Vascular Normalization by ROCK Inhibitor: Therapeutic Potential of Ripasudil (K-115) Eye Drop in Retinal Angiogenesis and Hypoxia

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Purpose. In this study, we investigated the therapeutic potential of a Rho-associated coiled-coil–containing protein kinase (ROCK) inhibitor ripasudil (K-115) eye drop on retinal neovascularization and hypoxia.

Methods. In vitro, human retinal microvascular endothelial cells (HRMECs) were pretreated with ripasudil and then stimulated with VEGF. ROCK activity was evaluated by phosphorylation of myosin phosphatase target protein (MYPT)-1. Endothelial migration and cell viability were assessed by cell migration and MTT assay, respectively. The concentration of ripasudil in the retina was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In vivo, normal saline, 0.4%, or 0.8% ripasudil were administered three times a day to mice with oxygen-induced retinopathy (OIR). The areas of neovascularization and avascular retina were also quantified with retinal flat-mounts at postnatal day (P) 15, P17, or P21. The retinal hypoxic area was evaluated using hypoxia-sensitive drug pimonidazole by immunohistochemistry at P17. The vascular normalization was also evaluated by immunohistochemistry at P17.

Results. Ripasudil but not fasudil significantly reduced VEGF-induced MYPT-1 phosphorylation in HRMECs at 30 μmol/L. Ripasudil significantly inhibited VEGF-induced HRMECs migration and proliferation. The concentration of ripasudil in the retina was 3.8 to 10.4 μmol/L and 6.8 to 14.8 μmol/L after 0.4% and 0.8% ripasudil treatment, respectively. In the 0.4% and 0.8% ripasudil treated OIR mice, the areas of neovascularization as well as avascular area in the retina was significantly reduced compared with those of saline-treated mice at P17 and P21. Pimonidazole staining revealed that treatment with 0.4% and 0.8% ripasudil significantly inhibited the increase in the hypoxic area compared with saline. 0.8% ripasudil could cause intraretinal vascular sprouting and increase retinal vascular perfusion.

Conclusions. Novel ROCK inhibitor ripasudil eye drop has therapeutic potential in the treatment of retinal hypoxic neovascular diseases via antiangiogenic effects as well as vascular normalization.

Keywords: rho kinase, diabetic retinopathy, VEGF, neovascularization, MYPT-1, pimonidazole

Retinal hypoxia is often found in retinal diseases such as retinopathy of prematurity (ROP) and diabetic retinopathy (DR).[1] The hypoxia and the accompanying neovascularization are major causes of vitreous hemorrhage and tractional retinal detachment, which are a leading cause of vision loss.

Vascular endothelial growth factor (VEGF) has been revealed to play a central role in hypoxia-related neovascularization of retinal diseases.[2] In recent years, anti-VEGF agents have been widely used for these pathological conditions.[3] Intravitreal injections of anti-VEGF therapy was reported to inactivate retinal angiogenesis via vessel contraction and an anti-inflammatory effect.[4–6] However, intravitreal injections of anti-VEGF therapy have several drawbacks such as high cost, ocular pain, endophthalmitis, cardiac infarction, and cerebral stroke.[3] Furthermore, several studies have reported that anti-VEGF treatment could cause retinal ischemia and fibrovascular membrane contraction.[7,8]

Rho-associated coiled-coil–containing protein kinase (ROCK) is a downstream effector of the small GTP-binding protein RhoA.[9,10] ROCK is known to be activated by various cytokines including VEGF.[11,12] ROCK is implicated in various cardiovascular diseases such as arteriosclerosis, vasospasm, and hypertension.[13] Selective ROCK inhibitor fasudil has already been used in clinical practice for these disease states.[14] Fasudil is also reported to dilate retinal vessels and attenuate retinal ischemia in rats.[15] Our studies have demonstrated the role of ROCK activation in the pathogenesis of diabetic retinopathy such as retinal endothelial injury and proliferative membrane contraction in vitro and in vivo.[16–18] Furthermore, we previously reported ROCK inhibition blocked VEGF-induced

angiogenesis in a corneal model.[12] Recently, we also demonstrated that ROCK is a key molecule in exudative AMD.[19] In our clinical observations with DR patients, intravitreal ROCK inhibitor fasudil combined with anti-VEGF agents also improved diabetic macular edema, which was refractory to anti-VEGF therapy.[20] From these results, ROCK is expected to be a novel therapeutic target for retinal diseases.

A novel, potent and selective ROCK inhibitor, ripasudil hydrochloride hydrate (K-115), has been developed and the steric affinity of the enzyme for ROCK was enhanced by the drug's structural change.[21] The enzyme inhibitory effect of ripasudil is approximately 5 to 10 times higher than previous ROCK inhibitors such as fasudil. It is recently reported that ripasudil has neuroprotective effects for retinal ganglion cells after systemic administration.[22] A previous study with radiolabeled drug revealed that ripasudil could reach the retina and choroid after eye drop administration in rabbits.[21] However, it has not been examined if ripasudil topical treatment shows the inhibitory effect on retinal disorders. In this study, we evaluate the effect of topical ripasudil treatment on pathological retinal neovascularization and hypoxia in the mouse oxygen-induced retinopathy (OIR) model.

Materials and Methods

Materials

Primary normal human retinal microvascular endothelial cells (HRMECs) and CSC (Cell System Corporation) Complete Medium were purchased from Cell Systems (Kirkland, WA, USA). Ripasudil, a novel ROCK inhibitor, was provided from Kowa Company, Ltd. (Nagoya, Japan). Fasudil was obtained from LC Laboratories (Woburn, MA, USA). Human recombinant VEGF was acquired from R&D Systems (Minneapolis, MN, USA). For Western blotting, primary antibody rabbit phosphomyosin phosphatase target protein (MYPT)-1 (Thr853) and rabbit anti–MYPT-1 were obtained from Cell Signaling (Beverly, MA, USA). The rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). For the evaluation of retinal hypoxia, pimonidazole (Hypoxyprobe) and primary antibody (rabbit anti-pimonidazole antisera) were purchased from Hypoxyprobe, Inc. (Burlington, MA, USA). Alexa Fluor 488-conjugated anti-rabbit pAb, Alexa Fluor 546-conjugated anti-rat pAb and DAPI (4,6-diamidino-2 phenylindole) were obtained from Molecular Probes (Eugene, OR, USA). Alexa Fluor 647-conjugated anti-rabbit pAb was obtained from Life Technologies (Gaithersburg, MD, USA). FITC-conjugated anti-lectin antibody and fluorescence labeled concanavalin A were obtained from Vector Laboratories (Burlingame, CA, USA). Rat anti-mouse CD31 antibody was obtained from BD Bioscience (San Jose, CA, USA). Anti-NG2 chondroitin sulfate proteoglycan antibody was obtained from Millipore (Billerica, MA, USA).

ROCK Activity in HRMECs

Human retinal microvascular endothelial cells were cultured in CSC Complete Medium before experiments. Human retinal microvascular endothelial cells were seeded in a 6-well plate at 2×10^6 cells/well and incubated for 12 hours to adhere to the wells. To elucidate the effect of ripasudil on ROCK activation, ripasudil was added to the wells at the final concentrations of 0.3, 3, and 30 μmol/L, and fasudil was added at a final concentration of 30 μmol/L, and the cells were incubated for 3 hours. The ROCK inhibitors were prepared with distilled water as the solvent of the compound. Distilled water was also used as control. Then, human recombinant VEGF was added at the final concentration of 25 ng/mL. The supernatant of the medium was removed 3 hours later and HRMECs lysates were prepared for Western blotting. The whole-cell lysate sample in lysis buffer were resolved by electrophoresis, followed by Western blotting on Whatman PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). MYPT-1 is a downstream target of ROCK. ROCK activity is generally quantified by the amount of phosphorylated MYPT-1. Phospho MYPT-1 and GAPDH were detected by the binding of the primary antibody rabbit phospho MYPT-1 (1:1000) and the rabbit GAPDH (1:1000), and the subsequent binding of the secondary antibody anti-rabbit HRP conjugate secondary antibody (1:4000; Bio-rad, Hercules, CA, USA) as previously described.[15] The membranes were also reblotted with rabbit anti-MYPT-1 (1:2000). The ROCK activity was calculated as a percentage of the value of phospho MYPT-1/GAPDH of each group when the value of phospho MYPT-1/GAPDH of a sample without VEGF stimulation was defined as 100%. The ROCK activity in HRMECs without ripasudil was set as 100% (control value), and that without VEGF stimulation was set as 0% (normal value). The reaction rate (% of control) of each concentration of ripasudil was calculated, and the 50% inhibitory concentrations (IC50) were determined.

