Involvement of endogenous nitric oxide in angiotensin II-induced activation of vascular mitogen-activated protein kinases

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Zhang G-X, Nagai Y, Nakagawa T, Miyanaka H, Fujisawa Y, Nishiyama A, Izuishi K, Ohmori K, Kimura S. Involvement of endogenous nitric oxide in angiotensin II-induced activation of vascular mitogen-activated protein kinases. Am J Physiol Heart Circ Physiol 293: H2403–H2408, 2007. First published July 6, 2007; doi:10.1152/ajpheart.00288.2007.—Angiotensin II (ANG II) is a powerful activator of mitogen-activated protein (MAP) kinase cascades in cardiovascular tissues through a redox-sensitive mechanism. Nitric oxide (NO) is considered to antagonize the vasoconstrictive and proatherosclerotic actions of ANG II. However, the role of endogenous NO in ANG II-induced redox-sensitive signal transduction is not yet clear. In this study using catheterized, conscious rats, we examined the effects of NO synthase inhibition on ANG II-induced aortic MAP kinase phosphorylation. Furthermore, we also examined the effects of NO synthase inhibition on ANG II-induced aortic MAP kinase phosphorylation. Additionally, we demonstrated that acute intravenous administration of N°-nitro-l-arginine methyl ester (l-NAME; 5 mg/kg) enhanced phosphorylation of aortic MAP kinases extracellular signal regulated kinase (ERK) 1/2 and p38, which were suppressed only partially by a superoxide dismutase mimetic (Tempol), whereas ANG II-induced MAP kinase phosphorylation was markedly suppressed by Tempol. FK409, a NO donor, had little effect on vascular MAP kinase phosphorylation. On the other hand, acute exposure to a vasoconstrictor dose of ANG II (200 ng·kg⁻¹·min⁻¹) iv failed to enhance phosphorylation of aortic MAP kinases in the chronically l-NAME-treated rats, whereas the vasoconstrictor effect of ANG II was not affected by l-NAME treatment. Furthermore, three different inhibitors of NO synthase suppressed, in a dose-dependent manner, ANG II-induced MAP kinase phosphorylation in rat vascular smooth muscle cells, which was closely linked to superoxide generation in cells. These results indicate the involvement of endogenous NO synthase in ANG II-induced signaling pathways, leading to activation of MAP kinase, and that NO may have dual effects on the vascular MAP kinase activation associated with redox sensitivity.

oxidative stress; N°-nitro-l-arginine methyl ester; extracellular signal-regulated kinase 1/2; p38

mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK) 1/2, p38 MAP kinase, and c-Jun NH2-terminal kinase (JNK), participate in pathological vascular remodeling and are implicated in the development of arteriosclerosis and hypertension (14). Several reports have suggested that reactive oxygen species (ROS) play a critical role in activation of these MAP kinases (11). We have recently demonstrated that acute intravenous infusion of angiotensin II (ANG II) or phenylephrine, an α-adrenergic agonist, strongly stimulates phosphorylation of MAP kinases in the aorta of conscious rats, and this is suppressed by simultaneous treatment with a membrane-permeable superoxide dismutase mimetic, 4-hydroxy-2,6,6-tetramethylpiperidine-N-oxyl (Tempol), indicating that redox-sensitive activation of MAP kinases also takes place in blood vessels in vivo (7, 23).

Nicotine oxide (NO) is a widespread vasoactive molecule and can stimulate soluble guanylate cyclase, the mechanism that accounts for its biological responses. Several roles of NO, including vascular relaxation and inhibitory actions on leukocyte adhesion to endothelium (9), platelet aggregation (18), and smooth muscle proliferation (6), are considered antagonistic to those of ANG II in a variety of vascular functions. However, the possible role of NO in regulation of vascular MAP kinase is controversial. Kubo et al. (10) have shown that sodium nitroprusside, a NO donor, antagonizes ANG II-induced ERK1/2 activation, and N°-nitro-l-arginine methyl ester (l-NAME), a nonspecific NO synthase inhibitor, increases ERK1/2 activity in intact rat aortic segments. Palen et al. (13) have recently shown that NO can dephosphorylate basal ERK1/2 in rat vascular smooth muscle cells in a cGMP-dependent fashion. Meanwhile, Gu et al. (5) have demonstrated that NO donor S-nitroso-N-acetylpenicillamine increases p21 protein expression through an ERK activation pathway. Bauer et al. (1) have demonstrated that the antiproliferative effect of NO on vascular smooth muscle cells is mediated by ERK1/2 activation. Furthermore, Mizuno et al. (12) have demonstrated, using human pulmonary vascular smooth muscle cells, that exogenous NO transiently activates ERK via induction of p53 and then suppresses it via inactivation of the Ras/Raf cascade. Pinzar et al. (15) have recently shown that l-NAME suppresses ANG II-stimulated ERK phosphorylation in rat vascular smooth muscle cells. Although these studies indicate a complexity of NO-mediated interaction, through different signal transduction pathways leading to MAP kinase activation, few studies have investigated the possible effect of NO on vascular MAP kinases at the whole-animal level from the viewpoint of ROS sensitivity.

