Rho-Kinase inhibition decreases focal cerebral ischemia-induced glial activation in rats

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ABSTRACT

BACKGROUND: Rho-kinase inhibition in a rat middle cerebral artery occlusion (MCAO) model is reported to improve neurological functions and decrease infarction size.

OBJECTIVE: The objective of this study is to investigate the underlying mechanisms of such improvement by evaluating the effects of Rho-kinase inhibition on astrocytes and microglial accumulation and activation in this condition.

METHODS: Adult male Sprague-Dawley (SD) rats were used to generate the MCAO model, which received an I.P injection of a chemical Rho-kinase inhibitor (Fasudil- 5 mg/kg/day) or vehicle (PBS) for 2 and 4 days.

RESULTS: Fasudil treatment significantly decreased the stroke volumes and water content in the lesion areas, as revealed by MRI. Immunostaining and Western blotting results demonstrated that Fasudil significantly decreased the levels of Aquaporin-4, a water channel protein. The number of GFAP$^+$ astrocytes and Iba-1$^+$ macrophage/microglia was decreased in the lesion areas. Proinflammatory transcription factor NF-κB protein levels were decreased in the Fasudil group 2 days after MCAO. Also, proinflammatory mediators including TNF-α, IL-1β, and iNOS levels were decreased. In vitro migration study using a human microglial cell line (HMO6) confirmed the inhibitory effects of Fasudil on the process. Fasudil also decreased combined IL-1β and IFNγ-induced NF-κB nuclear translocation in HMO6. Moreover, Fasudil transiently decreased combined IL-1β and IFNγ-induced iNOS, TNFα, and IL-1β mRNA levels in HMO6.

CONCLUSION: Our study demonstrates the inhibitory effects of Rho-kinase on NF-κB-mediated glial activation and cerebral edema, which might be a promising therapeutic target in acute cerebral ischemia conditions.

KEYWORDS: cerebral ischemia, rho-kinase, microglia, astrocytes, cytokines, aquaporin-4

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Introduction

Focal brain ischemia causes energy depletion-dependent necrotic cell death in the area where there is a complete loss of blood supply.[1,2] Near the vicinity of the ischemic area, the so-called ischemic border zone (IBZ), blood supply is decreased.[1,3] In this area, slow and gradual cell death occurs for a relatively prolonged period, and ultimately a mature ischemic lesion is formed.[3,4] By effective modulation of the lesion pathophysiology, much of the dying cells of IBZ appear to be salvageable,[4] hence an attractive target for stroke therapy. Several studies provide evidence that neuroinflammation plays an important role in this type of delayed cell death.[5-7] After ischemia, several tissue-resident and infiltrating immune-competent cell types including neutrophils, macrophages/microglia, T-cells, and astrocytes are accumulated and activated in and around the lesion area.[8] These immune cells modulate delayed cell death by producing inflammatory mediators, further activating inflammatory cells, affecting apoptotic pathways, controlling cerebral edema, scavenging necrotic tissue, and producing neurotropic factors.[9-14] These diverse functions are implicating the immune cells as a double-edged sword having both beneficial effects including necrotic tissue scavenging and neurotrophic factors production, and deleterious effects, such as excessive production of proinflammatory mediators.

Several reports are suggesting that the modulation of the functions of immune cells, such as accumulation, activation, and production of inflammatory mediators might be an effective means for the management of cerebral ischemia.[15] Methods including cell therapy, gene manipulations, or the use of small molecules are employed to control the inflammatory condition in stroke.[16-20] For instance, reducing leukocyte infiltration through adhesion

molecules inhibition, or MCP-1 gene knockout improves neurological and pathophysiological outcomes after such injury.[21,22] In previous studies, we demonstrated that cell therapy like mesenchymal stem cell transplantation effectively reduces inflammatory cell infiltration and activation, and causes neurological improvement.[23] However, due to the beneficial effects of inflammatory cells, total depletion of these cell types appears to worsen the lesion.[24] Therefore, well-controlled regulations of the inflammatory cell population and proinflammatory mediators' expression might be effective for the management of cerebral ischemia pathology. For this matter, desired control of the accumulation and activation status of inflammatory cells by modulating the Rho-kinase-dependent pathways could be important.

Rho-kinases are implicated in several pathophysiological processes, encompassing cellular apoptosis, growth, metabolism, and movement via control of actin cytoskeletal assembly and cell contraction, and gene expression.[25] For example, by inhibiting myosin phosphatase, Rho-kinase increases phosphorylation of the myosin light chain, resulting in smooth muscle cell contraction and vasospasm.[26,27] Its activity is found to be involved in macrophage and neutrophil migration,[28,29] possibly through cytoskeletal rearrangement and adhesion molecule expression. Moreover, Rho-kinase inhibition can increase anti-inflammatory cytokine production and phagocytosis activities of macrophages.,[30,31] indicating its role in M2-type differentiation. Since the accumulation and M2-differentiation of macrophage/microglia play an important role in ischemia-induced neuroinflammation, Rho-kinase inhibition might yield beneficial effects in this condition. Indeed, Rho-kinase expression is increased in experimental ischemia conditions, especially during early time points, and inhibition of Rho-kinase activity results in an improvement in cerebral ischemia, pathologically and functionally.[32-34] Besides, its activity is found to be increased in the leukocytes of stroke patients. In a prospective placebo-controlled double-blind trial Fasudil, a Rho-kinase inhibitor shows promising results in the management of stroke.[35,36] Although it has been demonstrated that the underlying mechanisms of these beneficial effects are mediated through iNOS inhibition and increased eNOS expression with resultant increased blood flow,[34] in the view of the effects of Rho-kinase on the inflammatory system, a comprehensive study on the effects of Fasudil on cerebral ischemia-induced neuroinflammation is required.

In this study, we aimed to investigate the detailed effects of Fasudil on cerebral ischemia-induced neuroinflammation, at the level of immune cellular accumulation and proinflammatory gene induction. Since neuroinflammation plays a vital role during the acute stage, we evaluated the effects of Fasudil 2 and 4 days after the generation of a rat stroke model.

