Fasudil inhibits LPS-induced migration of retinal microglial cells via regulating p38-MAPK signaling pathway

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Purpose: To investigate the effect and possible molecular mechanisms of fasudil on retinal microglial (RMG) cell migration.

Methods: Primary cultured RMG cells were incubated with lipopolysaccharide (LPS), fasudil, and/or SB203580 (a p38 inhibitor). RMG cell motility was determined with the scratch wound assay and the Transwell migration assay. The phosphorylation of p38 and levels of matrix metalloproteinase 2 (MMP-2) and MMP-9 were measured with western blot.

Results: In the scratch-induced migration assay, as well as in the Transwell migration assay, the results indicated that LPS stimulated the migratory potential of RMG cells and fasudil significantly reduced LPS-stimulated RMG cell migration in a concentration-dependent manner. However, fasudil had no effect on RMG cell migration in the absence of LPS stimulation. Moreover, fasudil reduced the level of phosphor-p38 mitogen-activated protein kinase (p-p38-MAPK) in a concentration-dependent manner, without effects on the levels of phospho-p44/42 (p-ERK1/2) and phospho-c-Jun N-terminal kinase (p-JNK). Cotreatment with SB203580 (a p38 inhibitor) and fasudil resulted in the synergistic reduction of MMP-2, MMP-9, and p-p38-MAPK, as well as a reduction in the LPS-stimulated migration capabilities of the RMG cells, suggesting fasudil suppresses the LPS-stimulated migration of RMG cells via directly downregulating the p38-MAPK signaling pathway.

Conclusions: Our studies indicated that fasudil inhibited LPS-stimulated RMG cell migration via suppression of the p38-MAPK signaling pathway.

One of the first responses of the retina and the optic nerve to disease, inflammation, and injury features prominent involvement of retinal microglia (RMG) cells, the primary resident immune cells [1,2]. Functionally, RMG cells regulate retinal neuron growth and are active phagocytes, eliminating dying photoreceptor cells [1]. In pathological conditions, RMG cells, which are mainly located in the inner retina, are rapidly activated in response to various pathogenic contexts [1]. These activated RMG cells retract their branches, become amoeboid, and migrate toward the injury area, where they influence local cell damage [1]. Despite the cells’ importance, the mechanisms controlling trigger microglial cell migration remain poorly understood.

Modulating the migration of microglial cells might create a niche environment for reduction of tissue damage [3,4]. Mitogen-activated protein kinases (MAPKs) are a highly conserved family of serine and threonine protein kinases that participate in intracellular signaling, such as proliferation, differentiation, cellular stress responses, and apoptosis [5]. p38-MAPKs are a class of MAPKs that are activated by various environmental stresses and inflammatory cytokines [6]. The migration of microglial cells in the retina requires specific intracellular signaling cascade activations, among which the p38-MAPK signaling pathway has been well demonstrated to play crucial roles [7].

The forward-propelling machinery for microglia cell migration requires dissolution of the extracellular matrix (ECM) [8,9]. The breakdown of the ECM is controlled by matrix metalloproteinases (MMPs) [9]. The expression of MMPs, produced in microglia at sites of inflammation upon activation (such as lipopolysaccharide, LPS), has been investigated in various studies [10]. In particular, the secreted MMP-2 and MMP-9 seem to be crucial modulators [10,11].

Microglia cell migration relies on dynamic remodeling of the actin cytoskeleton [12]. This remodeling, in turn, is regulated by Rho kinase (ROCK) [13]. In a previous study, fasudil hydrochloride (fasudil), a potent ROCK inhibitor, was found to improve the pathology in brain ischemia, Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease, as well as brain neurotrauma [14,15]. In addition, fasudil can protect the retina from ischemia-reperfusion injury by inhibiting retinal ganglion cell (RGC) apoptosis and inducible nitric oxide synthase expression [16,17]. Additionally, fasudil has a therapeutic potential for ocular angiogenic diseases via blockade Rho-kinase signaling and extracellular...
signal-related kinase and Akt signaling [18]. Furthermore, previous studies have demonstrated that fasudil attenuates the apoptosis of RGCs and ameliorates damage of the optic nerve in traumatic optic neuropathy by inhibiting the Rho signaling pathway in vitro and in vivo [19,20]. Interestingly, fasudil can affect microglia polarization and plasticity in vitro and in vivo [21]. However, the effects and the mechanism of fasudil on the migration of microglia remains largely unknown. The aim of this study, therefore, was to test the hypothesis that administration of fasudil might inhibit the migration of primary RMG cells via regulating the p38-MAPK signaling pathway in vitro.

