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Abstract—Chronic inhibition of endothelial NO synthesis by the administration of N\textsuperscript{3}-nitro-L-arginine methyl ester (L-NAME) to rats induces early vascular inflammation (monocyte infiltration into coronary vessels and monocyte chemoattractant protein-1 expression) as well as subsequent arteriosclerosis. The small GTPase Rho controls cell adhesion, motility, and proliferation and is activated by several growth factors such as angiotensin II. We investigated the effect of a specific inhibitor of Rho-kinase, Y-27632, in rats treated with L-NAME to determine the role of the Rho/Rho-kinase pathway in the development of arteriosclerosis. We found here increased activity of Rho/Rho-kinase after L-NAME administration and its prevention by angiotensin II type 1 receptor blockade. Hydralazine or lecithinized superoxide dismutase (l-SOD) did not affect Rho/Rho-kinase activity. Co-treatment with Y-27632 did not affect the L-NAME–induced increase in cardiovascular tissue ACE activity or L-NAME–induced decrease in plasma NO concentrations, but did prevent the L-NAME–induced early inflammation and late coronary arteriosclerosis. In addition, Y-27632 prevented the increased gene expression of monocyte chemoattractant protein-1 and transforming growth factor-B1 as well as cardiac fibrosis and glomerulosclerosis. These findings suggest that increased activity of Rho/Rho-kinase pathway mediated via the angiotensin II type 1 receptor may thus be important in the pathogenesis of early vascular inflammation and late remodeling induced by chronic inhibition of NO synthesis. The beneficial effects of Rho-kinase inhibition are not mediated by restoration of NO production. The Rho-kinase pathway could be a new therapeutic target for treatment of vascular diseases. (Hypertension. 2002;39:245-250.)

Key Words: Rho-kinase ■ arteriosclerosis ■ remodeling ■ nitric oxide ■ angiotensin

Recent studies have shown that the small GTPase Rho controls multiple cell functions—such as adhesion, proliferation, or migration through the actin cytoskeleton reorganization—or some kinase cascade activation.\textsuperscript{1–4} Because angiotensin II\textsuperscript{5,6} or endothelin-1\textsuperscript{7} has been shown to activate the Rho pathway, its role in the pathogenesis of vascular disorders has become a focus of interest. Indeed, inhibitors of Rho or Rho-kinase (a major target of Rho) have been shown to decrease systolic blood pressure in hypertensive rats,\textsuperscript{8} to reduce brain infarct size after middle cerebral artery occlusion in mice,\textsuperscript{9} and to suppress neointimal formation after balloon injury.\textsuperscript{10}

We have recently reported that chronic inhibition of nitric oxide synthesis by the administration of 78 N\textsuperscript{3}-nitro-L-arginine methyl ester (L-NAME) induces early inflammation (monocyte infiltration and monocyte chemoattractant protein-1 [MCP-1 expression])\textsuperscript{11} and late cardiovascular remodeling in rats.\textsuperscript{12} The importance of our observation is supported by the fact that the adhesion of mononuclear cells to, and their infiltration into, the blood vessel wall are assumed to be crucial early arteriosclerotic events.\textsuperscript{13} We have further demonstrated that ACE inhibition or angiotensin II type 1 receptor blockade prevents such vascular inflammation and subsequent arteriosclerosis in the rat model, suggesting that the increase in angiotensin II activity plays a primary role in the development of such vascular inflammatory and proliferative disorders.\textsuperscript{14–16} However, no direct evidence for the role of Rho/Rho-kinase pathway in the formation of such vascular disorders has not been addressed.

A potent and specific inhibitor of Rho-kinase, Y-27632,\textsuperscript{8} may be a useful tool for investigating the functional importance of Rho-kinase in vivo. Thus, the purpose of this study is to investigate the role of Rho-kinase in the development of early vascular inflammation and late arteriosclerosis in a rat model of chronic inhibition of NO synthesis.
Methods

An Animal Model of Chronic Inhibition of NO Synthesis

Seven groups of 20-week-old Wistar-Kyoto rats (WKY) were studied. The control group received untreated chow and drinking water. The second group (L-NAME) received L-NAME in the drinking water (1 mg/mL). The third group (L+Y10) received L-NAME in the drinking water and a specific inhibitor of Rho-kinase Y-27632 (10 mg/kg per day by an osmotic minipump). The fourth group (L+Y30) received L-NAME in the drinking water and Y-27632 (30 mg/kg per day by an osmotic minipump). The minipump was implanted in the peritoneal cavity under anesthesia 2 days before the initiation of treatment. The fifth group (L+Hyd) received L-NAME and hydralazine (12 mg/kg per day) in the drinking water. The sixth group (L+TCV) received L-NAME and the angiotensin II type 1 receptor antagonist TCV-116 (10 mg/kg per day) in the drinking water. The seventh group (L+1-SOD) received L-NAME in the drinking water and superoxide dismutase17 (1-SOD, 3000U/kg per day IV).