Cell Viability

Human retinal microvascular endothelial cells were seeded in a 96-well plate at 1×10^3 cells/well and were incubated for 12 hours to adhere to the wells. To examine the effect of ripasudil on HRMECs viability, ripasudil was added at final concentrations of 3 and 30 μmol/L, and fasudil was added at a final concentration of 30 μmol/L, and the plates were incubated for 3 hours. Human recombinant VEGF was added at a final concentration of 25 ng/mL and incubated for 24 hours. MTT assay was performed by using MTT reagent from Cell Quaint-MTT Assay Kits (BioAssay Systems, Hayward, CA, USA), and incubated for 4 hours. The medium was removed and was replaced by an MTT solubilization solution, then the mixture was allowed to react for 30 minutes at room temperature. The color reaction was measured by reading the absorbance at 540 nm on a plate reader. Cell viability was evaluated as a percentage of absorbance of each group when the absorbance of a sample without VEGF stimulation was defined as 100%.

Cell Migration

Human retinal microvascular endothelial cells were seeded in a 96-well plate equipped with a cell seeding stopper of Oris Universal Cell Migration Assembly Kit (PLATYPUS Technologies, Madison, WI, USA) at 1×10^5 cells/well and were allowed to adhere to the wells with 12 hours of preincubation. Ripasudil was added at final concentrations of 3 and 30 μmol/L, and fasudil was added at a final concentration of 30 μmol/L, and the plates were incubated for 3 hours. The stopper was removed, and human recombinant VEGF was added at a final concentration of 25 ng/mL and incubated for 24 hours. Calcein-AM was added to the plate and incubated for 30 minutes. Measurements were made at the excitation wavelength at 490 nm and the fluorescence wavelength at 540 nm using a fluorescence plate reader. Cell migration was evaluated as the percentage of fluorescence intensity of each group when the fluorescence of a sample without VEGF stimulation was defined as 100%.

Concentration of Ripasudil in the Posterior Segment of Mice

This experiment was performed to confirm the presence of the pharmacologically active form of ripasudil in the retina and the

choroid after instillation to neonatal mice. A single dose of 0.4% ripasudil ophthalmic solution or a single dose of 0.8% ripasudil ophthalmic solution was instilled into the eyes of C57BL/6J mice (Charles River Japan, Inc., Yokohama, Japan) on postnatal day 12 (P12). Mice were euthanized at 15 and 30 minutes and 1, 4, and 8 hours, and both eyes were enucleated. Each enucleated eyeball was washed quickly in physiologic saline and the anterior eye was cut out with scissors, and then the retina and choroid were harvested. After the samples of retina and choroid were homogenized and deproteinized with acetonitrile, the concentration of ripasudil was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Mouse Model of OIR and Eye Drop Treatment

C57BL/6JJcl mice were purchased from Kyudo Company (Tosu, Saga, Japan). All experimental procedures on the animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The retinal nonperfusion area or neovascular lesions in the angiogenesis model has been commonly evaluated by flat-mount. Oxygen-induced retinopathy was induced in C57BL/6JJcl mice as described.[23] Mice were reared in a 75 ± 2% oxygen air from P7, and then moved into room air on P12. Then, a physiological saline solution, a 0.4% ripasudil ophthalmic solution (12.37 mM), a 0.4% fasudil ophthalmic solution (13.73 mM), or a 0.8% ripasudil ophthalmic solution (24.74 mM) was applied to both eyes 3 times daily for 3, 5, or 9 days (P12–P14, P12–P16, or P12–P20, respectively). The amount of administered eye drop was approximately 38 µl. On P17, ROCK activity in the retina was evaluated by Western blotting, and retinal avascular area, neovascular area, and the hypoxic area were evaluated on flat-mount. On P15 or P21, retinal avascular area and neovascular area were also evaluated.

Intravitreal Treatment

Vehicle, ripasudil, or fasudil that was dissolved with 0.1-mL intraocular irrigating solution (Opeguard-MA; Senju Pharmaceutical, Osaka, Japan) at a final intravitreal concentration of 0 (vehicle) or 30 µmol/L were injected intravitreally at P12, just prior to being exposed to the hyperoxygen atmosphere, and at P15. Eyes were enucleated at P17.

ROCK Activity in OIR Retina

Retinal lysates (P17) for Western blotting were prepared as previously described.[19] The cell lysate samples were resolved by electrophoresis followed by Western blotting as described above. The ROCK activity was calculated as a percentage of the value of phospho MYPT-1/total MYPT-1 of each group when the value of phospho MYPT-1/total MYPT-1 of a sample with saline treatment was defined as 100%.

Evaluation of the Retinal Avascular Area and Neovascular Area by Flat-Mount

Mice were euthanized with an overdose of pentobarbital, and both eyes were enucleated. The eyes were fixed with 4% paraformaldehyde (PFA) at 4°C for 1 hour. Then, the corneal limbus was incised circumferentially, and the cornea and the iris were removed from the eyeball. Subsequently, after fixation with 4% PFA at 4°C for 1 hour, the lens, the sclera, and the choroid were removed, and the retina was isolated from the eye cup. Then the retina was additionally fixed with 4% PFA at 4°C for 3 hours, and then washed. Tissue was blocked (1% BSA, 0.5% Triton X-100 in PBS) at 4°C for 1 hour, and treated with 0.7% FITC-conjugated anti-lectin antibody in PBS (Vector Laboratories) at 4°C overnight. Tissues were washed four times for 20 minutes in PBS. Flat-mount retinal preparations were prepared by making four to six radial incisions to the eye cup. Retinal flat-mounts were prepared on glass slides using a mount Perma-fluor (Lab Vision Corporation, Fremont, CA, USA). The flat mounts were examined by fluorescence microscopy and digital images were recorded using a fluorescent microscope (BZ-9000; KEYENCE, Tokyo, Japan) with standardized illumination and contrast. The ratios of the avascular area and the neovascular area against the area of the whole retina were calculated according to the equations below using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The obtained values were converted to percentages by defining the value for the physiological saline-treated group as 100%. Avascular area represents the area of avascular area/area of the whole retina. Neovascular area represents the neovascular area/area of the whole retina.

Evaluation of Retinal Hypoxic Area by Hypoxyprobe

The mice (P17) were treated with intraperitoneal administration of pimonidazole at 80 mg/kg. Forty-five minutes after, the mice were euthanized with an overdose of pentobarbital. The eyes were enucleated and fixed with 4% PFA at 4°C for 1 hour. The corneal limbus was then incised circumferentially, and the cornea and the iris were removed from the eyeball. Subsequently, after fixation with 4% PFA at 4°C for 1 hour, the lens, the sclera, and the choroid were removed, and the retina was isolated from the eye cup. Then the retina was additionally fixed with 4% PFA at 4°C for 3 hours, and then washed. Tissue was blocked with 3% skim milk for 30 minutes, and treated with a primary antibody (rabbit anti-pimonidazole antisera) at 4°C overnight. After treatment with a secondary antibody (Alexa Fluor 488-conjugated anti-rabbit pAb) at room temperature for 1 hour, counterstaining of nuclei was performed with DAPI. For quantification, the flat mounts were prepared as described above. The flat mounts were examined by fluorescence microscopy and digital images were recorded using a fluorescent microscope, and the hypoxic area was measured using ImageJ software.

Physiological Vascular Development

For eye drop treatment, the eye lid was forcibly opened and a physiological saline solution or a 0.8% ripasudil ophthalmic solution was applied to both eyes three times daily from P4 to P6. At P7, the flat mounts were prepared for the estimation of vascular area as described above.

Evaluation of Vascular Normalization in OIR Retina

Oxygen-induced retinopathy mice with saline or 0.8% ripasudil treatment three times a day for 5 days (P12–P16) were anesthetized deeply (P17). The mice were perfused with fluorescence labeled concanavalin A (100 µg/mL in PBS) through the left ventricle and both eyes were then enucleated. Retinal flat mounts were prepared as mentioned previously. Endothelial cells were labeled with CD31 (1:100) and pericytes were labeled with NG2 (1:100). The flat mounts were examined by a confocal microscope (A1; Nikon, Tokyo, Japan) with standardized illumination and contrast. A z stacks of 55 photomicrographs 0.5 µm in thickness were taken, extending through 27 µm of the retinal vasculature. Three-dimensional (3D) reconstruction and analysis of z stacks were performed with NIS-Element (Nikon).

