Rho-Kinase Mediates Angiotensin II–Induced Monocyte Chemoattractant Protein-1 Expression in Rat Vascular Smooth Muscle Cells

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Abstract—Recently, it was shown that Rho-kinase plays an important role in blood pressure regulation. However, it is not known whether Rho-kinase is involved in atherogenesis. Monocyte chemoattractant protein-1 (MCP-1) is an important chemokine that regulates monocyte recruitment and atherogenesis. Therefore, we examined the role of Rho and Rho-kinase in the angiotensin (Ang) II–induced expression of MCP-1. Ang II dose- and time-dependently enhanced the expression of MCP-1 mRNA and the protein production in vascular smooth muscle cells. CV11974, an Ang II type 1 receptor (AT₁-R) specific antagonist inhibited the enhancement of MCP-1 expression by Ang II, suggesting that the effect of Ang II is mediated by the AT₁-R. Botulinum C3 exotoxin, a specific inhibitor of Rho, suppressed Ang II–induced MCP-1 production. To examine the role of Rho-kinase in Ang II–induced MCP-1 expression, we used adenovirus-mediated overexpression of the dominant negative mutant of Rho-kinase (AdDNRhoK) or Y-27632, a specific inhibitor of Rho-kinase. Both AdDNRhoK and Y-27632 strongly inhibited Ang II–induced MCP-1 expression. Although inhibition of extracellular signal–regulated protein kinase (ERK) by PD 098,059 also inhibited Ang II–induced MCP-1 expression, Y-27632 did not affect Ang II–induced activation of ERK. These results indicate that Rho-kinase plays a critical role in Ang II–induced MCP-1 production independent of ERK. The Rho–Rho-kinase pathway may be a novel target for the inhibition of Ang II signaling and the treatment of atherosclerosis.

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Key Words: angiotensin II ■ peptides ■ muscle, smooth, vascular ■ receptors, angiotensin ■ kinase

Angiotensin (Ang) II has been known to regulate blood pressure, fluid homeostasis, and electrolyte balance.1 Recent studies have shown that Ang II plays an important role in atherogenesis as well. The physiological functions of Ang II are transmitted into target cells through its specific receptor located in the plasma membrane. Although there are 2 isoforms for the Ang II receptor, the Ang II type 1 receptor (AT₁-R)² and the Ang II type 2 receptor (AT₂-R),³ most of the cardiovascular effects of Ang II are ascribed to AT₁-R. Vascular smooth muscle cells (VSMCs) express AT₁-R, and Ang II induces the production of growth factors and extracellular matrices through this receptor. We have recently reported that Ang II induced interleukin-6 production in VSMCs and have proposed that the Ang II–induced cytokine plays an important role in the progression of atherosclerosis.⁴

Invasion of monocytes into the blood vessel wall is one of the early steps in the development of atherosclerosis. Various cytokines, such as monocyte chemoattractant protein-1 (MCP-1),⁵ macrophage inflammatory protein-1,⁶ and RANTES (regulated on activation, normal T–expressed and –secreted),⁷ are known to regulate the movement of monocytes. Among these factors, MCP-1 is one of the most potent chemoattractants for monocytes or macrophages in vitro and in vivo. MCP-1 expression is induced in response to tumor necrosis factor-α or γ-interferon,⁸ thrombin,⁹ or interleukin-1β¹⁰ in VSMCs. Pathological conditions such as hypercholesterolemia and vascular injury also induce expression of the MCP-1 gene in the vascular wall. Recent studies have suggested that MCP-1 is critical for the progression of atherosclerosis. Targeted disruption of the receptor for MCP-1 (CCR2) attenuated the development of atherosclerosis when the mice were crossed with apoE knockout mice that develop severe atherosclerosis.¹¹

Rho-kinase, identified as a downstream target of Rho A, has been shown to phosphorylate the myosin-binding subunit of myosin light chain phosphatase and enhance smooth muscle contraction.¹²,¹³ Y-27632, a specific inhibitor of Rho-kinase, is reported to reduce blood pressure in hypertensive rats but not in normotensive rats.¹⁴ Recently, a role of Rho A in Ang II–induced actin organization in cardiac myocytes has been reported.¹⁵ However, the role of Rho and Rho-kinase in Ang II–
induced gene expression has not been determined. In the present study, we examined whether Rho and Rho-kinase were involved in Ang II–induced MCP-1 expression in VSMCs.

