Involvement of Rho-Kinase–Mediated Phosphorylation of Myosin Light Chain in Enhancement of Cerebral Vasospasm

Motohiko Sato, Eiichi Tani, Hirokazu Fujikawa, Kozo Kaibuchi

Abstract—Subarachnoid hemorrhage (SAH) often induces a long-term narrowing of the cerebral artery called cerebral vasospasm. Myosin light chain (MLC) in the spastic basilar artery was reported previously to be phosphorylated by Ca\(^{2+}\)/calmodulin-dependent MLC kinase. Because Rho-kinase, which is activated by the small GTPase Rho, phosphorylates not only MLC but also myosin phosphatase at its myosin-binding subunit (MBS), thus inactivating myosin phosphatase, we examined whether Rho-kinase is involved in the development of vasospasm. Cerebral vasospasm was produced in the canine basilar artery by a 2-hemorrhage method, and vasoconstrictions were induced by topical application of 80 mmol/L KCl or 0.5 μmol/L serotonin to the canine basilar artery exposed transclinically. The phosphorylation of MLC in the basilar artery was increased concurrently with an enhancement in the intensity of vasospasm with the passage of time after SAH. In addition, Rho-kinase in the basilar artery was activated concurrently with an increase in the phosphorylation of MBS at Ser854 in vasospasm. The Rho-kinase activation levels in vasospasm on days 0 and 2 were comparable to those in KCl- and serotonin-induced sustained vasoconstriction, respectively, and those in vasospasm on day 7 were markedly high. The topical application of Y-27632, a specific inhibitor of Rho-kinase, to the exposed spastic basilar artery on day 7 induced a dose-dependent dilation, and the intensities of vasospasm and the phosphorylation of MBS and MLC were simultaneously decreased by 10 μmol/L Y-27632, although the decrease in MBS phosphorylation was more marked than the decrease in MLC phosphorylation. These results indicate that the activation of Rho-kinase and the phosphorylation of MLC and MBS occur concomitantly during vasospasm induced by SAH and suggest that Rho-kinase is involved in the enhancement of cerebral vasospasm in addition to Ca\(^{2+}\)/calmodulin-dependent MLC kinase by increasing the phosphorylation of MLC directly or indirectly as a result of the inhibition of myosin phosphatase by its phosphorylation. (Circ Res. 2000;87:195-200.)

Key Words: vasospasm ■ Rho-kinase ■ myosin light chain ■ phosphorylation ■ dogs

The management of patients with acutely ruptured intracranial aneurysms should achieve 2 major goals: (1) prevention of subsequent bleeding and (2) prevention and treatment of cerebral vasospasm, which usually develops between 4 and 14 days after subarachnoid hemorrhage (SAH). Despite the improvements in management of aneurysmal SAH, vasospasm remains an important cause of the morbidity and mortality accompanying this disease.\(^1\) Recently, it has been reported that μ-calpain, but not m-calpain, in the canine basilar artery is continuously activated in vasospasm,\(^2,3\) indicating a continuous rise of intracellular Ca\(^{2+}\) levels, because μ- and m-calpains are activated at 1 μmol/L and 1 mmol/L Ca\(^{2+}\) levels, respectively.\(^4\) Myosin light chain (MLC) in the spastic canine basilar artery has been shown to be phosphorylated by Ca\(^{2+}\)/calmodulin (CaM)-dependent MLC kinase,\(^5,6\) and the phosphorylation of MLC in the spastic anterior spinal artery is increased.\(^7\) In addition, the phosphatase activity toward myosin in the basilar artery is decreased in vasospasm.\(^8\) Because the use of a Ca\(^{2+}\) indicator in experiments of smooth muscle contraction revealed that the force/Ca\(^{2+}\) ratio in smooth muscle contraction is variable, the Ca\(^{2+}\)/CaM-dependent MLC kinase pathway cannot solely account for the mechanisms of agonist-induced or GTP-γ-S–induced increase in the force/Ca\(^{2+}\) ratio, so-called Ca\(^{2+}\) sensitization.\(^9–14\) Therefore, an additional mechanism that can regulate Ca\(^{2+}\) sensitization of vascular smooth muscle has been considered. With the use of membrane permeabilization of smooth muscle, the possibility that monomeric Ras family G proteins, such as Rho, contribute to Ca\(^{2+}\) sensitization of smooth muscle was demonstrated.\(^13,15,16\) In permeabilized smooth muscle cells, GTP-γ-S induces MLC phosphorylation at submaximal Ca\(^{2+}\) concentration by inhibiting the dephosphorylation of MLC,\(^10,13\) presumably by activating Rho.\(^17\) Recently, Rho-kinase, which is activated by Rho,\(^16,18,19\) has been reported to phosphorylate not only MLC stoichiometrically at the site that is phosphorylated by Ca\(^{2+}\)/CaM-dependent MLC kinase\(^20\) but also myosin phosphatase at its myosin-binding subunit (MBS), thus inactivating it in vitro.\(^21\)

