Long-Term Inhibition of Rho-kinase Suppresses Neointimal Formation After Stent Implantation in Porcine Coronary Arteries—Involvement of Multiple Mechanisms

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Objective—We recently demonstrated that Rho-kinase, an effector of the small GTPase Rho, is substantially involved in the pathogenesis of arteriosclerosis. In this study, we examined whether Rho-kinase is also involved in in-stent restenosis and if so, what mechanism is involved.

Methods and Results—Pigs underwent stent implantation in the left coronary artery with or without administration of fasudil (30 mg/kg/d PO), a specific Rho-kinase inhibitor, starting 2 days before the procedure for a duration of 4 weeks. At day 28, reductions in coronary diameter and neointimal formation associated with macrophage accumulation, collagen deposition, and transforming growth factor (TGF)-β1 expression were noted at the stent site, and all were significantly suppressed by fasudil. At day 7, fasudil significantly increased the frequency of TUNEL-positive apoptotic cells, while it tended to reduce that of bromodeoxyuridine-positive proliferating cells in the neointima. Western blot analysis at day 7 demonstrated that phosphorylations of the ezrin/radixin/moesin family (a marker of Rho-kinase activity in vivo) and protein expression of monocyte chemoattractant protein-1 and bcl-2 were upregulated at the stent site and were significantly suppressed by fasudil.

Conclusions—These results indicate that long-term inhibition of Rho-kinase suppresses in-stent neointimal formation by multiple mechanisms, including reduced vascular inflammation, enhanced apoptosis, and decreased collagen deposition. (Arterioscler Thromb Vasc Biol. 2004;24:1-9.)

Key Words: Rho-Kinase \textbullet} Stents \textbullet} Inflammation \textbullet} Apoptosis \textbullet} Collagen

Although the use of stents has dramatically increased in interventional cardiology, in-stent restenosis continues to be a serious problem and is more troublesome when it occurs.\textsuperscript{1,2} Furthermore, pharmacological approaches have generally been unsuccessful in suppressing in-stent restenosis except for a recent promising outcome with a drug-eluting stent.\textsuperscript{3,4} However, late neointimal catch-up remains a potential adverse outcome with the stent-based drug delivery.\textsuperscript{5} More recently, extracellular matrix accumulation has been recognized as a very important component of in-stent restenosis in the chronic phase after stent implantation.\textsuperscript{6} Since drug-eluting stents have several limitations for a defined period of time with kinetics and are expensive, there may become a need for systemic therapies to maintain neointimal inhibition, including matrix metabolism.\textsuperscript{7,8}

Accumulating evidence has demonstrated that Rho-kinase, an effector of the small GTPase Rho, plays an important role in adhesion, migration, proliferation, and cytokinesis of vascular smooth muscle cells (VSMCs) and other vascular wall cells.\textsuperscript{9–11} Rho-kinase is substantially involved in the signal transduction initiated by angiotensin II,\textsuperscript{12} platelet-derived growth factor (PDGF),\textsuperscript{13} thrombin,\textsuperscript{14} and endothelin-1,\textsuperscript{15} all of which may play an important role in the pathogenesis of restenosis,\textsuperscript{16} especially in that of in-stent restenosis.\textsuperscript{17–20} We recently demonstrated that neointimal formation after balloon injury was significantly inhibited by in vivo gene transfer of dominant-negative Rho-kinase in porcine femoral arteries.\textsuperscript{21} A similar finding was also noted in the rat carotid artery with Rho-kinase inhibitor Y-27632, although the relative contribution of inhibition of VSMC proliferation and enhancement of VSMC apoptosis to the inhibitory effect of a Rho-kinase inhibitor remains to be elucidated.\textsuperscript{22,23} Furthermore, involvement of Rho-kinase in the extracellular matrix deposition, especially that of collagen, a major component of the neointima,\textsuperscript{6} remains unknown. There are also some
differences in the restenosis mechanisms between balloon angioplasty and stent implantation.24-29

In the present study, we examined whether long-term inhibition of Rho-kinase suppresses in-stent restenosis in porcine coronary arteries, and if so, what mechanism is involved. For this purpose, we employed long-term oral treatment with fasudil, which we found metabolized to hydroxyfasudil, a specific Rho-kinase inhibitor, after oral administration.30

Materials and Methods
This study was reviewed and approved by the Ethical Committee on the Animal Experiments of the Kyushu University Graduate School of Medical Sciences.

