Involvement of Apoptosis Signal-Regulating Kinase-1 on Angiotensin II-Induced Monocyte Chemoattractant Protein-1 Expression

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**Objective**—Monocyte chemoattractant protein 1 (MCP-1) could contribute to enhanced leukocyte recruitment and activation resulting in chronic tissue damage. However, little is known about the molecular mechanisms of cardiac MCP-1 expression. To elucidate these molecular mechanisms, angiotensin II-induced expression of MCP-1 was examined in cultured rat neonatal ventricular cardiomyocytes and fibroblasts by adenovirus gene transfer.

**Methods and Results**—MCP-1 mRNA increased 3.6-fold in cardiac fibroblasts at 3 hours after 100 nmol/L angiotensin-II stimulation (P<0.01), whereas MCP-1 mRNA in cardiomyocytes was unchanged. Angiotensin II significantly enhanced JNK, p38MAPK, and nuclear factor-κB (NF-κB) activities of cardiac fibroblasts. Wild-type ASK-1 increased MCP-1 expression of cardiac fibroblasts, whereas dominant negative mutant of ASK-1 (DN-ASK), dominant negative mutant of p38MAPK (DN-p38MAPK), and pyrrolidine dithiocarbamate significantly inhibited such expression. The increased MCP-1 mRNA expression in wild-type ASK-1 transfected fibroblasts was inhibited by cotransfection with adenovirus expressing DN-p38MAPK. On the contrary, the decreased MCP-1 mRNA expression in DN-ASK transfected cells was increased by cotransfection with adenovirus expressing constitutively active MKK6.

**Conclusion**—Angiotensin II induced MCP-1 gene expression in cardiac fibroblasts. The angiotensin II-induced activation of ASK-1 followed by p38MAPK and NF-κB signaling in cardiac fibroblasts is partially involved in myocardial MCP-1 expression. (Arterioscler Thromb Vasc Biol. 2004;24:270-275.)

**Key Words:** apoptosis signal-regulating kinase-1 ■ monocyte chemoattractant protein-1 ■ cardiac fibroblast ■ angiotensin II ■ p38 MAPK

Monocyte chemoattractant protein 1 (MCP-1) is a chemokine produced in a variety of cells in response to injury or exposure to other cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, or interferon-γ, and has been implicated in deterioration in heart failure. MCP-1 contributes to enhanced leukocyte recruitment and activation resulting in chronic tissue damage. However, little is known about the molecular mechanisms of cardiac MCP-1 expression.

Accumulating evidence shows that angiotensin II plays an important role in modulating the cardiac remodeling and that angiotensin-converting enzyme inhibitor (ACEI) and angiotensin II type 1 receptor blocker (ARB) are well known to be effective in improving hemodynamics and preventing cardiac remodeling. Angiotensin II causes cardiac remodeling accompanied by myocardial cellular phenotypic changes and gene expression. Recent studies suggest that angiotensin II may play an important role in the regulation of MCP-1 expression in vascular smooth muscle cells and may thereby affect vascular remodeling. Although there is substantial evidence of an important role for angiotensin II in cardiac remodeling, the physiological importance of angiotensin II in modulating MCP-1 expression and the molecular mechanisms of myocardial MCP-1 expression remain unclear.

Extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38MAPK, which all belong to the mitogen-activated protein kinase (MAPK) family, are commonly activated by myocardial remodeling-related molecules, and play the central role in the initiation of cellular responses, including cellular gene expression, growth, or apoptosis. Apoptosis signal-regulating kinase-1 (ASK-1) was recently identified as a reactive oxygen species-sensitive MAPK kinase, which activates JNK and p38MAPK. Recently, we have reported that ERK, JNK, and p38MAPK are...
activated in nonischemic myocardium after myocardial infarction (MI). However, the role of these activated signals in myocardial cells remains to be fully elucidated. Therefore, a clarification of the role of ASK-1 and MAPK in the process of cardiac MCP-1 expression induced by angiotensin II is important in order to understand cardiac remodeling.