**METHODS**

*Cell cultures:* All animals were purchased from the Guangdong Provincial Center for Animal Research in Guangzhou, China. The research protocol was approved by the Animal Care Committee of the Zhongshan Ophthalmic Center at Sun Yat-sen University in China. All experiments on animals were handled in accordance with the ARVO Statement on Use of Animals in Ophthalmic and Vision Research. RMG cells were isolated from Newborn Sprague-Dawley rats (5 to 20 days old) as previously described [22]. A total of 40 rats were used in our study. Briefly, the eyes were enucleated, and the retina was removed carefully without contamination. Retinal tissues were dissociated by trituration and incubation at 37°C in 2% papain (Roche, Nutley, NJ) in Hanks Balanced Salt Solutions (HBSS). Suspended cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Invitrogen Corporation, Carlsbad, CA) + 10% fetal calf serum (FCS; Gibco, Invitrogen) supplemented with 1 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF; Sigma-Aldrich, St. Louis, MO) and allowed to grow at 37 °C in 5% CO2 in 75-cm2 flasks pre-coated with poly-D-lysine. The cells were grown to confluence, and RMG cells were found distributed on the top of the cell layer and could be detached by shaking the flask. The detached cells, comprising 90% microglia, were then cultured in 100-mm dishes at low density. Each microglial cell divided over the next 2-3 weeks to form individual colonies of adherent cells. Individual cell clusters, comprising solely microglia, were trypsinized inside a colony cylinder and cultured in a new 75-cm2 flask. The purity of the microglia in cultures was identified by staining with the microglial marker OX42 (Abcam, Cambridge, UK). The morphology of microglia in culture was carefully examined by phase-contrast and fluorescence microscopy.

*MTT cell viability assays:* The standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Roche, Mannheim, Germany] assay was used as the indicator for RMG cells survival ability in accordance with previously described protocols [23]. The cells were treated with fasudil (Sigma Chemical Co., St. Louis, MO) at serially diluted concentrations of 10, 25, and 50 μM, Lipopolysaccharides (LPS; Sigma, Deisenhofen, Germany; 1 μg/ml) or SB203580 (Sigma; a p38 inhibitor; 20 μM) for 24 h. Then, the cells were washed twice with PBS and incubated with 5 mg/ml MTT solution for 4 h at 37 °C and 5% CO2. Afterwards, 100 μl of DMSO (Sigma) solution was added and the optical density was measured by spectrophotometry at 570 nm with a microplate reader.

*Transwell migration assay:* In this study, the Costar Transwell System (8-μm pore size polycarbonate membrane, Costar, Cambridge, MA) was used to evaluate RMG cells migration. RMG cells in 0.2 ml serum-free medium were seeded in the upper well and 0.6 ml 1 μg/ml LPS, 50 μM fasudil, 1 μg/ml LPS + fasudil (10, 25 or 50 μM), 1 μg/ml LPS + 20 μM SB203580 or 1 μg/ml LPS + 50 μM fasudil + 20 μM SB203580 were added to the lower chamber. At the end of a 12 h incubation period at 37 °C, the non-migrating cells on the upper side of the membrane were removed with a cotton swab. Migrated cells on the lower side of the membrane were fixed with methanol for ten minutes and stained with Mayer’s Hematoxylin (Dakocytomation, Glostrup, Denmark) for 20 min. Photomicrographs of five randomly chosen fields were taken (Olympus CK2; Tokyo, Japan) and cells were enumerated to calculate the average number of cells that had migrated. All migrated cells were counted, and the results are presented as the mean ± SD of triplicates.

*Scratch wound assay:* RMG cells were seeded in 6-well plates in monolayer until approximately 95% confluent prior to the migration assay. Then, the medium was replaced with serum-free medium to ensure that no proliferation occurred during the experiments. A scratch wound was created by using a 200 μl pipette tip on confluent cells monolayers. The cells were treated with 1 μg/ml LPS, 50 μM fasudil, 1 μg/ml LPS + fasudil (10, 25 or 50 μM), 1 μg/ml LPS + 20 μM SB203580 or 1 μg/ml LPS + 50 μM fasudil + 20 μM SB203580 for 12 h. Following treatment, digital photographs of the cells that had migrated into the open wound were taken.