TUNEL Assays

Eyes of P17 control mice and OIR mice treated with saline or 0.8% ripasudil treatment (10 eyes each) were enucleated and embedded in OCT compound (4583; Sakura Finetech, Tokyo, Japan) and kept at –80°C until sectioning. Then, 10-μm thick sections were cut with a cryostat and placed on glass slides. Apoptotic cells were detected by the Apop Tag Plus fluorescein In Situ Apoptosis Detection Kit (S7110; Millipore, MA, USA) according to instructions of the manufacturer. We compared the number of apoptotic cells in the P17 control mice with that in the OIR mice with saline or 0.8% ripasudil treatment three times a day for 5 days from P12. Because the number of cells in each slide varied depending on the cutting angle, the number of apoptotic cells also varied. To avoid this sampling artifact, three sections for each eye specimen were randomly selected (10 eyes for each group).

Statistical Analysis

All results were expressed as mean ± SEM. All statistical analysis was performed with JMP version 10 (SAS Institute, Inc., Cary, NC, USA). Before statistical analysis, normal distribution was tested. In vitro, with regard to statistical analysis, the comparison between the VEGF-nontreated normal group and the VEGF-treated control group was made using the Student's *t*-test, and the comparison between the treatment groups and the VEGF-treated control group was made using the Dunnett's multiple comparison test. In vivo, the Dunnett's multiple comparison test was used for statistical analysis.

Results

Suppression of VEGF-Induced ROCK Activation by Ripasudil in Retinal Endothelial Cells

It is unknown whether VEGF causes ROCK activation in retinal endothelial cells. We first examined if VEGF could induce the activation of ROCK signaling in HRMECs. To examine the effect of ripasudil on VEGF-induced ROCK activation, Western blotting was performed with phosphorylated MYPT-1, a downstream target of ROCK (Fig. 1A). The quantitative analysis showed significant increase in ROCK activity by VEGF stimulation could be observed compared to the unstimulated control group (1.79-fold, $P < 0.05$; Fig. 1B). We next examined the effect of ripasudil as well as fasudil on VEGF-induced ROCK activation in HRMECs (Fig. 1, $n = 10$ each). Ripasudil tended to inhibit VEGF-induced ROCK activation from 0.3 μmol/L (28.3% reduction of the increase in VEGF-treated controls compared with the unstimulated control group, NS, not significant), 3 μmol/L (75.9% reduction, NS), and significantly inhibited at 30 μmol/L (112.7% reduction, $P < 0.05$; Fig. 1B). On the other hand, at 30 μmol/L fasudil showed less but not significant reduction in phosphorylated MYPT-1 in HRMECs. The IC50 of ripasudil for ROCK activity in HRMEC was 1.1 μmol/L. This result indicates that ripasudil had inhibitory effects for VEGF-induced activation of ROCK.

Suppression of VEGF-Induced Cell Properties by Ripasudil

To investigate the impact of ripasudil on VEGF-induced retinal endothelial cell properties, we examined HRMECs viability and migration with the MTT assay and the migration assay, respectively. The effects of ripasudil on cell viability were shown in Figure 1C ($n = 5$ each). Cell proliferation was increased by VEGF stimulation compared with the nontreated normal group (1.43-fold, $P < 0.01$). Ripasudil showed a significant inhibitory activity against cell proliferation induced by VEGF stimulation at concentrations of 3 μmol/L (130.2% reduction of the increase in VEGF-treated controls compared with the nontreated normal group, $P < 0.05$) and 30 μmol/L (148.7% reduction, $P < 0.05$). Fasudil at a concentration of 30 μmol/L also demonstrated significant inhibition (138.1% reduction, $P < 0.05$). The effects of ripasudil on cell migration were shown in Figure 1D ($n = 6$ each). A significant increase in the cell migration by VEGF stimulation was observed compared with the nontreated control group (1.74-fold, $P < 0.01$). Ripasudil at concentrations of 3 μmol/L and above showed a significant inhibitory activity against cell migration induced by VEGF stimulation compared with the nontreated control group. Ripasudil significantly inhibited VEGF-induced cell migration at 3 μmol/L (61.4% reduction of the increase in VEGF-treated controls compared with the nontreated normal group, $P < 0.05$) and 30 μmol/L (101.3% reduction, $P < 0.01$). Fasudil at a concentration of 30 μmol/L also showed significant inhibition (54.3% reduction, $P < 0.05$). These results revealed that ripasudil could inhibit both VEGF-induced proliferation and migration by retinal endothelial cells.

Inhibitory Effect of Ripasudil in ROCK Activation of OIR Mice

We next examined the effect of ripasudil eye drop on ROCK activation in the OIR mouse retina. First of all, to confirm that the drug could reach the posterior segment of the eye in sufficient concentration after the topical treatment, the concentration of the drug was measured in the retina as well as the choroid from neonatal mice using LC-MS/MS. The concentration of ripasudil in the retina was 3.8 to 10.4 μmol/L and 6.8 to 14.8 μmol/L after topical 0.4% and 0.8% ripasudil treatment, respectively (Fig. 2A). The choroidal concentration was 50.5 to 107.7 μmol/L and 69.8 to 209.3 μmol/L after 0.4% and 0.8% ripasudil treatment, respectively (Fig. 2B). Furthermore, we confirmed that the phosphorylation of MYPT-1 in the retina of the OIR mice was downregulated by ripasudil eye drop treatment (Fig. 2C). Treatment with 0.4% ripasudil ophthalmic solution tended to inhibit ROCK activation (13.1% reduction compared with the vehicle-treated group, NS), and treatment of 0.8% ripasudil ophthalmic solution significantly inhibited ROCK activation in the OIR mice retina (34.5% reduction, $P < 0.05$; Fig. 2D). Next, to examine the effect of fasudil eye drop, we tried to prepare 0.4% and 0.8% fasudil ophthalmic solution. However, fasudil could not be dissolved completely at a concentration of 0.8% at 5°C (data not shown). Therefore, we examined the effect of 0.4% fasudil eye drop on ROCK activation in the retina of OIR mice. Western blots showed that 0.4% fasudil eye drop treatment did not affect ROCK activation (Figs. 2E, 2F). These data indicate that 0.8% ripasudil but not 0.4% fasudil topical treatment by eye drop penetrated to the retina at sufficiently high concentrations to inhibit ROCK activation in the OIR mouse model.

Impact of Ripasudil Eye Drop on the Retinal Avascular Area and Neovascular Area

To examine the effect of ripasudil eye drop on the retinal avascular area as well as neovascular area in the OIR mouse model, we administered ripasudil eye drop three times a day in OIR mice (P12–P16). The retinal avascular area at P17 in the OIR mice was significantly attenuated at 0.4% ripasudil ophthalmic solution (23.0% reduction compared with saline-treated group, $P < 0.01$) and 0.8% ripasudil ophthalmic solution (41.8% reduction, $P < 0.01$; Figs. 3A, 3B, $n = 11$–14 each). Moreover, treatment with 0.4% ripasudil ophthalmic solution and 0.8% ripasudil ophthalmic solution significantly inhibited the increase in the neovascularization area compared to saline-treated group at P17 (17.8% and 29.9% reduction, $P < 0.05$ and $P < 0.01$, respectively; Figs. 3A, 3C, $n = 11$–14 each). However, treatment of 0.4% fasudil eye drop did not affect the avascular area or neovascularization in the OIR model (Figs. 3D–F). These data suggest that ripasudil eye drops but not