Materials and methods

Focal cerebral ischemia model

All animals were used according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, and guidelines of the Institute of Experimental Animals of Shimane University. The experimental protocols and procedures were approved by the Ethical Committee of Shimane University (approval code: IZ30-3). Adult male Sprague-Dawley rats (CLEA Japan, Inc Tokyo, Japan) of 8-10 weeks weighing between 250-280 g were used to generate the middle cerebral artery occlusion (MCAO) model as previously described.[14] Briefly, the animals were anesthetized with 4.0% halothane, and the right common carotid, external carotid, and internal carotid arteries were exposed via a ventral midline incision. A 4-0 monofilament nylon suture (Nescosuture, Tokyo, Japan), having a tip rounded by silicon (Xantopren L blue, Germany) coating, was inserted through the right external carotid artery, and moved into the internal carotid artery to block the origin of the right middle cerebral artery for 60 minutes Then the rats were re-anesthetized, and the filament was removed. After recovery from anesthesia, rats were returned to their cages. Rectal temperature was maintained at 37°C throughout the surgical procedure utilizing a feedback-regulated heating system. A total of 42 rats were used to generate the MCAO model. Among them, 4 rats died within the study period (mortality rate of about 9.5%), and 8 rats were excluded (5 rats due to insufficient neurological deficit after 1 day, and 3 rats due to intracerebral hemorrhage) from the study. The schematic representation of the study design is shown in the supplemental Figure 1.

Evaluation of neurological performance

Neurological performance was evaluated before and 1, 2, 3, and 4 days after MCAO using a modified neurological severity scoring (mNSS) system.[37] The mNSS is a composite of the motor, sensory, balance tests, and reflexes, and graded on a scale of 0 to 22, where the points were counted for the inability to perform a test, or alteration of a tested reflex. Therefore, an increasing score indicates the severity of the neurological deficit.

Intraperitoneal injection of Rho-kinase inhibitor (Fasudil)

A Rho-kinase inhibitor, Fasudil (Asahi Kasei Pharma Corporation, Japan), was dissolved in PBS at a 4 mg/ml concentration. After MCAO, rats were randomly divided into 1) Fasudil or 2) vehicle group (n = 15 in each group), and received the drug (5 mg/kg) or vehicle (PBS) intraperitoneally. Each group was further subdivided into 2 subgroups, a) Day 2 (total n = 20, PBS = 10 and Fasudil = 10) and b) Day 4 (total n = 10; PBS = 5 and Fasudil = 5). The administration of the drug started 1 hour after reperfusion and continued as a single daily injection till the day of sacrifice.

MRI

A T2-weighted MRI image of the whole brain (n = 5/group) was acquired with a 1.5 T MRI system (Mrmini SA; DS Pharma Biomedical, Osaka, Japan) on Day 4 after MCAO. The area of

the infarct region in 5 slices of the MRI image was measured using ImageJ (NIH), expressed as % of the contralateral side, averaged, and considered as the lesion size of the animal.

Tissue preparation and immunohistochemical analysis

At 2 and 4 days after MCAO, the rats were deeply anesthetized with 5% isoflurane and the brains were fixed by transcardial perfusion of normal saline, followed by 4% paraformaldehyde (PFA) in PBS. The rat brains were removed, cryoprotected, sectioned, and frozen on dry ice. Coronal tissue sections 10 μm thick were cut with a cryostat. After quenching endogenous peroxidase activity, the sections were incubated in a blocking solution containing 10% normal goat or horse serum. The sections were incubated with a primary antibody against a microglia-specific ionized calcium-binding molecule-1 (Iba-1, 1:500, rabbit, Abcam, Waltham, MA, USA), glial fibrillary acidic protein (GFAP, rabbit, DAKO, Carpinteria, CA, USA), rat CD68 (ED-1, 1:100, GeneTex, Hsinchu, Taiwan, R.O.C) inducible nitric oxide synthase (iNOS, 1:100, rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA), aquaporin 4 (AQP4, 1:100, rabbit, Santa Cruz), NF-κB (p65, 1:100, rabbit, Santa Cruz), tumor necrosis factor α (TNF-α; 1:100, rabbit, Santa Cruz), CREB-1 (1:100, rabbit, Santa Cruz) or interleukin-1β (IL-1β; 1:100, rabbit, Santa Cruz). After incubation with primary antibody, sections were incubated with Texas red- or FITC-conjugated species-specific IgG (1:100, Santa Cruz). The immunoreactive proteins were analyzed with a fluorescence microscopy system (Nikon, ECLIPSE, E600, Tokyo, Japan). Immune reaction positive cells in the coronal brain sections at similar positions were counted in 5 random microscopic fields of the cortical core and the IBZ area at ×400 magnification in a blinded manner. The average of such immune reaction positive cells was represented as the cell population of that rat.

Cell culture

A human microglial cell line, HMO6, was established by isolating microglia from human fetal telencephalon tissue and immortalizing it using a retroviral vector encoding v-myc.[38] HMO6 cells were cultured in DMEM supplemented with antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin), L-glutamine, and 10% FCS. During in vitro stimulations, HMO6 was cultured in DMEM containing 1% FCS, L-glutamine, and antibiotics. DMEM, FCS, antibiotics, and L-glutamine were purchased from Invitrogen (ThermoFisher, Waltham, MA, USA)

Immunocytochemistry of myosin light chain phosphorylation

HMO6 cells were cultured on a sterile glass coverslip in a complete cell culture medium. After appropriate treatment, cells on the coverslip were washed and fixed with 4% paraformaldehyde. The cells were incubated in a blocking solution containing 10% normal goat serum and .2% Triton-X in PBS for 30 minutes. Then the cells were incubated for 60 minutes with polyclonal goat anti-MLC2 or polyclonal goat anti-pMLC2 antibody (1:100, Santa Cruz), followed by biotinylated anti-goat IgG (1:200, Santa Cruz). Then the coverslips were incubated with an avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA), and the immunoreaction products were visualized with 3,30-diaminobenzidine (DAB, Sigma, St Louis, MO, USA). The immunoreactive cells were analyzed by light microscopy.

Migration assay

Migration assay was performed using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD, USA) and a 5-μm pore membrane (Costar, High Wycombe, England) coated with fibronectin (6.5 μg/mL, Sigma) as described previously.[39] Briefly, MIP-1α was diluted in DMEM containing 1% FCS, and 28 μL of each dilution was applied in triplicate into the lower wells of the chemotaxis chamber. The filled lower chamber was then overlaid with the membrane with the coated surface facing downwards, and the top chamber was assembled to form wells. Cell suspension (5×10^5/mL) in DMEM containing 1% FCS was applied (50 μL) in the upper wells. After incubation, the migrated cells on the fibronectin-coated lower surface of the membrane were fixed with methanol and stained with Harris' hematoxylin (Sigma). Migrated cells were counted in 5 microscopic fields per well at ×400 magnification.

