Role of neuronal NO synthase in regulating vascular superoxide levels and mitogen-activated protein kinase phosphorylation

Guo-Xing Zhang1*, Shoji Kimura2, Koji Murao3, Juichiro Shimizu1, Hiroko Matsuyoshi1, and Miyako Takaki1

1Department of Physiology II, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan; 2Department of Pharmacology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe Miki-cho, Kita-gun, Kagawa 761-0793, Japan; and 3Division of Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe Miki-cho, Kita-gun, Kagawa 761-0793, Japan

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Aims The present study is designed to investigate the role of neuronal nitric oxide synthase (nNOS) in the regulation of vascular mitogen-activated protein kinase (MAPK) activity under basal and angiotensin II (Ang II)-stimulated conditions.

Methods and results Incubation with a potent nNOS inhibitor (L-VNIO) significantly increased superoxide (O2•−) levels, with increased MAPK phosphorylation, in isolated aorta and vascular smooth muscle cells (VSMCs) from wild-type mice. Both increases were inhibited by the superoxide dismutase mimetic, tempol, but not by the peroxynitrite scavenger, FeTPPS. The levels of O2•− and MAPK phosphorylation were higher in aorta from nNOS−/− mice than from wild-type mice. These parameters were suppressed by tempol and oxypurinal (a xanthine oxidase inhibitor). In isolated VSMCs or aorta from wild-type mice, Ang II stimulation markedly increased the levels of O2•− and MAPK phosphorylation. L-VNIO significantly reduced Ang II-induced increase of these parameters. Apocynin, an NAD(P)H oxidase inhibitor, further inhibited Ang II-induced increase of these parameters compared with the L-VNIO-treated group. FeTPPS did not suppress the Ang II-induced increase of O2•− levels, but markedly inhibited Ang II-induced MAPK phosphorylation. In contrast to the wild-type, in isolated aorta or VSMCs from nNOS−/− mice, Ang II failed to increase O2•− levels and MAPK phosphorylation.

Conclusion Under basal conditions, nNOS-derived NO acting as antioxidant reduces O2•− accumulation and suppresses vascular MAPK phosphorylation. Under Ang II-stimulated conditions, NAD(P)H oxidase-derived O2•−, inducing nNOS uncoupling, potentiates the Ang II-induced increase of O2•− generation. The generated O2•− may react with NO to form peroxynitrite (ONOO−). Both O2•− and ONOO− participate in Ang II-induced activation of vascular MAPK.

KEYWORDS Neuronal nitric oxide synthase; Angiotensin II; Superoxide; Mitogen-activated protein kinase (MAPK)

1. Introduction

Increased angiotensin II (Ang II) production in all vascular wall cells is implicated in the pathogenesis of vascular diseases, such as hypertension, atherosclerosis, and diabetes.1,2 A major mechanism underlying Ang II-induced vascular injury involves generation of reactive oxygen species (ROS), such as O2•− and H2O2, and ROS-mediated activation of mitogen-activated protein kinase (MAPK), mainly including ERK1/2, p38, JNK, and ERK5.3,4 Although membrane-bound NAD(P)H oxidase is generally accepted as the source of Ang II-induced ROS generation, the generated ROS activates mitochondrial KATP channels to induce ROS burst in the mitochondria, which activates down-stream signalling pathways, including the MAPK cascade in the cardiovascular tissue.7,9 Recently, we have reported that Ang II-induced O2•− generation and subsequent vascular MAPK activation in rat vascular smooth muscle cells (VSMCs) is attenuated by nitric oxide synthase (NOS) inhibition with Nω-nitro-L-arginine methyl ester (L-NAME).10 This report was consistent with a previous study showing that Ang II-induced ERK phosphorylation in rat VSMCs was suppressed by L-NAME.11 However, the mechanism is still unknown.

