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Full paper

Effects of ripasudil, a ROCK inhibitor, on retinal edema and nonperfusion area in a retinal vein occlusion murine model

Q1 Yoshifumi Hida, Shinsuke Nakamura, Anri Nishinaka, Yuki Inoue, Masamitsu Shimazawa*, Hideaki Hara

Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, Gifu, Japan

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A B S T R A C T

Rho-associated coiled-coil containing protein kinase (ROCK) inhibitors are used to treat glaucoma patients and have protective effects on ischemic states. However, it is poorly understood how the ROCK pathway affects the pathological signs of retinal vein occlusion (RVO). In this study, we evaluated the effects of ripasudil, a ROCK inhibitor, on a murine RVO model. *In vivo*, RVO was induced by retinal vein laser irradiation in mice, and evaluated with ripasudil. *In vitro*, the effects of ripasudil were examined on tight junction protein integrity in human retinal microvascular endothelial cells (HRMECs). Moreover, we investigated the expression level of the phosphorylated myosin phosphatase target protein (MYPT)-1 after administration of ripasudil. Ripasudil significantly prevented deterioration, such as retinal edema, reduced the size of the nonperfusion area, and improved retinal blood flow. Ripasudil treatment inhibited disintegration of ZO-1 in HRMECs. Administration of ripasudil suppressed retinal phosphorylation of MYPT-1 in a murine RVO model. These findings indicate that ripasudil might be as a possible therapeutic agent for RVO.

Introduction

Retinal vein occlusion (RVO) is caused by obstruction of retinal vessels from high blood pressure and arteriosclerosis.[1] The number of RVO patients is about 16 million, and the prevalence of RVO is 2.1% in a general Japanese population aged 40 years or older.[2] In clinical studies, RVO patients have retinal edema and nonperfusion areas, which cause loss of visual function.[3,4] Anti-vascular endothelial growth factor (VEGF) antibodies, such as bevacizumab and ranibizumab have been used for RVO. Aflibercept which is a recombinant fusion protein comprised of VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2 and the Fc region of the human IgG1 immunoglobulin have been also used to treat RVO. The retinal edema and nonperfusion areas were ameliorated by the administration of anti-VEGF compounds.[3,5–7] However, some patients have repeat recurrences of macular edema and develop nonperfusion areas despite administration of anti-VEGF antibody.[8] Moreover, the anti-VEGF antibody is a symptomatic therapy with physical and economic burden from continuous intravitreal administration. Therefore, it is important to investigate alternative therapies or combination therapy for RVO patients.

Rho-associated coiled-coil containing protein kinase (ROCK) is an intracellular serine–threonine kinase. ROCK has been identified as a target for the small molecule GTP-binding protein Rho, and has two isoforms – ROCK1 and ROCK2.[9] The ROCK pathway regulates myosin-actin interactions and the migration of endothelial cells under physiological conditions.[9–11] In pathological conditions, excessive ROCK activity is involved in vascular endothelial cell damage, which causes cerebrovascular and cardiovascular diseases such as ischemic stroke or vasospastic angina.[12,13] Because ROCK1 and ROCK2 exist in various tissues including the retina,[14] ROCK inhibitors can reduce several eye diseases such as glaucoma and diabetic retinopathy.[15] ROCK inhibitors reduce the development of macular edema by normalizing retinal vasopermeability in diabetic retinopathy patients.[15,16]

The ROCK inhibitor ripasudil is approved to treat glaucoma in Japan,[15] and ripasudil has a neuroprotective effect on TNF-induced

* Corresponding author. Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu, 501-1196, Japan. Fax: +81 58 230 8126.
E-mail address: shimazawa@gifu-pu.ac.jp (M. Shimazawa).
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axon loss.[17] Moreover, ripasudil treatment reduces corneal edema and suppresses apoptosis of corneal endothelial cells.[18] Various ocular disease models are associated with the ROCK pathway.[14,19,20] For example, in the oxygen-induced retinopathy model, ripasudil suppresses not only retinal angiogenesis but also decreases the avascular area.[19] However, the relationship between the ROCK pathway and pathological signs of RVO is poorly understood.

We investigated the effects of ripasudil on edema and nonperfusion area to clarify the relationship between the ROCK pathway and RVO in a murine RVO model.

Materials and methods

Animals

Eight-week-old male ddY mice were purchased from Japan SLC (Hamamatsu, Japan) and housed at 23 ± 3 °C under controlled lighting conditions (12 h/12 h: light/dark). All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Analysis was conducted blindly by Ms. Anri Nishinaka and evaluation was done by Mr. Yoshifumi Hida.

