European Journal of Pharmacology 594 (2008) 77–83

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Demonstration of elevation and localization of Rho-kinase activity in the brain of a rat model of cerebral infarction

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ARTICLE INFO

Article history:
Received 30 November 2007
Received in revised form 24 June 2008
Accepted 22 July 2008
Available online 31 July 2008

Keywords:
ROCK (Rho-kinase)
Cerebral infarction
Cerebral thrombosis
Protein kinase
MBS (myosin-binding subunit)
MLC (myosin light chain)
(Rats)

ABSTRACT

Evidence that Rho-kinase is involved in cerebral infarction has accumulated. However, it is uncertain whether Rho-kinase is activated in the brain parenchyma in cerebral infarction. To answer this question, we measured Rho-kinase activity in the brain in a rat cerebral infarction model. Sodium laurate was injected into the left internal carotid artery, inducing cerebral infarction in the ipsilateral hemisphere. At 6 h after injection, increase of activating transcription factor 3 (ATF3) and c-Fos was found in the ipsilateral hemisphere, suggesting that neuronal damage occurs. At 0.5, 3, and 6 h after injection of laurate, Rho-kinase activity in extracts of the cerebral hemispheres was measured by an ELISA method. Rho-kinase activity in extracts of the ipsilateral hemisphere was significantly increased compared with that in extracts of the contralateral hemisphere at 3 and 6 h but not 0.5 h after injection of laurate. Next, localization of Rho-kinase activity was evaluated by immunohistochemical analysis in sections of cortex and hippocampus including infarct area 6 h after injection of laurate. Staining for phosphorylation of myosin-binding subunit (phospho-MBS) and myosin light chain (phospho-MLC), substrates of Rho-kinase, was elevated in neuron and blood vessel, respectively, in ipsilateral cerebral sections, compared with those in contralateral cerebral sections. These findings indicate that Rho-kinase is activated in neuronal and vascular cells in a rat cerebral infarction model, and suggest that Rho-kinase could be an important target in the treatment of cerebral infarction.

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1. Introduction

Cerebral infarction results from a reduction in cerebral blood flow, and is frequently caused by occlusion of cerebral arteries by either an embolus or local thrombosis. In cerebral infarction, the brain is damaged by a primary insult, impairment of cerebral blood flow, as well as secondary injury, such as inflammation by neutrophils and macrophages migrating into ischemic brain (Dirnagl et al., 1999).

Rho-kinase, one of the downstream effectors of Rho, is a serine/threonine kinase that is activated by binding to the active GTP-bound form Rho. Rho-kinase exists as two isomers, Rho-kinase α/ROK α/ROCK2 and Rho-kinase β/ROK β/ROCK1 (Wettschureck and Offermanns, 2002), which are known to phosphorylate various substrates, including MBS (myosin-binding subunit) of myosin phosphatase, ERM (ezrin–radixin–moesin) family proteins, myosin light chain (MLC), and LIM kinase (Fukata et al., 2001). We have reported that administration of fasudil and hydroxy fasudil, which are Rho-kinase inhibitors, improves neurological deficits and reduces the volume of infarction in models of cerebral infarction (Satoh et al., 1999, 2001; Toshima et al., 2000). These findings suggest that Rho-kinase contributes to cerebral infarction. Recent studies have demonstrated that Rho-kinase plays important roles in various cellular functions (Amano et al., 2000; Riento and Ridley, 2003; Shimokawa and Rashid, 2007) including cell motility and mobility, actin cytoskeleton organization, contraction of vascular vessels, and retraction of neurites, which may be involved in the pathogenesis of cerebral infarction. It has been demonstrated that levels of Rho are up-regulated in the brains of patients who have died following focal cerebral infarction (Brabeck et al., 2003) and in mouse brains after middle cerebral artery occlusion (Trapp et al., 2001), suggesting up-regulation of Rho/Rho-kinase signaling. However, there is little direct evidence of activation of Rho-kinase itself following cerebral infarction, nor has the location of the Rho-kinase activity in brain parenchyma following infarction been determined. In the present study, to address these problems, we measured Rho-kinase activity directly using brain extracts and determined where MBS and MLC phosphorylations occur using brain sections in a rat model of cerebral infarction.

