Inhibition of Myosin Phosphatase by Upregulated Rho-Kinase Plays a Key Role for Coronary Artery Spasm in a Porcine Model With Interleukin-1β

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Background—We recently demonstrated that the Rho-kinase–mediated pathway plays an important role for coronary artery spasm in our porcine model with interleukin-1β (IL-1β). In this study, we examined whether or not Rho-kinase is upregulated at the spastic site and if so, how it induces vascular smooth muscle hypercontraction.

Methods and Results—Segments of the left porcine coronary artery were chronically treated from the adventitia with IL-1β–bound microbeads. Two weeks after the operation, as reported previously, intracoronary serotonin repeatedly induced coronary hypercontractions at the IL-1β–treated site both in vivo and in vitro, which were markedly inhibited by Y-27632, one of the specific inhibitors of Rho-kinase. Reverse transcription–polymerase chain reaction analysis demonstrated that the expression of Rho-kinase mRNA was significantly increased in the spastic compared with the control segment. Western blot analysis showed that during the serotonin-induced contractions, the extent of phosphorylation of the myosin-binding subunit of myosin phosphatase (MBS), one of the major substrates of Rho-kinase, was significantly greater in the spastic than in the control segment and that the increase in MBS phosphorylations was also markedly inhibited by Y-27632. There was a highly significant correlation between the extent of MBS phosphorylations and that of contractions.

Conclusions—These results indicate that Rho-kinase is upregulated at the spastic site and plays a key role in inducing vascular smooth muscle hypercontraction by inhibiting myosin phosphatase through the phosphorylation of MBS in our porcine model. (Circulation. 2000;101:1319-1323.)

Key Words: vasospasm muscle, smooth kinase myosin phosphorylation

The clinical importance of coronary artery spasm in the pathogenesis of ischemic heart disease is now widely accepted.1–4 However, the intracellular mechanism for the spasm still remains to be elucidated. We previously developed a porcine model of coronary spasm in which the spasm was repeatedly induced by serotonin or histamine at the atherosclerotic lesions made by a combination of endothelial injury and high-cholesterol feeding.5–7 We subsequently developed a porcine model in which long-term adventitial treatment with interleukin-1β (IL-1β), one of the major inflammatory cytokines, induces arteriosclerotic changes and vasospastic responses of the coronary artery.8–13 Because the histological changes and vasospastic responses in our porcine models are similar to those observed in humans,14,15 our models may be useful to examine the molecular mechanism of the spasm in humans.6,8,9

Phosphorylation of myosin light chain (MLC) is one of the most important steps for vascular smooth muscle contraction.16,17 The classic concept of the mechanism of vascular smooth muscle contraction includes an activation of MLC kinase (MLCK) that leads to the phosphorylation of MLC and subsequent smooth muscle contraction.18 However, because the intracellular Ca²⁺ concentrations were not always proportional to the levels of MLC phosphorylation and smooth muscle contraction, an additional mechanism to regulate Ca²⁺ sensitivity has been proposed.19 Recently, evidence for the involvement of the small GTPase Rho in Ca²⁺ sensitivity in smooth muscle contraction was reported from several laboratories.20–22 The molecular mechanism of MLC phosphorylation regulated by Rho was largely unknown, but recent analyses revealed that Rho regulates MLC phosphorylation through its...
target protein, Rho-kinase, and the myosin-binding subunit (MBS) of MLC phosphatase (MLCPh).23,24 Indeed, studies in vitro suggested that Rho activates Rho-kinase, which then phosphorylates MBS and results in the inhibition of MLCPh.23 We have recently demonstrated in our porcine model with IL-1β that MLC phosphorylations (on stimulation by serotonin) are enhanced at the spastic site12,13 and that hydroxyfasudil, a specific Rho-kinase inhibitor, exerts an inhibitory effect on the spasm both in vivo and in vitro.13 However, the molecular mechanism for the spasm in our model remains to be elucidated.

This study was thus designed to examine whether or not Rho-kinase is upregulated at the spastic site and if so, how it induces vascular smooth muscle hypercontraction.

Results

In Vivo Study
Two weeks after the operation, serotonin 10 μg/kg IC repeatedly caused hyperconstriction at the IL-1β–treated site in vivo (Figures 1 and 2). Pretreatment with Y-27632 did not significantly change the baseline heart rate or blood pressure (data not shown). Y-27632 dose-dependently inhibited serotonin-induced coronary hyperconstriction at the IL-1β–treated site in vivo, whereas at the control site, its inhibitory effect on serotonin-induced constriction was not evident (Figures 1 and 2).

Organ Chamber Experiment
Serotonin 1 μmol/L induced a contraction in the IL-1β–treated and control coronary segments without endothelium, which rapidly developed and reached a maximum after 5 to 8 minutes. Serotonin caused hypercontractions in the IL-1β–treated segments compared with the control segments, which were markedly inhibited by Y-27632 (Figure 3).

RT-PCR Analysis
The expected sizes of the bands for Rho-kinase were detected in both the spastic and control coronary segments. However, the density of PCR products from Rho-kinase mRNA (nor-
malized to that from β-actin mRNA) was significantly higher in the spastic than in the control segment (Figure 4).