Although NOS has been demonstrated to generate nitric oxide (NO) by formation of L-citrulline from L-arginine under physiological conditions, increasing evidence also suggested that NOS function is affected by exposure to ROS, resulting in decreased NO generation and enhanced
ROS generation. Three types of NOS have been identified: NOS I (neuronal NOS, nNOS), NOS II (inducible NOS, iNOS), NOS III (endothelial NOS, eNOS). In vasculature, nNOS and eNOS are constitutively expressed, and they are tightly regulated by calcium. In bovine aortic endothelial cells, eNOS is uncoupled due to tetrahydrobiopterin (BH4) deficiency induced by NAD(P)H oxidase-derived ROS under basal conditions and this ROS down-regulates dihydrofolate reductase (DHFR) expression in response to Ang II stimulation. Although iNOS is stimulated by cytokine and microbial endotoxin to generate NO unrelated to intracellular calcium, it has been suggested that iNOS is involved in Ang II-induced ERK phosphorylation in VSMCs.

Recently, nNOS has been shown to exert vasculoprotective effects in carotid artery ligation and balloon injury models. It was also reported that nNOS can be activated by platelet-derived growth factor through MEK/ERK pathway in mouse aorta and by Ang II in VSMCs from spontaneously hypertensive rats. However, the role of nNOS condition in O2 generation and MAPK activation under Ang II-stimulated was still unknown. Therefore, in the present study, we used pharmacological inhibition of nNOS and genetic deletion of nNOS to investigate the possible roles of nNOS in the regulation of O2 generation and MAPK activity under basal and Ang II-stimulated conditions.

2. Methods

2.1 Vascular rings experiments

Mice homozygous for targeted disruption of the nNOS gene (nNOS−/−) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and its littermate age-matched C57BL/6J mice (CLEA, Japan) were used as wild-type in this study. All surgical and experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa University and conformed to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mice were anaesthetized with pentobarbital sodium (50 mg/kg IP) and thoracic aortas were dissected free from surrounding tissue and cut into 3-4 mm long rings. Rings were incubated at 37°C in serum-free Dulbecco’s modified Eagle’s medium with N5-(1-imino-3-butenyl) ornithine, a potent nNOS inhibitor (L-VNIO, 10 μM) for 30 min with or without pre-treatment by tempol (a superoxide dismutase mimetic, 10 μM) or oxypurinyl (a xanthine oxidase (OXO) inhibitor, 10 μM) or 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrinato iron(III) (a per-oxynitrite scavenger, FeTPPS, 10 μM). In additional experiments, rings were stimulated by angitensin II (Ang II, 100 nM) for 30 min with or without pre-treatment by L-VNIO (10 μM) or apocynin (NAD(P)H oxidase inhibitor 10 μM) or FeTPPS (10 μM). Tempol or oxypurinyl were given 30 min before L-VNIO incubation, and L-VNIO, apocynin, and FeTPPS were also given 30 min before Ang II incubation.

2.2 Cell culture

Mouse VSMCs prepared by the explant method from the descending thoracic aorta of 4-week-old male mouse were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. Cells were incubated at 37°C in 5% CO2 in air. Mouse VSMCs were authenticated by using immunohistochemical staining for smooth muscle α-actin (Sigma). Cultures showing 95% staining for α-actin between passages three and seven were used. Prior to the experiments, cells were serum free for 24 h.

2.3 Detection of O2 levels in mouse vascular smooth muscle cells and aortas

Intracellular superoxide anion levels in mice VSMCs were determined by dihydroethidium (DHE) staining method as described previously. After treatment with L-VNIO or Ang II, mice VSMCs were incubated with 5 μM DHE for 10 min at 37°C. Cells were washed with PBS, DHE fluorescent images of mice VSMCs were visualized with a confocal microscope (Bio-Rad, CA, USA) by excitation at 405 nm and emission at 420 nm for detection of DHE-loaded cells, as stained in blue, and by excitation at 488 nm and emission at 610 nm for detection of oxidized DHE product ethidium, as stained in red. Densitometry analysis was performed after various treatments. Data were represented as fold change compared with wild-type control group.

