of Interactions Between Ripasudil and Pilocarpine**

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<th>Measurements</th>
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*+, significant interaction; +, slight interaction; −, no interaction; PD, pupil diameter.

* Ripasudil relaxed CM contraction induced by pilocarpine.
To investigate why the two drugs interfere in IOP reduction, we next explored the effects of these drugs on TM pathophysiology, including cytoskeletal rearrangements and myofibroblast-like transition. TGFβ2 was used to mimic glaucoma of HTM cells.7,8 As shown in Figure 2, collagen gel contraction and M3-HTM cells was completely blocked by the ROCK inhibitor but not pilocarpine, which also did not abrogate the ripasudil effect. Next, we explored TGFβ2-induced cytoskeletal rearrangements (including actin stress fiber assembly, vinculin recruitment, and myofibroblast-like transition). TGFβ2-induced cytoskeletal and fibrotic changes were completely abrogated by the ROCK inhibitor (Fig. 3). Pilocarpine induced cell contraction and did not affect the TGFβ2-dependent contraction, cytoskeletal rearrangements, or myofibroblast-like transition of HTM cells, even when added with ripasudil (Figs. 3, 4).

The cytoskeleton is the key framework for many cell activities, and cytoskeletal alterations may effectively control aqueous humor outflow resistance.7,8 To clarify the molecular mechanisms of such cytoskeletal interference, and drug dose-dependency, we explored the phosphorylation status of the MLC and coflin, both of which play crucial roles in cytoskeletal regulation: phosphorylation stabilizes actin filaments and triggers stress fiber formation. Ripasudil significantly inhibited TGFβ2-dependent MLC and coflin phosphorylation, correlating with the decrease in actin stress fiber levels evident on immunostaining. In contrast, pilocarpine per se did not exert any significant effect, but compromised the effects of 10 μM ripasudil on TGFβ2-dependent MLC and coflin phosphorylation when concomitantly applied (Fig. 5). However, no significant interference was apparent between 30 μM ripasudil and pilocarpine. Thus, dose levels must be carefully considered in clinical situations.

We also assessed the effect of the ROCK inhibitor on pilocarpine-induced contraction of CM. As was noted in TM cells, higher concentrations of ripasudil completely relaxed CM contraction (Fig. 6). Ripasudil has been reported to bind strongly to melanin, and significantly higher ripasudil levels (approximately 16-fold those in the aqueous humor) have been observed in the iris-ciliary body of pigmented rabbits, even after a single instillation.19 Therefore, it is possible that relaxation induced by ripasudil overcomes CM contraction induced by pilocarpine. The actual drug concentrations may differ in vivo; further studies are required to determine whether our in vitro observations are relevant in vivo.

The SCE is known to be an important ocular component producing outflow resistance against aqueous humor in the conventional outflow route, and junctional protein complexes in SCE cells create a barrier against aqueous humor outflow. The Rho/ROCK signaling pathway is reportedly involved in regulating SCE permeability.12,18 In this study, to evaluate the barrier function of SCE, we measured FITC-dextran permeability and TEER in a confluent SCE cell monolayer, and observed that treatment with ripasudil increased the permeability and decreased the TEER when compared with control levels (Fig. 7), which was consistent with previous reports.12,18 As expected, pilocarpine did not affect permeability or TEER, which is reasonable because pilocarpine is known to expand SC by the contraction of the CM in the eye.29,35 However, we found that a decrease of TEER induced by ripasudil was significantly attenuated by concomitant treatment with pilocarpine. Although the precise mechanisms should be further explored, this observation may be important for clinical practice because it suggests that pilocarpine may have effects against SCE cells without the involvement of CM contraction.

Collectively, our results suggest that signals downstream of TGFβ2 and M3 are shared (at least in terms of Rho activation) in HTM cells and CM, and possibly in SCE cells.40–42 Many studies have found that TGFβ2-induced changes in the fibrogenic and contractile properties of HTM cells are mediated in part by activation of Rho and Rho-kinase, which may explain the pathobiology of IOP elevation in glaucoma patients.27–29,34–36 Because ROCK inhibitors regulate Rho/ROCK-dependent phosphorylation of the MLC kinase, TGFβ2- or pilocarpine-induced contraction of the TM or CM may be almost completely inhibited by a ROCK inhibitor. ROCK inhibitors are known to relax the smooth muscles; therefore, we assumed if such an inhibitor relaxes the CM, iris sphincter muscles might be affected by the drug. However, the ROCK inhibitor did not affect the pupil diameter in human eyes (Fig. 1B). One reason for this may be that the local doses afforded by eye drops may differ from the doses used in vitro. Another reason may be a difference in pharmacological affinity of the ROCK inhibitor for TM and CM, and iris sphincter muscles. Pilocarpine reportedly produces a greater TM contraction than a CM contraction. CM contraction depends almost entirely on calcium, but TM contraction uses both calcium-dependent and calcium-independent pathways.25,26 Additionally, higher levels of mRNAs for ROCK and ROCK substrates were found in the TM when compared with CM.30 and Y27632 is known to have a faster and more potent effect on TM cells when compared with CM.47 The detailed expression of ROCK in iris tissue and effects of ROCK inhibitor on iris sphincter muscles are unclear, and further studies will be needed.

Our study had several limitations. First, we studied only short-term responses to the drugs. Both pilocarpine and ROCK inhibitors can affect the extracellular matrix,28,36,42,48 so a longer period of evaluation after dosing is required. Second, the doses we used in vitro may differ from those in the eye. Thus, some serious issues may yet require attention. Third, we only evaluated the IOP and pupil diameter in healthy volunteers, with relatively lower baseline IOP. Tissue responses to pilocarpine or ROCK inhibitors may differ in the presence and absence of ocular hypertension, and such future studies would be of particular interest. Finally, in vitro studies allowed us to observe the cellular functions of conventional outflow tissues. In the future, optical coherence tomography should be used to analyze the in vivo interactions of the TM, SC, and CM.

In conclusion, the interactions of two glaucoma drugs, pilocarpine and a ROCK inhibitor, both affecting the conventional outflow pathway, were explored using in vivo IOP measurements in human eyes and in vitro cell and tissue culture studies. Pilocarpine and the ROCK inhibitor did not additively reduce IOP. Pilocarpine inhibited the effects of the ROCK inhibitor in TM cells, SCE cells, and CM. These findings suggest that modification of Rho/ROCK signals may be a feasible means by which to increase conventional outflow.

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