Animal Preparation
Thirty-four domestic male pigs (aged 2 to 3 months and weighing 25 to 30 kg) were used. They were divided into 2 groups: the control group was treated with aspirin (325 mg/d) and ticlopidine (500 mg/d) alone (n = 17) and the fasudil group with oral administration of fasudil (30 mg/kg/d, Asahi Kasei Company) in addition to the antiplatelet therapy (n = 17). The oral administration of fasudil started 2 days before the procedure and continued until the follow-up period. We performed coronary angiography, intravascular ultrasound (IVUS) imaging, and histological study at 4 weeks (n = 6 each). We performed immunostaining for bromodeoxyuridine (BrdU) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) (n = 5 each), and Western blot analysis for substrates of Rho-kinase at 1 week (n = 6 each).

Stent Implantation
Nitroglycerin (10 μg/kg IC) was administered prior to angiography. A stainless-steel stent (3.0 \text{ mm}) was implanted to either the left anterior descending (LAD) or the left circumflex coronary (LCx) arteries.31 A segment with a mean coronary diameter of 2.3 mm was selected by using quantitative coronary angiography with a stent-to-artery ratio of approximately 1:3. A balloon catheter mounted with a stent was then advanced to the pre-selected coronary segments for deployment over a standard guide wire in a blind manner without knowledge about the fasudil treatment. The balloon catheter was inflated at 8 atm for 30 seconds once and was then slowly withdrawn, leaving the stent in place.31

Coronary Angiography
Left coronary angiography was performed before, immediately after, and 4 weeks after the stent implantation. Among the 12 animals that underwent angiography, 4 received a stent for LAD and 2 for LCx in the control group, while in the fasudil group, 3 received a stent for LAD and 3 for LCx. A preshaped Judkins catheter was inserted into the right or left carotid artery, and coronary angiography in a left anterior oblique view was performed.31,32 Arterial pressure, heart rate, and ECG were continuously monitored and recorded on a recorder.

Left coronary angiography was performed in a left anterior oblique projection.31,32 The measured coronary luminal diameters included mean reference diameter (proximal + distal) reference diameters/2, mean diameter of the stent site at full expansion, stent-to-artery ratio ([2/1]), and minimal diameter of the stent site at follow-up.31

Coronary IVUS
To assess the extent of neointimal formation in vivo, we performed IVUS 4 weeks after the stent implantation, as previously described.31

Histopathological Study
For histological analysis, the heart was excised 4 weeks after stent implantation; the left coronary artery was perfused with 10% formalin at 120 mm Hg and fixed for 24 hours. The dissected whole artery was embedded in methylmethacrylate, leaving the stent wires intact to minimize potential artifacts from cutting the wires.4 After polymerization, stented segments were cut into 3 blocks (proximal, middle, and distal portion) using a rotating saw with diamond edge. The blocks were cut into thin sections (4 to 5 μm) with tungsten carbide blades using a microtome (Leica). The sections were then stained with van Gieson elastic stains. Morphometric analysis of the neointima from photomicrographs was performed for vessel injury score and neointimal area, which was determined by subtracting the lumen area from the area enclosed by the internal elastic lamina.30,33 Mean value of the injury score and neointimal area from the 3 blocks was used for analysis.

Immunohistochemistry
Immunostaining was performed with a specifically designed kit with a tyramide signal amplification (Dako). The antibodies used in this study included monoclonal antibodies to human macrophages (AM-3K, Transgenic) and human α-smooth muscle actin (1A4, Dako), polyclonal antibodies to transforming growth factor (TGF)-β1 (Santa Cruz Biotechnology), and Dolichos biflorus agglutinin for porcine endothelium (Sigma),34 and nonimmune mouse IgG (Dako). We semi-quantitatively assessed the extent of macrophage accumulation using a conventional scale (0, no cells; 1, scattered cells; 2, focal deposits; and 3, diffuse intense infiltration)26 and that of re-endothelialization as the percentage of circumference covered by the endothelium (1, <25%; 2, 25% to 75%; and 3, >75%).4

