Rho-Associated Protein Kinase Contributes to Early Atherosclerotic Lesion Formation in Mice

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Abstract—Members of the Rho family of small GTPases have been recently implicated in inflammatory signaling. We examined the effect of in vivo inhibition of Rho kinase on atherogenesis in mice. Low-density lipoprotein receptor (LDLR) knockout (KO) mice fed a cholate-free high-fat diet received daily intraperitoneal injection of saline (n=8, control group) or Y-27632 (30 mg/kg, n=9), a specific Rho kinase inhibitor. After 9 weeks, Y-27632 treatment resulted in significant in vivo inhibition of Rho kinase activity (P=0.004). Body weights, arterial blood pressures, and plasma cholesterol levels were comparable in both groups. Atherosclerotic lesion size in the aortic sinus and thoracic aorta of mice treated with Y-27632 was reduced by respectively 35% and 29% in comparison with the saline-treated animals (P=0.006 and P=0.03, respectively). This was associated with a significant reduction in T lymphocyte accumulation (P=0.035) and expression of p65 subunit of NF-κB within plaques (P<0.05). In vitro, treatment with Y-27632 inhibited p65 phosphorylation and degradation of IkBα in mouse peritoneal macrophages and significantly inhibited concanavalin A–induced proliferation of spleen-derived T cells (P<0.001). In conclusion, inhibition of Rho kinase significantly limits early atherosclerotic plaque development in the LDLR KO mice. This study identifies Rho kinase inhibitors as potential candidates for the treatment of atherosclerosis. (Circ Res. 2003;93:884-888.)

Key Words: atherosclerosis ■ inflammation ■ lymphocytes ■ signal transduction ■ nuclear factor-κB

Atherosclerosis is the leading cause of cardiovascular morbidity and mortality in the world. Recent consistent studies from several groups have elucidated the critical role of inflammation in the development and complications of atherosclerosis. However, despite the increasing knowledge regarding the role of inflammation in atherogenesis, the precise intracellular transduction pathways involved in this process remain unknown. Recent studies reported enhanced activation of the transcription factor nuclear factor-κB (NF-κB) after a high-fat cholate-free diet in LDLR KO mice, but its direct role or that of other signaling pathways in atherogenesis was not assessed.

Small G proteins of the Rho family, initially described as key regulators of the actin cytoskeleton, are now known to play a major role in many biological processes including calcium sensitization of smooth muscle contraction, migration, gene transcription, proliferation, and transformation. They have also been implicated in the regulation of signal transduction cascades related to inflammation, particularly the JNK and NF-κB pathways. These cellular functions and transduction pathways may be relevant to the atherogenic process. However, although a role for Rho/Rho kinase activation has been recently reported in experimental models of vascular inflammation or injury, hitherto no information is available regarding the role of Rho kinase in atherosclerosis. Therefore, we examined the effects of in vivo inhibition of the Rho effector, Rho kinase, on the development of early atherosclerosis in LDLR KO mice.

Materials and Methods

Mice

Seventeen 10-week-old C57BL/6J LDLR KO mice (kindly provided by Dr Francis Bayard, Toulouse, France) were placed on a high-fat (15% cacao butter, 1.25% cholesterol), cholate-free diet (UAR, France) with free access to food and water. One group of mice (n=9) received daily intraperitoneal injection of Y-27632 (30 mg/kg) for 9 weeks. Another group of mice (n=8) received daily saline injections and served as control. Carotid arterial pressures were recorded invasively at the time of euthanasia using an intravascular catheter and a Gould apparatus. This study was conducted in accordance with both institutional guidelines and those formulated by the European community for experimental animal use.