In this study using catheterized, conscious rats, we examined 1) the acute effect of NO synthase inhibition on aortic MAP kinase phosphorylation in the presence or absence of radical scavengers and 2) the involvement of endogenous NO in ANG II-induced aortic MAP kinase phosphorylation. Furthermore, we also examined the effects of NO synthase inhibition on ANG II-induced MAP kinase phosphorylation and superoxide generation using cultured rat vascular smooth muscle cells.

METHODS

Animal preparation. Nine-wk-old male Sprague-Dawley rats were used. All animals were allowed to recover overnight after catheterization.
ization, and all hemodynamic measurements were performed on conscious rats (23). The femoral arterial catheter was connected to a pressure transducer, and mean arterial blood pressure and heart rate were continuously recorded on a multichannel polygraph recorder. In the acute experiment, L-NAME was administered intravenously at a dose of 5 mg/kg. ANG II and FK409 (Fujisawa Pharmaceutical, Osaka, Japan), a NO donor, were infused at a rate of 200 ng·kg⁻¹·min⁻¹ and 0.1 mg·kg⁻¹·min⁻¹ for 30 min, respectively. Tempol (Sigma) and 3-carboxy-2,2,5,5-tetramethyl-1-pyrroldinioxy (3-CP, Sigma), a structurally related and inactive compound of Tempol, were given intravenously at a priming dose of 30 mg/kg, followed by infusion at a rate of 0.5 mg·kg⁻¹·min⁻¹. Treatment with Tempol or 3-CP was started 10 min before the administration of L-NAME or ANG II. Thirty min after treatment, the thoracic aorta was removed, quickly frozen in liquid nitrogen, and stored at −80°C.

In a separate experiment, rats received chronic L-NAME treatment (1 g/l in drinking water) for 7 days. ANG II was infused into rats chronically treated with L-NAME for 30 min, with a similar protocol to that of the acute experiment above. All surgical and experimental procedures were approved by the Kagawa University Animal Care and Use Committee (ACUC) and performed according to the Kagawa University ACUC guidelines.

Cell culture. Rat vascular smooth muscle cells prepared by the explant method from the descending thoracic aorta of 4-wk-old male rats were incubated at 37°C in 5% CO₂ in air. Rat vascular smooth muscle cells were prepared by the explant method from the descending thoracic aorta of 4-wk-old male rats. L-NAME was given 30 min before the exposure to 100 nM ANG II. Thirty min after treatment, the thoracic aorta was removed, quickly frozen in liquid nitrogen, and stored at −80°C. In the acute experiment, rats received chronic L-NAME treatment (1 g/l in drinking water) for 7 days. ANG II was infused into rats chronically treated with L-NAME for 30 min, with a similar protocol to that of the acute experiment above. All surgical and experimental procedures were approved by the Kagawa University Animal Care and Use Committee (ACUC) and performed according to the Kagawa University ACUC guidelines.

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**RESULTS**

**Effects of acute L-NAME administration on arterial blood pressure and heart rate in conscious rats.** Changes of mean blood pressure and heart rate are summarized in Table 1. Intravenous injection of L-NAME (5 mg/kg) increased mean arterial blood pressure from 103 ± 4 to 141 ± 5 mmHg within 10 min and decreased heart rate from 320 ± 14 to 244 ± 17 beats/min. Tempol or 3-CP alone did not alter mean arterial blood pressure and heart rate during the experimental period (data not shown), and simultaneous administration of Tempol or 3-CP had no significant effects on the hemodynamic changes induced by L-NAME. Intravenous infusion of FK409 (0.1 mg·kg⁻¹·min⁻¹) decreased mean blood pressure by 22 mmHg.

