Endothelium-Derived Nitric Oxide Regulates Postischemic Myocardial Oxygenation and Oxygen Consumption by Modulation of Mitochondrial Electron Transport

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Background—Nitric oxide (NO) production is increased in postischemic myocardium, and NO can control mitochondrial oxygen consumption in vitro. Therefore, we investigated the role of endothelial NO synthase (eNOS)—derived NO on in vivo regulation of oxygen consumption in the postischemic heart.

Methods and Results—Mice were subjected to 30 minutes of coronary ligation followed by 60 minutes of reperfusion. Myocardial oxygen tension (PO2) was monitored by electron paramagnetic resonance oximetry. In wild-type, N-nitro-L-arginine methyl ester (L-NAME)–treated (with 1 mg/mL in drinking water), and eNOS knockout (eNOS−/−) mice, no difference was observed among baseline myocardial PO2 values (8.6±0.7, 10.0±1.2, and 10.1±1.2 mm Hg, respectively) or those measured at 30 minutes of ischemia (1.4±0.6, 2.3±0.9, and 3.1±1.4 mm Hg, respectively). After reperfusion, myocardial PO2 increased markedly (P<0.001 versus baseline in each group) but was much lower in L-NAME–treated and eNOS−/− mice (17.4±1.6 and 20.4±1.9 mm Hg) than in wild-type mice (46.5±1.7 mm Hg; P<0.001). A transient peak of myocardial PO2 was observed at early reperfusion in wild-type mice. No reactive hyperemia was observed during early reperfusion. Endothelial NO decreased the rate-pressure product (P<0.05), upregulated cytochrome c oxidase (CcO) mRNA expression (P<0.01) with no change in CcO activity, and inhibited NADH dehydrogenase (NADH-DH) activity (P<0.01) without alteration of NADH-DH mRNA expression. Peroxynitrite-mediated tyrosine nitration was higher in hearts from wild-type mice than in eNOS−/− or L-NAME–treated hearts.

Conclusions—eNOS-derived NO markedly suppresses in vivo O2 consumption in the postischemic heart through modulation of mitochondrial respiration based on alterations in enzyme activity and mRNA expression of NADH-DH and CcO. The marked myocardial hyperoxygenation in reperfused myocardium may be a critical factor that triggers postischemic remodeling. (Circulation. 2005;111:2966-2972.)

Key Words: nitric oxide • enzymes • free radicals • reperfusion • ischemia

Nitric oxide (NO) produced by NO synthase (NOS) is an important regulator of vascular and cardiac function. NO released from the vascular endothelium by endothelial NOS (eNOS) is the major source of NO in the myocardium under physiological conditions.1 It has also been demonstrated that eNOS is present in cardiac myocytes.2 NO has been reported to regulate myocardial oxygen consumption.3,4 Endothelial dysfunction has been demonstrated after ischemia/reperfusion in the coronary circulation, which has been attributed to a decrease in eNOS-derived NO.5–7 However, increased NO formation during ischemia or the early period of reperfusion has also been shown to occur in hearts subjected to global ischemia by our laboratory and others.8–12 eNOS-derived NO has been reported to regulate oxygen consumption in vitro,13,14 but controversy remains about the role of NO in regulating oxygen consumption in vivo under normal physiological conditions.15,16 Mitochondrial dysfunction is a characteristic of myocardial ischemia/reperfusion injury.17 Inhibition of mitochondrial respiration could be induced by either NO or its derivatives. At low levels, NO competes with O2 and reversibly inhibits the respiratory chain at the site of cytochrome c oxidase (CcO). With high-level production of NO along with superoxide, as occurs during postischemic reperfusion, the potent oxidant peroxynitrite is formed; this can induce inhibition of many mitochondrial components, especially complex I and complex II, and appears to be irreversible.1,3 It has been demonstrated that peroxynitrite is produced during reperfu-

Received December 2, 2004; revision received January 14, 2005; accepted February 17, 2005.

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Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.104.527226

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sion of ischemic hearts in vitro; however, questions remain about the role of NO or peroxynitrite in the regulation of myocardial oxygenation during myocardial ischemia/reperfusion in vivo and the mechanisms involved.

