Effect of Fasudil, a Selective Inhibitor of Rho Kinase Activity, in the Secondary Injury Associated with the Experimental Model of Spinal Cord Trauma

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ABSTRACT

Rho kinase (ROK) may play an important role in regulating the biological events of cells, including proliferation, differentiation, and survival/death. Blockade of ROK promotes axonal regeneration and neuron survival in vivo and in vitro, thereby exhibiting potential clinical applications in spinal cord damage and stroke. The aim of this experimental study was to determine the role of ROK signaling pathways in the inflammatory response, in particular in the secondary injury associated with the experimental model of spinal cord trauma. The injury was induced by application of vascular clips to the dura via a four-level T5 to T8 laminectomy in mice. Fasudil was administered in mice (10 mg/kg i.p.) 1 and 6 h after the trauma. The treatment with fasudil significantly decreased 1) histological damage; 2) motor recovery; 3) nuclear factor-κB (NF-κB) expression; 4) ROK activity; 5) inflammation (caspase-1 and NOD-like receptor family, pyrin domain-containing 3 expression); 6) production of proinflammatory cytokine such as tumor necrosis factor and interleukin-1β (IL-1β); 7) neutrophil infiltration; 8) nitrotyrosine and poly-ADP-ribose formation; 9) glial fibrillary acidic protein expression; 10) apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling staining, FAS ligand expression, and Bax and Bcl-2 expression); and 11) mitogen-activated protein kinase activation (phospho-extracellular signal-regulated kinase and phospho-c-Jun NH2-terminal kinase expression). Our results indicate that inhibition of ROK by fasudil may represent a useful therapeutic perspective in the treatment of inflammation associated with spinal cord trauma.

Introduction

Individuals paralyzed by spinal cord injury (SCI) are left with one of the most physically disabling and psychologically devastating conditions known to humans. More than 10,000 North Americans, most of them younger than age 30 years, experience such an injury each year (Nobunaga et al., 1999). Although medical, surgical, and rehabilitative care for SCI has an enormous economic impact, its complex pathophysiology leads to difficulty in finding a suitable therapy (Stover and Fine, 1987). Typically, the center of the spinal cord injury is predominantly characterized by necrotic death. The primary injury refers to the mechanical damage leading to direct cell death and bleeding. Further progressive destruction of the tissue surrounding the necrotic core is known as secondary injury (Beattie et al., 2000). which is determined by a large number of vascular, biochemical, and cellular cascades including breakdown of the blood-spinal cord barrier with edema formation, ischemia, and hypoxia, release of vasoactive substances leading to alteration of spinal cord perfusion, excitotoxicity leading to Ca2+-dependent, glutamate-associated neuronal cell death, formation of free radicals and nitric oxide (NO), damage of mitochondria with energy depletion, the invasion and activation of inflammatory cells (such as neutrophils, resident microglia, peripheral

ABBREVIATIONS: SCI, spinal cord injury; NO, nitric oxide; ROS, reactive oxygen species; IκB, inhibitor of nuclear factor-κB; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; NLRP3, NOD-like receptor family, pyrin domain-containing 3; ROK, Rho kinase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; MPO, myeloperoxidase; BMS, Basso Mouse Scale; PAR, poly-ADP-ribose; GFAP, glial fibrillary acidic protein; P-JNK, phospho-c-Jun NH2-terminal kinase; CNS, central nervous system; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; P-ERK, phospho-extracellular signal-regulated kinase; n-MYPT1, myosin phosphate target subunit-1; Y27632, (-)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexancarboxamide dihydrochloride; ONOO-, peroxynitrate; PARP, poly-ADP-ribose polymerase.
Macrophages, and astrocytes), which secrete lytic enzymes and cytokines contributing to further tissue damage, apoptosis of oligodendrocytes, and neurodegeneration (Hausmann, 2003).

Neutrophils are the first inflammatory cells to arrive at the site of injury in non-neuronal and neuronal tissue. Neutrophils are involved in the modulation of the secondary injury by release of neutrophil proteases and reactive oxygen species (ROS) (Hausmann, 2003), which activate transcription factors such as nuclear factor-κB (NF-κB) that plays a central and crucial role in inducing the expression of inflammatory cytokines (Chen et al., 2004). Increased ROS production is also implicated in the development of cellular hypertrophy and remodeling, at least in part through activation of redox-sensitive protein kinases such as members of the mitogen-activated protein kinase (MAPK) superfamily (Li et al., 2002). In addition, the generation of ROS also seems crucial for the activation of the NLRP3 inflammasome (Dostert et al., 2009).

Ras-homologous (Rho) signaling pathways, which probably have homeostatic functions under normal physiological conditions, appear to be most highly activated under conditions of inflammation and injury. Whereas their recruitment may be of benefit for initiation of protective responses, their sustained activation may have pathological consequences (Seaholtz and Brown, 2004).