In the present study, in order to clarify the potentially important control mechanisms of myocardial MCP-1 expression, constitutively active ASK-1 (ΔN), wild-type ASK-1 (WT-ASK), dominant negative mutant of ASK-1 (DN-ASK), wild-type JNK (WT-JNK), dominant negative mutant of JNK (DN-JNK), dominant negative mutant of p38MAPK (DN-p38MAPK), and constitutively active MKK6 were overexpressed by adenovirus gene transfer into cultured rat neonatal ventricular fibroblasts, and the effects on angiotensin II-induced MCP-1 expression in cardiac fibroblasts were examined. These studies demonstrated that there is a key role for ASK-1 and MAPK as a mediator of myocardial MCP-1 expression by angiotensin II.

**Methods**

**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM) and FBS were purchased from Sigma; PD98059, a specific inhibitor of ERK, was obtained from Cell Signaling Technology (Beverly, MA); Y27632, a specific inhibitor of Rho-kinase, and pyrrolidine dithiocarbamate (PDTC) were obtained from Calbiochem (San Diego, CA).

**Cell Cultures**

Primary culture of rat neonatal ventricular myocytes and fibroblasts were prepared as previously reported. In brief, cardiac ventricles from 1- to 2-day-old Wistar rats were separated, minced, and dispersed with 80 U/mL collagenase IV and 0.6 mg/mL pancreatin. Then, a discontinuous gradient of Percoll was prepared. The myocytes were incubated on uncoated 10-cm culture dishes for 30 minutes to remove any remaining nonmyocytes, and the nonattached viable cells were plated on gelatin-coated culture dishes at a density of 3.0 x 10⁴ cells/cm² with DMEM supplemented with 10% FCS for 36 hours. The fibroblasts were incubated on uncoated dishes with DMEM supplemented with 10% FCS for 30 minutes, washed with phosphate buffered saline. Then, HRP-conjugated mouse anti rat MCP-1 antibody. The incubation was performed at 37°C overnight and centrifuged to obtain the pellet. The pellet was suspended with 50 µL kinase buffer containing 100 µmol/L ATP (kinase reaction buffer), and 2 µg Elk-1 fusion protein for ERK assay or 2 µg ATF-2 fusion protein for p38MAPK assay. For JNK assay, 500 µL cell lysate containing 500 µg total protein was added to 20 µL of c-Jun fusion protein beads, incubated at 4°C overnight, centrifuged, and the resulting pellet was suspended with 50 µL kinase reaction buffer. These reactions were performed at 30°C for 30 minutes, terminated by addition of SDS sample buffer. Phosphorylated substrates were analyzed with Western blotting using phospho-Elk-1 antibody for kinase assay of ERK, phospho-ATF-2 antibody for kinase assay of p38MAPK, and phospho-c-Jun antibody for kinase assay of JNK.

**Northern Blot Analysis**

The method of the total RNA extraction was described previously. Northern blot analyses were performed with cDNA probes for mouse MCP-1 and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described. The densities of individual mRNA bands were measured using a bioimaging analyzer (BAS-2000, Fuji Photo Film, Tokyo, Japan).

**Kinase Assays for ERK, p38, and JNK**

Kinase assays of ERK, p38MAPK, and JNK were performed using commercial kits (Cell Signaling Technology). Briefly, 500 µL cell lysate containing 200 µg total protein was incubated with 20 µL of immobilized phospho-p44/p42 MAP kinase monoclonal antibody for ERK assay or immobilized phospho-p38MAPK monoclonal antibody for p38MAPK assay. The mixtures were then incubated at 4°C overnight and centrifuged to obtain the pellet. The pellet was suspended with 50 µL kinase buffer containing 100 µmol/L ATP (kinase reaction buffer), and 2 µg Elk-1 fusion protein for ERK assay or 2 µg ATF-2 fusion protein for p38MAPK assay. For JNK assay, 500 µL cell lysate containing 500 µg total protein was added to 20 µL of c-Jun fusion protein beads, incubated at 4°C overnight, centrifuged, and the resulting pellet was suspended with 50 µL kinase reaction buffer. These reactions were performed at 30°C for 30 minutes, terminated by addition of SDS sample buffer. Phosphorylated substrates were analyzed with Western blotting using phospho-Elk-1 antibody for kinase assay of ERK, phospho-ATF-2 antibody for kinase assay of p38MAPK, and phospho-c-Jun antibody for kinase assay of JNK.

**Electrophoretic Mobility Shift Assay for NF-κB**

For the electrophoretic mobility shift assay, nuclear protein extracts were prepared as described. The sequence of the double-stranded oligonucleotide used in the present study was as follows: consensus nuclear factor-κB (NF-κB), 5′-AGTTGAGGGACTTCCAGGAAGC-3′.

