J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-016-1537-3 36(1):31-36,2016

ROCK Inhibition with Fasudil Promotes Early Functional Recovery of Spinal Cord Injury in Rats by Enhancing Microglia Phagocytosis*

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Summary: Emerging evidence indicates that microglia activation plays an important role in spinal cord injury (SCI) caused by trauma. Studies have found that inhibiting the Rho/Rho-associated protein kinase (ROCK) signaling pathway can reduce inflammatory cytokine production by microglia. In this study, Western blotting was conducted to detect ROCK2 expression after the SCI; the ROCK Activity Assay kit was used for assay of ROCK pathway activity; microglia morphology was examined using the CD11b antibody; electron microscopy was used to detect microglia phagocytosis; TUNEL was used to detect tissue cell apoptosis; myelin staining was performed using an antibody against myelin basic protein (MBP); behavioral outcomes were evaluated according to the methods of Basso, Beattie, and Bresnahan (BBB). We observed an increase in ROCK activity and microglial activation after SCI. The microglia became larger and rounder and contained myelin-like substances. Furthermore, treatment with fasudil inhibited neuronal cells apoptosis, alleviated demyelination and the formation of cavities, and improved motor recovery. The experimental evidence reveals that the ROCK inhibitor fasudil can regulate microglial activation, promote cell phagocytosis, and improve the SCI microenvironment to promote SCI repair. Thus, fasudil may be useful for the treatment of SCI.
Key words: Rho/ROCK; microglia; spinal cord injury; phagocytosis

The pathophysiology of trauma-induced spinal cord injury (SCI) is believed to involve two stages[1]. Although the primary mechanical damage is not amenable to treatment, secondary damage, such as vascular ischemia, edema, inflammatory reactions, electrolyte disorders, free radical formation, and apoptosis, is manageable.

Microglia play an important role in the SCI-related secondary damage. After SCI, microglia, the macrophages of the central nervous system, secrete inflammatory cytokines to trigger inflammatory responses. This early inflammatory response is thought to be detrimental[2, 3]. Upon SCI, the microglia constantly monitor the microenvironment for toxic substances and noxious processes, respond to extracellular signals, engulf cellular debris and toxic substances, and secrete trophic factors to provide neuroprotection[4, 5]. The regulation of microglia phagocytosis may create a better environment that encourages SCI repair.

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*This project was supported by grants from Natural Science Foundation of China (No. 81471200 and No. 81000521), National Basic Research Program of China (No. 2011CB504403) and the Young Medical Scientists Project of Wuhan (No. 2013-12).

Rho-associated protein kinase (ROCK), a well-characterized downstream effector of Rho, includes two highly homologous isoforms, ROCK1 and ROCK2[6, 7]. Recently, ROCK has attracted attention for its potency in regulating cell activation. Phagocytosis is regulated by the cytoskeleton through myosin light chain kinase (MLCK) and ROCK, which regulates cell morphology[8, 9]. Accumulating evidence suggests that ROCK inhibition may play an active role in cell morphology and phagocytosis[10–13]. Understanding the effects of ROCK inhibition on microglia phagocytosis and differentiation may be particularly important in gaining insight into the role of ROCK in SCI. On the basis of previous studies, we were led to hypothesize that the regulation of ROCK signalling may influence the activation of microglia and associated phagocytosis to ameliorate secondary damage after SCI. To test the assumption, in this study, we examined ROCK expression, microglial activation, phagocytosis, and morphological changes in rats with traumatic SCI after blocking ROCK with fasudil, a ROCK inhibitor that has been widely used for studying the function of ROCK signalling[14–17].

1 MATERIALS AND METHODS

1.1 Surgical Procedures and Reagent Delivery

All experimental procedures were performed in accordance with protocols approved by the Governmental

Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technolgoy, Wuhan, China. Intraoperatively, rats were placed on a warming pad to maintain a body temperature of 37.0±0.5°C. After the SCI, the animals were returned to individual cages with access to sufficient water and food. After the operation, each animal received a daily penicillin injection (200 000 U per rat, i.m.) for three days in a row.

Adult Sprague-Dawley male rats (weighing 260 to 300 g) were randomly assigned to one of three experimental groups: sham-treated, vehicle-treated, and fasudil-treated groups. Traumatic SCI was induced by employing the weight-drop technique, as described previously[18]. First, rats were anesthetized with ketamine (75 mg/kg) and xylazine (20 mg/kg) by intraperitoneal injection. Then, the T_{11} spinal cord was exposed by spinal laminectomy, and a moderate intensity weight drop (10 g×12.5 cm) was performed by using a MASCIS Impactor II (New York University, USA). Rats in the sham-treated group underwent similar procedures as the vehicle-treated group, expect for the weight-drop step.

