Attenuation of Myocardial Ischemia/Reperfusion Injury by Superinduction of Inducible Nitric Oxide Synthase

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Background—Nitric oxide (NO) has been implicated as a mediator in myocardial ischemia/reperfusion (I/R) injury, but its functional properties have been conflicting. We investigated whether NO has a protective role against I/R injury.

Methods and Results—Using endothelial NO synthase knockout (eNOS KO) mice, inducible NOS KO mice, the NO donor S-nitroso-N-acetylpenicillamine (SNAP), and the NOS inhibitor N-iminoethyl-L-ornithine (L-NIO), we performed studies of isolated perfused hearts subjected to 30 minutes of global ischemia followed by reperfusion. After 60 minutes of reperfusion, nitrite levels in the coronary effluent in the SNAP and eNOS KO groups were significantly elevated compared with other groups. Immunoblot and immunohistochemistry showed that iNOS was markedly induced in the eNOS KO hearts. Under spontaneous beating conditions during reperfusion, increased NO activity was correlated with a prevention of the hyperdynamic contractile response and enhanced myocardial protection, as evidenced by a reduction in myocardial injury and infarct size. During prolonged reperfusion, SNAP-treated hearts were able to preserve contractile functions for 180 minutes, whereas L-NIO–treated hearts showed a sustained deterioration in contractility.

Conclusions—NO protects against I/R injury by preventing the hyperdynamic response of isolated perfused hearts during early reperfusion. In the eNOS KO hearts, a paradoxical increase in NO production was seen, accompanied by a superinduction of iNOS, possibly due to an adaptive mechanism. (Circulation. 2000;101:2742-2748.)

Key Words: nitric oxide • nitric oxide synthase • ischemia • reperfusion • hemodynamics

Nitric oxide (NO) can be produced by essentially all cell types in the heart and is known to have profound effects on cardiac function. NO can be generated by NO synthases (NOS), which catalyze the conversion of L-arginine to NO and L-citrulline. All 3 known NOS isoforms can be expressed in the heart, including neuronal NOS (nNOS, NOS 1), endothelial NOS (eNOS, NOS 3), and inducible NOS (iNOS, NOS 2). nNOS and eNOS are mostly calcium/calmodulin-dependent and usually express constitutively, whereas iNOS is independent of intracellular calcium concentration and typically is expressed only when cells are stimulated by microbial or immunological stimuli. Under physiological conditions in the heart, NO maintains coronary vasodilatory tone, inhibits platelet aggregation, and inhibits the adhesion of neutrophils to vascular endothelium. Moreover, NO has negative inotropic effects on cardiomyocytes.

Ischemia/reperfusion (I/R) injury to the heart occurs after myocardial infarction, shock, and transplantation, and the role of NO in myocardial damage and dysfunction remains controversial. Several investigations have reported that the administration of NO donors prevents I/R injury. Approaches to remove NO by pharmacological inhibition of NOS and transgenic iNOS or eNOS knockout (KO) mice have also been shown to exacerbate I/R injury in the heart. In contrast to these findings, other studies using pharmacological inhibition of NOS and eNOS KO mice showed protective effects against I/R injury in the heart. Comparisons between these studies are difficult because of differences in agents and study design. Furthermore, the approaches using KO animals have assumed a reduction in NO synthesis.

Experiments carried out here used the isolated perfused Langendorff preparation to permit the evaluation of the effect of short-term I/R on myocardial dysfunction and damage. This approach permitted the assessment of NO synthesis in isolation of circulating cellular sources. Furthermore, use of hearts from eNOS and iNOS KO mice permitted an evaluation of the consequence of the absence of these specific NOS isoforms. We report here that NO protects against I/R injury by preventing the hyperdynamic response in our model during early reperfusion and that eNOS deficiency results in a rapid superinduction of iNOS, leading to myocardial protection.