Total RNA isolation and real-time PCR

The mRNA expression of proinflammatory mediators was analyzed by quantitative real-time PCR.[23] After appropriate stimulation, total RNA was isolated from HMO6 cells using RNA STAT reagent (Tel-Test, Friendswood, Tx, USA) according to the manufacturer's instructions. Two μg of total RNA was reverse transcribed using reverse transcriptase enzyme (ReverTraAce, Toyobo, Osaka, Japan), and quantitative real-time PCR was done with SyBr green real-time PCR system (Applied Biosystem, Warrington WA1 4SR, UK) and gene-specific primers, using an ABI Prism 7300 Sequence Detection System (Applied Biosystem).

Tissue preparation for western blot analysis

Two days after MCAO, rats were deeply anesthetized and transcardially perfused with normal saline.[23] The brains were removed, and 5 mm coronal sections were made starting from 2 mm anterior to bregma to 3 mm posterior to bregma. The first 2 mm of brain tissue was fixed with 4% paraformaldehyde (PFA), cryoprotected, and used to assess the infarction size by Hematoxylin-Eosin staining. The cortical portion of remaining 3 mm tissue sections then subdivided into core, IBZ and contralateral parts, homogenized in ice-cold RIPA buffer (PBS, pH 7.4, 1% Nonidet p-40, .5% sodium deoxycholate, .1%SDS,

10 mg/ml PMSF, and 1 mg/ml aprotinin) (1:10 wt-vol) and stored at -70°C for further use.

Preparation of nuclear extract

After appropriate treatment, nuclear extracts were prepared, as described previously.[40] In a brief, cells were harvested by scraping in ice-cold PBS and centrifuge at 1500 rpm for 5 minutes at 4°C. The cells were incubated in 2 packed cell volume of hypotonic buffer A (10 mM HEPES [pH 8.0], 1.5 mM $MgCl_2$, 10 mM KCl, .5 mM dithiothreitol [DTT], 200 mM sucrose, .5 mm phenylmethanesulfonyl fluoride [PMSF], 1 mg/ml each of leupeptin and aprotinin, .5% Nonidet P-40) for 5 minutes at 4°C. Then the nuclei were pelleted by centrifuging the samples at 10,000 rpm for 5 seconds. The nuclei were dissolved in 2/3rd packed cell volume of extraction buffer C (20 mm HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mm NaCl, .2 mM EDTA, .5 mM PMSF, 1.0 mM DTT, 1.0 mg/ml each of leupeptin and aprotinin) and incubated on a rocking platform for 30 minutes at 4°C. Then the samples were centrifuged at 15,000 rpm for 10 minutes, the supernatants were collected and stored at -70°C until further use.

Western blot analysis

Forty to eighty μg of nuclear extracts or brain tissue samples were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The target proteins were detected with the following antibodies: anti-AQP-4 rabbit polyclonal IgG, anti-CREB-1 rabbit polyclonal IgG, anti-NF-κB (p65) rabbit polyclonal IgG, anti-IRF-1 mouse monoclonal IgG and anti-TNFα rabbit polyclonal IgG. All antibodies were purchased from Santa Cruz. After stripping, the same membrane was used to detect β-Actin (mouse monoclonal IgG, Santa Cruz). Immunoreactive proteins were detected using an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) according to the manufacturer's protocol.

Statistical analysis

The numerical data are expressed as mean ± SD. To determine the statistical significance of the results, student's t-test, one-way ANOVA followed by Student-Newman-Keuls multiple comparisons, or Scheffe post hoc test was employed (SPSS). The statistical significance level was set at $P < .05$.

Results

Assessment of neurological performance, infarct volume, and cellular apoptosis

All animals showed normal neurological functions before the generation of MCAO. One day after MCAO all animals, irrespective of groups, showed similar neurological deficits (mNSS= 14-18). As shown in Figure 1A, neurological performances were improved in the Fasudil groups from Day 2 onwards after MCAO compared to the PBS group, which became statistically significant on Day 3 and Day 4 (Figure 1A).

Next, the effect of Fasudil on infarction size was evaluated. The MRI results demonstrated that in the Fasudil group, infarct

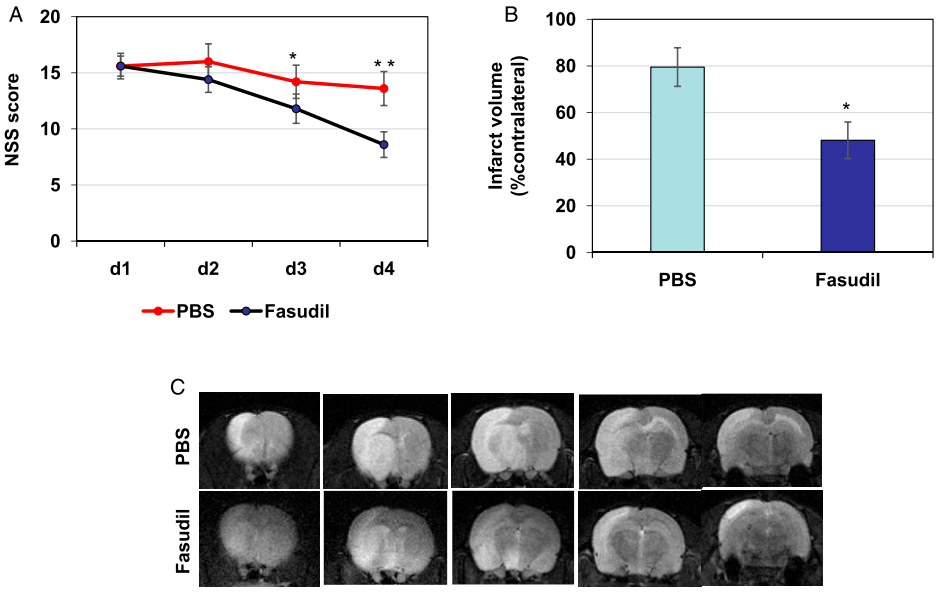

Figure 1. Effects of Fasudil on neurological functions and infarct volume in MCAO rats. (A) Neurological performance was assessed by a modified neurological score system (mNSS), as described in the Materials and Methods. The data presented here are the mean ± SD of 5 rats in a group. (B) The infarction size was evaluated by T2 weighted MRI. The infarction volume was calculated as % volume of the contralateral area. The data presented here are the mean ± SD of 5 rats in a group. In (C), representative multi-slice MRI images of the brains of PBS and Fasudil-treated MCAO rats are shown. Statistical significance is denoted as follows; *$P < .05$, **$P < .01$ vs PBS group at the same time point.

volume was significantly decreased at Day 4 (infarct size- PBS group 79.1 ± 8.3 vs Fasudil group 48.1 ± 7.8% of contralateral hemisphere, $P < .01$) after MCAO (Figure 1B). Moreover, T2 weighted images of PBS groups were brighter, suggesting that the edema was decreased in the Fasudil group (Figure 1C).

