The Anti-Inflammatory Effect of Ripasudil (K-115), a Rho Kinase (ROCK) Inhibitor, on Endotoxin-Induced Uveitis in Rats

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PURPOSE. To investigate the anti-inflammatory properties of ripasudil, a Rho kinase (ROCK) inhibitor, using endotoxin-induced uveitis (EIU) in rats.

METHODS. Endotoxin-induced uveitis was induced by footpad injection of lipopolysaccharide (LPS). Ripasudil was administered intraperitoneally 1 hour before and after LPS injection. The aqueous humor was collected 24 hours after injection, and the infiltrating cells, protein concentration, and levels of monocyte chemotactic protein-1 (MCP-1) were determined. The mRNA levels of II-1b, IL-6, TNF-a, and MCP-1 in the retina and ICB were determined. A mouse macrophage cell line, RAW264.7, was stimulated with LPS in the presence or absence of ripasudil, and the expression of MCP-1 and nuclear translocation of nuclear factor (NF)-kB was analyzed.

RESULTS. Ripasudil significantly reduced infiltrating cells and protein exudation in the aqueous humor, as well as the number of infiltrating cells in the ICB and adherent leukocytes in retinal vessels in EIU. Additionally, the protein level of MCP-1 in the aqueous humor and mRNA levels of II-1b, IL-6, TNF-a, and MCP-1, and intercellular adhesion molecule-1 in the ICB and retina were suppressed by ripasudil. The production of MCP-1 and nuclear translocation of NF-kB in RAW264.7 cells were also suppressed by ripasudil.

CONCLUSIONS. The Rho/ROCK pathway plays a role in adhesion molecule expression and inflammatory cell infiltration in EIU, and ripasudil is a potent anti-inflammatory agent against ocular inflammatory diseases, including acute uveitis and possibly uveitic glaucoma.

Keywords: Rho kinase (ROCK), endotoxin-induced uveitis, ripasudil, inflammation, glaucoma eye drop
CaMKIIz were 0.051, 0.019, 2.1, 27, and 0.37 μM, respectively, while the IC50 of other ROCK inhibitors such as Y27632 and fasudil were 2 to 18 times higher than that of ripasudil. This high selectivity contributes to the safety profile of ripasudil because different protein kinases have structurally similar active binding sites yet regulate diverse signaling pathways. Several studies have recently reported that ripasudil is a safe topical agent for IOP reduction in the treatment of glaucoma patients. In addition, it has been reported that ripasudil exhibits neuroprotective effects for retinal ganglion cells following systemic administration in the rat optic nerve crush model. A previous study with the radiolabeled drug showed that ripasudil reached the retina and choroid after eye drop administration in rabbits. However, it is not known if ripasudil treatment inhibits ocular inflammation in the anterior chamber or retina, and the role of the Rho/ROCK pathway in ocular inflammation and leukocyte adhesion in EIU has not yet been clarified. In the present study, we assessed the anti-inflammatory properties of ripasudil in the rat EIU model and in LPS-stimulated mouse macrophage-like RAW264.7 cells. Intercellular adhesion molecule-1 (ICAM-1), MCP-1, and other proinflammatory cytokines were characterized to determine the role of Rho/ROCK signaling during adhesion and inflammatory infiltration.

Materials and Methods

Animals

Male, 6-week-old Wistar rats (160–170 g) were purchased from Tokyo Laboratory Animals Science (Tokyo, Japan). Animals were maintained in standard animal cages under constant 12-hour light/dark cycles. Food and water were available ad libitum. All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health Guiding Principles for the Care and Use of Animals (DHEW Publication, NIH 80–23), and were approved by the Institutional Animal Research Committee of the University of Tokyo.

