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Original Article

Fasudil hydrochloride could promote axonal growth through inhibiting the activity of ROCK

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Abstract: Objective: This study aims to investigate the neuroprotective effect of Rho kinase inhibitor fasudil hydrochloride in ischemia/reperfusion injury N2a neuron. Methods: In vitro, N2a cells induced by ischemia and ischemia-reperfusion were treated with fasudil hydrochloride, cell damage was analyzed by MTT. On the other hand, the cytoskeleton of N2a cells was scanned through immunofluorescence techniques by Confocal Laser Microscopy which stained with FITC-phalloidin for F-actin visualization. Results: The activation of ROCK-II increased significantly in the damaged local during the following phase of ischemia/reperfusion injury. Ischemia induced a striking reorganization of actin cytoskeleton with a weakening of fluorescent intensity of the peripheral filament actin bands and formation of the long and thick stress fibers, but pretreatment of Fasudil hydrochloride could reversed the changes of ultra-structure on the cellular surface. MTT assay showed that Fasudil hydrochloride could prolong the survival time of the N2a cells after mimic ischemia-reperfusion for 24 h. Conclusions: The activation of ROCK-II has an exceptional hoist after ischemia/reperfusion injury, it is likely to induce the collapse of the growth cone through MLC-P. Fasudil hydrochloride could promote axonal growth on inhibitory of ROCK activity.

Keywords: Fasudil hydrochloride, ROCK, ischemia/reperfusion injury, neuroprotection

Introduction

Fasudil hydrochloride (Hexahydro-1-(5-isoquinolinylsulfonyl)-1H-1, 4-diazepine monohydrochloride; also known as HA 1077) is a new type of isoquinoline sulfonamide derivatives. At present, it is only used in clinic as selective inhibitors of Rho kinase for preventing and improving the cerebral vasospasm after subarachnoid hemorrhage and symptoms of cerebral ischemia. However, recent studies found that it can promote the survival of neural stem cells, axonal regeneration and differentiation of bone marrow mesenchymal cell into neurons [1, 2]. Yamashita [3] observed that fasudil hydrochloride can effect on neurons directly by reducing the activity of Rho kinase (ROCK) and protect neuronal ischemic damage in persistent model of cerebral ischemia. ROCK is the main effector molecules of RhoA, while the three important molecules Cdc42, Rac1 and RhoA of Rho GTPases is a molecular switch mediating cytoskeletal reorganization of neuronal actin. The RhoA regulated by repulsive guidance signal of micro environment is a key molecule mediating axon retraction. The structural basis of axon collapse retraction after nerve cell damage is the retraction and collapse of cytoskeleton.

In this study, we investigated the expression of ROCK-I and ROCK-II and the phosphorylation of its downstream substrate myosin light chain (MLC) in neuron ischemia and reperfusion injury model in vitro adding fasudil hydrochloride to intervene. We also explored neuroprotective mechanism of fasudil hydrochloride by inhibiting the RhoA/ROCK pathway involved in axonal retraction.

Materials and methods

Culture of murine neuroblastoma cell lines N2a (N2a/wt)

Wild-type murine neuroblastoma cell lines (N2a/wt) were gifted by Professor Chen Juan (Department of Molecular Biology, Tongji Medical College of Huazhong University of Science and Technology). They were cultured with medium containing 50% DMEM, 50% OPTI-MEM and

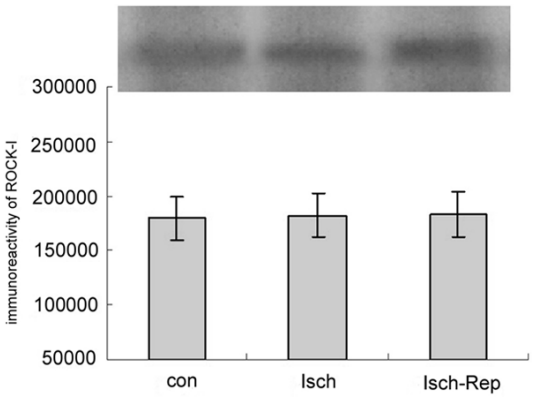

Figure 1. Western Blotting of ROCK-I (ROK β) in N2a cells. Con: control group; Isch: ischemia group; Isch-Rep: ischemia reperfusion group. There was no difference between the groups ($P > 0.05$).

