Role of Rho-Associated Kinase in Neointima Formation After Vascular Injury

Rei Shibata, MD; Hisashi Kai, MD, PhD; Yukihiko Seki, MD; Seiya Kato, MD, PhD; Minoru Morimatsu, MD, PhD; Kozo Kaibuchi, MD, PhD; Tsutomu Imaizumi, MD, PhD

Background—The Rho/Rho-associated kinase (Rho-kinase) system is implicated in various cellular functions, including migration, proliferation, and apoptosis. Because a possible role of the system is suggested in neointima formation after vascular injury, we sought to examine whether a new specific Rho-kinase inhibitor, Y27632, prevents neointima formation of the balloon-injured rat carotid artery, and if so, to investigate the effects of Y27632 on migration, proliferation, and apoptosis of smooth muscle cells (SMCs) in the injured artery.

Methods and Results—Y27632 was administered intraperitoneally from 1 day before to 14 days after vascular injury. Treatment with Y27632 inhibited phenylephrine-induced Rho-kinase activation in the carotid artery on the basis of immunoblotting against the phosphorylated myosin-binding subunit of myosin phosphatase. Y27632 markedly prevented neointima formation at days 7 and 14. In controls, BrdU-proliferating and TUNEL-apoptotic SMCs were transiently and coincidentally increased in the neointima, with a peak at day 7. Y27632 significantly increased the neointimal TUNEL-apoptotic SMCs at days 7 and 14, but not BrdU-proliferating SMCs. Y27642 significantly decreased the number of intimal SMCs at day 4, while not affecting the number of BrdU or TUNEL SMCs. Reendothelialization after balloon injury was not significantly affected by Y27632 at days 7 and 14.

Conclusions—Y27632 inhibited neointima formation by enhancing SMC apoptosis and probably by suppressing early SMC migration. Therefore, a role of Rho-kinase is suggested in neointima formation after vascular injury. (Circulation. 2001;103:284-289.)

Key Words: kinases n neointima n apoptosis n migration

The small GTPase Rho is implicated in a variety of physiological functions associated with actomyosin-based cellular processes such as cell adhesion, cytokinesis, cell motility, migration, and contraction. Among several putative Rho effectors, the Rho-associated kinase (Rho-kinase/ROK/ROCK) family plays major roles in Rho-induced actin reorganization, such as focal adhesion and stress fiber formation. Also, Rho-kinase phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase, inhibits phosphatase activity, and thereby enhances the myosin light chain (MLC) phosphorylation level and subsequently myosin-based contractility in smooth muscle cells (SMCs). Furthermore, although the precise mechanism remains unclarified, there is increasing evidence that Rho has abilities to activate proliferation signals as well as signals that regulate apoptosis, either positively or negatively.

Neointima formation after balloon injury in the rat carotid artery is the best-studied model of vascular remodeling after vascular injury. Balloon injury triggers medial SMC replication that peaks within a few days after injury, SMC migration into the intima beginning at day 4, and SMC proliferation in the neointima, with the cell number reaching a maximum at day 14. The SMC accumulation in the neointima is theoretically the sum of cell migration and proliferation as well as apoptotic cell death, and an alteration in any of these events could affect neointima formation. Therefore, it is plausible that the Rho/Rho-kinase system plays a role in neointima formation after vascular injury. However, little is known about the significance of the Rho/Rho-kinase system in vivo, because specific Rho inhibitors, such as botulinum C3 ectoenzyme and Rho–GDP dissociation inhibitor (Rho-GDI), are not permitted for in vivo use.

Y27632, a specific inhibitor for the Rho-kinase family, has been shown to suppress the Rho/Rho-kinase–mediated stress fiber formation in cultured SMCs and the phenylephrine-induced contraction of vascular strips. Furthermore, systemic administration of Y27632 induced significant and persistent decreases in blood pressure in various hypertensive rat models. These results suggest not only that a Rho-kinase–mediated mechanism contributes to blood pressure regulation but also that Y27632 is a valuable tool for investigating the Rho-kinase function of vascular cells in vivo.
and its pathophysiological implications. The aims of this study were to examine whether Y27632 prevents neointima formation after vascular injury and if so, to investigate the possible mechanisms of the inhibitory effect of Y27632.

