A Defect of Neuronal Nitric Oxide Synthase Increases Xanthine Oxidase–Derived Superoxide Anion and Attenuates the Control of Myocardial Oxygen Consumption by Nitric Oxide Derived From Endothelial Nitric Oxide Synthase

Shintaro Kinugawa, Harer Huang, Ziping Wang, Pawel M. Kaminski, Michael S. Wolin, Thomas H. Hintze

Abstract—Endothelial nitric oxide synthase (eNOS) plays an important role in the control of myocardial oxygen consumption (MVO₂) by nitric oxide (NO). A NOS isoform is present in cardiac mitochondria and it is derived from neuronal NOS (nNOS). However, the role of nNOS in the control of MVO₂ remains unknown. MVO₂ in left ventricular tissues from nNOS−/− mice was measured in vitro. Stimulation of NO production by bradykinin or carbachol induced a significant reduction in MVO₂ in wild-type (WT) mice. In contrast to WT, bradykinin- or carbachol-induced reduction in MVO₂ was attenuated in nNOS−/−. S-methyl-L-thiocitrulline, a potent isoform selective inhibitor of nNOS, had no effect on bradykinin-induced reduction in MVO₂ in WT. Bradykinin-induced reduction in MVO₂ in eNOS−/− mice, in which nNOS still exists, was also attenuated. The attenuated bradykinin-induced reduction in MVO₂ in nNOS−/− was restored by preincubation with Tiron, ascorbic acid, Tempol, oxypurinol, or SB203850, an inhibitor of p38 kinase, but not apocynin. There was an increase in lucigenin-detectable superoxide anion (O₂⁻) in cardiac tissues from nNOS−/− compared with WT. Tempol, oxypurinol, or SB203850 decreased O₂⁻ in all groups to levels that were not different from each other. There was an increase in phosphorylated p38 kinase normalized by total p38 kinase protein level in nNOS−/− compared with WT mice. These results indicate that a defect of nNOS increases O₂⁻ through the activation of xanthine oxidase, which is mediated by the activation of p38 kinase, and attenuates the control of MVO₂ by NO derived from eNOS. (Circ Res. 2005;96:355–362.)

Key Words: endothelial nitric oxide synthase ■ neuronal nitric oxide synthase ■ superoxide anion ■ p38 ■ oxygen consumption

Nitric oxide (NO) attenuates mitochondrial respiration by nitrosylating the iron-sulfur centers of aconitase, complexes I and II of the electron transport chain, and through a very potent reversible alteration in the activity of cytochrome c oxidase.1–3 We and others have shown that NO can modulate mitochondrial respiration and tissue oxygen consumption in whole body,4 heart, skeletal muscle, and kidney both in vivo5–8 and in vitro.8–10 Furthermore, we have shown that NO derived from endothelial NO synthase (eNOS) plays an important role in these processes.11

Immunohistochemical studies have shown that a NOS is present in the mitochondria.12–14 Giulivi et al have provided evidence for the production of NO by intact, purified mitochondria using two spectroscopic techniques.15–18 In other laboratories, the production of NO by mitochondria has been shown by formation of L-citrulline from radiolabeled L-arginine.12,19,20 Furthermore, Giulivi et al have reported that mitochondrial NOS was identified as neuronal NOS (nNOS) with two posttranslational modifications in isolated mitochondria from rat liver. Kanai et al identified mitochondrial NOS as nNOS in the isolated cardiac mitochondria from nNOS wild-type (WT) and knockout (nNOS−/−) mice. Thus, nNOS may provide a local source of NO, which can modulate mitochondrial respiration and myocardial oxygen consumption (MVO₂). The role of nNOS in the regulation of MVO₂ remains to be elucidated.

French et al showed that the local production of NO by mitochondrial NOS is not significant and does not contribute to the regulation of mitochondrial function using isolated porcine cardiac mitochondria. Our previous study showed that bradykinin (BK) had no inhibitory effect on MVO₂ in tissues obtained from eNOS-deficient mice, in which nNOS still exists.11 Those data suggest that NO derived from nNOS does not directly contribute to the inhibition of mitochondrial respiration. Very recently, Khan et al have been shown that deficiency of nNOS leads to profound increase in xanthine oxidase (XO)–mediated superoxide anion (O₂⁻) production without affecting XO mRNA or protein abundance, which
depresses myocardial excitation-contraction coupling in a manner reversible by XO inhibition with allopurinol. Thus, we hypothesized that a defect of nNOS increases XO-derived \( \text{O}_2^- \) production, which decreases NO bioavailability, and attenuates the control of MVO\(_2\) by NO derived from eNOS.

