Evidence for the effect of nitric oxide (NO) on mitochondrial function was reported before the discovery of NO by Granger et al. in 1980. Their initial observations demonstrated that activated mouse peritoneal macrophages severely inhibited O\textsubscript{2} 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.\textsuperscript{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.\textsuperscript{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 O\textsubscript{2} consumption. For instance, L-arginine analogues, which are nonspecific inhibitors of the 3 isoforms of nitric oxide synthase (NOS),\textsuperscript{7} increase O\textsubscript{2} consumption in whole body,\textsuperscript{8} heart, skeletal muscle, and kidney both in vivo\textsuperscript{9-12} and in vitro.\textsuperscript{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 O\textsubscript{2} consumption by NO. However, we have yet to determine which isoform of NOS regulates mitochondrial O\textsubscript{2} 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 O\textsubscript{2} consumption.
Baseline Tissue O₂ Consumption in Normal C57BL/6x129 and Different Groups of Genetically Altered Mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6x129</th>
<th>iNOS (−/−)</th>
<th>eNOS (−/+−)</th>
<th>eNOS (+/+)</th>
<th>eNOS (+/−)</th>
<th>eNOS (−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue weight, mg</td>
<td>38±2</td>
<td>60±12</td>
<td>40±3</td>
<td>43±2</td>
<td>41±3</td>
<td>39±3</td>
</tr>
<tr>
<td>Age, wk</td>
<td>8±10</td>
<td>8±10</td>
<td>8±10</td>
<td>19±3</td>
<td>19±3</td>
<td>9±3</td>
</tr>
<tr>
<td>Baseline O₂ consumption, mmol · min⁻¹ · g⁻¹</td>
<td>160±12</td>
<td>176±13</td>
<td>140±8</td>
<td>207±15</td>
<td>191±22</td>
<td>186±12</td>
</tr>
<tr>
<td>n</td>
<td>19</td>
<td>11</td>
<td>19</td>
<td>11</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

Tissue weight, age, and baseline O₂ consumption from 5 groups are shown in the absence or presence of NOS inhibitors. NLA or L-NAME: control C57BL/6x129, iNOS (−/−), eNOS (+/−), eNOS (+/−), and eNOS (−/−) mice. Baseline O₂ consumption was not statistically different between groups and neither treatment with NLA nor L-NAME (both at 10⁻⁴ mol/L) affected baseline O₂ consumption.

Materials and Methods

Animals Studied

iNOS-deficient mice were bred by pairing homozygous mutant iNOS (−/−) mice originally developed by MacMicking et al. C57BL/6x129 mice obtained from the Jackson Laboratories were used as wild-type controls. Heterozygous eNOS (+/−) mice, originally developed by Shesely et al., 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, 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 (mmol/L) 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) to equilibrate for at least 2 hours. 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 (10⁻⁵ to 10⁻⁴ mol/L) of bradykinin (Sigma) or S-nitroso-N-acetyl-L-arginine (SNAP, Sigma) on O₂ uptake were studied in the absence or presence of NOS inhibitors (10⁻⁴ mol/L) N-nitro-L-arginine (NLA) 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. 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 and was expressed as nanomoles of O₂ consumed per minute per gram of tissue.

Figure 1. Effect of cumulative doses of bradykinin on O₂ consumption in the absence or presence of NOS inhibition with NLA (10⁻⁴ mol/L) in myocardial tissue slices from normal C57BL/6x129 and iNOS-deficient mice. A, Bradykinin caused dose-dependent decreases in O₂ consumption in normal mice (filled triangle, n=15), and this effect was significantly attenuated by NLA (open triangle, n=6). *P<0.05 vs normal C57BL/6x129 mice in the absence of NLA. B, Similarly, bradykinin caused dose-dependent decreases in O₂ consumption in iNOS-deficient mice (filled square, n=15), an effect that was also inhibited by NLA (open square, n=6). *P<0.05 vs iNOS (−/−)-deficient mice in the absence of NLA, 2-way ANOVA, followed by Student-Newman-Keuls.
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⁻⁳ 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 (10⁻³ 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/6j 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 (+/-) in the absence of L-NAME; **P<0.05 compared with either 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 [ie, 10⁻⁴ mol/L, eNOS (+/+)--; -27±10% versus eNOS (+/-): -30±6% versus eNOS (-/-): -31±6%; P=NS, 1-way ANOVA].

