p38 Mitogen-Activated Protein Kinase Contributes to the Diminished Aortic Contraction by Endothelin-1 in DOCA-Salt Hypertensive Rats

Bokyung Kim, Junghwan Kim, Young M. Bae, Sung I. Cho, Seong C. Kwon, Jin Y Jung, Jung-C. Park, Hee Y. Ahn

Abstract—We investigated whether the diminished contractile responsiveness to endothelin-1 (ET-1) is associated with the altered activation of mitogen-activated protein kinase (MAPK) in aortic smooth muscles from deoxycorticosterone acetate (DOCA)-salt hypertensive rats. ET-1 dose-dependently increased contractions in aortic smooth muscle strips, and the contractions were significantly attenuated in tissues from DOCA-salt hypertensive rats compared with those from sham-operated rats. The phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 was elevated by ET-1, with the magnitude and time-course being similar between strips. Although ET-1 also increased the phosphorylation of p38 MAPK in both strips, the increment was markedly lower in the strips from DOCA-salt hypertensive rats compared with sham-operated controls. 5-Hydroxytryptamine (5-HT) increased vascular contraction and phosphorylation of both MAPK isoforms; these were greater in DOCA-salt hypertensive rats than in sham-operated rats. ET-1 also increased the phosphorylation of caldesmon, an actin-binding protein, in sham-operated and DOCA-salt hypertensive rats. However, the increment was markedly lower in the strips from DOCA-salt hypertensive rats compared with sham-operated controls. The phosphorylation of MAPK isoforms and caldesmon elevated by ET-1 was inhibited by PD098059, an inhibitor of ERK1/2 kinase, and SB203580, an inhibitor of p38 MAPK, respectively. These results suggest that ET-1 and 5-HT induce contraction by activating the MAPK pathway in rat aortic smooth muscle and that the diminished responsiveness to ET-1 in the DOCA-salt hypertensive rat may be, in part, mediated by the decrease of caldesmon phosphorylation after the decreased activation of p38 MAPK. (Hypertension. 2004;43:1-6.)

Key Words: endothelin ■ hypertension ■ vasoconstriction ■ deoxycorticosterone ■ protein kinases

Endothelin-1 (ET-1) is an important vasoconstrictor that contributes to vascular disorders, including hypertension, vascular remodeling, and coronary artery disease. ET-1 induces vascular smooth muscle contraction. It is widely accepted that smooth muscle contraction is triggered by intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)), released from intracellular Ca\(^{2+}\) stores and from the extracellular space. The increased [Ca\(^{2+}\)]\(_{i}\) can phosphorylate the 20-kDa myosin light chain (MLC) by activating MLC kinase, and this initiates smooth muscle contraction. Previous reports have shown that ET-1 induces a sustained contraction, which results from the increase of [Ca\(^{2+}\)]\(_{i}\), in isolated vascular smooth muscle. In addition to the [Ca\(^{2+}\)]\(_{i}\)-MLC kinase pathway, a number of intracellular signal molecules, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), phosphatidylinositol 3 kinase (PI3K), and Rho kinase, play important roles in the regulation of smooth muscle contraction. ET-1 can also stimulate these kinases.

MAPK is a family of serine/threonine-specific protein kinase, consisting of three isoforms: extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK). MAPK plays a central role in intracellular signal transduction initiated by extracellular stimuli, including growth factors, neurotransmitters, and hormones. ERK1/2 is activated by receptor agonists, including angiotensin II and phenylephrine, which induce smooth muscle contraction. ET-1 also increases the activity of ERK1/2 in vascular smooth muscle. There is accumulating evidence that the MAPK pathway is closely linked to modulating the intensity of contraction in vascular smooth muscle. Moreover, the inhibition of p38 MAPK diminishes contractility in smooth muscle cells, indicating that p38 MAPK, as well as ERK1/2, can contribute to the elevation of contraction. In DOCA-salt hypertensive rats, altered reactivity of blood vessels is often associated with the elevation of systolic blood pressure.
contractile responses of vascular smooth muscle to vasoconstrictors, including 5-hydroxytryptamine (5-HT) and norepinephrine, are significantly increased in hypertensive animals.\textsuperscript{16,24–26} In contrast, the contractile response to ET-1 is significantly diminished in the DOCA-salt hypertensive rat compared with the normotensive rat.\textsuperscript{27,28} Furthermore, the lower density of ET-A receptor on vascular smooth muscle has been reported in DOCA-salt hypertensive rats.\textsuperscript{27} In addition, the increment of [Ca\textsuperscript{2+} ]\textsuperscript{i} induced by ET-1 also decreased in vascular smooth muscle from DOCA-salt hypertensive rats.\textsuperscript{28,29} Although the changes in the receptor number and intracellular Ca\textsuperscript{2+} levels have been reported, the clear reason for this diminution in response to ET-1 in DOCA-salt hypertensive rats has not been elucidated.