Extracellular superoxide anion production in aortic rings was determined by the lucigenin-enhanced chemiluminescence (LC) method as described previously. Briefly, after L-VNIO or Ang II treatment, aortic rings were incubated in chilled bicarbonate buffer composed of (in mmol/L) 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 25.0 NaHCO3, 5.5 glucose, and 0.026 EDTA. The buffer was then aerated continuously with 95% O2 and 5% CO2 to maintain a pH of 7.4, and allowed to equilibrate for 30 min at 37°C. After equilibration, the tissue samples were rinsed with pre-warmed (37°C) modified Krebs–HEPES buffer composed of (in mmol/L) 119 NaCl, 20 HEPES, 4.6 KCl, 1.0 MgSO4, 0.15 Na2HPO4, 0.4 KH2PO4, 25 NaHCO3, 1.2 CaCl2, and 5.5 glucose (pH 7.4). The rings were then placed in 1 mL of Krebs–HEPES buffer containing 10 μmol/L lucigenin and equilibrated in the dark for 10 min at 37°C. LC was recorded every 30 s for 15 min with a luminescence reader (BLR-301; Aloka) and data were expressed as counts per minute per milligram of dry tissue weight (× 103 CPM/mg).

2.4 Measurements of phosphorylation of ERK1/2, p38, and JNK mitogen-activated protein kinase, and expression of iNOS, eNOS, nNOS, superoxide dismutase, xanthine oxidase, and xanthine dehydrogenase

Total and phosphorylated ERK1/2, p38, and JNK MAPK, and expression of iNOS, eNOS, nNOS, superoxide dismutase, xanthine oxidase, and xanthine dehydrogenase were determined by western blotting, as previously described. Briefly, aortic rings or VSMCs, which were treated with or without L-VNIO or Ang II, were homogenized in cold-lysis buffer (Bio-Rad) or directly lysed in the lysis buffer. Equal amount of protein from each homogenate or lysate was resolved by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Hybond TM-ECL; Amersham Pharmacia Biotech). The membranes were blocked for 2 h at room temperature with 5% skimmed milk in PBS and 0.1% Tween 20. The blots were incubated overnight with 1:1000 diluted primary antibodies: anti-ERK1/2, anti-p38, anti-JNK, anti-phospho-ERK1/2, anti-phospho-p38, and anti-phospho-JNK (Cell Signaling Tech), anti-iNOS, anti-eNOS, anti-nNOS, anti-SOD1-3, anti-JNK, and xanthine dehydrogenase (XDH) were analysed by western blotting, as previously described. Briefly, aortic rings or VSMCs, which were treated with or without L-VNIO or Ang II, were homogenized in cold-lysis buffer (Bio-Rad) or directly lysed in the lysis buffer. Equal amount of protein from each homogenate or lysate was resolved by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Hybond TM-ECL; Amersham Pharmacia Biotech). The membranes were blocked for 2 h at room temperature with 5% skimmed milk in PBS and 0.1% Tween 20. The blots were incubated overnight with 1:1000 diluted primary antibodies: anti-ERK1/2, anti-p38, anti-JNK, anti-phospho-ERK1/2, anti-phospho-p38, and anti-phospho-JNK (Cell Signaling Tech), anti-iNOS, anti-eNOS, anti-nNOS, anti-SOD1-3, anti-JNK, and xanthine dehydrogenase (XDH) were analysed by western blotting, as previously described.