Fasudil treatment decreased water channel protein Aquaporin 4 levels in MCAO brains.

Water channel protein Aquaporin 4 (AQP4) plays an important role in the development of cerebral edema in stroke condition.[41,42] Hence, we decided to check its regulation in MCAO conditions. Our immunostaining results showed that AQP4 was expressed around the vessel-like structures, which was decreased in the core and ischemic boundary zone (IBZ) areas in the Fasudil group (Figure 2A). Then, we quantified AQP4 protein levels by Western blotting. The results showed that the M1 isoform of AQP4 was significantly decreased in the core and IBZ regions in the Fasudil group compared to the PBS group, whereas the M23 isoform was not changed much (Figure 2B and C).

Accumulation of microglia/macrophage and astrocytes was inhibited by Fasudil

Macrophage/microglial accumulation in the MCAO rat brain was evaluated by Iba-1 immunostaining. On Day 2 and Day 4 after MCAO, Iba-1$^+$ cells were detected in the core and the IBZ (Figure 3A and B). Fasudil significantly decreased Iba1$^+$ macrophage/microglial accumulation both in the core and the IBZ on Day 4 after MCAO (Figure 3C).

Accumulation of the astrocytes was evaluated by GFAP immunofluorescence staining. Very few GFAP$^+$ cells were detected in the core area both on Day 2 and Day 4 after MCAO (data not shown), which were mainly found in the IBZ. GFAP$^+$ cells in IBZ were significantly decreased in the Fasudil group on Day 4 after MCAO (Figure 3D and E).

Effects of Fasudil on transcription factors expression in MCAO rats

To investigate the effects of Fasudil on inflammatory gene transcription in MCAO conditions, we analyzed the protein level of several transcription factors in the MCAO rat brain. Our Western blotting results showed that on Day 2 after MCAO, NF-κB protein levels were decreased in the core region of the Fasudil group compared to their PBS counterparts (Figure 4A and B). Conversely, CREB-1 and IFR-1 levels were not decreased in the same region of the Fasudil-treated rats (Figure 4A and B). Double immunofluorescence staining revealed that both NF-κB and CREB-1 were expressed in the ED-1$^+$ macrophage/microglia in the core region (Figure 4C).

Effects of Fasudil on the levels of proinflammatory mediators in MCAO rats

To investigate the effects of Fasudil on inflammatory mediators in MCAO conditions, we analyzed the protein levels of IL-1β, TNF-α, and iNOS in the MCAO rat brain by

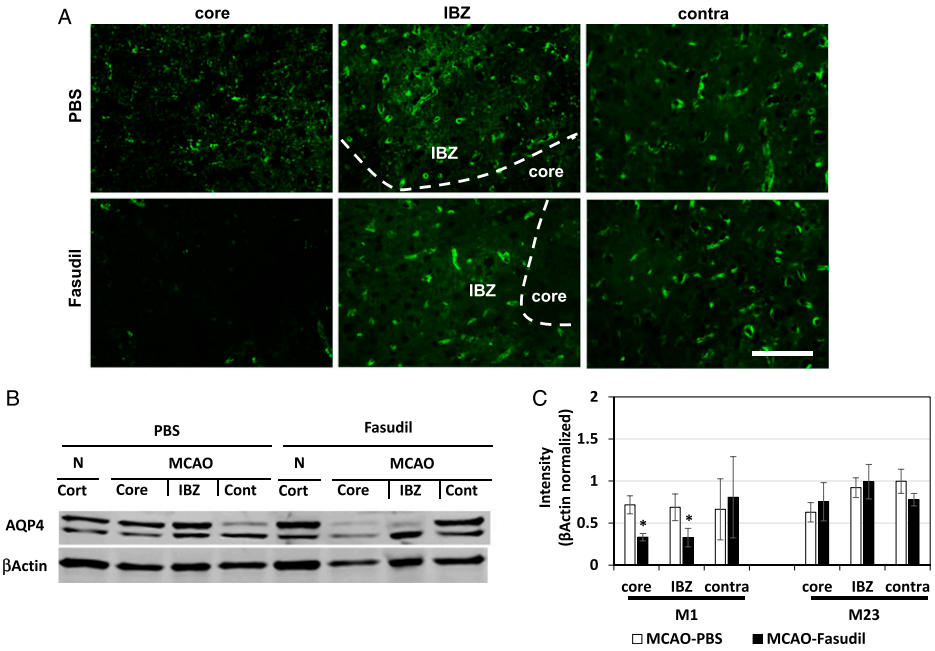

Figure 2. Effects of Fasudil on water channel protein AQP4 levels in MCAO rat brain. The levels of AQP4 protein in MCAO rat brains were evaluated by immunofluorescence staining and Western blotting. In (A), representative photomicrographs of AQP4 immunofluorescence staining are shown. Representative Western blotting data are shown in (B). βActin was used as the loading control. AQP4 protein levels were quantified by densitometry and normalized with that of βActin. The normalized levels of AQP4 are shown in (C). The data presented here are the mean ± SD of 5 rats in a group. Statistical significance is denoted as follows; *p<.05 vs PBS group.