Tension Experiments

The aorta were properly dissected in situ and collected in physiological saline solution (PSS, in mmol/L: 130 NaCl, 5.6 KCl, 1.5 MgCl₂, 2 CaCl₂, 11 glucose, 10 Tris, pH 7.4 with HCl) cleaned of fat
and adherent connective tissue, and cut in rings. The endothelium was carefully removed by gently rubbing the intimal surface with the tip of small forceps. The absence of endothelium was then checked in each ring by the inability of carbachol to relax phenylephrine-induced contraction. All the processes required to prepare arterial rings were performed in minimal time, and the duration between euthanasia and the tension studies ranged between 15 and 30 minutes. Endothelium-denuded smooth muscle rings were suspended under isometric conditions and connected to a force transducer in organ baths filled with Krebs-Henseleit solution maintained at 37°C, and equilibrated with 95% O2/5% CO2. The preparations were initially placed under a resting tension of 1000 mg, left to equilibrate for 1 hour, and washed at 20-minute intervals, before stimulation with contracting agonists.

Analysis of Atherosclerotic Lesions
Plasma cholesterol and HDL were measured with a commercially available Cholesterol kit (Sigma). Morphometric and immunohistochemical studies were performed in the aortic sinus and the thoracic aorta (spanning from the brachiocephalic artery to the renal arteries and including the first 3 mm2 of the brachiocephalic artery). Cryostat sections (10 μm) from the aortic sinus were processed for detection of lipid deposition using Oil red, detection of collagen using Sirius red, and immunohistochemical studies as previously described. Staining for the p65 subunit of the transcription factor NF-κB (antibody directed against p65 (Chemicon), and anti-CD3 (Dako); and anti-CD30 from Sigma) and for the nucleus using DAPI were performed in the aortic sinus and the thoracic aorta (spanning from the brachiocephalic artery to the renal arteries and including the first 3 mm2 of the brachiocephalic artery). Cryostat sections (10 μm) from the aortic sinus were processed for detection of lipid deposition using Oil red, detection of collagen using Sirius red, and immunohistochemical studies as previously described. The sections were stained with specific primary antibodies: anti-mouse macrophage, clone MOMA2 (BioSource), phosphatase alkaline-conjugated anti-α-actin (Sigma), and anti-CD3 (Dako); and anti-CD30 from Sigma) as previously described. Staining for the p65 subunit of NF-κB was performed using a specific goat anti-p65 antibody (Santa Cruz) or a biotinylated mouse monoclonal antibody directed against p65 (Chemicon). Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins. Quantification of stained sections was done blindly using computer assisted-image quantification (NS15000, Microvision), whereas CD3 or p65-positive cells were microscopically counted in a blinded manner as previously described. At least 5 sections taken 80 μm apart were stained per animal and were used for quantification.

Cell Culture, Activation, and Proliferation
T cells were extracted from the spleen of 5 LDLR KO mice as previously described. For the cell proliferation assay, spleen-derived T cells were incubated with concanavalin A (conA) (0.5 μg/mL-Sigma) in the presence or absence of Y-27632 (0.15 to 20 μmol/L) in 96 well plates. The cells were stimulated for 72 hours, and 1 μCi of [3H]thymidine (Perkin Elmer) was added for the last 10 hours of cell culture. Proliferation was also assessed by a direct count derived T cells were incubated with concanavalin A (0.5 μg/mL-Sigma) in the presence or absence of Y-27632 (0.15 to 20 μmol/L). In vitro treatment of aortic rings with Y-27632 (10 μmol/L) inhibited the NA (10 μmol/L)-induced contraction by 78±2% in control mice but only by 12±8% in Y-27632-treated mice (P<0.0001). These data indicate that Rho/Rho kinase–mediated Ca2+ sensitization of the contraction was inhibited in Y-27632–treated mice, strongly suggesting effective in vivo blockade of Rho kinase activity.