EIU Induction and the Administration of Ripasudil

Rats were anesthetized by intramuscular injection of a mixture of ketamine and xylazine. Endotoxin-induced uveitis was induced by footpad injection of 200-μg LPS from Escherichia coli O55 (100 μg each footpad; Wako Pure Chemicals, Osaka, Japan), diluted in 0.1-mL PBS. An aliquot of 0.1 mL of ripasudil (20 mg/mL; Kowa Company, Tokyo, Japan) and vehicle (PBS; Wako Pure Chemicals) was intraperitoneally injected 1 hour before and after LPS administration.

Histology

The rats were euthanized 24 hours after LPS injection, and the eyes were enucleated immediately, stored in 4% paraformaldehyde in 0.1 M PBS (Wako Pure Chemicals, Osaka, Japan), and embedded in paraffin. Sagittal sections (5 μm) were cut through the optic nerve head and stained with hematoxylin and eosin (Wako Pure Chemicals). The eyes were enucleated and stored in 4% paraformaldehyde in 0.1 M PBS for 15 minutes. The retinas were dissected and flat-mounted on glass slides containing a drop of VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) to stain adherent leukocytes and conjugated concanavalin-A (40 μg/mL in PBS; Vector Laboratories, Burlingame, CA, USA) to stain adherent leukocytes and vascular endothelial cells. Unbound concanavalin-A was removed with an additional perfusion with 30 mL of PBS. The eyes were enucleated and stored in 4% paraformaldehyde in 0.1 M PBS 3 hours after LPS injection and for the total RNA was extracted from the iris ciliary body (ICB) and retina using ISOGEN (Nippon Gene, Tokyo, Japan). Complementary DNA was prepared using the ReverTra Ace qPCR RT Master Mix with genomic DNA remover (Toyobo, Osaka, Japan). The qPCR was performed using the Thermal Cycler Dice Realtime System (Takara Bio, Shiga, Japan) using SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio). The values for each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used in qPCR were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Gapdh</td>
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<td>5′-ACCACGTGCATGCTGTCGTCAG-3′</td>
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<tr>
<td>mouse Mcp-1</td>
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<td>5′-CCGGAGAGGAGACTTCCTCCTG-3′</td>
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<tr>
<td>mouse Il-1</td>
<td>5′-GGCATGCATGCTGTCGTCAG-3′</td>
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<tr>
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<td>5′-ACAGTGCATGCTGTCGTCAG-3′</td>
<td>5′-ATTCCTAACAGAAGAAGAAGAAG-3′</td>
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Quantitative Real-Time PCR (qPCR)

The rats were euthanized 3 hours after LPS injection, and the total RNA was extracted from the iris ciliary body (ICB) and retina using ISOGEN (Nippon Gene, Tokyo, Japan). Complementary DNA was prepared using the ReverTra Ace qPCR RT Master Mix with genomic DNA remover (Toyobo, Osaka, Japan). The qPCR was performed using the Thermal Cycler Dice Realtime System (Takara Bio, Shiga, Japan) using SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio). The values for each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used in qPCR were as follows:

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Cell Culture and LPS Stimulation

The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM; high glucose; Wako Pure Chemicals) containing 10% (vol/vol) fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO, USA), supplemented with penicillin (100 units/mL), streptomycin (0.1 mg/mL), and maintained at 37°C in a humidified incubator containing 5% CO2. The cells were pretreated with 1-, 10-, or 100-μM ripasudil for 1 hour and stimulated with 10 ng/mL LPS. The levels of MCP-1 in the culture medium were assessed by ELISA 24 hours after LPS exposure.