The goals of our experiments were as follows: (1) to determine whether NO-dependent control of MVO\(_2\) is altered in nNOS\(^{-/-}\) compared with WT mice; (2) to determine whether \( \text{O}_2^- \) production is increased and \( \text{O}_2^- \) is associated with NO-dependent control of MVO\(_2\) in nNOS\(^{-/-}\); and (3) to determine the responsible mechanism for \( \text{O}_2^- \) production in nNOS\(^{-/-}\).

**Materials and Methods**

**Animal Studied**
Mice (8 to 10 weeks old) homozygous for targeted disruption of the nNOS gene (nNOS\(^{-/-}\), n=46), the eNOS gene (eNOS\(^{-/-}\), n=8), and wild-type control mice (WT, C57BL/6J×129 F2 hybrids, n=24) were purchased from Jackson Laboratories (Bar Harbor, Me). All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the current National Institutes of Health and American Physiological Society Guidelines for the Use and Care of Laboratory Animals.

**Preparation of Cardiac Muscle Tissues and Measurement of MVO\(_2\)**
MVO\(_2\) was measured in vitro as we described previously.\(^{11,24}\) Mice were anesthetized with pentobarbital sodium (50 mg/kg IP), and hearts were removed immediately. The left ventricle was bisected such that each piece of muscle contained the septum, free wall, and apex. The muscle tissues were incubated in Krebs solution (mol/L: 118 NaCl, 4.7 KCl, 1.5 CaCl\(_2\), 25 NaHCO\(_3\), 1.1 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), and 5.6 glucose) at 37°C for 2 hours and bubbled continuously with 20% \( \text{O}_2 \)-5% \( \text{CO}_2 \)-75% \( \text{N}_2 \). Each piece of tissue was placed in a stirred bath with 3 mL of air-saturated Krebs solution containing 10 mmol/L HEPES (pH 7.4). The bath was sealed using a Clark-type platinum oxygen electrode (Yellow Springs Instruments) that was connected to an oxygen monitor (model YSI 5331). Oxygen uptake by tissues was recorded. Tissue respiration was continuously monitored with 20% \( \text{O}_2 \)-5% \( \text{CO}_2 \)-75% \( \text{N}_2 \), and the percentage of change in baseline MVO\(_2\).

**Experimental Protocols**

**Inhibition of MVO\(_2\) by Endogenous NO in WT and nNOS\(^{-/-}\) Mice**
BK or carbachol (CCh) stimulates kinin B2-receptors and muscarinic receptors respectively on the endothelium to cause NO production. After baselines were recorded, cumulative concentrations of BK at 10\(^{-7}\) to 10\(^{-4}\) mol/L were added to the chambers in the presence or absence of 10\(^{-6}\) mol/L N\(^{-2}\)-nitro-L-arginine methyl ester (L-NAME). In separate experiments, the effects of BK (10\(^{-7}\) to 10\(^{-4}\) mol/L) on MVO\(_2\) in WT and nNOS\(^{-/-}\) were studied after 30 minutes incubation of 10\(^{-2}\) mol/L Tiron, 10\(^{-3}\) mol/L ascorbic acid, 10\(^{-3}\) mol/L 4-Hydroxy-2,2,6,6-tetramethyl-piperidine 1-oxyl (Tempol), 10\(^{-4}\) mol/L oxytpurinol, an inhibitor of XO, 10\(^{-3}\) mol/L apocynin, an inhibitor of NAD(P)H oxidase activation, or 5×10\(^{-5}\) mol/L SB203580, an inhibitor of p38 kinase.

**Inhibition of MVO\(_2\) by Exogenous NO in WT and nNOS\(^{-/-}\) Mice**
S-nitroso-N-acetylpenicillamine (SNAP) was used as a NO donor. After baselines were recorded, cumulative concentrations of SNAP at 10\(^{-7}\) to 10\(^{-5}\) mol/L were added to the chambers.