Immediately after SCI induction, a subcutaneous osmotic pump (Model 1007D, Alzet, USA) was placed close to the injury site for intrathecal reagent infusion. Before implantation, the pump was filled with 100 μL saline (sham-treated and vehicle-treated) or fasudil (15 μg/μL, 180 μg/day), connected to a 1.5-mm long PE-10 tube, and then pre-incubated for over 6 h at 37°C[19]. At 1, 3, 7, and 14 days after the infusion, the pump was removed and the spinal cords were extracted for the experiment.

1.2 Immunocytochemical Staining

Anesthetized rats were transcardially infused with saline, and then by ice-cold Zamboni's fixative. Spinal cord tissues containing the injury site were extracted, fixed in Zamboni's fixative for 24 h, cryoprotected in 30% sucrose/0.1 mol/L PBS for 3 days at 4°C, and finally cut longitudinally into 10-μm sections for fluorescent staining.

Tissue sections were washed in PBS, incubated with 5% bovine serum albumin in PBS at room temperature for 1 h to block non-specific antibody binding, incubated overnight with anti-CD11b and anti-ROCK2 at 4°C, and then incubated with goat anti-rabbit IgG antibodies and goat anti-mouse IgG antibodies conjugated to (CY3, 1:200 and FITC, 1:200; Jackson ImmunoResearch Laboratories Inc., USA) and 4', 6-diamidino-2-phenylindole (DAPI, 10 μg/mL) for 10 min. The microglia were morphologically examined under a fluorescence microscope.

1.3 Western Blotting

After the rats were transcardially infused with saline, the spinal cord tissue (1.5-mm long, with the injury site centered) of the rats was quickly removed and homogenized by sonication in RIPA lysis buffer. The lysates were centrifuged at 12 000 g at 4°C for 15 min, and the supernatants were collected for protein determination by using a BCA kit. A 40-μg sample of protein from each rat was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the separated proteins were transferred to nitrocellulose membranes (Schleicher & Schull, USA). The membranes were blocked in 5% (w/v) non-fat milk in TBST [10 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.02% (v/v) Tween-20, pH 7.5] and incubated with primary antibodies overnight at 4°C, including anti-ROCK2 (1:500, Cell Signaling Technology, USA) and anti-β-actin (1:1000, Santa Cruz Biotechnology, USA). Odyssey secondary antibodies (IRDye 800-conjugated goat anti-rabbit IgG) were added according to the manufacturer's instructions. The immunoreactivity of the protein bands was quantitatively determined by using an Odyssey IR imaging system (LI-COR Biosciences Inc., USA) and expressed as the mean absorbance (*A*). The level of each protein was normalized to that of β-actin (i.e., *A* of target protein/*A* of related β-actin) prior to statistical analysis.

1.4 Electron Microscopy

At the 1st, 3rd and 7th day post-infusion, the injured edge of the spinal cords (approximately 2–5 mm^3 in size) were extracted, placed on ice, and fixed in glutaric silane solution. Electron microscopy (FEI Tecnai G2 12, The Netherlands) was performed by the Morphology Laboratory of Tongji Medical College.

1.5 Apoptosis Detection and Behavioral Measurement

At day 1 post-infusion, the pump was removed and spinal cord tissues containing the injury site were extracted, fixed in cold isopentane, and cut longitudinally into 10-μm sections for apoptosis detection. The apoptosis test was carried out in accordance with the instructions of the apoptosis assay kit (*In Situ* Cell Death Detection Kit, TUNEL; Roche Diagnostics, USA).

Behavioral outcomes were evaluated according to the methods of Basso, Beattie, and Bresnahan (BBB)[20] 1, 3, 7 and 14 days after the SCI by two trained investigators blinded to the experimental grouping.