Determination of Rho Translocation and Rho-Kinase Activation

For Rho translocation analyses, 5 rats in 5 groups (control, L-NAME, L+Hyd, L+TCV, L+1-SOD) were killed on the third day of treatment. For Rho-kinase activation analyses,18 5 rats in 4 groups (control, L-NAME, L+Y10, L+Y30, L+1-SOD) were sacrificed on the third day of treatment.

Histopathology and Immunohistochemistry

On the third day or 28th day of treatment, the heart and kidney were fixed with methacarn solution.11,19 Heart sections were immunostained with mouse anti-rat monoclonal antibody against human α-smooth muscle actin (α-SMA), rabbit polyclonal antibody against human α-smooth muscle actin (α-SMA), rabbit polyclonal antibody against human nuclear factor (NF)-κB p50 subunit (Santa Cruz), or nonimmune mouse (rabbit) IgG (Zymed). The slides were washed and incubated with the biotinylated, affinity-purified secondary antibody (rabbit anti-mouse IgG or goat anti-rabbit IgG).

Medial thickening (the wall-to-lumen ratio) and perivascular fibrosis of the coronary arterial wall,11 myocardial reparative fibrosis,14 and glomerular sclerosis (glomerular injury scale29) were studied in each groups of animal as described.

Northern Blot Analysis

Northern blot hybridization was performed as we described previously.11 The cDNA probes used were as follows: a rat MCP-1 cDNA, a rat transforming growth factor-β1 (TGF-β1) cDNA, and a mouse GAPDH. Relative amounts of TGF-β1 and MCP-1 mRNA were normalized against the amount of GAPDH mRNA.

Results

Systolic Blood Pressure and Tissue ACE Activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>ACE Activity, nmol · mg⁻¹ · h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td>125±4</td>
<td>126±3</td>
</tr>
<tr>
<td>L+Y10</td>
<td>130±4</td>
<td>159±3*</td>
</tr>
<tr>
<td>L+Y30</td>
<td>124±4</td>
<td>151±3*</td>
</tr>
<tr>
<td>L+Hyd</td>
<td>127±3</td>
<td>130±2†</td>
</tr>
<tr>
<td>L+TCV</td>
<td>129±3</td>
<td>127±5†</td>
</tr>
<tr>
<td>L+1-SOD</td>
<td>124±4</td>
<td>160±5*</td>
</tr>
</tbody>
</table>

Values are mean±SE. NM indicates not measured.

*P<0.05 vs control, †P<0.05 vs L-NAME.

Cardiac tissue and thoracic aorta were isolated and the ACE activity was measured using fluorometric assay.14,15

Plasma NOX Concentration

Plasma NOx concentration was measured by a fluorometric assay.

Statistical Analysis

Data are expressed as the mean±SE. Statistical differences were determined by ANOVA and Bonferroni’s multiple comparison test. A level of P<0.05 was considered statistically significant.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

RhoA Translocation and MBS phosphorylation

To test whether Rho/Rho-kinase system is upregulated, immunoblot analysis of RhoA was performed. Compared with the control group, the L-NAME, L+Y10, and L+Y30 groups had greater systolic arterial pressures on days 3, 14, or 28 of treatment (Table). Co-treatment with 30 mg of Y27632 significantly decreased the L-NAME–induced rise in systolic arterial pressure, whereas 10 mg of Y27632 had no effect. The concomitant administration of hydralazine or TCV-116 prevented L-NAME–induced rise in systolic arterial pressure on day 3 (Table). Treatment with l-SOD did not affect such hypertensive changes (Table).