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These findings in a cell-free system, plus the reports of G-protein–mediated Ca\(^{2+}\) sensitization of smooth muscle contraction,\(^{13,14,22,23}\) suggest that Rho-kinase may induce contraction and concomitant MLC phosphorylation of the smooth muscle.\(^{24,25}\) In the present study, we examine whether Rho-kinase is involved in the development of cerebral vasospasm in the presence of elevated intracellular Ca\(^{2+}\) levels.

**Materials and Methods**

Cerebral vasospasm was produced in dogs by an injection of 5 mL fresh autogenous arterial blood into the cisterna magna, followed by another injection 2 days later. Vasocontractions were induced by a topical application of 80 mmol/L KCl and 0.5 \(\mu\)mol/L serotonin for 15 minutes (KCl-15 and serotonin-15 subgroups, respectively) or 90 minutes (KCl-90 and serotonin-90 subgroups, respectively) to the canine basilar artery exposed via a translavical route. Vasospasm and vasocontraction of the basilar artery were confirmed by angiography or by use of a surgical microscope. The animals were euthanized after a perfusion with 500 mL physiological salt solution, and the basilar artery was removed together with the brain. In the vasospasm group, the blood clot around the basilar artery and its branches was carefully removed without any mechanical stimulation given to the arteries. After it was washed briefly with physiological salt solution, the basilar artery was quickly frozen in liquid nitrogen until used.

The phosphorylations of MLC and MBS in myosin phosphatase and the activity of Rho-kinase were studied. The phosphorylation of MLC was examined by urea-glycerol gel electrophoresis and immunoblot analysis with anti-MLC antibody, and phosphorylated MLC was expressed as a percentage of total MLC (ie, unphosphorylated MLC plus phosphorylated MLC). Rho-kinase activity was examined by Rho-kinase assay and immunoblot analysis with anti–glutathione-S-transferase-bovine Rho-kinase antibody and calculated by dividing the densimetric values of Rho-kinase activity by those of the immunoreactive Rho-kinase level. The phosphorylation of MBS was probed separately with anti-pS854 antibody, which was prepared against the synthetic phosphopeptide pS854 (CREKRR phosphoS\(^854\)TGVSF) as an antigen, and with anti–glutathione-S-transferase-MBS-COOH terminal antibody and calculated by dividing the densimetric values of the phosphorylation levels of MBS by those of the immunoreactive levels of MBS. Finally, after the basilar artery in vasospasm on day 7 was exposed transclivally, it was treated by a topical 30-minute application of successively increasing concentrations of Y-27632, a specific Rho-kinase inhibitor. At the end of experiment, 10 mmol/L EGTA was added, and the reduced diameter of the spastic basilar artery was expressed as a percentage of the relaxation induced by EGTA.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

![Figure 1](http://circres.ahajournals.org/article-figures/196-fig1.png)

**Figure 1.** Bar graph shows mean percentage of the caliber of canine basilar artery on angiography in vasospasm on day 0 (D-0), day 2 (D-2), and day 7 (D-7), which was expressed as a percentage of the control caliber (C). Vertical lines indicate SEM (n=5 per group).

![Figure 2](http://circres.ahajournals.org/article-figures/196-fig2.png)

**Figure 2.** Line graph shows the time course of the mean percentage of the caliber of the canine basilar artery in 5 dogs in the KCl and serotonin subgroups. Vertical bars indicate SEM. The vasoconstriction in both the KCl and serotonin subgroups increased over a period of 45 minutes after treatment and was sustained thereafter.

![Figure 3A](http://circres.ahajournals.org/article-figures/196-fig3a.png)

**Figure 3A.** Phosphorylation of MLC measured by immunoblotting after glyceral-urea polyacrylamide gel electrophoresis shows nonphosphorylated (MLC-P0) and monophosphorylated (MLC-P1) forms of the 20-kDa MLC in the basilar artery and immunoreactive MLC-P1 increases in density in vasospasm on D-0, D-2, and D-7 (n=5 in each group).

