Cardiac Myocyte–Specific Expression of Inducible Nitric Oxide Synthase Protects Against Ischemia/Reperfusion Injury by Preventing Mitochondrial Permeability Transition

Matthew B. West, PhD*; Gregg Rokosh, PhD*; Detlef Obal, MD; Murugesan Velayutham, PhD; Yu-Ting Xuan, PhD; Bradford G. Hill, PhD; Rachel J. Keith, MS; Jürgen Schrader, MD; Yiru Guo, MD; Daniel J. Conklin, PhD; Sumanth D. Prabhu, MD; Jay L. Zweier, MD; Roberto Bolli, MD; Aruni Bhatnagar, PhD

Background—Inducible nitric oxide synthase (iNOS) is an obligatory mediator of the late phase of ischemic preconditioning, but the mechanisms of its cardioprotective actions are unknown. In addition, it remains unclear whether sustained elevation of iNOS in myocytes provides chronic protection against ischemia/reperfusion injury.

Methods and Results—Constitutive overexpression of iNOS in transgenic mice (α-myosin heavy chain promoter) did not induce contractile dysfunction and did not affect mitochondrial respiration or biogenesis, but it profoundly decreased infarct size in mice subjected to 30 minutes of coronary occlusion and 24 hours of reperfusion. In comparison with wild-type hearts, isolated iNOS-transgenic hearts subjected to ischemia for 30 minutes followed by 40 minutes of reperfusion displayed better contractile recovery, smaller infarct size, and less mitochondrial entrapment of 2-deoxy-[3H]-glucose. Reperfusion-induced loss of NAD+ and mitochondrial release of cytochrome c were attenuated in iNOS-transgenic hearts, indicating reduced mitochondrial permeability transition. The NO donor NOC-22 prevented permeability transition in isolated mitochondria, and mitochondrial permeability transition–induced NAD+ loss was decreased in wild-type but not iNOS-null mice treated with the NO donor diethylene triamine/NO 24 hours before ischemia and reperfusion ex vivo. iNOS-mediated cardioprotection was not abolished by atacryloside. Reperfusion-induced production of oxygen-derived free radicals (measured by electron paramagnetic resonance spectroscopy) was attenuated in iNOS-transgenic hearts and was increased in wild-type hearts treated with the mitochondrial permeability transition inhibitor cyclosporin A.

Conclusions—Cardiomyocyte-restricted expression of iNOS provides sustained cardioprotection. This cardioprotection is associated with a decrease in reperfusion-induced oxygen radicals and inhibition of mitochondrial swelling and permeability transition. (Circulation. 2008;44:1970-1978.)

Key Words: infarction ■ ischemia ■ nitric oxide ■ nitric oxide synthase

Myocardial ischemic injury is attenuated in hearts subjected to brief bouts of ischemia before the onset of sustained ischemia (ischemic preconditioning). Both early and delayed phases of ischemic preconditioning have been described.1-3 The early phase occurs immediately after the preconditioning stimulus but disappears within 1 to 2 hours. The protective effects of early preconditioning have been attributed to posttranslational modification of proteins, particularly kinase-mediated protein phosphorylation events.3 Late preconditioning becomes manifest 12 to 24 hours after ischemia, lasts 3 to 4 days, and is associated with increased synthesis of cardioprotective proteins.1 Although the signaling pathways involved in triggering cardioprotection have been extensively characterized,1-3 the metabolic basis for the antischismic phenotype of the preconditioned heart remains obscure.

Editorial p 1915
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Mechanistic studies of late preconditioning have demonstrated that nitric oxide (NO) plays a central role in mediating cardioprotection. According to the NO hypothesis of late preconditioning, increased generation of NO from endothelial...
NO synthase (NOS) on day 1 triggers multiple signaling pathways.1,4 These lead to the upregulation of a number of proteins, including inducible NOS (iNOS), which in turn mediate the cardioprotective effects of late preconditioning 24 hours later (day 2).1,4 The postulated dual role of NO—as both a trigger of late preconditioning on day 1 and a mediator on day 2—is based on the observations that pretreatment with NO donors protects the heart from ischemia (24 hours later). NOS inhibitors given on day 1 abolish the development of delayed cardioprotection, and iNOS inhibitors given on day 2 abrogate the infarct-sparing effect of late preconditioning.1,4 The obligatory role of iNOS also is supported by the observation that targeted deletion of the iNOS gene abrogates late preconditioning induced by a variety of stimuli, including ischemia, adenosine A1 agonists, opioid δ agonists, endotoxin derivatives, and exercise, suggesting that iNOS is the final common effector of cardioprotection.1,4,5 Collectively, these data support a key role of iNOS-derived NO in mitigating ischemic injury. Nevertheless, it is unclear whether this protection is mediated by a myocyte-specific increase in iNOS and whether continuous expression of iNOS can confer chronic protection against ischemia/reperfusion (I/R) injury. These issues are important considerations in developing therapeutically viable antiischemic strategies and in understanding the mechanisms underlying the cardioprotective effects of iNOS.