Proliferation and Apoptosis
A segment 2 to 3 mm long was cut from the midportion of the stented artery using fine scissors. Stent wires were carefully removed under a dissecting microscope before paraffin embedding.31 The sections were subjected to BrdU and TUNEL stainings to identify proliferating and apoptotic cells, respectively. BrdU (50 mg/kg) was intravenously injected three times at 24, 16, and 8 hours before necropsy; BrdU-positive cells were detected by the LSAB method and counterstained with hematoxylin.33 Apoptotic cells were detected with an apoptosis kit (Wako) with porcine small intestine as a positive control. A total cell number and a number of BrdU-positive and TUNEL-positive cells in high-power field were counted in 3 randomly selected fields of each section.31 A number of BrdU-positive and TUNEL-positive cells were expressed as BrdU index and TUNEL index (BrdU- or TUNEL-positive cells/total cells ×100), respectively.31

Collagen Deposition
Collagen deposition was measured on the entire neointima 4 weeks after stent implantation when it became evident. To avoid color balance variation, Sirius red staining of all sections was performed at the same time. Then, once a standard for the particular slide/section was set by polarization microscopy, all the sections from the different groups of animals were photographed with the same strength of light by digital image capture.36

Western Blot Analysis
Stented coronary segments were subjected to SDS-PAGE immunoblot analysis at 1 week, as described previously.32 Phosphorylation of the ezrin/radixin/moesin (ERM) substrates of Rho-kinase was measured, using a rabbit polyclonal antibody to phosphorylated human moesin (Thr558), which also binds to phosphorylated ezrin (Thr567) and radixin (Thr564). Monocyte chemoattractant protein (MCP)-1 (R&D Systems) and bcl-2 (Roche Diagnostics) were also evaluated.

Statistical Analysis
Results are expressed as means±SEM. Throughout the text and figures, n represents the number of animals tested. Comparison between the control and the fasudil groups was performed by an unpaired, two-tailed t test. Multiple comparisons were made by
analysis of variance followed by Scheffé post hoc test. A probability value of \( p < 0.05 \) was considered to be statistically significant.

**Results**

**Coronary Angiography**

Before stent implantation, there was no significant difference in coronary diameter (mm) between the control (2.33±0.31) and the fasudil (2.31±0.03) groups (Figure 1 and Figure I, available online at http://atvb.ahajournals.org). Similarly, there was no significant difference in the diameter (mm) at full stent expansion (2.95±0.02 versus 2.97±0.02) or stent-to-artery ratio (1.27±0.02 versus 1.29±0.02) between the 2 groups. Four weeks after the stent implantation, coronary diameter (mm) at the stent site was significantly decreased in the control group (1.65±0.11) but remained unchanged in the fasudil group (2.23±0.12) (Figure 1 and Figure I). There was no significant change in mean arterial pressure throughout the experiment in both groups (data not shown).

**IVUS Analysis**

IVUS analysis demonstrated that the extent of neointimal formation (as expressed by percentage of neointimal area to the area covered by stent) was significantly less in the fasudil group (36.6±3.7) than in the control group (50.2±4.7) (\( p < 0.05 \)).

**Histology**

Neointimal area (mm\(^2\)) was significantly less in the fasudil group (2.2±0.2) than in the control group (3.1±0.3) (\( p < 0.05 \)), while injury score was comparable between the control (1.13±0.11) and the fasudil (1.20±0.09) groups (Fig. 2). Endothelialization score was also comparable between the control (2.8±0.2) and the fasudil (2.8±0.2) groups.

**Rho-Kinase Activity**

The extent of ERM family phosphorylation was significantly increased at the stent site in the control group and significantly suppressed in the fasudil group (Figure 3).

**Vascular Inflammation**

At 4 weeks, macrophage accumulation was noted in the neointima and to a greater extent, in the adventitia in the control group and was significantly suppressed in the fasudil group (Figure 4 and Figure I, available online at http://atvb.ahajournals.org). At 1 week, MCP-1 protein expression increased and was again significantly reduced by fasudil (Figure III, available online at http://atvb.ahajournals.org).