Retinal vein occlusion (RVO) model

The RVO murine model was produced previously.[21,22] Briefly, we anesthetized mice with a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer, Healthcare Osaka, Japan) by intramuscular injection. We injected rose bengal (8 mg/mL; Wako, Osaka, Japan) into the tail vein. Pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceuticals Co., Ltd.). After several minutes, we performed laser irradiation 10 to 20 times per vein and three veins for mice were applied to the branch vein of the right eye of each animal (3 disc diameters from the optic nerve center). The image-guided laser system (532 nm) attached to a Micron IV retinal imaging microscope (Phoenix Research Laboratories, Inc., Pleasanton, Calif., USA) was used at 50-mW power, 5000-ms duration, and 50-μm size.

Drug administration

Ripasudil was obtained from Kowa Pharmaceutical Co. Ltd. (Nagoya, Japan). Mice received an intravitreal injection of ripasudil (50 pmol/2 μL) in their right eye immediately, 3 and 12 h after laser irradiation. Sterile 34-gauge needles (Terumo, Tokyo, Japan) were attached to fine-bore tubes (Natsume Seisakusho, Tokyo, Japan) and filled with ripasudil. A microsyringe was attached to the opposite side of the tube. The limbus of the cornea was pierced toward the posterior segment with a needle, and then 2 μL of ripasudil was administered into the vitreous cavity using a microscope. For buffer control experiments, mice were injected intravitreally with the same volume of phosphate buffered saline (PBS) into their right eye. After intravitreal injection, the sclera was disinfected with 0.5% levofloxacin (Santen Pharmaceuticals Co., Ltd.).

Histological analysis

Mice were euthanized by cervical dislocation, and each eye was enucleated. The eyes were immersed in a fixative solution containing 4% paraformaldehyde (PFA) for 48 h at 4 °C. Six paraffin-embedded sections (5 μm) were stained for hematoxylin and eosin. All images were taken using a fluorescence microscope (BZ-710; Keyence, Osaka, Japan). The edema induced by retinal vein occlusion was assessed using three stained sections from each eye for morphometric analysis. We measured the thickness of the inner nuclear layer (INL) at 240-μm intervals from the optic disc toward the periphery by ImageJ (National Institutes of Health, Bethesda, MD, USA).

Imaging of the retinal nonperfusion area

Ripasudil was injected into the right eye immediately after laser irradiation, and the retina was removed 1 day after laser irradiation. Mice were injected with 0.5 mL of 20 mg/mL fluorescein-conjugated dextran (Sigma–Aldrich Corp., St. Louis, MO, USA) dissolved in PBS into the tail vein before sampling. The eyes were removed and fixed in 4% PFA for 7 h to prepare retinal flat-mounts. Images of attached retinas were taken with Metamorph (Universal Imaging Corp., Downingtown, PA, USA). We measured the size of the retinal nonperfusion area using ImageJ processing software.

Blood flow measurements determined by laser speckle flowgraphy

The blood flow of the optic nerve head (ONH) was measured by laser speckle flowgraphy (LSFG; Softcare Co., Ltd., Fukuoka, Japan) as previously reported in detail.[21] Blood flow was measured as the mean blur rate (MBR), which is an index of the relative blood flow velocity. The MBR images were acquired continuously at a rate of 30 frames per second over a time period of about 4 s. The measured fundus area was approximately 3.8 × 3 mm (width × height) with an estimated tissue penetration of 0.5–1 mm. The blood vessels and tissue regions in the ONH area were automatically detected by the LSFG Analyzer software (version 3.1.14.0; Software Co., Ltd.). The MBR in different regions of the ONH was divided into three parameters: the MBR of the total area (MA) was calculated as the average MBR over the ONH, the MBR of the vessel region (MV) was calculated as the average over the vessel region, and the MBR of the tissue region (MT) was calculated as the average MBR obtained by subtracting the blood vessel region from the total ONH region.

Cell culture

Primary human retinal microvascular endothelial cells (HRMECs) were obtained from DS Pharma Biomedical (Osaka, Japan) and cultured in CSC Complete Recombinant Medium (DS Pharma Biomedical) with culture boost (growth factors) (DS Pharma Biomedical) at 37 °C in a humidified atmosphere of 5% CO_2.

Monolayer permeability assay

HRMECs were plated at a density of 1.5 × 10^5 cells/mL onto Transwell ® inserts (Corning, Inc., Canton, NY, USA). Culture medium was replaced every other day. The cells were further cultured until transendothelial electrical resistance (TEER) plateaued. TEER was measured using an Epithelial Volt-Ohm Meter (Millicell ERS-2, Millipore, Merck Millipore Co., Darmstadt, Germany) and a cup-shaped electrode (Endohm-6, World Precision Instruments, Inc., Sarasota, FL, USA). After TEER plateaued, cells were pre-treated with ripasudil or solvent (PBS) for 1 h and incubated with human recombinant VEGF (100 ng/ml) (R&D Systems, Inc., Boston, MA, USA) or medium for 1 day. We measured TEER 1 day after VEGF exposure. The values of TEER are given as $\Omega\ cm^2$ and calculated by the following equation:

Tissue TEER ($\Omega\ cm^2$) = (sample well resistance − blank well resistance) × monolayer area.