Therefore, a series of studies were performed in wild-type and eNOS knockout (eNOS$^{-/-}$) mice with in vivo electron paramagnetic resonance (EPR) oximetry that enables real-time monitoring of myocardial PO$_2$. We observed that eNOS-derived NO has a major role in the in vivo regulation of myocardial O$_2$ levels and oxygen consumption in the postischemic heart. The mechanism of this regulation was determined to be through modulation of mitochondrial NADH dehydrogenase (NADH-DH) and CcO function.

**Methods**

**Animals**

Male wild-type C57BL/6 and eNOS$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Me). Wild-type mice were randomly selected to be administered 1 mg/mL N-nitro-L-arginine methyl ester (L-NAME; Sigma) in drinking water for 3 days before experiment (L-NAME–treated group). All procedures were performed with the approval of the Institutional Animal Care and Use Committee at The Ohio State University, Columbus, Ohio, and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

**In Vivo Myocardial Ischemia/Reperfusion Model**

The in vivo ischemia/reperfusion model was performed by a technique similar to that described previously.18 Mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Atropine (0.05 mg SC) was administered to reduce airway excitation. Animals were intubated and ventilated with room air (tidal volume 250 µL, 120 breaths/min) with a mouse respirator (Harvard Apparatus). A left intercostal thoracotomy was performed. The left anterior descending coronary artery was ligated with a 7-0 silk suture. After 30 minutes of ischemia, the occlusion was released, and reperfusion was confirmed visually. Sham-operated mice underwent the same surgery minus the coronary artery ligation. Rectal temperatures of the mice were maintained at 37°C by a thermo heating pad. In all 3 groups of mice, similar basal heart rates were observed, with values of 300 to 400 bpm, typical for anesthetized mice.

**EPR Oximetry**

Lithium octa-N-butoxy-naphthalocyanine (LiNc-BuO) was used as a probe for EPR oximetry. The O$_2$ response of LiNc-BuO showed good linearity from 0 to 760 mm Hg with a sensitivity of 8.5 mg/mm Hg.$^{19}$ After thoracotomy, ~10 µg of LiNc-BuO crystals loaded in a 25-gauge needle was implanted into the myocardium of the area at risk. After 10 minutes, the mouse was placed into the EPR system with its heart close to the loop resonator. EPR spectra of LiNc-BuO crystals were obtained with the use of a custom-made L-band spectrometer with frequency 1.1 GHz, microwave power 16 mW, modulation field 0.07 G, and scan width 5 G.$^{20,21}$ The implanted crystals in mice were confirmed by histology to be located at the midmyocardium.

**Measurement of Myocardial Tissue Blood Flow**

After thoracotomy, the fourth rib was removed. An optic suction probe (P10d, Moor Instruments) connected to a laser Doppler perfusion monitor (Moor Instruments) was placed on the area at risk. Before and during coronary occlusion and reperfusion, regional myocardial blood flow was monitored continuously and is presented as a percentage of the baseline before ischemia.

**Measurements of Mean Arterial Pressure, Heart Rate, and Rate-Pressure Product**

The right carotid artery was cannulated with a 1.4F Millar tip transducer catheter (model SPR-261) connected to PowerLab system for continuous monitoring of mean arterial pressure and heart rate. Rate-pressure product was calculated by the equation rate-pressure product (mm Hg/min) = mean arterial pressure × heart rate.

**Activities of CcO and NADH-DH**

Frozen myocardial tissue, obtained from the risk region, was homogenized in ice-cold HEPES buffer (3 mmol/L, pH 7.2) containing sucrose (0.25 mol/L), EGTA (0.5 mmol/L), and protease-inhibitor cocktail (1:40, Sigma). CcO activity was measured in the presence of phosphate buffer (50 mmol/L, pH 7.4) and reduced cytochrome c (60 µmol/L, Sigma).$^{22}$ NADH-DH activity was measured in the presence of Tris-HCl buffer (20 mmol/L, pH 8.0), NADH (150 µmol/L, Sigma), and coenzyme Q$_1$ (100 µmol/L, Sigma).$^{23}$ The extinction coefficients, $\epsilon$ 550 nm = 18.5 mmol/L$^{-1}$ . cm$^{-1}$ for cytochrome c and $\epsilon$ 340 nm = 6.22 mmol/L$^{-1}$ . cm$^{-1}$ for NADH, were used for activity calculation. Protein concentration of the tissue homogenate was measured by BCA assay (PIERCE Biotechnology).

