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

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Fischer 344 rats, the model of accelerated aging, was reduced and associated with NAD(P)H oxidase-generated O2- . Hintze’s laboratory (22) also reported that NO-dependent control of VO2 was attenuated in cardiac muscle from heterozygous manganese superoxide dismutase gene knockout (SOD2+/−) mice, which was reversed by the freely membrane-permeable O2- scavenger Tiron. Our previous studies have shown that O2- plays an important role in the regulation of NO-dependent control of VO2.

Phagocyte-type NAD(P)H oxidases have been shown to play an important role in the production of O2- in the cardiovascular system (13). NAD(P)H oxidase activity is increased by stimuli such as ANG II, and NAD(P)H oxidases are implicated in ANG II-induced hypertension (26), vascular smooth muscle (33, 34) and cardiac hypertrophy (4, 6), endothelial dysfunction (16), and atherosclerosis (13). NAD(P)H oxidase comprises a membrane-bound p22phox/gp91phox (or Nox) subunits and cytosolic p47phox, p67phox, and rac-1 subunits (2). A gp91phox-containing oxidase is known to be expressed in endothelium (3, 10), fibroblasts (34), and cardiomyocytes (21). Kinugawa et al. (18) found that ANG II at pathophysiological concentrations stimulates an increase in O2- through the activation of NAD(P)H oxidase and attenuates NO-dependent control of myocardial oxygen consumption (MV02) in cardiac muscle from normal dogs, a phenomenon we termed “endothelial stunning.” The role of a gp91phox-containing NAD(P)H oxidase in ANG II-induced attenuation of NO-dependent control of VO2 in cardiac muscle has not been studied. We hypothesized that ANG II would not stimulate O2- production via the NAD(P)H oxidase in gp91phox(−/−) mouse heart to alter the biological activity of NO.

METHODS

Animals Studied

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 MV02

MV02 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 tissue samples were incubated in Krebs solution (mmol/l: 118 NaCl, 4.7 KCl, 1.5 CaCl2, 25 NaHCO3, 1.1 MgSO4, 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^-7 mol/l were added to the chambers in the presence or absence of 10^-4 mol/l N^6-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^-7 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^-7 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 at 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.

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

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
MV˙O2 is enhanced in gp91phox(-/-) or kidneys (Fig. 6A)
from gp91phox(-/-) mice. The center of the abscess was
composed of massive liquid necrosis containing neutrophil
debris. Fibrous tissue surrounded the abscess forming a mem-
brane (Fig. 5A). Obvious fibrosis and invasion of chronic
inflammatory cells were observed around abscesses (Fig. 5B).
On the other hand, no inflammation was observed in the hearts
inflammatory cells with necrosis were found in livers (Fig. 5A).
Nodes of abscess, up to 11 mm in diameter, developed in all
livers from gp91phox(-/-) mice, including some neutrophils, and chronic
fibrosis and invasion of chronic inflammatory cells were observed in
the hearts (Fig. 6A) or kidneys (Fig. 6B) from gp91phox(-/-) mice.

**DISCUSSION**

In the present study, we demonstrated that BK-induced
reduction in MV˙O2 was enhanced in gp91phox(-/-) compared with
WT mice. L-NAME, a nonselective inhibitor of NO
synthase, inhibited BK-induced reduction in MV˙O2 in WT and
gp91phox(-/-) mice. Apocynin or Tiron slightly but signifi-
cantly enhanced BK-induced reduction in MV˙O2 in WT mice. We also showed that lucigenin (5 × 10^-6 mol/l)-detectable O2-
is produced less in gp91phox(-/-) mice than in WT mice. Therefore, these findings suggest that NO-dependent control of
MV˙O2 is enhanced in gp91phox(-/-) mice, and the enhanced
NO-dependent control of MV˙O2 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(-/-) compared with rings from WT mice. They also have shown that O2- production was lower in aortic rings from
gp91phox(-/-) than rings from WT mice. These findings from
studies that used aortic rings from gp91phox(-/-) and WT mice support the findings of our study that used heart tissues.
ANG II (10^-8 mol/l) attenuated BK-induced reduction in
MV˙O2 and resulted in significant increases in O2- 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 O2- production through the activation of
NAD(P)H oxidase via ANG II type 1 receptor and attenuates
NO-dependent relaxation in aortic rings from WT mice but not
from gp91phox(-/-) mice. Therefore, gp91phox-containing NAD(P)H oxidase plays an important role in
oxygen radical production and cardiac pathophysiology in-
duced by ANG II.

All vascular and cardiac cells have the capacity to generate
O2- in response to ANG II via different NAD(P)H oxidase
isofoms. The Nox homolog gp91phox is expressed in the
endothelium (3, 10), the vascular smooth muscle (15), fibro-
blasts (34), and cardiomyocytes (21). Nox4 is reported to be
expressed throughout the vessel wall (32), and Nox1 expres-
sion is restricted to the smooth muscle cells (19). In the
present study, we have shown that ANG II had no effect
on O2- production and NO-dependent control of MV˙O2 in

**Histological Examination**

Nodes of abscess, up to 11 mm in diameter, developed in all
lungs from gp91phox(-/-) mice. The center of the abscess was
composed of massive liquid necrosis containing neutrophil
debris. Fibrous tissue surrounded the abscess forming a mem-
brane (Fig. 5A). Obvious fibrosis and invasion of chronic
inflammatory cells were observed around abscesses (Fig. 5A).
Moderate inflammation was also found in all livers from
gp91phox(-/-) mice, including some neutrophils, and chronic
fibrosis and invasion of chronic inflammatory cells were found in livers (Fig. 5B).

**Fig. 2. Effects of 10^-9 mol/l ANG II on BK-induced reduction in MV˙O2 in
WT mice in absence or presence of inhibitor. Shown are WT (○, n = 14); WT
with 10^-8 mol/l ANG II (●, n = 15); WT with 10^-6 mol/l ANG II + 10^-5
mol/l apocynin (▲, n = 5); WT with 10^-3 mol/l ANG II + 10^-2 mol/l Tiron
(△, n = 4). **P < 0.01 vs. WT; ††P < 0.01 vs. WT with 10^-9 mol/l ANG II.**
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 control of MV˙O₂.
control of \( MV_{O2} \) in response to ANG II. Alternatively, our data may indicate that Nox1 or Nox4 are not involved in ANG II-induced \( O_2 \) 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 opportunis-

27). A deficiency of NAD(P)H oxidase leads to the lack of complex responsible for generating the respiratory burst (25, 27). Actually, it has been reported that pulmonary abscesses occur in gp91phox(-/-) mice. Our histological examination showed that the develop-

ment 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 \( O_2^- \), 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 sub-

unit of NAD(P)H oxidase plays a pivotal role in the production of \( O_2^- \). 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 \( O_2^- \) 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 \( MV_{O2} \) was enhanced in gp91phox(-/-) mice compared with WT mice, and ANG II had no effect in NO-dependent control of \( MV_{O2} \) and \( O_2^- \) production in gp91phox(-/-) mice. These results indicate that the gp91phox subunit of NAD(P)H oxidase plays a pivotal role in \( O_2^- \) 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.

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