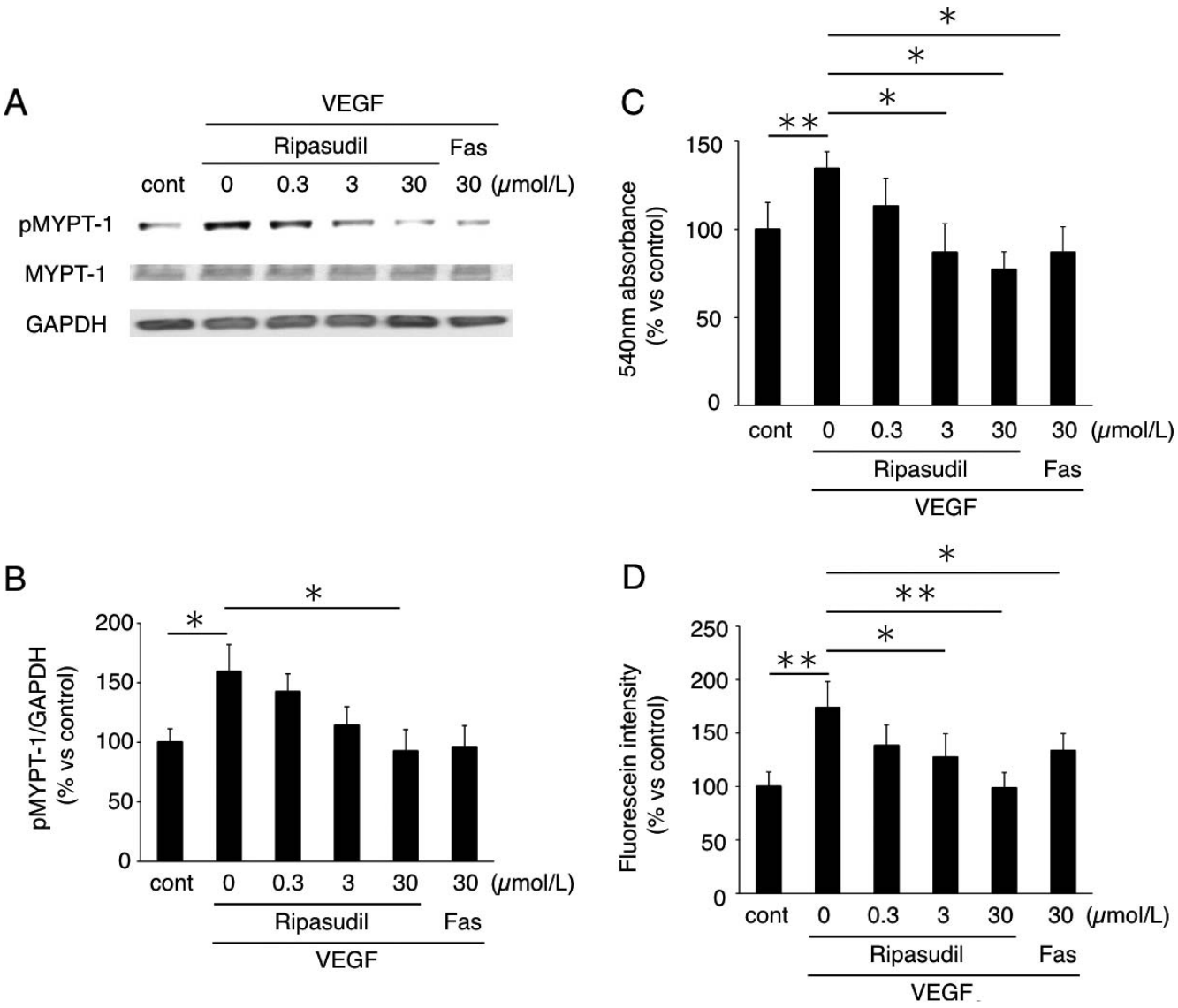

FIGURE 1. Inhibitory effect of ripasudil on VEGF-induced ROCK activation, viability, and migration in retinal endothelial cells. (**A**) Representative Western blot of antiphospho–MYPT-1, anti–MYPT-1, and anti-GAPDH with ripasudil or fasudil treatment in VEGF-stimulated HRMECs. Human retinal microvascular endothelial cells were treated with vehicle, ripasudil, or fasudil at the indicated concentration and stimulated with human VEGF (25 ng/mL). Lane loading differences were normalized by reblotting the membranes with an Ab against GAPDH. (**B**) Average signal intensities quantified and expressed as percentage of the ratio of control without any stimulation. Values are means ± SEM from 10 independent experiments. (**C**) MTT assay by HRMECs (1×10^3 cells) with vehicle, ripasudil, or fasudil at the indicated concentration and stimulated with human VEGF (25 ng/mL) for 24 hours (OD 540 nm; $n = 5$). $^*P < 0.05$; $^{**}P < 0.01$. (**D**) The in vitro cell migration was measured using the Oris 96-well cell migration assay kit. Migration by HRMECs (1×10^5 cells) with vehicle, ripasudil, or fasudil at the indicated concentration and stimulated with human VEGF (25 ng/mL) for 24 hours. Quantitation of the migrated cell number by fluorescence intensity ($n = 6$). $^*P < 0.05$; $^{**}P < 0.01$.

fasudil eye drops could have an antiangiogenic effect as well as the beneficial impact on the avascular area during retinal angiogenesis.

Impact of Intravitreal ROCK Inhibitor Treatment on the Retinal Avascular Area and Neovascular Area

To examine the effect of intravitreal ROCK inhibitor treatment on the retinal avascular area as well as neovascular area in the OIR mouse model, we administered ripasudil as well as fasudil in OIR mice at 30 μmol/L concentration, which was previously shown to inhibit ROCK activation in different animal models.[19,24] Intravitreal treatment of both ROCK inhibitors inhibited the retinal avascular area (fasudil, 33.9% reduction and ripasudil, 32.3% reduction, $P < 0.05$) as well as neovascular area significantly at P17 (fasudil, 45.4% and ripasudil, 47.0% reduction, respectively, $P < 0.05$; Supplementary Fig. S1).

Impact of Ripasudil Eye Drop on Hypoxic Area of the OIR Retina

To investigate if ripasudil topical treatment could minimize the extent of hypoxia in the retina, we performed the immunohistochemistry of the hypoxia-sensitive drug pimonidazole in OIR mice with or without the treatment. Pimonidazole serves to define and visualize tissue hypoxia. In the OIR mice retina, pimonidazole staining was detected in the inner layer at the