Small (21-kDa) GTPases of the Rho family and one of their effectors, Rho kinase (ROK), are known to act as molecular switches controlling several critical cellular functions, such as actin cytoskeleton organization, cell adhesion, migration, ROS formation, and apoptosis, as well as cytokinesis and oncogenic transformation (Riento and Ridley, 2003; Bokoch, 2005). There are two isoforms of ROK, known as ROK I and II. ROK I shows the highest expression level in non-neuronal tissues, whereas ROK II is preferentially expressed in the brain (Wang et al., 2011). Moreover, ROK inhibitors have been shown to be effective against reperfusion injury in the liver (Shiotani et al., 2004), heart (Bao et al., 2004), tissue fibrosis (Bourgier et al., 2005), cerebral ischemia (Satoh et al., 2001), and pulmonary hypertension (Abe et al., 2004).

1-(5-Isoquinolinesulfonyl)-homopiperazine hydrochloride (fasudil, HA-1077) is a specific ROK inhibitor (He et al., 2008) and is the first kinase inhibitor drug used in a clinical setting in Japan (Shibuya et al., 1992). Fasudil has been used for years for the treatment of subarachnoid hemorrhage, and its safety for clinical use is well established. Fasudil has been reported to inhibit NF-κB signaling after infection with the human immunodeficiency virus (Sato et al., 1998). NF-κB is normally sequestered in the cytoplasm, bound to regulatory proteins IκB. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IκB is phosphorylated by the enzyme IκB kinase (Bowie and O’Neill, 2000). The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus and to active genic transcription of inflammatory proteins.

The aim of the present study was to determine the role of ROK signaling pathways in the inflammatory response, in particular in the secondary injury associated with spinal cord trauma.

Materials and Methods

Animals. Male Adult CD1 mice (25–30 g; Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations for protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the European Union regulations (O.J. of E.C. L 358/1, 12/18/1986).

SCI. Mice were anesthetized using chloral hydrate (400 mg/kg b.wt.). We used the clip compression model described by Rivlin and Tator (1978). A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away, exposing T5 to T8 vertebrae. The spinal cord was exposed via a four-level T5 to T8 laminectomy, and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. In the injured groups, the cord was compressed for 1 min. After surgery, 1.0 ml of saline was administered subcutaneously to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27°C for a survival period of 10 days. Food and water were provided to the mice ad libitum. During this time period, the animals’ bladders were manually voided twice a day until the mice were able to regain normal bladder function. Sham-injured animals were subjected to laminectomy only.

Experimental Design. Mice were randomized into four groups of 20 mice/group (n = 80 total animals). Forty mice were sacrificed at 24 h after SCI to evaluate the various parameters, whereas the other 40 were observed until 10 days after SCI to evaluate the motor score. Sham animals were subjected to the surgical procedure except that the aneurysm clip was not applied, and they were treated intraperitoneally with vehicle (saline) or fasudil (10 mg/kg) 1 and 6 h after the surgical procedure. The remaining mice were subjected to SCI (as described above) and treated with an intraperitoneal bolus of vehicle (saline) or fasudil 1 and 6 h after SCI. The dose was chosen on the basis of recent studies (Ding et al., 2011; Ma et al., 2011).

Light Microscopy. Spinal cord tissues were taken at 24 h after trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin-embedded and cut into 5-μm-thick sections. Tissue sections (thickness 5 μm) were deparaffinized with xylene, stained with hematoxylin and eosin (H&E) or with silver impregnation for reticulum, and studied using light microscopy (Dialux 22; Leitz, Wetzlar, Germany). The segments of each spinal cord were evaluated by an experienced histopathologist. Damaged neurons were counted, and the histopathology changes of the gray matter were scored on a six-point scale (Sirin et al., 2002): 0, no lesion observed; 1, gray matter contained 1 to 5 eosinophilic neurons; 2, gray matter contained 5 to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one-third of the gray matter area); 5, moderate infarction (one-third to one-half of the gray matter area); and 6, large infarction (more than one-half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion.

Measurement of Spinal Cord TNF-α and IL-1β Levels. Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized in phosphate-buffered saline (PBS) containing 2 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO) as described previously, and tissue TNF-α and IL-1β levels were evaluated. The assay was performed by using a colorimetric, commercial kit (Calbiochem-Novabiochem, San Diego, CA) according to the manufacturer’s instructions. All TNF-α and IL-1β determinations were performed in duplicate serial dilutions.

Myeloperoxidase Activity. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined in the spinal cord tissues as described previously (Mullane,
1989) at 24 h after SCI. After SCI, spinal cord tissues were obtained and weighed, and each piece was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000g and 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37°C and was expressed as units of MPO per milligram of protein.

Grading of Motor Disturbance. The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the Basso Mouse Scale (BMS) (Basso et al., 2006).