Effect of Rho Kinase Inhibition on Atherosclerotic Lesion Size and Composition
All animals were healthy during the whole experiment and no clinical or pathological signs of toxicity were observed. After 9 weeks of treatment with either Y-27632 or saline, animal weights (24.7±1.5 versus 25.0±1.2 g, respectively), total cholesterol (394.7±45.6 versus 402.0±23.6 mg/dL, respectively), and high-density lipoprotein serum levels (49.1±9.9 versus 67.7±10.4 mg/dL, respectively) were not different between the 2 groups. However, analysis of aortic sinus sections stained with Oil red revealed significant differences in lipid deposition and atherosclerotic plaque size (Figure 1). Morphometric analysis showed that administration of the Rho kinase inhibitor for 9 weeks induced a significant 35% reduction in lesion size compared with control mice (137 332±16 516 versus 212 502±15 937 μm2; P=0.006). Lesion size in the aortic arch and thoracic aorta was also significantly reduced (29% reduction) in mice treated with Y-27632 compared with saline-treated animals (9.2±1.4% versus 12.9±0.5%, respectively; P=0.03). These findings underscore the role of endogenous Rho kinase activity in the early development of atherosclerosis. We next examined plaque composition. We found that relative macrophage, smooth muscle cell, or collagen accumulation, expressed as the percentage per lesion cross-sectional area occupied by these plaque components, was not different between Y-27632–treated and saline-treated animals (macrophages, 42.5±3.4% versus 43.9±2.2%, respectively; smooth muscle cells, 10.3±1.4% versus 8.5±1.4%, respectively; collagen, 16.2±3.1% versus 16.0±1.9%, respectively). It is noteworthy that the number of CD3-positive T lymphocytes per lesion area was reduced by 40% (P<0.05) in Y-27632–treated mice compared with controls (Figure 2), suggesting a
T lymphocyte effect of Y-27632. Interestingly, we found a significant 45% reduction in the expression of p65 NF-κB subunit in atherosclerotic lesions of mice treated with Y-27632 compared with controls (P<0.05; Figure 2).

The reduction in T cell infiltration and expression of p65 subunit of NF-κB in plaques of mice treated with Y-27632 may be an indirect consequence of reduced plaque development. Therefore, we examined the direct effect of Y-27632 on T lymphocyte proliferation and activation of NF-κB in vitro. As shown in Figure 3, Y-27632 was found to exert a profound inhibitory and dose-dependent effect on concanavalin A (conA)-induced proliferation of spleen-derived T lymphocytes (P<0.001 for the global comparison). These results were confirmed by a direct cell count and by analysis of the decay in CFSE intensity on FACS (data not shown). In addition, incubation of Y-27632 with LPS-activated or interleukin-1 (IL-1)–activated peritoneal macrophages resulted in a clear inhibition of iκBα degradation and p65 phosphorylation (Figure 4).

Discussion

Despite the significant progress in our understanding of the pathophysiology of atherosclerosis, little is known about the role of specific transduction pathways in the development and progression of this serious disease.

Atherosclerosis is an inflammatory disease of the arterial wall. Our interest in the role of Rho kinase in atherosclerosis is based on recent observations suggesting a role for Rho/Rho kinase system in biological pathways that may be related to the atherosclerotic process. In endothelial cells, Rho/Rho kinase activation was reported to play a role in oxidized LDL-induced endothelial cell contractility17 and in the modulation of endothelial fibrinolytic activity.18 In vascular smooth muscle cells, the Rho/Rho kinase system is involved in proliferation/migration,19,20 and angiotensin II–induced expression of monocyte chemoattractant protein–1 (MCP-1) and plasminogen activator inhibitor type–1.21 In fibroblasts and inflammatory cells, activation of Rho signaling pathways has been shown to induce the transcriptional activity of serum response factor, activator protein–1, and NF-κB, which are potentially important in atherogenesis.5–9,23 In addition, in

![Figure 1. Reduction of atherogenesis by Y-27632 treatment.](image1)

Representative photomicrographs (lipid staining) of aortic sinus from mice treated with saline (a) or Y-27632 (b). c, Quantitative computer-assisted image analysis of lesion size in the aortic sinus of saline or Y-27632–treated mice. Data represent mean values with SEM. **P=0.006. Original magnification, ×40.