**MBS Phosphorylations**

The extent of MBS phosphorylation was measured when the serotonin-induced contraction of each ring (without endothelium) reached a maximum. Western blot analysis showed that on stimulation by serotonin, MBS phosphorylation was significantly increased in the IL-1β-treated coronary segment compared with the control segment (Figure 5). In the spastic coronary segments, the enhanced MBS phosphorylation was markedly inhibited by Y-27632 to levels under control conditions (Figure 5). Importantly, there was a highly significant positive correlation between the extent of MBS phosphorylations and that of serotonin-induced contractions (Figure 6).

**Discussion**

The novel findings of the present study were that (1) the expression of Rho-kinase was upregulated at the spastic coronary segment, (2) coronary spasm was associated with an enhanced MBS phosphorylation that should have resulted in the inhibition of MLCPh, and (3) Rho-kinase mediated this MBS phosphorylation, resulting in the occurrence of smooth muscle hypercontraction. Thus, the present study clearly demonstrates that enhanced inhibition of MLCPh by upregulated Rho-kinase plays a key role in the molecular mechanisms of coronary spasm in our porcine model (Figure 7).

**Inhibition of MLCPh Through MBS Phosphorylation in the Spastic Coronary Artery**

We recently reported that coronary artery spasm is associated with an enhanced and sustained MLC monophosphorylation and the appearance of MLC diphosphorylation.12,13 The level of MLC phosphorylation is determined by a balance between MLC phosphorylation by MLCK and dephosphorylation by MLCPh.17,25 Seto et al previously suggested that the generation of diphosphorylated MLC may be caused in part by inhibition of MLCPh in smooth muscle cells. They also showed that treatment with calyculin A, an inhibitor of phosphatases, including MLCPh,27,28 potently induced MLC diphosphorylation in smooth muscle cells without an increase...
in intracellular Ca\(^{2+}\) levels.\(^{26}\) Furthermore, we recently found that direct increase in the intracellular Ca\(^{2+}\) levels by calcium ionophore does not result in an increase in diphosphorylated MLC (unpublished data). These lines of evidence suggest that inhibition of MLCPh activity is essential for the occurrence of coronary artery spasm.

In the present study, MBS phosphorylation at the IL-1\(\beta\)–treated site in response to serotonin was significantly increased, suggesting that MLCPh activity is significantly suppressed in the spastic compared with the control segment, resulting in an increase in MLC phosphorylations on stimulation by serotonin (Figure 7). In contrast, the extent of the MBS phosphorylations under control conditions was comparable between the spastic and the control segments, which was consistent with our previous findings that the extents of MLC monophosphorylation under control conditions were comparable between the 2 sites.\(^{12,13}\)

**Enhanced MBS Phosphorylation Caused by Upregulated Rho-Kinase**

In the present study, we demonstrated that the expression of Rho-kinase mRNA was significantly upregulated in the spastic compared with the control segment. Smooth muscle MLCPh consists of a 38-kDa catalytic subunit, the 130-kDa MBS, and a 21-kDa subunit.\(^{29,30}\) MBS serves as a targeting subunit of MLCPh to myosin and enhances the activity of the enzyme toward myosin.\(^{29}\) Recently, we reported that Rho-kinase phosphorylates MBS and reduces the MLCPh activity in vitro.\(^{23}\) When an activated mutant of Rho was expressed in NIH3T3 fibroblasts, the extent of MLC phosphorylation was increased together with an increase in MBS phosphorylation.\(^{23}\) Taken together, these findings suggest that MBS phosphorylation is mediated by Rho-kinase, resulting in inhibition of MLCPh and a subsequent increase in MLC phosphorylations (Figure 7).

Indeed, in the present study, the hypercontractions to serotonin were dose-dependently inhibited by Y-27632, one of the specific inhibitors of Rho-kinase both in vivo and in vitro.\(^{31}\) Furthermore, there was a highly significant positive correlation between the extent of MBS phosphorylations and that of the serotonin-induced contractions. We also recently demonstrated that hydroxyfasudil, another specific inhibitor of Rho-kinase, also dose-dependently inhibited the hypercontractions to serotonin both in vivo and in vitro.\(^{13}\) Thus, it is highly possible that in our porcine model, upregulated Rho-kinase inhibits MLCPh through MBS phosphorylation, resulting in the occurrence of coronary artery spasm (Figure 7).

We have previously shown in the present model that the coronary constriction in response to prostaglandin \(\text{E}_2\) is resistant to the blockade of protein kinase \(\text{C}\) (PKC) and is not augmented at the IL-1\(\beta\)–treated site, whereas that to serotonin or histamine is sensitive to the blockade of PKC and is augmented at the IL-1\(\beta\)–treated site.\(^{10}\) Thus, the PKC-mediated pathway for vascular smooth muscle contraction is apparently involved in the molecular mechanism for coronary artery spasm in our model, although the relationship between PKC and Rho-kinase remains to be elucidated (Figure 7).

Although Rho-kinase is one of the major regulators of vascular smooth muscle contraction,\(^{13,22,23,32}\) other regulators, including the C-kinase–potentiated inhibitor of myosin phosphatase (CPI 17),\(^{33,34}\) arachidonic acid,\(^{35}\) might also be involved in smooth muscle hypercontraction (Figure 7). The possible involvement of those mechanisms in the pathogenesis of coronary spasm remains to be examined.

In summary, we were able to demonstrate that Rho-kinase is upregulated at the spastic site and mediates coronary spasm by inhibiting MLCPh through its MBS phosphorylation. The detailed molecular mechanism(s) for the upregulation of Rho-kinase in the inflammatory/arteriosclerotic coronary segment remains to be examined in a future study.

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**References**


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