**Methods**

**Vascular Injury Model and Morphometry**

All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in conformity with institutional guidelines. Male Wistar rats (400 to 450 g; Japan SLC Inc; Shizuoka, Japan) were anesthetized with sodium pentobarbital (50 mg/kg), and then balloon injury of the left carotid artery was performed as described previously.13 Sham-operated rats underwent the same operation, except that the balloon was not inserted. Y27632 was supplied by Yoshitomi Pharmaceutical Industries. Y27632 (15 mg/ kg) or vehicle (saline) was administered intraperitoneally twice a day from 1 day before balloon injury for 4, 7, or 14 consecutive days. Blood pressure in conscious, unrestricted rats was measured by the tail-cuff method. After the rat was killed with an overdose of pentobarbital, the carotid artery was perfusion-fixed at 100 mm Hg with 4% paraformaldehyde, excised, and embedded in paraffin. Three individual sections (3 μm) from the middle of injured segments were stained with hematoxylin/eosin in each rat (n=5). The medial and intimai areas were measured with a computerized digital image analysis system and averaged in 3 independent sections.

**Detection of DNA Synthesis In Vivo and Apoptosis**

In vivo bromodeoxyuridine (BrdU) labeling was performed to identify replicating cells in balloon-injured artery by detection of the DNA synthesis with a Cell Proliferation kit (Amersham Pharmacia Biotech): 0.5 mg/kg BrdU, a thymidine analogue, was injected intraperitoneally 24 hours before preparation of the artery, and BrdU incorporated into replicating cells was detected immunohistochemically with an anti-BrdU antibody. In each serial section, apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method using Apop Tag (Intergen). The counterstain was done with 0.5% methyl green. In each experimental setup, rat small intestine and testis were used as positive controls. A negative control was incubated without the TdT. Double immunohistostaining with α-smooth muscle actin showed that these BrdU+ or TUNEL+ cells were SMCs. The total, BrdU+, and TUNEL+ nucleated SMCs were counted at a magnification of ×400 by 3 observers in a blinded manner, and the values obtained by the 3 observers were averaged in each section. The quantitative analysis was performed in 5 independent sections in each rat (n=5). The numbers of BrdU+ and TUNEL+ SMCs were expressed as BrdU labeling and TUNEL indexes (BrdU+ and TUNEL+ SMCs/total nucleated SMCs×100), respectively.

**Number of SMCs in Early Neointimal Lesions**

The number of SMCs in the injured artery was counted at day 4 by the modified method of Prescott et al. Briefly, the cross sections were subjected to immunohistostaining against α-smooth muscle actin with a commercially available detection system (DAKO) and counterstained with hematoxylin. The number of nuclei that were accompanied by α-smooth muscle actin–positive cytoplasm was counted at a magnification of ×400 in 10 independent sections from each rat (n=5) by an observer in a blinded fashion.

**Analysis of Reendothelialization**

Planimetric analysis of reendothelialization after balloon injury was performed with a computerized digital image analysis system by an observer in a blinded manner. Briefly, 30 minutes before rats (n=5) were killed, they received an intravenous injection of 6 mL of 0.5% Evans blue dye (Sigma) to identify areas of nonendothelialized artery with a blue stain. After perfusion-fixation at 100 mm Hg with 100% methanol, the injured artery was excised, incised longitudinally, and then photographed with a dissecting microscope. The initially injured area was defined as the total surface of the harvested arterial segment. The total harvested segment corresponded to the total length of the injured segment; in each case, this length was similarly defined proximally by the carotid bifurcation and distally by the edge of the omohyoid muscle. The reendothelialized area was defined as the area not stained with Evans blue dye.Extent of reendothelialization (% reendothelialization) was expressed as a percentage of the initially injured area.