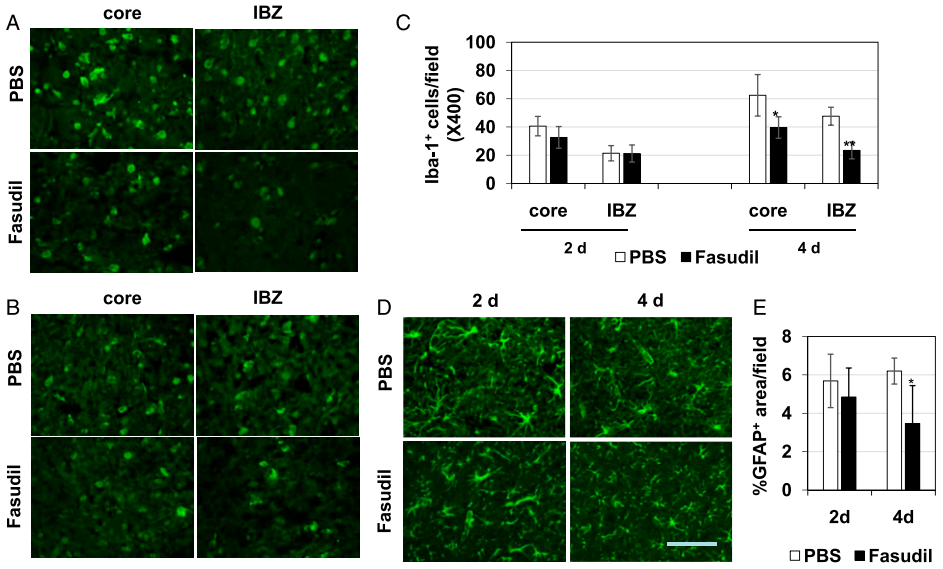

Figure 3. Effects of Fasudil on macrophage/microglia and astrocytes accumulation in MCAO rat brain. The macrophage/microglia accumulation was evaluated by Iba-1 (A and B), and astrocytes accumulation by GFAP (D) immunostaining, as described in the Materials and Methods. Iba-1-positive cells in a rat were counted randomly in 5 microscopic fields of a tissue section in the cortical core (A) and IBZ (B) areas at ×400 magnification, and the average was considered as the cell count of the animal. The data presented in (C) are the mean ± SD of 5 rats in a group. GFAP-positive cells in a rat were counted randomly in 5 microscopic fields of a tissue section in the cortical IBZ areas at ×400 magnification and the average was considered as the cell count of the animal. The data presented in (E) are the mean ± SD of 5 rats in a group. Statistical significance is denoted as follows; **P* < .05, ***P* < .01 vs PBS group of same cell type at same time point.

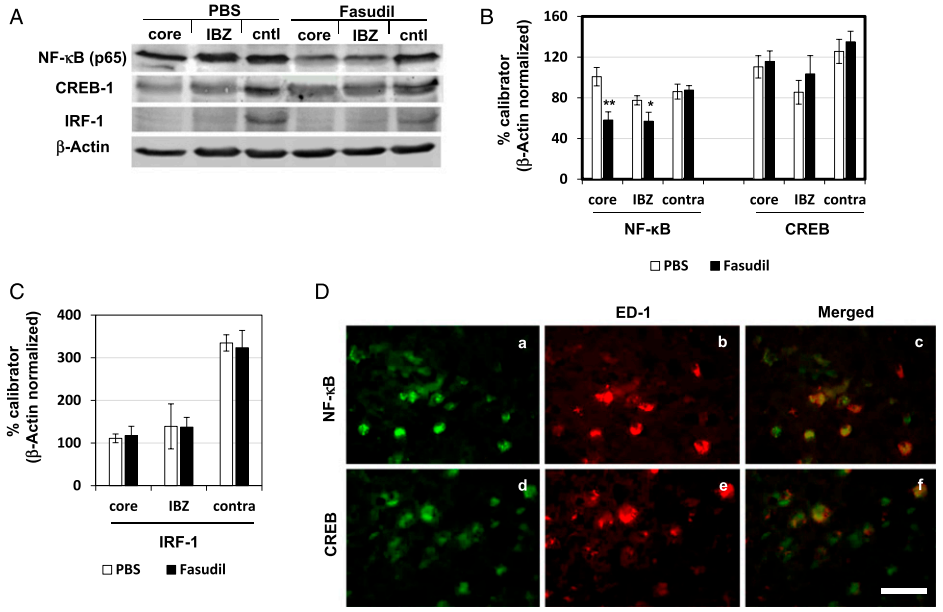

Figure 4. Effects of Fasudil on transcription factors expression in MCAO rats. Two days after MCAO, the protein levels of transcription factors in the core, IBZ, and contralateral (ctl) cortical areas of PBS- and Fasudil-treated rats were analyzed by Western blotting, as described in the Materials and Methods. A representative Western blotting data of 1 rat each of PBS- and Fasudil-treated group are shown in (A). βActin was used as a loading control. In (B), βActin normalized average (n = 5 in each group) densitometric data of NF-κB and CREB-1 Western blotting are shown, and that of IRF-1 is shown in (C). The data was expressed as a % calibrator, where 1 core sample of a PBS-treated rat was served as such; and presented here are the average ±SD of 5 rats in a group. (D) NF-κB and CREB-1 localization in the core region were analyzed by ED-1 and NF-κB, or ED-1 and CREB-1 double immunofluorescence staining. ED-1$^+$ cells are visualized by Texas Red (b and e), and NF-κB$^+$ (a) and CREB-1$^+$ (d) cells are visualized by FITC conjugated species-specific secondary antibodies. Merged photomicrographs of NF-κB and CREB-1 with ED-1 are shown in (c) and (f), respectively. Bar=50 μm.