2.5 Statistical analysis

Values are expressed as mean ± SEM. Statistical significance between more than two groups was tested using two-way ANOVA followed by the Newman–Keuls test or unpaired two-tailed Student’s t-test. P < 0.05 was considered to indicate statistical significance.
3. Results

3.1 Effects of L-VNIO, tempol, or FeTPPS on superoxide level, and of tempol or FeTPPS on L-VNIO-induced superoxide level, or of L-VNIO, tempol, or oxypurin on nNOS deletion-induced superoxide level

Intracellular O$_2^-$ levels were measured by DHE staining in wild-type mouse VSMCs, showing that acute inhibition of nNOS by L-VNIO for 30 min significantly increased the O$_2^-$ level to $3.5 \pm 0.43$-fold of wild-type control (P < 0.05 vs. wild-type control) and tempol significantly reduced it to $1.3 \pm 0.24$-fold of wild-type control (P < 0.05 vs. L-VNIO group), but the O$_2^-$ level was not reduced by FeTPPS ($3.1 \pm 0.31$-fold of wild-type control) (Figure 1A and B). The extracellular O$_2^-$ level was measured in wild-type mouse aortic tissue, showing that L-VNIO significantly increased the O$_2^-$ levels from $13.5 \pm 1.6$ to $21.7 \pm 2.21 \times 10^3$ CPM/mg (P < 0.05) and tempol significantly reduced it to $10.8 \pm 1.2 \times 10^3$ CPM/mg (P < 0.05 vs. L-VNIO group), but FeTPPS did not reduce it ($20.5 \pm 2.3 \times 10^3$ CPM/mg) (Figure 1C).

It has been shown that genetic deficiency of nNOS leads to a marked increase in the O$_2^-$ level in cardiac tissue post-transcriptional regulation of XO. 19 The intracellular O$_2^-$ level in VSMCs from nNOS$^{-/-}$ mice was $4.2 \pm 0.36$-fold greater than wild-type control (P < 0.05). Tempol and oxypurin significantly reduced intracellular O$_2^-$ level to $0.95 \pm 0.17$ and $1.25 \pm 0.21$-fold of wild-type control, respectively (P < 0.05 vs. nNOS$^{-/-}$ control), but L-VNIO did not reduce it ($3.8 \pm 0.57$-fold of wild-type control) (Figure 1A and B). Similarly, the extracellular O$_2^-$ level in nNOS$^{-/-}$ mice aortic tissues was significantly higher than that of wild-type mice ($19.1 \pm 1.88$ vs. $13.5 \pm 1.6 \times 10^3$ CPM/mg, P < 0.05). Tempol and oxypurin significantly reduced it to $9.5 \pm 1.32$ and $10.5 \pm 1.34 \times 10^3$ CPM/mg, respectively, (P < 0.05), but L-VNIO did not reduce ($21.1 \pm 1.88 \times 10^3$ CPM/mg) (Figure 1C).

3.2 Effects of tempol or FeTPPS on L-VNIO-induced phosphorylation of ERK1/2, p38, and JNK MAPK, and of tempol or oxypurin on nNOS deletion-induced the mitogen-activated protein kinase phosphorylation

Acute inhibition of nNOS with L-VNIO significantly increased phosphorylation levels of ERK1/2, p38, and JNK MAPK to $2.4 \pm 0.21$, $3.1 \pm 0.25$, and $2.6 \pm 0.21$-fold, respectively, of wild-type control in wild-type mice VSMCs (P < 0.05). Tempol significantly decreased all the three phosphorylation levels to $1.1 \pm 0.1$, $1.2 \pm 0.25$, and $1.1 \pm 0.1$-fold of wild-type control, respectively (P < 0.05 compared with L-VNIO group), but FeTPPS did not decrease them ($2.8 \pm 0.1$, $3.3 \pm 0.23$, and $3.1 \pm 0.1$-fold of wild-type control, respectively) (Figure 2A and B). Data from aortic rings were similar to those from VSMCs (data not shown).

Genetic deletion of nNOS resulted in high phosphorylation levels of ERK1/2, p38, and JNK MAPK in VSMCs of nNOS$^{-/-}$ mice to $2.8 \pm 0.14$, $2.8 \pm 0.13$, and $3.1 \pm 0.34$-fold, respectively, of wild-type control (P < 0.05 vs. wild-type control). Tempol and oxypurin reduced the MAPK phosphorylations to $1.1 \pm 0.09$, $1.2 \pm 0.13$, and $1.2 \pm 0.12$-fold of wild-type control and $0.98 \pm 0.1$, $1.3 \pm 0.23$, and $1.1 \pm 0.1$-fold of wild-type control, respectively (P < 0.05 vs. nNOS$^{-/-}$ control), but L-VNIO did not reduce them ($2.5 \pm 0.37$, $2.9 \pm 0.24$, $2.9 \pm 0.36$-fold of wild-type control, respectively) (Figure 2B). Similar data were also obtained from aortic rings (data not shown).