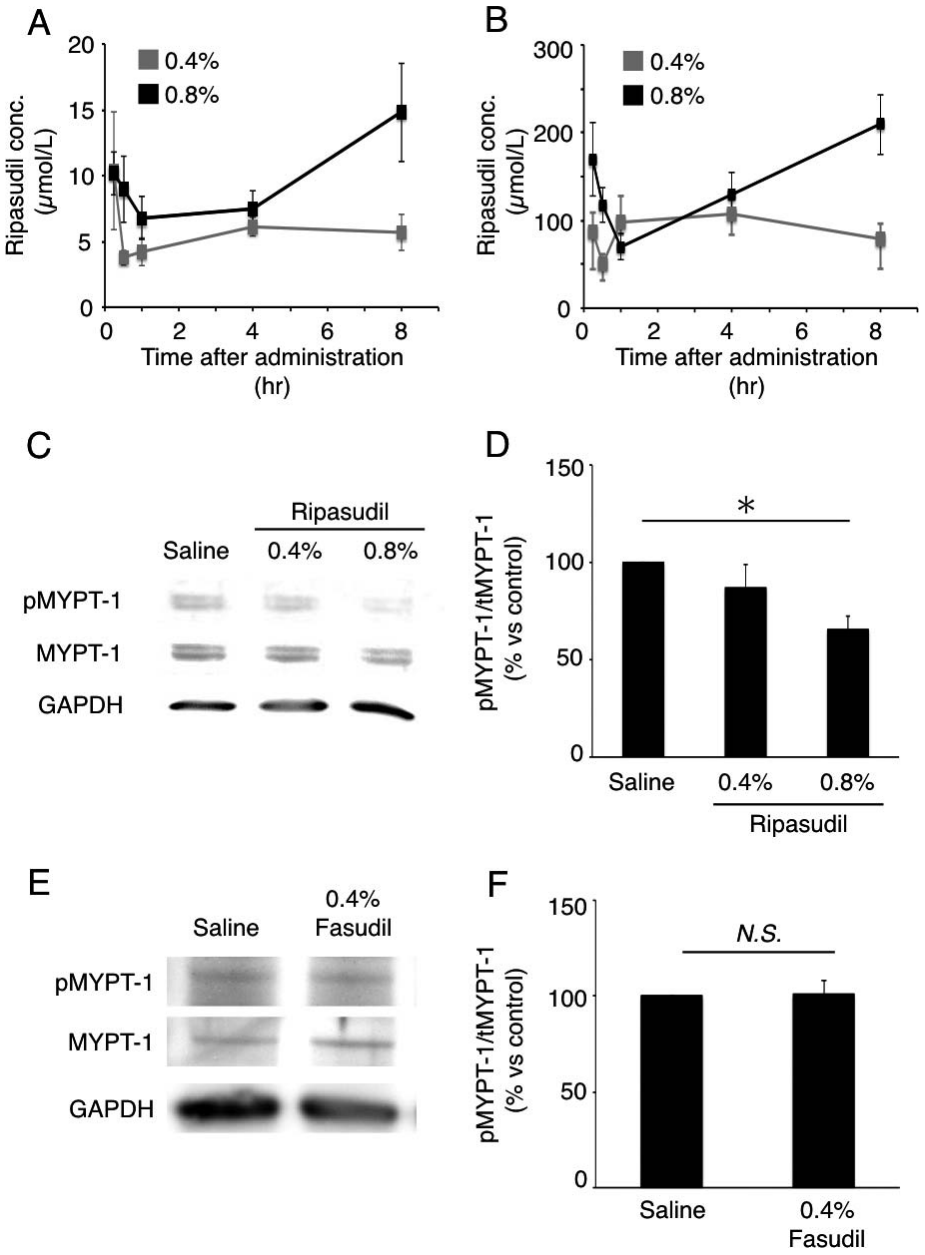

FIGURE 2. Inhibitory effect of topical ripasudil but not fasudil in ROCK activation of OIR mice. (**A**, **B**) Eye tissue concentration-time profile of ripasudil after single instillation to normal mice on P12 in (**A**) retina and (**B**) choroid. Each point represents the mean ± SEM ($n = 5$–6). (**C**, **D**) Western blot analysis in whole-cell lysates of OIR retina with saline, 0.4% ripasudil or 0.8% ripasudil treatment three times a day for 5 days (P17). (**C**) Representative Western blot of anti–phospho MYPT-1, anti–MYPT-1, and anti-GAPDH in retinal samples from OIR model. (**D**) Average signal intensities quantified and expressed as percentage of the ratio of control with saline treatment. Values are means ± SEM from 12 independent experiments. (**E**, **F**) Western blot analysis in whole-cell lysates of OIR retina with saline, 0.4% fasudil treatment three times a day for 5 days (P17). (**E**) Representative Western blot of anti–phospho MYPT-1, anti–MYPT-1, and anti-GAPDH in retinal samples from OIR model. (**F**) Average signal intensities quantified and expressed as percentage of the ratio of control with saline treatment. Values are means ± SEM from 10 independent experiments. $^*P < 0.05$ versus saline group.

level of the ganglion cell layer and in cells within the inner nuclear layer (Fig. 4A), whereas pimonidazole was not detected in untreated healthy control mice (Fig. 4B). To perform the quantitative analysis, we stained the whole mount of OIR retina with pimonidazole. Treatment with 0.4% and 0.8% ripasudil ophthalmic solution significantly inhibited the increase in the hypoxic area compared with the treatment with saline (Fig. 4C). These results indicate ripasudil eye drop treatment could inhibit the hypoxia that induces retinal angiogenesis.

Time-Dependent Impact of Ripasudil Eye Drop on the Retinal Avascular Area

The OIR model is known to show that proliferation of neovascularization with normal vessel regrowth occurs at P12 to P17, whereas regression of neovascularization with normal vessel regrowth occurs at P17 to P25.[23] To confirm the observation that ROCK inhibition could cause blocking of neovascularization, reduced avascular area, and reduced hypoxic area at the same time (P17), we first investigated time-dependent

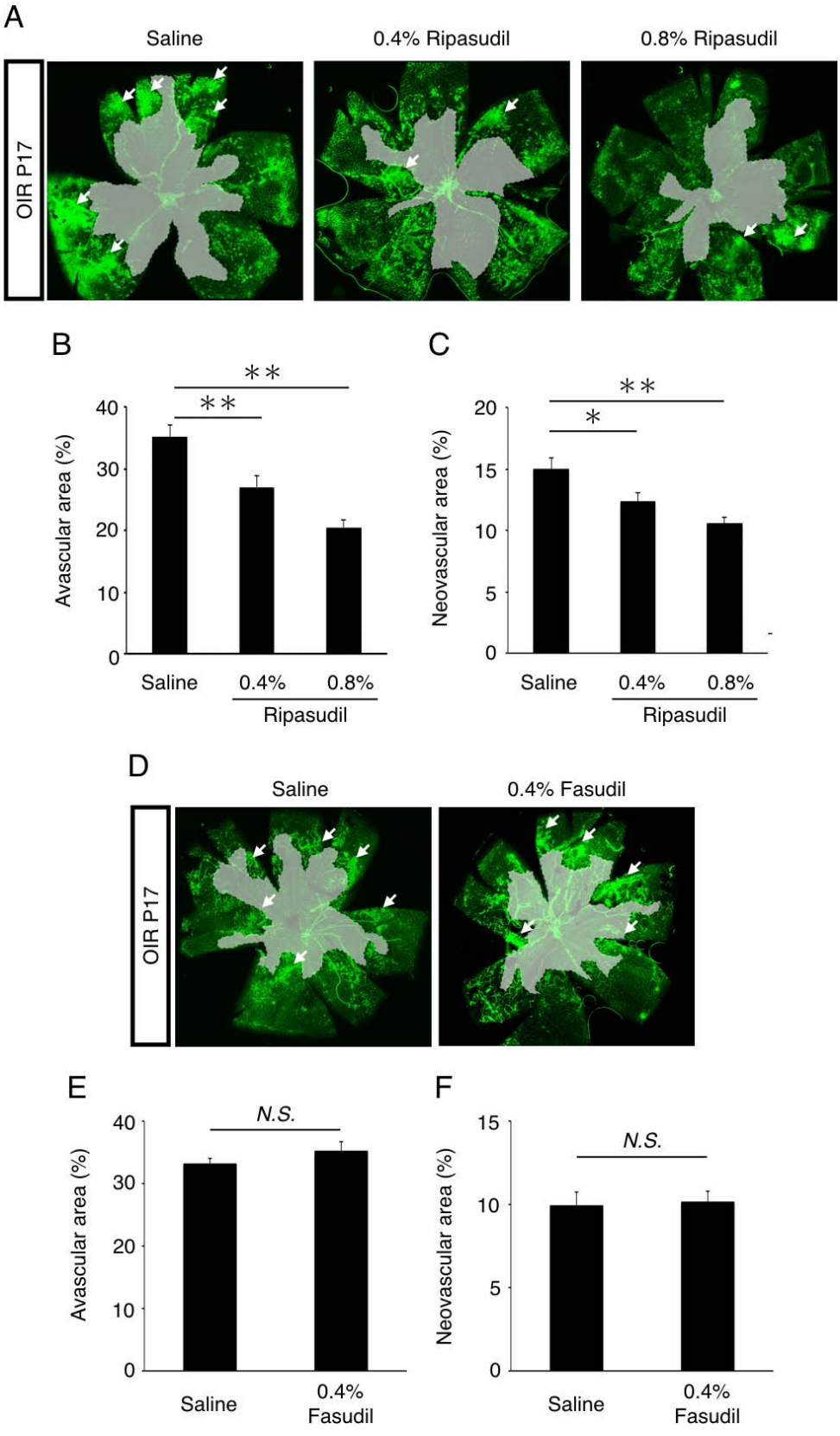

Figure 3. Impact of ripasudil eye drop on the retinal avascular area and neovascular area in OIR model. (**A**) Representative images of a flat-mounted OIR retina with saline, 0.4%, or 0.8% ripasudil treatment three times a day for 5 days (P17). *Arrows* indicate retinal neovascularization. *Gray areas* indicate avascular area. (**B**, **C**) Quantitation of avascular area (**B**) or neovascular area (**C**) with saline, 0.4%, or 0.8% ripasudil treatment (P17; $n = 11$–14). (**D**) Representative images of a flat-mounted OIR retina with saline or 0.4% fasudil treatment three times a day for 5 days (P17). *Arrows* indicate retinal neovascularization. *Gray areas* indicate avascular area. (**E**, **F**) Quantitation of avascular area (**E**) or neovascular area (**F**) with saline or 0.4% fasudil treatment (P17; $n = 13$–14). Values are means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group.

