Activation of Rho-associated kinase during augmented contraction of the basilar artery to serotonin after subarachnoid hemorrhage

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Watanabe, Yoshimasa, Frank M. Faraci, and Donald D. Heistad. Activation of Rho-associated kinase during augmented contraction of the basilar artery to serotonin after subarachnoid hemorrhage. Am J Physiol Heart Circ Physiol 288: H2653–H2658, 2005. First published January 21, 2005; doi:10.1152/ajpheart.00923.2004.—Delayed cerebral vasospasm after subarachnoid hemorrhage (SAH) may be due, in part, to altered regulation of arterial smooth muscle contraction. Contraction of cerebral arteries to serotonin is augmented after experimental SAH. We hypothesized that activation of Rho-associated kinase (Rho kinase) contributes to augmented contraction of cerebral arteries to serotonin after SAH. Autologous arterial blood (SAH) or artificial cerebrospinal fluid (control) was injected into the cisterna magna of anesthetized rabbits. At 2 days after injection, the basilar artery was excised and isometric contraction of arterial rings was recorded. Maximum contraction of the basilar artery to serotonin was augmented about fourfold in SAH compared with control rabbits (P < 0.01). Contraction to histamine was similar in the two groups. Fasudil hydrochloride (3 μmol/l), an inhibitor of Rho kinase, markedly attenuated serotonin-induced contraction. Fasudil had little effect on contractions induced by histamine or phorbol 12,13-dibutyrate. In addition, phosphorylation of myosin phosphatase, a major target of Rho kinase in regulation of smooth muscle contraction, in the basilar artery was examined by Western blotting. In basilar arteries of SAH, but not control, rabbits, serotonin increased phosphorylation of myosin phosphatase about twofold at Thr853 of the myosin-targeting subunit. These results suggest that enhanced activation of Rho kinase contributes to augmented contraction of the basilar artery to serotonin after SAH.

myosin phosphatase; myosin-targeting regulatory subunit-1; phosphorylation; rabbits

Delayed cerebral vasospasm is a major complication after subarachnoid hemorrhage (SAH). Cerebral vasospasm may involve multiple and complex mechanisms, including altered regulation of vascular smooth muscle contraction (1, 29).

Agonist-induced contraction is enhanced in cerebral arteries after experimental SAH (2, 11, 14, 21, 34). In the basilar artery of rabbits, contraction to serotonin is greatly augmented after SAH (4, 18, 32, 34). It is of interest that serotonin-induced contraction is also enhanced in coronary vasospasm in pigs, and contraction in this setting is mediated by activation of Rho-associated kinase (Rho kinase) in smooth muscle (7).

Rho kinase is a key mediator of agonist-induced smooth muscle contraction (31). Myosin phosphatase is thought to be the primary target of Rho kinase in regulation of smooth muscle contraction. Rho kinase inhibits phosphatase activity of the catalytic subunit of myosin phosphatase by phosphorylat-

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brate for 45 min. Contractile responses were recorded during treatment with serotonin (1 × 10^-9–3 × 10^-6 mol/l) or histamine (1 × 10^-8–3 × 10^-5 mol/l). Contraction is expressed as a percentage of the response to 40 mmol/l KCl.

Some vessel rings were pretreated with fasudil hydrochloride (3 × 10^-6 mol/l) for 20 min, and responses to serotonin or histamine were recorded in the presence of fasudil. In preliminary experiments, we found that inhibitory effects on serotonin-induced contraction were nearly maximal at 3 × 10^-6 mol/l fasudil in SAH animals. Contraction is expressed as a percentage of the response to 40 mmol/l KCl recorded in the absence of fasudil, because contraction to 40 mmol/l KCl was reduced in the presence of fasudil (3 × 10^-6 mol/l). In preliminary experiments, inhibition of KCl-induced contraction by fasudil was similar in control and SAH animals (77.7 ± 7.1 and 82.3 ± 3.9%, n = 4). It has been reported that contraction of vascular smooth muscle induced by a high concentration of potassium is inhibited by Rho kinase inhibitors (15, 23).

We also examined effects of fasudil on contraction induced by phorbol 12,13-dibutyrate (PDBu), an activator of protein kinase C (PKC), because fasudil may inhibit PKC at higher concentrations (17). Contraction of basilar arteries of normal rabbits was recorded during treatment with PDBu (1 × 10^-9–10^-7 mol/l) in the presence or absence of fasudil (3 × 10^-6 mol/l). Arterial rings were pretreated with indomethacin (10^-6 mol/l for 1 h), because preliminary experiments indicated that contraction produced by PDBu was very small, presumably because of release of endothelial prostacyclin (3, 20) or other products of cyclooxygenase activity.