Analysis of Adherent Leukocytes in Retinal Vessels

To analyze the effect of ripasudil on adherent retinal leukocytes in the EIU model, a perfusion labeling technique was used, based on a previous report with slight modifications. The rats were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine 24 hours after LPS injection, then perfused with 30 mL of PBS (13.2 mL/min) to remove the blood, followed by 20 mL of fluorescein isothiocyanate (FITC)-conjugated concanavalin-A (40 μg/mL in PBS; Vector Laboratories, Burlingame, CA, USA) to stain adherent leukocytes and vascular endothelial cells. Unbound concanavalin-A was removed with an additional perfusion with 30 mL of PBS. The eyes were enucleated and stored in 4% paraformaldehyde in 0.1 M PBS 3 hours after LPS injection, and the concentration of MCP-1 in the aqueous humor samples was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of MCP-1 in the aqueous humor samples was measured using a mouse/rat MCP-1 ELISA kit (R&D systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

The Number of Infiltrating Cells and the Concentrations of Protein and MCP-1 in the Aqueous Humor

Rats were euthanized 24 hours after LPS injection, and the aqueous humor was collected by anterior chamber puncture of the eye with a 30-G needle. The aqueous humor sample was suspended in an equal amount of 0.4% Trypan Blue solution and counted using a TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA). The total protein concentration in the aqueous humor sample was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of MCP-1 in the aqueous humor samples was measured using a mouse/rat MCP-1 ELISA kit (R&D systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

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Anti-Inflammatory Effect of ROCK Inhibitor on EIU

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Cell Viability Assay

Cells (5 × 10^4 cells/well) were placed in a 96-well plate and incubated at 37°C for 24 hours. The medium was then changed to media containing 0 to 100 μM ripasudil. After the cells were incubated at 37°C for 24 hours, the cell viability was assayed using the Premix WST-1 Cell Proliferation Assay System (Takara Bio) according to the manufacturer’s instructions.

Nuclear Translocation of NF-κB p65 in RAW 264.7 Cells

One hour after LPS stimulation, fixed with 4% paraformaldehyde in 0.1 M PBS for 15 minutes, blocked with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 10 minutes, and permeabilized with a 0.05% Triton X-100 solution in PBS. The permeabilized cells were incubated with anti-NF-κB p65 antibody (1:100, sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The cells were then washed three times with PBS, incubated with Alexa 488-conjugated anti-mouse immunoglobulin G secondary antibodies (1:200, A11029; Thermo Fisher Scientific) for 1 hour at room temperature with 4',6-diamidino-2-phenylindole (1 μg/mL; Dojindo Molecular Tech, Rockville, MD, USA), washed three times with PBS, and embedded with VECTASHIELD Mounting Medium (Vector Laboratories). The number of cells with nuclear translocations was counted using a fluorescence microscope (Keyence) and expressed as the percentage of translocated cells compared with the total number of cells.

Statistical Analysis

Data are expressed as the means ± SD. Statistical analyses were performed using a 2-tailed Student’s t-test. A value of P less than 0.05 was considered statistically significant.

RESULTS

Histopathologic Findings

Histologic examinations were performed to examine the anti-inflammatory effect of ripasudil in the LPS-induced rat model of EIU. As shown in Figure 1, severe cellular infiltration was observed in the anterior chamber and ICBs in the LPS + vehicle group 24 hours after LPS injection, while inflammatory cells in the ripasudil-treated groups were significantly decreased (Fig. 1C). No inflammation in the anterior chamber or ICB was observed histopathologically in rats treated with PBS + vehicle (Fig. 1A).

The Effect of Ripasudil on Infiltrating Cells and Protein Levels in the Aqueous Humor

To confirm the anti-inflammatory effects of ripasudil in EIU, we measured the number of infiltrating cells and protein concentrations in the aqueous humor. As shown in Figure 2, no cells were detected in the aqueous humor of the PBS + vehicle group. Twenty-four hours after LPS injection, the number of infiltrating cells increased in the LPS + vehicle group.
The Effect of Ripasudil on the Concentration of MCP-1 in the Aqueous Humor

A wide variety of inflammatory mediators such as TNF-α, prostaglandin-E2, IL-1β, IL-6, and MCP-1 have been reported to affect the development of the EIU model.5,10,26,27 The effect of ripasudil pretreatment on the concentration of MCP-1 in the aqueous humor was examined by ELISA. Monocyte chemotactic protein-1 in the aqueous humor was not detectable in the PBS + vehicle group. Data are expressed as the means ± SD (n = 5–8). *P < 0.05.