**Assay of CcO mRNA and NADH-DH mRNA by Real-Time Polymerase Chain Reaction**

Total RNA was extracted with TRIzol reagent (Gibco RBL) from the frozen myocardial tissue. Total RNA (1 µg) was reverse transcribed into first-strand cDNA with random hexamers (Life Technologies). The primer sequences (sense/antisense) selected from the Genbank mRNA sequence of mouse by use of Primer Express software (Applied Biosystems) were the subunit Vb of CcO (CcO Vb): 5'-CCCTAACAGAAGAGACGC-3'/5'-CTGCACCCAGTGGG-G3'; NADH-DH 1α: 5'-CACGTTGAACAAACAGGGC-3'/5'-CATAACTGTTGCAGCTGCT-3'; and GAPDH: 5'-ATGCACACA-GTCCATGCATAC-3'/5'-TGTTGAAAGTCGAGGACACAC-ACCT-3'. Real-time polymerase chain reaction (PCR) was performed with the use of SYBR green PCR master mix (Applied Biosystems). Relative quantification of different genes was normalized with GAPDH as an endogenous control. The specificity of the amplification product was confirmed by regular PCR.$^{24}$

**Immunohistochemistry for Nitrotyrosine**

Mouse heart injected with peroxynitrite (1 mmol/L, 0.05 mL; Cayman Chemical Co) into the left ventricle was taken as the positive control. Immunohistochemistry was performed as described previously by our laboratory.$^8$ Briefly, the formalin-fixed paraffin-embedded tissue sections were incubated with rabbit polyclonal anti-nitrotyrosine antibody (Upstate, 1:400), then with the biotinylated secondary, and finally with the tertiary, ExtrAvidin alkaline phosphatase (1:800). Immunohistochemical staining was performed as a positive control.

**Statistical Analysis**

Two-way ANOVA was used for data analysis of PO$_2$, blood flow, heart rate, mean arterial pressure, and rate-pressure product and 1-way ANOVA for that of enzyme activity and PCR; these were followed by Newman-Keuls multiple-comparison test among the groups. Data were represented as mean±SEM. A value of $P<0.05$ was considered significant.

**Results**

**Myocardial Tissue PO$_2$**

In wild-type, L-NAME–treated, and eNOS$^{-/-}$ mice, myocardial tissue PO$_2$ dropped from baseline values of 8.6±0.7, 10.1±1.2, and 10.0±1.2 to 1.4±0.6, 2.3±0.9, and 3.1±1.4 mm Hg, respectively ($P<0.001$ within each group) at 30 minutes of coronary ligation (Figure 1). Reperfusion resulted in a marked hyperoxygenation state during reperfu-
sion \( (P < 0.001 \text{ versus baseline within each group}) \); however, the hyperoxygenation level was significantly lower in L-NAME–treated \( (17.4 \pm 1.6 \text{ mm Hg}) \) and eNOS\(^{-/-}\) \( (20.4 \pm 1.9 \text{ mm Hg}; P < 0.001) \) mice than in wild-type mice \( (46.5 \pm 1.7 \text{ mm Hg}) \). A transient peak of myocardial \( PO_2 \) \( (46.5 \pm 1.7 \text{ mm Hg}) \) was observed at 12.5 minutes of reperfusion, followed by a constant high level in wild-type mice but not in L-NAME–treated or eNOS\(^{-/-}\) mice.