In the present study, because the MAPK pathway is an important mediator of vascular contraction, we hypothesized that this pathway regulates the diminished contractile response to ET-1 during DOCA-salt hypertension. To test this hypothesis, we examined the roles of MAPK isoforms and the decreased responsiveness to ET-1 in contraction between sham-operated and DOCA-salt hypertensive rats.

Methods

Animals
All experiments were performed in accordance with the institutional guidelines of Konkuk University, Korea. Male Sprague-Dawley rats (190 to 200 g; Daehan Biolink, Korea) were uninephrectomized and, after 1 week, received a silicon rubber implant impregnated with DOCA (200 mg kg\textsuperscript{-1}) subcutaneously under intramuscular anesthesia (35 mg kg\textsuperscript{-1} ketamine/5 mg kg\textsuperscript{-1} xylocaine). DOCA-salt hypertensive rats received 0.9% NaCl plus 0.2% KCl drinking water. Systolic blood pressure was directly determined using a tonometer of Konkuk University, Korea. Male Sprague-Dawley rats (190 to 200 g; Daehan Biolink, Korea) were uninephrectomized and, after 1 week, received a silicon rubber implant impregnated with DOCA (200 mg kg\textsuperscript{-1}) subcutaneously under intramuscular anesthesia (35 mg kg\textsuperscript{-1} ketamine/5 mg kg\textsuperscript{-1} xylocaine). DOCA-salt hypertensive rats received 0.9% NaCl plus 0.2% KCl drinking water. A control group (sham-operated rat) was also uninephrectomized and operated without receiving an implant. Sham-operated rats received normal tap water. All animals were fed standard laboratory rat chow and had ad libitum access to both food and water. Systolic blood pressure was directly determined using a pressure transducer (Statham P23XL; Viggo Spectramed) at the common carotid artery under ketamine/xylazine anesthesia. At 4 weeks after the silicon rubber implantation, the blood pressure was significantly higher in DOCA-salt hypertensive rats (187±7 mm Hg, n=25) than in sham-operated rats (119±2 mm Hg, n=27).

Measurement of Isometric Contraction

Rats were euthanized by stunning and bled; the thoracic aorta was removed, cut into strips (3×8 mm), and the endothelium was removed. Isometric muscle contraction was recorded, as described previously.\textsuperscript{30}

Measurement of Protein Phosphorylation

Aortic strips were isolated and snap-frozen in liquid N\textsubscript{2}, after treatment with various stimulants for different times. The samples were then homogenized in sample buffer containing 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L EGTA, 20 mmol/L β-glycerophosphate, 1 mmol/L NaF, 2 mmol/L Na\textsubscript{3}VO\textsubscript{4}, 5 μg/mL aprotinin, 5 μmol/L leupeptin, 1% Triton-X 100, 10% glycerol, 300 μmol/L phosphoenolpyruvate, 5 mmol/L diithothreitol, and 150 mmol/L NaCl. The homogenate was centrifuged at 14 000g for 10 minutes at 4°C, and the supernatant was collected.\textsuperscript{30–32} Proteins separated with SDS-PAGE were transferred to polyvinylidene fluoride membranes (Millipore). These were incubated with phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk for 60 minutes, and then incubated with individual rabbit anti-phosphorylated MAPK and antiphosphorylated caldesmon antibodies diluted 1:1000 to 1:5000 overnight at 4°C. In some experiments, the membrane was stripped and then reprobed with nonphosphorylated antibodies for detection of total protein expression. After incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000) for 60 minutes, the blots were developed using an enhanced chemiluminescence detection system (Amersham). Antibody-specific bands were quantified using an image analyzer (BioRad).