**Effects of Acute Inhibition of nNOS on MVO\(_2\) in WT Mice**
To assess the effects of acute inhibition of nNOS, cardiac muscle from WT mice was preincubated with 10\(^{-3}\) mol/L S-methyl-l-thiocitrulline (SMTC), a potent isoforome selective inhibitor of nNOS,\(^{25}\) and 2×10\(^{-4}\) mol/L 7-Nitroindazole (7-NI), a relatively selective inhibitor of nNOS,\(^{26}\) for 30 minutes. After baselines were recorded, cumulative concentrations of BK at 10\(^{-7}\) to 10\(^{-4}\) mol/L were added to the chambers.

**Inhibition of MVO\(_2\) by Endogenous NO in WT and eNOS\(^{-/-}\) Mice**
After baselines were recorded, cumulative concentrations of BK at 10\(^{-7}\) to 10\(^{-4}\) mol/L were added to the chambers in the presence or absence of 10\(^{-3}\) mol/L L-NAME or 10\(^{-2}\) mol/L Tiron.

**O\(_2^-\) Production**
The chemiluminescence elicited by O\(_2^-\) in the presence of lucigenin (5×10\(^{-6}\) mol/L) was measured in cardiac tissues from WT and nNOS\(^{-/-}\) mice as described previously.\(^{27,28}\) Approximately 30 mg of left ventricle were prepared in the same manner as the measurement of MVO\(_2\), and then incubated in 5 mL of air-saturated Krebs solution containing 10 mmol/L HEPES (pH 7.4) for 30 minutes at room temperature. The samples were then placed into scintillation vials containing 5×10\(^{-6}\) mol/L lucigenin in a final volume of 1 mL Krebs solution. Photon counting was used to quantitate chemiluminescence. Vials containing all components with the exception of left ventricles were counted and these blank values subtracted from the chemiluminescence signals obtained from left ventricle. To validate that the chemiluminescence signals we measured are derived from O\(_2^-\), the experiments were performed in the presence of 10\(^{-3}\) mol/L Tempol. In separate experiments, O\(_2^-\) production in left ventricle was determined in presence of 10\(^{-4}\) mol/L oxytpurinol, 10\(^{-5}\) mol/L apocynin, 10\(^{-4}\) mol/L L-NAME or 5×10\(^{-5}\) mol/L SB203580.

**Immunoblotting for eNOS, iNOS, XO, Phosphorylated p38 Kinase, and Total p38 Kinase Protein in Cardiac Muscle**
eNOS, iNOS, XO, phosphorylated p38 kinase, and total p38 kinase protein in cardiac muscle were measured by Western blotting analysis with a monoclonal antibody to eNOS, iNOS (Transduction Laboratories), XO and xanthine dehydrogenase (XDH, NeoMark- ers), p38 kinase, and phosphorylated form of p38 kinase (Cell Signaling Technology) followed by densitometry as described previously.\(^{24,29}\) β-Actin was used to normalize the amount of protein loaded.

**Chemicals**
All drugs were purchased from Sigma Chemical Co.

**Data Analysis**
All data are presented as mean±SE. Comparisons of O\(_2^-\) production were made using one-way ANOVA followed by Scheffé’s t test. The changes in MVO\(_2\), caused by BK, CCh, or SNAP were analyzed using repeated measures 2-way ANOVA followed by Scheffé’s t test. Statistical significance of differences for baseline MVO\(_2\) in cardiac muscle and protein level of XO/XDH, eNOS, iNOS, total p38 kinase, or phosphorylated p38 kinase was determined with unpaired t test. Significant changes were considered at a value of P<0.05.

**Results**
Baseline MVO\(_2\) in WT and nNOSKO Mice
Baseline MVO\(_2\) was not different in any groups in the absence or presence of L-NAME, SMTC, Tempol, ascorbic acid, Tiron, oxytpurinol, or apocynin (data not shown).
MVO$_2$ in WT and nNOS$^{-/-}$ Mice in Response to BK, CCh, or SNAP

Cumulative dose of BK (Figure 1A) or CCh (Figure 1B) caused concentration-dependent reduction in MVO$_2$ in WT mice. BK-induced reduction in MVO$_2$ was attenuated by l-NAME (data not shown). The extent of BK- or CCh-induced reduction in MVO$_2$ was significantly less in nNOS$^{-/-}$ than WT mice (Figure 1A and 1B). In contrast to BK or CCh, there was no difference in SNAP-induced reduction in MVO$_2$ between WT and nNOS$^{-/-}$ mice (Figure 1C).