3.3 Expression of eNOS, iNOS, SOD1-3, xanthine oxidase, and xanthine dehydrogenase proteins in aorta from wild-type and nNOS$^{-/-}$ mice

Expression of eNOS and iNOS protein levels in aortic tissue were not different between wild-type and nNOS$^{-/-}$ mice (Figure 3A and B). As expected, nNOS protein was not detected in nNOS$^{-/-}$ mouse aorta, but it was detected in wild-type mouse aorta (Figure 3C). There were no differences in SOD1-3, XO, and XDH protein levels between wild-type and nNOS$^{-/-}$ mice (Figure 3D and E).

3.4 Effects of L-VNIO, FeTPPS, or apocynin and genetic deletion of nNOS on Ang II-increased O$_2^-$ level

DHE staining results showed that Ang II significantly increased the intracellular O$_2^-$ level to $4.7 \pm 0.34$-fold of wild-type control in VSMCs from wild-type mice (P < 0.05). L-VNIO markedly reduced Ang II-increased intracellular O$_2^-$ level to $2.3 \pm 0.24$-fold of wild-type control (P < 0.05 vs. Ang II wild-type group). Apocynin further decreased it to $1.2 \pm 0.21$-fold of wild-type control (P < 0.05 vs. Ang II-L-VNIO wild-type group), but FeTPPS did not reduce it ($4.5 \pm 0.39$-fold of wild-type control) (Figure 4A and B). Ang II stimulation also significantly increased extracellular O$_2^-$ level in aorta of wild-type mice from $13.5 \pm 1.62$ to $30.7 \pm 1.21 \times 10^3$ CPM/mg (P < 0.05). Similar results were obtained in aorta. L-VNIO and apocynin significantly reduced Ang II-increased extracellular O$_2^-$ levels to $21.8 \pm 1.23 \times 10^3$ CPM/mg (P < 0.05 vs. Ang II wild-type group) and $10.1 \pm 0.88 \times 10^3$ CPM/mg (P < 0.05 vs. Ang II wild-type group and Ang II+L-VNIO wild-type group), respectively, but FeTPPS did not reduce it ($29.9 \pm 2.1 \times 10^3$ CPM/mg) (Figure 4C).

Although VSMCs and aorta from nNOS$^{-/-}$ mice also showed high levels of intracellular and extracellular O$_2^-$, which is similar to results from cardiac tissues, neither intracellular nor extracellular O$_2^-$ levels were further increased in response to Ang II stimulation in VSMCs ($3.9 \pm 0.33$ vs. $3.5 \pm 0.43 \times 10^3$ CPM/mg) or aorta ($21.5 \pm 1.34$ vs. $19.1 \pm 1.88 \times 10^3$ CPM/mg), respectively (Figure 4B and C).

3.5 Effects of L-VNIO, FeTPPS, or apocynin and genetic deletion of nNOS on Ang II-induced phosphorylation levels of ERK1/2, p38, and JNK mitogen-activated protein kinase

