Rapid Communications

Endogenous Endothelial Nitric Oxide Synthase–Derived Nitric Oxide Is a Physiological Regulator of Myocardial Oxygen Consumption


Abstract—Our objective was to determine the precise role of endothelial nitric oxide synthase (eNOS) as a modulator of cardiac O2 consumption and to further examine the role of nitric oxide (NO) in the control of mitochondrial respiration. Left ventricle O2 consumption in mice with defects in the expression of eNOS [eNOS (−/−)] and inducible NOS [iNOS (−/−)] was measured with a Clark-type O2 electrode. The rate of decreases in O2 concentration was expressed as a percentage of the baseline. Baseline O2 consumption was not significantly different between groups of mice. Bradykinin (10−4 mol/L) induced significant decreases in O2 consumption in tissues taken from iNOS (−/−) (−28±4%), wild-type eNOS (+/+)(−22±4%), and heterozygous eNOS (+/−)(−22±5%) but not homozygous eNOS (−/−)(−3±4%) mice. Responses to bradykinin in iNOS (−/−) and both wild-type and heterozygous eNOS mice were attenuated after NOS blockade with N-nitro-L-arginine methyl ester (L-NAME) (−2±5%, −3±2%, and −6±5%, respectively, P<0.05). In contrast, S-nitroso-N-acetyl-penicillamine (SNAP, 10−4 mol/L), which releases NO spontaneously, induced decreases in myocardial O2 consumption in all groups of mice, and such responses were not affected by L-NAME. In addition, pretreatment with bacterial endotoxin elicited a reduction in basal O2 consumption in tissues taken from normal but not iNOS (−/−)−deficient mice. Our results indicate that the pivotal role of eNOS in the control of myocardial O2 consumption and modulation of mitochondrial respiration by NO may have an important role in pathological conditions such as endotoxemia in which the production of NO is altered. (Circ Res. 1999;84:840-845.)

Key Words: endothelial nitric oxide synthase–derived nitric oxide ■ cardiac oxygen consumption

Evidence for the effect of nitric oxide (NO) on mitochondrial function was reported before the discovery of NO by Granger et al1 in 1980. Their initial observations demonstrated that activated mouse peritoneal macrophages severely inhibited O2 consumption in numerous tumor cell lines obtained from different tissues and animal species in cultures by an unknown mechanism. Evidence now suggests that the macrophage-induced cytotoxic effect on mitochondrial metabolism is NO related.2,3 NO inhibits 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.4–6 Recently, we and others have provided direct evidence to suggest that under physiological conditions NO plays a modulatory role on mitochondrial respiration and tissue O2 consumption. For instance, L-arginine analogues, which are nonspecific inhibitors of the 3 isoforms of nitric oxide synthase (NOS),7 increase O2 consumption in whole body,8 heart, skeletal muscle, and kidney both in vivo9–12 and in vitro.12–14 We have interpreted our previous studies to suggest that endothelial nitric oxide synthase (eNOS), the most highly expressed isoform of NOS in vascular tissue under physiological conditions, is responsible for the control of tissue O2 consumption by NO. However, we have yet to determine which isoform of NOS regulates mitochondrial O2 consumption, because almost all cells are capable of expressing all 3 different NOS isoforms. Studies of the effects of bacterial endotoxins have attributed a substantial role for inducible nitric oxide synthase (iNOS) in the development of shock and perhaps other pathological states. To address the role of NO in both physiological and pathophysiological states in the control of mitochondrial respiration, we used tissues from mice deficient in iNOS and eNOS and 3 additional groups, ie, control C57BL/6x129, control outbred wild-type eNOS (+/+), and heterozygous eNOS (+/−) mice to better understand the sources of NO responsible for the control of O2 consumption.
Materials and Methods

Animals Studied

iNOS-deficient mice were bred by pairing homozygous mutant iNOS (−/−) mice originally developed by MacMicking et al.15 C57BL/6x129 mice obtained from the Jackson Laboratories were used as wild-type controls. Heterozygous eNOS (+/−) mice, originally developed by Shesely et al.,16 were interbred to generate eNOS wild-type (+/−), heterozygotes (+/−), and homozygous mutant (−/−) mice. The eNOS mice were genotyped by Southern analysis of DNA as described previously,16 with the eNOS wild-type used as littermate controls for the eNOS (+/−) and (−/−) mice. 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 Tissue Slices and Measurement of Tissue O2 Consumption