Several mechanisms could account for the antiischemic actions of NO. These include regulation of mitochondrial respiration,6 antioxidant protection,7 activation of the mitochondrial KATP channels,8 and inhibition of cell death pathways.9,10 Because of the central role of mitochondria in governing cell death/survival decisions,11,12 it seems likely that the mechanism of NO protection may be related to mitochondrial injury. Indeed, mitochondrial swelling is the first sign of irreversible ischemic damage,13 and multiple cardioprotective signaling pathways converge on the mitochondria.14 Induction of mitochondrial permeability transition (MPT), in particular, has been suggested as the defining event in myocardial reperfusion injury.15,17 and activation of cell death pathways.11,12 Therefore, we hypothesized that the beneficial actions of iNOS stem from mitochondrial protection. To test this hypothesis, we investigated whether chronic cardiomyocyte-specific expression of iNOS affects mitochondrial permeability and the generation of oxygen-derived free radicals in hearts subjected to I/R. The results demonstrate, for the first time, that cardiomyocyte-restricted expression of iNOS is sufficient to confer chronic cardioprotection and that transgenic upregulation of cardiac iNOS decreases free radical generation, which in turn prevents MPT and swelling and chronically protects the heart against I/R injury. Preliminary findings of this study have been reported.18

**Methods**

Detailed methodology is provided in the online Data Supplement. Adult C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Me). The iNOS-transgenic mice express the iNOS gene specifically in cardiomyocytes under the control of the α-myosin heavy chain promoter.19 These mice are healthy, and their cardiovascular function is normal.18 Baseline 2-dimensional echocardiography (Toshiba T380 Powervision) was performed as previously described.20

**Ischemic Preconditioning and Acute Myocardial Infarction In Vivo**

The murine model of ischemic preconditioning and infarction has been described previously.21 Briefly, mice were preconditioned with a sequence of six 4-minute occlusion/4-minute reperfusion cycles. Control mice were subjected to sham operation. Myocardial infarction was produced by subjecting mice to a 30-minute coronary occlusion followed by 24 hours of reperfusion.

**MPT Measurement**

Induction of MPT was assessed by the mitochondrial uptake of 2-deoxy-[3H]-glucose (DOG),16,22 NADH measurement,15 and appearance of cytochrome c in the cytosol.12 Hearts were excised and perfused in the Langendorff mode at constant flow (3 mL/min) to ensure consistent perfusion. After 20 minutes of equilibration, the hearts were perfused in the recirculating mode with 50 mL modified Krebs-Henseleit buffer containing 0.5 mmol/L 2-[3H]-DOG (0.1 μCi/ml) for 20 minutes. Perfusion was then returned to normal for 10 minutes, and the hearts were subjected to 30 minutes of ischemia. After 15 minutes of reperfusion, mitochondria were isolated, and the radioactivity associated with the mitochondrial fraction was measured by scintillation counting. To ensure equal yield of mitochondria, citrate synthase activity was measured by colorimetric assay (Sigma-Aldrich, St Louis, Mo). Retention of [H]-DOG was calculated by dividing the radioactivity in the mitochondria by the total radioactivity recovered from the tissue.

**Electron Paramagnetic Resonance Spin Trapping Measurement of Oxygen Radicals**

After intraperitoneal induction of anesthesia (pentobarbital sodium, Abbott Laboratories, Abbott Park, Ill; 5 mg), the heart was excised, and the ascending aorta was cannulated and perfused at a constant flow (2 mL/min) with Krebs-Henseleit buffer. After ischemia, the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; 1 mol/L; Dojindo Laboratories, Kumamoto, Japan) in buffer containing 100 μmol/L diethylenetriaminepentaacetic acid (Sigma-Aldrich), was administered immediately on reperfusion with the trap infused at a rate of 100 μL/min through a side arm located close to the heart. During reperfusion, periodic collections of the effluent were made until 5 minutes of reperfusion at indicated intervals. On sample collection, each tube was immediately frozen in liquid nitrogen. Electron paramagnetic resonance spectra were recorded as described previously.23,24