Ripasudil Ameliorates Leukocyte Adhesion in Retinal Vessels

It is well known that ICAM-1 and MCP-1 have significant effects on cell adhesion, proliferation, and inflammatory cell infiltration.28 The qPCR results showed that ripasudil significantly suppressed the LPS-induced mRNA levels of MCP-1 in both the ICB and retina. To determine whether ripasudil attenuated the LPS-induced adherent leukocytes in retinal vessels, a perfusion labeling technique with FITC-conjugated concanavalin-A was performed. Figure 6 shows that adherent leukocytes in retinal vessels were very rarely observed in the PBS + vehicle group (Fig. 6A) 24 hours after treatment. Injection of LPS increased the number of adherent leukocytes in retinal vessels (325.5 ± 146.2 cells; n = 8; Fig. 6B) compared with the PBS + vehicle group, and pretreatment with ripasudil significantly reduced the LPS-induced adherent leukocytes in...
retinal vessels (122.0 ± 89.0 cells; n = 9; P < 0.01; Figs. 6C, 6D).

The Effects of Ripasudil on Cell Viability and MCP-1 Concentration in LPS-Stimulated RAW 264.7 Cells
The in vivo results above confirmed that ripasudil effectively inhibited LPS-regulated inflammatory responses in the rat model of EIU. It was therefore important to examine whether ripasudil suppressed the inflammatory responses in LPS-activated RAW264.7 macrophage cells. In addition, to examine the cytotoxicity of ripasudil on RAW 264.7 macrophage cells, the cells were treated with various concentrations of ripasudil, and cell survival was determined using the MTT assay. Figure 7 shows that LPS stimulation resulted in increased expression of MCP-1 protein in RAW 264.7 cells, and treatment with ripasudil inhibited this increase in a dose-dependent manner (Fig. 7). Furthermore, the viability of RAW264.7 cells incubated with 100-μM ripasudil for 48 hours was not decreased (data not shown).

The Suppressive Effect of Ripasudil on LPS-Induced Nuclear Translocation of NF-κB in RAW 264.7 Cells
It is well known that during an inflammatory response, expression of several important proinflammatory genes, such as cyclooxygenase-2, inducible nitric oxide synthase, and matrix proteinases is controlled by signaling downstream of NF-κB expression.29 It was previously reported that ROCK inhibition reduced LPS-induced kidney injury, in part by attenuation of NF-κB p65 activation.30 We therefore determined the ability of ripasudil to inhibit LPS-stimulated nuclear translocation of NF-κB p65 in RAW 264.7 macrophages. To observe the reduction in translocation of nuclear transcription factors, the intracellular behavior of these factors was directly analyzed using a fluorescence microscope. Figure 8A shows that ripasudil treatment significantly decreased LPS-induced nuclear translocation of the NF-κB p65 subunit from the cytosolic space. At 100 μM, the translocation of NF-κB was significantly reduced compared with the vehicle-treated cells with LPS. Cell counting showed that LPS treatment induced nuclear translocation of the NF-κB p65 subunit (49.2 ± 14.4%; n = 4) and ripasudil significantly inhibited this LPS-induced nuclear translocation (22.5 ± 11.0%; n = 4; P < 0.05; Fig. 8B). These results suggest that ripasudil is a negative regulator of LPS-stimulated nuclear translocation of NF-κB in RAW 264.7 macrophages.