**Proliferation and Apoptosis**

In the intact artery, neither BrdU-positive nor TUNEL-positive cells were detected in the intima or the media (data not shown). Although statistically insignificant, BrdU index (%) tended to be reduced in the fasudil group (23.2±2.0) compared with the control group (33.2±5.0) at 1 week (\( p = 0.10 \)). In contrast, TUNEL index at 1 week was significantly increased in the fasudil group (55.5±5.4) compared with the control group (33.8±4.6) (\( p < 0.05 \)) (Figure 5). Bcl-2 protein expression increased in the control group, which was significantly downregulated by the fasudil treatment (Figure IV, available online at http://atvb.ahajournals.org).

**Collagen Deposition**

Picrosirius red polarization showed that neointimal collagen content (%) was significantly less in the fasudil group (24.1±3.1) than in the control group (43.6±3.2) (\( p < 0.01 \)) (Figure 6). Immunostaining for TGF-β1 revealed reduced TGF-β1 immunoreactivity in the fasudil group rather than in the control group (Figure 6).

**Side Effects**

In the present study, no appreciable side effects, such as weight loss, diarrhea, or blood abnormalities, were noted in the fasudil group (data not shown).

**Discussion**

The novel findings of the present study were: (1) Rho-kinase activity was enhanced at the stent implantation site associated with neointimal formation and (2) long-term
inhibition of Rho-kinase with fasudil significantly suppressed the neointimal formation by multiple mechanisms, including inhibition of vascular inflammation, enhanced apoptosis, and reduced collagen deposition (Figure V, available online at http://atvb.ahajournals.org). To the best of our knowledge, this is the first report that demonstrates the involvement of Rho-kinase in in-stent restenosis and thereby the potential usefulness of a Rho-kinase inhibitor to prevent the disorder.

Increased Rho-Kinase Activity by Stent Implantation

We previously demonstrated that both expression and activity of Rho-kinase increase after balloon injury\(^2\) and on stimulation by an inflammatory cytokine\(^5\) in pigs in vivo. The present study also demonstrated that stent implantation increased Rho-kinase activity. Recent studies in vitro have shown that Rho-kinase and its substrates mediate actin cytoskeleton organization, cell adhesion and migration, and cytokinesis,\(^9\) all of which may be involved in in-stent neointimal

Figure 2. Photomicrographs (van Gieson elastic staining) of stented coronary segments 4 weeks after stent implantation in the control and the fasudil groups. Calibration, 1 mm.
formation. Although a molecular mechanism for the upregulation of Rho-kinase by stent implantation remains to be elucidated, it has recently been demonstrated that various vasoactive factors enhance Rho-kinase activity in vitro. Indeed, we have recently demonstrated that Rho-kinase plays an important role in angiotensin II-induced MCP-1 expression in cultured rat VSMCs. Various growth factors (e.g., PDGF) and cytokines, angiotensin-II, endothelin-1, and thrombin may all be involved in restenosis after angioplasty. Importantly, all of them could upregulate Rho-kinase. Thus, it is highly possible that Rho-kinase plays an important role in the pathogenesis of in-stent restenosis (Figure V).

Mechanism for the Inhibitory Effect of Fasudil on Neointimal Formation After Stent Implantation
The present study demonstrated that multiple mechanisms are involved in the inhibitory effect of fasudil on neointimal formation after stent implantation, including inhibition of vascular inflammation, enhanced apoptosis, and reduced collagen deposition.

In-stent restenosis is characterized by prolonged and pronounced inflammation. In this study, the long-term treatment with fasudil suppressed macrophage accumulation, not only around stent struts but also in the adventitia. We previously demonstrated that long-term treatment with fasudil significantly suppresses macrophage accumulation at the adventitia and subsequent coronary vascular lesion formation in porcine coronary arteries in vivo. Two mechanisms may be involved for the anti-inflammatory effect of fasudil. First, fasudil may directly inhibit macrophage chemotaxis. Second, fasudil may inhibit the expression of proinflammatory molecules such as MCP-1. Importantly, the inhibitory effect of fasudil on the MCP-1 expression at the stent site (70% reduction) is equivalent to that of sirolimus-coated stent. It is thus highly possible that reduced MCP-1 expression resulted in decreased macrophage accumulation at 4 weeks after stent implantation.