Methods

Mice
All mice used in the experiments were male, 2 to 3 months of age, weighing 24 to 26 g each. Animals were housed individually and...
provided food and water ad libitum. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” and the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratory (Wilmington, Mass). Mice homozygous (−/−) for KO of the eNOS gene (parental background strains of 129 and C57BL/6) were a gift from Edward G. Shesely (University of North Carolina, Chapel Hill). eNOS KO mice had been backcrossed onto the C57BL/6 background 6 times. Mice with targeted KO (−/−) of the iNOS gene were prepared as described and were a gift from Drs Carl Nathan and John Mudgett (Merck Research, Rahway, NJ). iNOS KO mice had been backcrossed onto the C57BL/6 background through 4 generations.

Isolated Mouse Heart Preparation
Mice were anesthetized with an injection of sodium pentobarbital (50 mg/kg IP), and heparin (500 U/kg) was administered at the same time. The heart was excised immediately after thoracotomy and placed into cold perfusion buffer. The aorta was cannulated, and the heart was perfused in a nonrecirculating Langendorff mode at constant coronary pressure of 70 mm Hg with Krebs-Henseleit bicarbonate buffer (KHB) containing (in mmol/L) NaCl 118, KCl 4.6, MgSO4 1.2, NaHCO3 24.9, CaCl2 2.5, KH2PO4 1.2, glucose 10, and EDTA 0.5, equilibrated with 95% O2, 5% CO2 (pH 7.4, 37°C). A small incision was made at the trunk of the pulmonary artery to drain coronary effluent. The effluent was collected before ischemia and during reperfusion and stored at −20°C for subsequent measurement of creatine kinase (CK) and nitrite.

Measurement of Isovolumic Contractile Performance
A water-filled latex balloon (Hugo Sachs) was inserted through the mitral valve into the left ventricle. The balloon was connected to a pressure transducer for continuous recording of left ventricular performance. The balloon was inflated to set left ventricular end-diastolic pressure (LVEDP) at 8 mm Hg for all hearts, and the balloon volume was then held constant. Contractile performance data were collected with a commercially available data acquisition system (MacLab ADInstruments).

Nitrite Measurement
After release, NO reacts with O2 to form the stable metabolite nitrite. Nitrite concentrations were measured by the Griess reaction to estimate the total amounts of NO production as previously described.

Immunohistochemistry
After 60 minutes of reperfusion, some hearts were fixed in 4% paraformaldehyde and embedded in paraffin, and 5-μm sections were cut. Polyclonal anti-iNOS antibody (Calbiochem) and monoclonal anti-eNOS antibody (Transduction Laboratories) were used as a primary antibody. Immunohistochemistry was carried out with the ABC staining system (Santa Cruz Biotechnology, Inc) according to the manufacturer’s protocol.

Immunoblot Analysis
Some hearts were rapidly frozen in liquid nitrogen and stored at −80°C. Protein extracts were prepared according to the method of Gödecke et al. Protein (100 μg) was separated on 10% SDS-polyacrylamide gels and electrophoressed to a nitrocellulose membrane. iNOS and eNOS were detected by monoclonal antibodies (Transduction Laboratories). Protein bands were visualized by use of the SuperSignal chemiluminescence detection system (Pierce).

Measurement of Myocardial Injury
To estimate myocardial injury, 2 parameters of myocardial damage, CK release and infarct size, were measured. CK release was estimated in the effluent collected from the hearts before ischemia and during reperfusion. Collected effluent was assayed by the Central Laboratory in the University of Pittsburgh Medical Center. The results were expressed as total CK release from the heart after the indicated time periods. Measurement of infarct size was performed according to Yoshida et al.

Experimental Protocols
Protocol A: 60-Minute Reperfusion Experiment
Eighteen WT mice, 12 eNOS KO mice, and 6 iNOS KO mice were used. After stabilization, preischemic baseline contractile function was measured. Thereafter, global ischemia under normothermia was induced for 30 minutes by clamping of the aortic cannula, followed by 60 minutes of reperfusion. WT mice were randomized into 3 groups, and eNOS KO mice into 2 groups. The groups received bolus injections (2 mL) of vehicle (KHB; n = 6 in each of the WT, eNOS KO, or iNOS KO mice), S-nitroso-N-acetylpenicillamine (SNAP, 1 mmol/L; n = 6 in WT mice), N-iminoethyl-L-ornithine (L-NIO, 1 mmol/L; n = 6 in the WT or eNOS KO mice) into the side branch of the aortic cannula just after the aortic clamping.