To demonstrate the specificity of NF-κB DNA-protein binding, the reactions were performed in the presence of nonlabeled consensus oligonucleotide competitors, and a supershift assay was carried out using rabbit polyclonal IgG against Rel family proteins (p50 and p65; Santa Cruz Biotechnology, Santa Cruz, CA) as described.

**Measurement of Rat MCP-1 in Cell Culture Media**

The cell culture medium of nonstimulated or angiotensin II-stimulated cardiac fibroblasts was collected and centrifuged at 3000 rpm for 5 minutes. MCP-1 was measured by a sandwich ELISA, using Rat MCP-1 EIA kit (Immuno-Biological Laboratories, Gunma, Japan). Briefly, serial dilutions of recombinant rat MCP-1 and test samples were applied to a microtiter plate, which was coated with rabbit anti-rat MCP-1 antibody. The incubation was performed at 37°C for 60 minutes, followed by a wash with 0.05% Tween 20 in phosphate buffered saline. Then, HRP-conjugated mouse anti rat MCP-1 antibody was added. After 30 minutes incubation, the plate was washed. Tetra methyl benzidine buffer was added to the plate for 30 minutes. The reaction was stopped by the addition of 0.5mol/L H₂SO₄. The color was read spectrophotometrically at 450 nm.

**Statistics**

The results are expressed as mean±SEM. Statistical significance was determined using ANOVA and Student-Newman-Keuls test. Differences were considered statistically significant when P<0.05.

**Results**

**Angiotensin II Induced MCP-1 mRNA Expression and Activities of MAPK in Cardiac Fibroblasts**

As shown in Figure 1, angiotensin II did not change MCP-1 mRNA expression in cultured cardiomyocytes. However, angiotensin II significantly increased MCP-1 mRNA expres-
sion in cardiac fibroblasts (3.6-fold at 3 hours, \( P < 0.01 \) vs at 0 hour). Figure 1 (available online at http://atvb.ahajournals.org) shows that angiotensin II enhanced MAPK activities in cardiac fibroblasts. In angiotensin II-treated cardiac fibroblasts, ERK, JNK, and p38MAPK activities increased 2.3-fold at 5 minutes, 4.8-fold at 30 minutes, and 7.0-fold at 5 minutes, respectively. These results demonstrate that angiotensin II significantly increased MCP-1 mRNA expression in cardiac fibroblasts.

**Effects of Inhibition of MAPK on MCP-1 Expression in Cardiac Fibroblasts**

Figure II (available online at http://atvb.ahajournals.org) shows MAPK activities in cardiac fibroblasts treated with PD98059 or transfected with adenovirus expressing DN-JNK, DN-p38MAPK. PD98059, DN-JNK, and DN-p38MAPK significantly inhibited the activation of ERK, JNK, and p38MAPK, respectively. As shown in Figure 2, angiotensin II stimulation resulted in a statistically significant increase in MCP-1 mRNA expression in nontreated cardiac fibroblasts (6.9-fold, \( P < 0.01 \)) and in fibroblasts transfected with adenovirus expressing lacZ (7.0-fold, \( P < 0.01 \)). Interestingly, PD98059 and DN-p38MAPK attenuated an increase in MCP-1 mRNA expression by angiotensin II (4.2- and 2.4-fold, respectively, \( P < 0.01 \)). On the other hand, DN-JNK enhanced MCP-1 mRNA expression in fibroblasts treated with or without Angiotensin II.