Ang II significantly increased phosphorylation levels of ERK1/2, p38, and JNK MAPK in VSMCs of wild-type mice to $6.5 \pm 0.54$, $4.3 \pm 0.34$, and $6.4 \pm 0.54$-fold wild-type control (P < 0.05). L-VNIO or FeTPPS markedly reduced Ang II-increased phosphorylations of ERK1/2, p38, and JNK MAPK to $3.2 \pm 0.32$, $2.1 \pm 0.34$, and $2.45 \pm 0.43$-fold of wild-type control or $2.8 \pm 0.37$, $1.9 \pm 0.27$, and $2.4 \pm 0.53$-fold of wild-type control, respectively (P < 0.05 vs. Ang II wild-type group). However, apocynin further decreased Ang II-increased MAPK phosphorylation to $1.2 \pm 0.14$, $1.2 \pm 0.2$, and
Figure 1 Effects of tempol (Tem, 10 μM) or FeTPPS (10 μM) on L-VNIO (10 μM)-induced superoxide level or L-VNIO, Tem, or oxypurinal (Oxy, 10 μM) on nNOS deletion-induced superoxide level in isolated mouse vascular smooth muscle cells and aorta. (A) Representative fluorescence images of vascular smooth muscle cells after 30 min exposure to L-VNIO or pre-treated with Tem or FeTPPS from wild-type (WT) mice and those after 30 min exposure to L-VNIO, Tem, or Oxy from nNOS−/− mice are shown. (B) Intracellular superoxide level shown by intensity of ethidium (oxidized form of DHE) fluorescence of vascular smooth muscle cells after 30 min exposure to L-VNIO or pre-treated with Tem or FeTPPS from WT mice and that after 30 min exposure to L-VNIO, Tem, or Oxy from nNOS−/− mice are shown. Mean value in WT control (Con) is expressed as 1. (C) Extracellular superoxide level from aortic rings after 30 min incubation with L-VNIO or pre-treated with Tem or FeTPPS from WT mice and that after 30 min incubation to L-VNIO, Tem, or Oxy from nNOS−/− mice are shown. Data are presented as mean ± SEM of six different experiments. *P < 0.05 vs. WT Con. †P < 0.05 vs. L-VNIO. §P < 0.05 vs. nNOS−/− Con.
1.2 ± 0.21-fold of wild-type control (P < 0.05 vs. Ang II wild-type group and Ang II+L-VNIO wild-type group) (Figure 5A and B).

High-level phosphorylations of ERK1/2, p38, and JNK MAPK were observed in VSMCs from nNOS−/− mice as mentioned earlier. Similar to O₂⋅⁻ generation in response to Ang...
levels of MAPK phosphorylation were not further increased in VSMCs (3.2 ± 0.53-, 2.6 ± 0.35-, and 3.0 ± 0.3-fold of wild-type control). Similar results were also observed from aortic rings (data not shown).

4. Discussion
In the present study, we have investigated the effects of acute nNOS inhibition and genetic deletion of nNOS on vascular superoxide generation and MAPK phosphorylation. Our experimental data clearly demonstrate that nNOS acts as an antioxidant in the vascular system under physiological conditions and that genetic deletion of nNOS stimulates XO-mediated O$_2^-$ generation without affecting XO protein expression. Furthermore, nNOS enhances Ang II-induced increases of vascular O$_2^-$ levels and MAPK phosphorylation, whereas genetic deletion of nNOS blunts Ang II-induced increases of O$_2^-$ levels and MAPK phosphorylation. Based on these findings, propose a possible mechanism, by which O$_2^-$ from NAD(P)H oxidase elicited by Ang II may trigger nNOS
uncoupling to generate $\text{O}_2^-$. The generated $\text{O}_2^-$ may react with NO to form $\text{ONOO}^-$ and thus these generated $\text{O}_2^-$ and $\text{ONOO}^-$ may result in phosphorylation of vascular MAPK. The cascade of intracellular signal transduction pathways illustrated in Figure 6 represents a novel mechanism for Ang II-induced vascular diseases such as hypertension, diabetes, atherosclerosis, and myocardial infarction.\textsuperscript{20-22}