Figure 1. Nitrite level in coronary effluent during reperfusion.
Coronary effluent was collected at various times before and during reperfusion. Nitrite was measured to estimate total amount of NO production. Each nitrite assay was determined in duplicate. Detection limit in this assay is 0.08 μmol/L. In WT/L-NIO, WT, and eNOS KO/L-NIO groups, nitrite was not detected throughout reperfusion, and it was detected only at 1, 5, and 10 minutes after reperfusion in iNOS KO group. Results are expressed as mean ± SEM for n = 6 in each group.

Figure 2. NOS induction after postischemic reperfusion in iNOS and eNOS KO hearts. Immunoblot of iNOS (top) and eNOS (bottom). Whole hearts were homogenized, and extracted protein was electrophoresed in 10% SDS-polyacrylamide gels and subjected to immunoblot analysis using mouse monoclonal anti-iNOS or anti-eNOS antibody. C indicates control hearts; IR, hearts subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion. Blot is representative of experiment performed in 2 hearts per group.
Protocol B: 3-Hour Reperfusion Experiment

Eighteen WT hearts were prepared according to protocol A. When subjected to normothermic ischemia, the hearts were separated into 3 groups (n=6 in each), and vehicle, L-NIO (1 mmol/L), or SNAP (1 mmol/L) was administered, respectively. After 60 minutes of reperfusion, the constant-coronary-pressure Langendorff mode was switched to the constant-coronary-flow mode with the set volume at that time. There was no significant difference in coronary flow among the groups. Hemodynamic parameters were recorded as described above. In a separate control group, the hearts of WT mice (n=6) were perfused continuously with KHB for 210 minutes after stabilization to serve as a time-matched control.

Figure 3. Immunohistochemistry with monoclonal anti-iNOS and anti-eNOS antibody for hearts subjected to I/R. After 60 minutes of reperfusion, hearts were fixed with 4% paraformaldehyde and prepared for 5-μm sections. Photomicrographs were taken at magnification of ×200. Anti-iNOS staining is shown in WT before ischemia (A) and hearts after I/R of eNOS KO (B), WT (C), and iNOS KO (D). Anti-eNOS staining is shown in WT before ischemia (E) and hearts after I/R of eNOS KO (F), WT (G), and iNOS KO (H). Figures are representative of 3 separate sets of experiments.

Statistical Analysis

The results are presented as mean±SEM. Data were analyzed by ANOVA with StatView software (SAS institute Inc). A value of P<0.05 was considered to be statistically significant.

Results

NO Production and NOS Induction in NOS KO Mice

To determine the production of NO and induction of NOS in mouse hearts exposed to ischemia/reperfusion injury, we used an isolated heart perfused in retrograde Langendorff mode.
The hearts were divided into 6 groups: group 1, WT hearts (WT group); group 2, WT treated with the NO donor SNAP (WT/SNAP); group 3, WT treated with the NOS inhibitor L-NIO (WT/L-NIO); group 4, iNOS KO hearts (iNOS KO); group 5, eNOS KO hearts (eNOS KO); and group 6, eNOS KO treated with L-NIO (eNOS KO/L-NIO). Levels of the stable NO metabolite nitrite were measured in coronary effluent during reperfusion in protocol A. As shown in Figure 1, the expected increase in nitrite levels in WT/SNAP was observed. Nitrite was not detected in the WT, WT/L-NIO, or eNOS KO/L-NIO group during reperfusion. Low levels of nitrite were detected in iNOS KO for the first 10 minutes after reperfusion. Interestingly, elevated nitrite levels were detected immediately after reperfusion in eNOS KO, and detectable levels were measured for the remainder of the experiment.