1.6 Quantification of Myelin Loss and Cavity Area

The animals in the sham-, vehicle-, and fasudil-treated groups were sacrificed 14 days after the injury (n=4 in each group). The cavity area was measured in the spinal cord tissue. Sagittal cryostat sections (10-μm in thickness) were stained with myelin basic protein (MBP, 1:200; Boster, China) for detection of myelin loss, and the area that was not stained was identified as the cavity area. Every tenth section in each animal was selected, and the images were captured using an Olympus BX51 fluorescent microscope, Japan. The maximum area of the cavity in the sagittal sections containing the lesion epicenter and the total T_{10}-segment cavity were measured with an NIH image processing and analysis program.

1.7 Statistical Analysis

Statistical differences between the groups (defined as P<0.05) were evaluated by employing a one-way ANOVA, followed by Tukey's *post hoc* test. The data were presented as $\bar{x}\pm s_x$. The statistical analyses were detailed in the paper.

2 RESULTS

2.1 Fasudil Reduces ROCK Activity after SCI

Western blotting was conducted to detect ROCK2 expression after the SCI, and the ROCK Activity Assay kit (Millipore, USA) was used for detection of ROCK activity. At the 1st, 3rd and 7th day after the SCI, ROCK expression showed no significant difference among the sham-, vehicle- and fasudil-treated groups (fig. 1A and 1B, n=4). However, as compared with the sham-treated group, ROCK activity was significantly increased in the

vehicle-treated group and significantly reduced in the fasudil-treated group 1, 3 and 7 day(s) after the SCI (fig. 1C, n=4, P<0.05 or P<0.01).

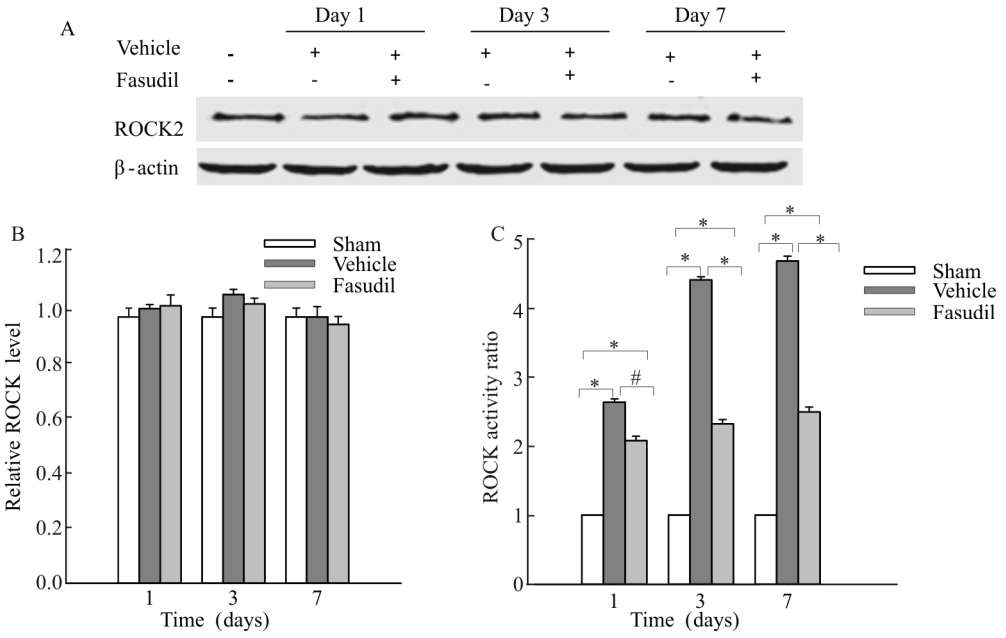

Fig. 1 Inhibitory effect of fasudil on ROCK expression and activity after SCI

A: A representative Western blot reveals changes in ROCK2 expression in response to SCI and fasudil treatment at 1st, 3rd, and 7th day post-injury; B: statistical analysis of ROCK2 protein expression (n=4); C: statistical analysis of ROCK2 activity 1, 3 and 7 days post-injury (n=4, $^{*}P$<0.01, $^{\#}P$<0.05)

2.2 Fasudil Promotes Microglia Phagocytosis after SCI

Next, microglia were morphologically examined by using the CD11b antibody. In the sham-treated group, the microglia remained branched and small (fig. 2A1), and ROCK2 expression was observed in a variety of cells (fig. 2A2 and 2A3). After the SCI, the lesions were surrounded by round or amoeboid-shaped activated microglia that expressed ROCK2 (fig. 2B1–2B3; 3 days post-injury). Compared with the vehicle-treated group, the microglia in the fasudil-treated group were more abundant and larger (fig. 2C1–2C3). Electron microscopy revealed that the microglia activated macrophages that had engulfed phospholipids and cell pieces after the SCI. Three days after the injury, many macrophages containing phospholipid-like materials were observed on the edge of the lesion. In addition, non-engulfed phospholipid-like debris and erythrocytes were observed (fig. 3A1). Compared with the vehicle-treated group, more macrophages containing phospholipid-class material on the edge of the lesion, more phospholipid-class material in the macrophages, and less non-engulfed phospholipid-class debris and erythrocytes were observed in the fasudil-treated group (fig. 3A2). These results indicate that fasudil promotes the phagocytosis of microglia after SCI.

