gp91phox-containing NAD(P)H oxidase mediates attenuation of nitric oxide-dependent control of myocardial oxygen consumption by ANG II

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Kinugawa, Shintaro, Juhua Zhang, Eric Messina, Erin Walsh, Harer Huang, Pawel M. Kamiński, Michael S. Wolin, and Thomas H. Hintze. gp91phox-containing NAD(P)H oxidase mediates attenuation of nitric oxide-dependent control of myocardial oxygen consumption by ANG II. Am J Physiol Heart Circ Physiol 289: H862–H867, 2005. First published March 18, 2005; doi:10.1152/ajpheart.00076.2005.—We have previously reported that ANG II stimulation increased superoxide anion (O2•−) through the activation of NAD(P)H oxidase and inhibited nitric oxide (NO)-dependent control of myocardial oxygen consumption (MV̇O2) by scavenging NO. Our objective was to investigate the role of NAD(P)H oxidase, especially the gp91phox subunit, in the NO-dependent control of MV̇O2. Mice homozygous for targeted disruption of the gp91phox gene (gp91phox−/−) were not altered by ANG II. There was a decrease in lucigenin (5 × 10−6 mol/l)-detectable O2•− in gp91phox−/− mice compared with WT mice. ANG II resulted in significant increases in O2•− production in WT mice, which was inhibited by coincubation with Tiron or apocynin. However, ANG II had no effect on O2•− production in gp91phox−/− mice. Histological examination showed that the development of abscesses and/or the invasion of inflammatory cells occurred in lungs and livers but not in hearts and kidneys from gp91phox−/− mice. These results indicate that the gp91phox subunit of NAD(P)H oxidase mediates O2•− production through the activation of NAD(P)H oxidase and attenuation of NO-dependent control of MV̇O2 by ANG II.

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 (7, 8, 11). We and others have shown that NO can modulate mitochondrial respiration and tissue oxygen consumption (MV̇O2) in the whole body, heart, skeletal muscle, and kidney both in vivo (5, 17, 20, 30) and in vitro (20, 29, 35). Furthermore, Loke et al. (23) have shown that NO derived from both in vivo (5, 17, 20, 30) and in vitro (20, 29, 35). Further-

Mice homozygous for targeted disruption of the gp91phox gene [background C57 black 6, gp91phox−/−, n = 23 mice] and age-matched wild-type control mice (WT, n = 27 mice) were purchased from Jackson Laboratories. 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’s Guidelines for the Care and Use of Laboratory Animals.

Preparation of Cardiac Muscle Tissues and Measurement of MV̇O2

MVO2 was measured in vitro as Hintze et al. (22, 23) described previously. Mice were anesthetized with pentobarbital sodium (50 mg/kg ip), and hearts were removed immediately. The atria, right
ventricle together with connective tissues, and fat and large coronary arteries were discarded. The left ventricle was then bisected such that each piece of muscle contained the septum, free wall, and apex. The muscle tissues were incubated in Krebs solution (mmol/l): 118 NaCl, 4.7 KCl, 1.5 CaCl2, 25 NaHCO3, 1.2 KH2PO4, and 5.6 glucose) at 37°C for 2 h and bubbled continuously with 20% O2-5% CO2-75% N2. At the end of the incubation period, each piece of tissue was placed in a stirred bath with 3 ml of air-saturated Krebs bicarbonate 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 calculated as the rate of decrease in oxygen concentration, assuming an initial oxygen concentration of 224 μmol/ml, and was expressed as nanomoles of oxygen consumed per minute per gram of tissue. The effect of all drugs on tissue oxygen uptake is expressed as a percentage of change in baseline MV˙O2.

**Experimental Protocols**

*Inhibition of MV˙O2 by endogenous NO in WT and gp91phox(−/−) mice.* Bradykinin (BK) stimulates kinin B2-receptors on the endothelium to cause NO production. After baselines were recorded, cumulative concentrations of BK at 10−10 to 10−5 mol/l were added to the chambers in the presence or absence of 10−4 mol/l Nω-nitro-L-arginine methyl ester (L-NAME).

*Effects of ANG II on MV˙O2 in WT and gp91phox(−/−) mice.* Muscle segments were incubated with 10−8 mol/l ANG II 30 min before measurements of MV˙O2 as previously described (18). In separate experiments, the effects of ANG II on MV˙O2 were also studied in the presence of 10−2 mol/l Tiron or 10−5 mol/l apocynin, an inhibitor of NAD(P)H oxidase activation. After baselines were recorded, cumulative concentrations of BK at 10−10 to 10−5 mol/l were added to the chambers.