Figure 1. Effects of cumulative dose of BK (A), CCh (B), and SNAP (C) on MVO$_2$ in tissues. ○, WT (n=8 in BK, n=6 in CCh, n=5 in SNAP); ●, nNOS$^{-/-}$ (n=34 in BK, n=6 in CCh, n=5 in SNAP). **P<0.01 vs WT.

Effects of Acute Inhibition of nNOS on MVO$_2$ in WT Mice

Activation of kinin-B$_2$ receptors by BK leads to the elevation of intracellular calcium to produce NO through the activation of eNOS. However, this pathway could lead to NO production through the activation of nNOS. To investigate whether NO derived from nNOS directly affect MVO$_2$, we examined the effects of SMTC and 7-NI on MVO$_2$ in WT mice. 10$^{-5}$ mol/L SMTC and 2×10$^{-3}$ mol/L 7-NI had no effect on BK-induced reduction in MVO$_2$ in WT mice (Figure 2).

Effects of O$_2^*$ on MVO$_2$ in nNOS$^{-/-}$ Mice

NO derived from nNOS had no direct effect on the control of MVO$_2$. Thus, we hypothesized that NO bioavailability might be reduced in nNOS$^{-/-}$ mice. Because it is well known that O$_2^*$ reacts rapidly with NO and reduces NO bioavailability,$^{30}$ we investigated whether O$_2^*$ is associated with the attenuated BK-induced reduction in MVO$_2$ in nNOS$^{-/-}$ mice. The attenuated control of MVO$_2$ in nNOS$^{-/-}$ mice was restored by preincubation with Tiron, a freely membrane-permeable O$_2^*$ scavenger (Figure 3A). The restored control of MVO$_2$ in nNOS$^{-/-}$ mice by Tiron was attenuated by coincubation of l-NAME (at 10$^{-4}$ mol/L, BK, -26±2% in nNOS$^{-/-}$) with Tiron versus -13±4% in nNOS$^{-/-}$ with Tiron and l-NAME, P<0.01). BK-induced reduction in MVO$_2$ in WT mice was not affected by preincubation with Tiron (at 10$^{-4}$ mol/L BK, -26±2% in WT versus -27±2% in WT with Tiron;

Figure 2. Effects of 10$^{-5}$ mol/L SMTC and 2×10$^{-5}$ mol/L 7-NI on BK-induced reduction in MVO$_2$ in tissues from WT mice. ○, WT (n=8); ●, WT with 10$^{-5}$ mol/L SMTC (n=7); □, WT with 2×10$^{-5}$ mol/L 7-NI.

Figure 3. Effects of cumulative dose of BK on MVO$_2$ in tissues from nNOS$^{-/-}$ mice in absence and presence of inhibitor (A). ○, nNOS$^{-/-}$ (n=34); ●, nNOS$^{-/-}$ with 10$^{-2}$ mol/L Tiron (n=5); ○, nNOS$^{-/-}$ with 10$^{-3}$ mol/L ascorbic acid (n=5); ●, nNOS$^{-/-}$ with 10$^{-3}$ mol/L Tempol (n=5). Effects of oxypurinol on BK-induced changes in MVO2 in tissues from nNOS$^{-/-}$ mice (B). ○, nNOS$^{-/-}$ (n=25); ○, nNOS$^{-/-}$ with 10$^{-4}$ mol/L oxypurinol (n=5); ●, nNOS$^{-/-}$ with 10$^{-5}$ mol/L apocynin (n=5). **P<0.01 vs nNOS$^{-/-}$. 

Figure 3. Effects of cumulative dose of BK on MVO$_2$ in tissues from nNOS$^{-/-}$ mice in absence and presence of inhibitor (A).

- ○: nNOS$^{-/-}$ (n=34)
- ●: nNOS$^{-/-}$ with 10$^{-2}$ mol/L Tiron (n=5)
- ○: nNOS$^{-/-}$ with 10$^{-3}$ mol/L ascorbic acid (n=5)
- ●: nNOS$^{-/-}$ with 10$^{-3}$ mol/L Tempol (n=5)

Effects of oxypurinol on BK-induced changes in MVO2 in tissues from nNOS$^{-/-}$ mice (B).

- ○: nNOS$^{-/-}$ (n=25)
- ○: nNOS$^{-/-}$ with 10$^{-4}$ mol/L oxypurinol (n=5)
- ●: nNOS$^{-/-}$ with 10$^{-5}$ mol/L apocynin (n=5)
nNOS effect on MVO_2 in nNOS was attenuated by coincubation of L-NAME (at 10^-4 mol/L) and BK, 10^-5 mol/L L-NAME (n=5); ⋄, eNOS^-/- with 10^-4 mol/L L-NAME (n=5); ▴, eNOS^-/- with 10^-2 mol/L Tiron (n=5). **P<0.01 vs WT.