2.3 Fasudil Reduces Apoptosis of Tissue Cells

Microglia phagocytosis enhanced by fasudil exerts a positive effect on SCI repair. TUNEL was used to detect tissue cell apoptosis. In the sham-treated group, practically no spinal cord was stained by TUNEL (fig. 4A). One day after injury, a significant amount of TUNEL staining was observed around the injury site (fig. 4B). As compared with the vehicle-treated group, the expression of TUNEL in the fasudil-treated group was down-regulated 1 day after injury (fig. 4C and 4D, n=4, P<0.01).

2.4 Fasudil Reduces Myelin Loss and Improves Motor Function after SCI

Myelin staining was performed by using an antibody against MBP in the lesion area of the spinal cord epicenter in the sham-treated, vehicle-treated, and fasudil-treated groups. Fig. 5A1 shows that normal myelin appeared intact in the spinal cord tissue of the sham-treated group. However, at the 14th post-injury day, normal-appearing myelin was significantly decreased in the vehicle-treated group (fig. 5A2). This decrease was attenuated in the fasudil-treated group (fig. 5A3), indicating that the fasudil treatment could effectively protected against post-SCI demyelination (fig. 5A and 5B, n=4, P<0.01).

In addition to the morphological observations, functional recovery also was evaluated (fig. 5C, n=4, P<0.01). All the rats had severe and uniform functional deficits 1 day after the SCI. Behavioral improvement was observed after the treatment with vehicle. Fasudil treatment, lasting for 14 days, progressively reduced the functional deficits 7 and 14 days after fasudil treatment, and the deficit amelioration was statistically significant as compared with vehicle-treated rats.

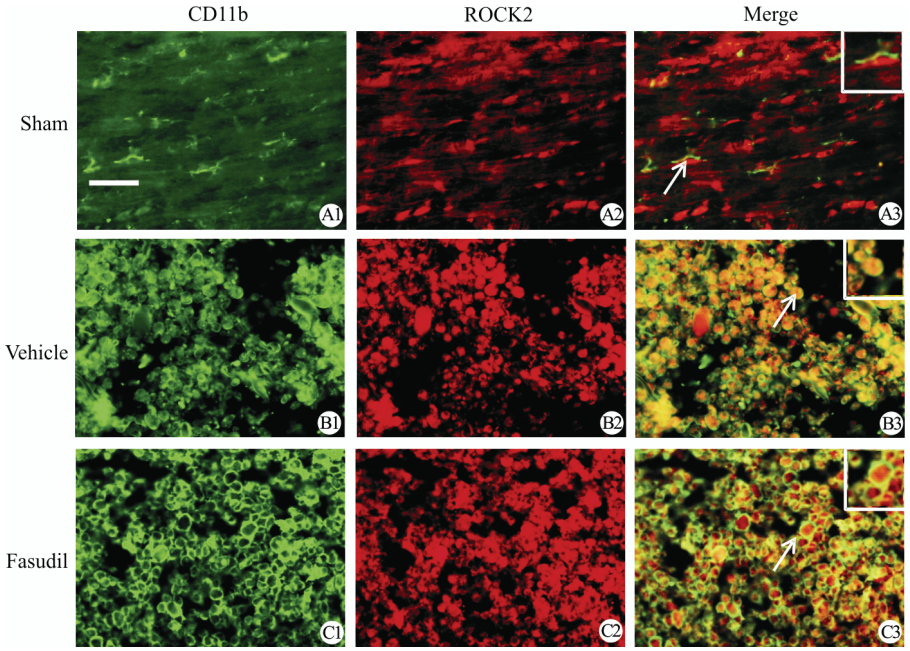

Fig. 2 Fasudil promotes microglia phagocytosis after SCI

An assessment of microglia CD11b (green) and the expression of ROCK2 (red) by immunocytochemical double-staining in (A) sham-treated; (B) vehicle-treated; and (C) fasudil-treated rats. The scale bar = 25 μm. White arrows indicate the expression of ROCK2 in microglia.