*O2− Production*

The chemiluminescence elicited by O2− in the presence of lucigenin (5 × 10−6 mol/l) was measured in cardiac tissues from WT and gp91phox(−/−) mice as Mohazzab et al. (24) described previously. Muscle segments were incubated with 10−8 mol/l ANG II 30 min before measurements of chemiluminescence. In separate experiments, the effects of ANG II on O2− production were also studied in the presence of 10−2 mol/l Tiron or 10−5 mol/l apocynin, an inhibitor of NAD(P)H oxidase activation. After baselines were recorded, cumulative concentrations of BK at 10−10 to 10−5 mol/l were added to the chambers.

*Histological Examination*

Hearts, lungs, livers, and kidneys were removed from WT or gp91phox(−/−) mice, preserved in a 10% formalin solution for 48 h, and embedded in paraffin. Tissue blocks were sectioned to 4-μm thick. Hematoxylin and eosin slides were then prepared using standard methods from the *Handbook of Histopathological and Histochemical Techniques* (7a.) as well as an H2500 microwave processor (Energy Beam Sciences; Agawam, MA).

*Chemicals*

All drugs were purchased from Sigma Chemical, and Krebs bicarbonate was used to dissolve drugs and as a vehicle.

*Data Analysis*

All data are presented as means ± SE. The slope of the line relating oxygen concentration and time (MV˙O2) was measured, and data are expressed as a percentage of change in the slope because there were no differences in the baseline in any group. Comparisons of O2− production were made using one-way ANOVA followed by Scheffé’s t-test. The changes in MV˙O2 caused by BK were analyzed by using repeated measures two-way ANOVA followed by Scheffé’s t-test.

Fig. 1. Effects of cumulative doses of bradykinin (BK) on myocardial oxygen consumption (MV˙O2) in hearts. Shown are wild-type mice (WT, ●, n = 14); WT with Nω-nitro-L-arginine methyl ester (L-NAME) (●, n = 4); mice homozygous for targeted disruption of the gp91phox gene [gp91phox(−/−)], □, n = 14), gp91phox(−/−) with L-NAME (●, n = 4). **P < 0.01 vs. WT; ††P < 0.01 vs. gp91phox(−/−).

Statistical significance of differences for baseline MV˙O2 in cardiac muscle was determined with an unpaired t-test. Significant changes were considered at a value of P < 0.05.

**RESULTS**

*Baseline MV˙O2 in WT and gp91phox(−/−) Mice*

Baseline MV˙O2 was not different in any groups in the absence or presence of L-NAME, Tiron, or apocynin (data not shown).

*MV˙O2 in WT and gp91phox(−/−) Mice in Response to BK*

Cumulative doses of BK (Fig. 1) caused concentration-dependent reduction in MV˙O2 in WT mice. The extent of BK-induced reduction in MV˙O2 was significantly larger in gp91phox(−/−) than WT mice (Fig. 1). BK-induced reduction in MV˙O2 was attenuated by L-NAME (Fig. 1). BK-induced reduction in MV˙O2 in WT mice was slightly but significantly enhanced by Tiron or apocynin (at 10−5 mol/l BK, −26 ± 1% in WT mice vs. −32 ± 1% in WT mice with Tiron, P < 0.01, vs. −29 ± 1% in WT with apocynin, P < 0.01).

*Effects of ANG II on MV˙O2 in WT and gp91phox(−/−) Mice*

BK-induced reduction in MV˙O2 in WT mice was significantly attenuated by preincubation with 10−8 mol/l ANG II (Fig. 2). The inhibitory effects of ANG II on MV˙O2 in response to BK were completely restored by coincubation with Tiron or apocynin (Fig. 2). In contrast to WT mice, BK-induced reduction in MV˙O2 in gp91phox(−/−) mice was not affected by preincubation with 10−8 mol/l ANG II (Fig. 3).

*O2− Production in Cardiac Muscle from WT and gp91phox(−/−) Mice*

BK-induced reduction in MV˙O2 in WT mice was significantly attenuated by preincubation with 10−8 mol/l ANG II (Fig. 2). The inhibitory effects of ANG II on MV˙O2 in response to BK were completely restored by coincubation with Tiron or apocynin (Fig. 2). In contrast to WT mice, BK-induced reduction in MV˙O2 in gp91phox(−/−) mice was not affected by preincubation with 10−8 mol/l ANG II (Fig. 3).