NO generated from NOS by catalysing L-arginine to L-citrulline under physiological conditions, is a ubiquitous molecule. Its roles in vascular relaxation, inhibition of leukocyte
adhesion to the endothelium, platelet aggregation, and smooth muscle proliferation are considered to be beneficial to the cardiovascular system under physiological conditions.\textsuperscript{14,15} Until now, most observations have focused on the roles of iNOS- and eNOS-derived NO in the vascular system. Following the discovery of the universal existence of nNOS in mitochondria\textsuperscript{23,24} and the sarcoplasmic reticulum (SR),\textsuperscript{25} increasing evidence has suggested that nNOS also plays critical roles in the cardiovascular system.

nNOS co-localizes with XO in the cardiac SR, and nNOS-derived NO acts as a direct antioxidant in the regulation of XO activity.\textsuperscript{19} Moreover, nNOS-derived NO exerts vasculoprotective effects in carotid artery ligation and balloon injury models.\textsuperscript{16} Moreover, it has been demonstrated that the

**Figure 5** Effects of acute nNOS inhibition by L-VNIO (10 \(\mu\)M), genetic deletion of nNOS, and NAD(P)H oxidase inhibition by Apo (10 \(\mu\)M) on Ang II (100 nM)-induced ERK1/2, p38, and JNK mitogen-activated protein kinase phosphorylation in vascular smooth muscle cells. (A) Representative blots of phospho-ERK1/2, p38, and JNK, and total ERK1/2, p38, and JNK after treatment with Ang II for 30 min or pre-incubated with L-VNIO, FeTPPS (10 \(\mu\)M), or Apo from wild-type (WT) and nNOS\textsuperscript{2/2} mice. (B) Densitometry analysis of phosphorylated mitogen-activated protein kinase. Values of each phosphorylated protein were normalized by each total protein, and mean value in WT Con is expressed as 1. White bar represents data of phospho-ERK1/2, grey bar represents phospho-p38, and black bar represents phospho-JNK. Data are presented as mean ± SEM of 10 different experiments. *\( P < 0.05\) vs. WT Con. †\( P < 0.05\) vs. Ang II. §\( P < 0.05\) vs. Ang II + L-VNIO.
The balance of antioxidant and oxidant systems plays a critical role in the regulation of vascular diseases. Several oxidant-generating systems that produce ROS in the vasculature, including the mitochondrial respiration chain, cyclooxygenases, lipoxygenases, and cytochrome P450 monooxygenases, have been generally recognized. The most relevant...
sources of ROS related to vascular diseases appear to be XO, NAD(P)H, and uncoupled eNOS. Our data reported here demonstrate that acute inhibition of nNOS by L-VNIO increases vascular superoxide and MAPK phosphorylation, suggesting that under basal conditions, nNOS-derived NO acts directly as an antioxidant to suppress vascular superoxide generation (such as from XO). Thereafter, it inhibits the vascular MAPK activity and protects vascular system from various diseases such as hypertension, diabetes, and atherosclerosis.

Genetic deletion of a target protein provides an alternative approach to pharmacology for the investigation of the role of proteins. Data from genetic deletion of nNOS have demonstrated that XO activity in cardiomyocytes from nNOS−/− mice is up-regulated without increase of XO protein expression under basal conditions. Our present results show that the vascular superoxide generation was increased after genetic deletion of nNOS, suggesting that the vascular superoxide generation is inhibited by nNOS or nNOS-derived NO. Furthermore, the vascular superoxide generation was attenuated by the XO inhibitor, suggesting the up-regulation of the vascular XO activity. This is consistent with the observation in cardiac tissue from nNOS−/− mice. Up-regulation of XO activity without an increase in XO protein expression also may be related to increased superoxide induced by acute nNOS inhibition in wild-type mice, although mechanisms for up-regulation of XO activity are still unknown.

The beneficial roles of NOS and NOS-derived NO in cardiovascular system are well accepted; however, excessively high levels of NO can be potentially toxic due to its oxidative reaction products, such as ONOO−, which can injure the cardiovascular system. In addition to the generation of NO, NOS can become 'uncoupled' and preferentially synthesizes superoxide which often has opposing effects to NO. Fully coupled NOS catalyses formation of nitric oxide (NO), L-citrulline, NADP+, and water from L-arginine, NADPH, and oxygen. Uncoupled or partially coupled NOS catalyses the synthesis of ROS such as O2−, H2O2, and ONOO−, depending on the availability of cofactor tetrahydrobiopterin (BH4) and L-arginine during catalysis. A mounting evidence indicates that ROS-induced NOS uncoupling can, in turn, generate ROS, resulting in a vicious circle.