Immunohistochemical Localization of TNF-α, IL-1β, Nitrotyrosine, PAR, FAS Ligand, Bax, Bel-2, GFAP, and P-JNK. At 24 h after SCI, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde, and 8-mm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the sections in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin-biotin peroxidase complex (DBA, Milan, Italy). Sections were incubated overnight with the following: (1) goat polyclonal anti-TNF-α antibody (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); (2) rabbit polyclonal anti-IL-1β (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.); (3) rabbit polyclonal anti-Bax (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.); (4) rabbit polyclonal anti-Bcl-2 (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.); (5) goat polyclonal anti-PAR antibody (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.); (6) mouse monoclonal anti-Fas ligand (1:100 in PBS, w/v) (Monosan, Uden, The Netherlands); (7) mouse monoclonal anti-GFAP (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.); (8) rabbit polyclonal anti-nitrotyrosine (1:250 in PBS, w/v) (Millipore Corporation, Billerica, MA); and (9) mouse monoclonal anti-P-JNK (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and DBA. The counter stain was developed with diaminobenzidine (brown color) and nuclear staining was performed with diaminobenzidine. The number of TUNEL-positive cells per high-power field was counted in 5 to 10 fields for each coded slide.

Western Blot Analysis for IkB-α, NF-κB p65, Caspase-1, NLRP3, p-MYPT1, Bax, Bel-2, P-ERK, and P-JNK Kinases. Cytosolic and nuclear extracts were prepared as described previously (Bethea et al., 1998) with slight modifications. Spinal cord tissues from each mouse were suspended in extraction buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, and 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged for 10 min at 1000g for 10 min and 4°C. Supernatants represented the cytosolic fraction. The pellets containing enriched nuclei were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 20 μM leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000g and 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. The levels of IkB-α, caspase-1, myosin-binding subunit, p-MYPT1, NLRP3, Bax, Bel-2, P-ERK, and P-JNK were quantified in the cytosolic fraction from spinal cord tissue collected 24 h after SCI, and NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1× PBS and 5% (w/v) nonfat dried milk for 40 min at room temperature and subsequently probed with specific antibodies IkB-α (1:1000; Santa Cruz Biotechnology, Inc.), anti-Bax (1:500; Santa Cruz Biotechnology, Inc.), anti-Bcl-2 (1:500; Santa Cruz Biotechnology, Inc.), anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology, Inc.), anti-phospho-MYPT1 antibody (1:5000; Millipore Corporation), anti-NLRP3 (1:200; Santa Cruz Biotechnology, Inc.), anti-caspase-1 p10 (1:200; Santa Cruz Biotechnology, Inc.), anti-P-ERK (1:500; Santa Cruz Biotechnology, Inc.), or anti-P-JNK (1:500; Santa Cruz Biotechnology) in 1× PBS, 5% w/v nonfat dried milk, and 0.1% Tween 20 at 4°C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin (1:1000; Santa Cruz Biotechnology, Inc.).

The relative expression of the protein bands of IkB-α (~37 kDa), NF-κB p65 (~65 kDa), p-MYPT1 (~130 kDa), caspase-1 (~46 kDa), NLRP3 (~120 kDa), Bax (~23 kDa), Bel-2 (~29 kDa), P-ERK (~44 kDa), and P-JNK (~46 kDa) was quantified by densitometric scanning of the X-ray films with a imaging densitometer (GS-700; BioRad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM, White Plains, NY).

Materials. Fasudil was obtained from LC Laboratories (Woburn, MA). All compounds were obtained from Sigma-Aldrich (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter, Rome, Italy).

Statistical Evaluation. All values in the figures and text are expressed as mean ± S.E.M. (Streit et al., 1998) of n observations. For the in vivo studies, n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments (histological or immunohistochemical coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. The results were analyzed by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. P < 0.05 was considered significant. BMS scale data were analyzed by the Mann-Whitney test and was considered significant at P < 0.05.

Results

Fasudil Reduces the Severity of Spinal Cord Trauma. The severity of the trauma at the level of the perilesional area, assessed by the presence of edema as well as alteration of the white matter and infiltration of leuko-
cytes, was evaluated 24 h after injury by hematoxylin and eosin staining. Significant damage was observed in the spinal cord tissue collected from SCI mice (Fig. 1, B and B1) compared with that from sham-operated mice (Fig. 1, A and A1). Significant protection against SCI was observed in fasudil-treated mice (Fig. 1, C and C1). The histological score (Fig. 1D) was evaluated by an independent observer.

To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the modified BMS hindlimb locomotor rating scale score was evaluated. Whereas motor function was only slightly impaired in sham mice, mice subjected to SCI had significant deficits in movement (Fig. 1E). Fasudil treatment significantly ameliorated the functional deficits induced by SCI (Fig. 1E).

Effect of Fasudil on Astrocytic Activation. Astrocytes are the major glial cell population within the CNS. After severe activation, astrocytes secrete various neurotoxic substances and express an enhanced level of GFAP, which is considered a marker protein for astrogliosis (Eng and Ghirnikar, 1994). To investigate the cellular mechanisms by which treatment with fasudil may attenuate astrocytic activation during spinal cord injury, we also evaluated GFAP expression by immunohistochemistry. Spinal cord sections from sham-operated mice did not stain for GFAP (Fig. 2, A and D), whereas spinal cord sections obtained from SCI mice exhibited positive staining for GFAP (Fig. 2, B and D). Fasudil treatment reduced the degree of positive staining for GFAP in the spinal cord of mice subjected to SCI (Fig. 2, C and D).