Phosphorylation of MYPT-1. Phosphorylation of MYPT-1 was examined by Western blotting, as described previously (7). In this study, we examined two different phosphorylation sites by Rho kinase, Thr696 and Thr853 (numbered on the basis of the human MYPT-1 sequence), which are reported to be phosphorylated during agonist-induced contraction of vascular smooth muscle (6, 9, 19, 26).

In organ baths, rings of the basilar artery from control or SAH rabbits were incubated for 3 min with serotonin (10^-6 mol/l) or vehicle. The vessel rings were quickly removed from the organ bath and frozen in dry ice-cold acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol. After they were washed three times with acetone containing 10 mM dithiothreitol, the vessel rings were dried and stored at -80 °C until they were used. Basilar arteries from two rabbits were pooled for one assay.

Stored vessel rings were cut into small pieces in sample lysis buffer (2% SDS, 10% glycerol, and 0.05 mol/l Tris-HCl, pH 6.8). After centrifugation of the sample at 12,000 g for 20 min, supernatant was collected and protein concentration was measured by Lowry's assay (Bio-Rad). Protein (30 μg) was electrophoresed in 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Gels were run in triplicate, and each membrane was probed with polyclonal antibodies against MYPT-1, MYPT-1 phosphorylated at Thr696, or MYPT-1 phosphorylated at Thr853. The immunoblot was incubated with chemiluminescent substrate (Femto Maximum Sensitivity, Pierce) and exposed to X-ray film. MYPT-1 and MYPT-1 phosphorylated at Thr696 or Thr853 were detected as single bands. Molecular weight of the detected proteins was ~130,000, which is consistent with that of MYPT-1. Density of bands was analyzed using NIH Image software. Data for density were normalized to the value for vehicle-treated vessel rings of the control group. The ratio of phosphorylated to total MYPT-1 was calculated for each phosphorylation site.

Drugs. Goat anti-MYPT-1 and phosphospecific antibodies against MYPT-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fasudil hydrochloride was provided by Asahi Kasei (Shizuoka, Japan).

Statistical analysis. Values are means ± SE. Two-way repeated-measures ANOVA was used for comparison of concentration-response curves among groups. Student's t-test was used for comparison of data from Western blot analysis. *P < 0.05 was accepted as statistically significant.

RESULTS

Experimental SAH. Experimental SAH was produced in 29 rabbits. In 21 of these rabbits, a subarachnoid clot covered the ventral surface of the brain stem. The other animals, which died immediately after the injection of blood (n = 2) or had no blood in the subarachnoid space on postmortem examination (n = 6), were excluded from further analyses. All control rabbits injected with artificial CSF into the cisterna magna tolerated the procedure (n = 21).

Effect of SAH on vasomotor function of the basilar artery. The magnitude of contraction induced by 40 mmol/l KCl was similar in arterial rings of control and SAH rabbits (1.28 ± 0.12 and 1.30 ± 0.08 g, respectively, n = 8). Histamine produced a dose-dependent contraction, which was similar in control and SAH rabbits (Fig. 1A). Contraction to serotonin was greatly augmented in SAH rabbits, with the maximum contraction increasing about fourfold compared with control animals (*P < 0.01; Fig. 1B).

Effect of fasudil on contraction. In control rabbits, there was a small but significant inhibitory effect of fasudil on contraction induced by histamine (Fig. 2A). In contrast, serotonin-

Fig. 1. Effects of subarachnoid hemorrhage (SAH) and artificial cerebrospinal fluid (control) on vasomotor function of the basilar artery in rabbits. A: contraction to histamine, expressed as percentage of response to 40 mmol/l KCl (n = 5). B: contraction to serotonin (n = 6). **P < 0.01 (2-way repeated-measures ANOVA).
induced contraction was virtually abolished in control rabbits (Fig. 2B). Fasudil did not inhibit contraction induced by the PKC activator PDBu (Fig. 3).

After SAH, the response to serotonin was profoundly inhibited by fasudil (Fig. 4). Fasudil tended to have minor inhibitory effects on contraction to histamine after SAH, but any effect was not statistically significant.