Materials

Polyclonal antiphosphorylated and nonphosphorylated ERK1/2 antibodies, Triton-X 100, and diithothreitol were purchased from Promega. Polyclonal antiphosphorylated and nonphosphorylated p38 MAPK antibodies were purchased from Cell Signaling. Polyclonal antiphosphorylated and nonphosphorylated caldesmon was purchased from Upstate. DOCA, ET-1, 5-HT, Na\textsubscript{3}VO\textsubscript{4}, β-glycerophosphate, Na\textsubscript{2}SO\textsubscript{4}, phenylmethylsulfonyl fluoride, leupeptin, and aprotinin were purchased from Sigma. P0098509 and SB203580 were purchased from Tocris.

Data Analysis

The results of experiments are expressed as means±SEMs. Unpaired Student t tests were used to compare the data, and P<0.05 was considered significantly different.

Results

ET-1 induced sustained contractions in dose-dependent manners in both sham-operated and DOCA-salt hypertensive rats (Figure 1A). The response to ET-1 was significantly decreased in the experimental rats compared with sham-operated controls. 5-HT dose-dependently increased muscle contraction in both strips, and the contractions were significantly greater in DOCA-salt hypertensive rats than in sham-operated rats (Figure 1B). The results from mechanical study are consistent with earlier studies.\textsuperscript{24,27,28}

To determine whether MAPK influences receptor agonist-induced responses, the activity of MAPK was measured using phosphorylated MAPK antibodies in aortic smooth muscles from sham-operated and DOCA-salt hypertensive rats. In the quiescent state without any stimulant, the phosphorylation of ERK1/2 was significantly greater in DOCA-salt hypertensive rat (182.0±19.3% of sham-operated rat, n=5). ET-1 (30

Figure 1. Contractile responses to ET-1 and 5-HT in aortic smooth muscle strips from sham-operated and DOCA-salt hypertensive rats. The muscle strips from sham-operated and DOCA-salt hypertensive rats were stimulated repeatedly with 70 mmol/L K\textsuperscript{+}. After the response to high K\textsuperscript{+} was determined, ET-1 (0.1 to 100 mmol/L) or 5-HT (0.1 mmol/L to 100 μmol/L) was applied cumulatively. Dose–response curves of the contraction induced by ET-1 (A) and 5-HT (B). The contractile levels of 70 mmol/L K\textsuperscript{+} before the treatment of vasoconstrictors were defined as 100%. Each point represents the mean±SEM of 6 to 14 experiments. *Significant difference from the results of strips from sham-operated rat (P<0.05).


nmol/L) increased the phosphorylation of ERK1/2 in a time-dependent manner in both strips, and reached a maximum at 15 minutes (Figure 2A and B). The magnitudes and time-course of phosphorylation of ERK1/2 elevated by ET-1 did not differ between strips. The treatment of muscle strips with 5-HT (10 nmol/L) increased the phosphorylation of ERK1/2 in both strips (Figure 2C). The increase in ERK1/2 phosphorylation by 5-HT was significantly greater in DOCA-salt hypertensive rats than in sham-operated rats (Figure 2D). The level of phosphorylated ERK1/2 in the quiescent state was defined as 100%. *Significant difference from the results of strips from sham-operated rat (P<0.05).

PD098059 (50 μmol/L), an inhibitor of ERK1/2 kinase, partially attenuated the sustained contraction induced by ET-1 (30 nmol/L) in sham-operated (64.0%±1.2% of ET-1-induced contraction, n=4) and DOCA-salt hypertensive rats (33.6%±11.6% of ET-1-induced contraction, n=4: Figure 4A). SB203580 (50 μmol/L), an inhibitor of p38 MAPK, strongly attenuated ET-1-induced contraction to 13.7%±4.8% (n=5) and 5%±1.3% (n=5) in sham-operated and DOCA-salt hypertensive rats, respectively (Figure 4B).