In immunoblot analysis of iNOS and eNOS proteins (Figure 2), iNOS was not detectable in iNOS KO and eNOS was not detectable in eNOS KO, as expected. Interestingly, iNOS was dramatically induced in eNOS KO after 60 minutes of reperfusion. This induction was probably responsible for the NO measured as nitrite levels in eNOS KO. iNOS was not detected or induced in WT. A baseline-level eNOS protein was detected in WT and iNOS KO. There was also a remarkable induction of eNOS protein in iNOS KO after reperfusion. Immunohistochemistry confirmed that iNOS was expressed in the myocardium after reperfusion in eNOS KO with the appearance of iNOS immunoreactivity in myocytes (Figure 3). WT and iNOS KO did not express iNOS protein after reperfusion. eNOS protein was detected in the endothelial cells and myocytes in WT and iNOS KO.

**Myocardial Damage Measurement**

In this study, the whole ventricle was regarded as the area of risk, because 30 minutes of global ischemia was applied. The area that was not stained with tetrazolium red was calculated as infarct area. As shown in Figure 4, the mean values of infarct area in WT/SNAP and eNOS KO were significantly lower than that in WT (16.5±4.9% and 20.0±7.7% versus 30.2±3.25%), whereas WT/L-NIO showed a significantly greater area of infarction (50.2±1.1%). The apparent cardioprotection observed in eNOS KO was completely abolished by L-NIO administration (infarct area with L-NIO, 40.2±9.1%, versus without L-NIO, 20.0±7.7%).

CK release in coronary effluent from the heart was not detectable before ischemia. After 60 minutes of reperfusion, the increase of CK release was seen in all groups; however, it was significantly lower in WT/SNAP and eNOS KO than in WT (Figure 5). Administration of L-NIO in WT and eNOS KO resulted in a significant increase in CK release. Taken together, these results suggest that increased NO availability through provision of an exogenous NO donor or from superinduction of iNOS in the eNOS KO hearts exerts a protective effect in this isolated heart model.

**Hemodynamic Analysis**

**Basal Function**

Basal functions of the perfused hearts are summarized in the Table. There were no significant differences in contractile functions among the groups.

**Protocol A: 60-Minute Reperfusion Experiment**

All hearts were beating spontaneously after 30 minutes of global ischemia followed by reperfusion. After 60 minutes of reperfusion, all the groups showed an ≈30% decrease in heart rate and ≈15% decrease in coronary flow compared with preischemic value. No significant difference was found in heart rate and coronary flow among the groups at 60 minutes (data not shown). A significant decrease in recovery of left ventricular peak systolic pressure (LVSP) and +dP/dt was seen in the WT/SNAP and eNOS KO compared with the...
other groups \( (P < 0.05, \text{Figure 6, A and B}) \). This was ultimately correlated with myocardial protection by a decrease in CK release and infarct sizes (Figures 4 and 5). Administration of the NOS inhibitor L-NIO showed significantly increased recovery of \( +\text{dP/dt} \) at 60 minutes \( (P < 0.05, \text{Figure 6B}) \). In all groups, LVEDP was gradually elevated during ischemia to 25 mm Hg (300% of preischemic value) when reperfusion started (data not shown). After reperfusion, LVEDP gradually decreased in all groups, as shown in Figure 6C; however, WT/SNAP and eNOS KO showed significantly improved diastolic relaxation, as indicated by lower LVEDP at 60 minutes of reperfusion. These results demonstrate that NO prevents the hypercontractile response during the early phase of reperfusion and lowers the ventricular wall stress.

**Protocol B: 3-Hour Reperfusion Experiment**

Next, we performed experiments on isolated perfused hearts for 180 minutes of reperfusion. The hearts were perfused as in protocol A until 60 minutes of reperfusion. At that point, to eliminate the possible differences in coronary flow, the Langendorff mode was changed from a constant-pressure mode to a constant-coronary-flow mode. A separate group of hearts was perfused continuously with KHB without ischemia for 210 minutes to serve as a time-matched control. In this control group, functional parameters did not deviate 15% throughout perfusion (data not shown). LVSP and \( +\text{dP/dt} \) were markedly higher during the first 60 minutes in WT and WT/L-NIO. These values drastically decreased over time, in contrast to the marked stability in these parameters exhibited by WT/SNAP (Figure 7, A and B). In addition, SNAP improves myocardial diastolic relaxation by reducing LVEDP throughout 180 minutes of reperfusion (Figure 7C). These results suggest that NO has a role in preventing the hypercontractile response during the first 60 minutes of reperfusion, ultimately leading to a protection against myo-