Effect of Fasudil on IkB-α Degradation and NF-κB p65 Activation. We evaluated IkB-α degradation and NF-κB p65 activation by Western blot analysis to investigate the cellular mechanisms by which treatment with fasudil may attenuate the development of SCI.

A basal level of IkB-α was detected in the spinal cord from sham-operated animals (Fig. 3A), whereas IkB-α levels were

![Fig. 1. Effect of fasudil treatment on histological alterations of spinal cord tissue 24 h after injury. B and B1, significant damage to the spinal cord of mice subjected to SCI mice at the perilesional area was assessed by the presence of edema as well as alteration of the white matter 24 h after injury. Of note, significant protection from the SCI was observed in the tissue collected from fasudil-treated mice (C and C1) compared with that from sham-operated mice (A and A1). The histological score was determined by an independent observer. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all animals in each group. Values shown are means ± S.E.M. of 10 mice for each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI (D). The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the Basso Mouse Scale (Basso et al., 2006). Treatment with fasudil reduced the motor disturbance after SCI. Values shown are means ± S.E.M. of 10 mice for each group. **, P < 0.01 versus SCI (E). wm, white matter; gm, gray matter; ND, not detectable.](image-url)
substantially reduced in SCI mice (Fig. 3A). Fasudil administration prevented the SCI-induced IκB-α degradation (Fig. 3A). In addition, NF-κB p65 levels in the nuclear fractions from spinal cord tissue were also significantly increased at 24 h after SCI compared with those in the sham-operated mice (Fig. 3B). Fasudil treatment reduced the levels of NF-κB p65 as shown in Fig. 3B.

**Effect of Fasudil on Caspase-1 and NLRP3 Expression.** The NLRP3 inflammasome is a caspase 1-containing cytosolic protein complex that is essential for processing and secretion of IL-1β. Thus, to investigate the cellular mechanisms by which treatment with fasudil may attenuate the inflammasome activation after SCI, we also evaluated caspase-1 and NLRP3 expression by Western blot. In spinal cord tissue homogenates after SCI, a significant increase in caspase-1 and NLRP3 expression was observed in SCI mice (Fig. 4, A and B). Treatment of mice with fasudil significantly reduced caspase-1 and NLRP3 expression (Fig. 4, A and B). No expression was observed in sham animals (Fig. 4, A and B).

**Fasudil Modulates the Expression of TNF-α and IL-1β and MPO Activity.** To test whether fasudil modulates the inflammatory process through the regulation of secretion of proinflammatory cytokines, we analyzed spinal cord levels of TNF-α and IL-1β (Fig. 5, G and H). A substantial increase in TNF-α and IL-1β production was found in spinal cord tissue samples collected from SCI mice 24 h after SCI (Fig. 5, G and H). Spinal cord levels of TNF-α and IL-1β were significantly attenuated by the intraperitoneal injection of fasudil (Fig. 5, G and H). Spinal cord sections were also taken at 24 h after SCI to determine the immunohistological staining for TNF-α and IL-1β expression. Spinal cord tissues obtained from sham-operated mice did not stain for TNF-α and IL-1β (Fig. 5, A, D, and I). A substantial increase in TNF-α and IL-1β expression was found in inflammatory cells as well as in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected from SCI mice 24 h after SCI (Fig. 5, B, E, and I). Fasudil treatment significantly...
Fig. 5. Effects of fasudil on TNF-α and IL-1β expression and MPO activity. A substantial increase in TNF-α (B) and IL-1β (E) expression was found in inflammatory cells in nuclei of Schwann cells in white matter (wm) and gray matter (gm) of the spinal cord tissues from mice at 24 h after SCI compared with that from sham groups (A and D). Spinal cord levels of TNF-α (C) and IL-1β (F) were significantly attenuated in fasudil-treated mice. In addition, a substantial increase in TNF-α (G) and IL-1β (H) production was found in spinal cord tissue collected from SCI mice at 24 h. Spinal cord levels of TNF-α and IL-1β were significantly attenuated by fasudil treatment (G and H). Densitometry analysis of immunocytochemistry photographs (5 photos from each sample collected from all mice in each experimental group) for TNF-α and IL-1β (I) from spinal cord tissues was assessed. The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. *, $P < 0.01$ versus sham + fasudil; **, $P < 0.01$ versus sham; °, $P < 0.01$ versus SCI + vehicle. After the injury, MPO activity in spinal cord from SCI mice was significantly increased at 24 h after the damage compared with that in sham groups (L). Intraperitoneal treatment with fasudil significantly attenuated neutrophil infiltration. Data are means ± S.E.M. of 10 mice for each group. ***, $P < 0.01$ versus sham; °, $P < 0.01$ versus SCI + vehicle. ND, not detectable.
reduced the degree of positive staining for these proinflammatory cytokines (Fig. 5, C, F, and I).

In this study, we also investigated the effect of the treatment of fasudil on the infiltration of neutrophils by measuring tissue MPO activity. MPO activity was significantly elevated in the spinal cord at 24 h after injury in mice subjected to SCI compared with sham-operated mice (Fig. 5L). In fasudil-treated mice, the MPO activity was significantly attenuated compared with that observed in SCI (Fig. 5L).