*Western blot analysis:* Total cells were harvested after treatment, washed twice with cold PBS, and lysed in ice-cold radioimmunoprecipitation buffer containing protease inhibitors [phenylmethylsulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology, Haimen, China)]. Lysates were centrifuged at 12,000 g at 4 °C for 15 min, and the supernatant was collected. Total protein concentration was quantified using the bicinchoninic acid (BCA; Beyotime
Institute of Biotechnology) protein assay kit (Sigma) [24]. The proteins were separated electrophoretically using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Beyotime Institute of Biotechnology) and then the gel was transferred onto polyvinylidene fluoride (PVDF; Beyotime Institute of Biotechnology) membranes. The membranes were subsequently blocked with the use of 5% milk in Tris Buffered saline Tween (TBST; Beyotime Institute of Biotechnology) for 1 h and incubated overnight with antibodies against p38 (Cell Signaling Technology, Beverly, MA), p-p38 (Cell Signaling Technology), p44/42 (Erk1/2) (Cell Signaling Technology), phospho-ERK1/2 (p-ERK1/2) (Cell Signaling Technology), c-Jun N-terminal kinase (JNK; Cell Signaling Technology), phospho-JNK (p-JNK; Cell Signaling Technology), MMP-2 (Abcam), MMP-9 (Abcam) or β-actin (Abcam) in TBST containing 5% defatted milk at 4 ºC followed by incubation with horseradish peroxidase-linked secondary antibodies (Abcam) at 4 ºC for additional 1 h. The immunobands were detected with an enhanced chemiluminescence kit (Amersham, ECL Plus, Freiburg, Germany), and the intensity was measured using ImageJ software (NIH, Bethesda, MD).

Statistical analysis: Experiments were repeated three times, and data are presented as the means ± standard deviation (SD). Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL). One-way analysis was used for a comparison among the experimental conditions and a P-value <0.05 was regarded as significant.

RESULTS

Fasudil does not affect the viability of RMG cells: Primary RMG cells were identified with immunofluorescence staining of OX42 (Figure 1). The cytotoxicity of fasudil was evaluated with an MTT assay. As shown in Figure 2A, fasudil (0, 10, 25, 50 μM) did not affect the cell viability of the RMG cells. Similarly, fasudil (0, 10, 25, 50 μM) did not significantly affect the viability of the RMG cells when the cells were incubated with or without LPS (1 μg/ml) for 24 h (Figure 2B).

Fasudil suppresses the LPS-stimulated phosphorylation of p38-MAPK in RMG cells: In human microglial cells, activation of the p38 signaling pathway is crucial for the migration process [6]. The results showed that the expression of phospho-p38 MAPK (p-p38-MAPK) was significantly elevated in LPS-stimulated RMG cells. However, fasudil reduced the level of p-p38-MAPK in a concentration-dependent manner but not total p38-MAPK. Additionally, the cells were pretreated with a p38 inhibitor (SB203580, 20 μM) for 30 min and then incubated in the presence or absence of fasudil (50 μM) for 6 h. We found that treatment with SB203580 and fasudil further inhibited the expression of p-p38-MAPK in the RMG cells (Figure 3). The activation of other MAPKs was also examined by assessing the phosphorylation of ERK1/2 and JNK. The expression of the p-ERK1/2 and p-JNK was significantly elevated in the LPS-stimulated RMG cells. However, the phosphorylation of ERK1/2 and JNK induced by LPS was not significantly affected by fasudil (50 μM) pretreatment (Figure 4).

Fasudil inhibits the migration of RMG cells: To assess the effect of fasudil on RMG cell motility, we performed a scratch wound assay and a Transwell migration assay. In the scratch-induced migration assay, representative microscopic images

Figure 1. Primary RMG cells were identified with immunofluorescence staining of OX42. A: OX42 (red). B: Nuclear Hoechst staining (blue). C: The purple color visualized in the merged images represents the colocalization of OX42 with nuclear Hoechst staining. Scale bars are equivalent to 25 μm.
indicated that LPS enhanced the migratory potential of the RMG cells compared to the control. Treatment with fasudil significantly reduced cell motility in the coculture with LPS substantially in a concentration-dependent manner. However, fasudil had no effect on RMG cell migration in the absence of LPS. The inhibition effect of fasudil was similar to that of SB203580, a p38-MAPK specific inhibitor. Furthermore, RMG cells that were pretreated with SB203580 (20 μM) for 30 min and then incubated in the presence or absence of fasudil (50 μM) for 12 h further reduced the LPS-stimulated RMG cell migration (Figure 5).