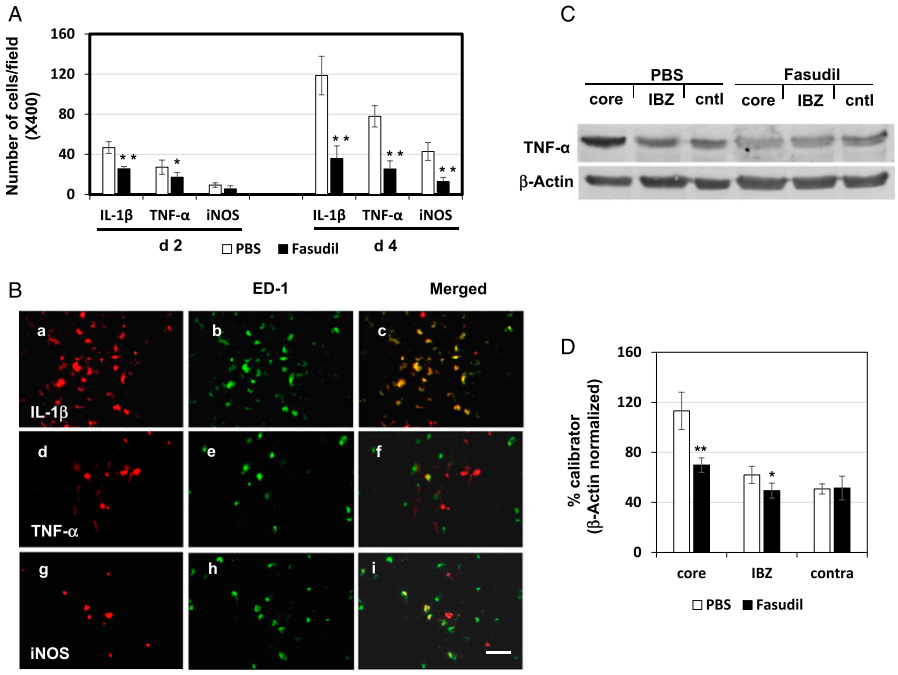

Figure 5. Expression and localization of NF-κB dependent proinflammatory mediators and the effects of Fasudil on their expression in MCAO rat brain. Proinflammatory mediators including IL-1β, TNFα, and iNOS levels were evaluated by fluorescence immunohistochemistry, as described in the Materials and Methods. (A) Immune reactive cells in the cortical core area of a rat brain were counted in 5 random microscopic fields of a tissue section at ×400 magnification and averaged. The data presented here are the mean ± SD of 5 rats in a group. (B) The localization of proinflammatory mediators in the core area of day 4 PBS rat brains was evaluated by double immunofluorescence staining of ED-1 (as macrophage/microglia cells marker) and IL-1β, TNFα, or iNOS (B). ED-1$^+$ macrophage/microglia were visualized by FITC (b, e and h), and IL-1β$^+$ (a), TNFα$^+$ (d), and iNOS$^+$ (g) cells by Texas red conjugated species-specific IgG. The localization of IL-1β$^+$ (c), TNFα$^+$ (f), and iNOS$^+$ (i) cells were analyzed by merging the photomicrograph of FITC and Texas-Red stained pictures at the same position. Levels of TNF-α were quantified by Western blotting. A representative Western blotting data are shown in (C). βActin was used as a loading control. βActin normalized average (n=5 in each group) densitometric data of Western blotting are shown in (D). The data was expressed as a % calibrator, where 1 core sample of a PBS-treated rat was served as such; and presented as the average ±SD of 5 rats in a group. Statistical significance is denoted as follows; *P < .05, **P < .01 vs PBS group at the same time point; $^{\#}P$ < .01 vs PBS group at day 2.

immunofluorescence staining. The immunostaining results demonstrated that on Day 4 after MCAO, IL-1β, TNF-α, and iNOS positive cell number was decreased in the lesion areas of the Fasudil group compared to the PBS group (Figure 5A). Double immunofluorescence results showed that ED-1$^+$ macrophage/microglia expressed IL-1β, TNF-α, and iNOS in the core areas (Figure 5B). Western blotting results showed that on Day 2 after MCAO, TNF-α levels were significantly decreased in the core and IBZ regions of the Fasudil group (Figure 5C and D).

Microglial cell migration was inhibited by Fasudil

To assess whether decreased Iba-1$^+$ macrophage/microglia accumulation in the lesion areas of the Fasudil group was due to decreased migration, we investigated the effect of Fasudil on macrophage inflammatory protein 1α (MIP-1α)-induced migration of an immortalized human microglial cell line HMO6. Preliminary time course experiments at 1, 2, 4, and 6 hours showed a time-dependent increase in migrated cell number. Further experiments were performed at a single time point of 4 hours. Dose-response experiments demonstrated that migrated cell number was increased up to 40 ng/ml of MIP-1α, making a typical bell-shaped migration curve (Figure 6A). Fasudil significantly decreased MIP-1α-induced HMO6 cell migration in a dose-dependent manner (Figure 6B).

Myosin light chain (MLC) phosphorylation is essential for cytoskeleton rearrangement and cell migration.[29] To check whether Rho-kinase affects these events during microglia migration, MIP-1α-induced MLC phosphorylation in HMO6 was investigated by immunocytochemical analysis. MIP-1α treatment with or without Fasudil for 1 hour did not change the total MLC level in HMO6 cells (Figure 6C-a, b and c). MIP-1α treatment markedly increased the phosphorylated form of MLC (pMLC) level in HMO6. However, when pretreated with Fasudil for 30 minutes, MIP-1α failed to increase the pMLC level in HMO6 (Figure 6C- d, e, and f).

Cytokine-induced transcription factors activation and proinflammatory gene expression were modulated by Rho-kinase inhibition in a microglial cell line

The inhibitory effect of Fasudil on microglial migration raises the possibility that decreased NF-κB protein levels in the Fasudil group might be due to decreased number of macrophage/microglia cells in the lesion areas. Moreover, changing the protein level might not be sufficient, because activation and nuclear translocation of NF-κB are necessary to

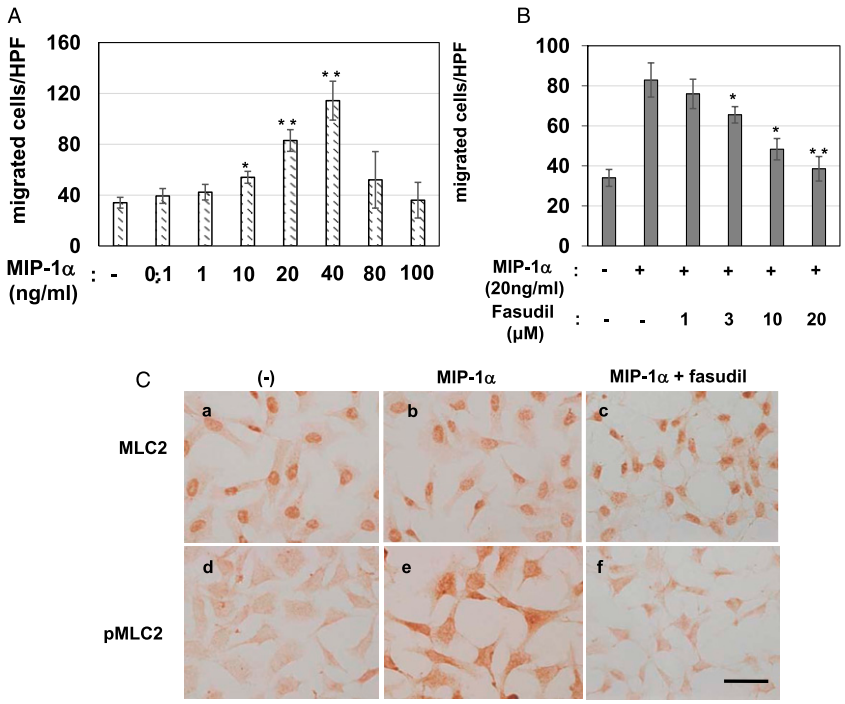

Figure 6. Effects of Fasudil on MIP-1α-induced microglia migration and myosin light chain (MLC) phosphorylation. MIP-1α-induced migration of a human microglial cell line, HMO6 was evaluated in a 48-wells micro-chemotactic chamber. To identify the effective dose of MIP-1α, a dose-dependent migration assay was performed (A). The dose-dependent effects of Fasudil on MIP-1α-induced HMO6 migration are presented in (B). In every experiment, each condition was evaluated in triplicate, and migrated cells were counted at high magnification in 5 random microscopic fields. The data presented here are the mean ± SD of 4 experiments. MLC2 phosphorylation in HMO6 was evaluated by total MLC2 and phosphorylated MLC2 (pMLC2) immunocytochemistry. Representative photomicrograph of immunocytochemistry of total MLC2 (a, b and c) and pMLC2 (d, e, and f) are shown in (C). Statistical significance is denoted as follows; **P* < .05, ***P* < .01 vs MIP-1α (-) condition (A), and MIP-1α (+) Fasudil (-) condition (B).