### Basal Parameters of Isolated Perfused Hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>LVSP, mm Hg</th>
<th>(+\text{dP/dt}, \text{mm Hg/s})</th>
<th>(-\text{dP/dt}, \text{mm Hg/s})</th>
<th>Coronary Flow, mL/min</th>
<th>Heart Rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>62.7±2.8</td>
<td>2800±132</td>
<td>1935±93</td>
<td>2.75±0.23</td>
<td>465±17</td>
</tr>
<tr>
<td>WT/SNAP</td>
<td>63.5±5.6</td>
<td>2852±142</td>
<td>2241±89</td>
<td>3.04±0.22</td>
<td>468±10</td>
</tr>
<tr>
<td>WT/L-NIO</td>
<td>59.9±5.2</td>
<td>2847±163</td>
<td>2122±103</td>
<td>2.95±0.19</td>
<td>476±9</td>
</tr>
<tr>
<td>eNOS KO</td>
<td>59.0±3.4</td>
<td>2758±132</td>
<td>1827±108</td>
<td>2.77±0.27</td>
<td>475±11</td>
</tr>
<tr>
<td>eNOS KO/L-NIO</td>
<td>61.2±4.9</td>
<td>2804±155</td>
<td>1944±96</td>
<td>2.84±0.17</td>
<td>472±10</td>
</tr>
<tr>
<td>eNOS KO</td>
<td>59.9±3.5</td>
<td>2954±169</td>
<td>2280±119</td>
<td>3.17±0.28</td>
<td>467±13</td>
</tr>
</tbody>
</table>

\(+\text{dP/dt}\) indicates maximal \( \text{dP/dt} \); \(-\text{dP/dt}\), minimal \( \text{dP/dt} \). After stabilization, basal parameters of isolated perfused hearts were measured in 18 WT mice, 12 eNOS KO mice, and 6 iNOS mice. WT mice were randomized into 3 groups, and eNOS KO mice into 2 groups. The groups received bolus injections (2 mL) of vehicle (KHB; \( n = 6 \) in each of the WT, eNOS KO, or iNOS KO mice), SNAP (1 mmol/L; \( n = 6 \) in WT mice), or L-NIO (1 mmol/L; \( n = 6 \) in each of the WT or eNOS KO mice) into the side branch of the aortic cannula just after aortic clamping. Results represent mean±SEM of 6 animals.

**Figure 6.** Cardiac performances after 60 minutes of reperfusion. Cardiac contractile performance was measured with a water-filled latex balloon inserted in left ventricle. LVSP (A), \(+\text{dP/dt}\) (B), and LVEDP (C) are shown as % preischemic value. Results are expressed as mean±SEM for 6 animals in each group. Significant differences are indicated at 60 minutes of reperfusion. *\( P < 0.05 \) vs WT.

**Figure 7.** Cardiac performances and CK release after 180 minutes of reperfusion. Hearts were subjected to 30 minutes of ischemia followed by 180 minutes of reperfusion. LVSP (A), \(+\text{dP/dt}\) (B), and LVEDP (C) are shown as % preischemic value. Total CK release (D) is measured as described in Methods. Results are expressed as mean±SEM for 6 animals in each group. Significant differences are indicated at 60 and 180 minutes of reperfusion. *\( P < 0.05 \) vs WT.
cardiac dysfunction. Consistent with this, NO has a protective function in the heart in this model by decreasing myocyte injury, as shown by a decrease in CK release in the coronary effluent (Figure 7D).