**Effects of acute L-NAME administration on the phosphorylation of aortic MAP kinase in conscious rats.** Previously, we demonstrated that ANG II and phenylephrine, an α-adrenergic receptor agonist, stimulated aortic MAP kinase phosphorylation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>−10 min</th>
<th>−5 min</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
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<tbody>
<tr>
<td>Control</td>
<td>102 ± 4</td>
<td>102 ± 4</td>
<td>103 ± 4</td>
<td>102 ± 4</td>
<td>102 ± 3</td>
<td>105 ± 3</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>L-NAME</td>
<td>340 ± 8</td>
<td>342 ± 8</td>
<td>341 ± 11</td>
<td>341 ± 12</td>
<td>345 ± 10</td>
<td>342 ± 10</td>
<td>342 ± 10</td>
</tr>
<tr>
<td>Tempol + L-NAME</td>
<td>104 ± 3</td>
<td>104 ± 3</td>
<td>103 ± 4</td>
<td>121 ± 5*</td>
<td>141 ± 5*</td>
<td>140 ± 5*</td>
<td>141 ± 6*</td>
</tr>
<tr>
<td>3-CP + L-NAME</td>
<td>321 ± 16</td>
<td>322 ± 15</td>
<td>320 ± 14</td>
<td>268 ± 8*</td>
<td>243 ± 10*</td>
<td>241 ± 12</td>
<td>241 ± 12</td>
</tr>
<tr>
<td>ANG II</td>
<td>105 ± 3</td>
<td>105 ± 3</td>
<td>104 ± 3</td>
<td>128 ± 5*</td>
<td>145 ± 8*</td>
<td>145 ± 6*</td>
<td>146 ± 6*</td>
</tr>
<tr>
<td>Tempol + ANG II</td>
<td>335 ± 2</td>
<td>331 ± 9</td>
<td>331 ± 9</td>
<td>266 ± 5*</td>
<td>255 ± 9</td>
<td>250 ± 9*</td>
<td>249 ± 9*</td>
</tr>
<tr>
<td>FK409</td>
<td>107 ± 3</td>
<td>106 ± 4</td>
<td>107 ± 6</td>
<td>149 ± 3*</td>
<td>152 ± 3*</td>
<td>155 ± 5*</td>
<td>152 ± 1*</td>
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Values are means ± SE. Mean blood pressure (top values, in mmHg) and heart rate (bottom values, in beats/min) in each group. Tempol or 3-carboxy-2,2,5,5-tetramethyl-1-pyrroldinioxy (3-CP) was administered 10 min before N⁵-nitro-l-arginine methyl ester (L-NAME). *P < 0.05 vs. values at 0 min.
tion in a redox-sensitive manner in conscious rats (3). Figure 1 shows the effects of L-NAME or FK409 administration on aortic MAP kinase phosphorylation compared with the effect of ANG II. L-NAME alone significantly increased the phosphorylation levels of aortic ERK1/2 and p38 MAP kinase 3.5- and 3.3-fold, respectively, compared with saline-infused control rats. Simultaneous administration with Tempol significantly, but only partially, suppressed the L-NAME-induced increases in phosphorylated MAP kinase in the aorta, whereas, consistent with our previous study, ANG II-induced phosphorylation of aortic MAP kinase was markedly suppressed by Tempol treatment.

FK409 did not affect the basal level of phosphorylated MAP kinase in the aorta.

**Effects of chronic L-NAME treatment on ANG II-induced aortic MAP kinase phosphorylation.** To investigate the role of endogenous NO in ANG II-induced signal transduction, the phosphorylated levels of aortic MAP kinases were analyzed using rats chronically treated with L-NAME. Mean blood pressure in rats treated with L-NAME for 7 days was significantly elevated (121 ± 4 mmHg compared with 102 ± 2 mmHg in the sham-operated control group). After the start of ANG II infusion (200 ng·kg⁻¹·min⁻¹) to rats

Fig. 1. Effects of N⁵-nitro-L-arginine methyl ester (L-NAME), angiotensin II (ANG II), and FK409 on aortic mitogen-activated protein (MAP) kinase phosphorylation. The phosphorylated forms of ERK1/2 and p38 MAP kinases were analyzed in the aorta obtained 30 min after treatment. Top: representative blots. Bottom: densitometric analysis of the phosphorylated forms of MAP kinase. The mean value of each phosphorylated protein in the saline-infused control rats is represented as 1. Data are presented as means ± SE of 4 or 5 rats. *P < 0.05 vs. the control rats. †P < 0.05 vs. the L-NAME- or ANG II-treated rats.