Similar to the scratch assays, the Transwell migration assay over a period of 12 h showed that the migratory capacity of the RMG cells was not altered by fasudil alone; however, cotreatment with LPS decreased the number of migrating cells substantially in a concentration-dependent manner. In addition, treatment with the p38-MAPK specific
inhibitor SB203580 and fasudil together further reduced LPS-stimulated RMG cell migration (Figure 6).

**Fasudil reduces the expression of MMP-2 and MMP-9:** The expression of MMP-2 and MMP-9 plays an important role in microglial cell migration [9]. The expression of MMP-2 and MMP-9 was significantly increased in the LPS-stimulated RMG cells. However, the elevated expression of both MMPs was inhibited by fasudil in a concentration-dependent manner. This inhibition effect was similar to that of the p38-MAPK specific inhibitor SB203580. Furthermore, the RMG cells were pretreated with SB203580 (20 μM) for 30 min and then incubated in the presence or absence of fasudil (50 μM) for 6 h. The results show that treatment with SB203580 and fasudil together further reduced the expression of MMP-2 and MMP-9 (Figure 7).

**DISCUSSION**

The model of LPS-stimulated RMG cells used in this study helped determine whether fasudil affected the migration of primary RMG cells via the p38-MAPK signaling pathway. Stimulation with LPS enhanced RMG cell motility, which was associated with elevated expression of p-p38-MARK, MMP-2, and MMP-9. However, treatment with fasudil can significantly inhibit RMG cell migration in the scratch wound assay and Transwell migration assay. Additionally, this inhibitory effect of fasudil was associated with reduced expression of p-p38-MARK, MMP-2, and MMP-9. Furthermore, SB203580, a p38 inhibitor, was used to confirm the role of the p38 signaling pathway in LPS-stimulated RMG cell migration. SB203580 suppressed the LPS-stimulated expression of p-p38-MAPK, MMP-2, and MMP-9 and enhanced these effects of fasudil, suggesting that fasudil inhibits LPS-stimulated RMG cell migration via directly suppressing the p38 signaling pathway.

Microglia are intrinsic immune cells in the central nervous system and possess neurotoxic and neuroprotective functions [25]. These cells perform dynamic immune surveillance and respond to any kind of pathology with a reaction termed microglial activation. Under physiological conditions, microglia exist in a resting state with ramified morphology with protruding processes that continually survey their microenvironment. In response to pathological stimuli, microglial processes rapidly activate and autonomously converge on the damaged site to perform the function of macrophages. However, mounting evidence has also suggested that microglia can promote inflammation and act in a neurotoxic role to progress neuron damage in
Figure 5. Fasudil inhibits the migration of LPS-stimulated RMG cells, as shown with the scratch wound assays. A: Fasudil (0–50 μM) inhibits the migratory potential of lipopolysaccharide (LPS)-stimulated retinal microglial (RMG) cells, as shown by the scratch wound assays. The cells were pretreated with SB203580 (20 mM) for 30 min and then incubated in the presence or absence of fasudil (50 μM) for 12 h in the LPS-stimulated RMG cells. Micrographs from one representative experiment of three independent experiments are shown. The results are expressed as mean ± standard deviation (SD). *p<0.05 compared with untreated cells, **p<0.05 compared with LPS-stimulated cells.
severe injury or neurodegenerative diseases. In the retina, substantive evidence similarly demonstrates that microglial cells play an important role in the development of various retinal injuries and diseases, such as age-related macular degeneration (AMD) [26,27], light-induced retinal degeneration [28], diabetic retinography (DR) [29], glaucoma [30], and endotoxin-induced uveitis [31]. The migration of RMG cells is a hallmark of inflammation. Increased RMG cell migration in response to specific chemoattractants has been found, but less is known about the signal transduction pathways that mediate their effects. According to the present study results, activation of the p38-MAPK signaling pathway appears to be involved in LPS-induced migration of retinal microglial cells and fasudil can inhibit LPS-induced migration of retinal microglial cells by suppressing the p38-MAPK signaling pathway.