**Statistical Analysis**

Data are reported as mean±SEM. Comparisons between 2 groups were performed with unpaired Student’s t tests. Comparisons among multiple groups or between 2 groups at multiple time points were performed by either 1-way or 2-way ANOVA, as appropriate, followed by paired or unpaired Student’s t tests with the Bonferroni correction. For comparing wild-type (WT) and iNOS-transgenic mice at multiple time points, a repeated-measures ANOVA was used.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Constitutive Expression of iNOS Protects Against I/R Injury**

Previous studies have shown that the late phase of ischemic preconditioning is associated with selective upregulation of iNOS and that targeted disruption of iNOS abrogates the infarct-sparing effect of late preconditioning.1,4,5 Nevertheless, it remains unclear whether continuous expression of iNOS in cardiac myocytes can establish a state of chronic cardioprotection and enhance ischemic tolerance. Accordingly, to test the duration and cellular dependence of iNOS-mediated cardioprotection, we used 12- to 20-week-old...
iNOS-transgenic mice (20 to 30 g) in which the iNOS gene is under the control of the cardiac myocyte–specific α-myosin heavy chain promoter. Despite an increase in cardiac iNOS, these mice are healthy and breed normally. Heger et al have also reported that cardiomyocyte-specific iNOS expression does not affect the ratio of left ventricular (LV) to body weight or the heart rate, and although cardiac output and mean arterial pressures were mildly depressed, there was no overt hypertrophy or heart failure. In agreement with these observations, we found no significant differences in echocardiographically assessed cardiac function between hearts of iNOS-transgenic and WT mice (the Table). The iNOS-transgenic hearts, however, displayed a slight concentric hypertrophy as evidenced by increased anterior and posterior wall thickness and elevated LV mass with no changes in LV diastolic or systolic dimension (the Table).

Cardiac homogenates from iNOS-transgenic mice displayed an ~400-fold increase in iNOS protein expression and an ~3-fold increase in NOx content compared with WT littermates. Although it has been reported that NO triggers mitochondrial biogenesis, we found no difference in the size distribution or the number of mitochondria in electron micrographs from WT and iNOS-transgenic hearts (Figure I of the online Data Supplement). The expression of cytochrome c and cytochrome c oxidase subunit IV also was similar between WT and iNOS-transgenic hearts (supplemental Figure II), and there was no difference in the ratio of the mitochondrial and nuclear DNA content between WT and iNOS-transgenic hearts (supplemental Figure II), indicating that mitochondrial synthesis is not stimulated by a chronic increase in iNOS abundance and activity. Rates of state 3 and 4 respiration and the ADP/O ratios measured in mitochondria isolated from iNOS-transgenic and WT mice were similar (supplemental Figure III), and no difference was observed in basal reactive oxygen species (ROS) production or ROS production after inhibition of complex I and III (supplemental Figure IV). Small increases in the abundance of complex II and IV, but not complex I, III, and V, were observed (supplemental Figure III). The reasons for the increase in complex II and IV are unclear, but because no changes in respiration and ROS generation were observed, it appears unlikely that these changes significantly affect mitochondrial function.

To examine the effects of chronic iNOS overexpression on ischemic tolerance, WT and iNOS-transgenic mice were subjected to 30 minutes of coronary occlusion followed by 24 hours of reperfusion. The iNOS-transgenic mice and their WT littermates were not different with respect to the size of the risk region (35±3 vs 41±2 mg); however, as shown in Figure 1, the infarct was significantly smaller in iNOS-transgenic hearts compared with WT hearts (25±3% versus 58±4% of the region at risk; P<0.05). These observations indicate that cardiomyocyte-specific iNOS expression produces a chronically protected cardiac phenotype.

### Table. Cardiac Parameters in iNOS-TG and WT Mice

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<th>M-Mode Echocardiogram Parameter</th>
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<th>iNOS-TG</th>
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<tr>
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<td>116±3.4*</td>
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<tr>
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<td>8.3±0.4</td>
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</tbody>
</table>

*P<0.05 vs WT; n=12 to 14.