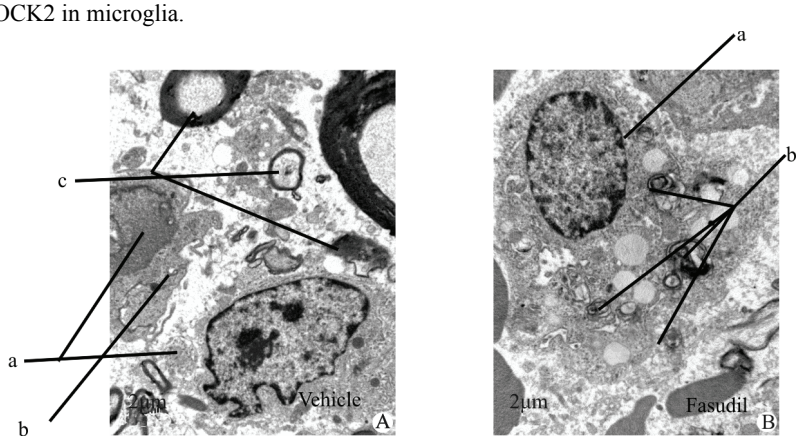

Fig. 3 Electron microscopic detection of microglia phagocytosis

Representative electron microscopy results reveal the changes in microglia in response to vehicle treatment (A) and fasudil treatment (B) at 3 days post-injury. a: activated microglia; b: macrophages containing phospholipid-class material; and c: remaining debris and erythrocytes

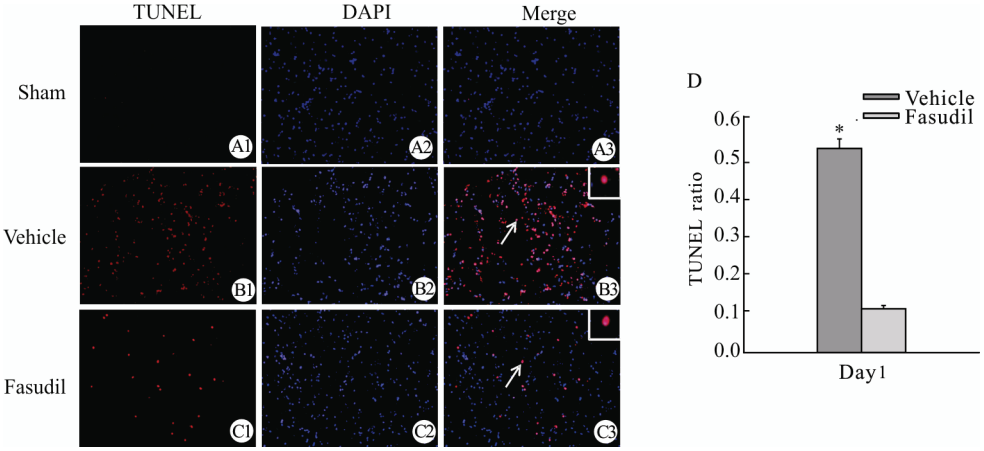

Fig. 4 Fasudil reduces tissue cell apoptosis.

TUNEL expression in the (A) sham-treated group; (B) the vehicle-treated group; and (C) the fasudil-treated group; TUNEL (red)/DAPI (blue). The scale bar=50 μm. D: statistical analysis of TUNEL expression at 1st day post-injury (n=5, $^{*}P$<0.01). The white arrows indicate apoptotic cells.

