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Increased Leukocyte ROCK Activity in Patients After Acute Ischemic Stroke

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Abstract

BACKGROUND—Rho-kinase (ROCK) is a downstream effector of Rho GTPase that is known to regulate various pathological processes important to the development of ischemic stroke, such as thrombus formation, inflammation, and vasospasm. Inhibition of ROCK leads to decreased infarct size in animal models of ischemic stroke. This study tests the hypothesis that ROCK activity increases during the acute phase of ischemic stroke.

METHODS—Serial blood samples were drawn from 10 patients with acute ischemic stroke presenting within 24 hours of symptom onset and with NIHSS scores ≥ 4 . Samples were taken at 24, 48, and 72 hours. Leukocyte ROCK activity was determined by immunoblotting leukocyte lysates with antibodies to the phosphorylated form of myosin-binding subunit (P-MBS) of myosin light chain phosphatase (MLCP). MBS and P-MBS contents were normalized to α -tubulin, and ROCK activity was expressed as the ratio of P-MBS to MBS. ROCK activities in these 10 patients were compared to baseline ROCK activities in 10 control subjects without acute illness and matched for sex, age, and number of vascular risk factors using a two-tailed Student's t-test.

RESULTS—The mean NIHSS score in patients with stroke was 15.4. ROCK activity was significantly increased at 24 and 48 hours in patients after acute ischemic stroke when compared to control values, with peak elevations at 48 hours after stroke onset. There was no apparent correlation between ROCK activity and stroke severity based on NIHSS.

CONCLUSIONS—Leukocyte ROCK activity is increased in patients after acute ischemic stroke with maximal activity occurring about 48 hours after stroke onset. These findings suggest that activation of ROCK may play a role in the pathogenesis of ischemic stroke in humans.

Keywords

Acute ischemic stroke; ROCK; Rho-kinase; fasudil

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Introduction

Stroke pathophysiology includes thrombus formation, vascular occlusion and the initiation of the ischemic cascade, and secondary vascular events that may prevent the reperfusion of cerebral microvessels even after large artery recanalization through spontaneous or therapeutic clot lysis, the no-reflow phenomenon. There is growing evidence that structural damage to vessel walls is not sufficient to explain this phenomenon (Fischer 1977) and that local thrombus formation, inflammation, and vasospasm may all contribute to this process (Kloner 1974, Mori 1992, Hillegas 2001, Eeckhout 2001, Rezkalla 2002).

Recent studies suggest that abnormal ROCK function may contribute to the pathogenesis of ischemic stroke (Shimokawa, 2002). The Rho/ROCK pathway regulates actin cytoskeleton organization, cell adhesion, and motility (Fukata et al., 2001) and is involved in platelet aggregation and activation (Yang et al., 2004). In the vascular wall, ROCK mediates vascular smooth muscle contraction, and elevated ROCK activity contributes to abnormal smooth muscle contraction observed in cerebral and coronary vasospasm (Masumoto et al., 2002; Sato et al., 2000) and hypertension (Masumoto et al., 2001). In addition, ROCK could also regulate vascular tone and blood flow indirectly through negative effects on eNOS expression and activity (Shin et al., 2007; Takemoto et al., 2002; Wolfrum et al., 2004) or via direct effects on the central nervous system (Ito et al., 2003; Ito et al., 2004). Indeed, inhibition of ROCK leads to increase in cerebral blood flow and decrease in cerebral infarct size via upregulation of eNOS (Rikitake et al., 2005).

Inflammation is also a well-recognized component of the ischemic cascade. Leukocytes have been shown to migrate to and enter sites of acute infarction, and this inflammatory reaction likely contributes to the early progression of infarction. ROCK is involved in vascular inflammation (Noma et al., 2008) and remodeling (Kataoka et al., 2002), ischemia-reperfusion injury (Bao et al., 2004; Ikeda et al., 2003; Wolfrum et al., 2004), and atherosclerosis (Mallat et al., 2003; Miyata et al., 2000; Wang et al., 2008). The mechanism is due, in part, to the upregulation of the expression of a variety of genes, which are pertinent to vascular function and stroke, such as monocyte chemoattractant protein-1 (MCP-1) (Funakoshi et al., 2001) and plasminogen activator inhibitor-1 (PAI-1) (Takeda et al., 2001). Indeed, ROCK is upregulated by inflammatory stimuli, such as angiotensin II and interleukin-1 β (Hiroki et al., 2004).

