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Full Paper

c-Jun N-terminal Kinase Contributes to Norepinephrine-Induced Contraction Through Phosphorylation of Caldesmon in Rat Aortic Smooth Muscle

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Abstract. Vascular smooth muscle contraction is mediated by activation of extracellular signal-regulated kinase (ERK) 1/2, an isoform of mitogen-activated protein kinase (MAPK). However, the role of stress-activated protein kinase/*c-Jun* N-terminal kinase (JNK) in vascular smooth muscle contraction has not been defined. We investigated the role of JNK in the contractile response to norepinephrine (NE) in rat aortic smooth muscle. NE evoked contraction in a dose-dependent manner, and this effect was inhibited by the JNK inhibitor SP600125. NE increased the phosphorylation of JNK, which was greater in aortic smooth muscle from hypertensive rats than from normotensive rats. NE-induced JNK phosphorylation was significantly inhibited by SP600125 and the conventional-type PKC (cPKC) inhibitor Gö6976, but not by the Rho kinase inhibitor Y27632 or the phosphatidylinositol 3-kinase inhibitor LY294002. Thymeleatoxin, a selective activator of cPKC, increased JNK phosphorylation, which was inhibited by Gö6976. SP600125 attenuated the phosphorylation of caldesmon, an actin-binding protein whose phosphorylation is increased by NE. These results show that JNK contributes to NE-mediated contraction through phosphorylation of caldesmon in rat aortic smooth muscle, and that this effect is regulated by the PKC pathway, especially cPKC.

Keywords: *c-Jun* N-terminal kinase, mitogen-activated protein kinase, vascular smooth muscle contraction, caldesmon, hypertension

Introduction

Increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) stimulates smooth muscle contraction. Within smooth muscle cells, Ca^{2+} binds to calmodulin, a Ca^{2+}-binding protein and subsequently activates myosin light chain kinase, causing the phosphorylation of myosin light chain and force development (1). In addition to Ca^{2+}-mediated vascular contraction, several families of protein kinases are activated by increases in $[Ca^{2+}]_i$ or other signal molecules in smooth muscle cells, including protein kinase C (PKC), Rho kinase, and mitogen-activated protein kinase (MAPK), which are believed to play important roles in vascular contraction (2, 3).

MAPK is a family of serine/threonine-specific protein kinases consisting of three isoforms: extracellular signal-regulated kinase (ERK), p38 MAPK, and stress-activated protein kinase/*c-Jun* N-terminal kinase (JNK) (4). A growing body of evidence suggests that ERK1/2 is activated by receptor agonists such as angiotensin II, phenylephrine, 5-hydroxytryptamine, and endothelin-1, all of which induce smooth muscle contraction (5 – 7). We have shown previously that receptor agonists induce vascular contraction and increase ERK1/2 activity and that these responses are inhibited by ERK inhibitors but

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not by chelating extracellular Ca^{2+}, implying that MAPK is involved in vasoconstriction through a Ca^{2+}-independent pathway (2, 8). Moreover, stimulation of MAPK also increases the phosphorylation of the actin-binding protein caldesmon, an effect that is abolished by the MAPK inhibitors (2). These results demonstrate that vasoconstrictors induce MAPK-mediated contraction by regulating actin-binding proteins in vascular smooth muscle. Furthermore, inhibitors of p38 MAPK also attenuate vascular smooth muscle contraction mediated by the receptor agonists that increase p38 MAPK activity (2). These results imply that the p38 MAPK pathway, as well as ERK 1/2, is closely linked to the contraction process in vascular smooth muscle. We hypothesized that JNK might contribute to the smooth muscle contraction in a similar manner, although no previous reports have addressed the role of JNK in vasoconstriction.

In various cellular systems, the activation upstream of JNK results in its phosphorylation at Thr^{183} and Tyr^{185} and enhances the kinase activity (9). Activated JNK phosphorylates and activates *c-Jun*, which induces gene transcription. Previous data suggest that the signaling mediated by JNK plays a key role in vascular proliferation, apoptosis, and differentiation (9, 10). JNK activation contributes to cell morphogenesis and motility, including migration in vascular smooth muscle (11, 12). Although previous data demonstrate that JNK contributes to the physiology and pathophysiology of vascular function, the effect of JNK on vascular smooth muscle contraction and its mechanism of action are not understood. We determined the role of JNK in, and the possible underlying mechanisms responsible for, receptor agonist-induced contraction in rat vascular smooth muscle.