**Methods**

**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM) and FBS were purchased from Gibco-BRL. Ang II was purchased from the Peptide Institute. BSA was purchased from Sigma Chemical Co. (α-32P)dCTP was obtained from DuPont-NEN. CV11974, a specific AT1-R antagonist, was obtained from Takeda Chemical Industries, Ltd. PD123319, a specific AT2-R antagonist, was obtained from Warner-Lambert, Park Davis Co. Adenovirus vector expressing the dominant negative mutant of Rho-kinase (AdDNRhoK) has been described previously.16 Botulinum C3 exotoxin, a specific inhibitor of Rho, was purchased from Calbiochem. Y-27632, a specific inhibitor of Rho-kinase, was obtained from Yoshitomi Chemical Industries, Ltd. Antibodies against the phosphorylated form of extracellular signal–regulated protein kinase (ERK) and ERK were obtained from New England Biolabs. Unless indicated otherwise, other chemical reagents were purchased from Wako Pure Chemicals.

**Cell Culture**

VSMCs were isolated from the thoracic aortas of Sprague-Dawley rats and maintained as described previously.4 Passages 5 through 12 were used for the experiments. Cells were grown to confluence in DMEM with 10% FBS, growth-arrested in DMEM with 0.1% BSA for 2 days, and then used for the experiments.

**Infection of AdDNRhoK**

Confluent VSMCs were washed with PBS twice and incubated with AdDNRhoK at a multiplicity of infection (MOI) of 1 to 30 in PBS for 2 hours at room temperature. Cells were washed 3 times with PBS and incubated in DMEM with 0.1% BSA for 48 hours. Then the cells were stimulated with Ang II.

**Northern Blot Analysis**

Total RNA was prepared by an acid guanidinium–phenol–chloroform extraction method.17 Northern blot analyses of MCP-1 mRNA and 18S rRNA were performed as described previously.4 The radioactivity of the hybridized band of MCP-1 mRNA or rRNA was measured by a Phosphorimager (FUJIFILM). The medium of nonstimulated or Ang II–stimulated VSMCs was collected and centrifuged at 12 000 rpm for 1 minute. The supernatant was stored at –70°C until assay. A sandwich ELISA for MCP-1 (Quantikine Rat MCP-1, R&D Systems) was performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech). It has been shown that phosphorylation of ERK is associated to the activation.19 Therefore, phosphorylation was taken as a measure of enzymatic activity. The membranes were stripped by incubation in a buffer containing 2% SDS, 100 mMol/L Tris-HCl, pH 7.4, and 100 mMol/L mercaptoethanol at 70°C for 1 hour and reprobed with an antibody against ERK.

**Statistical Analysis**

Statistical analyses were performed by 1-way ANOVA and a multiple comparison (Fisher exact) test if appropriate. A value of P<0.05 was considered to be significant.

**Results**

**Ang II Increased MCP-1 Production**

VSMCs were stimulated with Ang II, and MCP-1 protein in the supernatant was measured by sandwich ELISA. As shown in Figure 1A, Ang II dose-dependently increased MCP-1 production at 12 hours of stimulation. CV11974 (10−5 mol/L) inhibited the enhancement of MCP-1 protein expression by Ang II (10−7 mol/L). However, PD 123,319 (10−7 mol/L) did not show a significant effect (Figure 1B).

**Expression of MCP-1 mRNA by Ang II**

Next, we examined the effect of Ang II on MCP-1 mRNA expression. Figure 2A shows the time course of Ang II (10−7 mol/L)–induced MCP-1 mRNA expression. The expression of MCP-1 mRNA reached peak at ~3 to 6 hours after stimulation and then decreased. VSMCs were incubated with Ang II (10−7 mol/L) for 2 days, and then used for the experiments. Cells were grown to confluence in DMEM with 10% FBS, growth-arrested in DMEM with 0.1% BSA for 2 days, and then used for the experiments.