DISCUSSION
This study investigated the anti-inflammatory effect of the ROCK inhibitor, ripasudil, in the rat EIU model. Pretreatment with ripasudil reduced cellular infiltration and protein concentration, as well as the concentration of MCP-1 in the anterior chamber, and decreased mRNA levels of the adhesion molecule, ICAM-1, and proinflammatory mediators both in the ICB and retina. Pretreatment with ripasudil also suppressed LPS-induced retinal leukocyte adhesion in the retina. In addition, ripasudil significantly inhibited the LPS-induced...
FIGURE 5. The effect of ripasudil on mRNA levels of proinflammatory mediators in the retina. The effects of ripasudil on LPS-induced mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, and ICAM-1 in the retina were evaluated by qPCR. Lipopolysaccharide treatment significantly induced elevated mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, and ICAM-1 at 3 hours after LPS injection, and pretreatment with ripasudil significantly suppressed these changes. *P < 0.05. Data are representative of three independent experiments.

FIGURE 6. The effect of ripasudil on retinal leukocyte adhesion. The effects of ripasudil on LPS-induced adherent leukocytes in retinal vessels were investigated using a perfusion labeling technique. (A) No adherent leukocytes were observed in the PBS + vehicle control group. (B) The injection of LPS increased the adherent leukocytes in retinal vessels, and (C) pretreatment with ripasudil significantly reduced the adherent leukocytes in the retinal vessels. Data are expressed as the means ± SD (n = 3–9; **P < 0.01) (D).
activation of MCP-1 expression and nuclear translocation of NF-κB in RAW 264.7 cells. Together, these results demonstrated that inhibition of Rho/ROCK signaling by ripasudil ameliorated cell infiltration and subsequent inflammation both in the anterior and posterior segments of the eye, and suppressed the development of EIU.3

Extensive inflammation accompanied by tissue injury and abnormal vascular permeability is known to result in tissue damage, and inflammatory cell infiltration is a key event in the onset of uveitis.5 In the inflammatory response, it is well known that ICAM-1 and MCP-1 have a significant effect on cell adhesion, proliferation, and inflammatory cell infiltration, as is shown in the systemic organ such as renal failure.28 ICAM-1 precedes the transendothelial migration of inflammatory cells from the capillary bed into tissues.41 It is also known that MCP-1 rapidly causes rolling monocytes to adhere firmly onto monolayers, where it plays a role in monocyte recruitment.24 It has been previously reported that macrophage migration to endothelial cells requires the Rho/ROCK pathway during inflammatory infiltration, and ROCK inhibition has a significant effect on the expression of adhesion molecules and chemokines.30 However, there have been no reports concerning the role of the Rho/ROCK pathway in ocular inflammation and leukocyte adhesion or infiltration in EIU. The present study firstly showed that LPS-induced ICAM-1 and MCP-1 expression was significantly suppressed by the ROCK inhibitor, ripasudil, in the aqueous humor (Fig. 3), ICB (Fig. 4), and retina (Fig. 5), which is consistent with the results reported by other studies in other systemic organs.14,33 Taken together, the present study suggests the involvement of Rho/ROCK pathway in the ocular inflammation and that ROCK inhibition decreases inflammation by reducing leukostasis in the EIU animal model in vivo, possibly due to the inhibition of ICAM-1 and MCP-1 expression, as shown in vitro. In addition, Figure 6 shows that inflammation triggers synthesis of IL-1β, IL-6, and TNF-α, all of which contribute to the pathophysiology of EIU.4,5 To elucidate the anti-inflammatory mechanism of ripasudil, we therefore evaluated these proinflammatory mediators.54,55 According to previous reports of the contribution of cytokines, TNF-α and IL-6 are pleiotropic in EIU; both cytokines are proinflammatory and are produced principally by activated macrophages and monocytes, with some previous studies suggesting that these two cytokines have protective roles in ocular inflammation.56 However, it has been reported that injection of TNF-α or IL-6 into the vitreous of rats produces severe intraocular inflammation, and anti-TNF-α and IL-6 receptor therapy has been shown to be a useful strategy for managing uveitis; therefore, both chemokines are essential in the pathogenesis of EIU.57,58 The present study showed that ripasudil decreased these proinflammatory mediators both in the anterior chamber and retina (Figs. 3–5). These findings suggest that the mechanism of ripasudil suppression of EIU includes the prevention of cytokine and chemokine production.

