Inhibition of Rho-Associated Kinase Results in Suppression of Neointimal Formation of Balloon-Injured Arteries

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Background—Rho-associated kinase (ROCK), an effector of small GTPase Rho, regulates vascular tone via a calcium sensitization mechanism and plays a key role in the pathogenesis of hypertension. However, its role in vascular growth remains unclear.

Methods and Results—Y-27632, a specific ROCK inhibitor, and the overexpression of dominant-negative ROCK suppressed the mitogen-induced DNA synthesis of cultured vascular smooth muscle cells (VSMCs), which indicates the essential role of ROCK in the control of VSMC proliferation in vitro. Y-27632 also suppressed the chemotaxis of VSMCs. Male Wistar rats were systemically given Y-27632 (35 to 70 mg kg\(^{-1}\) day\(^{-1}\)) through an intraperitoneal infusion. The neointimal formation of balloon-injured carotid arteries was significantly suppressed in Y-27632–treated rats (intima/media ratio, 0.22 \(\pm\) 0.02) compared with vehicle-treated rats (intima/media ratio, 0.92 \(\pm\) 0.21) or hydralazine-treated rats with a similar blood pressure decrease (intima/media ratio, 1.03 \(\pm\) 0.15). The phosphorylation of myosin phosphatase and myosin light chain was elevated in injured arteries in a Y-27632–sensitive manner, indicating the augmentation of ROCK activity in neointimal formation. The downregulation of the cyclin-dependent kinase inhibitor p27kip1 in injured vessels was reversed by Y-27632 treatment, reflecting the antiproliferative effect of ROCK inhibition in vivo.

Conclusions—We conclude that ROCK plays a key role in the process of neointimal formation after balloon injury. Thus, the inhibition of ROCK may be a potential therapeutic strategy for treating vascular proliferative disorders and hypertension. (Circulation. 2000;101:2030-2033.)

Key Words: atherosclerosis ■ muscle, smooth ■ remodeling ■ signal transduction ■ hypertension

Elevated vascular tone contributes to the pathogenesis of hypertension. Rho-associated kinase (ROCK),\(^1\) a target of small GTPase Rho, regulates vascular contractility by increasing the level of phosphorylated myosin light chain and thereby elevating the calcium sensitivity of vascular smooth muscle cells (VSMCs).\(^2\) Recently, Uehata et al\(^3\) developed a potent, specific, ROCK inhibitor, Y-27632. The administration of Y-27632 to several hypertensive rat models markedly reduced systolic blood pressure (SBP), implicating ROCK as a key mediator in the pathogenesis of hypertension.\(^3\)

We and others have reported that regulators of vascular tone, such as angiotensin II or natriuretic peptides, are also involved in vascular growth.\(^4\) Thus, we postulated that intracellular mechanism(s) should exist that govern both vascular contraction and growth. Using Y-27632 and dominant-negative ROCK, the present study demonstrates that ROCK, the key regulator of vascular contraction, also controls vascular growth in vitro and in vivo.

Methods

Materials

Y-27632 was obtained from Yoshitomi Pharmaceutical Industries, Osaka, Japan. The pCAG-myc and pCAG-myc-KD-IA plasmids\(^1\) were a gift from T. Ishizaki and S. Narumiya (Kyoto University). The pEXV-myc-N19RhoA was from M. Symons (the Picower Institute for Medical Research), and the anti-MLC20 and anti-monophosphorylated (Ser19) MLC20 antibodies\(^5\) were from M. Seto (Asahi Chemical Industry). The anti-MYPT1 and anti-phospho-MYPT1 (pM133T695) antibodies\(^2\) were from M. Ito (Mie University).

DNA Synthesis and Cell Migration Assay

\(^3\)H-thymidine uptake and modified Boyden chamber analyses for human aortic smooth muscle cells (AOSMCs; Clonetics) were performed as described previously.\(^6\) Then, 24 hours after transfection using lipofectamine PLUS (Gibco-BRL), cells were stimulated with 5% FCS, 50 ng/mL platelet-derived growth factor-BB (PDGF-BB), or 50 \(\mu\)mol/L lysophosphatidic acid (LPA) for 16 hours. They were then pulsed with 30 \(\mu\)mol/L 5-bromodeoxyuridine (BrdU). Immunostaining for BrdU and myc-tag epitope were superimposed using a confocal laser scanning microscope.

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Animal Experiments

Animals were cared for according to the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (NIH Publication No. 85–23; revised 1985). Male Wistar rats (14 to 16 weeks old) were used in the study. ALZET osmotic pumps (2 MLA, Alza) were filled with vehicle (phosphate-buffer, n=6), hydralazine (2 mg · kg⁻¹ · day⁻¹, n=5) or Y-27632 (35 mg · kg⁻¹ · day⁻¹, n=4; 70 mg · kg⁻¹ · day⁻¹, n=4) and implanted intraperitoneally. Three days later, a balloon injury to the right carotid artery was made. On days 7 and 14 after the injury, rats were killed for morphometric and immunoblotting analyses, respectively.