**Measurement of MBS Phosphorylations**

After Y27632 or saline had been administered to rats twice a day for 4 days (n=5), the carotid artery was excised and cut into 3-mm-wide strips. Equal amounts of the strips were incubated in Hanks’ buffered salt solution with or without 10 μmol/L phenylephrine for 5 minutes (37°C). The reaction was stopped by immediately freezing the strips in dry ice/acetone. After tissue homogenization, protein extract was separated by 7.5% SDS-PAGE and subjected to immunoblotting with an anti–phosphorylated MBS antibody, and the signals were detected and analyzed with a chemifluorescence detection system and Fluorolimag (Amershams). The specificity of this antibody was described elsewhere.

**Statistical Analysis**

Statistical analysis was performed by unpaired Student’s t test or ANOVA followed by Scheffe’s F test. A value of P<0.05 was considered significant. The interobserver or intraobserver variation was <5% in each experiment.

**Results**

There were no significant differences in heart rate, blood pressure, or body weight between saline-treated control rats and Y27632-treated rats during the observation period, and apparently, no other evidence of an adverse effect of Y27632 was observed.

**Neointima Formation After Vascular Injury**

After vascular injury, SMCs were first observed in the intima at day 4, and thereafter, neointima formation progressed, resulting in thick myointima at day 14 (Figure 1A, top right). Y27632 remarkably prevented the neointima formation (Figure 1A, bottom left). The intima/media area ratio at days 7 and 14 was reduced by 43% and 74%, respectively, by Y27632 (P<0.05 and P<0.001, Figure 1B). The medial cross-sectional area did not differ between them. The intima and media remained intact in the sham-operated artery with Y27632 treatment.

**Cell Proliferation and Apoptosis in Injured Artery**

Serial arterial sections were subjected to in vivo BrdU labeling and the TUNEL method (Figures 2 and 3). In the intact artery, neither BrdU+ nor TUNEL+ cells were detected in the media and intima (Figures 2Aa and 3Aa). In controls, BrdU+ SMCs were scarcely observed in the intima at day 4, and a robust increase in neointimal BrdU+ SMCs was observed at day 7 (Figure 2Ab), returning to lower levels at day 14 (Figure 2Ad). Y27632 had no effect on the number of BrdU+ SMCs over the course of the study (Figure 2B). In controls, very small numbers of TUNEL+ SMCs were found in the intima at day 4, and TUNEL+ SMCs robustly increased in the neointima at day 7 (Figure 3Ab) and decreased to lower levels at day 14 (Figure 3Ad). When Y27632 was administered, although the TUNEL index at day 4 was similar to that
of controls, the increase in TUNEL<sup>+</sup> SMCs was enhanced at day 7 versus controls (<i>P</i>&lt;0.01) and was sustained at day 14 (<i>P</i>&lt;0.001, Figure 3B). SMC apoptosis was confirmed by typical electron microscopic features of apoptosis and the DNA ladder formation on genomic DNA gel electrophoresis (data not shown).

In the media, smaller numbers of BrdU<sup>+</sup> or TUNEL<sup>+</sup> SMCs were observed after vascular injury than in the intima, and Y27632 had no effect on BrdU labeling and TUNEL indexes. In the sham-operated artery, BrdU<sup>+</sup> or TUNEL<sup>+</sup> SMCs were not found during the observation period, irrespective of Y27632 treatment.

### Early Neointimal Lesion Formation

Consistent with earlier studies,<sup>8,9</sup> small numbers of SMCs were sporadically observed in the intima at day 4 (Table). Y27632 reduced the number of intimal SMCs at day 4 by 50% (<i>P</i>&lt;0.01), while not affecting BrdU labeling and TUNEL indexes (Table). The number of medial SMCs did not differ between the groups.