regulate NF-κB-dependent gene expression. As NF-κB and other transcription factors translocate to the nucleus after activation, we investigated whether Fasudil modulates the cytokine-induced nuclear accumulation of transcription factors in HMO6. The Western blotting results of HMO6 nuclear proteins revealed that combined IL-1β and IFNγ treatment resulted in increased nuclear translocation of NF-κB, which was inhibited by Fasudil (Figure 7A and B). Conversely, combined IL-1β and IFNγ treatment did not increase CREB-1 translocation. However, the addition of Fasudil significantly increased its translocation to the nucleus (Figure 7A and B). Similarly, real-time quantitative PCR results revealed that Rho-kinase inhibition by Fasudil transiently inhibited IL-1β and IFNγ-induced mRNA expression of NF-κB-dependent genes including IL-1β, TNF-α, and iNOS after 4 hours (Figure 7C and Table 1). After 24 hours stimulation, only TNF-α and iNOS were inhibited by Fasudil (Figure 7E and Table 1).

Discussion

Previous studies have demonstrated the functions of Rho-kinase, and the beneficial effects of its inhibition by Fasudil in cerebral ischemia.[33-36] Possible underlying mechanisms delineated are through improving endothelial dysfunction and cerebral blood flow, and reduction of neutrophil migration.[33,34] In the present report, we extended that observation, describing the contribution of Rho-kinase, hence Fasudil to the neuro-inflammatory system, essentially on astrocytes and macrophages/microglia accumulation and activation. Another important finding of this study is that Rho-kinase inhibition can decrease AQP4 expression in the MCAO condition. Since AQP4 inhibition was proved to be beneficial in the MCAO condition through decreasing cerebral edema, reducing its expression by Fasudil could provide a new target for stroke therapy.[41-43]

AQP4 is 1 of the main water-channel proteins expressed at the astrocyte end-feet that surround cerebral blood vessels. In several neurological conditions including cerebral ischemia, AQP4 expression is increased and contributes to the development of edema.[43] In the stroke condition, reducing its expression by gene knockout, or inhibiting its function appears to reduce the cerebral edema.[42,44] Our immunostaining results showed a dramatic reduction of AQP4 levels in the core region of Fasudil-treated rats, suggesting that reduced edema could be found in these rats. Indeed, MRI results indicated a reduction in water content. We also observed that on Day 4, AQP4 was almost undetectable in the core region of MCAO rats irrespective of treatment (data not shown). Such results

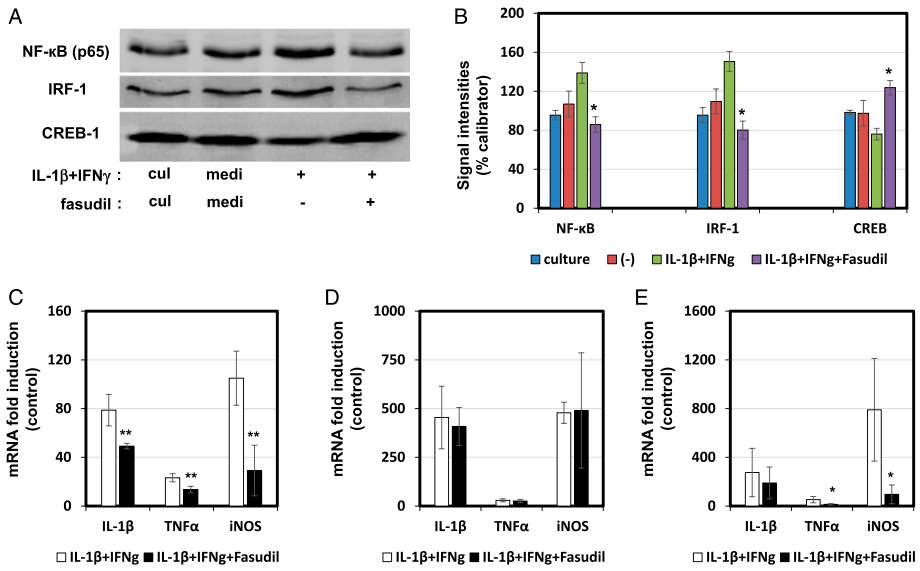

Figure 7. Effects of Rho-kinase inhibition on transcription factors activation and proinflammatory gene expression in a human microglial cell culture system. (A) HMO6 was treated with IL-1β (10 ng/ml) and IFNγ (10 ng/ml) with or without Fasudil (10 μM) for 1 h. Nuclear protein was isolated, and Western blotting was performed to analyze the translocation of NF-κB, CREB-1, and IRF-1. (B) The densitometric analysis of the nuclear accumulation of NF-κB, IRF-1, and CREB-1 was done and expressed as a % calibrator, where 1 of the untreated culture condition was served as such. (C) HMO6 cells were treated with IL-1β (10 ng/ml) and IFNγ (10 ng/ml) with or without Fasudil (10 μM) for 4 h (C), 8 h (D) and 24 h (E). Total RNA was isolated and real-time PCR was performed to analyze IL-1β, TNFα and iNOS mRNA expression. The results were calculated by the relative quantification method and presented as fold induction relative to a calibrator, where mRNA of an untreated condition was served as such. GAPDH mRNA level was used as a loading control. The numerical data presented here are the mean ± SD of 3 independent experiments. Statistical significance is denoted as follows; *$P < .05$ vs IL-1β and IFNγ-treated condition, **$P < .01$ vs IL-1β and IFNγ-treated condition.

Table 1. Effects of Fasudil on the mRNA expression of pro-inflammatory molecules.