Myocardial Tissue Blood Flow
To identify whether the myocardial hyperoxygenation during reperfusion was due to reactive hyperemia, myocardial tissue blood flow was measured in mice. Myocardial tissue blood flow was reduced to 14.2\( \pm 1.2\% \), 12.8\( \pm 0.9\% \), and 14.2\( \pm 1.8\% \) in wild-type, L-NAME–treated, and eNOS\(^{-/-}\) mice, respectively, at the end of 30 minutes of coronary ligation compared with preischemic baseline values (Figure 2). Flow was restored to 92.8\( \pm 2.8\% \), 81.8\( \pm 6.2\% \), and 86.0\( \pm 3.8\% \) of baseline levels during the first 3 minutes of reperfusion in wild-type, L-NAME–treated, and eNOS\(^{-/-}\) mice, respectively. No difference in myocardial blood flow was observed among the 3 groups, which suggests that the marked increase in \( PO_2 \) during the initial period of reperfusion was not due to hyperemia.

Mean Arterial Pressure, Heart Rate, and Rate-Pressure Product
At baseline and during reperfusion, mean arterial pressure was significantly higher, with no difference in heart rate, in L-NAME–treated and eNOS\(^{-/-}\) compared with wild-type mice \( (P < 0.05; \text{ Figure 3}) \). Rate-pressure product was significantly higher at baseline and during reperfusion in L-NAME–treated and eNOS\(^{-/-}\) mice than in wild-type mice \( (P < 0.05) \).

Activities of Myocardial CcO and NADH-DH
Activities of myocardial CcO and NADH-DH were measured to investigate the mechanism by which NO regulates \( O_2 \) consumption. The sham group was taken as the baseline control. At 60 minutes of reperfusion, no significant difference was found in CcO activity among the groups (Figure 4); however, NADH-DH activity was much lower in wild-type hearts than in sham, eNOS\(^{-/-}\), and L-NAME–treated hearts \( (P < 0.01) \). No significant difference was found in L-NAME–treated and eNOS\(^{-/-}\) hearts compared with the sham group.

mRNA Expressions of CcO and NADH-DH
To explore the regulation mechanisms of myocardial \( O_2 \) consumption at the gene level, mRNA expressions of CcO and NADH-DH were detected by real-time PCR. The sham group was taken as the baseline control. At 60 minutes of reperfusion, significant upregulation of CcO mRNA expression was detected in wild-type mice \( (P < 0.01) \) but not in L-NAME–treated or eNOS\(^{-/-}\) mice \( (P > 0.05) \). The extent of CcO mRNA upregulation was significantly lower in L-NAME–treated and eNOS\(^{-/-}\)
mice than in wild-type mice ($P<0.05$; Figure 5A). No marked difference for NADH-DH mRNA expression was found among the groups ($P>0.05$; Figure 5B).

**Immunohistochemical Staining of Nitrotyrosine**

Immunohistochemical staining of nitrotyrosine is a marker of peroxynitrite formation. Nitrotyrosine staining was seen throughout the vascular beds in the positive control (Figure 6A). No staining was detected in the negative control section (Figure 6B). Much stronger nitrotyrosine staining was seen in wild-type hearts (Figure 6C), but only weak staining was observed in eNOS$^{-/-}$ (Figure 6D) and L-NAME–treated (Figure 6E) hearts.

**Discussion**

In this study, we observed that marked myocardial hyperoxgenation occurs on reperfusion and is inhibited to a similar extent with lack of eNOS or NOS inhibition. This hyperoxgenation is accompanied by a lower rate-pressure product and is not due to reactive hyperemia. After 60 minutes of reperfusion, eNOS-derived NO upregulated CcO mRNA expression without a change in CcO activity and inhibited NADH-DH activity without alteration of NADH-DH mRNA expression. Peroxynitrite was produced more in hearts from wild-type mice than from eNOS$^{-/-}$ and L-NAME–treated mice. Thus, in vivo, eNOS-derived NO markedly suppressed oxygen consumption in postischemic myocardium through CcO and NADH-DH regulation of mitochondrial respiration during the early period of reperfusion.