Although inhibition of Rho/ROCK pathway leads to smaller cerebral infarct size in animal models of ischemic stroke, it is not known whether ROCK activity is elevated during the acute phase of ischemic stroke. Such information may be clinically useful in developing therapies for acute ischemic stroke. In this study, we tested the hypothesis that ROCK activity is elevated in patients in the days after ischemic stroke.

Results

The subjects in the control and stroke groups were well matched for sex, age, and number of stroke risk factors with no significant differences between the groups. The mean NIHSS score in patients with acute stroke was 15.4 (Table). Pre-incubation of P-MBS antibody with phosphopeptide antigen inhibited binding to human PMN extracts, while pre-incubation with non-phosphopeptide antigen and a saline control did not inhibit binding (Figure 1). Two control samples were excluded due to poor anti-tubulin staining of leukocyte samples. The 10 patients were compared to the 8 remaining control subjects. ROCK activity was significantly increased at 24 and 48 hours in patients after acute ischemic stroke when compared to control values with peak values at 48 hours after stroke onset (Figure 2). ROCK activity did not correlate with blood leukocyte counts on admission ($R^2=0.01$), consistent with normalization to α -tubulin content. Mean leukocyte ROCK activity levels measured as a ratio of phosphorylated to

unphosphorylated myosin-binding subunit were 1.55 ± 0.38 in control subjects. In the patients with acute stroke, the activity was slightly elevated compared to control subjects 24 hours after stroke onset (2.01 ± 0.50 , $p = 0.047$). ROCK activity rose further until 48 hours after stroke onset (2.60 ± 1.11 , $p = 0.021$), and then fell by 72 hours (2.18 ± 1.08 , $p = 0.193$) (Figure 2). ROCK activities did not correlate with stroke severity as assessed by NIHSS score ($R^2=0.02$).

Treatment of PMN cells from stroke and control subjects with the ROCK inhibitor fasudil ($1 \mu\text{M}$) caused about 20 - 30% inhibition of *in vitro* MBS phosphorylation (Figure 3). Fasudil inhibits MBS phosphorylation of PMN cells in a dose-dependent manner and $10 \mu\text{M}$ of fasudil almost completely inhibit it (data not shown).

Discussion

Peripheral leukocyte ROCK activity increased in patients in the days after acute ischemic stroke. The ROCK activity appears to peak between 24 and 72 hours after the onset of the stroke. These findings support the hypothesis that the activation of ROCK may play a role in the pathogenesis of acute ischemic stroke and suggest that therapies that can inhibit ROCK may be clinically useful in the treatment of acute ischemic stroke.

Peripheral leukocyte ROCK activation did not correlate with stroke size as measured by NIHSS scores in this study. ROCK activation is known to be nonspecific, occurring in many thrombotic, inflammatory, and malignant neoplastic disorders. Therefore, we eliminated patients with disorders, other than acute stroke, that would be expected to cause ROCK activation. NIHSS does not directly measure stroke size but depends also on the location of the infarcted tissue. The major limitation of this study is the lack of direct correlation with activation in CNS tissues at the site of the pathophysiologic events in stroke. In future studies we hope to look directly at the cell type localization and temporal profile of activation in the CNS in animals and stroke subjects.