**Western Blot Analysis**

VSMCs were lysed in a sample buffer (50 mMol/L NaCl, 30 mMol/L sodium phosphate, 50 mMol/L NaF, 5 mMol/L EDTA, 10 mMol/L Tris-HCl, pH 7.6, 1% Triton X, 0.5% pepstatin A, 0.2 U/mL aprotinin, 5 mMol/L leupeptin, and 1 mMol/L phenylmethlysulfonyl fluoride) after stimulation. Protein concentration was quantified by a BCA protein assay reagent (Pierce). Twenty micrograms of total protein was electrophoresed on 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon, Millipore Co) electrophoretically (100 V, for 1 hour). Detection of phosphorylated ERK was performed by using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech). It has been shown that phosphorylation of ERK is associated with the activation.19 Therefore, phosphorylation was taken as a measure of enzymatic activity. The membranes were stripped by incubation in a buffer containing 2% SDS, 100 mMol/L Tris-HCl, pH 7.4, and 100 mMol/L mercaptoethanol at 70°C for 1 hour and reprobed with an antibody against ERK.

**Figure 1.** MCP-1 in the supernatant of cultured rat VSMCs. VSMCs were grown to confluence in 24-well plates and growth-arrested in DMEM with 0.1% BSA for 2 days. Then VSMCs were stimulated with Ang II for 12 hours, and production of MCP-1 in the supernatant was measured by sandwich ELISA. A, VSMCs were cultured with various concentrations of Ang II. B, VSMCs were cultured with Ang II after pretreatment with either an AT1-R antagonist (CV11974, 10−5 mol/L) or an AT2-R antagonist (PD123319, 10−5 mol/L) for 30 minutes. C, VSMCs were infected with AdDNRhoK or AdlacZ or preincubated with Y-27632 (30 minutes), C3 exotoxin (48 hours), or PD98059 (30 minutes). Then, cells were stimulated with Ang II for 12 hours. *P<0.05 vs Control.
the various concentrations of Ang II indicated in Figure 2 for 6 hours. The expression of MCP-1 mRNA by Ang II stimulation was increased dose-dependently (Figure 2B). Preincubation with CV11974 (10^{-5} mol/L) did not affect the expression (Figure 2C). These results indicate that Ang II stimulates MCP-1 expression through AT_{1}-R in VSMCs and that the Rho-Rho-kinase pathway is critical for Ang II–induced MCP-1 expression.

Critical Role of Rho and Rho-Kinase in Ang II–Induced MCP-1 Expression

Recently, Ang II–induced Rho A activation was reported. Therefore, we examined whether inhibition of the Rho pathway affected Ang II–induced MCP-1 expression. Botulinum C3 exotoxin, a specific inhibitor of Rho, inhibited Ang II–induced MCP-1 production (Figure 1C).

Next, we investigated whether Rho-kinase, a target molecule of Rho A, is involved in Ang II–induced MCP-1 expression. VSMCs were infected with AdDNRhoK at 1 to 30 MOI or preincubated with Y-27632 (10^{-5} mol/L, 30 minutes). AdDNRhoK MOI-dependently suppressed the Ang II–induced MCP-1 mRNA expression (Figure 3A). Y-27632 also suppressed the Ang II–induced MCP-1 mRNA expression as well.

Y-27632 Did Not Affect Ang II–Induced ERK Activation

Previously, it has been reported that ERK is important for Ang II–induced MCP-1 expression. PD 098,059, a specific inhibitor of ERK kinase, inhibited Ang II–induced MCP-1 protein expression; however, the adenovirus vector expressing LacZ (AdLacZ, 30 MOI) that was used as a negative control did not show a significant effect. AdDNiRhok MOI-dependently suppressed the Ang II–induced MCP-1 mRNA expression (Figure 3C). Y-27632 also suppressed the Ang II–induced MCP-1 mRNA expression as well.