P'=NS). We also investigated the effects of ascorbic acid, another O2^- scavenger, or Tempol, a membrane-permeable superoxide anion dismutase mimetic. Again, the attenuated control of MVO_2 in nNOS^-/- mice was restored by preincubation with either drug (Figure 3A).

Effects of Oxypurinol or Apocynin on MVO_2 in nNOS^-/- Mice
O2^- is produced via several mechanisms including xanthine oxidase or NAD(P)H oxidase. Thus, we investigated the effects of oxypurinol, an inhibitor of XO, or apocynin, an inhibitor of NAD(P)H oxidase activation. The attenuated control of MVO_2 in nNOS^-/- mice was restored by preincubation with oxypurinol (Figure 3B), whereas apocynin had no effect on MVO_2 in nNOS^-/- mice (Figure 3B). Furthermore, the restored control of MVO_2 in nNOS^-/- mice by oxypurinol was attenuated by coincubation of L-NAME (at 10^-4 mol/L BK, 30±2% in nNOS^-/- with oxypurinol versus -14±3% in nNOS^-/- with oxypurinol and L-NAME; P<0.01). BK-induced reduction in MVO_2 in WT mice was not affected by preincubation with oxypurinol (at 10^-4 mol/L BK, -26±2% in WT versus -26±2% in WT with oxypurinol; P=NS).

MVO_2 in WT and eNOS^-/- Mice in Response to BK
To further investigate the role of nNOS in the control of MVO_2, we examined BK-induced reduction in MVO_2 in eNOS^-/- mice, in which nNOS still exists. The extent of BK-induced reduction in MVO_2 was significantly less in eNOS^-/- than WT mice (Figure 4). L-NAME had no effect on BK-induced reduction in MVO_2 in eNOS^-/- mice (Figure 4). In contrast to nNOS^-/-, the attenuated control of MVO_2 in eNOS^-/- was not restored by Tiron (Figure 4).

O2^- Production in Cardiac Muscle From WT and nNOS^-/- Mice
There was an increase in lucigenin (5×10^-6 mol/L)-detectable O2^- production in cardiac muscle from nNOS^-/- compared with WT (336±16 versus 194±17 CPM/mg tissue) mice. Tempol decreased O2^- in all groups to levels that were not different from each other (Figure 5). SMTC, acute inhibition of nNOS, did not affect O2^- production in WT mice (Figure 5). O2^- production in nNOS^-/- mice was decreased by coinubcation of oxypurinol, whereas it was unaffected by apocynin or L-NAME (Figure 5). O2^- production in WT mice was not affected by coinubcation of oxypurinol (Figure 5).

eNOS, iNOS, and XO Protein in Cardiac Muscle From WT and nNOS^-/- Mice
There was no difference in eNOS and iNOS protein levels between WT and nNOS^-/- mice (Figure 6A and 6B). Bands corresponding to both XO (130 and 100 kDa) and XDH (170 kDa) were present. There were no differences in XO/XDH protein levels between WT and nNOS^-/- mice (Figure 6C).

Phosphorylated Form of p38 Kinase and Total p38 Kinase Protein and the Effect of an Inhibitor of p38 Kinase on MVO_2 and O2^- Production in Cardiac Muscle From WT and nNOS^-/-
O2^- production in nNOS^-/- mice was increased, which was inhibited by oxypurinol, an inhibitor of XO. However, we could not find an increase of XO/XDH protein level. Therefore, XO activity is increased in nNOS^-/- mice, which should be controlled at a posttranslational level. Recently, Kayyali et al29 have shown that XO/XDH is phosphoprotein, and XO/XDH is phosphorylated in hypoxic rat pulmonary microvascular endothelial cell through a mechanism involving p38 kinase. First, we investigated whether p38 kinase is activated in heart from nNOS^-/- mice. Because p38 kinase becomes phosphorylated on activation, antibodies to phosphorylated form of p38 kinase were used. There was an increase in phosphorylated p38 kinase normalized by total p38 kinase protein level in nNOS^-/- compared with WT mice (Figure 7A and 7B). Next, we examined whether an activation of p38 kinase was associated with the attenuated NO-dependent control of MVO_2 or O2^- production in heart from nNOS^-/- mice. The attenuated control of MVO_2 was restored and lucigenin-detectable O2^- production was inhibited by prein-
cubation with SB203580, an inhibitor of p38 kinase, in nNOS−/− mice (Figure 7C and 7D).