Discussion
In this study, we investigated whether NO has a protective role against I/R injury with the use of pharmacological modulation of NOS and NOS KO mice. We clearly demonstrated that NO has a cardioprotective role against I/R injury. The role of NO against I/R injury has been discussed in numerous studies. In the heart, in vivo studies using NO donor or eNOS KO mice have demonstrated that NO inhibits neutrophil-mediated injury by inhibiting neutrophil adhesion to the endothelial cells and preserves endothelial function, resulting in myocardial protection.15,18,20 Ex vivo studies, however, have shown conflicting results. The mechanisms of NO in modulating cardiac function during I/R injury proposed in those studies are quite numerous. NO has been reported to exert multiple beneficial effects: inhibiting inositol-1,4,5-triphosphate and reducing calcium overload,14 mediating protein kinase C translocation,19 and inhibiting neutrophil-associated injury.20 On the contrary, NO also has been reported to react with superoxide to form peroxynitrite, which is considered cytotoxic.23

Therefore, we used an ex vivo system to remove the contribution of neutrophils and platelets to focus specifically on the effect of NO in the heart. Enhanced NO availability occurred in 2 of our experimental groups: first, the hearts receiving the NO donor, and second, the eNOS KO hearts that have a superinduced iNOS during I/R. Both groups showed a protection against the hypercontractile response during the early reperfusion period with spontaneous beating, accompanied by a reduction in myocardial damage. We also observed that in the hearts with reduced NO activity, they showed a heightened level of contractility in the early phase of reperfusion. Like the action of phospholamban, which serves as a brake for calcium release by the sarcoplasmic reticulum in the heart, NO plays a role as a brake for hypercontractility during early reperfusion periods and preserves myocardial viability. Peroxynitrite, recently considered an important mediator of tissue injury, is reported to have a cardioprotective role against I/R injury in physiologically relevant concentrations.32 This concentration-dependent effect of peroxynitrite might be able to explain, at least in part, the conflicting results of the role of NO against I/R injury in previous investigations.

Our results suggest that NO mediates energy balance in protecting the heart after I/R, which might be related to ATP synthesis and energy metabolism. Indeed, several investigations have shown that NO decreases myocardial O2 consumption by modulating mitochondrial respiration.33–36 Also, NO has other possible protective mechanisms in the heart: NO can inhibit apoptosis by nitrosylating active sites of cysteine residues in caspases.34 NO can also interact with ion channels on the plasma membrane or mitochondria and reduce Ca2+ increases.37

In our present study, we demonstrate with immunoblot and immunohistochemistry that iNOS was superinduced in the eNOS KO mice after I/R injury, resulting in increased NO activity, which is then responsible for the observed cardioprotection. The iNOS superinduction in eNOS KO hearts during I/R is most likely due to an adaptive mechanism in the homozygous eNOS-deleted mice. The mechanism of the upregulation of iNOS in the eNOS KO mice is unknown but could be due to redox stress associated with the absence of NO from eNOS. It is entirely possible that in the eNOS KO mice, an unidentified promoter for the iNOS gene could be activated in response to this redox stress. Recent studies showed that mice with a disruption of the iNOS gene can undergo tissue-specific transcription by a previously unidentified promoter region and translation of an iNOS-incompetent product.38 Also, there is a possibility that KO mice have other regulatory mechanisms, including posttranscriptional regulation or mRNA stability. Whether compensatory iNOS upregulation is a more generalized feature of eNOS deficiency is unclear.

In regard to cardiac performance, we found that hearts with reduced NO activity demonstrated a transient heightened contractile response in the early periods of reperfusion. These hearts are unable to maintain their contractile function for >60 minutes. Bolus administration of an NO donor before the ischemic period prevents the early hypercontractile response during reperfusion. Improved cardiac performance is correlated with a reduction in myocardial damage by all measured parameters.

Acknowledgments
This work was supported by a Thoracic Surgery Research Fellowship, American Heart Association Scientist Development Grant 9630257N, and National Institutes of Health grants (R01GM-44100, R01RR/AI-15187, and P41RR-03631).

References
2748 Circulation June 13, 2000


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Circulation. 2000;101:2742-2748
doi: 10.1161/01.CIR.101.23.2742

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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