MMPs, especially MMP-2 and MMP-9, play critical roles in the degradation of type IV collagen, a major constituent of the ECM, and are closely related to the migration of microglia [9]. Furthermore, MMPs are enzymes that
induce cellular morphologic changes that promote increased motility [25,32,33]. We demonstrated that fasudil reduced the expression of LPS-stimulated MMP-2 and MMP-9 in RMG cells. Consistently, previous studies showed that fasudil prevented injury to the human brain endothelial cells via the reduction of MMP-9 activity [34]. Importantly, it was reported that fasudil suppressed the expression and activity of MMP-2 and MMP-9 in RMG cells [34]. We found that fasudil and SB203580, a p38-MAPK specific inhibitor, show additive inhibitory effects on the expression of MMP-2 and MMP-9, suggesting that the expression of MMP-2 and MMP-9 was also controlled by the p38-MAPK signaling pathway and fasudil-suppressed RMG cell migration was correlated with modulation of MMP-2 and MMP-9 expression.

p38-MAPK, one of the families of intracellular signaling molecules that transduce extracellular responses, plays a well-known role in regulating the LPS-stimulated activation of microglia [38-40]. Accumulating evidence indicates that p38 is also essential for cell migration. The phosphorylation of p38-MAPK is involved in regulating the expression of MMPs, especially MMP-2 and MMP-9 [41-43], and thus promotes the degradation of ECM proteins and cellular morphologic changes, which leads to cell migration. To further explore the possible mechanism of fasudil in the inhibition of RMG cell migration, we detected the levels of phosphorylation of p38-MAPK in RMG cells. The results demonstrated that the p-p38-MAPK in cells treated with fasudil was significantly reduced relative to that in control cells, but not p38-MAPK. Fasudil combined with a p38 inhibitor (SB203580) significantly reduced RMG cell migration and was accompanied by downregulation of MMP-2 and MMP-9. Additionally, fasudil may affect different MAPKs in other cell types. Previous studies showed that fasudil pretreatment obviously inhibited the activation of p38 and JNK induced by LPS in LPS-induced apoptosis of rat pulmonary microvascular endothelial cells, whereas that of extracellular ERK1/2 was not affected [44]. In addition, fasudil inhibited phosphorylation of ERK1/2, JNK, and p38 in hepatic stellate cells [45]. In the present study, the results showed that fasudil, the selective ROCK inhibitor, reduced the expression of p-p38 in RMG cells treated with LPS but had no significant effect on the expression of p-ERK1/2 and p-JNK, suggesting that p-p38 instead of p-ERK1/2 and p-JNK might act as the downstream mediators of the ROCK signal pathway in RMG cells upon LPS exposure.

Cell migration primarily reflects the dynamic organization of the actin cytoskeleton, and ROCK critically regulates this process, which mediates the reorganization of stress fibers and focal adhesion as well as the rearrangement of filopodia [46]. Previous studies have shown that fasudil can effectively inhibit ROCK I and ROCK II expression in microglia [47]. The present study further showed that fasudil could inhibit retinal microglial migration. Meanwhile, it has been reported
that p38-MAPK is involved in cell migration and actin cytoskeleton reorganization. Inhibition of p38-MAPK activity and the subsequent phosphorylation could prevent actin cytoskeleton reorganization [48-50]. Furthermore, the low molecular weight GTP-binding proteins from the Rho subfamily such as Rho, Rac1, and Rit take part in the regulation of the p38-MAPK pathway as upstream molecules [51-53]. The present study indicated that SB203580 (a p38-MAPK inhibitor) could enhance the effect of retinal microglial migration produced by fasudil, suggesting fasudil directly suppresses the p38-MAPK signaling pathway. Further study is needed to clarify the mechanisms by which the ROCK and p38-MAPK pathways regulate retinal microglial migration, particularly in cross-talk among cellular signaling pathways.

Taken together, the results demonstrate the inhibitory effect of fasudil on the migration of RMG cells and the expression of MMP-2 and MMP-9. This inhibitory effect may be controlled by the p38 MAPK pathway. These findings reveal a new potential therapeutic application of fasudil for treating retinal neuroinflammation associated with RMG cell migration.

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