Discussion

We have demonstrated that BK- or CCh-induced reduction in MVO₂ was attenuated in nNOS−/− compared with WT mice. Acute inhibition of nNOS with SMTC or 7-NI did not affect BK-induced reduction in MVO₂ in WT mice. The attenuated control of MVO₂ in nNOS−/− mice was restored by preincubation with Tiron, Tempol, ascorbic acid, oxypurinol, or SB203580. There was an increase in lucigenin-detectable O₂⁻ production in cardiac muscle from nNOS−/− compared with WT mice, which was inhibited by oxypurinol or SB203580. There was no difference in eNOS, iNOS, and XO/XDH protein levels between WT and nNOS−/− mice. Very interestingly, there was an increase in phosphorylated p38 kinase protein level in nNOS−/− compared with WT mice. Therefore, we concluded that a defect of nNOS attenuated control of MVO₂ by NO derived from another source most likely eNOS, which is associated with an increase in O₂⁻ through the activation of XO, and XO was posttranslationally activated through a mechanism involving p38 kinase.

Cardiac nNOS has been found in nerve terminals, cardiac conduction tissue, sarcoplasmic reticulum, and mitochondria. However, the role that the subcellular localization of nNOS plays has not been well understood. For example, nNOS, probably in sarcoplasmic reticulum, plays an important role in the control of myocardial contraction and calcium cycling. Sears et al have reported that nNOS suppresses sarcoplasmic reticulum calcium release, and Khan et al have reported that nNOS plays a primary role in stimulating sarcoplasmic reticulum calcium cycling. In this regard, the role of nNOS remains highly controversial. Giulivi et al have provided evidence for the production of NO by intact, 

Figure 6. Representative Western blot (top) and densitometric analysis (bottom) for eNOS (A), iNOS (B), and Xanthine oxidase (130 and 100 kDa) and xanthine dehydrogenase (170 kDa) (C) protein in cardiac muscle from WT (n=4) and nNOS−/− (n=4) mice. Data are expressed as densitometric arbitrary units normalized by β-actin.

Figure 7. Representative Western blot (A) and densitometric analysis (B) for phosphorylated p38 (p-p38) and total p38 protein (t-p38) in cardiac muscle from WT (n=4) and nNOS−/− (n=4) mice. Data are expressed as densitometric arbitrary units normalized by total p38. Effects of cumulative dose of BK on MVO₂ in tissues from nNOS−/− mice in absence and presence of 5×10⁻⁷ mol/L SB203850 (C). ○, nNOS−/− with SB203850 (n=5); ●, nNOS−/− (n=34). O₂⁻ production as determined by lucigenin chemiluminescence in myocardial tissues from nNOS−/− mice in absence and presence of 5×10⁻⁷ mol/L SB203850 (D). *P<0.05 vs WT; ††P<0.01 vs nNOS−/−.
purified mitochondria using two spectroscopic techniques, and they have identified mitochondrial NOS as a nNOS with two posttranslational modifications in isolated mitochondria from rat liver. Kanai et al showed the similarity between mitochondrial NOS and nNOS in a study performed on isolated cardiac mitochondria from WT and nNOS mice. Given that the subcellular localization of nNOS exists in proximity to the regulatory site of MVO₂, nNOS may play an important role in the regulation of MVO₂.

We demonstrated that BK-induced reduction in MVO₂ was attenuated in nNOS⁻/⁻ compared with WT mice. l-NAME inhibited BK-induced reduction in MVO₂ in WT mice, whereas it did not affect that in nNOS⁻/⁻ (data not shown). These results suggest that NO-dependent control of MVO₂ is attenuated in nNOS⁻/⁻. We have reported that eNOS plays an important role in NO-dependent reduction of MVO₂ in WT mice. Therefore, these results suggest that the attenuated NO-dependent control of MVO₂ in nNOS⁻/⁻ mice is attributable to different mechanisms in nNOS⁻/⁻ and eNOS⁻/⁻.