There was a decrease in lucigenin (5 × 10−6 mol/l)-detectable O2− production in cardiac muscle from gp91phox(−/−) compared with WT mice (64 ± 16 vs. 109 ± 15 counts/min per milligram of tissue, Fig. 4). ANG II (10−8 mol/l) resulted in a significant increase in O2− production in WT mice, which was inhibited by coincubation with Tiron or apocynin (Fig. 4). In contrast to WT mice, ANG II had no effect on O2− production in gp91phox(−/−) mice (Fig. 4).
with WT mice. L-NAME, a nonselective inhibitor of NO synthase, inhibited BK-induced reduction in MV\textsuperscript{\textcircled{O}2} in WT and gp91phox\textsuperscript{-/-} mice (\(\Delta, n = 4\)). \(\Delta\Delta P < 0.01\) vs. WT; \(\uparrow\uparrow P < 0.01\) vs. WT with 10\textsuperscript{-8} mol/l ANG II.

**DISCUSSION**

In the present study, we demonstrated that BK-induced reduction in MV\textsuperscript{\textcircled{O}2} was enhanced in gp91phox\textsuperscript{-/-} mice compared with WT mice. L-NAME, a nonselective inhibitor of NO synthase, inhibited BK-induced reduction in MV\textsuperscript{\textcircled{O}2} in WT and gp91phox\textsuperscript{-/-} mice. Apocynin or Tiron slightly but significantly enhanced BK-induced reduction in MV\textsuperscript{\textcircled{O}2} in WT mice. We also showed that lucigenin (5 \(\times\) 10\textsuperscript{-6} mol/l)-detectable \(O_2^\cdot\) is produced less in gp91phox\textsuperscript{-/-} mice than in WT mice. Therefore, these findings suggest that NO-dependent control of MV\textsuperscript{\textcircled{O}2} is enhanced in gp91phox\textsuperscript{-/-} mice, and the enhanced NO-dependent control of MV\textsuperscript{\textcircled{O}2} is due to an increase in NO bioavailability. Jung et al. (16) have shown that endothelium-dependent relaxation was enhanced in aortic rings from gp91phox\textsuperscript{-/-} mice compared with rings from WT mice. They also have shown that \(O_2^\cdot\) production was lower in aortic rings from gp91phox\textsuperscript{-/-} than rings from WT mice. These findings from studies that used aortic rings from gp91phox\textsuperscript{-/-} and WT mice support the findings of our study that used heart tissues. ANG II (10\textsuperscript{-8} mol/l) attenuates BK-induced reduction in MV\textsuperscript{\textcircled{O}2} and resulted in significant increases in MV\textsuperscript{\textcircled{O}2} production in WT mice, which were inhibited by Tiron or apocynin. A previous study by Kinugawa et al. (18) has demonstrated that ANG II increases \(O_2^\cdot\) production through the activation of NAD(P)H oxidase via ANG II type I receptor and attenuates NO-dependent control of MV\textsuperscript{\textcircled{O}2} in heart tissues from normal dogs. The present results extend our previous observations. In contrast to WT mice, 10\textsuperscript{-8} mol/l ANG II had no effect in BK-induced reduction in MV\textsuperscript{\textcircled{O}2} and \(O_2^\cdot\) production in gp91phox\textsuperscript{-/-} mice. This suggests that attenuation of control of MV\textsuperscript{\textcircled{O}2} and \(O_2^\cdot\) production by ANG II is mediated by gp91phox-containing NAD(P)H oxidase. Jung et al. (16) showed that 3 \(\times\) 10\textsuperscript{-8} mol/l ANG II attenuates endothelium-dependent relaxation in aortic rings from WT mice but not from gp91phox\textsuperscript{-/-} mice. Bendall et al. (4) showed that chronic infusion of ANG II to WT mice increases NAD(P)H oxidase activity and \(O_2^\cdot\) production and induces cardiac hypertrophy but not in gp91phox\textsuperscript{-/-} mice. Therefore, gp91phox-containing NAD(P)H oxidase plays an important role in oxygen radical production and cardiac pathophysiology induced by ANG II.