![Figure 3B](http://circres.ahajournals.org/article-figures/196-fig3b.png)

**Figure 3B.** Densitometric quantification of MLC phosphorylation in vasospasm was standardized as the percentage of the total amount of immunostained MLC (MLC-P0 plus MLC-P1).
Results

Caliber of the Basilar Artery
No significant angiographic narrowing of the basilar artery was shown before the animals in the control group were killed. The percentage of caliber reduction, as shown in Figure 1, indicates the occurrence of vasospasm and the increase in its intensity with the lapse of time after SAH. The mean percent calibers in the KCl and serotonin groups decreased over a period of ~45 minutes after treatment and then stabilized, as shown in Figure 2.

Phosphorylation of MLC and Activation of Rho-Kinase Associated With Phosphorylation of MBS
Previously, we confirmed that MLC in the spastic basilar artery was phosphorylated by Ca\(^2+\)/CaM-dependent MLC kinase but not by protein kinase C.\(^5,6\) Therefore, we examined by glycerol-urea gel electrophoresis and subsequent immunoblot analysis whether the phosphorylation of MLC was increased in vasospasm. As shown in Figure 3, the mono-phosphorylation of MLC was increased with the passage of time in vasospasm. In addition, the Rho-kinase assay demonstrated a progressive increase in Rho-kinase activity in the basilar artery in vasospasm on days 0, 2, and 7, as shown in Figure 4A, but immunoreactive Rho-kinase levels were not changed (Figure 4B), demonstrating a significant increase in Rho-kinase activity in vasospasm with the lapse of time after SAH (Figure 4E). The Rho-kinase activities were increased slightly in the KCl-15 subgroup, moderately in the KCl-90 and serotonin-15 subgroups, and markedly in the serotonin-90 subgroup, as shown in Figure 4F. Thus, the Rho-kinase activities in vasospasm on days 0 and 2 were comparable to those in the KCl-90 or serotonin-15 subgroup and in the serotonin-90 subgroup, respectively. One of the major sites of phosphorylation of MBS by Rho-kinase has been identified as Ser854, and the antibody that specifically recognizes MBS phosphorylated at Ser854 has been developed.\(^26\) When MBS phosphorylation of myosin phosphatase was immunologically examined with use of an anti-pS854 antibody, the phosphorylation of MBS was shown to increase in vasospasm on days 0 and 2 and particularly on day 7 (Figure 4C), without any significant changes in the immunoreactive MBS level (Figure 4D), demonstrating a significant increase in MBS phosphorylation in vasospasm, particularly on day 7 (Figure 4E).

Dilation of Spastic Artery by Y-27632
Representative angiograms of the basilar artery in the spastic group on day 7 are shown in Figure 5. The mean ± SEM bars) and MBS phosphorylation (solid bars) in vasospasm on D-0, D-2, and D-7. MBS phosphorylation levels in vasospasm on D-7 were decreased markedly by a topical application of 10 \(\mu\)mol/L Y-27632 (Y) (C and E). The bar graph in panel F shows mean values of Rho-kinase activity in KCl and serotonin groups, demonstrating a slight increase in the KCl-15 subgroup, a moderate increase in the KCl-90 and serotonin-15 (S-15) subgroups, and a marked increase in the serotonin-90 (S-90) subgroup. The mean values of Rho-kinase activity and MBS phosphorylation were calculated by dividing the densitometric values of Rho-kinase activity and MBS phosphorylation levels by those of immunoreactive Rho-kinase and MBS levels, respectively.
diameter of the spastic basilar arteries on day 7 was reduced to 56 ± 3% of the control caliber (Figure 1). To define the involvement of Rho-kinase in the development of vasospasm, Y-27632, a specific inhibitor of Rho-kinase, was topically applied to the exposed spastic basilar artery on day 7 to examine the responses of caliber as well as the phosphorylation of MBS and of MLC in the spastic basilar artery. Before the topical application of Y-27632, the blood clot around the exposed spastic basilar artery was carefully removed to avoid inducing significant changes in the caliber of the spastic artery. The exposed spastic basilar artery was dilated dose-dependently by a topical application of Y-27632 at doses of 3 × 10⁻³ mol/L (d), 1 × 10⁻⁴ mol/L (e), 3 × 10⁻⁶ mol/L (f), 1 × 10⁻⁷ mol/L (g), 3 × 10⁻⁸ mol/L (h), and 1 × 10⁻⁹ mol/L (i) and of 10 mmol/L EGTA (j).