ET-1 (30 nmol/L) elicited ERK1/2 phosphorylation in strips from sham-operated (343.1%±26.2% of the resting state, n=6) and DOCA-salt hypertensive rats (282.0%±29.3% of resting state, n=6), and these responses were abolished by 10 μmol/L PD098059 in sham-operated (115.2%±19.7% of resting states, n=6) and DOCA-salt hypertensive rats (110.3%±21.8% of resting states, n=6: Figure 4C). ET-1 (30 nmol/L) also increased p38 MAPK phosphorylation in strips from sham-operated (415.6%±52.1% of resting state, n=5) and DOCA-salt hypertensive rats (207.0%±28.7% of resting state, n=5). These responses were also abolished by 10 μmol/L SB203580 in sham-operated (123.6%±14.3% of resting states, n=5) and DOCA-salt hypertensive rats (103.7%±11.8% of resting states, n=5: Figure 4D).

To evaluate whether the activation of the MAPK pathway regulates caldesmon, an actin-binding protein, we measured the phosphorylation of caldesmon using Ser189 phosphorylated h-caldesmon antibody. In the quiescent state without any stimulant, the phosphorylation of caldesmon did not differ between sham-operated and DOCA-salt hypertensive rats (120.2%±14.4% of sham-operated control, n=6). ET-1 (30 nmol/L) increased the phosphorylation of caldesmon,
Discussion

In the present study, we found that ET-1 activates MAPK pathways, ERK1/2 and p38 MAPK, resulting in increased contraction in aortic smooth muscles. The contractile responses and p38 MAPK phosphorylation by ET-1 were significantly attenuated in strips from DOCA-salt hypertensive rats compared with those from sham-operated rats. These results indicate that ET-1 and 5-HT evoke a sustained contraction resulting from the activation of p38 MAPK, as well as ERK1/2, in rat aortic smooth muscle. Furthermore, the diminution of ET-1–induced contraction in aortic strips from DOCA-salt hypertensive rats may be mediated, at least in part, by the reduced activation of p38 MAPK, but not that of ERK1/2. The p38 MAPK inhibitor, SB203580, completely inhibited both p38 MAPK phosphorylation and contraction by ET-1 in sham-operated and DOCA-salt hypertensive rats. Although PD098059, an inhibitor of ERK1/2 kinase, abolished ERK phosphorylation by ET-1, the contraction in response to ET-1 was not completely abolished by PD098059 in sham-operated and DOCA-salt hypertensive rats. These findings show that p38 MAPK activation is more important than that of ERK1/2 in ET-1–induced contraction of sham-operated and DOCA-salt hypertensive rats. Although previous reports showed that the ET-1–increased MAPK activity is slightly elevated in DOCA-salt hypertensive rats, the role of p38 MAPK has not been demonstrated in vascular smooth muscle. To our knowledge, this is the first study to show that altered activation of the p38 MAPK pathway mediates the diminished ET-1–induced contraction in muscle from DOCA-salt hypertensive rats.

Figure 4. Effects of inhibitors of MAPK on contractile response and MAPK phosphorylation elevated by ET-1 in aortic smooth muscles from sham-operated and DOCA-salt hypertensive rats. The strips were stimulated with ET-1 30 nmol/L for 15 minutes and then treated with PD098059 and SB203580 for 15 minutes, respectively. The phosphorylation of ERK1/2 (C) and p38 MAPK (D) were detected using phosphorylated MAPK antibodies as described in Figure 2 and 3. The levels of contractility (A and B) and MAPK phosphorylations (C and D) before treatment with inhibitors were defined as 100%. Each result represents the mean±SEM of 4 to 6 experiments. *Significant difference from the ET-1–stimulated strips (P<0.05).