All vascular and cardiac cells have the capacity to generate \(O_2^\cdot\) in response to ANG II via different NAD(P)H oxidase isoforms. The Nox homolog gp91phox is expressed in the endothelium (3, 10), the vascular smooth muscle (15), fibroblasts (34), and cardiomyocytes (21). Nox4 is reported to be expressed throughout the vessel wall (32), and Nox1 expression is restricted to the smooth muscle cells (19). In the present study, we have shown that ANG II had no effect on \(O_2^\cdot\) production and NO-dependent control of MV\textsuperscript{\textcircled{O}2} in...
gp91phox(−/−) mice. This means that Nox1 or Nox4 do not compensate for the absence of gp91phox in the response to ANG II. We previously found that a low concentration (10^{-10} to 10^{-8} mol/l) of ANG II dose dependently attenuated NO-dependent control of MV˙O2 in heart tissues from normal dogs (18), whereas a higher concentration of ANG II (10^{-6} to 10^{-4} mol/l) did not affect NO-dependent control of MV˙O2 (unpublished data). It has been shown that the action of the endothelial ANG II type 2 receptor blocks radical formation at higher concentrations of ANG II (28, 31), whereas this mechanism was not observed in smooth muscle cells (12). Therefore, our previous data may suggest that endothelial NAD(P)H oxidase plays an important role in O2⁻ production by ANG II in our model, but it may be altered if the ANG II type 2 receptor expression changes. Furthermore, we also showed that ANG II had no effect in the control of MV˙O2 induced by S-nitroso-N-acetyl penicillamine (SNAP), an NO donor. Our assumption is that SNAP releases NO at the same site that the NAD(P)H oxidase produces superoxide. These data suggest that O2⁻ formed through the activation of NAD(P)H oxidase by ANG II not only scavenges NO but may also impair eNOS-dependent NO formation. Our present and previous data indicate that endothelial gp91phox-containing NAD(P)H oxidase plays a pivotal role in O2⁻ production and attenuation of NO-dependent

Fig. 5. Photomicrographs of hematoxylin and eosin-stained lung (A) and liver (B) from a gp91phox(−/−) mouse. Centers of the abscesses in the lung were composed of massive liquid necrosis containing neutrophil debris. Fibrous tissue surrounded them forming abscess membranes. Some neutrophils and chronic inflammatory cells with necrosis were found in the liver.

Fig. 6. Photomicrographs of hematoxylin and eosin-stained heart (A) and kidney (B) from a gp91phox(−/−) mouse. No inflammation was observed in heart and kidney.
control of MVo2 in response to ANG II. Alternatively, our data may indicate that Nox1 or Nox4 are not involved in ANG II-induced O2 production.

It is well known that NAD(P)H oxidase is the enzyme complex responsible for generating the respiratory burst (25, 27). A deficiency of NAD(P)H oxidase leads to the lack of bactericidal and fungicidal mechanisms and causes opportunistic infections (25, 27). Chronic granulomatous disease in humans has been reported to result from a deficiency of NAD(P)H oxidase, especially a mutation in the gene for gp91phox (27). Therefore, gp91phox(−/−) mice have been used to study chronic granulomatous disease and the role of respiratory burst in host defense (25, 27). Actually, it has been reported that pulmonary abscesses occur in gp91phox(−/−) mice (9). Our histological examination showed that the development of abscesses and/or invasion of inflammatory cells occurred in lungs as well as livers from gp91phox(−/−) mice. Phagocytes in gp91phox(−/−) mice fail to produce O2-, and therefore the production of substances such as hydrogen peroxide is decreased. The injury in lungs and livers from gp91phox(−/−) mice provides evidence that the gp91phox subunit of NAD(P)H oxidase plays a pivotal role in the production of O2-. On the other hand, no injury was found in the hearts or kidneys from gp91phox(−/−) mice. These results suggest that the role of gp91phox and O2- produced by NAD(P)H oxidase is different among organs and does not influence the results of our study that used cardiac tissue.

In the present study, we demonstrated that NO-dependent control of MVo2 was enhanced in gp91phox(−/−) mice compared with WT mice, and ANG II had no effect in NO-dependent control of MVo2 and O2- production in gp91phox(−/−) mice. These results indicate that the gp91phox subunit of NAD(P)H oxidase plays a pivotal role in O2- production through the activation of NAD(P)H oxidase and participation in the regulation of NO-dependent control of myocardial oxygen consumption by ANG II. gp91phox subunit of NAD(P)H oxidase plays a protective role in host defense in lungs or livers and is an important mediator of ANG II in hearts.

GRANTS
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