EPR oximetry with the recently developed LiNc-BuO probe provides a sensitive technique for repetitive measurement of $P_{O_2}$ in capillaries, tissues, and cells. This study is the first to measure in vivo myocardial $P_{O_2}$ using this technique. To detect myocardial $P_{O_2}$ directly, EPR oximetry has advantages over microelectrode oximetry. The implanted probe can move freely with the beating heart, and myocardial $P_{O_2}$ can be monitored repetitively after the chest is closed. The baseline of $P_{O_2}$ in the myocardium was 8.6 to 10.1 mm Hg before coronary ligation, which is consistent with prior studies. 

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The nonzero myocardial Po2 during coronary ligation suggests the existence of collateral circulation, as also shown by the nonzero myocardial blood flow.

Coronary microvessels can regulate oxygen consumption through an NO-dependent mechanism.\textsuperscript{27} eNOS-derived NO plays an important role in the regulation of oxygen consumption in isolated cardiac tissue.\textsuperscript{14} The present study showed that baseline myocardial Po2 was not altered by L-NAME treatment or lack of eNOS, which suggests a minimal influence of basal NO release on oxygen consumption. This observation is consistent with the reports by Loke et al\textsuperscript{13} and Kojic et al.\textsuperscript{16} However, as would be expected with a lack of NOS-derived NO generation in eNOS\textsuperscript{+/−} or L-NAME–treated mice, systemic blood pressure is increased along with the rate-pressure product. This likely suggests that there is an increase in ATP generation for a given amount of O2 consumption in eNOS\textsuperscript{+/−} or L-NAME–treated hearts. This could occur either if NOS-derived NO decreases the efficiency of ATP generation for a given amount of O2 consumption or if NOS-derived NO serves to decrease ATP from nonmitochondrial pathways, such as glycolysis. Indeed, it has been reported that NO or NO-derived peroxynitrite can decrease myocardial efficiency and inhibit glycolysis;\textsuperscript{28,29} however, the present study is the first to provide in vivo evidence that basal eNOS-derived NO regulates myocardial efficiency.

Using a voltametric microelectrode technique, Al-Obaidi et al\textsuperscript{26} reported an increase in myocardial Po2 after reperfusion. An important observation in the present study was that myocardial hyperoxygenation induced by reperfusion was attenuated to a similar extent in L-NAME–treated and eNOS\textsuperscript{+/−} mice. Thus, in vivo, eNOS-derived NO markedly suppressed oxygen consumption in postischemic myocardium.

It has been reported that inhibition of NOS results in very little, if any, change in coronary blood flow.\textsuperscript{30} The present study showed that no reactive hyperemia was observed after reperfusion, and no difference in myocardial blood flow was found among wild-type, L-NAME–treated, and eNOS\textsuperscript{+/−} mice. Therefore, the hyperoxygenation in postischemic myocardium of wild-type mice results from the NO-mediated suppression of oxygen consumption.

In L-NAME–treated and eNOS\textsuperscript{+/−} mice, basal mean arterial pressures were ∼30% higher than in control wild-type mice, and rate-pressure product values were also higher. No significant differences in intras ischemic pressures or rate-pressure products were observed; however, higher values were seen during the first hour of reperfusion in L-NAME–treated and eNOS\textsuperscript{+/−} mice. Thus, the lack of eNOS-derived NO conferred a higher functional recovery.

The role of NO in myocardial ischemia/reperfusion has been controversial.\textsuperscript{9,31–35} Although early indirect studies reported a lack of NO production in the postischemic heart, subsequent direct measurements demonstrated that NO production is markedly increased during ischemia and the early minutes of reperfusion.\textsuperscript{6,8,9,12,31} The present study supports the concept that eNOS-derived NO in postischemic myocardium could increase to a pathological level for some time and induce a significant inhibition of oxygen consumption. The sharp peak of myocardial Po2 during the early minutes of reperfusion strongly suggests a transient reversible inhibition of oxygen consumption by eNOS-derived NO in wild-type mice. As recently reported, relative changes in myocardial oxygenation can trigger marked alterations in cellular phenotype and influence the process of ventricular remodeling.\textsuperscript{36}