2. Materials and methods

2.1. Induction of cerebral microthrombosis

All animal protocols were approved by the Committee on Ethics in Animal Experiments of Asahi Kasei Pharma and were performed in

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doi:10.1016/j.ejphar.2008.07.045

accordance with the Guidelines for Animal Experiments of the Asahi Kasei Pharma and for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. Male Sprague–Dawley rats (age, 8–9 weeks; weight, 245–341 g, Japan SLC) were used for the present study. Cerebral microthrombosis was induced by injection of sodium laurate into the left internal carotid artery as described previously (Toshima et al., 2000) with some modifications. In brief, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After exposure of the left common, external, and internal carotid arteries, the left external carotid, occipital, pterygopalatine, and common carotid arteries were ligated with 6-0 silk braid. A polyethylene catheter was inserted into the left common carotid artery. Sodium laurate (100 μg/body) dissolved in saline was injected into the internal carotid artery. The catheter was removed, and the internal common carotid artery was ligated at a position slightly distal to the site of injection. Sham operation was performed in the same manner but with saline injection.

2.2. Neurological examination and observation of infarct area

Neurological deficits such as hemiplegia were evaluated in a posture test (Bederson et al., 1986). In a postural reflex test, rats were tested for degree of abnormal posture when suspended by their tails 10 cm above the floor. They were scored according to the following criteria: Rats extended both forelimbs straight, no observable deficit: 0 (normal). Rats attached the right forelimb to the breast and extended the left forelimb straight: 1 (mild). Rats twisted the upper half of the body, in addition to the behavior in score 1: 2 (severe).

6 h after injection of laurate, brains were perfused with heparinized saline and subsequently with 4% phosphate-buffered paraformaldehyde and fixed until they were embedded in paraffin. Several serial coronal sections (5 μm thick) were obtained at −3 mm from the optic chiasm. The brain sections were stained with hematoxylin–eosin for observation of infarct.

2.3. Preparation of cerebral extracts

At 0.5, 3, and 6 h after injection of saline or laurate, brains were quickly removed under anesthesia and separated into the left and right cerebral hemispheres, and frozen in liquid nitrogen. The intact brains were harvested and frozen as those of the normal control group. The brain samples were stored at −80 °C until use. Cerebral hemispheres were homogenized in 7 ml of ice-cold extraction buffer (1% Triton X-100, 10 mmol/l $MgSO_4$, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.5 mol/l NaCl, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail 1, 1% phosphatase inhibitor cocktail 2, 20 mmol/l HEPES (pH 7.5)) with a Polytron homogenizer at 20,000 rpm for 1 min. The homogenate was centrifuged at 15,000 g for 20 min at 4 °C. The supernatant was taken as the cerebral hemisphere extract and stored at −80 °C until use. The protein concentration of extracts was measured with a BCA Protein Assay kit (PIERCE).

2.4. Measurement of MBS phosphorylation activity and Rho-kinase activity

Myosin-binding subunit (MBS, a substrate for Rho-kinase) was fixed on an ELISA plate (Nunc). A reaction mixture (75 μl; 100 μg/ml protein of cerebral extract, 100 μmol/l ATP, 1% Triton X-100, 10 mmol/l $MgSO_4$, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.1 mol/l NaCl, 0.1% BSA, 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail 1, 1% phosphatase inhibitor cocktail 2, 20 mmol/l HEPES (pH 7.5)) was added to each well of the MBS-fixed plate. To examine the effects of kinase inhibitors, 10 μmol/l of kinase inhibitor was added to the reaction mixture. After incubation for 20 min at 30 °C, an equal volume of 0.2 mol/l EDTA (pH 7.5) was added to stop the reaction. Phosphorylated MBS was detected with rabbit anti-phospho-MBS antibody and goat anti-rabbit IgG conjugated with HRP (Zymed). MBS phosphorylation activity was assessed by measuring the absorbance at 490 nm (A490) in a color reaction with *o*-phenylenediamine. Rho-kinase activity was evaluated as the difference in MBS phosphorylation activity in the presence and absence of 10 μmol/l fasudil.