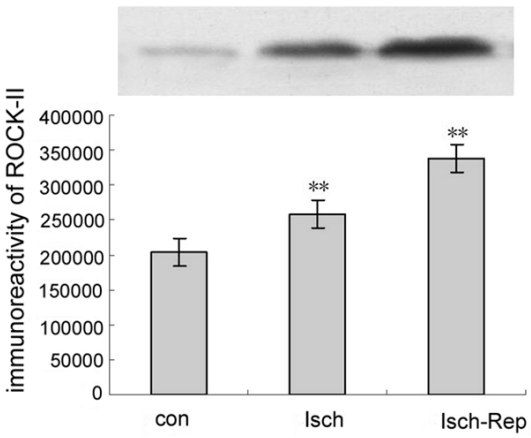

Figure 2. Western Blotting of ROCK-II (ROK α) in N2a cells. Con: control group; Isch: ischemia group; Isch-Rep: ischemia reperfusion group. **Compared with the control group, ROCK-II content increased significantly in ischemia group and ischemia reperfusion group ($P < 0.05$).

5% FBS (Gibco, USA), under 37°C, 5% CO_2 and saturated humidity conditions. The logarithmic growth phase cells growing to 70%~80% abundance were used to do experiments.

Establishment of ischemia and reperfusion model in vitro and experimental groups

The cell density was adjusted to be 1×10^5/ml and cultured in 96-well plates with 100 μl in each well. They were divided into control group, ischemia group, reperfusion group, ischemia with fasudil hydrochloride intervention group and reperfusion with fasudil hydrochloride intervention group. Each group has 6 wells. The medium of ischemia group were discarded when cells grow to 80% and the same amount of balanced salt solution including 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 0.9 mM $CaCl_2$ and 10 mg/l phenol red was added into them. They were cultured under 37°C, 5% CO_2 and 95% N_2 conditions for 120 min to simulate ischemia process. Then the balanced salt solution was changed to normal culture medium and the cells were cultured for 24 h under normal conditions to simulate reperfusion process. The intervention group was added 3 mmol/L of fasudil hydrochloride (Asahi Kasei Pharma Corporation, Nagoya Pharmaceuticals Plant).

Determination of ROCK-I and ROCK-II content with western blotting

Cells were collected after treatment and washed with cold PBS for 3 times. Then the cellular lysis buffer was added and incubated on ice for 30 min. The total proteins were extracted after centrifugation. Quantitative protein determination was done with BCA kit in accordance with the kit manual and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. The membrane containing the proteins was blocked with 5% milk/TBS for 1 h at room temperature, ROk α and ROK β polyclonal antibody (1:250, Santa Cruz, USA) were added into them respectively and then donkey anti-goat IgG-HRP (1:5000, Santa Cruz, USA) was added. They were stained with ECL enhanced chemiluminescence detection kit (Pierce, USA). The protein bands were scanned.

Detection of myosin light chain (MLC) phosphorylation with western blotting

The methods were similar with the above. The first antibodies were rabbit anti rat myosin light chain phosphorylation antibody p-MLC (Thr18/Ser19, 1:500, Santa Cruz, USA) and MLC polyclonal antibody (1:500, Sigma, USA).

Detection of cellular damage with MTT methods

The cell density was adjusted to be 1×10^5/ml and cultured in 96-well plates with 100 ul in each well. A total of 10 ul 10 mg/ml four methyl thiazolyl blue (MTT, Amersco, USA) was added into each well and the cells were cultured for 24 h. Then medium was discarded and 200 ul of

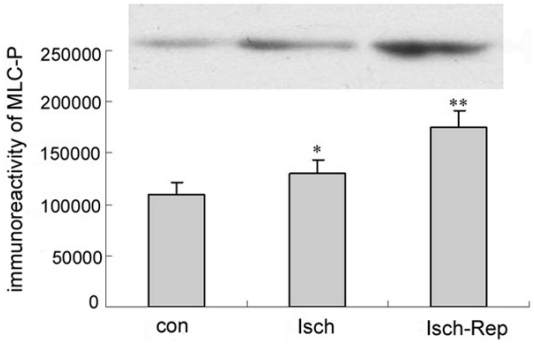

Figure 3. Western Blotting of MLC phosphorylation in N2a cells. Con: control group; Isch: ischemia group; Isch-Rep: ischemia reperfusion group. Compared with control group, MLC phosphorylation in damaged neuron presented a gradual upward trend with time (*P < 0.05, **P < 0.01).

