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. 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. 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. Because the histological changes and vasospastic responses in our porcine models are similar to those observed in humans, our models may be useful to examine the molecular mechanism of the spasm in humans.

Phosphorylation of myosin light chain (MLC) is one of the most important steps for vascular smooth muscle contraction. 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. 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. Recently, evidence for the involvement of the small GTPase Rho in Ca2+ sensitivity in smooth muscle contraction was reported from several laboratories. 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). Indeed, studies in vitro suggested that Rho activates Rho-kinase, which then phosphorylates MBS and results in the inhibition of MLCPh. We have recently demonstrated in our porcine model with IL-1β that MLC phosphorylations (on stimulation by serotonin) are enhanced at the spastic site and that hydroxyfasudil, a specific Rho-kinase inhibitor, exerts an inhibitory effect on the spasm both in vivo and in vitro. 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-
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. The level of MLC phosphorylation is determined by a balance between MLC phosphorylation by MLCK and dephosphorylation by MLCPh. 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, potently induced MLC diphosphorylation in smooth muscle cells without an increase

Figure 4. RT-PCR analysis for Rho-kinase mRNA expression in control and IL-β-treated coronary segments. Results are expressed as mean ± SEM.

Figure 5. Western blot analysis for MBS phosphorylation of porcine coronary artery with or without serotonin (1 μmol/L). MBS phosphorylation was significantly increased in response to serotonin in IL-1β-treated segment compared with control segment. Y-27632 significantly suppressed MBS phosphorylation in response to serotonin in IL-1β-treated segment. Results are expressed as mean ± SEM.

Figure 6. Correlation between extent of MBS phosphorylations (arbitrary units) and that of serotonin-induced contractions (percent of contraction to 62 mmol/L KCl). There was a highly significant positive correlation between the 2 values.

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

Figure 3. Inhibitory effect of Y-27632 on serotonin (1 μmol/L)-induced contractions in vitro. Y-27632 inhibited serotonin-induced hypercontractions of IL-1β-treated segment, and it also significantly inhibited contractions in control segment. Results are expressed as mean ± SEM.
Enhanced MBS Phosphorylation Caused by Upregulated Rho-Kinase

In the present study, we demonstrated that 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. MBS serves as a targeting subunit of MLCPh to myosin and enhances the activity of the enzyme toward myosin. Recently, we reported that Rho-kinase phosphorylates MBS and reduces the MLCPh activity in vitro. 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. 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. 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. 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 F2α is resistant to the blockade of protein kinase C (PKC) and is not augmented at the IL-1β-treated site, whereas that to serotonin or histamine is sensitive to the blockade of PKC and is augmented at the IL-1β-treated site. 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, several other regulators, including the C-kinase–potentiated inhibitor of myosin phosphatase (CPI 17) and arachidonic acid, 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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