Materials and Methods

Preparation of muscle strips and measurement of isometric contraction

All experiments were performed in accordance with the institutional guidelines of Konkuk University, Korea. Male Sprague Dawley rats weighing 190 – 200 g (n = 56) were used for this study. Rats were stunned by a blow and bled by cutting the carotid arteries, and the thoracic aorta was isolated and cut into 2 – 3-cm-wide strips. The endothelium was removed by gently rubbing the inner surface of the vessel with a cotton thread moistened with physiological salt solution (136.9 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23.8 mM $NaHCO_3$, 5.5 mM glucose, and 0.01 mM EDTA). Strips were attached to a holder with a resting tension of 10 mN. After equilibration for 20 min in the physiological salt solution, strips were repeatedly exposed to a 70 mM K^+ solution until the response became stable. The high concentration of K^+ was prepared by replacing the NaCl in the physiological salt solution with an equimolar amount of KCl. These solutions were saturated with a mixture of 95% O_2 and 5% CO_2 at 37°C and pH 7.4. Muscle contraction was recorded isometrically with a force-displacement transducer (FT03; Grass, West Warwick, RI, USA) connected to a recording system (ADInstruments, Sydney, Australia).

Preparation of hypertensive rats

Male Sprague Dawley rats (160 – 170 g) were uninephrectomized and received a silicon rubber implant impregnated with deoxycorticosterone acetate (DOCA, 200 mg/kg) subcutaneously under intramuscular anesthesia (35 mg/kg ketamine and 5 mg/kg xylazine). DOCA-salt hypertensive rats received 0.9% NaCl plus 0.2% KCl drinking water solution. A control group (sham-operated rats) was also uninephrectomized and operated on 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 measured directly using a pressure transducer (Statham P23XL; Viggo Spectramed, Oxnard, CA, USA) at the common carotid artery under ketamine-xylazine anesthesia. Four weeks after the silicon rubber implantation, systolic blood pressure was significantly higher in DOCA-salt hypertensive rats (182 ± 9 mmHg, n = 4) than in sham-operated rats (122 ± 7 mmHg, n = 4).

Measurement of protein phosphorylation

Aortic strips were isolated and prepared as described for the contraction measurement experiments and were snap-frozen in liquid N_2 after treatment with various stimulants for different times. The strips were then homogenized in sample buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EGTA, 20 mM β-glycerophosphate, 1 mM NaF, 2 mM Na_3VO_4, 5 μg/ml aprotinin, 5 μM leupeptin, 1% Triton-X 100, 10% glycerol, 300 μM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 150 mM NaCl. The homogenate was centrifuged at 14,000 × g for 10 min at 4°C and the supernatant was collected. Protein concentrations were determined using a colorimetric protein assay kit (BioRad, Hercules, CA, USA). Samples were diluted 1:1 (vol:vol) with sodium dodecyl sulfate (SDS) sample buffer containing 40 mM Tris-HCl (pH 6.8), 8 mM EGTA, 4% 2-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, and 4% SDS, and then they were

boiled for 5 min.

Equal amounts (30 – 50 μg/lane) of protein were separated on a 10% SDS-polyacrylamide gel. Electrophoretically separated proteins were transferred to a polyvinylidene fluoride membrane (Amersham, Buckinghamshire, UK). Membranes were incubated for 60 min in phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk and then incubated with individual antibodies diluted 1:1000 – 5000 overnight at 4°C. Following incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000, Amersham) for 60 min, blots were developed using an enhanced chemiluminescence detection system (Amersham). Antibody-specific bands were quantified using an image analyzer (BioRad).

Materials

Norepinephrine (NE) and DOCA were purchased from Sigma (St. Louis, MO, USA). SP600125, LY294002, Y27632, and GF109203X were purchased from Tocris (Bristol, UK). Thymeleatoxin (THX) and Gö6976 were purchased from Calbiochem (La Jolla, CA, USA). Polyclonal anti-phosphorylated and anti-nonphosphorylated JNK antibodies were purchased from Cell Signaling (Beverly, MA, USA). Polyclonal anti-phosphorylated and nonphosphorylated caldesmon antibodies were purchased from Upstate (Lake Placid, NY, USA).

Data analysis

The results of experiments are expressed as means ± S.E.M. Unpaired Student's *t*-tests were used to compare the data of two groups. Values were considered significantly different at $P<0.05$.