Mice were anesthetized with pentobarbital sodium (65 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. Tissues were then incubated in Krebs bicarbonate solution containing NaCl 118, KCl 4.7, CaCl2 1.5, NaHCO3 25, KH2PO4 1.2, MgSO4 1.1, and glucose 5.6 at 37°C, bubbled with 21% O2/5% CO2/74% N2 (pH 7.4) to equilibrate for at least 2 hours. 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 O2 electrode (Yellow Springs Instruments) that was connected to an O2 monitor (model YSI 5331); hence, the uptake of O2 by the tissue was recorded. Increasing concentrations (10−2 to 10−4 mol/L) of bradykinin (Sigma) or L-nitroso-N-acetyl-penicillamine (SNAP, Sigma) on O2 uptake were studied in the absence or presence of NOS inhibitors (10−4 mol/L) N-nitro-L-arginine (LNAME) or N-nitro-L-arginine methyl ester (L-NAME). Sodium cyanide (10−3 mol/L, Sigma), an inhibitor of complex IV of the electron transport chain, was given at the end of each experiment to confirm that changes in O2 consumption originated from mitochondria.

Pretreatment of Lipopolysaccharide in Normal and iNOS (−/−) Mice

In the lipopolysaccharide (LPS)-treated mice, Escherichia coli LPS (serotype 026:B6, Sigma) was administered at 0.75 mg/kg IP. After 3 to 4 hours, mice were anesthetized, blood samples were collected by cardiac puncture, and hearts were removed and prepared as described in the previous section.

Measurement of Plasma Nitrate/Nitrite

Plasma nitrate/nitrite (NOx) assay was performed using a method previously described by our laboratory.17 Briefly, after centrifugation, plasma was incubated in an air-tight tube containing Aspergillus nitrate reductase to reduce nitrate to nitrite, then converting nitrite into NO by the addition of hydrochloric acid. Gaseous NO produced was then injected into a NO chemiluminescence analyzer (Sievers, Inc). The NO content was determined by combining with ozone to produce photons that are directly proportional to the amount of NO injected; hence, the NOx in the plasma sample.

Calculation of Tissue O2 Consumption and Statistical Analysis

Tissue respiration was calculated as the rate of decrease in O2 concentration, assuming an initial O2 concentration of 224 nmol/mL,18 and was expressed as nanomoles of O2 consumed per minute per gram of tissue.
The effect of bradykinin or SNAP on tissue O₂ uptake is expressed as a percentage of change in baseline O₂ consumption. All data in the Table, text, and figures are presented as mean±SEM. Statistical analysis on baseline O₂ consumption was performed using 1-way ANOVA followed by Tukey test for multiple comparisons, and the changes in O₂ consumption caused by bradykinin or SNAP were analyzed using 2-way ANOVA followed by multiple comparison using Student-Newman-Keuls method.

**Results**

**Baseline Cardiac Tissue O₂ Consumption in Normal and Genetically Altered Mice**

Baseline tissue O₂ consumption was not different in any group in the absence or presence of NOS inhibitors NLA or L-NAME (Table).

**Effects of Cardiac Tissue O₂ Consumption in Response to Bradykinin and SNAP in Normal Mice**

Cumulative doses of bradykinin (10⁻⁷ to 10⁻⁴ mol/L) in tissues from control mice caused concentration-dependent decreases in O₂ consumption (Figure 1A). These responses were attenuated after blockade of bradykinin B₂ receptors with the B₂ receptor antagonist HOE-140 (10⁻³ mol/L) (10⁻⁵ mol/L, control: -25±4% versus HOE-140: 7±11%, P<0.05, t test, n=5). Bradykinin-induced reduction in O₂ consumption was attenuated by NLA (10⁻⁵ mol/L, control: -31±4% versus NLA: -12±4%, P<0.05; Figure 1A). Administration of SNAP (10⁻⁴ mol/L) decreased tissue O₂ consumption in control C57BL/6x129 mice by 29±4%. In contrast to bradykinin, responses to SNAP were not affected by NLA (data not shown).

**Effects of Cardiac Tissue O₂ Consumption in Response to Bradykinin and SNAP in iNOS (−/−) and eNOS (−/−)-Deficient Mice**

Bradykinin caused concentration-dependent decreases in O₂ consumption in tissues from iNOS-deficient mice (10⁻⁴ mol/L, iNOS: -28±4%; Figure 1B), and this was reduced in the presence of NLA (Figure 1B). Similarly, administration of bradykinin decreased O₂ consumption in tissues from both wild-type and heterozygous eNOS mice [10⁻⁴ mol/L, eNOS (+/+) -20±4% versus eNOS (+/-): -22±6%; Figure 2A and 2B] in a concentration-dependent manner. These responses were significantly attenuated by L-NAME (Figure 2A and 2B). In contrast, bradykinin had no inhibitory effect on O₂ consumption in tissues from eNOS (−/−) mice (n=10). D, In contrast, the NO donor SNAP caused dose-dependent decreases in O₂ consumption in tissues from eNOS (−/−) mice (n=8). *P<0.05 vs respective eNOS (+/+) or eNOS (+/-), 2-way ANOVA, followed by Student-Newman-Keuls.