In the present study, fasudil significantly enhanced apoptosis, a consistent finding with a previous study. However, the molecular mechanism for the proapoptotic effect of fasudil remains to be elucidated. In the present study, we demonstrated that downregulation of anti-apoptotic protein bcl-2 is involved in the proapoptotic effect of fasudil. Although statistically insignificant, fasudil also tended to reduce cellular proliferation. While it has been controversial whether an antiproliferative effect is involved in the antiatherogenic effect of a Rho-kinase inhibitor, the present results suggest that such effect may not play a central role in the present porcine model, although this point remains to be examined in a future study. Fasudil did not affect the extent of re-endothelialization in vivo, a consistent finding with a previous report on Y-27632. Thus, it is suggested that Rho-kinase is not involved in endothelial regeneration after vascular injury.

Finally, the importance of extracellular matrix formation is recognized as a key component of in-stent restenosis. Stent implantation causes a significant increase in collagen synthesis and TGF-β expression compared with balloon angioplasty alone. In the present study, we demonstrated the abundant collagen deposition associated with TGF-β1 expression in all layers of stented coronary arteries. TGF-β is one of the most potent stimuli for collagen synthesis and may contribute to the formation of restenotic lesions. A substantial portion of the neointima consists of matrix rather than cells. Thus, a strategy to inhibit TGF-β may be useful in preventing in-stent neointimal formation. In this study, we were able to demonstrate for the first time that long-term treatment with fasudil significantly suppresses collagen deposition in the neointimal lesion after stent implantation due, at least in part, to the inhibition of TGF-β1 expression.
Possible Side Effects of Fasudil
In the present study, no appreciable side effects were observed in the fasudil group, and fasudil had no effects on arterial pressure or nonstented coronary artery. We have recently demonstrated that fasudil is well tolerated without any serious side effects in patients with angina. Thus, fasudil may be a safe drug, although caution should be made when used clinically.

Limitations of the Study
Several limitations of the present study should be mentioned. First, the present study was performed in the normal porcine coronary artery without preexisting intimal thickening. Thus, the inhibitory effects of fasudil need to be confirmed in animal models with atherosclerotic coronary lesions. Second, cell-specific Rho-kinase expression was not examined. However, based on our recent findings and the present results with stainings for BrdU, TUNEL and macrophages, we consider that Rho-kinase was expressed mainly in macrophages and VSMCs. Third, although we confirmed the inhibitory effect of fasudil (hydroxyfasudil) on Rho-kinase in the present study, other unknown effects of this agent might be involved. Fourth, it remains to be examined how long fasudil should be continued to prevent neointimal formation after coronary stenting or whether lesion development is permanently suppressed by some period of treatment with fasudil. Finally, there are some differences in the mechanism between atherosclerosis and restenosis, including severity of injury, time course of the response, cellular/extracellular elements, and relation to lipids. Thus, an agent that may have beneficial effects in atherosclerosis may not be equally effective in the prevention of in-stent restenosis.

Figure 4. Inhibitory effects of fasudil on macrophage accumulation at the stented porcine coronary arteries. Photomicrographs show immunostaining for macrophages 4 weeks after stent implantation in the control and the fasudil groups. Calibration, 100 μm. N indicates neointima; M, media; A, adventitia. *stent strut.
In summary, the present results indicate that long-term inhibition of Rho-kinase suppresses in-stent restenosis by multiple mechanisms, suggesting the potential usefulness of a Rho-kinase inhibitor to prevent the disorder (Figure V).

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Figure 6. Inhibitory effects of fasudil on collagen deposition at the stented porcine coronary segments at 4 weeks after stent implantation. Sirius red stainings without (left) and with (middle) polarized light show more thickened neointima and abundant collagen deposition, respectively, in the control group compared with the fasudil group. Right, TGF-β1 immunoreactivity was detected in the neointima and the adventitia in the control group and was almost undetectable in the fasudil group. Calibration, 400 μm. N indicates neointima; M, media; A, adventitia; *stent strut.
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