2.5. Quantitation of transcription factors, Rho-kinase and Rho by Western blot analysis

The amounts of activating transcription factor 3 (ATF3), c-Fos, c-Jun, actin, Rho-kinase α, Rho-kinase β, RhoA, and RhoB in cerebral extracts were evaluated by Western blot analysis. Equal amounts of cerebral extracts were subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and treated with primary antibodies, including rabbit-anti-ATF3 (Santa Cruz), -c-Fos (Cell Signaling), -c-Jun (Cell Signaling), -RhoA (Santa Cruz), -RhoB (Santa Cruz) or mouse-anti-Rho-kinase α (BD Bioscience), -Rho-kinase β (BD Bioscience), -actin (Sigma) and then secondary antibody, HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (GE Healthcare). The band of each protein was detected with a chemiluminescent method using SuperSignal West-Dura (PIERCE). The image of bands was scanned in a scanner (CanoScan 5000F, Canon). The intensity of bands was analyzed with Image J (National Institution of Health).

2.6. Immunohistochemistry for phospho-MBS and -MLC in cerebral cortex and hippocampus

6 h after injection of laurate, brains were perfused with heparinized saline and subsequently with 4% phosphate-buffered paraformaldehyde and fixed until they were embedded in paraffin. Several serial coronal sections (5 μm thick) were obtained at −3 mm from the optic chiasm. Sections were deparaffinized and heated (95 °C for 30 min) in antigen retrieval buffer (10 mmol/l citric acid, 0.1% Tween20, pH 6.0). The sections were incubated with the following primary antibodies: rabbit anti-phospho-MBS, mouse anti-phospho-MLC, mouse anti-neuron-specific nuclear protein (NeuN; Chemicon), rabbit anti-neurofilament L (NF; Cell Signaling), rabbit anti-glial fibrillary acidic protein (GFAP; Cell Signaling) or rabbit anti-von Willebrand factor (vWF; Chemicon) antibody. Anti-NeuN, NF, GFAP and vWF antibodies were used for a marker of neuronal cell, neurofilament, glia and blood vessel, respectively. The sections were then incubated with fluorescence-conjugated secondary antibody (Alexa Fluor 488 for green and/or 568 for red, Molecular Probes). In the case of immunostaining of phospho-MLC, the sections were incubated with goat anti-mouse IgG conjugated with HRP (Zymed) and developed with a Metal Enhanced DAB Substrate Kit (PIERCE) according to the manufacturer's protocols. Counter staining was performed with hematoxylin (Dako). These signals were observed under a fluorescent microscope (AX80, Olympus).

2.7. Determination of K_i values of Rho-kinase inhibitors

The values of the inhibition constants (K_i) of fasudil, hydroxy fasudil, and (*R*)-(+)-*trans*-*N*-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) for MBS phosphorylation by recombinant Rho-kinases (Rho-kinase α and Rho-kinase β) were determined. Fixed MBS on an ELISA plate was phosphorylated by recombinant Rho-kinase for 10 min at 30 °C under the following conditions: 6.25–400 μmol/l ATP, 0.1 mol/l NaCl, 5 mmol/l $MgSO_4$, 5 mmol/l dithiothreitol, and 20 mmol/l HEPES (pH 7.5). After the reaction was stopped with EDTA, phosphorylated MBS was detected by the method described above. K_i values were calculated by Lineweaver–Burk analysis for fasudil and hydroxy fasudil, and from the IC_{50} at 100 μmol/l ATP for Y-27632.

2.8. Materials

Fasudil and hydroxy fasudil were synthesized in Asahi Kasei Pharma. (Y-27632 (Rho-kinase inhibitor), 3-[1-[3-(Amidinothio)propyl-1*H*-indol-

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