Results

Effects of JNK inhibition on NE-induced contractions

To evaluate the role of JNK on vascular contraction, we assessed the effects of the selective JNK inhibitor SP600125 on NE-induced contraction in rat aortic smooth muscle (13). NE (0.1 nM – 10 μM) induced aortic contraction in a dose-dependent manner. Pretreatment with 1 – 100 μM SP600125 for 30 min significantly inhibited NE-induced contraction in a dose-dependent manner (Fig. 1C). High K^+ (10 – 70 mM) induced contraction in a dose-dependent manner. In contrast, SP600125 (10 – 100 μM) did not show an inhibition of contraction induced by high K^+ (Fig. 1D).

Effects of JNK inhibition on NE-mediated JNK phosphorylation

To clarify the role of JNK on the NE-induced response, we examined the effects of NE and SP600125 on JNK activity measured in rat aortic smooth muscle using anti-phosphorylated JNK antibodies. NE (10 μM) increased the phosphorylation of JNK, which reached a maximum at 15 min (277 ± 3.6% of resting level, n = 4; Fig. 2A). In the quiescent state, SP600125 (10 μM) did not alter the resting phosphorylation of JNK. Pretreatment with SP600125 (10 μM) for 30 min inhibited the NE-induced phosphorylation of JNK to 185 ± 8.6% of

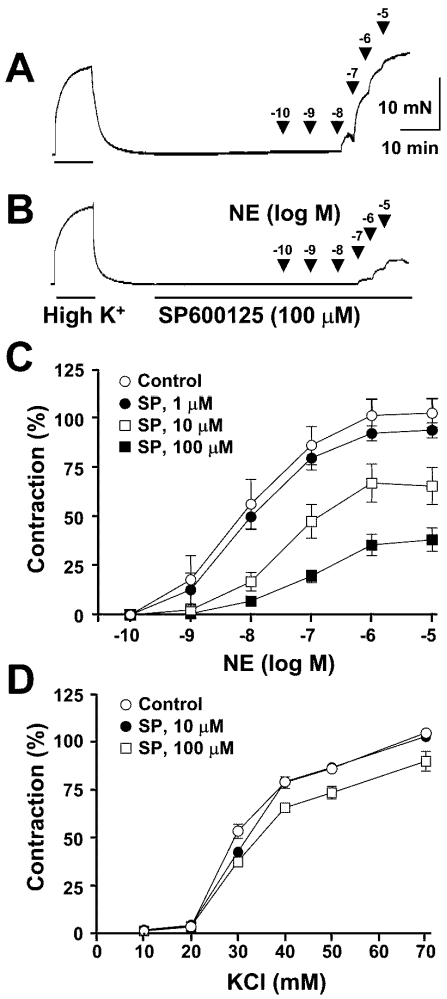

Fig. 1. Effects of SP600125 on contraction in aortic smooth muscle strips. After the treatment with (B) or without (A) SP600125 (100 μM) for 30 min, NE (0.1 nM – 10 μM) was applied cumulatively. Dose-response curves of the contraction induced by NE (C) and high K^+ (D) in SP600125 (SP, 1 – 100 μM)-pretreated strips. The contractile level of 70 mM K^+ before treatment with NE (n = 8) or high K^+ (n = 4) was defined as 100%.