**Effects of LPS Treatment on Cardiac Tissue O₂ Consumption in Normal and iNOS (−/−)-Deficient Mice**

Administration of endotoxin significantly lowered resting myocardial O₂ consumption in normal mice (Figure 3A). This effect was accompanied by an increase in levels of NOx (Figure 3B). In contrast, endotoxin treatment had no effect on either resting O₂ consumption or plasma NOx in iNOS (−/−)-deficient mice (Figure 3A and 3B).

**Discussion**

In the present study, we have demonstrated that the constitutive isoform of eNOS plays a pivotal role in the regulation of myocardial tissue O₂ consumption. These findings support our speculation in previous studies that NO released from the vascular endothelium not only controls tissue blood flow but also affects tissue respiration. Our data show that the ability...
Baseline myocardial tissue O2 consumption was significantly reduced from iNOS-deficient mice. Baseline O2 consumption was significantly lower compared with O2 consumption in tissue taken from the hearts of mice lacking bradykinin receptor–mediated mechanism or exogenous NO inhibits myocardial O2 consumption. The B2 kinin receptor–mediated mechanism is supported by our more recent data showing that the inhibitory effect of bradykinin on O2 consumption was abolished in hearts from mice lacking bradykinin B2 receptors.20

When the involvement of specific NOS isoforms in the regulation of myocardial O2 consumption was examined, similar concentrations of bradykinin were tested in tissues from iNOS- and eNOS-deficient mice. The loss of bradykinin but not SNAP responses on tissue O2 consumption in eNOS (−/−) mice suggests that the bradykinin-induced response requires the expression of eNOS to produce NO to regulate mitochondrial respiration. Although there is evidence suggesting that iNOS may be expressed in tissues or cells under normal conditions (ie, in the absence of bacterial endotoxins or inflammatory cytokine activation) in the kidney21 and even in the heart,22 iNOS is not likely to be the mediator of cardiac tissue respiration, because bradykinin suppressed O2 consumption in tissues lacking iNOS. Furthermore, bradykinin B2 receptor activation leads to the elevation of [Ca2+], to stimulate NO synthesis and release in neurons expressing the NOS enzyme. However, if nNOS were responsible for the modulation of mitochondrial respiration, bradykinin administration would have decreased O2 consumption in eNOS (−/−)–deficient mice. These findings strongly suggest that eNOS plays a major role in the physiological regulation of mitochondrial respiration, and the contribution of iNOS and/or nNOS toward myocardial tissue respiration is probably negligible under physiological conditions.

iNOS is well documented in the pathogenesis of sepsis and endotoxemia, conditions of elevated NO levels that contribute to LPS-induced hypotension and mortality. To test the possibility that large amounts of NO produced by iNOS regulate tissue O2 consumption and perhaps play an important pathological role, endotoxin was administered to C57BL/6×129 mice and to iNOS (−/−) mice, and cardiac tissue O2 consumption was measured. Endotoxin treatment significantly reduced basal myocardial tissue O2 consumption, but not in iNOS (−/−)–deficient mice, indicating that iNOS may be important in the pathogenesis of endotoxemia and sepsis.

The blockade of endogenous NO production using NOS inhibitors NLA or L-NAME had no effect on baseline tissue O2 consumption in normal or genetically altered mice. This observation is consistent with our previous reports of isolated tissue O2 consumption,12–14 suggesting that in the absence of shear stress or a chemical stimulus, the influence of basal release of NO on tissue O2 consumption is minimal. When baseline myocardial O2 consumption was analyzed across the different strains of mice, we found that baseline O2 consumption in tissue from the iNOS (−/−) mice is significantly lower compared with O2 consumption in tissue taken from the wild-type eNOS (+/+) mice. This difference could not be explained by the possible upregulation of eNOS/neuronal nitric oxide synthase (nNOS) after iNOS gene deletion, as reported by Meng et al19 in pial arterioles. This should have resulted in enhanced NO production and lowered baseline O2 consumption; however, NOS inhibition with NLA in our study had no effect on baseline O2 consumption. The difference may be due to a different number of mitochondria per cell, a genetically determined value. In the murine model, we demonstrate that endogenous NO released through a B2 kinin receptor–mediated mechanism or exogenous NO inhibits myocardial O2 consumption. The B2 kinin receptor–mediated mechanism is supported by our more recent data showing that the inhibitory effect of bradykinin on O2 consumption was abolished in hearts from mice lacking bradykinin B2 receptors.20