Statistical Analysis

The data were expressed as means±SEM. Statistical differences were determined by ANOVA. P<0.05 was considered significant.

Results

The Effect of ROCK Inhibition on Proliferation and Migration of Cultured VSMCs

Pretreatment with Y-27632 resulted in a dose-dependent (0.3 to 30 μmol/L) inhibition of the DNA synthesis of cultured human AOSMCs. A dose of 10 μmol/L, Y-27632 suppressed the ³H-thymidine uptake induced by serum, PDGF-βB, or LPA to 46%, 48%, and 23% of controls, respectively. At 10 μmol/L, Y-27632 reportedly inhibits ROCK activity by 90%, with minimal effects on other kinases (such as protein kinase C, CAMP-dependent protein kinase, or myosin light chain kinase [MLCK]). Transient expression of dominant-negative ROCK (KD-IA) or RhoA (N19 RhoA) also significantly suppressed mitogen-induced DNA synthesis, as assessed by BrdU positivity (Figure 1). Moreover, Y-27632 dose-dependently suppressed the chemotactic motility of AOSMCs. Pretreatment with 10 μmol/L Y-27632 suppressed PDGF-BB- and LPA-induced chemotaxis by 28% and 36%, respectively.

Suppression of Neointimal Formation by Y-27632

Continuous systemic delivery of Y-27632 at doses of 35 and 70 mg · kg⁻¹ · day⁻¹ resulted in a decrease of SBP by 20 to 30 mm Hg compared with control rats throughout the observation period. A comparable SBP decrease was also achieved by hydralazine treatment. The steady-state level of Y-27632 in carotid arteries was 5 to 6 times higher than the plasma level (for 35 mg · kg⁻¹ · day⁻¹, plasma: 1.25±0.21 μmol/L, tissue: 7.15±1.40 μmol/kg; for 70 mg · kg⁻¹ · day⁻¹, plasma: 3.65±0.29 μmol/L, tissue: 20.18±2.68 μmol/kg). Thus, the vascular Y-27632 level achieved in the present study seems optimal for effective and specific ROCK inhibition, as inferred from the in vitro data above and previous reports. Y-27632 administration resulted in neither abnormal laboratory data nor systemic adverse effects, such as body weight loss or diarrhea.

Y-27632 treatment dramatically reduced neointima formation 14 days after balloon injury in a dose-dependent manner (Figure 2 and Table). With the higher dose, the

![Figure 1](http://circ.ahajournals.org/)

**Figure 1.** Dominant-negative Rho and ROCK inhibit DNA synthesis of VSMCs. VSMCs were transfected with mock (pCAG-myc), dominant-negative ROCK (pCAG-myc-KD-IA), or RhoA (pEXV-myc-N19RhoA) expression plasmids. After 24 hours, the cells were stimulated with serum, PDGF-βB, or LPA and pulsed with BrdU. a, Double immunostaining for myc-tag (red) and BrdU (green). Magnification ×100. b, BrdU positivity of transfected VSMCs. *P<0.05 and **P<0.01 versus corresponding mock-transfected cells.

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Y-27632 inhibits neointimal formation after balloon injury. Figure shows representative cross-sections of noninjured (a) or balloon-injured carotid arteries (b through e) treated with vehicle (b), hydralazine (c), or Y-27632 (d, 35 mg · kg⁻¹ · day⁻¹; e, 70 mg · kg⁻¹ · day⁻¹). The sections were stained with the Elastica Van Gieson method. Scale bar in e=0.2 mm. f, Immunoblotting of vessel extracts. Seven days after injury, extracts (50 μg of protein) from noninjured (first lane) and injured arteries (second lane, vehicle; third lane, Y-27632-treated) were subjected to immunoblotting with anti-pM133T(695), anti-MYPT1, anti-phospho-MLC20, anti-MLC20, anti-p27kia, or anti-p21cip1 antibodies. Molecular size is indicated on the left.
blockade was profound (76% decrease in intima/media ratio). Hydralazine treatment did not prevent neointima formation, which suggests that the effect of Y-27632 was not exerted via the lowering of SBP. Considering the optimal tissue level achieved in this study, Y-27632 is thought to have effects locally. However, the involvement of other systemic effects remains to be determined. A significantly smaller number of VSMCs were found in Y-27632–treated vessels sections than in sections treated with vehicle (740 ± 21 versus 1337 ± 54 nuclei/section for 70 mg · kg⁻¹ · day⁻¹ Y-27632 versus vehicle), indicating that the inhibition of VSMC proliferation should contribute, at least in part, to the effect of Y-27632. Fasudil, another inhibitor of protein kinases including ROCK, suppresses VSMC migration in vivo. Considering its in vitro suppression of VSMC migration and proliferation, Y-27632 likely suppressed neointima formation through the inhibition of both the migration and proliferation of VSMCs in vivo.