**Fasudil Reduces the Expression of MAPKs.** To investigate the cellular mechanisms by which treatment with fasudil may attenuate the development of spinal cord injury, we also evaluated the activation of MAPKs such as P-ERK by Western blot and P-JNK by immunohistochemistry and by Western blot. Spinal cord sections from sham-operated mice did not stain for P-JNK (Fig. 6, A and D), whereas spinal cord sections obtained from SCI mice exhibited positive staining for P-JNK (Fig. 6, B and D). Fasudil treatment reduced the degree of positive staining for P-JNK in the spinal cord of mice subjected to SCI (Fig. 6, C and D). In addition, in spinal cord tissue homogenates after SCI, a significant increase in P-ERK and P-JNK expression was observed in SCI mice (Fig. 6, E and F). Treatment of mice with fasudil significantly reduced P-ERK and P-JNK expression (Fig. 6, E and F).

**Effect of Fasudil on ROK Activity.** Because Rho kinase inhibits myosin phosphatase by phosphorylating its myosin-binding subunit, MYPT1 (Sharpe and Hendry, 2003), we measured phosphorylated levels of MYPT1 in spinal cord tissues as a marker of Rho kinase activity. Western blot analysis revealed that the levels of MYPT1 phosphorylation in spinal cord tissues were markedly increased in mice subjected to SCI, indicating that Rho kinase was activated after trauma. The increase in spinal cord tissues of MYPT1 phosphorylation was prevented by treatment with fasudil (Fig. 7).

**Effects of Fasudil on Nitrotyrosine and PAR Formation.** Spinal cord sections from sham-operated mice did not stain for nitrotyrosine and PAR (Fig. 8, A, D, and G), whereas spinal cord sections obtained from SCI mice exhibited positive staining for nitrotyrosine and PAR (Fig. 8, B, E, and H). Treatment of mice with fasudil significantly reduced nitrotyrosine and PAR expression (Fig. 8, C, F, and I).

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**Fig. 6.** Effect of fasudil on P-JNK and P-ERK expression. A, spinal cord sections from sham-operated mice did not stain for P-JNK. B, SCI caused positive staining for P-JNK at 24 h after trauma. C, treatment with fasudil significantly reduced the degree of positive staining for P-JNK. D, densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for JNK from spinal cord tissues. The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of the total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. *, P < 0.01 versus sham; **, P < 0.01 versus SCI + vehicle. E and F, in addition, representative Western blots showing no significant P-JNK and P-ERK expression in spinal cord tissues obtained from sham-treated animals. A significant increase in P-JNK and P-ERK was observed in the spinal cord from mice subjected to SCI. On the contrary, fasudil treatment prevented the SCI-induced expression of these proteins. Moreover, the relative expression of the protein bands was standardized for densitometric analysis to β-actin levels. ***, P < 0.01 versus sham; **, P < 0.01 versus SCI + vehicle. wm, white matter; gm, gray matter; ND, not detectable.
B, E, and G). The positive staining was mainly localized in inflammatory cells as well as in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues. Fasudil treatment reduced the degree of positive staining for nitrotyrosine and PAR (Fig. 8, C, F, and G) in the spinal cord.

**Fig. 7.** Effect of fasudil on MYPT1 phosphorylation (ROK activity). ROK activity was measured by phosphorylation of MYPT1. No increase of MYPT1 phosphorylation was observed in sham animals. Spinal cord phosphorylated levels of MYPT1 were significantly increased in mice subjected to SCI. Treatment with fasudil attenuated SCI-induced MYPT1 phosphorylation. β-Actin was used as internal control. The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels is expressed as the mean ± S.E.M. from n = 5/6 spinal cords for each group. ***, P < 0.01 versus sham; *, P < 0.01 versus SCI vehicle. wm, white matter; gm, gray matter; ND, not detectable.

**Fig. 8.** Effects of fasudil on nitrotyrosine and PAR formation. A, spinal cord sections from sham-operated mice did not stain for nitrotyrosine. B, sections obtained from vehicle-treated animals after SCI demonstrate positive staining for nitrotyrosine mainly localized in inflammatory cells, in nuclei of Schwann cells in the white matter (wm) and gray matter (gm). C, fasudil treatment (10 mg/kg 1 and 6 h after SCI induction) reduced the degree of positive staining for nitrotyrosine in the spinal cord. E, in addition, immunohistochemistry for PAR, an indicator of in vivo PARP activation, revealed the occurrence of positive staining for PAR localized in nuclei of Schwann cells in white matter and gray matter of the spinal cord tissues from SCI mice. D, spinal cord sections from sham-operated mice also did not stain for PAR. F, fasudil treatment reduced the degree of positive staining for PAR in the spinal cord. G, densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for nitrotyrosine and PAR from spinal cord tissues was performed. The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. ***, P < 0.01 versus sham; *, P < 0.01 versus SCI vehicle. ND, not detectable.
Effects of Fasudil on FAS Ligand Expression. Immunohistological staining for FAS ligand in the spinal cord was also determined 24 h after injury. Spinal cord sections from sham-operated mice did not stain for FAS ligand (A), whereas a substantial increase in Fas-ligand expression was found in inflammatory cells in nuclei of Schwann cells in white matter (wm) and gray matter (gm) of the spinal cord tissues from SCI mice at 24 h after SCI (B). C, spinal cord levels of Fas ligand were significantly attenuated in fasudil-treated mice compared with those of SCI animals. G, densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Fas ligand from spinal cord tissues was performed. The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. D, moreover, almost no apoptotic cells were detected in the spinal cord from sham-operated mice. E, at 24 h after the trauma, SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments. F, in contrast, tissues obtained from mice treated with fasudil demonstrated no apoptotic cells or fragments. H, the number of TUNEL-positive cells per high-power field was counted in 5 to 10 fields for each coded slide. The figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. ND, not detectable.