Increasing evidence suggests that Rho/ROCK signaling has important vascular effects that are relevant to myocardial infarction and ischemic stroke, and many of these effects have been successfully blocked by pharmacological inhibition of ROCK. In a swine model, coronary vasospasm has been shown to correlate with myosin light chain phosphorylation, and that this phosphorylation and the associated vasospasm could be blocked by inhibitors of ROCK activation (Katsumata et al., 1997; Shimokawa et al., 1999). In humans, fasudil is metabolized to hydroxyfasudil, a potent inhibitor of ROCK. Intravenous fasudil and its active metabolite hydroxyfasudil have also been shown to reduce pacing-induced myocardial ischemia and to increase coronary blood flow in a canine model (Sato et al., 2001). Also in animal models of ischemic stroke, fasudil has been shown to decrease hyperviscosity induced by acute stroke, to prevent chemical-induced basilar and middle cerebral artery vasoconstriction, to reduce the accumulation of neutrophils in ischemic brain, and to inhibit the activation of NADPH oxidase and the generation of oxygen free radicals in activated neutrophils (Asano et al., 1991; Hitomi et al., 2000; Miyata et al., 2000; Sato et al., 2001). ROCK inhibition has also been shown to reduce infarct size and to improve neurological function in a rat model and to decrease delayed hippocampal neuronal degeneration in a gerbil model of ischemic stroke (Asano et al., 1991; Sato et al., 1999; Sato et al., 2001; Toshima et al., 2000). ROCK mediates the effects of many other vasoactive substances, such as endothelin-1, sphingosine 1-phosphate, and nitric oxide (Endres et al., 1998; Scherer et al., 2002; Tosaka et al., 2001). A recent report suggests that estrogen-induced inhibition of ROCK activation, but not expression, may contribute to the protection from vascular disease in pre-menopausal women (Chrissobolis et al., 2004). Rho/ROCK signaling inhibition by HMG-CoA reductase inhibitors (statins) offers a potential mechanism for some of the cholesterol-independent effects of these agents (Hernandez-Perera et al., 2000; Laufs and Liao, 2000). Finally, one small clinical trial has suggested a clinical

benefit of the ROCK inhibitor fasudil when given within 48 hours of stroke onset (Shibuya et al., 2005). Inhibition activity for ROCK by fasudil 1 μ M, as used in this study, would be expected to be close to inhibition by serum concentration of fasudil plus hydroxyfasudil in clinical use in Japan for patients with cerebral vasospasm following subarachnoid hemorrhage.

This study provides the first evidence of ROCK activation in patients with acute stroke. There is strong evidence for the importance of Rho signaling in the events leading to arterial occlusion in the obstruction to flow after re-canalization as well as in the cellular and molecular events of the ischemic cascade. Further studies are required to determine how the Rho/ROCK pathway and its interactions with other signaling pathways affect the components of the neurovascular unit such as endothelial, neuronal, and glial cells, that determines stroke outcomes. It is hoped that these insights will lead to the development of ROCK inhibitors, which might have therapeutic benefits in patients with acute ischemic stroke.

Experimental Procedure

Subjects

Patients with acute ischemic stroke were identified based on symptoms and signs of a sudden acute focal neurological deficit. Subjects were enrolled if they were ≥ 18 years old, symptom onset was within 24 hours, the NIHSS score was ≥ 4 , and they signed informed consent. Stroke patients were excluded from enrollment if they presented in coma or with a rapidly improving neurological deficit, if head CT or MRI demonstrated intracranial hemorrhage or a diagnosis other than ischemic stroke to explain the neurological deficit, or if they had had an ischemic stroke within the prior year. Stroke and control subjects were excluded if they had any of the following active medical conditions: inflammatory disease, infection, cancer, bleeding from the gastrointestinal or urogenital tracts or lungs or another site, a high risk of hemorrhage based on history of prior hemorrhage or bleeding diathesis, or hematocrit $< 30\%$. Based on clinical considerations, all patients had been excluded from acute thrombolytic therapies before enrollment. The control subjects were recruited from the Harvard Cooperative Program on Aging subject registry. Control subjects were matched for age, sex, and number of major stroke risk factors (hypertension, diabetes mellitus, hypercholesterolemia, smoking, coronary heart disease, peripheral arterial disease, and atrial fibrillation). The study protocol was approved by the Human Research Committee at Brigham and Women's Hospital.

Procedures

Ten patients with acute ischemic stroke presenting within 24 hours of symptom onset and with NIHSS scores ≥ 4 were enrolled. Ten case-matched control subjects were enrolled. NIHSS was performed on admission on stroke subjects and at the time of blood sampling in control subjects. Serial blood samples were taken from stroke subjects 24, 48, and 72 hours after stroke onset. Polymorphonuclear leukocytes (PMNs) from blood samples were isolated by density gradient centrifugation using Mono-Poly Resolving Medium (Dainippon Pharmaceutical Co). PMN cells were suspended in Hank's balanced salt solution (Gibco) and counted by a hemacytometer. The PMN cells were fixed in 10% trichloroacetic acid solution with 10 mM dithiothreitol. After centrifugation, the PMN cells were stored at -80°C until performing Western blot analysis.