3-CP, 3-carboxy-2,2,5,5-tetramethyl-1-pyrroldinoyloxyl.

Fig. 2. Effects of chronic L-NAME treatment on ANG II-induced hypertension and aortic MAP kinase phosphorylation. A: mean arterial blood pressure was monitored during ANG II infusion. The data used were obtained at the end of a 30-min ANG II (200 ng·kg⁻¹·min⁻¹) or saline infusion. B: phosphorylated ERK1/2 and p38 MAP kinases were analyzed in the aorta obtained 30 min after treatment. B, top: representative blots. B, bottom: densitometric analysis of phosphorylated MAP kinase. The mean value of each phosphorylated protein in the saline-infused control rats is represented as 1. Data are presented as means ± SE of 4 or 5 rats. *P < 0.05 vs. the saline-infused rats in each group. †P < 0.05 vs. the rats without L-NAME treatment.
chronically treated with l-NAME, mean blood pressure increased rapidly by 41 mmHg, and this vasoconstrictive effect of ANG II was not affected by chronic l-NAME treatment (Fig. 2A).

The effect of chronic NO synthase inhibition on ANG II-induced vascular MAP kinase activation is shown in Fig. 2B. Aortic MAP kinases in rats chronically treated with l-NAME returned to basal levels. Interestingly, an addition of ANG II to chronic treatment with l-NAME failed to enhance aortic phosphorylation of ERK1/2 and p38 MAP kinases.

NO synthase inhibition suppresses ANG II-induced MAP kinase activation in rat vascular smooth muscle cells. In rat vascular smooth muscle cells, a 30-min exposure to 100 nM ANG II increased phosphorylation of ERK1/2 and p38 MAP kinases approximately eight and five times more than the basal level, respectively (Fig. 3). l-NAME, l-NMMA, and l-NNA suppressed ANG II-induced phosphorylation at doses >0.1 mM, with the exception of ERK1/2 suppression by l-NAME, which was only significant at a dose of 1 mM. No NO synthase inhibitor alone had any significant effect on the basal phosphorylation of ERK1/2 and p38 MAP kinases in rat vascular smooth muscle cells.

l-NAME suppresses superoxide generation stimulated by ANG II in rat vascular smooth muscle cells. To gain insights about the relationship between NO and vascular MAP kinase activation, we studied the effects of l-NAME on superoxide generation using DHE-loaded rat vascular smooth muscle cells. As shown in Fig. 4, ANG II increased fluorescence intensity of ethidium, indicating an enhancement of superoxide generation in rat vascular smooth muscle cells during the incubation time determined. The enhanced superoxide generation by ANG II was apparently suppressed in the presence of l-NAME. It is of note that l-NAME alone slightly, but significantly, increased ethidium fluorescence (Fig. 4B).

**DISCUSSION**

The vascular actions of NO play an important role in regulating physiological vascular tone and in the pathophysiological modulation of vascular smooth muscle cell prolifer...
DHE-loaded cells
Ethidium
Merged

Control
ANG II
L-NAME
L-NAME + ANG II

A

B

Fig. 4. Effect of l-NAME on ANG II-induced superoxide generation in rat vascular smooth muscle cells. Superoxide generation of rat vascular smooth muscle cells was visualized by the dihydroethidium (DHE) fluorescence method. l-NAME (1 mM) was given 30 min before exposure to 100 nM ANG II. A: representative fluorescence images after 30 min exposure to ANG II are shown. B: ratio of fluorescence intensity of ethidium (oxidized form of DHE) to DHE at 30 min after ANG II exposure. Data are presented as means ± SE of 4 different cover slides. *P < 0.05 vs. cells without ANG II. †P < 0.05 vs. cells without l-NAME treatment.

tion, which are both mediated through cGMP-dependent and -independent signaling. Our experimental data clearly demonstrated that acute intravenous administration of l-NAME elicited increases of phosphorylated ERK1/2 and p38 MAP kinases in the aorta of conscious rats. On the contrary, ANG II-induced augmentation of phosphorylation of aortic MAP kinases was markedly suppressed during chronic NO synthase inhibition with l-NAME. The latter finding was supported by in vitro experiments using cultured rat vascular smooth muscle cells, showing that pretreatment of l-NAME or other NO synthase inhibitors suppressed, in a dose-dependent manner, ANG II-induced ERK1/2 and p38 MAP kinase phosphorylation, which was closely related to superoxide production. These data indicate that NO has dual effects on vascular MAP kinase activation associated with redox sensitivity.