MRNA	4 H		8 H		24 H	
	IL-1β + IFNγ	IL-1β+ IFNγ + FASUDIL	IL-1β + IFNγ	IL-1β + IFNγ+FASUDIL	IL-1β + IFNγ	IL-1β + IFNγ + FASUDIL
IL-1β	78.7 ± 2.2	49.2 ± 12.9[b]	454.5 ± 160.5	408.6 ± 97.3	2759 ± 1986	1893 ± 1316
TNF-α	23.2 ± 3.3	13.6 ± 2.7[b]	29.5 ± 7.6	25.8 ± 8.4	52.7 ± 25.8	14.4 ± 6.3[a]
iNOS	105 ± 22.2	29.2 ± 20.8[b]	490.6 ± 295.6	478.6 ± 54.3	7909 ± 4217	964.2 ± 774.3[a]

A human microglial cell line (HMO6) was stimulated with IL-1β and IFNγ (10 ng/ml each) with or without Fasudil (10 μM) for indicated times and the mRNA expression of IL-1β, TNFα, and iNOS were measured by quantitative real-time PCR. Results were normalized with corresponding GAPDH mRNA and calculated as fold induction relative to unstimulated conditions and expressed as mean ± SD of 3 independent experiments. The statistical significance was denoted as follows.
[a]$p < .05$.
[b]$p < .01$ vs IL-1β and IFNγ condition.

indicate that the rats might enter an edema resolution phage after Day 4.[45] In addition to cerebral edema, AQP4 has roles in neuroinflammation and glial activation by increasing the expression of osteopontin, GFAP, and tenacin.[46,47] Hence, Fasudil-mediated regulation of AQP4 could be an important modulator of stroke pathology via the regulation of neuro-inflammation along with edema. The Western blotting results demonstrated that the M1 isoform of AQP4 was inhibited by Fasudil in the brains of MCAO rats. Although we did not explore the reason for decreased M1 isoform, the possible cause could be altered translational regulation by Rho-kinase.[48] Such altered ratio of M1 and M23 isoform might be important for the formation of water channels and regulation of edema.

After cerebral ischemia, activated microglia and astrocytes had been observed to take part in acute neuroinflammatory processes, and thereby neuronal cell death.[6-10] Besides them, infiltrating neutrophils and macrophages are demonstrated to be involved in this process, as evidenced by the fact that decreasing the number of these immune cells confers favorable effects.[8-10] However, total depletion of macrophages/microglia proved to be detrimental for ischemia-like neurodegenerative condition,[24] suggesting a controlled number of immune cell population might be beneficial. Although we did not find any

morphological differences in immune cell types between the groups, the Fasudil group showed decreased macrophages/microglia and astrocytes accumulation; which might be responsible for the beneficial effects. It is believed that microglia respond to injury in the early phase, increase their number, and then infiltrating macrophages migrate to the injury site. Interestingly, Fasudil treatment decreased Iba-1$^+$ cells number at day 4 in the core and the IBZ; suggesting that Fasudil might be involved in the regulation of activation and migration of microglia/macrophage cells. Indeed, a previous report has demonstrated the inhibitory effects of Fasudil on macrophage migration in a porcine model of coronary vascular lesion.[49] Rho-kinase is known to inhibit myosin phosphatase activities, leading to increased phosphorylation of the myosin light chain; and ultimately affects the cell migration including smooth muscle cells and neutrophils.[26,27] However, the effects of Rho-kinase on cell migration appear to be cell-type specific. Our in vitro experiments demonstrated that Fasudil inhibited MIP-1α-induced microglia migration along with myosin light chain phosphorylation. These results are highlighting the significance of Rho-kinase-mediated myosin phosphorylation in microglia migration.

One of the principal events of ischemic brain injury is the expression of proinflammatory mediators by the inflammatory cells at the lesion site.[50,51] The number of macrophages/microglia, which can express proinflammatory mediators, was increased time-dependently in a similar fashion as proinflammatory mediators positive cells. In the Fasudil group, both inflammatory cells and proinflammatory mediators including IL-1β-, TNFα- and iNOS-positive cell numbers were decreased along with NF-κB. These findings raise the possibility that decreased NF-κB and proinflammatory mediators in this condition might be due to decreased inflammatory cell accumulation. However, in vitro inhibition studies with microglia cells culture demonstrated the critical function of Rho-kinase on nuclear translocation of NF-κB and proinflammatory gene expression, suggesting a combined effect of Fasudil on macrophages/microglia accumulation and activation results in a decreased level of proinflammatory mediators. Several previous studies have also shown the involvement of Rho-kinase in proinflammatory gene expression by inflammatory cells in many types of disease settings including rheumatoid arthritis, and inflammatory bowel disease through influencing intracellular signaling pathways involving MAP kinase and NF-κB.[50,51] NF-κB-mediated neuroinflammatory signals are known to be activated in stroke. A lot of signals including Rho-kinase can activate NF-κB.[52,53] In stroke, Rho-kinase is activated, raising the possibility that it may contribute to the activation of NF-κB. Therefore, Rho-kinase inhibition by Fasudil could be important for the regulation of neuroinflammation through NF-κB in stroke conditions. Although the protein levels of CREB and IRF-1 were not changed by Fasudil in MCAO rat brains, in vitro experiments showed that it can increase CREB nuclear translocation, and inhibits IRF-1 nuclear translocation in a microglial cell line. CREB is known to induce an anti-inflammatory condition through regulating anti-inflammatory cytokines and inhibiting NF-κB, and IRF-1 is a proinflammatory transcription factor.[54,55] Therefore, the overall function of the Fasudil could induce an anti-inflammatory condition that might provide the beneficial effects seen in the stroke animal model.

Our study provides an important insight into the role of Rho-kinase in MCAO conditions. However, some limitations of the study should be noted. First, we evaluated water-content changes in the infarction areas indirectly using MRI. A detailed study on the time-dependent changes of AQP4 and the state of edema in Fasudil-treated MCAO rats was not conducted. Also, how AQP4 expression is regulated in MCAO conditions, and the role of Rho-kinase in that regulation is important. In this matter, a detailed study of edema development, AQP4 expression, and Rho-kinase signaling in the MCAO condition would be interesting. Second, in vitro studies showed an increase in CREB nuclear translocation when microglia cells were treated with Fasudil. But in the rat MCAO model, CREB was not increased at protein levels after 2 days of Fasudil treatment. Since CREB showed neuroprotective effects through nuclear translocation and induction of downstream gene expression, the in vitro results could be important. Hence, a detailed study of Fasudil-mediated time-dependent changes in CREB nuclear translocation and downstream gene expression in the MCAO condition is warranted. Finally, this study lacks the data on the delayed effects of Fasudil on MCAO pathology, which is important to know its full potential as a therapy for stroke.

In conclusion, the present study demonstrated a comprehensive mechanism of Fasudil-mediated inhibition of neuroinflammation and cerebral edema after transient ischemic stroke. The inhibition of Rho-kinase in the early stage of ischemic stroke might provide a promising therapeutic approach to improve functional neuronal outcomes and reduce the morbidity and mortality of stroke patients.

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