### Reendothelialization in Injured Artery

Effects of Y27632 on reendothelialization were evaluated by planimetric analysis performed with Evans blue dye staining (Figure 4A). The initially injured area was equivalent in Y27632-treated and control rats (Figure 4Ba). Controls showed progressive reendothelialization, with % reendothelialization of 20% and 43% at days 7 and 14, respectively (Figure 4Bb), consistent with the previous studies.<sup>17</sup> Y27632 had no significant effects on reendothelialized area or % reendothelialization at day 7 or 14 (Figure 4Bc).

### MBS Phosphorylation Levels

Phosphorylation levels of MBS, a specific substrate of activated Rho-kinase,<sup>6</sup> were examined to evaluate the Rho-kinase activity of the carotid artery. Denoted doses of Y27632 or saline were administered to rats twice a day for 4 days. In the vascular strips obtained from controls, phosphorylated...
MBS was not detected at baseline, and ex vivo phenylephrine stimulation markedly induced MBS phosphorylation (Figure 5). In the vascular strips from Y27632-treated rats, there was no baseline MBS phosphorylation, and the ex vivo phenylephrine-induced MBS phosphorylation was dose-dependently inhibited by Y27632.

**Discussion**

The present study demonstrated that a Rho-kinase inhibitor, Y27632, prevented neointima formation after vascular injury. At day 4, Y27632 reduced the number of intimal SMCs but did not affect SMC replication and apoptosis. Thereafter, Y27632 enhanced neointimal SMC apoptosis. Reendothelialization after injury was not significantly affected by Y27632 at day 7 or 14.

Y27632, a pyrimidine derivative, is a new specific inhibitor of the Rho-kinase family. Y27632 is competitive for the ATP-binding site of Rho-kinase and is very selective for Rho-kinase; its affinity for Rho-kinase is 200 times and 2000 times higher than that for protein kinase C or A and MLC kinase, respectively. Y27632 inhibits the Rho-kinase–mediated processes, including MLC phosphorylation, which enhances myosin-based contractility, in SMCs. Accordingly, Y27632 can induce SMC relaxation and suppression of hypertension and can inhibit migration of cultured SMCs. In contrast, Y27632 has no effects on other Rho-family GTPases, such as Rac and Cdc42. As shown in Figure 5, the phenylephrine-induced MBS phosphorylation was inhibited by Y27632, indicating that the arterial Rho-kinase activity was inhibited by Y27632. In this study, the dose of Y27632 used was similar to that showing antihypertensive effects in several hypertensive rat models. Previous studies reported that Y27632 had little effect on blood pressure in normotensive rats. Consistent with these
enhanced SMC apoptosis by Y27632 was observed specifically in the neointima. Although the mechanism remains unclarified, this may be related to the phenotypic difference and the possible differential susceptibilities to apoptosis between medial and neointimal SMCs, as suggested by Pollman et al.\textsuperscript{22,23}

Another possible mechanism of the inhibitory effect of Y27632 would be the prevention of SMC migration from the media. There is no available method to estimate SMC migration in vivo at the later phase, when SMC replication has actively occurred. Thus, the effects of Y27632 on early neointimal lesion formation were evaluated at day 4. Intimal SMCs were first observed at day 4, as earlier studies reported.\textsuperscript{8,9} The intimal SMCs at day 4 are considered to derive primarily from medial SMC migration into the intima and, to a lesser extent, from the initial proliferation of migrated SMCs.\textsuperscript{14,24,25} Y27632 reduced the number of intimal SMCs without any effects on SMC replication and apoptosis at day 4 (Table). Accordingly, it seems plausible that the reduction in neointimal SMCs by Y27632 would be mainly due to decreased early SMC migration. This is in accord with a recent observation that Y27632 suppressed thrombin-induced migration of cultured SMCs.\textsuperscript{18} At present, however, it remains unclear not only how far the early suppression of SMC migration contributed to the Y27632-induced prevention of neointima formation observed at days 7 and 14 but also whether the inhibitory effects of Y27632 on SMC migration continued up to the later phase of neointima formation.