**Fig. 9.** Effect of fasudil on FAS ligand expression and on TUNEL-like staining in the perilesional spinal cord tissue. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for Fas ligand and TUNEL staining. Spinal cord sections from sham-operated mice did not stain for FAS ligand (A), whereas a substantial increase in Fas-ligand expression was found in inflammatory cells in nuclei of Schwann cells in white matter (wm) and gray matter (gm) of the spinal cord tissues from SCI mice at 24 h after SCI (B). C, spinal cord levels of Fas ligand were significantly attenuated in fasudil-treated mice compared with those of SCI animals. G, densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Fas ligand from spinal cord tissues was performed. The assay was performed by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. D, moreover, almost no apoptotic cells were detected in the spinal cord from sham-operated mice. E, at 24 h after the trauma, SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments. F, in contrast, tissues obtained from mice treated with fasudil demonstrated no apoptotic cells or fragments. H, the number of TUNEL-positive cells per high-power field was counted in 5 to 10 fields for each coded slide. The figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. ND, not detectable.

**Effects of Fasudil in the Apoptosis in Spinal Cord after Injury.** To test whether spinal cord damage was associated with cell death by apoptosis, we also measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detected in the spinal cord from sham-operated mice (Fig. 9, D and H). At 24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 9, E and H). In contrast, tissues obtained from mice treated with fasudil demonstrated no apoptotic cells or fragments (Fig. 9, F and H).

**Western Blot Analysis and Immunohistochemical Analysis for Bax and Bcl-2.** At 24 h after SCI, the appearance of proapoptic protein, Bax, in spinal cord homogenates was investigated by Western blot. Bax levels were appreciably increased in the spinal cord from mice subjected to SCI (Fig. 10H). On the contrary, fasudil treatment prevented the SCI-induced Bax expression (Fig. 10H). Bcl-2 expression in homogenates from spinal cord of each of the mice was also analyzed by Western blot. A basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Fig. 10I). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice (Fig. 10I). Treatment of mice with fasudil significantly blunted the SCI-induced inhibition of antiapoptotic protein expression (Fig. 10I).

Moreover, samples of spinal cord tissue were taken at 24 h after SCI to determine immunohistological staining for Bax
Spinal cord sections from sham-operated mice did not stain for Bax (A), whereas SCI caused, at 24 h, an increase in Bax expression (B). C, fasudil treatment reduced the degree of positive staining for Bax in the spinal cord. On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues from sham-operated mice (D), whereas the staining was significantly reduced in SCI mice (E). F, fasudil treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice. G, densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Bax and for Bcl-2 from spinal cord tissues was performed. The assay was performed out by using Optilab Graftech software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. H, in addition, representative Western blots showing no significant Bax expression in spinal cord tissues were obtained from sham-treated animals. Bax levels were appreciably increased in the spinal cord from SCI mice. On the contrary, fasudil prevented SCI-induced Bax expression. I, moreover, a basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice. Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice. Fasudil treatment significantly reduced the SCI-induced inhibition of Bcl-2 expression. Moreover, the relative expression of the protein bands was standardized for densitometric analysis to β-actin levels. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. wm, white matter; gm, gray matter; ND, not detectable.

**Fig. 10.** Effect of fasudil on expression of Bax and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (A), whereas SCI caused, at 24 h, an increase in Bax expression (B). C, fasudil treatment reduced the degree of positive staining for Bax in the spinal cord. On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues from sham-operated mice (D), whereas the staining was significantly reduced in SCI mice (E). F, fasudil treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice. G, densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Bax and for Bcl-2 from spinal cord tissues was performed. The assay was performed out by using Optilab Graftech software on a Macintosh personal computer (CPU G3-266). Data are expressed as a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissue sections collected from all the animals in each group. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. H, in addition, representative Western blots showing no significant Bax expression in spinal cord tissues were obtained from sham-treated animals. Bax levels were appreciably increased in the spinal cord from SCI mice. On the contrary, fasudil prevented SCI-induced Bax expression. I, moreover, a basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice. Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice. Fasudil treatment significantly reduced the SCI-induced inhibition of Bcl-2 expression. Moreover, the relative expression of the protein bands was standardized for densitometric analysis to β-actin levels. **, P < 0.01 versus sham; *, P < 0.01 versus SCI + vehicle. wm, white matter; gm, gray matter; ND, not detectable.

and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (Fig. 10, A and G), whereas spinal cord sections obtained from SCI mice exhibited positive staining for Bax (Fig. 10, B and G). Fasudil treatment reduced the degree of positive staining in Bax-expressing spinal cord of mice subjected to SCI (Fig. 10, C and G). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Fig. 10, D and G), whereas in SCI mice the staining was significantly reduced (Fig. 10, E and G). Fasudil treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from mice subjected to SCI (Fig. 10, F and G).