### Table: Response of Exposed Basilar Artery to Topical Application of Y-27632 on Day 7

<table>
<thead>
<tr>
<th>Relaxation After Y-27632, %</th>
<th>0.01 μmol/L</th>
<th>0.3 μmol/L</th>
<th>1 μmol/L</th>
<th>3 μmol/L</th>
<th>10 μmol/L</th>
<th>30 μmol/L</th>
<th>100 μmol/L</th>
<th>ED₅₀, μmol/L</th>
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<tbody>
<tr>
<td>Dog 1</td>
<td>13.1</td>
<td>30.9</td>
<td>41.6</td>
<td>55.6</td>
<td>72.4</td>
<td>80.6</td>
<td>88.1</td>
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<tr>
<td>Dog 2</td>
<td>10.6</td>
<td>23.8</td>
<td>35.3</td>
<td>51.2</td>
<td>67.3</td>
<td>76.9</td>
<td>85.8</td>
<td>2.39</td>
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<tr>
<td>Dog 3</td>
<td>12.7</td>
<td>29.4</td>
<td>38.3</td>
<td>52.1</td>
<td>69.5</td>
<td>76.5</td>
<td>87.3</td>
<td>2.25</td>
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<tr>
<td>Mean ED₅₀</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.25 ± 0.28</td>
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</tr>
</tbody>
</table>

### Figure 5. Representative vertebral angiograms of a canine basilar artery before (a) and 7 days after (b) blood injection. The exposed spastic basilar artery (c) is dilated in a dose-dependent manner by topical application of Y-27632 at doses of 3 × 10⁻³ mol/L (d), 1 × 10⁻⁴ mol/L (e), 3 × 10⁻⁶ mol/L (f), 1 × 10⁻⁷ mol/L (g), 3 × 10⁻⁸ mol/L (h), and 1 × 10⁻⁹ mol/L (i) and of 10 mmol/L EGTA (j).

### Figure 6. Topical application of 10 μmol/L Y-27632 (Y) and 10 mmol/L EGTA to the basilar artery in vasospasm on D-7 decreases the density of MLC-P1 (n=3 in each group). A, Phosphorylation of MLC is measured by immunoblotting after glycerol-urea polyacrylamide gel electrophoresis. B, Densitometric quantification of MLC phosphorylation levels after the topical application of Y-27632 and EGTA to the spastic basilar artery on D-7 is standardized as the percentage of the total amount of immunostained MLC (MLC-P0 plus MLC-P1).

### Discussion

The phosphorylation of MLC in the basilar artery examined by glycerol-urea gel electrophoresis and subsequent immunoblot analysis demonstrated an increase in vasospasm with the passage of time after SAH. At the same time, Rho-kinase was activated, and the phosphorylation of MBS was elevated in the basilar artery in vasospasm. Recently, Kimura et al²¹ have shown that activated Rho-kinase phosphorylates MBS of myosin phosphatase, thereby leading to a decrease in myosin phosphatase activity in vitro. The decrease in activity of myosin phosphatase in the spastic basilar artery reported previously⁸ is consistent with the present increase in MBS phosphorylation levels. It has been shown that MBS is phosphorylated and that myosin phosphatase activity is inactivated during the action of thromboxane A₂ in platelets, and both reactions are blocked by prior treatment of platelets with Clostridium botulinum C3 toxin, which interferes with Rho.
functions. Similar observations have been made in the endothelial cells responding to thrombin. In addition, the activated Rho-kinase stoichiometrically phosphorylates MLC at the Ser19 residue, which is the site phosphorylated by Ca\(^{2+}\)/CaM-dependent MLC kinase. Thus, the activated Rho-kinase in vasospasm appears to induce MLC phosphorylation by the direct phosphorylation and/or inactivation of myosin phosphatase. It may be noted that the direct phosphorylation of MLC by Rho-kinase in smooth muscle remains to be elucidated, because GTP-γ-S induces minimal MLC phosphorylation in permeabilized smooth muscle in the presence of EGTA. Because the level of MBS phosphorylation in vasospasm on day 7 is much higher than that on day 2, Rho-kinase may play more critical roles at the later stage in vasospasm.