**Phosphorylation of MYPT-1.** Phosphorylation of MYPT-1 at Thr⁶⁹⁶ and Thr⁸⁵³ was detected by Western blotting using antibodies against phosphorylated MYPT-1 specific for the phosphorylation sites (Fig. 5A). Expression levels of MYPT-1 protein in the basilar artery were similar in SAH and control rabbits (Fig. 5B). There was no significant change in phosphorylation levels of MYPT-1 at Thr³⁶⁸ in control and SAH rabbits (Fig. 5C). In contrast, phosphorylation levels at Thr⁸⁵³ were increased to about twofold in SAH animals (*P < 0.05; Fig. 5D). There was no significant change in phosphorylation levels at Thr⁶⁹⁶ in control animals (Fig. 5D).

**DISCUSSION**

Major findings of this study are that augmented contraction to serotonin of the basilar artery after SAH was strongly inhibited by fasudil, a Rho kinase inhibitor, and that phosphorylation of MYPT-1 after serotonin was greater in SAH than in control animals. These results suggest that enhancement of activation of Rho kinase contributes to augmentation of contraction to serotonin in the basilar artery after SAH.

**Role of Rho kinase in contraction to serotonin.** The Rho kinase pathway is involved in contraction of normal cerebral arteries induced by serotonin (24). In this study, serotonin-induced contraction was abolished by fasudil in control rabbits, suggesting a major role of Rho kinase in serotonin-induced contraction in the basilar artery of rabbits. In addition, augmented contraction to serotonin after SAH was strongly inhibited by fasudil. In contrast to serotonin-induced contraction, histamine-induced contraction did not increase after SAH and was not inhibited by fasudil. Thus Rho kinase has a critical role in augmentation of contraction of the basilar artery to serotonin after SAH.

**Effect of SAH on Rho kinase.** In the basilar artery of dogs, activity of Rho kinase and phosphorylation of MYPT-1 (at Ser⁸⁵², which is another Rho kinase phosphorylation site) were elevated in the basal condition in SAH animals or after stimulation with serotonin in normal animals (24). In this study, basal activity of Rho kinase does not appear to be elevated after SAH, because baseline levels of phosphorylation of MYPT-1 were not increased after SAH. Contrasting findings may be explained by differences in species (dogs vs. rabbits), severity in vasospasm after SAH (more severe in dogs than in rabbits), phosphorylation sites (Ser⁸⁵² vs. Thr⁶⁹⁶ and Thr⁸⁵³), and experimental conditions (analysis immediately after excision of the vessels vs. after incubation in organ baths).

Our study focused on agonist-stimulated activation, not on basal activity, of Rho kinase in the basilar artery after SAH. We observed that phosphorylation of MYPT-1 induced by serotonin was enhanced in basilar arteries from SAH animals. A similar finding has been demonstrated in a model of coronary vasospasm (7). These findings support the hypothesis, based on effects of inhibitors of Rho kinase on vasomotor...
responses, that enhanced activation of Rho kinase contributes to augmented contraction to serotonin. 

Mechanisms of enhancement of Rho kinase activation. Mechanisms of augmentation of serotonin-induced, Rho kinase-mediated contraction are not clear. In cerebral arteries of rats after SAH, RhoA (a monomeric GTP-binding protein that activates Rho kinase) and Rho kinase are upregulated at the mRNA level (16). In contrast, Rho kinase protein is not increased in dogs after SAH (24). Upregulation of the serotonin 1B receptor on smooth muscle cells after SAH may contribute to enhanced activation of Rho kinase (2).

Endothelium-derived nitric oxide (NO) may inhibit the RhoA/Rho kinase pathway in smooth muscle (31). Thus, if NO-mediated signaling in the basilar artery is impaired after SAH, Rho kinase may be activated. There is evidence for impairment of the NO/cGMP pathway in the basilar artery after
SAH (30). Because serotonin may release NO from the endothelium in the basilar artery of normal rabbits (25), reduced bioavailability of endothelium-derived NO after SAH may contribute to enhanced activation of Rho kinase and augmented contraction induced by serotonin.

Contraction of cerebral arteries to other vasoconstrictors, including norepinephrine and PGE$_2$, is also augmented after SAH in rabbits (11, 34). Contraction of vascular smooth muscle induced by these agonists may involve activation of the RhoA/Rho kinase pathway (6, 22). Thus we speculate that subcellular mechanisms that enhance activation of Rho kinase may act as a common pathway for augmentation of contraction of cerebral arteries to various agonists after SAH.