**Discussion**

Spinal cord injury is a highly debilitating pathology. The pathological events after acute SCI are divided into two chronological phases (Tator and Fehlings, 1991). The traumatic mechanical injury to the spinal cord that is incurred after blunt impact and compression is called the “primary injury”; it causes the death of a number of neurons that cannot be recovered and regenerated. The events that characterize this successive phase to mechanical injury are called “secondary damage.” The secondary damage is determined by a large number of cellular, molecular, and biochemical cascades.

The Rho family of small GTPases is a group of 20- to 40-kDa monomeric G proteins that can regulate a number of cellular biological functions, including actin stress fiber formation, focal adhesion, motility, aggregation, proliferation, and transcription (Burridge and Wennerberg, 2004). Regulation of these cellular functions by Rho is mainly dependent on the activation of its downstream effector, ROK (Burridge and Wennerberg, 2004). It is also involved in the regulation of...
several aspects of innate immunity, including leukocyte chemotaxis, phagocytosis, and ROS formation (Riento and Ridley, 2003; Bokoch, 2005). Rho GTPases have been implicated in the modulation of NF-κB activation and T-cell proliferation (Tharaux et al., 2003). It has also been reported that inhibition of Rho kinase suppresses NF-κB activation and IκB phosphorylation and degradation in peripheral blood mononuclear cells from patients with Crohn’s disease (Segain et al., 2003).

The aim of the present study was to determine the effect of fasudil, a ROK inhibitor, in the modulation of secondary injury associated with SCI. We show here that SCI resulted in edema and loss of myelin in lateral and dorsal funiculi. This histological damage was associated with the loss of motor function. In this study, we demonstrated that administration of fasudil inhibits the development of SCI through its effects on the NF-κB activation pathway and also possibly through other pathways (Okamoto et al., 2010).

In this study, we report that SCI was associated with significant IκB-α degradation as well as increased nuclear expression of p65 in spinal cord tissue at 24 h after injury. Fasudil significantly reduced IκB-α degradation as well as NF-κB translocation. A direct consequence of the inhibitory effect of fasudil on NF-κB activation is reduction of proinflammatory cytokine secretion (He et al., 2008). We have clearly confirmed a significant increase in TNF-α and IL-1β during SCI. On the contrary, no significant expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from mice subjected to SCI mice that received fasudil. An in vitro study also demonstrated that treatment with fasudil or (+)-(R)-trans-4-(1-aminoethyl)-N-[4-pyridyl]cyclohexanecarboxamide dihydrochloride (Y27632) decreased production of TNF-α, IL-1β, and IL-6 by synovial membrane cells, peripheral blood mononuclear cells, and fibroblast-like synoviocytes from patients with active rheumatoid arthritis (He et al., 2008).

IL-1β is produced in large amounts by infiltrating macrophages and neutrophils, is initially expressed in its proform, and is only converted to a biologically active form after proteolytic cleavage by the protease caspase-1 (Thornberry et al., 1992). Caspase-1 is activated in the cytosol in a multi-protein scaffold termed the inflammasome (Martinon et al., 2002), which forms only in response to different danger signals (Miao et al., 2008). The best characterized inflammasome is the NLRP3 (also known as NALP3 and cryopyrin) inflammasome. It comprises the NLR protein NLRP3, the adapter ASC, and pro-caspase-1 (Lamkanfi et al., 2009). Thus, caspase-1 activation is a central regulator of the innate immune defense. Recent work has indicated that the activation of Rho GTPases, in particular Rac1 and possibly Cdc42, might represent a novel type of signaling input that can activate caspase-1 signaling (Müller et al., 2010). In that regard, the treatment with a ROK inhibitor, fasudil, could interfere with activation or assembly of the inflammasome, but the mechanism is still unclear. Activation of Rho A leads to stimulation of Rho kinase, which can phosphorylate and subsequently inactivate the myosin light chain phosphatase favoring actin-myosin interaction and cell contraction (da Silva-Santos et al., 2009). Because Rho kinase inhibits myosin phosphatase by phosphorylating its myosin-binding subunit, MYPT1 (Sharpe and Hendry, 2003), we measured phosphorylated levels of MYPT1 in spinal cord tissues as a marker of Rho kinase activity and we showed that spinal cord injury is associated with increases in ROK activity and that fasudil treatment markedly attenuated ROK activity.