Inflammatory and Proliferative Changes on Day 3

Three days after L-NAME treatment was started, the rats in the L-NAME group had marked infiltration of ED1-positive monocytes into the intima and adventitia of their coronary
arteries (Figure 2). Spindle-shaped, fibroblast-like cells positive for α-SMA (myofibroblasts), which might have been derived from fibroblast or vascular smooth muscle cells, were also seen in perivascular area of coronary arteries. To assess the proliferation, PCNA staining was performed. Nuclear staining for PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, and monocytes or myofibroblast-like cells in the L-NAME group. To examine NF-κB activity, NF-κB/p50 immunostaining was performed (Figure 2). In L-NAME group, a considerable number of endothelial cells and medial smooth muscle cell showed intense nuclear staining for NF-κB. No such inflammatory and proliferative changes were observed in the control group. In rats treated with L-NAME plus Y-27632, such inflammatory and proliferative changes were markedly suppressed. When ED1-positive monocytes or PCNA-positive cells were counted, the number of immunopositive cells per section was significantly greater in the L-NAME group than in the control group (Figure 3A). The increases in ED1-positive cells and PCNA-positive cells were both significantly reduced by treatment with the low and high doses of Y-27632 (Figure 3A). As we previously reported,12,16 these inflammatory and proliferative changes were not reduced by hydralazine but were prevented by TCV-116 (data not shown). Treatment with l-SOD markedly reduced such inflammatory and proliferative changes (data not shown).

Vascular Remodeling, Myocardial Remodeling, and Glomerular Injury on Day 28
Compared with control group, medial thickening (the wall-to-lumen ratio) and perivascular fibrosis of coronary arteries were significantly greater in the L-NAME group. Co-treatment with the low and high doses of Y-27632 inhibited the L-NAME–induced medial thickening and perivascular fibrosis (Figures 2B and 3B). The increase in cardiac fibrosis induced by L-NAME administration was also reduced by the low and high doses of Y-27632 (Figure 2C). Treatment with l-SOD prevented such vascular and myocardial remodeling (Figure 3A through 3C).

Compared with control group, the glomerular injury score was significantly greater in the L-NAME group. Co-treatment with the low and high doses of Y-27632 significantly inhibited the L-NAME–induced glomerulosclerosis (Figure 3D).

Expression of TGF-β1 and MCP-1 mRNA on Day 3
As we have previously shown,11,19 the cardiac TGF-β1 and MCP-1 mRNA levels were significantly greater in the L-NAME group (Figure 4). The increased expressions of TGF-β1 and MCP-1 mRNA were both prevented by treatment with the low and high doses of Y-27632.

Tissue ACE Activity on Day 3
Compared with control group, the cardiac and aortic tissue ACE activities were significantly greater in the L-NAME group (Table). Co-treatment with the low and high doses of Y-27632 did not inhibit the increases in cardiac and aortic tissue ACE activities.

Plasma NOx Concentration
Plasma NOx concentration was significantly decreased in the L-NAME group (1.0±0.1 μmol/L, P<0.01 versus control) compared with the control group (2.6±0.3 μmol/L). Treatment of Y-27632 partially restored serum NOx level in L+Y10 (1.3±0.1 μmol/L, P=0.05 versus L-NAME group, P<0.01 versus control) and L+Y30 groups (1.4±0.1 μmol/L, P=0.05 versus L-NAME group, P<0.01 versus control).

Discussion
We have demonstrated herein that (1) membrane translocation of Rho is increased in cardiac tissues from the L-NAME group through stimulation of angiotensin II type 1 receptor; (2) treatment with a Rho-kinase inhibitor Y-27632 markedly inhibited early inflammatory changes as well as late cardiovascular remodeling; and (3) Y-27632 did not restore NO production after L-NAME administration. Our present findings suggest that activation of Rho to Rho-kinase pathway may be essential in the development of cardiovascular disorders in this model.

Rho has been shown to translocate from the soluble to the particulate fraction on its activation.21 In this study, we found the increased membrane translocation of RhoA protein in the heart of L-NAME group, indicating RhoA activation in cardiac tissue. This RhoA activation was prevented by angiotensin II type 1 receptor blockade but not with hydral-
azine. We also found that treatment with Y-27632 did not affect the increase in tissue ACE activity after inhibition of NO synthesis. Previously, we demonstrated in our rat model that the increased angiotensin II activity caused by overexpression of tissue ACE mediates early inflammation and later cardiovascular remodeling.14,15 Prior in vitro studies showed angiotensin II–induced Rho pathway activation in several cell types.5,6 Overall, it is suggested that an increase in angiotensin II activity mediated via type 1 receptor caused cardiac Rho activation in this model in vivo.