**DN-ASK Inhibited MCP-1 mRNA Expression Through p38MAPK in Cardiac Fibroblasts**

Figure III (available online at http://atvb.ahajournals.org) shows the effects of WT-ASK and DN-ASK on the MAPK activities in cardiac fibroblasts. WT-ASK and DN-ASK did not change the activities of ERK with or without angiotensin II. WT-ASK significantly activated JNK and p38MAPK in cardiac fibroblasts treated with or without angiotensin II. DN-p38MAPK significantly reduced the activation of p38MAPK by WT-ASK. DN-ASK significantly inhibited the activation of JNK and p38MAPK by angiotensin II. Although constitutively active MKK6 did not change the activities of JNK, it enhanced the activation of p38MAPK with or without DN-ASK. Figure 3 shows that DN-p38MAPK inhibited an increase in the MCP-1 mRNA expression of cardiac fibroblasts treated with adenovirus expressing WT-ASK. Furthermore, constitutively active MKK6 enhanced MCP-1 mRNA expression in cardiac fibroblasts treated with adenovirus expressing DN-ASK. In addition, WT-JNK inhibited MCP-1 mRNA expression by angiotensin II in cardiac fibroblasts (Figure IV, available online at http://atvb.ahajournals.org). Y27632 (10 \( \mu \)mol/L), a specific inhibitor of Rho-kinase, significantly inhibited MCP-1 mRNA expression by angiotensin II. However, Y27632 did not change the increased expression of MCP-1 in fibroblasts treated with adenovirus expressing WT-ASK or constitutively active MKK6 (Figure V, available online at http://atvb.ahajournals.org).

**DN-ASK and DN-p38MAPK Inhibited Transcriptional Activity of NF-κB in Cardiac Fibroblasts**

Figure 4A shows the transcriptional activities of NF-κB with or without angiotensin II in cardiac fibroblasts. LacZ,
PD98059, and DN-JNK did not change the activities of NF-κB. Activation of ASK by WT-ASK enhanced the activity of NF-κB with or without angiotensin II. DN-ASK and DN-p38MAPK reduced the increased activities of NF-κB by angiotensin II. PDTC (50 μmol/L), an NF-κB inhibitor by blocking the dissociation of the NF-κB-IB complex, significantly inhibited MCP-1 mRNA expression by angiotensin II in both nontreated cardiac fibroblasts and in fibroblasts transfected with adenovirus expressing WT-ASK. (Figure 4B)

DN-p38MAPK and DN-ASK Inhibited MCP-1 Protein Synthesis in Cardiac Fibroblasts

As shown in Figure VI (available online at http://atvb.aha-journals.org), angiotensin II stimulation resulted in a statistically significant increase in MCP-1 protein synthesis in nontreated cardiac fibroblasts (1.6-fold, P<0.01) and in fibroblasts transfected with adenovirus expressing lacZ (1.7-fold, P<0.01). DN-p38MAPK and DN-ASK inhibited an increase in MCP-1 expression by angiotensin II (1.0- and 1.0-fold, P<0.01). DN-JNK, ΔN, and WT-ASK enhanced MCP-1 protein synthesis in fibroblasts treated with or without angiotensin II.

Discussion

In the present study, we have demonstrated that angiotensin II did not increase the MCP-1 mRNA expression in cardiomyocytes, whereas it did increase such expression in cardiac fibroblasts. Angiotensin II-induced expression of MCP-1 in cardiac fibroblasts was partially mediated by activation of ASK-1 and p38MAPK pathways.

Growing evidence supports the finding that inflammatory responses may induce the development of cardiac remodeling. MCP-1 is a chemokine produced in a variety of cells in response to injury or exposure to other cytokines. MCP-1 contributes to enhanced leukocyte recruitment and...
activation, resulting in chronic tissue damage. Elevated circulating levels of MCP-1 in congestive heart failure have been reported, not only in animals but also in humans. Moreover, it has been reported that there is increased expression of IL-1β, IL-6, and TNF-α in noninfarcted myocardium after MI, suggesting the possible implication of cytokines in cardiac remodeling. In our preliminary experiment, MI increased myocardial MCP-1 expression in noninfarcted myocardium of rat heart, and the expression was prevented by ACEI and ARB (data not shown). Taken together with previous findings, the enhanced MCP-1 mRNA in noninfarcted myocardium accompanied by cardiac remodeling encouraged us to examine the mechanisms of MCP-1 expression in both cardiomyocyte and cardiac fibroblasts. In the present study, angiotensin II could induce the upregulation of MCP-1 mRNA in cardiac fibroblasts, but not in cardiomyocytes. Because similar responses in cardiomyocytes and cardiac fibroblasts by angiotensin II have been observed in angiotensin II-induced cardiomyocyte hypertrophy, our results suggest that cardiac fibroblasts may play an important role in cardiac MCP-1 expression by angiotensin II. Therefore, we examined angiotensin II-induced MCP-1 expression in detail using cardiac fibroblasts.