Several studies also showed that fasudil markedly reduced the endotoxin-induced increase of MPO activity, indicating an inhibitory effect of fasudil on leucocyte accumulation in endotoxemic liver injury (Thorlacius et al., 2006). Here, we report that SCI was associated with a significant increase in neutrophil infiltration measured by MPO activity, whereas in fasudil-treated mice, the MPO activity was significantly attenuated compared with that observed in SCI.

The initiation of inflammatory responses in the CNS is also related to activation of MAPKs, and their activation would determine neuronal death or survival on certain occasions. Previous studies showed that the expression of activated ERK1/2 and p38 MAPK in microglia/macrophages may play a key role in production of CNS inflammatory cytokines and free radicals, such as NO (Choi et al., 2003). We confirm here that SCI leads to substantial expression of P-ERK and P-JNK in spinal cord tissues at 24 h after SCI; on the contrary, fasudil treatment decreases P-ERK and P-JNK expression in treated mice. Chen et al. (2009) have also shown that fasudil effectively suppressed serotonin-induced pulmonary artery smooth muscle cell proliferation and cell cycle progression, which was associated with inhibition of JNK activation, ERK translocation to nucleus, and subsequent c-fos and c-jun expression.

Among the reactive oxygen species, peroxynitrite (ONOO−) is known to play an important role in local and systemic inflammatory responses as well as neurodegenerative disease (Xu et al., 2001). It is one of a number of toxic factors produced in the spinal cord tissues after SCI (Xu et al., 2001) that probably contributes to secondary neuronal damage through pathways resulting from the chemical modification of cellular proteins and lipids. To probe the pathological contributions of ONOO− to secondary damage after SCI, we have used the appearance of nitrotyrosine staining in the inflamed tissue. We observed that the immunoassaying of nitrotyrosine is reduced in mice subjected to SCI treated with fasudil compared with mice subjected to SCI. A recent study also demonstrated that the administration of fasudil inhibited the activity of ROK in brain tissue and cultured microglia and protected hippocampal neurons, reducing the proinflammatory factors such as NO, IL-1β, IL-6, and TNF-α in a vivo model of hypoxia/reoxygenation injury (Ding et al., 2010). Several lines of evidence clearly demonstrated that NO also plays a key role in regulating the expression of GFAP in astrocytes (Brahmachari et al., 2006). Although activated astrocytes secrete different neurotrophic factors for neuronal survival, it is believed that rapid and severe activation augments/initiates an inflammatory response, leading to neuronal death and brain injury (Tani et al., 1996). Astrocytes react to various neurodegenerative insults rapidly, leading to vigorous astrogliosis (Eng et al., 1992). For this reason, in this study we also evaluated by immunohistochemical analysis the expression of GFAP, a marker of astrocytic activation, and we observed high expression in mice subjected to SCI compared with fasudil-treated mice.

A novel pathway of inflammation associated to SCI, governed by the nuclear enzyme PARP has been proposed in relation to hydroxyl radical and peroxynitrite-induced DNA
single strand breakage (Szabó and Dawson, 1998). Continuous or excessive activation of PARP produces extended chains of ADP-ribose (PAR) on nuclear proteins and results in a substantial depletion of intracellular NAD⁺ and subsequently ATP, leading to cellular dysfunction and, ultimately, cell death (Chiarugi, 2002). We demonstrate here that fasudil reduced the increase in PARP activation in the spinal cord in animals subjected to SCI.

Apoptosis is an important mediator of secondary damage after SCI (Beattie et al., 2002). In an effort to prevent or diminish levels of apoptosis, we demonstrate that treatment with fasudil attenuates the degree of apoptosis, measured by the TUNEL detection kit, in the spinal cord after the damage. Wang et al. (2005) have also reported that fasudil, a Rho kinase inhibitor, could attenuate angiotensin II-induced abdominal aortic aneurysm formation by inhibiting vascular wall apoptosis and extracellular matrix proteolysis. A recent study also determined whether the ROK inhibitor, fasudil, inhibited ischemic neuronal apoptosis through the phosphorylation and tenasin homolog deleted on chromosome 10/Akt/signal pathway in vivo (Wu et al., 2012). Here, we demonstrated that treatment with fasudil reduced Bax expression, whereas, on the contrary, the expression of Bel-2 is much higher in mice treated with fasudil. Some authors have also shown that FAS and p75 receptors are expressed on oligodendrocytes, astrocytes, and microglia in the spinal cord after SCI (Ackery et al., 2006). Therefore, Fas ligand plays a central role in apoptosis induced by a variety of chemical and physical insults (Dosreis et al., 2004). In the present study, we found that fasudil treatment leads to a substantial reduction of Fas ligand activation.

Finally, in this study we demonstrate that fasudil treatment significantly reduced SCI-induced spinal cord tissue alteration and improved motor function. The results of the present study enhance our understanding of the role of ROK activation in the pathophysiology of spinal cord cell and tissue injury after trauma, implying that inhibitors of the ROK such as fasudil may be useful in the therapy of spinal cord injury and inflammation.

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