The burst of NO generation could react with superoxide, generating reactive nitrogen species including peroxynitrite, and cause a nonselective irreversible inhibition of many mitochondrial components.\textsuperscript{3} Many of the toxic actions of NO are not directly due to NO itself but are mediated via production of peroxynitrite.\textsuperscript{37} Both high flux of NO and peroxynitrite formation under hypoxic conditions have been reported to promote cell death via mitochondrial damage and mitochondrial-independent mechanisms.\textsuperscript{38} Peroxynitrite is produced during acute reperfusion after global ischemia.\textsuperscript{8,39} The present study demonstrated that nitrotyrosine staining is much stronger in myocardium of wild-type mice than in that
of eNOS$^{-/-}$ and L-NAME–treated mice. Increased peroxynitrite could lead to inhibition of oxygen consumption in wild-type mice compared with eNOS$^{-/-}$ and L-NAME–treated mice. The similar degree of nitrotyrosine staining between eNOS$^{-/-}$ and L-NAME–treated hearts suggests that eNOS–derived NO is responsible for the generation of peroxynitrite during the early period of reperfusion.

CcO VIb is encoded on the nuclear DNA and imported into mitochondria after their synthesis in the cytosol. Expression of CcO VI–encoding gene is dependent on $O_2$ and heme availability. NO itself exerts an acute, potent, and reversible inhibition on CcO in competition with $O_2$. The present study demonstrated decreased upregulation of CcO VIb mRNA expression without change of CcO activity in postischemic myocardium of L-NAME–treated and eNOS$^{-/-}$ mice. These data are consistent with a prior study in mouse macrophages in which NO upregulated CcO I mRNA expression and protein levels without alteration in CcO activity. In postischemic myocardium, upregulated CcO mRNA expression leading to CcO synthesis may compensate for NO-mediated inhibition of CcO to maintain a constant capacity of CcO for oxidative phosphorylation.

It was reported that mitochondrial NADH-DH can be inhibited irreversibly by peroxynitrite. The activity of NADH-DH was decreased by global ischemia and further decreased by reperfusion in isolated rat hearts. Decreased mRNA of NADH-DH in HeLa cells due to hypoxia recovered with reexposure to normoxia. The present study demonstrated that NADH-DH activity was decreased without change of its mRNA expression in postischemic myocardium, but the decreased NADH-DH activity was prevented with L-NAME treatment or deficiency of eNOS. Therefore, eNOS-derived NO contributed to the inhibition of NADH-DH activity, but no compensatory mechanism occurred for NADH-DH at the mRNA level, as occurred for CcO. The attenuated NADH-DH activity paralleled the much stronger nitrotyrosine staining in wild-type mice, consistent with a role of peroxynitrite in regulation of mitochondrial respiration in postischemic myocardium.

In conclusion, coronary endothelial dysfunction was reported in postischemic myocardium, but increased in vivo NO production occurs during the early period of reperfusion. eNOS-derived NO with its derivative, peroxynitrite, suppresses oxygen consumption through CcO and NADH-DH regulation of mitochondrial respiration, which results in marked myocardial hyperoxygenation after reperfusion. This myocardial hyperoxygenation may be a critical factor influencing postischemic remodeling.

Acknowledgments
This work was supported by NIH grants HL63744, HL65608, and HL38324, as well as American Heart Association grant 0435299N (Dr He). We thank Drs Aiwen Zhang, and Yong Xia for assistance with real-time PCR. Dr Zhao is from Changzheng Hospital, Second Military Medical University, Shanghai, China.

References

Figure 6. Representative immunostaining of nitrotyrosine in mice subjected to 30 minutes of coronary ligation followed by 60 minutes of reperfusion ($\times$200). A, Positive control; strong nitrotyrosine staining in vascular beds is present. B, Negative control. C, Strong staining in wild-type heart. D, Weak staining in eNOS$^{-/-}$ heart. E, Weak staining in L-NAME–treated heart.


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Circulation. 2005;111:2966-2972
doi: 10.1161/CIRCULATIONAHA.104.527226
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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