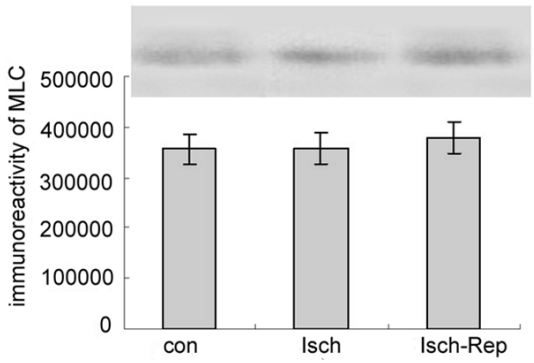

Figure 4. Western Blotting of MLC non-phosphorylation in N2a cells. Con: control group; Isch: ischemia group; Isch-Rep: ischemia reperfusion group. There was no change in the groups (P > 0.05).

two dimethyl sulfoxide (DMSO) were added into wells and mixed carefully. The absorbance (OD) at 570 nm wavelength was measured with automatic enzyme immunoassay instrument and the experiments were repeated for 3 times.

Staining of F-actin with FITC-phalloidin conjugate

Plates were washed with ice-cold PBS for two times and fixed with the ice-cold 4% paraformaldehyde for 15 min. The cells were permeabilized with PBS-0.1% Triton X-100 for 15 min at room temperature after being washed three times with PBS for 5 min each. Then they were blocked with PBS containing 3% BSA for 1 h at room temperature. Filamentous actin was stained with 320 nmol/L FITC-phalloidin conjugate solution (Sigma) in PBS for 2 h at 4°C. After several washes in PBS to remove unbound phalloidin conjugate, they were observed under Fluorescence microscopy (Olympus, Japan).

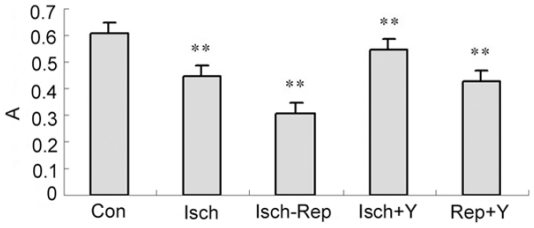

Figure 5. Protection of Fasudil on N2a cells. Con: control group; Isch: ischemia group; Isch-Rep: ischemia reperfusion group. Isch+Y: ischemia with fasudil hydrochloride intervention group; Rep+Y: reperfusion with fasudil hydrochloride intervention group. **Fasudil could significantly improve the 24 h survival rate of N2a cells of ischemia and reperfusion group (P < 0.05).

Statistical analysis

All the experimental data were analyzed by SPSS18.0. The comparison between two groups was carried out by t-test. Differences among multiple experimental groups were analyzed by One-way ANOVA. P < 0.05 was considered to be statistically significant differences.

Results

Changes of ROCK-I and ROCK-II content

After ischemia for 120 min and ischemia reperfusion injury for 24 h, there was no significant differences of ROCK-I content between ischemia group, ischemia reperfusion group and control group (P > 0.05). However, ROCK-II content increased significantly in ischemia group and ischemia reperfusion group (P < 0.05) (**Figures 1**, **2**).

Changes of MLC phosphorylation

Compared with control group, MLC phosphorylation in damaged neuron presented a gradual upward trend with time (P < 0.05). However, there was no change in the expression of myosin light chain protein (P > 0.05) (**Figures 3**, **4**).

Effect of fasudil hydrochloride on survival ability of N2a cells of ischemia and reperfusion

Fasudil could significantly improve the 24 h survival rate of N2a cells of ischemia and reperfusion group (P < 0.05) (**Figure 5**).