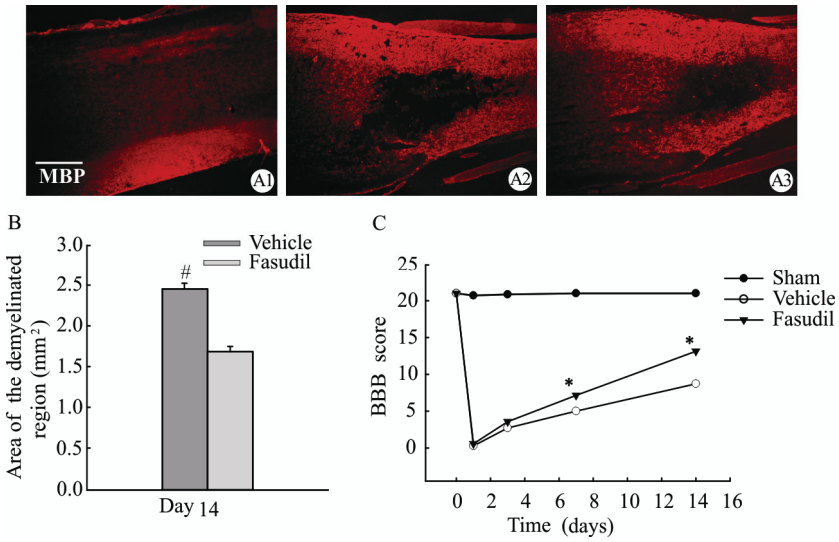

Fig. 5 Fasudil promotes functional recovery.

A: Representative pictures of MBP staining show the changes in the cavity area in sham-treated (A1), vehicle-treated (A2), and fasudil-treated (A3) rats at 14th day post-injury. The scale bar=1 mm. B: statistical analysis of the cavity area 14 days post-injury; C: Temporal changes in behavioral outcomes were evaluated by the BBB score (n=4, $^{*}P$<0.01)

3 DISCUSSION

This study has demonstrated that microglial activation was accompanied by Rho/ROCK signaling activation *in vivo* and that ROCK blockade by fasudil increased the phagocytic function of microglia, reduced cellular apoptosis, and promoted morphological and functional recovery of rats after SCI.

The experiment revealed that spinal cord lesions were surrounded by round- or amoeboid-shaped activated microglia. After SCI, the activation of normally quiescent microglia is accompanied by changes in morphology, motility, proliferation, expression of specific cell surface molecules, and the release of cytokines and chemokines to become so-called "reactive microglia" [21]. Reactive microglia are considered to be at the center of the injury cascade[22]. Through the release of molecules such as TNF-α, IL-1β, nitric oxide, and reactive free radicals, microglia aggravate early post-injury necrotic cell death and promote cell apoptosis, tissue edema, and axonal degeneration[23–25]. Activated microglia, however, also produce damage as they engulf tissue fragments and clean up the microenvironment of the spinal cord lesion. The rapid and efficient phagocytosis of apoptotic cells plays a critical role in the prevention of secondary necrosis and inflammation, tissue remodeling and immune response regulation, all of which could promote repair of injured spinal cord[26, 27].

Therefore, this study aimed to modulate microglial activation to simultaneously promote favorable functions and minimize harmful functions to encourage post-SCI recovery. The cellular inflammatory response, a detrimental factor of microglia activation, has been reported to be markedly reduced after Rho/ROCK was inhibited both in primary microglia cultures and *in vivo*[16, 28], suggesting that ROCK might be a potential therapeutic target. In this study, we observed an elevated expression of ROCK2 in activated microglia, and blocking Rho/ROCK activation led to an increase in microglia phagocytosis. Given the wide distribution and multiple functions of ROCK, other mechanisms may also be involved in this improvement. For example, ROCK activates astrocytes and increases the expression of neurite growth-inhibitory chondroitin sulfate proteoglycans to regulate astrocyte-derived G-CSF and improve neurite outgrowth and myelination[29–32].

SCI is a catastrophic injury that includes multiple events. The results obtained from animal experiments may provide only partial information about the mechanisms underlying the observed phenomenon, and some limitations remain. As a newly recognized therapeutic target, the Rho/ROCK signaling is thought to be of neuroprotective nature. However, negative evidence also exists. For example, it was reported that delayed treatment with Rho-kinase inhibitor did not enhance axonal regeneration or functional recovery after SCI in rats[33, 34]. Thus, further studies are warranted to examine the intricate post-SCI regulation of Rho/ROCK.

In summary, this study suggests that Rho/ROCK signaling is essential for microglial activation and phagocytosis, and ROCK might be a potential therapeutic target for the treatment of SCI. Rats which sustained spinal cord trauma could be effectively treated by fasudil, a potent Rho/ROCK blocker that up-regulates microglial phagocytosis. The fact that the Rho/ROCK blocker fasudil has been already used clinically for the treatment of ischemic cerebrovascular disease makes this therapy a particularly attractive candidate for clinical trials looking for better treatment modalities for SCI.

Conflict of Interest Statement

The authors declare no conflict of interest.

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(Received June 6, 2015; revised Oct. 13, 2015)