Angiotensin II has been shown to activate various protein kinase pathways through angiotensin II type 1 receptor. It is well known that angiotensin II activates ERK, JNK, and p38MAPK, which are critical protein kinases for cell growth, cell death, and gene expression. Recently, the role of ERK in the induction of angiotensin II-induced MCP-1 expression in rat vascular smooth muscle cells has been reported. On the other hand, the roles of JNK and p38MAPK in myocardial MCP-1 expression remain to be fully determined. Furthermore, as an important upstream cascade of MAPK, ASK-1 has been shown to activate JNK and p38MAPK. Recent work demonstrated that ASK-1 is involved in G-protein-coupled receptor-agonist induced NF-κB activation and the resulting cardiomyocyte hypertrophy. However, the role of ASK-1 is still controversial, and the downstream target gene of ASK-1 for cardiac remodeling remains to be fully determined.

For the inhibition of ERK activity, PD98059 can inhibit the MCP-1 mRNA expression in cardiac fibroblasts as it does in vascular smooth muscle cells. In the present study, we used an adenoviral expression system to overexpress MAPK family-related molecules in cardiac fibroblasts to stimulate and/or inhibit ASK-1, JNK, and p38MAPK. As shown in the results, our findings demonstrate that DN-p38MAPK can suppress MCP-1 mRNA expression enhanced by angiotensin II, and constitutively active MKK6 can enhance this expression, thereby suggesting an important role of p38MAPK for MCP-1 expression in cardiac fibroblasts. Moreover, activation of ASK-1 by adeno virus gene transfer enhanced MCP-1 mRNA expression, and DN-ASK inhibited an increase in mRNA expression, thereby suggesting the involvement of ASK-1 in angiotensin II-induced MCP-1 expression in cardiac fibroblasts. The increased MCP-1 mRNA expression in WT-ASK transfected fibroblasts was inhibited by cotransfection with adenovirus expressing constitutively active MKK6. These results suggest that activation of p38MAPK through ASK-1 signaling, rather than activation of JNK, preferentially activates MCP-1 mRNA expression in cardiac fibroblasts. Furthermore, NF-κB is reported to be important for regulating MCP-1 expression. In the present study, DN-ASK and DN-p38MAPK inhibited the NF-κB activities by angiotensin II and pyrrolidine dithiocarbamate inhibited MCP-1 mRNA expression by angiotensin II, thereby suggesting the important role of NF-κB through ASK-1 and p38MAPK signaling on MCP-1 expression in cardiac fibroblasts. Although activation of JNK is reported to be important for MCP-1 gene expression in other type of cells, angiotensin-II-induced JNK activation has no marked effects on MCP-1 expression in the present study. Because DN-p38MAPK inhibited and DN-JNK did not inhibit the activation of NF-κB by angiotensin II in the present study, one of the reasons for different effects between JNK and p38MAPK on MCP-1 expression may be different NF-κB activation in cardiac fibroblasts. Furthermore, recent study shows that Rho-kinase mediates angiotensin-II-induced MCP-1 expression in rat vascular smooth muscle cells. Although Rho-kinase also play an important role in angiotensin II-induced MCP-1 mRNA expression in cardiac fibroblasts, Y27632 could not reduce the increased MCP-1 mRNA expression in WT-ASK or constitutively active MKK6 transfected cells. These results suggest that the increased MCP-1 mRNA expression following activation of ASK-1 and p38MAPK may be related to mechanisms downstream of Rho-kinase. MCP-1 protein synthesis is also inhibited by adenovirus transfer of DN-p38MAPK and DN-ASK, and was enhanced by DN-JNK, DN, and WT-ASK, thereby confirming the roles of ASK-1 and p38MAPK on MCP-1 expression in cardiac fibroblasts. PD98059 also completely inhibited the MCP-1 protein synthesis, thereby
suggesting the possibility of interaction between downstream signaling of ERK and p38MAPK for MCP-1 synthesis. In conclusion, we put forth evidence that angiotensin II increased MCP-1 mRNA expression in cardiac fibroblasts, but not in cardiomyocytes. As shown in Figure 5, angiotensin II-induced expression of MCP-1 in cardiac fibroblasts was partially mediated by activation of ASK-1, p38MAPK, and NF-κB. The results of our study suggest that the activation of p38MAPK and NF-κB, through ASK-1 signaling by angiotensin II in cardiac fibroblasts, is involved in myocardial MCP-1 expression and may be associated with cardiac remodeling. However, further work is needed to demonstrate the direct in vivo evidence of the effects of MCP-1 expression on cardiac remodeling.

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