Reendothelialization is one of the important aspects of the response to balloon injury.\textsuperscript{8,17} Accordingly, we evaluated the effects of Y27632 on reendothelialization at days 7 and 14. Y27632 did not affect the extent of reendothelialization after vascular injury (Figure 4). Thus, the inhibitory effects of Y27632 on neointima formation were not attributed to reendothelialization at least by day 14.

Recently, there have been increasing lines of evidence that Rho-kinase inhibitors may be a new category of agents that prevent a number of vascular responses to injury. A less specific Rho-kinase inhibitor than Y27632, fasudil, which is known to be an MLC kinase inhibitor, inhibited migration of cultured rabbit SMCs and reduced neointima formation by enhancing SMC loss without affecting cell proliferation in balloon-injured rabbit carotid artery.\textsuperscript{26} In porcine coronary artery showing the inflammatory/arteriosclerotic remodeling induced by interleukin-1β treatment, increased Rho-kinase activity was documented, and hydroxyfasudil, a derivative of fasudil, suppressed serotonin-induced vasospasm at the remodeling site.\textsuperscript{27} This, taken together with the present study using a more specific Rho-kinase inhibitor, Y27632,\textsuperscript{12} indicates that the Rho-kinase–mediated pathway may play important roles in vascular responses to various kinds of vascular injury that is implicated in the pathogenesis of arteriosclerosis through the mechanisms of not only vascular remodeling but also vasomotion control.

**Limitations of This Study**

First, at present, no direct method is available to assay Rho-kinase activity in the vascular samples. Also, no rescue experiment was done on Y27632-treated rats. Thus, it remains possible that inhibition of other serine-threonine protein kinases in addition to Rho-kinase may have contributed to the effects of...
Y27632 observed in the present study. Second, because Rho is not the only possible activator of Rho-kinase, it is possible that upstream pathways other than Rho would activate Rho-kinase in injured artery. Third, because Y27632 was systemically administered for a long time in the present study, the possibility of the involvement of indirect systemic effects, such as the neurohumoral effects, cannot be excluded. Fourth, we used BrdU labeling and the TUNEL method for the cell kinetic study, because these methods are commonly accepted as the most reliable methods currently available in vivo. However, quantitative analysis using these methods is potentially limited, because both the methods are based on immunohistochemical techniques. Furthermore, recent studies have suggested that the TUNEL method does not necessarily provide a specific marker of apoptosis, and the TUNEL phenomenon may also occur in other types of cell death, including necrosis and oncosis, and even in nonapoptotic nuclei with abundant RNA transcription and splicing or with DNA repair synthesis. Thus, it is conceivable that the present study based on the TUNEL method may have overestimated the true incidence of apoptotic SMCs in injured artery irrespective of Y27632 treatment. In addition, another methodological limitation of the TUNEL method is the lack of appropriate standardization. These issues should be addressed in future studies. Finally, future studies should evaluate whether Y27632 remains effective in the chronic phase of vascular remodeling later than day 14.

In conclusion, a Rho-kinase inhibitor, Y27632, inhibited early neointimal lesion formation, probably by suppressing early SMC migration into the intima and prevented neointima formation in the later phase by enhancing neointimal SMC apoptosis. Thus, a role of Rho-kinase in neointima formation is suggested by regulation of SMC migration and apoptosis. Finally, the present study may provide insight into the possible treatment strategy to prevent progression of atherosclerosis by inducing apoptosis in neointimal SMCs during the remodeling process after vascular injury.

Acknowledgments

This study was supported in part by a Kimura Memorial Heart Foundation research grant. We thank Kaoru Moriyama for technical assistance.

References

Role of Rho-Associated Kinase in Neointima Formation After Vascular Injury
Rei Shibata, Hisashi Kai, Yukihiro Seki, Seiya Kato, Minoru Morimatsu, Kozo Kaibuchi and Tsutomu Imaizumi

Circulation. 2001;103:284-289
doi: 10.1161/01.CIR.103.2.284

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/103/2/284

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/