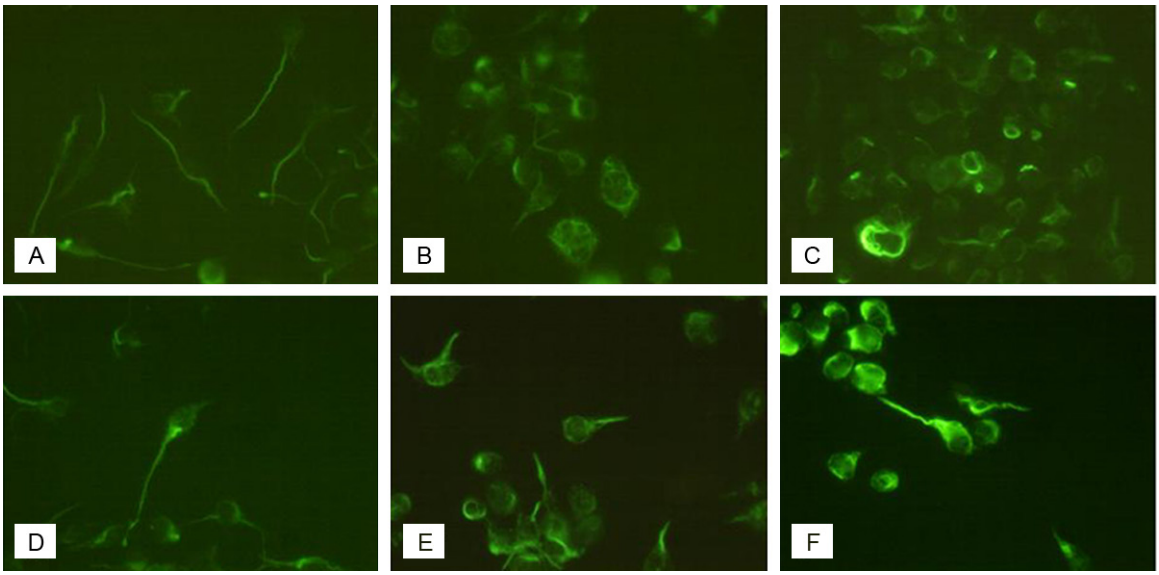

Figure 6. F-actin cytoskeleton of N2a cells inducing by ischemia-reperfusion stained with FITC-conjugated phalloidin. A: Normal culture. F-actin was mainly distributed in the cellular periphery, the short and thin stress fibers were seen in cytoplasm occasionally; B: Cultured under ischemia for 120 min. A lot of stress fibers were seen in cytoplasm and axonal retraction appeared; C: Changed to normal culture for 24 h. The peripheral actin ribbon and characteristics of neurons disappeared, Fuzzy F-actin; D: Pretreatment with Fasudil for protection and cultured under ischemia for 120 min. A small amount of stress fibers appeared in cytoplasm. The peripheral actin ribbon was clear and smooth but no obvious axonal retraction; E: Cultured under ischemia with Fasudil intervention for 120 min and changed to normal culture for 24 h. Neuronal characteristics existed; F: Adding Fasudil after cultured under ischemia for 120 min. Axon still existed and filopodia appeared in cell membrane.

Cytoskeleton changes of neuronal fibrous actin (F-actin)

Normal neurons' F-actin was mainly distributed in the cellular periphery, axon or dendrite, which forming the peripheral actin ribbon. The short and thin stress fibers were seen in cytoplasm occasionally. A lot of stress fibers were seen in cytoplasm and axonal retraction appeared after culture with ischemia for 120 min. The peripheral actin ribbon and characteristics of neurons disappeared after changing to normal culture, cells were prone to die. If they were pretreated with fasudil hydrochloride, a small amount of stress fibers appeared in cytoplasm. The peripheral actin ribbon was clear and smooth but no obvious axonal retraction. The situation was significant improved if adding fasudil hydrochloride after ischemia culture, axon still existed and filopodia appeared in cell membrane (**Figure 6**).

Discussion

One common injury mechanism of secondary nerve injury caused by many pathological factors such as injury, inflammation, ischemia, tumor or degeneration is ischemia reperfusion. Previous studies [6, 7] showed that the expression level of RhoA increased significantly in 8 hours after spinal cord injury although it was low in normal spinal cord, it reached the peak three days later and continued high expression in 4 weeks, which provided the basis for the application of Rho kinase inhibitors in the treatment of nervous system injury [4, 5].