The topical application of 10 μmol/L Y-27632 to the spastic basilar artery on day 7 induced a concurrent decrease in contraction and phosphorylation of MBS and MLC. Y-27632 is a specific inhibitor of Rho-kinase and selectively inhibits agonist (including serotonin)–induced smooth muscle contraction by inhibiting Ca\(^{2+}\) sensitization (K, 0.14 for Rho-kinase, K, 26 for protein kinase C, and K, >250 for MLC kinase). The concentration of Y-27632 used in the present study inhibits GTP-γ-S–induced contraction of permeabilized strips of rabbit mesentery artery by ≈80% and phenylephrine-induced contraction of rabbit aortic strips by ≈90% but has little effect on Ca\(^{2+}\)–induced and calyculin A–induced contraction of permeabilized strips of rabbit mesentery artery. Rho-kinase inhibition by Y-27632 in vasospasm has demonstrated that MBS phosphorylation is more decreased than is MLC phosphorylation and that the MLC phosphorylation induced by Ca\(^{2+}\)/CaM-dependent MLC kinase in vasospasm because of the continuous elevation of intracellular Ca\(^{2+}\) levels may not be affected by Y-27632. Therefore, the Y-27632–specific MLC phosphorylation in vasospasm may be mediated by Rho-kinase mainly through the inhibition of myosin phosphatase. Thus, although the intracellular Ca\(^{2+}\) level is elevated in vasospasm, the activation of Rho-kinase associated with the elevation of MBS phosphorylation and the Y-27632–sensitive MLC phosphorylation in the spastic basilar artery suggests the involvement of Rho-kinase in the enhancement of vasospasm, namely, a Ca\(^{2+}\) sensitization mechanism.

The present study (together with the previous studies) indicates that the Rho–Rho-kinase pathway is activated during the development of cerebral vasospasm. How is the Rho–Rho-kinase pathway activated? Several recent studies imply that some trimeric G-protein–coupled receptors, including lysophosphatidic acid, thrombin, and serotonin receptors, are linked to the Rho–Rho-kinase pathway. The α subunits from G, G, G, and G are postulated to activate Rho by regulating GDP/GTP exchange factors (GEFs) for Rho. For G, a direct interaction with a specific Rho-GEF, p115-RhoGEF, is known to enhance exchange activity and Rho-GTP binding. Although the mechanism by which SAH activates the Rho–Rho-kinase pathway remains to be clarified, it is tempting to speculate that certain ligands for the G-protein–coupled receptors, which are produced after SAH, activate Rho (and subsequently Rho-kinase) and enhance cerebral vasospasm. The activation of the tyrosine kinase pathway in vasospasm suggests an involvement of G-protein–coupled receptors in addition to receptor protein tyrosine kinase. The monophosphorylation of MLC in the spastic basilar artery on day 7 was almost completely inhibited by the topical application of 10 mmol/L EGTA. Because the concentration of EGTA used in the present study induces cytosolic Ca\(^{2+}\) levels to nominally zero, Ca\(^{2+}\)/CaM-dependent MLC kinase in the spastic basilar artery on day 7 could be almost completely inactivated when EGTA was applied topically. In contrast, because Rho-kinase is theoretically insensitive to 10 mmol/L EGTA, Rho-kinase in the spastic basilar artery on day 7, after topical application of EGTA, would be still capable of phosphorylating not only MLC but also myosin phosphatase, thus inactivating it. Therefore, the nominally zero phosphorylation of MLC in the spastic basilar artery on day 7 induced by the topical application of EGTA would be mediated mainly by the complete inhibition of Ca\(^{2+}\)/CaM-dependent MLC kinase, although we could not rule out the possibility that Rho-kinase activity is inhibited under the conditions.

The intracellular Ca\(^{2+}\) levels in the canine basilar artery were transiently elevated only at the beginning of serotonin-induced contraction. Therefore, serotonin-induced contraction is mainly dependent on the activation of Rho-kinase, according to the evidence of marked relaxation of serotonin-induced smooth muscle contraction by Y-27632. In contrast, the intracellular Ca\(^{2+}\) levels in the spastic basilar artery are continuously increased according to the evidence of calpain activation, and the MLC phosphorylation in the spastic basilar artery is reported to be mediated by Ca\(^{2+}\)/CaM-dependent MLC kinase. However, the maximum response of the spastic basilar artery to ML-9, a selective inhibitor of Ca\(^{2+}\)/CaM-dependent MLC kinase, was less than that to EGTA, suggesting that mechanisms other than the participation of Ca\(^{2+}\)/CaM-dependent MLC kinase (eg, involvement of Rho-kinase) operate in vasospasm. Therefore, the present study suggests that Rho-kinase works synergistically with Ca\(^{2+}\)/CaM-dependent MLC kinase in vasospasm and that the vascular smooth muscle cells need such a 2-pronged approach on MLC phosphorylation to sustain the uninterrupted increase in cellular contractility characteristic of vasospasm. It remains to be determined to what extent Rho-kinase and Ca\(^{2+}\)/CaM-dependent MLC kinase are involved in the development of vasospasm and to detect other mechanisms responsible for the development of vasospasm. However, from the therapeutic point of view, it is noted that the topical application of EGTA to the spastic basilar artery demonstrates the maximal relaxation compared with other reagents, such as inhibitors of Rho-kinase or Ca\(^{2+}\)/CaM-dependent MLC kinase.

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