Recently, we have reported that NO-dependent control of MVO₂ was attenuated in heterozygous manganese superoxide anion dismutase mice and was reversed by the freely membrane-permeable O₂⁻ scavenger Tiron. O₂⁻ reacts rapidly with NO, reducing NO bioavailability. In states where NO production is not altered, increased O₂⁻ may reduce NO bioavailability. We examined whether O₂⁻ is associated with the attenuated NO-dependent control of MVO₂ in nNOS⁻/⁻ mice. Interestingly, Tiron, ascorbic acid, or Tempol reversed the attenuated BK-induced reduction in MVO₂ in nNOS⁻/⁻ mice. Furthermore, there was an increase in lucigenin-detectable O₂⁻ production in cardiac muscle from nNOS⁻/⁻ compared with WT mice. We also investigated whether acute inhibition of nNOS by SMTC increases O₂⁻ production in heart tissue from WT mice. SMTC did not affect O₂⁻ production in WT mice. These data suggest that chronic deletion of nNOS is essential for an increase in O₂⁻ production. In contrast to nNOS⁻/⁻, Tiron did not reverse the attenuated control of MVO₂ in nNOS⁻/⁻ mice. This finding clearly demonstrates that the attenuated control of MVO₂ is attributable to different mechanisms in nNOS⁻/⁻ and eNOS⁻/⁻.

O₂⁻ is produced via several mechanisms including XO, NAD(P)H oxidase, or eNOS. The attenuated NO-dependent control of MVO₂ in nNOS⁻/⁻ mice was restored by oxypurinol, but not apocynin. Furthermore, the increase in lucigenin-detectable O₂⁻ in nNOS⁻/⁻ mice was also inhibited by oxypurinol, but not by apocynin or l-NAME. These results strongly suggest that O₂⁻, which is produced through the activation of XO, plays an important role in the attenuation of NO-dependent control of MVO₂ in nNOS⁻/⁻ mice. We also investigated the expression of XO/XDH protein by Western blotting using commercially available monoclonal antibody. There was no difference in XO/XDH protein level between WT and nNOS⁻/⁻ mice.

Activation of kinin-B₂ receptors by BK or muscarinic receptors by CCh leads to the elevation of intracellular calcium to produce NO through the activation of eNOS. However, this pathway could lead to NO production through eNOS receptors by CCh leads to the elevation of intracellular vascular endothelium, and 10⁻⁵ mol/L SMTC attenuated BK-induced reduction in MVO₂ in WT mice. The extent of BK-induced reduction in MVO₂ was significantly less in eNOS⁻/⁻ than WT mice and l-NAME had no effect on BK-induced reduction in MVO₂ in eNOS⁻/⁻ mice. These results suggest that NO production from nNOS is not responsible for the control of MVO₂. This finding is compatible with studies by French et al and us. Recently, we have reported that NO-dependent control of MVO₂ was attenuated in heterozygous manganese superoxide anion dismutase mice and was reversed by the freely membrane-permeable O₂⁻ scavenger Tiron. O₂⁻ reacts rapidly with NO, reducing NO bioavailability. In states where NO production is not altered, increased O₂⁻ may reduce NO bioavailability. We examined whether O₂⁻ is associated with the attenuated NO-dependent control of MVO₂ in nNOS⁻/⁻ mice. Interestingly, Tiron, ascorbic acid, or Tempol reversed the attenuated BK-induced reduction in MVO₂ in nNOS⁻/⁻ mice. Furthermore, there was an increase in lucigenin-detectable O₂⁻ production in cardiac muscle from nNOS⁻/⁻ compared with WT mice. We also investigated whether acute inhibition of nNOS by SMTC increases O₂⁻ production in heart tissue from WT mice. SMTC did not affect O₂⁻ production in WT mice. These data suggest that chronic deletion of nNOS is essential for an increase in O₂⁻ production. In contrast to nNOS⁻/⁻, Tiron did not reverse the attenuated control of MVO₂ in nNOS⁻/⁻ mice. This finding clearly demonstrates that the attenuated control of MVO₂ is attributable to different mechanisms in nNOS⁻/⁻ and eNOS⁻/⁻.