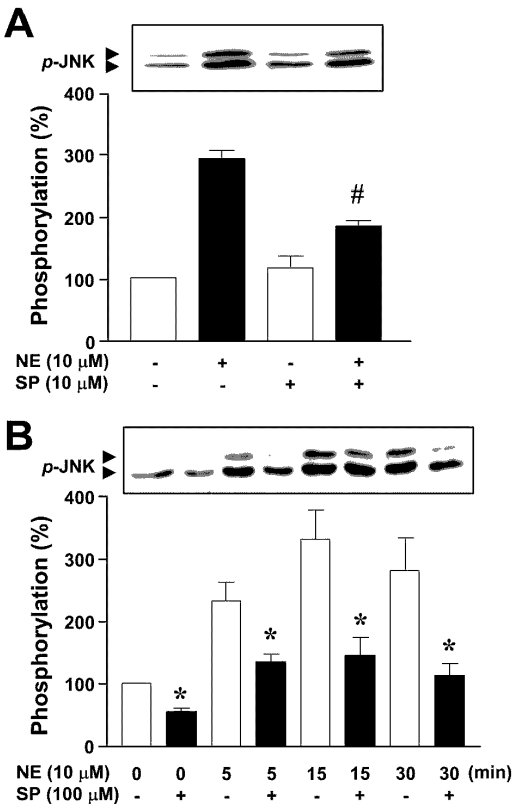

Fig. 2. Effects of NE and SP600125 on JNK phosphorylation in aortic smooth muscle. A: After the treatment with or without SP600125 (SP, 10 μM) for 30 min, NE (10 μM) was applied for 15 min. B: After the treatment with or without SP600125 (SP, 100 μM) for 30 min, NE (10 μM) was applied for 5, 15, or 30 min, cumulatively. The phosphorylation of JNK was demonstrated using a phosphorylated JNK antibody. Statistical analysis of the level of phosphorylated JNK was performed on four independent experiments. The level of phosphorylated JNK in the quiescent state of control strips was defined as 100%. *,# denote significant differences from control values and NE-stimulated state, respectively (P<0.05).

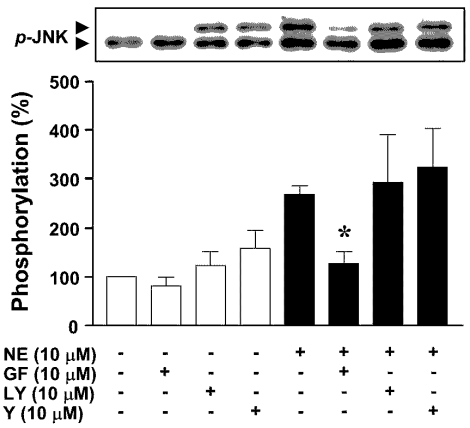

Fig. 3. Effects of kinase inhibitors on JNK phosphorylation elevated by NE in aortic smooth muscle. The strips were treated with GF109203X (GF, 10 μM), LY294002 (LY, 10 μM), or Y27632 (Y, 10 μM) for 30 min and then stimulated with NE 10 μM for 15 min. The phosphorylation of JNK was detected using phosphorylated JNK antibodies. The level of JNK phosphorylation before treatment with inhibitors was defined as 100% (n = 4). * denotes significant difference from the NE-stimulated strips (P<0.05).

the control value (n = 4, Fig. 2A). SP600125 (100 μM, 30 min) inhibited the NE-induced phosphorylation of JNK to 115 ± 29% of the control value at 15 min (n = 4, Fig. 2B). In the absence of NE, treatment with SP600125 (100 μM) for 30 min slightly inhibited the resting phosphorylation of JNK (54 ± 5% of resting level, n = 4; P<0.05; Fig. 2B). The total expression of JNK, determined using an anti-nonphosphorylated JNK antibody, was not altered by treatment with NE or SP600125 (data not shown).

Kinase pathways contribute to JNK phosphorylation

We examined the upstream of the JNK pathway response to NE in rat aortic smooth muscle. The PKC inhibitor GF109203X (10 μM) inhibited the NE-induced phosphorylation of JNK (127 ± 26% of control, n = 4; Fig. 3). In contrast, 10 μM LY294002 or 10 μM Y27632, inhibitors of phosphatidylinositol 3-kinase (PI3K) and Rho kinase, respectively, did not attenuate the NE-induced phosphorylation of JNK in rat aortic smooth muscle (Fig. 3). These kinase inhibitors did not change the resting level of JNK phosphorylation (Fig. 3). These results imply that PKC is involved in the JNK-mediated contraction elicited by NE in rat aortic smooth muscle. To determine the specific PKC isoforms involved in JNK activation, we assessed the effect of THX, a selective activator of cPKC, on the phosphorylation of JNK. THX (1 μM) increased the phosphorylation of JNK in rat aortic smooth muscle (Fig. 4A). The cPKC inhibitor Gö6976 (1 μM) inhibited the THX-induced increase in JNK phosphorylation. Gö6976 (1 μM) also inhibited the phosphorylation of JNK induced by NE in aortic smooth muscle (Fig. 4B).