![Figure 2. Representative photomicrographs of cellular staining for CD3 (arrows in a) and p65 subunit of NF-κB (arrows in b) in plaques of LDLR KO mice. L denotes the lumen. Immunostainings for T lymphocytes and p65 subunit were performed as described in Materials and Methods. Original magnification: a, ×400; b, ×200. c, Effect of Y-27632 treatment on lesion expression of p65 NF-κB subunit and T cell content. Quantitative computer-assisted image analysis was used to determine the number of p65-positive cells (black bars) or infiltrating T lymphocytes (gray bars) per mm² in aortic sinus lesions of control (cont) or Y-27632–treated mice. Data represent mean values with SEM. *P<0.05.)](image2)
vivo studies have recently identified a role for Rho/Rho kinase activation in experimental models of vascular inflammation or injury,\textsuperscript{10–13} but no information is available regarding the role of Rho kinase in experimental models of atherosclerosis. Interestingly, the antiinflammatory properties of 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors may be mediated, at least in part, by inhibition of Rho proteins isoprenylation, thus preventing membrane attachment of Rho proteins and the subsequent activation of downstream effectors such as Rho kinase.\textsuperscript{24,25} However, no data are available regarding the role of Rho kinase in mediating the reported effects of statins on plaque size or composition. Based on these observations, our aim was to assess the direct role of Rho kinase activity on the development of atherosclerosis.

LDLR KO mice were treated with the well-characterized inhibitor of Rho kinase activity, Y-27632 compound.\textsuperscript{6} In this study, we used a previously published dose\textsuperscript{14} (30 mg/kg daily for 9 weeks) that resulted in a marked inhibition of in vivo Rho kinase activity as shown by the strong decrease in NA-induced contraction in aortas of Y-27632–treated mice. This in vivo inhibition of Rho kinase activity was associated with a significant reduction in atherosclerotic lesion size in 2 lesion-prone arterial sites. Our results obtained in a well-validated mouse model of atherosclerosis suggest that endogenous Rho kinase activity contributes to early lesion formation in this complex disease. The role of Rho kinase in more advanced stages of atherosclerosis remain to be determined. The antiatherogenic mechanisms of Y-27632 remain speculative and may involve many of the pathophysiological processes cited above. However, our observations that Y-27632 inhibits LPS- and IL-1β–induced NF-κB activation in both peritoneal mouse macrophages (Figure 4 of the present study) and human peripheral blood mononuclear cells in vitro\textsuperscript{26} point to a potentially important role for NF-κB modulation in this context. This is supported by our finding of a significant reduction in p65 expression in lesions of Y-27632–treated mice. Although changes in p65 expression do not reflect NF-κB activation, the pattern of p65 staining within the plaques (Figure 2b) highly suggested a nuclear-associated staining. In contrast to staining for p65, staining for phosphorylated p65 was much harder to detect within the plaques. However, we have been able to detect phosphorylated p65 in 3 out of 6 plaques from saline-treated mice but failed to detect any phosphorylated p65 in plaques of Y-27632 mice (data not shown).

Another observation made in plaques of Y-27632–treated mice was the significant reduction in the number of T lymphocytes per lesion area, which is compatible with an antiproliferative effect of Y-27632 on T lymphocytes. This is in agreement with recent studies showing an important modulatory role of Rho/Rho kinase system in T lymphocyte homeostasis\textsuperscript{37–31} and is directly supported by our observation in the present study (Figure 3) and a recent report by Thaara\textsuperscript{29–30} of a profound inhibitory effect of Y-27632 on conA-induced T cell proliferation in vitro. However, a direct causal link between T cell reduction and changes in plaque size has not been demonstrated in this study.

The effects of Y-27632 were obtained at doses shown to be responsible for specific inhibition of Rho kinase.\textsuperscript{6,33} Inhibition of other kinases is rarely observed using Y-27632, the affinity of Rho-associated kinase for Y-27632 being at least 20 times higher than that for two other kinases, citron kinase and protein kinase PKN.\textsuperscript{34} Nevertheless, a small in vivo contribution of Rho effector kinases other than Rho-associated kinase to the early development of atherosclerosis cannot be ruled out.

In conclusion, we show for the first time that endogenous Rho kinase activity contributes to the development of early atherosclerosis in mice, possibly through its modulatory activity on NF-κB activation and T lymphocyte proliferation. Rho kinase activity should be considered as a novel target in the treatment of atherosclerosis.

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