Discussion

The major findings of the present study are that Ang II induces MCP-1 expression through AT_{1}-R in VSMCs and that the Rho–Rho-kinase pathway is critical for Ang II–induced MCP-1 expression.
Recently, it has been reported that Rho A plays an important role in Ang II–induced premyofibril formation by Ang II. However, the pathway linking AT₁-R and Rho A is not clear. Generally, there are few data involving the mechanism of activation of the Rho A guanine nucleotide exchange factor that activates Rho GTPase by extracellular ligand.22 Lysophosphatidic acid was reported to activate Rho through Gα13, whereas thrombin activated Rho through Gα12. It has been suggested that Gα directly binds to the Rho A guanine nucleotide exchange factor. Recently, it has been reported that AT₁-R is coupled with Gα13 in rat portal vein myocytes. Although it is not clear whether the same G protein is coupled with AT₁-R in VSMCs, this pathway may link AT₁-R to Rho A. Another possibility is the epidermal growth factor (EGF) receptor–dependent pathway, because lysophosphatidic acid–induced Rho activation was blocked by a dominant negative version of the EGF receptor. Transactivation of the EGF receptor by Ang II through AT₁-R is also reported to play a critical role in the signaling of AT₁-R. Therefore, it may be possible that the EGF receptor–dependent pathway mediates Ang II–induced Rho A activation. However, further investigation is necessary to clarify the mechanisms for Ang II–induced Rho A and Rho-kinase activation.

Recently, a role of ERK in the induction of Ang II–induced MCP-1 expression has been reported. We confirmed the results of the previous report. Stretch-induced ERK activation was blocked by C3 exotoxin or Y-27632, suggesting that the Rho–Rho-kinase pathway regulates stretch-induced ERK activation. However, we showed that Y-27632 did not affect the Ang II–induced ERK activation, and the result is consistent with that of the previous report. These data suggest that Ang II differentially activates the Rho-kinase and ERK pathways. Although the possibility that the ERK pathway activates the Rho–Rho-kinase pathway is not excluded at this point, such an interaction has not been reported.

Rho has been shown to activate serum response factor–dependent transcription and to activate nuclear factor (NF)-κB, a transcription factor. It has been shown that Ang II–induced MCP-1 expression is mediated by NF-κB because Ang II–induced MCP-1 expression was inhibited by pyrrolidine dithiocarbamate (PDTC), which is an antioxidant and an inhibitor of NF-κB as well. However, Chen et al.13 failed to observe the effect of PDTC on Ang II–induced MCP-1 expression. We also failed to observe the effect of PDTC on Ang II–induced MCP-1 expression in VSMCs (data not shown), suggesting that NF-κB may not play a dominant role in the induction of MCP-1 by Ang II. The role of serum response factor in MCP-1 gene transcription has not yet been reported. Therefore, further investigation is necessary to determine the Ang II–activated and Rho-kinase–dependent transcription factor that enhances MCP-1 gene transcription.

An increasing body of evidence suggests that monocytes and macrophages play an important role in the progression of atherosclerosis and in plaque instability as well. Disruption of the MCP-1 receptor caused attenuation of atherosclerosis when these mice were crossed with apoE knockout mice. These results suggest the critical role of MCP-1 in atherogenesis. We demonstrated in the present study that Ang II–induced MCP-1 expression in VSMCs via ERK and Rho-kinase pathways. Therefore, it is possible that the beneficial effects of the ACE inhibitor, at least in part, are derived from the inhibition of Ang II–induced cytokine production, as shown in the present study and our previous report. Recently, Uehata et al.14 reported that Y-27632 decreased blood pressure in hypertensive rats and proposed that Rho-kinase may be a target molecule for the treatment of hypertension. Our data suggest that inhibition of the Rho-kinase pathway by Y-27632 or AdDNRhoK may suppress the progression of atherosclerosis by inhibition of MCP-1 expression. Therefore, Rho-kinase may be a novel target for the treatment of high blood pressure and atherosclerosis as well.

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