We further examined Rho-kinase activity by measuring MBS phosphorylation level. As demonstrated previously by other investigators,10,22 our present data suggest specific suppression of cardiovascular Rho-kinase activity by Y-27632. Thus, it is reasonable to assume that Y-27632 prevented cardiovascular remodeling (coronary vascular medial thickening, perivascular and myocardial fibrosis, and glomerulosclerosis) in our model by inhibiting the increased Rho-kinase activity in a site-specific manner. This conclusion may be supported by a recent report demonstrating that treatment with statins, a nonselective inhibitor of Rho, attenuated angiotensin II–induced cardiovascular remodeling.23

Because Y-27632 is reported to decrease systolic blood pressure in hypertensive rats,8 the beneficial effects of Y-27632 might result from the decrease in systolic arterial pressure after L-NAME administration. In the present study, however, a low dose of Y-27632 did not affect the systolic loading conditions but partly inhibited early inflammation, cardiovascular remodeling, and glomerulosclerosis. We have previously shown that normalization of L-NAME–induced systolic hypertension by hydralazine did not affect both early inflammation and late cardiovascular remodeling.12 We measured plasma NOx concentrations and found that inhibition of NO synthesis was not restored by Y-27632. Thus, it is likely that the cardiovascular and renal protective effects of Y-27632 may not be explained totally by its antihypertensive effect or by restoration of NO production.

Figure 2. Histopathological and immunohistochemical photomicrographs of coronary artery sections from the control group, L-NAME group, L+Y10 or L+Y30 group. A, Coronary artery sections from a control rat, a rat receiving L-NAME, and a rat receiving L-NAME+Y-27632 for 3 days are stained with Hematoxylin-eosin (HE) or stained immunohistochemically for ED1, PCNA, α-SMA, or NF-κB/p50. Bar, 50 μm. B, Coronary arteries sections stained with Masson’s trichrome stain from a control rat, a rat receiving L-NAME, and a rat receiving L-NAME+Y-27632 at 30 mg/kg per day for 28 days. The bar indicates 100 μm.
Another important feature emerged in the present study is that the treatment with Y-27632 also prevented proliferative disorders (the number of PCNA-positive cells) and increased gene expression of MCP-1 and TGF-β1. Proliferating monocytes, endothelial cells, and/or smooth muscle cells are capable of producing growth-promoting factors. We have previously shown that MCP-1 mediates early inflammations (monocyte recruitment) and subsequent vascular medial thickening and that TGF-β1 mediates perivascular and myocardial fibrosis in this rat model. Therefore, we hypothesized that Rho and Rho-kinase activation increased gene expression and activity of MCP-1 and TGF-β1, and thus caused early inflammation and proliferative disorders and subsequent cardiovascular remodeling. Treatment with Y-27632 thereby prevented such pathological disorders by blocking gene expression and biological activity of MCP-1 and TGF-β1.

However, the precise mechanism by which Rho-kinase activation leads to increased gene expression of MCP-1 or TGF-β1 is unclear. It is reported that angiotensin II–induced expression of MCP-1 or TGF-β1 gene is transcriptionally regulated, respectively, by NF-κB and AP-1, and that Rho may activate such transcriptional factors. In this study, we have shown that Y-27632 prevented immunohistochemically documented NF-κB activation in the coronary arteries. Therefore, it is possible to assume that increased transcription mediated by Rho to Rho-kinase pathway might be involved in the pathogenesis of increased gene expression of MCP-1 and TGF-β1 after chronic inhibition of NO synthesis. Additional studies are needed to prove this claim.

We have previously demonstrated that oxidative stress participates importantly in the development of early inflammation, NF-κB activation and cardiovascular remodeling. Thus, we examined the role of oxidative stress and found that oxidative stress might participate independent of the Rho pathway or locate downstream to the Rho system.

In conclusion, our present data suggests that the Rho to Rho-kinase pathway plays an essential role in the pathogenesis of early vascular inflammation and late cardiovascular remodeling induced by chronic inhibition of NO synthesis. Rho or Rho-kinase system has been shown to be involved in various cardiovascular disorders such as hypertension, vascular remodeling after balloon injury, brain ischemia, and vasospasm. These data suggest that Rho to Rho-kinase pathway will be a new target for prevention and treatment of cardiovascular diseases.

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References


Important Role of Rho-kinase in the Pathogenesis of Cardiovascular Inflammation and Remodeling Induced by Long-Term Blockade of Nitric Oxide Synthesis in Rats
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