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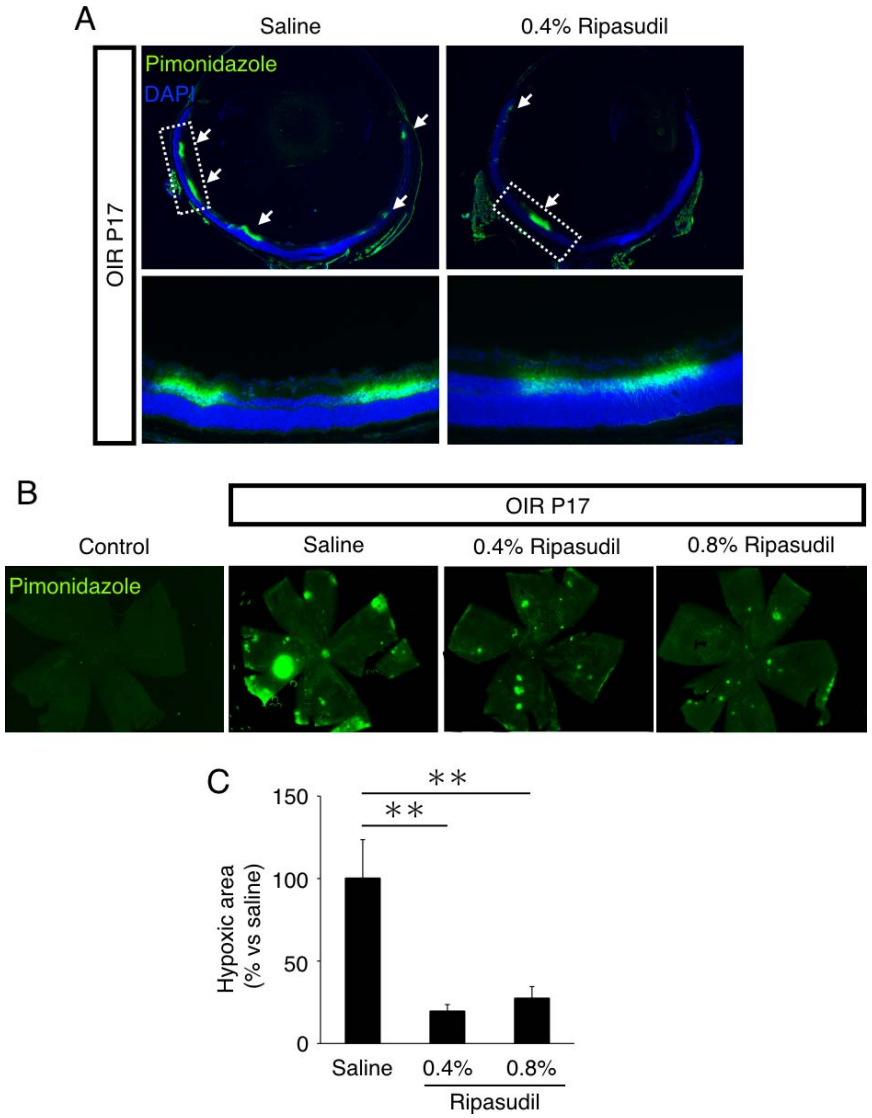

Figure 4. Impact of ripasudil eye drop on hypoxic area of the OIR retina. (**A**) Representative cross-sectional images of antipimonidazole (hypoxia) staining from the retina of OIR mice with saline or 0.4% ripasudil. *Arrows* indicate stained hypoxic areas. *Lower panels* (*dotted line square area* at the high magnification) shows pimonidazole staining was detected in the inner retinal layers. (**B**) Representative flat-mount images of antipimonidazole (hypoxia) staining from the retina of healthy control mouse, the retina of OIR mouse with saline, 0.4%, or 0.8% ripasudil. (**C**) Quantitation of pimonidazole positive areas with saline, 0.4%, or 0.8% ripasudil treatment (P17; $n = 10$–11). Values are means ± SEM; $^{**}P < 0.01$ versus saline group.

efficacy of ripasudil in the OIR model. Ripasudil ophthalmic solution treatment at 0.8% (P12–P14) did not affect avascular area at P15. The treatment showed slight but not significant inhibitory effect on neovascular area. On the other hand, the avascular and neovascular area at P21 was significantly reduced by 0.8% ripasudil eye drop (P12–P21; Figs. 5A–C). Furthermore, 0.8% ripasudil had no effect on physiological vascular development at P7 (Figs. 5D, 5E). These data confirm ROCK inhibition by ripasudil might inhibit neovascularization from a relatively earlier stage and promote normal vessel regrowth at the late phase during pathological conditions but not physiological conditions.

Vascular Normalization by Ripasudil Eye Drop in the OIR Retina

In cancer, it has been reported that certain antiangiogenic therapy could cause "vascular normalization" in tumor.[25] Furthermore, the concept has been recently reported in ischemic retinopathy.[26] Therefore, we hypothesized that reduced hypoxic area with blocking of neovascularization in ROCK inhibition could be due to vascular normalization in the OIR retina. The vertical location of blood vessels in saline- and ripasudil-treated retina was examined. The confocal images showed neovascular tufts existed at the higher level (to the vitreous side) than intraretinal vasculature in both groups (Figs. 6A, 6B). On the other hand, most vascular tips could be observed at the lower level than vascular tufts in the ripasudil-treated retina, whereas vascular tips in control retina were at the higher level (Fig. 6A). To confirm vascular normalization by ripasudil treatment, the pericyte coverage and perfusion area in retinal vessels were examined with NG2 immmunohistochemistry and lectin perfusion, respectively. The percentage of pericyte coverage (NG2+ area/CD31+ area) was significantly higher in ripasudil-treated retinas than in saline-treated retinas.

The percentage of perfused blood vessels (Lectin+ area/CD31+ area) in ripasudil-treated retina was also significantly

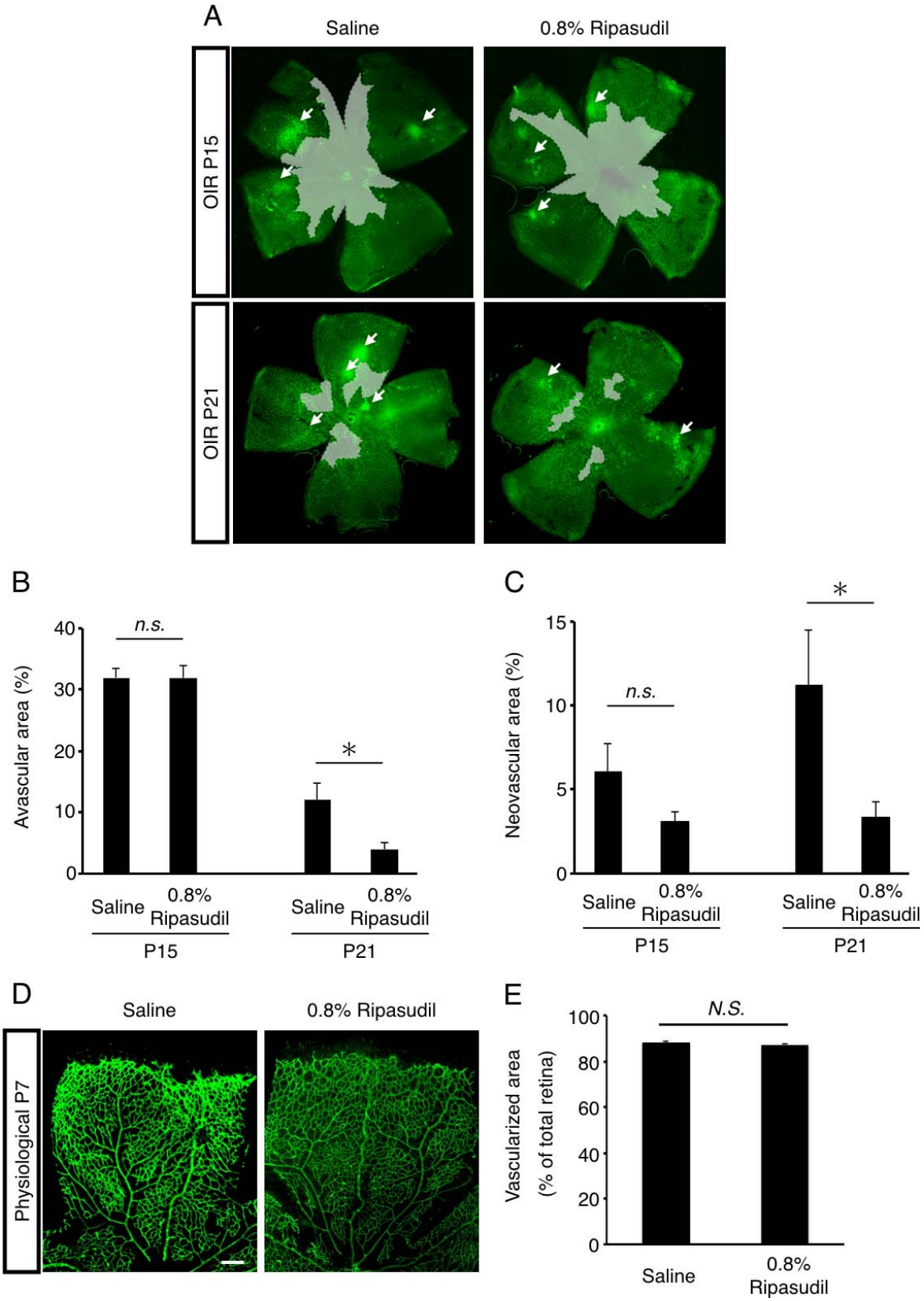

Figure 5. Impact of ripasudil eye drop on vascular development in pathological and physiological conditions. (**A**) Representative images of a flat-mounted OIR retina with saline or 0.8% ripasudil treatment three times a day from P12 (P15 and P21). *Arrows* indicate retinal neovascularization. *Gray areas* indicate avascular area. (**B**, **C**) Quantitation of avascular area with saline or 0.8% ripasudil treatment (P15 and P21; $n = 8$–10). (**D**) Representative images of a flat-mounted WT retina with saline, 0.8% ripasudil treatment three times a day for 3 days (P7). *Scale Bar*: 200 μm. (**E**) Quantitation of vascularized area with saline or 0.8% ripasudil treatment (P7; $n = 8$). Values are means ± SEM; $^*P < 0.05$ versus saline group.