Consistent with our previous reports, the increased phosphorylation of vascular ERK1/2 and p38 MAP kinases by acute ANG II challenge was almost completely reversed by simultaneous treatment with Tempol (23). On the other hand, the increase in these phosphorylated MAP kinases by acute l-NAME treatment was not as sensitive to Tempol, indicating that there may be different and complex pathways involved in activation of vascular MAP kinases by acute exposure to l-NAME. Kubo et al. (10) have demonstrated that ERK1/2 phosphorylation in intact rat aortic segments is increased by endothelium denudation or by l-NAME treatment, which indicates a role for endothelial cell-derived NO in vascular ERK1/2 regulation. However, in this study we found that a NO donor, at a large dose that elicited a reduction in arterial blood pressure of ~20 mmHg, did not affect the phosphorylation of aortic (and also cardiac; data not shown) MAP kinases in conscious rats. Therefore, despite much in vitro evidence supporting a suppressive role of NO in vascular MAP kinase activation, exogenous NO given intravenously may have little effect on vascular MAP kinase activity under conscious physiological conditions.

Another important finding of this study is that ANG II is no longer effective at inducing aortic MAP kinase phosphorylation under conditions of NO synthase inhibition with chronic l-NAME treatment, indicating that NO synthase potentiates the vascular MAP kinase activation in response to acute ANG II stimulation. Phosphorylated MAP kinase levels in the aorta returned to baseline after 7 days treatment with l-NAME, although arterial blood pressure remained at a significantly elevated level under chronic NO synthase inhibition. Vasoconstrictor activity of ANG II was not affected by chronic NO synthase inhibition. Although Epstein et al. (4) have shown the involvement of ERK1/2 kinase in vasoconstriction, our results indicate that MAP kinase signal transduction and vasoconstrictor mechanisms are regulated differently.

Not only vascular endothelial cells but also vascular smooth muscle cells possess active NO synthase (3). To assess the possibility that endogenous NO synthase in vascular smooth muscle cells contributes to ANG II-induced vascular MAP kinase activation, we examined the effects of NO synthase inhibition on MAP kinase phosphorylation. We found that l-NAME and other NO synthase inhibitors, l-NMMA and l-NNA, could suppress ANG II-induced ERK1/2 and p38 MAP kinase phosphorylation in rat vascular smooth muscle cells in a dose-dependent fashion. Sensitivity to l-NAME treatment was greater for p38 than ERK1/2 MAP kinases, the former of which belongs to the group of stress-activated MAP kinase such as JNK (11). We have previously demonstrated that ROS sensitivity of ANG II-induced phosphorylation is greater in p38 or JNK than in ERK1/2 MAP kinases (8). Therefore, it can be speculated that NO participates in the redox-sensitive signal transduction from AT1 receptor to MAP kinase activation. We also demonstrated that superoxide-specific fluorescence of ethidium was greatly increased by ANG II stimulation in DHE-loaded rat vascular smooth muscle cells, which was strongly suppressed in the presence of l-NAME. Interestingly, l-NAME treatment alone slightly, but significantly, enhanced ethidium fluorescence compared with the control cells. These results indicate that NO synthase may affect superoxide production differently in basal and stimulated conditions.

Increased vascular production of ROS may reduce the action of endogenous NO, which has been considered as a major mechanism of maintenance of high blood pressure (19). Con-
comitantly, although endothelium NO synthase may be activated and counteracts ANG II-induced vasoconstriction (17) in response to ANG II stimulation, ANG II stimulates peroxynitrite through the interaction of superoxide and NO in endothelial cells (2, 16). Peroxynitrite stimulation in phosphorylation of MAP kinase in vascular smooth muscle cells has already been demonstrated (22). Although we did not define directly whether ANG II activated vascular MAP kinase via the peroxynitrite pathway in this study, both superoxide and peroxynitrite might be involved in the process of redox-sensitive MAP kinase activation in vasculature. Alternatively, NO synthases themselves may catalyze the uncoupling reduction of oxygen, leading to the generation of superoxide (21, 22). Further studies are necessary to evaluate regulatory mechanisms of MAP kinase activity by NO synthase inhibition in the vasculature.

GRANTS

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REFERENCES