O₂⁻ is produced via several mechanisms including XO, NAD(P)H oxidase, or eNOS. The attenuated NO-dependent control of MVO₂ in nNOS⁻/⁻ mice was restored by oxypurinol, but not apocynin. Furthermore, the increase in lucigenin-detectable O₂⁻ in nNOS⁻/⁻ mice was also inhibited by oxypurinol, but not by apocynin or l-NAME. These results strongly suggest that O₂⁻, which is produced through the activation of XO, plays an important role in the attenuation of NO-dependent control of MVO₂ in nNOS⁻/⁻ mice. We also investigated the expression of XO/XDH protein by Western blotting using commercially available monoclonal antibody. There was no difference in XO/XDH protein level between WT and nNOS⁻/⁻ mice. Therefore, the increased production of O₂⁻ is attributable to an increase in XO activity, which should be controlled at a posttranslational level.

Khan et al have reported that XO-mediated O₂⁻ production is increased in nNOS⁻/⁻ without affecting XO mRNA and protein abundance, and enhanced XO activity inhibits myocyte contractility in nNOS⁻/⁻. They concluded that nNOS directly interacts with XO and represents an important antioxidant system, inhibiting XO activity. On the other hand, recently, Kayyali et al have shown that XO/XDH is phosphorylated and the activity of XO is increased in hypoxic rat pulmonary microvascular endothelial cell through a mechanism involving p38 kinase. Thus, we hypothesized that an increase in O₂⁻ production through the activation of XO in nNOS⁻/⁻ is associated with p38 kinase pathway. There was an increase in phosphorylated p38 kinase protein level in nNOS⁻/⁻ compared with WT mice, whereas there was no difference in total p38 kinase protein level between nNOS⁻/⁻ and WT mice. Furthermore, the attenuated control of MVO₂ was restored and lucigenin-detectable O₂⁻ production was
inhibited by preincubation with SB203580 in nNOS \(^{-/-}\) mice. Therefore, our data suggest that the activation of XO in nNOS \(^{-/-}\) is attributable to phosphorylation through p38 kinase pathway. p38 kinase has been reported to be activated by various cellular stresses (ie, reactive oxygen species, hypoxia/reoxygenation, hypoxic shock, or proinflammatory cytokines) or stimulation of G protein–coupled receptor agonist.\(^{36}\) It remains unknown, however, why chronic deletion of nNOS leads to the activation of p38 kinase.

There are several limitations that should be acknowledged in this study. There was a discrepancy in NO-dependent control of MVO\(_{2}\) and O\(_2\)\(^{-}\) production between chronic effect of nNOS gene deletion and acute effect of nNOS inhibitor. This discrepancy may be attributable to the term of a lack of nNOS or an incomplete inhibition by nNOS by inhibitors. However, the mechanism regarding this discrepancy remains unknown. Phosphorylation and activity of XO have never been directly measured. However, both oxypurinol and SB203580 restored the attenuated NO-dependent control of MVO\(_{2}\) and inhibited O\(_2\)\(^{-}\) production in nNOS \(^{-/-}\). These results strongly support our conclusions.

Figure 8 shows schematic representation of the results in the present study. We have demonstrated that NO-dependent control of MVO\(_{2}\) was attenuated in nNOS \(^{-/-}\) compared with WT mice and this was restored by preincubation with Tiron, Tempol, ascorbic acid, oxypurinol, or SB203580. There was an increase in lucigenin-detectable O\(_2\)\(^{-}\) production in cardiac muscle from nNOS \(^{-/-}\) compared with WT mice, which was inhibited by oxypurinol or SB203580. We did not find a difference in XO/XDH protein levels, but there was an increase in phosphorylated p38 kinase protein level in nNOS \(^{-/-}\). Therefore, we conclude that a chronic defect of nNOS attenuate the control of MVO\(_{2}\) by NO derived from another source most likely eNOS, which is associated with an increase in O\(_2\)\(^{-}\) through the activation of XO, and XO was posttranslationally activated through a mechanism involving p38 kinase.

Acknowledgments

This study was supported by grants PO-1-HL-43023, HL-50412, HL-61290 (to T.H.H), HL-31069, and HL-66331 (to M.S.W.) from the National Heart, Lung, and Blood Institute.

References


A Defect of Neuronal Nitric Oxide Synthase Increases Xanthine Oxidase-Derived Superoxide Anion and Attenuates the Control of Myocardial Oxygen Consumption by Nitric Oxide Derived From Endothelial Nitric Oxide Synthase
Shintaro Kinugawa, Harer Huang, Ziping Wang, Pawel M. Kaminski, Michael S. Wolin and Thomas H. Hintze

Circ Res. 2005;96:355-362; originally published online January 6, 2005;
doi: 10.1161/01.RES.0000155331.09458.A7

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/96/3/355

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/