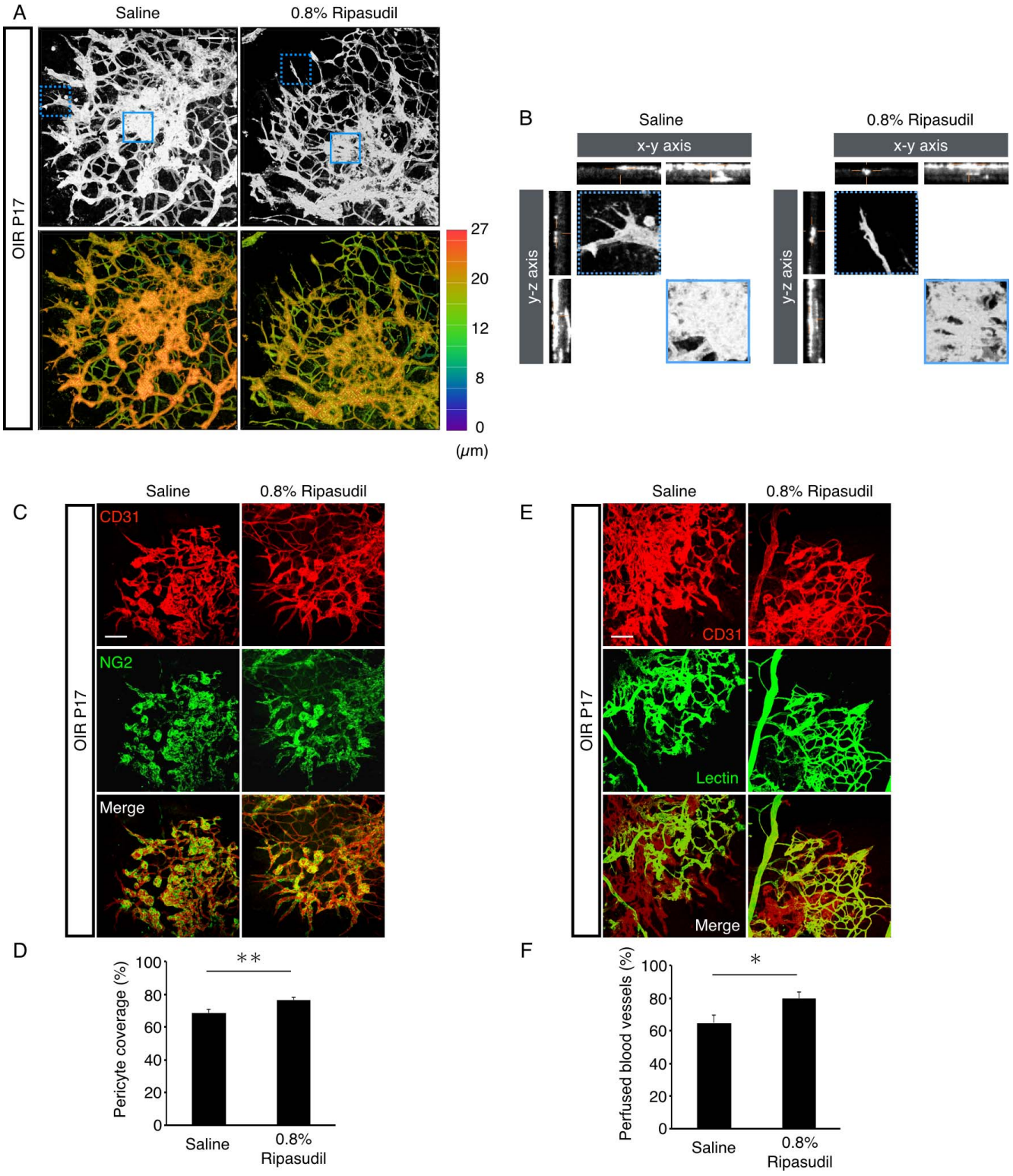

Figure 6. Vascular normalization by ripasudil eye drop in the OIR retina. (**A**) *Upper*: representative 3D images of a flat-mounted OIR retina from P17 OIR mice with saline or 0.8% ripasudil treatment three times a day for 5 days (P12–P16) with CD31 staining (*white*) in *x-y* axis. *Lower*: representative heat map of 3D images in *x-y* axis. *Scale bars*: 100 μm. (**B**) The photomicrographs are in *y-z* axis and *x-z* axis. *Blue squares* indicated neovascular tufts and *dotted line squares* indicated angiogenic fronts in (**A**). (**C**, **D**) Representative confocal images and quantitation of immunofluorescence staining for endothelial cells (CD31; *red*) and pericytes (NG2; *green*) in a flat-mounted OIR retina from P17 OIR mice with saline or 0.8% ripasudil treatment three times a day for 5 days ($n = 19$–20). Pericyte coverage index (%) was calculated from NG2+ area/CD31+ area. *Scale bars*: 100 μm. (**E**, **F**) Representative confocal images and quantitation of immunofluorescence staining for endothelial cells (CD31; *red*) and perfused blood vessels (FITC-lectin; *green*) in a flat-mounted OIR retina from P17 OIR mice with saline or 0.8% ripasudil treatment three times a day for 5 days ($n = 10$–13). Perfused blood vessels index (%) was calculated from lectin+ area/CD31+ area. *Scale bars*: 100 μm. Values are means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ versus saline group.

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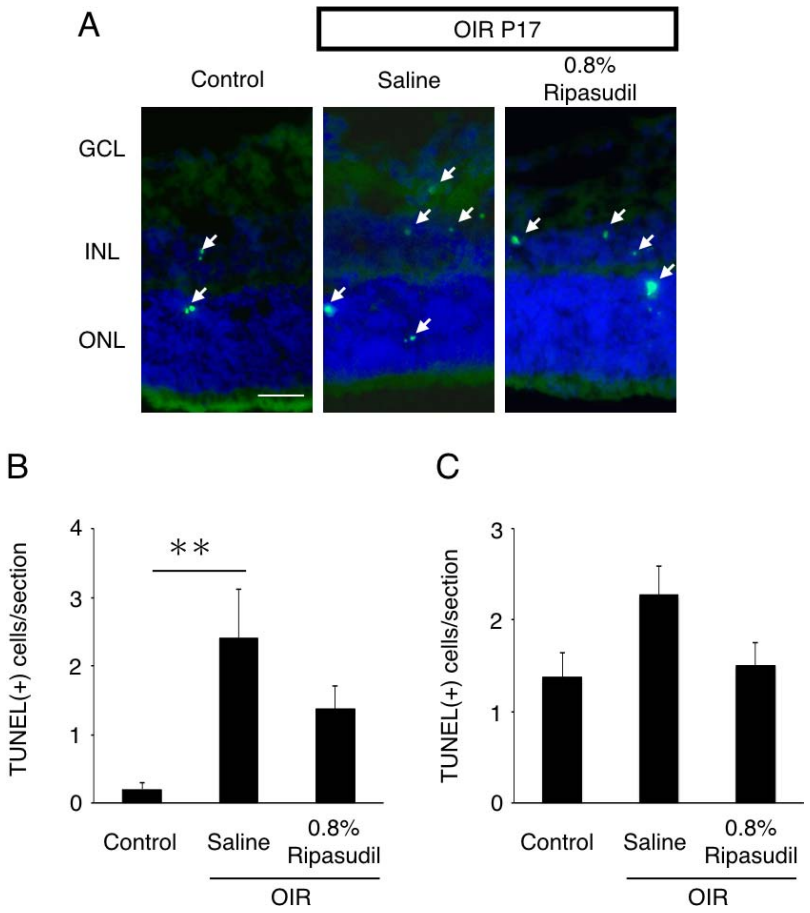

Figure 7. Impact of ripasudil eye drop on cell viability in the OIR retina. (**A**) Representative images of apoptotic cells in retinal sections from P17 control and OIR mice with saline, 0.8% ripasudil treatment three times a day for 5 days (P17) with TUNEL staining (*green*), and the nuclei counterstained with DAPI (*blue*). TUNEL-positive cells (*arrows*) were counted in retinal sections ([**B**] inner retina; [**C**] outer retina; $n = 30$). *Scale bar*: 50 μm. Values are means ± SEM; $^{**}P < 0.01$. ONL; outer nuclear layer, INL; inner nuclear layer, GCL; ganglion cell layer.

higher than saline-treated retina (Figs. 6C–F). This observation suggests that ripasudil causes vascular normalization with intraretinal vessel growth and reduced neovascular tufts.