The luminal narrowing of the injured vessels was also prevented by the administration of Y-27632. The areas surrounded by internal and external elastic laminae were enlarged by Y-27632 treatment when compared with vehicle (Table).

### Activation of ROCK in Balloon-Injured Arteries and its Reversal by Y-27632

ROCK inactivates myosin phosphatase through the specific phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Thr⁶⁹⁵, which results in an increase in the phosphorylated content of the 20-kDa myosin light chain (MLC20). As shown in Figure 2f, balloon injury increased the phosphorylation of both MYPT1 (at Thr⁶⁹⁵) and MLC20; this increase was almost reversed by Y-27632 treatment. MLC20 is also phosphorylated by MLCK. Y-27632 has a much higher inhibition constant (≥250 μmol/L) for MLCK than for ROCK (0.14 μmol/L). This, together with the specific phosphorylation of MYPT1 at Thr⁶⁹⁵ by ROCK, leads us to conclude that ROCK is activated in neointimal hyperplasia after balloon injury. Thus, it seems quite plausible that Y-27632 suppressed neointima formation by specifically inhibiting the augmented ROCK activity of VSMCs.

### Comparison of Cross-Sectional Areas of the Injured Arteries

<table>
<thead>
<tr>
<th></th>
<th>Noninjured</th>
<th>Vehicle</th>
<th>Hydralazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimal area</td>
<td>3.3±0.8</td>
<td>3.8±0.4</td>
<td>1.5±0.4†</td>
</tr>
<tr>
<td>Medial area</td>
<td>3.2±0.1</td>
<td>3.6±0.2</td>
<td>3.8±0.2*</td>
</tr>
<tr>
<td>Intima/media ratio</td>
<td>0.92±0.21</td>
<td>1.03±0.15</td>
<td>0.34±0.06†</td>
</tr>
<tr>
<td>IEL area</td>
<td>9.3±0.5</td>
<td>10.2±0.5</td>
<td>12.0±0.9†</td>
</tr>
<tr>
<td>EEL area</td>
<td>12.5±0.5</td>
<td>13.9±0.5</td>
<td>16.1±1.2†</td>
</tr>
<tr>
<td>Intima/media ratio</td>
<td>0.2±0.3</td>
<td>0.6±0.6‡</td>
<td>0.4±1.03‡</td>
</tr>
<tr>
<td>Areas are shown in 10⁻² mm². Values are indicated as mean±SEM. IEL indicates internal elastic lamina; EEL, external elastic lamina.</td>
<td></td>
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</tr>
<tr>
<td>*P&lt;0.05 vs noninjured; †P&lt;0.01 vs noninjured; ‡P&lt;0.05 vs vehicle; §P&lt;0.01 vs vehicle; ¶P&lt;0.05 vs hydralazine;</td>
<td></td>
<td>P&lt;0.01 vs hydralazine.</td>
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### Reversal of Injury-Induced Decrease of p27kip1 by Y-27632

Cyclin-dependent kinase inhibitors have been implicated in cell cycle regulation. Rho promotes cell proliferation through the titration of p27kip1. In addition, the downregulation of p27kip1 correlates well with VSMC proliferation in balloon-injured arteries; however, the involvement of p21waf1, another cyclin-dependent kinase inhibitor, is controversial. As shown in Figure 2f, balloon injury decreased the level of p27kip1 after 7 days, reflecting VSMC proliferation. p21waf1 was only faintly detectable in both noninjured and injured arteries. Y-27632 treatment almost reversed this decrease in levels of p27kip1, which suggests that the compound exerted its suppressive effect on neointimal formation via its antiproliferative action. Similar results were also obtained with cultured AOSMCs (data not shown).

### Discussion

Recently, it was reported that thrombin- or stretch-induced VSMC growth and migration are blocked by Y-27632. The present study has further strengthened the evidence for the involvement of ROCK in the regulation of VSMC proliferation in vitro. Moreover, we showed for the first time that ROCK activation is critical for neointima formation in vivo. Thus, ROCK regulates not only vascular tone but also vascular growth. The activation of ROCK may constitute a common component for the pathogenesis of both hypertension and vascular proliferative disorders. Inhibiting ROCK to block the intracellular signaling pathway for vascular tone and growth can be a potential therapeutic strategy for hypertension, atherosclerosis, and restenosis after angioplasty.

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