The structural basis of axons collapse after neuronal damage was the retraction and collapse of cytoskeleton. At present, it was found that the molecular switch to adjust the neuronal actin cytoskeleton was Cdc42, Rac1 and Rho, which were Rho subfamily members belonged to the GTP binding protein Ras superfamily. Rho was the key molecule [6, 7] and RhoA was its main subtype.

RhoA was activated to form RhoA-GTP and the main substrate was Rho associated kinase (ROCK), a kind of serine/threonine kinase and had two subtypes ROCK-I and ROCK-II. This experiment confirmed that ROCK-II of neural cells with ischemia reperfusion injury was activated and the phosphorylation of its down-

stream MLC increased. Hyperphosphorylation of MLC made calcium sensitization of the actin cytoskeleton and thus affected the polymerization and depolymerization of actin-globulin. The contractility of actin-myosin-II was changed leading to the growth cone collapse and axonal retraction ultimately, which was the ROCK pathway [8].

Fasudil hydrochloride, an inhibitor of Rho kinase, was effective for the treatment of many cardiovascular diseases, such as cerebral artery and coronary artery spasm, angina, hypertension, pulmonary hypertension and heart failure [9]. In this study we found that the survivability of N2a cells was significantly improved after adding fasudil hydrochloride. Immunofluorescence observation found that cytoskeleton reorganization, significant axonal retraction, a lot of stress fibers in cytoplasm, and fuzzy peripheral actin ribbon in anoxic cultured N2a cells. Cellular viability significantly decreased and the characteristics of neurons disappeared after reperfusion injury, cells were prone to die. However, the situation was significant improved, the axonal and neuronal collapse could be reversed if they were pretreated with fasudil hydrochloride, filopodia re-emerged. Therefore, we thought that fasudil hydrochloride had a wide application prospect in human central and peripheral nervous system injury protection and regeneration.

Many neuroprotective agents were effective in animal experiments but clinical invalid. Fasudil hydrochloride was also facing the embarrassing situation. It is effective administered intravenously or orally and had very short half-life of approximately 16 min. However, its blood brain barrier permeability was low and impeded the effectiveness in the central nervous system. Therefore, the development of fasudil liposome to increase the blood brain barrier permeability will be our further study.

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Disclosure of conflict of interest

None.

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References

[1] Ding J, Yu JZ, Li QY, Wang X, Lu CZ and Xiao BG. Rho kinase inhibitor Fasudil induces neuroprotection and neurogenesis partially through astrocyte-derived G-CSF. Brain Behav Immun 2009; 23: 1083-1088.

[2] Ding J, Li QY, Yu JZ, Wang X, Sun CH, Lu CZ and Xiao BG. Fasudil, a Rho kinase inhibitor, drives mobilization of adult neural stem cells after hypoxia/reoxygenation injury in mice. Mol Cell Neurosci 2010; 43: 201-208.

[3] Shimokawa H. Yamashita K, Kotani Y, Nakajima Y, Shimazawa M, Yoshimura S, Nakashima S, Iwama T and Hara H. Fasudil, a Rho kinase (ROCK) inhibitor, protects against ischemic neuronal damage in vitro and in vivo by acting directly on neurons. Brain Res 2007; 1154: 215-224.

[4] Conrad S, Schluesener HJ, Trautmann K, Joannin N, Meyermann R and Schwab JM. Prolonged lesional expression of RhoA and RhoB following spinal cord injury. J Comp Neurol 2005; 487: 166-175.

[5] Erschbamer MK, Hofstetter CP and Olson L. RhoA, RhoB, RhoC, Rac1, Cdc42, and Tc10 mRNA levels in spinal cord, sensory ganglia, and corticospinal tract neurons and long-lasting specific changes following spinal cord injury. J Comp Neurol 2005; 484: 224-233.

[6] Hall A and Nobes CD. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. Philos Trans R Soc Lond B Biol Sci 2000; 355: 965-970.

[7] Thies E and Davenport RW. Independent roles of Rho-GTPases in growth cone and axonal behavior. J Neurobiol 2003; 54: 358-369.

[8] Wettschureck N and Offermanns S. Rho/Rho-kinase mediated signaling in physiology and pathophysiology. J Mol Med (Berl) 2002; 80: 629-638.

[9] Hirooka Y and Shimokawa H. Therapeutic potential of rho-kinase inhibitors in cardiovascular diseases. Am J Cardiovasc Drugs 2005; 5: 31-39.