Nonspecific Effect of Ripasudil Eye Drop in the OIR Retina

Finally, to examine the nonspecific effect of ripasudil on cells other than vascular endothelial cells, we performed TUNEL staining in healthy retina or OIR retina with or without topical ripasudil treatment. TUNEL-positive apoptotic cells could be observed rarely in control retina (Fig. 7A). In the OIR model, the number of TUNEL-positive cells increased significantly in the inner retina but not in the outer retina (Figs. 7B, 7C). Ripasudil treatment at 0.8% did not affect the number of TUNEL-positive cells in the inner retina or the outer retina in the OIR model (Fig. 7). This data might suggest topical treatment of 0.8% ripasudil does not show apparent toxicity for the retinal cells in the OIR retina.

Discussion

Intravitreal injection of anti-VEGF agent is widely used because of the effectiveness for retinal vascular diseases.[27] However, anti-VEGF therapy for ischemic-related retinal angiogenesis could cause vessel contraction,[6] which may be associated with an increased risk of retinal arterial occlusions and tractional retinal detachment.[7,28] Moreover, depressed VEGF levels could also reduce the innate neuroprotective capabilities that directly impact neural cell survival.[29] Recently, we have been investigating the therapeutic potential of ROCK inhibitors in the treatment of retinal diseases.[12,16–19,24] In this study, we examined the effect of ROCK inhibitors ripasudil as well as fasudil in the mouse OIR model. Interestingly, in the OIR model, ROCK inhibitors demonstrate the inhibitory effect not only on retinal angiogenesis but also on avascular area. This present data shows ROCK inhibitor could have a beneficial impact on retinal hypoxia as well as angiogenesis in the retinal ischemia. On the other hand, our previous study concerning VEGF neutralizing antibody in OIR model revealed VEGF blockade could inhibit retinal angiogenesis but not avascular area.[4] ROCK inhibitors might have advantages over anti-VEGF therapy for preventing ischemia as well as the angiogenic response.

Surprisingly, this current OIR study showed ROCK inhibition could increase intraretinal vascularization by inhibiting preretinal angiogenesis, resulting in reduced hypoxic area. This data is contradictory to most reported data in OIR model such as VEGF inhibition.[4,30] Therefore, ROCK could play a different role in intraretinal physiological vascularization from VEGF. In fact, a recent paper using OIR model has shown that blockade of neuropilin-1 reduced both avascular area and neovascular area via ROCK inhibition.[31] This recent report supports our current data in terms of avascular and neovascular area. However, the ROCK-mediated mechanism has been unclear. In

this study, ROCK inhibition by ripasudil caused intraretinal vessel growth with reduced neovascular tufts. Because it has been reported that ROCK inhibitor treatment reduced VEGF expression in diabetic retina,[32] ripasidul might modulate VEGF concentration to the appropriate level, which does not affect physiological vascularization, resulting in vascular normalization.[25,26] A ROCK inhibitor, fasudil, has already been shown to improve ischemia in patients with acute ischemic stroke.[14] Nagaoka et al.[33] previously showed ROCK inhibition could cause retinal vessel dilation. This dilated effect on retinal vessels could also contribute to improvement of ischemia.

Furthermore, a few studies in animal models have shown the neuroprotective effect of ROCK inhibitor.[22,34] In this study, our TUNEL staining could confirm an increase of apoptotic cells in the inner retina of the OIR model, which corresponds with a previous report.[35] Topical 0.8% ripasudil treatment did not affect the number of apoptotic cells in the inner as well as the outer retina. This data suggests that ripasudil eye drop at this concentration did not have an untoward effect on the nonvascular retinal cells. Moreover, the neuroprotective effect on the retina was also reported in different models, as we could not observe significant inhibitory effect of 0.8% ripasudil topical treatment on the number of TUNEL-positive cells in the OIR model. Further investigation is necessary to explore the therapeutic potential of ripasudil eye drop as having a neuroprotective effect on ischemic neovascular diseases.

ROCK is known to play an important role in cell proliferation and migration.[9] We previously observed fasudil could inhibit VEGF-induced endothelial proliferation and migration with bovine retinal microvascular endothelial cells (BRECs) at concentrations of 10 and 30 μmol/L, respectively.[12] However, it had not been examined if ripasudil could affect the properties of human retinal endothelial cells. In this study, ripasudil showed a significant inhibitory activity against VEGF-induced HRMECs proliferation and migration at concentrations of 3 μmol/L and higher. Although we cannot compare the efficacy of these ROCK inhibitors in two independent studies because of the difference between bovine and human retinal endothelial cell, ripasudil might show a greater inhibitory effect on retinal endothelial cell properties than fasudil.

Ripasudil has been developed as an eye drop and received its first global approval in September 2014 for glaucoma and ocular hypertension in Japan (0.4% ophthalmic solution, Glanatec; Kowa Company, Ltd., Nagoya, Japan).[36,37] The steric affinity of the enzyme for ROCK is enhanced by the structural change in the molecule.[21] Ripasudil increases its enzyme affinity by methylation and halogenation. The enzyme inhibitory effect of ripasudil is approximately 5 to 10 times higher than previous ROCK inhibitors. Single dose administration of 1% ripasudil eye drops attains more than 3 μmol/L, and repeated twice a day administration attains more than 10 μmol/L in retinochoroidal tissue of Dutch rabbits (data not shown). A recent study with autoradiography showed marked distribution of radioactivity in the retina and choroid at 15 minutes and 1 hour after ripasudil topical instillation in rabbits.[21] Furthermore, our study with LC-MS/MS could confirm that the concentration of ripasudil in the mouse retina was 3.8 to 10.4 μmol/L and 6.8 to 14.8 μmol/L after the topical treatment of 0.4% and 0.8% ripasudil, respectively. Because an in vitro study revealed the IC50 of ripasudil for ROCK activity was 1.1 μmol/L, the drug concentration in the OIR retina was high enough to inhibit ROCK activity after the topical treatment of ripasudil. Although some ROCK inhibitors have been evaluated for their effect on retinal angiogenesis, topical treatment has not previously been evaluated. In this current study, because fasudil could not be dissolved completely at a concentration of 0.8% at 5°C (data not shown), the effect of only 0.4% fasudil eye drop could be investigated in this retinal angiogenic model. However, 0.4% fasudil eye drop did not show an inhibitory effect in this angiogenesis model as well as in ROCK activity. Fasudil might not reach the retina at the sufficient concentration. These data support the superiority of the therapeutic potential of ripasudil eye drops on retinal disorders.

The drug concentration at the target tissue is important for the pharmacological effects as well as adverse effects. In this study with mouse model, 0.8% ripasudil but not 0.4% inhibited phosphorylation of MYPT-1 in retinal tissue. However, 0.4% ripasudil topical treatment was sufficient to inhibit retinal angiogenesis, reduce retinal avascular area and reduce retinal hypoxia. Ripasudil is not a specific inhibitor of ROCK, as ripasudil also inhibits PKA, PKC, and CaMK,[21] the difference in the results after ripasudil treatment versus fasudil treatment may be due to an inhibitory effect on another pathway. Although the results obtained with ripasudil are largely consistent with the role of ROCK, definitive conclusions regarding the role of ROCK cannot be made from the current data.

We acknowledge the OIR model is not a model of diabetic retinopathy. Diabetic retinopathy is a complex pathogenesis that occurs after patients have diabetes for long time. Furthermore, various systemic conditions including hypertension and hyperlipidemia often contribute the pathogenesis.[38] Therefore, further investigation is needed to apply our current results to patients with diabetic retinopathy.

Our recent clinical study showed intravitreal injection of fasudil could improve refractory diabetic macular edema in spite of negative results with previous anti-VEGF agent treatment.[20] Furthermore, anti-VEGF treatment could cause fibrovascular membrane contraction in patients with proliferative diabetic retinopathy,[7] whereas our previous studies suggest ROCK inhibition blocks fibrovascular membrane.[16] Based on these findings, ROCK is a good therapeutic target for retinal diseases such as diabetic retinopathy. The results discussed above for these studies suggest that topically applied ripasudil may have clinical use for retinal vascular diseases.

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