Effects of JNK on actin-binding protein

To evaluate whether the activation of the JNK pathway regulates actin-binding protein, we measured the phosphorylation of caldesmon using a Ser^{789} phosphorylated *h*-caldesmon antibody. NE (10 μM) increased the phosphorylation of caldesmon (212 ± 19% of resting level, n = 4; Fig. 5A). Pretreatment with SP600125 (10 μM) inhibited the increase in caldesmon phosphory-

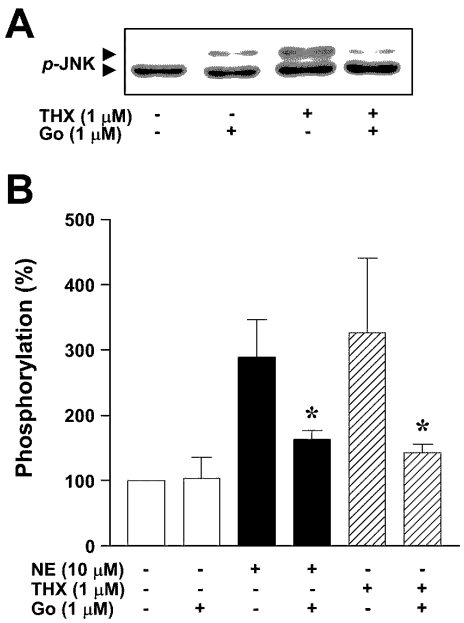

Fig. 4. Effects of stimulation and inhibition of cPKC on JNK phosphorylation in aortic smooth muscle. A: The strips were treated with Gö6976 (Go, 1 μM) for 30 min and then stimulated with THX (1 μM) or NE (10 μM) for 15 min. The phosphorylation of JNK was detected using phosphorylated JNK antibodies. B: Statistical results of levels of phosphorylated JNK were obtained from four independent experiments. The level of JNK phosphorylation before treatment with stimulators was defined as 100%. * denotes significant difference from the THX- or NE-stimulated strips (P<0.05).

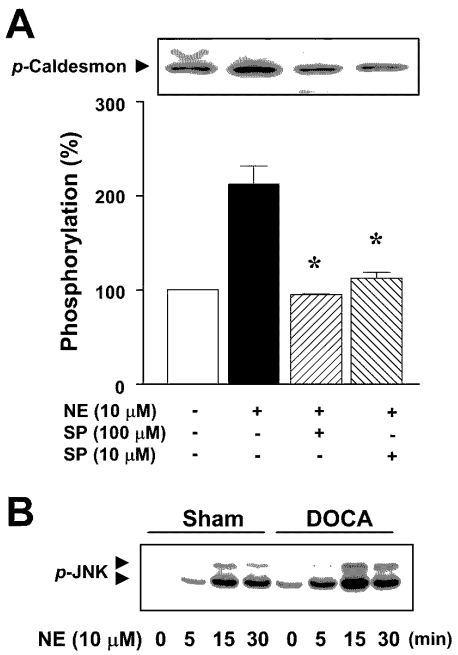

Fig. 5. Effects of NE on the caldesmon and JNK phosphorylation in aortic smooth muscle. A: Effects of NE on the phosphorylation of caldesmon in aortic smooth muscle. After treatment with or without SP600125 (SP, 10 and 100 μM) for 30 min, NE (10 μM) was applied for 15 min. The phosphorylation of caldesmon was detected using a Ser^{789} phosphorylated *h*-caldesmon antibody. The level of phosphorylated caldesmon in the quiescent state of control strips was defined as 100%. * denotes significant difference from the NE-stimulated strips (P<0.05). B: Differential phosphorylation of JNK between sham-operated and DOCA-salt hypertensive rats. The aortic strips were prepared as described in Fig. 2. Results are typical recordings obtained from three independent experiments.

lation by NE in rat aortic smooth muscle (113 ± 5.6% of resting level, n = 4; P<0.05; Fig. 5A). SP600125 (100 μM) also inhibited the phosphorylation of caldesmon induced by NE to 95 ± 1.7% of the resting level (n = 4; P<0.05; Fig. 5A).

JNK phosphorylation in vascular smooth muscle from hypertensive rats

To determine whether JNK influences hyperreactivity in hypertensive vessels, we analyzed the differences in JNK activity between sham-operated normotensive rats and DOCA-salt hypertensive rats. The phosphorylation of JNK was greater in aortic smooth muscle from DOCA-salt hypertensive rats than from sham-operated rats. NE (10 μM) increased JNK phosphorylation in aortic smooth muscle strips, and this response was greater in DOCA-salt hypertensive rats than in sham-operated rats (Fig. 5B).