Figure 3. Effect on baseline O2 consumption (A) and plasma NOx level (B) in the absence of LPS (−LPS) and presence of LPS (+LPS) in tissues of normal (C57BL/6×129) and iNOS (−/−)–deficient mice. Baseline O2 consumption was significantly lowered in tissues of LPS-treated normal mice than in those of the non–LPS-treated group. This reduction in myocardial O2 consumption was associated with a marked increase in plasma NOx level. In contrast, LPS treatment had no significant effect on either basal O2 uptake or plasma NOx level in iNOS (−/−)–deficient mice. *P<0.05 vs non–LPS-treated normal mice, t test (n=5 in each group).
develop significantly higher blood pressures, although there is evidence for "gene dosing."^{24}

Several lines of evidence support the findings in the present study: (1) the presence of a remarkably dense capillary network (more than a 100-fold greater than that of conduit vessels), (2) a short diffusion distance between capillaries and adjacent myocytes^{25,26} (<8 μm in human heart and a NO diffusion distance of 200 to 600 μm under physiological conditions,^{27} (3) immunohistochemical evidence of eNOS in capillaries,^{28} and (4) the ability of capillary endothelium to make NO.^{29} All these studies indicate that NO may readily diffuse from microvessels and affect mitochondrial function in surrounding myocytes.

There are several limitations in the present study. First, although our study provides strong evidence to support the role of capillary endothelium-derived NO in the modulation of mitochondrial respiration, we have yet to determine the cellular source of NO. We cannot rule out the possibility that eNOS from other cell types regulates mitochondrial respiration, given that agents well-known to activate the release of endothelium-derived NO such as bradykinin and carbachol also have direct physiological and metabolic actions on cardiac myocytes.^{30–33} More recently, the requirement of eNOS and caveolin interactions increasing cGMP level in the modulation of cholinergic control of cardiac function in myocytes has been demonstrated using transgenic mice lacking eNOS.^{34–36} In contrast to those findings, our previous data^{10,13,14} suggest that the effect of NO on mitochondria is cGMP independent. Furthermore, Kanai et al^{37} have reported in rat cardiac myocytes that NO is not released by bradykinin at a concentration of 10^{-5} mol/L (a concentration of bradykinin that inhibits O_2 consumption in the present study), carbachol, or shear stress. Apart from cardiac myocytes, the presence of eNOS has also been demonstrated in mitochondrial preparations from heart, skeletal muscle, kidney, and, more recently, from the liver.^{38–41} In addition, eNOS has even been suggested to play a role in O_2 consumption within mitochondria.^{39} Whether all of these sites are physiologically important sources of NO to control mitochondrial function requires further investigation. Second, the use of a Clark-type O_2 electrode to measure O_2 consumption in an isolated nonbeating myocardium and in the absence of blood and bloodborne products, may not truly reflect the magnitude of the effect of NO on myocardial O_2 consumption under in vivo conditions. Nevertheless, in vitro examination of tissue O_2 consumption eliminates neutrophilic and mechanical factors that may influence cardiac O_2 consumption to clearly demonstrate the direct effect of NO on mitochondrial respiration. The current findings are supported by our previous in vivo studies reporting that whole-body O_2 consumption or O_2 consumption across the skeletal muscle, heart, or kidney was elevated after the inhibition of NO synthesis. In addition, NO has a role in the regulation of cardiac O_2 consumption at increasing cardiac work loads due to exercise.^{11} Third, the use of normal C57BL6x129 mice instead of the age-matched wild-type iNOS (+/+ ) mice as controls could have influenced the results in the LPS study. However, the comparison of the effect of LPS on baseline O_2 consumption was made within the same strain. More importantly, the effect of bradykinin-induced reduction in O_2 consumption was similar in magnitude in the C57BL6x129 and iNOS (−/−) mouse hearts (eg, bradykinin decreased O_2 consumption by ~30% in both strains at 10^{-4} mol/L; Figure 1). This suggests that the tissue responsiveness to NO agonist in the modulation of O_2 consumption is not different between the 2 strains.

Despite these limitations, our study is the first to provide direct evidence that eNOS serves as an important source of NO to regulate parenchymal cell O_2 consumption through the activation of bradykinin receptors, which are found primarily on endothelial cells, in the heart and in other tissues as well. The control of O_2 consumption by NO may be altered therapeutically using NO donors,^{42} angiotensin-converting enzyme inhibitors,^{43} and possibly modified hemoglobin.^{44} In pathophysiological states such as endotoxemia and heart failure,^{45} in which NO production by the vasculature is altered, these alterations may contribute to the disease process.

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