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 exudation, and the expression of MCP-1 and NF-κB in the retina and ICBC were determined.

Results. Ripasudil significantly reduced infiltrating cells and protein exudation in the aqueous humor, as well as the number of infiltrating cells in the ICBC and adherent leukocytes in retinal vessels in EIU. Additionally, the protein level of MCP-1 in the aqueous humor and mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, and intercellular adhesion molecule-1 in the ICBC and retina were suppressed by ripasudil. The production of MCP-1 and nuclear translocation of NF-κB in ICBC 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

Uveitis is an inflammatory disease of the uvea triggered by various ocular pathological conditions, such as infections, autoimmune reactions, and injury, and is a major cause of vision loss worldwide. Visual impairment during uveitis can be caused by inflammation in the ocular tissues, secondary cataracts, and subsequent insults to retinal ganglion cells due to associated glaucoma. Several bioactive factors, such as cytokines and growth factors, are known to be major mediators of the inflammatory reactions in the pathology of uveitis. Macrophage activation by bacterial toxins such as lipopolysaccharide (LPS) induces secretion of several cytokines and chemokines, such as TNF-α, IL-6, IL-1β, and monocyte chemotactic protein (MCP-1), which are key inflammatory mediators in many chronic inflammatory diseases including uveitis. Corticosteroids are the most frequently used medications for uveitis, but long-term administration of steroids can result in many side effects, such as induction of glaucoma, formation of cataracts, and decreased resistance to infection. Endotoxin-induced uveitis (EIU) is a well-accepted animal model of acute anterior inflammation induced by LPS injection into footpads. It is characterized by the breakdown of the blood-ocular barrier and infiltration of leukocytes and proteins into the eye. In this model, LPS directly activates the vascular endothelium, macrophages, and other cell types, along with induction of various cytokines and other inflammatory mediators, such as TNF-α, IL-6, IL-1β, and MCP-1, and has long been used for the evaluation of drugs and for investigating the pathology of uveitis.

Rho-associated protein kinase (ROCK) is a serine/threonine protein kinase that is a downstream effector activated by the small GTP binding protein, Rho. It has been reported that ROCK is critical in controlling adhesion, migration, proliferation, cell apoptosis/survival, gene transcription, and differentiation. The endothelium plays a critical role in adhesion and migration of leukocytes, which require adhesion molecules and chemokines. Adhesion and migration of macrophages to the endothelium are important steps in the onset of inflammation, and Rho/ROCK signaling has been reported to play an important role in inflammation.

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 structural changes of the drug. In 2014, the first ROCK inhibitor was approved in Japan for the treatment of glaucoma. Ripasudil shows high selectivity for ROCK inhibition, especially ROCK 2. The 50% inhibitory concentrations (IC50s) of K-115 for ROCK 1, ROCK 2, PKCα, PKC, and
Anti-Inflammatory Effect of ROCK Inhibitor on EIU

CaMKIIz were 0.051, 0.019, 2.1, 27, and 0.37 μM, respectively, while the IC_{50} 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.18 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 in the anterior chamber after LPS injection, 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-M PBS. An aliquot of 0.1 M 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), and then embedded in paraffin. Sagittal sections (5 μm) were cut through the optic nerve head and stained with hematoxylin and eosin (Wako Pure Chemicals).

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.

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 for 15 minutes. The retinas were dissected and flat-mounted on glass slides containing a drop of VECTA-SHEILD Mounting Medium (Vector Laboratories). The number of adherent leukocytes in retinal vessels was counted using a fluorescence microscope (Keyence, Osaka, Japan).

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 (Toyo-bo, 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: rat Gapdh (forward, 5’-GGCATTCGCTCATTGACA-3’ and reverse, 5’-TGTGAGGGA GATGCTCAGG-3’); rat Il-1β (forward, 5’-AGGCTTCCTTGTG-3’ and reverse, 5’-ACATGTAGCATGCCTGTC-3’); rat Il-6 (forward, 5’-CCGGAGAGGAGACTTCACAG-3’ and reverse, 5’-ACAGTGCATCATGCCTGTC-3’); rat Tnf-α (forward, 5’-ACTCCCGAAGAGCAGAAC-3’ and reverse, 5’-CGAGCGGAGTGGAAGAGG-3’); and rat Mcp-1 (forward, 5’-ATGCAGTTAATGCCCCACTC-3’ and reverse, 5’-TTCCTATTTGGGTTCAACG-3’).

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.
Cell Viability Assay

Cells ($5 \times 10^4$ 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 (Fig. 1B), 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.

(9.5 ± 5.4 × 10^5 cells/mL; n = 8) and the group treated with ripasudil showed a significant reduction in the number of infiltrating cells in the aqueous humor (1.1 ± 1.3 × 10^5 cells/mL; n = 8; P < 0.01) compared with the LPS + vehicle group (Fig. 2A). In a similar manner, the protein concentration in the aqueous humor increased (14.0 ± 4.6 mg/mL; n = 8) with LPS injection from 1.0 ± 0.2 mg/mL (n = 5) in the PBS + vehicle group. Pretreatment with ripasudil significantly reduced the protein concentration in the aqueous humor to 6.7 ± 4.4 mg/mL in the LPS + ripasudil group (n = 8; P < 0.01; Fig. 2B).

The Effect of Ripasudil on the MRNA Levels of Adhesion Molecules and Proinflammatory Mediators in the ICB and Retina

We next performed qPCR to analyze the inhibitory effects of ripasudil on LPS-induced production of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, MCP-1, and ICAM-1 in the ICB and retina. These proinflammatory cytokines and the adhesion molecule play pivotal roles in inflammation as proinflammatory mediators, including the activation and migration of leukocytes and activation of the acute-phase response.5,10,26,27 Figures 4 and 5 show that LPS treatment significantly induced elevated mRNA levels of IL-1β, IL-6, TNF-α, MCP-1, and ICAM-1 at 3 hours after LPS injection in both the ICB and retina. Pretreatment with ripasudil significantly suppressed the LPS-induced mRNA levels of IL-1β, IL-6, TNF-α, and MCP-1, which preceded the migration of inflammatory cells in both the ICB and retina (Figs. 4, 5).

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 and ICAM-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 (188.4 ± 93.4 cells; n = 8; Fig. 6C).
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. It was previously reported that ROCK inhibition reduced LPS-induced kidney injury, in part by attenuation of NF-κB p65 activation. 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 retinal vessel (122.0 ± 89.0 cells; n = 9; P < 0.01; Figs. 6C, 6D).
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).
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.36 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 or anti–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.37,38 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-kB 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-kB activation. Several previous studies have reported that ROCK inhibition suppressed LPS-induced activation of NF-kB in several cells and tissues.40–42 Hence, the inhibition of NF-kB 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-kB activity (Fig. 8), suggesting that LPS-mediated proinflammatory mediator synthesis results, at least in part, from Rho/Rho-kinase pathway-dependent NF-kB 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, AMA0428, 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, 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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Anti-Inflammatory Effect of ROCK Inhibitor on EIU

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