**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
of bradykinin to reduce tissue O$_2$ consumption was abolished in the myocardial tissues taken from the hearts of mice lacking functional eNOS, but such responses were still present in tissues from the hearts of iNOS-deficient mice. Furthermore, induction of NO production by endotoxin significantly reduced basal myocardial tissue O$_2$ consumption from normal but not 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 O$_2$ consumption in normal or genetically altered mice. This observation is consistent with our previous reports of isolated tissue O$_2$ consumption, suggesting that in the absence of shear stress or a chemical stimulus, the influence of basal release of NO on tissue O$_2$ consumption is minimal. When baseline myocardial O$_2$ consumption was analyzed across the different strains of mice, we found that baseline O$_2$ consumption in tissue from the iNOS (-/-) mice is significantly lower compared with O$_2$ 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 al$^{19}$ in pial arterioles. This should have resulted in enhanced NO production and lowered baseline O$_2$ consumption; however, NOS inhibition with NLA in our study had no effect on baseline O$_2$ 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 B$_2$-kinin receptor-mediated mechanism or exogenous NO inhibits myocardial O$_2$ consumption. The B$_2$-kinin receptor-mediated mechanism is supported by our more recent data showing that the inhibitory effect of bradykinin on O$_2$ consumption was abolished in hearts from mice lacking bradykinin B$_2$ receptors.$^{20}$

When the involvement of specific NOS isoforms in the regulation of myocardial O$_2$ 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 O$_2$ 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 kidney$^{21}$ and even in the heart,$^{22}$ iNOS is not likely to be the mediator of cardiac tissue respiration, because bradykinin suppressed O$_2$ consumption in tissues lacking iNOS. Furthermore, bradykinin B$_2$ receptor activation leads to the elevation of [Ca$^{2+}$], 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 O$_2$ 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 O$_2$ consumption and perhaps play an important pathological role, endotoxin was administered to C57BL/6x129 mice and to iNOS (-/-) mice, and cardiac tissue O$_2$ consumption was measured. Endotoxin treatment significantly reduced basal myocardial tissue O$_2$ consumption from normal but not iNOS (-/-)-deficient mice, suggesting that enhanced NO formation by iNOS suppresses tissue O$_2$ consumption. Thus, iNOS may be only partly responsible for the sepsis-induced hypotension or myocardial dysfunction through the alteration in tissue respiration, because it has recently been demonstrated that mice deficient in the iNOS enzyme are still susceptible to LPS-induced mortality.$^{23}$

We noted that the inhibition of myocardial respiration was preserved after deletion of a single copy of the eNOS gene. Our findings are congruent with those of Shesely et al$^{16}$ who showed that heterozygous eNOS (+/-) mice have blood pressures similar to wild-type eNOS (+/+)- mice; both groups are in contrast to eNOS (-/-)-deficient mice that...
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 myocytes25,26 (<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 data10,13,14 suggest that the effect of NO on mitochondria is cGMP independent. Furthermore, Kani et al37 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 O2 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 O2 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 O2 electrode to measure O2 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 O2 consumption under in vivo conditions. Nevertheless, in vitro examination of tissue O2 consumption eliminates neurohormonal and mechanical factors that may influence cardiac O2 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 O2 consumption or O2 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 O2 consumption at increasing cardiac work loads due to exercise.11 Third, the use of normal C57BL/6x129 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 O2 consumption was made within the same strain. More importantly, the effect of bradykinin-induced reduction in O2 consumption was similar in magnitude in the C57BL/6x129 and iNOS (−/−) mouse hearts (eg, bradykinin decreased O2 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 O2 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 O2 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 O2 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,14 in which NO production by the vasculature is altered, these alterations may contribute to the disease process.

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References

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


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