Proteins in cell pellets were extracted by extraction buffer (8 M Urea, 2% sodium dodecyl sulfate, 5% sucrose, and 5% 2-mercaptoethanol). The cells extracts were subjected to SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with antibody to myosin-binding subunit (MBS), antibody to the phosphorylated form of myosin-binding subunit (P-MBS) or antibody to α -tubulin. The P-MBS antibody recognizes phosphorylated Thr(654) of MBS of myosin light chain phosphatase (MLCP), which is a selective target of

ROCK. Specificity of the P-MBS antibody was established by demonstrating the inhibition of binding to human PMN extracts after the antibody was pre-incubated with phosphopeptide antigen but not after pre-incubation with non-phosphopeptide antigen. Bands were visualized with the use of the ECL detection kit (Amersham). Quantification of each band was performed by densitometric analysis with the Imaging Densitometer (Bio Rad). MBS and P-MBS contents were normalized to α -tubulin content to standardize for differing leukocyte numbers, and ROCK activity was expressed as the ratio of P-MBS to MBS. The specificity of the ROCK assay was verified by *in vitro* treatment of samples with the ROCK inhibitor fasudil.

Materials

A polyclonal antibody against MBS phosphorylated at Thr654 (anti- P-MBS) was raised in New Zealand White rabbits, using a synthetic peptide corresponding to residues 648-660 (RQSRRSTQGVTLTC) of 130 kDa MBS containing phosphorylated Thr654 as the antigen. Anti-serum was purified through affinity columns. A polyclonal antibody against MBS (anti-MBS) was purchased from Covance. A monoclonal antibody against α -tubulin was purchased from Sigma. Synthetic peptides were purchased from Peptide Institute. Fasudil was from Asahi-Kasei Pharma.

Analysis

ROCK activities in these 10 patients were compared to baseline ROCK activities in 10 patients without acute illness and matched for sex, age, and number of vascular risk factors using a two-tailed Student's *t*-test. Correlation of ROCK activity to NIHSS and WBC counts was assessed by analysis of covariance.

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Figure 1. Characterization of anti-phosphorylated MBS antibody by immunoblot analysis
 (A) Extract of human PMN was separated on a SDS-gel, transferred to nitrocellulose paper, and then stained with anti-MBS antibody (lane1), anti-phosphorylated MBS (P-MBS) antibody (lane2) or Amido Black (lane3). (B) Before staining of human PMN extract with anti- P-MBS antibody, the antibody was preincubated with saline (lane1), 500 µg/ml of phosphopeptide antigen (residues 648-660 (RQSRRSTQGVTLTC) of 130 kDa MBS containing phosphorylated Thr654) (lane2), 500 µg/ml of non-phosphopeptide antigen (lane3).

Figure 2. ROCK Activity in PMNs from Acute Ischemic Stroke Patients

PMNs were isolated from stroke subjects 24, 48 and 72 h (hours) after stroke onset (Stroke) and control subjects matched age, sex and number of major stroke risk factors (Control). ROCK activity was determined by immunoblotting PMN lysates with antibodies to MBS and P-MBS. MBS and P-MBS contents were normalized to α -tubulin content. ROCK activity was expressed as the ratio of p-MBS to MBS. Data are mean $\pm$ S.D.(n=8-10). * $P < 0.05$, compared with control.

Figure 3. *in vitro* inhibition of MBS phosphorylation in PMNs by ROCK inhibitors

PMNs were isolated from stroke subjects (Stroke) 24 hours after stroke onset and control subjects (Control) matched age, sex and number of major stroke risk factors. PMN samples were incubated with saline or fasudil (1 μ M) for 5 min *in vitro* and fixed. ROCK activity was determined by immunoblotting PMN lysates with antibodies to MBS and p-MBS. MBS and p-MBS contents were normalized to α -tubulin content. ROCK activity was expressed as the ratio of p-MBS to MBS. Data are mean $\pm$ S.D.(n=8-10). * $P < 0.05$, ** $P < 0.01$, compared with each saline treatment.

Table

Baseline Characteristics

Characteristic	Control Group	Stroke Group	P-value
Female [No. (%)]	7 (88)	9(90)	NS
Age (mean)	67.6	66.2	0.72
No. of risk factors (mean)	2.3	2.4	0.90
NIHSS (mean)		15.8	--

NS = non-significant (Controls were matched by sex to cases.)