Discussion

We found that NE increased the activity of JNK and contraction in rat aortic smooth muscle. Moreover, the selective JNK inhibitor SP600125 inhibited NE-mediated contraction and JNK activity. These data suggest that NE-induced vascular contraction is mediated, at least in part, by the JNK pathway. Although SP600125 inhibits the bradykinin-mediated contraction in tracheal smooth muscle (14), the role of JNK in vascular smooth muscle contraction has not been reported. To our knowledge, this is the first study to show that the JNK pathway contributes to the NE-induced contraction in rat vascular aortic smooth muscle. NE also increases the activity of ERK and p38 MAPK and induces contraction in vascular smooth muscle, which is inhibited by kinase inhibitors (15, 16).

These results indicate that, in rat aortic smooth muscle, JNK can be activated in parallel with isoforms of MAPK kinase, including ERK1/2 and p38 MAPK, and that this activation results in vascular contraction.

Upstream signals including the Rho GTPase pathway and PI3K/protein kinase B pathway phosphorylate MAPK kinase kinase, and, in turn, can activate JNK via phosphorylation of MAPK kinase (10). PI3K activity is involved in growth factor-mediated contraction in vascular smooth muscle (17), and PI3K is upstream to the MAPK pathway in the regulation of basal vascular tone (18). In contrast, our data show that inhibition of PI3K did not attenuate the NE-induced phosphorylation of JNK. Moreover, the present and previous results show that the Rho kinase inhibitor Y27632 strongly inhibits receptor agonist-induced contraction without any change in the MAPK phosphorylation pathway (2, 3). Although the Rho and PI3K pathways can contribute to the regulation of NE-induced contraction in vascular smooth muscle, these pathways may be dissociated from MAPK-mediated NE-contraction. We have reported similar results in the regulation of basal tone in hypertensive rats (8).

PKC is involved in the signal transduction of a wide range of biological responses, including changes in cell morphology, proliferation, and differentiation (19). PKC is a major modulator of contraction, which is regulated by Ca^{2+}-dependent and independent mechanisms in vascular smooth muscle (20). PKC controls JNK activity as an upstream molecule (21). We found that NE-induced JNK activation was significantly inhibited by the PKC inhibitors Gö6976 and GF109203X. Moreover, the cPKC activator THX significantly increased JNK activity, and this effect was inhibited by cPKC inhibition in vascular smooth muscle (22). These results imply that PKC, especially cPKC, is an upstream activator of JNK in NE-induced vasoconstriction.

The present and previous studies showed that high K^+-induced contraction was not inhibited by treatment with JNK inhibitor and receptor agonist-mediated MLC phosphorylation was not inhibited by MAPK inhibitor (23). The activity of MAPK isoforms were not attenuated by the Ca^{2+} channel inhibitor in vascular smooth muscle (8). These results implying that the JNK-mediated contraction pathway may be dissociated with the Ca^{2+}-dependent contractile pathway. Moreover, the activation of ERK1/2 participates in smooth muscle contraction by inhibiting the hindrance of caldesmon (2). Our data show that NE increases the phosphorylation of *h*-caldesmon in aortic smooth muscle. The inhibitors of JNK inhibited the phosphorylation of caldesmon in response to NE. NE can induce a greater vascular contraction by a Ca^{2+}-independent mechanism (1). Moreover, caldesmon is a major modulator of Ca^{2+}-independent contraction in vascular smooth muscle (2). These results showed that NE stimulates the MAPK pathway, as well as Ca^{2+}-MLC phosphorylation, and subsequently phosphorylates caldesmon, causing force development. Altered responsiveness of vessels is often associated with the elevation of systolic blood pressure (6) and the contractile response of vascular smooth muscle to vasoconstrictors increases significantly in hypertensive animals (7). We have shown previously that the activities of MAPK, ERK1/2, and p38 MAPK and caldesmon phosphorylation are higher in vascular smooth muscle from hypertensive rats than in normotensive rats (2). Moreover, JNK can regulate caldesmon phosphorylation and this kinase activity increases in hypertensive rats. Taken together, these data suggest that increased activities of MAPK isoforms result in the phosphorylation of caldesmon and contribute to the elevated contractile responses in hypertensive rats.

In summary, our data demonstrate that the JNK pathway plays an important role in the contractile signaling initiated by NE in vascular smooth muscle. This JNK-mediated contraction is mediated by the phosphorylation of caldesmon, which is regulated by PKC, especially cPKC.

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