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 cytokine 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 I 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.

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. 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) and the Ang II type 2 receptor (AT2-R), 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. 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), macrophage inflammatory protein-1, and RANTES (regulated on activation, normal T–expressed and –secreted), 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, thrombin, or interleukin-1β 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. 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. Y-27632, a specific inhibitor of Rho-kinase, is reported to reduce blood pressure in hypertensive rats but not in normotensive rats. Recently, a role of Rho A in Ang II–induced actin organization in cardiac myocytes has been reported. 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 Cell Signaling Technology. 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 2-mercaptoethanol at 70°C for 1 hour and reprobed with an antibody against ERK.

**Cell Culture**

VSMCs were isolated from the thoracic aortas of Sprague-Dawley rats and maintained as described previously.4 Passages 3 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 quantified with a Mac BAS bioimaging analyzer (FUJIFILM).

**Quantification of Rat MCP-1 Protein by ELISA**

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 in the supernatant of cultured rat VSMCs.

**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 phenylmethylsulfonyl 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 a nitrocellulose membrane. The membranes were blocked with 0.2% of BSA in PBS. Then, serial dilutions of recombinant rat MCP-1 and test samples were applied to the plate. Briefly, a microtiter plate was coated with mouse anti-rat MCP-1 monoclonal antibody B4 or C4 overnight at 4°C. The plate was washed with 0.05% Tween-20 in Tris-buffered saline (TBS, pH 7.5) and blocked with 0.2% of BSA in PBS. Then, serial dilutions of recombinant rat MCP-1 and test samples were applied to the plate. The incubation was performed at 37°C for 90 minutes, followed by a wash with 0.05% Tween 20 in TBS twice. Then rabbit polyclonal anti-rat MCP-1 antibody was added. After 90 minutes of incubation, the plate was washed twice with TBS. The alkaline phosphatase–labeled anti-rabbit IgG(ab)2 (Vector Laboratories) and p-nitrophenyl phosphate (Sigma) were added to the plate and incubated at 37°C for 30 minutes. The color was read spectrophotometrically at 405 nm.

**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 ~5 to 6 hours after stimulation and then decreased. VSMCs were incubated with...
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) blocked the Ang II–induced MCP-1 mRNA expression. However, PD 123,319 (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 are consistent with the expression of MCP-1 protein in the supernatants.

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 30 MOI or preincubated with Y-27632 (10^{-5} mol/L), a specific inhibitor of Rho-kinase, and then stimulated with Ang II (10^{-7} mol/L) for 12 hours. As shown in Figure 1C, both AdDNRhoK and Y-27632 inhibited the 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. AdDNRhoK MOI-dependently suppressed the Ang II–induced MCP-1 mRNA expression (Figure 3). 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 expression (Figure 1C), confirming the previous report. Therefore, we examined whether the Rho-kinase pathway affects Ang II–induced ERK activation. The Ang II–induced ERK activation was not inhibited by Y-27632 (Figure 4).

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 Ang II–induced Rho A and Rho-kinase activation. VSMCs were cultured with Ang II (10^{-7} mol/L) for 5 minutes after pretreatment with or without Y-27632 (10^{-5} mol/L). Phosphorylation of ERK was detected by Western blot analysis using a phospho-specific ERK antibody (top). The membrane was stripped and reprobed with an ERK antibody (bottom). The same results were obtained in other independent experiments (n=3), and a representative autoradiograph is shown.

Recently, it has been reported that AT1-R is coupled with Gα13, which is a transcription factor. It has been shown that Ang II–induced MCP-1 expression was inhibited by pyrrolidone dithiocarbamate (PDTC), which is an antioxidant and an inhibitor of NF-κB as well. However, Chen et al. 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 our 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. 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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