Moreover, because NF-κB response elements are present in the promoters of TNF-α, IL-6, MCP-1, and ICAM-1 genes in the inflammatory response,39–41 and ripasudil significantly inhibits the levels of these proinflammatory cytokines, it is reasonable to postulate that ripasudil regulates the expression of these proteins by inhibiting NF-κB activation. Several previous studies have reported that ROCK inhibition suppressed LPS-induced activation of NF-κB in several cells and tissues.40,42,43 Thus, the inhibition of NF-κB activation and subsequent synthesis of inflammatory mediators can at least partially explain the beneficial effect of ripasudil in the treatment of EIU. The present study showed that inhibition of Rho/Rho kinase can attenuate LPS-induced NF-κB activity (Fig. 8), suggesting that LPS-mediated proinflammatory mediator synthesis results, at least in part, from Rho/Rho kinase pathway-dependent NF-κB activation.

This study had some limitations. We showed that the ROCK inhibitor significantly inhibited both anterior and posterior segment inflammation. Ripasudil significantly reduced adherent leukocytes in retinal vessels, and reduced MCP-1 and ICAM-1 mRNA expression in the retina, both of which have been reported to be related to leukocyte recruitment. The results of the present study are consistent with a previous report that involved intravitreal injection and reduced inflammation in the retina, and that showed another ROCK inhibitor, AMO428, significantly reduced retinal leukocyte adhesion in the streptozotocin-induced murine diabetic model.44 However, the present study administered the ROCK inhibitor systemically and did not investigate the topical administration of ripasudil, which is one of the limitations that should be further investigated in future studies. Because it has been previously reported that radiolabelled ripasudil reached the retina and choroid following the administration of eye drops in rabbits,15,24 there are enough possibilities that topical administration of ripasudil could attenuate ocular inflammation both in the anterior and posterior segments of the eye. In the future studies, further investigation with topical and intravitreal administration will be required to verify these possible effects. Additionally, the mechanism of regulation of the macrophage phenotype is unclear, and it will be important to determine the relationship between phenotypic conversion of macrophages and blockage of the Rho-ROCK pathway in ocular inflammation in future studies. Finally, the anti-inflammatory effect of ripasudil was investigated only in vitro and in an in vivo animal study. However, Yamada et al.45 recently reported that topical application of ripasudil significantly decreased intraocular pressure and aqueous flare in patients with anterior uveitis, glaucoma, and ocular hypertension, suggesting that the drug may result in favorable clinical effects in anterior uveitis. Although the relevant mechanisms were unclear, the present in vitro and animal studies provided insight into the anti-inflammatory effects of the drug, by revealing some of the underlying mechanisms. Regarding the consistency of the ripasudil concentration between clinical use and the in vivo
and in vitro experiments, it has been previously reported that a twice a day administration of 1% ripasudil eye drops resulted in greater than 10 μM of the drug in the retinochoroidal tissue of Dutch rabbits. Because 0.4% ripasudil is already clinically available as an anti-glaucoma drug in Japan,47,48 further studies with uveitis or uveitic glaucoma patients and precise evaluations will be needed to confirm the anti-inflammatory effects of the ROCK inhibitor.

In conclusion, our results showed that the ROCK inhibitor, ripasudil, attenuated LPS-induced ocular inflammation, suggesting that ROCK inhibitors play an anti-inflammatory role via suppression of ICAM-1 and MCP-1 expression, as well as via TNF-α/NF-κB inhibition, to inhibit leukocyte adhesion and inflammatory cell infiltration. Our findings reveal new insights into the mechanism of uveitis, and provide the basis for therapeutic strategies for the treatment of ocular inflammation, including uveitis and possibly uveitic glaucoma.

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