Role of site-specific phosphorylation of MYPT-1. Inactivation of myosin phosphatase appears to be an important mechanism of Rho kinase-mediated regulation of smooth muscle contraction (31). Rho kinase phosphorylates MYPT-1 at Thr$^{696}$ and inhibits activity of the catalytic subunit of myosin phosphatase (5). In some vessels, phosphorylation at this inhibitory site is increased by stimulation with PGE$_2$, in parallel with force development, and Rho kinase inhibitors reduce phosphorylation and contraction induced by PGE$_2$ (6, 26). Some studies, however, reported increased phosphorylation at Thr$^{853}$ (in the human MYPT-1 sequence), another phosphorylation site by Rho kinase, but not at the inhibitory Thr$^{696}$ site, during contraction induced by histamine or endothelin-1 in the portal vein and femoral artery of the rabbit (9, 19).

In this study, we used phosphorylation site-specific antibodies to examine phosphorylation of MYPT-1 at these two sites by Western blotting. We found site-specific differences in phosphorylation of MYPT-1. In the basilar artery of control and SAH rabbits, we could not detect an increase in phosphorylation levels of MYPT-1 at Thr$^{696}$ after application of serotonin. We observed, however, that phosphorylation at Thr$^{853}$ was significantly increased after application of serotonin in the basilar artery of SAH animals. The mechanism of lack of an increase in phosphorylation at Thr$^{696}$ is not clear. We speculate that the difference in pattern of phosphorylation of MYPT-1 may be related to differences in blood vessels and/or vasoconstrictor agonists.

In many studies, an increase in phosphorylation of MYPT-1 is detected at the peak of contraction of vascular smooth muscle. In this study, contraction of the basilar artery reaches the maximum level ~3 min after application of serotonin. In rabbit aorta, an increase in phosphorylation at Thr$^{696}$ of MYPT-1 was observed after application of PGE$_2$ for 1 or 15 min (6). Thus we speculate that an increase in phosphorylation at Thr$^{696}$ would be detected after 3 min of serotonin application, if the changes in phosphorylation levels occur at this site. We cannot exclude, however, the possibility that a 3-min application was not optimal for detecting an increase in phosphorylation at Thr$^{696}$.

In previous studies, it was suggested that phosphorylation at Thr$^{853}$ during agonist-induced contraction is mediated by Rho kinase in vascular smooth muscle (9, 19). Thus it is likely that increased phosphorylation at Thr$^{853}$ indicates greater activation of Rho kinase during serotonin-induced contraction after SAH. The role of phosphorylation of MYPT-1 at Thr$^{853}$ in regulation of smooth muscle contraction is not clear. Our data are not sufficient to explain the mechanisms downstream from Rho kinase that mediate augmented contraction induced by serotonin after SAH. There is, however, evidence in vitro for increased dissociation of MYPT-1 from myosin after phosphorylation of MYPT-1 at Thr$^{853}$ (33). Thus enhancement of phosphorylation at Thr$^{853}$ may contribute to augmented contraction of the basilar artery to serotonin after SAH as an indirect inhibitory mechanism on myosin phosphatase activity.

Another possibility is that enhancement of contraction is mediated by a pathway independent of phosphorylation of MYPT-1, such as inhibition of myosin phosphatase activity by a smooth muscle phosphoprotein, CPI-17. CPI-17 may be a downstream mediator of Rho kinase in agonist-induced smooth muscle contraction in some vascular tissue (8, 19).

Specificity of the effect of fasudil. Fasudil, especially at high concentrations, may reduce agonist-induced contraction of smooth muscle by inhibiting activity of PKC (17). This mechanism, however, seems unlikely in this study, because PDButreated contraction of the rabbit basilar artery was not inhibited by fasudil (at the concentration that inhibited serotonin-induced contraction).

We have considered the possibility that fasudil may affect the endothelium during serotonin-induced contraction. The role of endothelial Rho kinase in regulation of smooth muscle contraction is not clear. Rho kinase downregulates activity of endothelial NO synthase (eNOS) (13). It is not clear, however, whether inhibition of Rho kinase increases eNOS activity and alters vascular reactivity. In addition, inhibition of Rho kinase may negatively regulate agonist-induced activation of eNOS, because the RhoA/Rho kinase pathway may contribute to agonist-induced elevation of intracellular Ca$^{2+}$ concentration in endothelial cells (37).

In conclusion, results of this study suggest that enhanced activation of Rho kinase may contribute to augmented contraction of the basilar artery to serotonin after SAH in rabbits. Increased phosphorylation of MYPT-1 at Thr$^{853}$ may indicate indirect inhibition of myosin phosphatase, which would be expected to augment contraction to serotonin.

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