Brief (5 min) Ang II stimulation induces phosphorylation of ERK1/2 mediated via an iNOS- involved signalling pathway in vascular smooth muscles. Ang II stimulation for 24 h down-regulates DHFR in bovine endothelial cells, inducing rapid and transient activation of endothelial NAD(P)H oxidases responsible for the initial burst production of superoxide at 30 min and then inducing eNOS uncoupling contributed to the secondary peak of superoxide generation at 24 h. Ang II stimulation for 7 days induces oxidative, nitrosative stress and NOS uncoupling in the cardiovascular system. It is notable that Ang II-stimulated superoxide production in vitro is biphasic; the first phase occurs rapidly (peak at 30 s) and the second phase occurs later (peak at 30 min). This biphasic generation of superoxide is mediated via different upstream mediators. We have reviewed the possible mechanism for Ang II-induced ROS generation and MAPK activation. Ang II activates membrane-bound NAD(P)H oxidase to generate ROS, and ROS activates mitochondrial K_ATP channels to induce ROS bursts in the mitochondria. In the present study, pre-treatment with L-VNIO markedly suppressed superoxide generation induced by Ang II stimulation for a short time (30 min incubation), indicating that Ang II could induce nNOS uncoupling to enhance superoxide generation within this time frame. These results showed that the superoxide generation presented here corresponds to Ang II-induced 'second phase' as previously reported. Although mitochondria NOS (mtNOS) has been identified as nNOS, whether mitochondrial-specific nNOS uncoupling is responsible for Ang II-induced second phase of ROS generation and MAPK activation is still under speculation.

Based on recent observation showing that nNOS uncoupling could occur under superoxide stimulation, it is plausible that Ang II-induced nNOS uncoupling involves superoxide derived from NAD(P)H oxidase. In the present study, an NAD(P)H oxidase inhibitor, apocynin, further reduced the Ang II-induced superoxide generation, indicating the involvement of superoxide derived from NAD(P)H oxidase. This superoxide causes nNOS uncoupling to generate more amounts of superoxide, resulting in a vicious circle to enhance superoxide generation. Scavenging of ONOO− did not suppress Ang II-induced increase of O2− levels, but markedly reduced Ang II-induced MAPK phosphorylation, suggesting both O2− and ONOO− are involved in Ang II-induced vascular MAPK activation (Figure 6A).

Genetic deletion of nNOS showed no compensatory up-regulation of iNOS or eNOS under basal conditions, but did result in an increase of superoxide generation from XO. Upon Ang II stimulation, vascular superoxide generation did not further increase in aorta and VSMCs from nNOS−/− mice, indicating that Ang II could not further increase superoxide generation because at least superoxide generation induced by nNOS uncoupling was deficient. Of course, there are several other possibilities. One possibility is that XO-derived superoxide level reaches nearly maximum level in the vasculature. Another possibility is that a high level of superoxide may suppress the activation or translocation of protein kinase C (PKC) and/or suppress the Ang II-induced NAD(P)H oxidase activation (Figure 6B).

In conclusion, nNOS-derived NO acts directly as an antioxidant, reduces the superoxide accumulation, and suppresses vascular ERK1/2, p38, and JNK MAPK phosphorylation under physiological condition. In response to Ang II stimulation, NAD(P)H oxidase-derived superoxide may induce nNOS uncoupling, enhance Ang II-induced increase of superoxide generation, and participate in Ang II-induced activation of vascular MAPK. Our present study provides a novel mechanism of Ang II-induced cardiovascular diseases such as hypertension, diabetes, and atherosclerosis.

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