Figure 5. Effects of ET-1 on the phosphorylation of caldesmon in aortic smooth muscles from sham-operated and DOCA-salt hypertensive rats. The strips were pretreated with PD098059 (10 μmol/L) and SB203580 (10 μmol/L), respectively, for 60 minutes, and then treated with ET-1 30 nmol/L for 30 minutes. The total expressions of caldesmon were measured using a non-phosphorylated caldesmon antibody in both strips. The phosphorylation of caldesmon was detected using a Ser789 phosphorylated h-caldesmon antibody in both strips. The levels of caldesmon phosphorylation in resting states were defined as 100%. Each result represents the mean±SEM of 6 experiments. *Significant difference from the ET-1–stimulated strips (P<0.05).
DOCA-salt hypertensive rats showed decreased density of ET-A receptors.27,28 The increment of \([Ca^{2+}]_{i}\), to ET-1 was reduced in DOCA-salt hypertensive rats.28 In addition, ERK activity elevated by ET-1 was partially inhibited in the absence of extracellular \(Ca^{2+}\).36,37 Moreover, the activity of p38 MAPK, as well as ERK1/2, can be regulated by both \(Ca^{2+}\)-dependent and \(Ca^{2+}\)-independent mechanisms in vascular smooth muscle.17,38,39 Therefore, it can be assumed that the lowered activity of p38 MAPK cannot be associated with the decrease of \([Ca^{2+}]_{i}\), which may be mediated by decreased density of ET-A receptor, in DOCA-salt hypertensive rats. Furthermore, in the present study, despite alteration of the phosphorylation of p38 MAPK in DOCA-salt hypertensive rats, that of ERK1/2 by ET-1 did not differ between both strips, implying that the possibility of coupling between changed receptor properties and MAPK activity during DOCA-salt hypertension can be excluded. These results show that reduced p38 MAPK activity, as well as impaired \([Ca^{2+}]_{i}\),28 may be an alternative factor, which contributes to the lowered contractility to ET-1 in DOCA-salt hypertensive rats.

In the present study, the levels of MAPK in the quiescent state were significantly increased in the muscles of DOCA-salt hypertensive rats, which is consistent with previous reports on aortic smooth muscle from spontaneous and DOCA-salt hypertensive rats.18 Several vasoconstrictors, including 5-HT and norepinephrine, increased the contractility of MAPK in vascular smooth muscle, and the magnitude was greater in DOCA-salt hypertensive rats compared with normotensive rats.16,17,24–26 Furthermore, the application of MAPK inhibitor attenuated the systolic blood pressure in hypertensive rats.40 From these results, it can be assumed that the increased activity of MAPK may contribute to the elevation of blood pressure in DOCA-salt hypertensive rats. In contrast, the diminished responsiveness to ET-1 may act as a compensatory mechanism to the increased vascular resistance and blood pressure produced during DOCA-salt hypertension.

Previous studies have demonstrated that the activation of ERK1/2 participates in smooth muscle contraction by inhibiting the hindrance of caldesmon, an actin-binding protein, onto crossbridges.15–17 Although the total expression of h-caldesmon did not differ between sham-operated and DOCA-salt hypertensive rats, ET-1 increased the phosphorylation of h-caldesmon in both strips. The inhibitors of ERK1/2 and p38 MAPK inhibited the phosphorylation of caldesmon in response to ET-1, respectively. Moreover, the increment of h-caldesmon phosphorylation by ET-1 was significantly attenuated in strips from DOCA-salt hypertensive rats compared with sham-operated controls. These results indicate that p38 MAPK, as well as ERK1/2, increases the phosphorylation of caldesmon,41 and that the reduced activation of p38 MAPK to ET-1 contributes to the diminished caldesmon phosphorylation, which causes the diminution of the contraction in DOCA-salt hypertensive rats. Our current findings strongly support the hypothesis that part of the diminished responsiveness to ET-1 in DOCA-salt hypertensive rats may be caused by the decreased activation of p38 MAPK, but not caused by ERK1/2.

In summary, findings of the present study demonstrate that the MAPK pathway plays a central role in the contractile signaling initiated by ET-1 and 5-HT in vascular smooth muscle. The diminution of p38 MAPK activity contributes to the decreased contraction to ET-1 in the DOCA-salt hypertensive rats, and this downregulation may act a compensatory mechanism to the increased vascular resistance and blood pressure in DOCA-salt hypertensive rats. These studies suggest the roles of MAPK on the mineralocorticoid hypertension.
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