Figure 1. iNOS overexpression limits infarct size after I/R in vivo. WT and iNOS-transgenic hearts were subjected to a 30-minute coronary occlusion followed by 24 hours of reperfusion. Infarct size was measured by triphenyltetrazolium chloride staining and expressed as a percent of the risk region. *P<0.05 vs WT (n=10 to 12).
The greater functional recovery of iNOS-transgenic hearts was associated with a reduction in tissue injury (Figure 3A through 3F). Total lactate dehydrogenase release during reperfusion was greater in WT than in iNOS-transgenic hearts (465±35 versus 296±63 arbitrary units, respectively; \(P<0.05\); Figure 3A and 3C); creatine kinase release also was greater in WT (299±46 arbitrary units) than in iNOS-transgenic (165±27 arbitrary units; \(P<0.005\); Figure 3B and 3D) hearts. The reduction in tissue injury was confirmed by tetrazolium-based measurements of infarct size, which was reduced from 68±9% of the LV in WT to 43±6% in iNOS-transgenic hearts (\(P<0.05\); Figure 3E and 3F). Taken together, these data corroborate the in vivo results by demonstrating that constitutive overexpression of iNOS protects the heart against I/R injury in the same preparation that we used to assess MPT.

**Constitutive Expression of iNOS Prevents MPT**

Changes in MPT were determined by measuring the mitochondrial retention of DOG, the loss of NAD\(^+\), and the release of cytochrome c into the cytoplasm. Perfusion with aerobic buffer for 60 minutes led to basal retention of radioactivity in the mitochondrial fraction. This was significantly increased (\(P<0.05\)) in mitochondria isolated from WT hearts subjected to 30 minutes of ischemia followed by 15 minutes of reperfusion, indicating induction of MPT (Figure 4A). In contrast, mitochondria from iNOS-transgenic hearts subjected to I/R exhibited essentially no increase in radioactivity (Figure 4A). These observations indicate that MPT is inhibited in iNOS-transgenic hearts. In addition to MPT, loss of NAD\(^+\) is a measure of MPT.\(^{15}\) In WT hearts subjected to I/R, the NAD\(^+\) content was 36% of that in nonischemic hearts. No decrease in NAD\(^+\) was observed in iNOS-transgenic hearts (Figure 4B). Additionally, after I/R, the cytoplasmic cytochrome c level was significantly less in iNOS-transgenic than in WT hearts (Figure 4C). Collectively, all 3 indices indicate a decrease in MPT in iNOS-transgenic hearts subjected to I/R.

To determine whether MPT also is prevented in hearts preconditioned in vivo, we used a pharmacological model of preconditioning.\(^{26}\) For this, WT mice received 4 intravenous injections of 0.1 mg/kg diethylenglycylamine (DETA)/NO; 24 hours later, the hearts were excised and subjected to 30 minutes of ischemia followed by 30 minutes of reperfusion. Treatment with DETA/NO attenuated NAD\(^+\) depletion compared with vehicle-treated hearts (supplemental Figure V). Compared with vehicle-treated hearts, hearts preconditioned with DETA/NO also showed less oxidative stress as measured by the accumulation of protein adducts of the lipid peroxidation product 4-hydroxy-trans-2-nonenal (supplemental Figure V). DETA/NO pretreatment did not prevent NAD\(^+\) depletion in hearts from iNOS-null mice (supplemental Figure V), indicating that inhibition of MPT after pharmacological preconditioning is mediated by iNOS.

**NO Prevents Permeability Transition in Isolated Mitochondria**

Having observed that increased iNOS expression is associated with a decrease in MPT during I/R, we examined whether NO directly prevents MPT. For this, isolated mitochondria were treated in suspension with Ca\(^{2+}\). As shown in Figure 5, addition of Ca\(^{2+}\) led to an abrupt decrease in light absorbance (as a result of increased light scattering), indicating mitochondrial swelling. No change in absorbance was observed in the absence of Ca\(^{2+}\). The Ca\(^{2+}\)-induced decrease in absorbance was inhibited by cyclosporin A, which prevents opening of the permeability pore, and cyanide (NaCN), which inhibits cytochrome c oxidase (Figure 5A and 5B). This decrease in absorbance was accompanied by depolarization (loss of \(\Delta \psi\)). Mitochondrial membrane potential also was abolished by NaCN, and Ca\(^{2+}\) did not cause additional depolarization or loss of absorbance in NaCN-poisoned mitochondria. Taken together, these data demonstrate that the high negative potential generated in well-energized (metabol-
ically active) mitochondria favors Ca\(^{2+}\) uptake and that excessive accumulation of Ca\(^{2+}\) triggers MPT.

Preincubation with the NO donor spermine NONOate (NOC-22) prevented Ca\(^{2+}\)-induced loss of absorbance in mitochondrial suspension (Figure 5). Similar to NaCN, NOC-22 induced depolarization, and the mitochondria depolarized with NOC-22 were insensitive to Ca\(^{2+}\). Using a t\(_{1/2}\) of 230 minutes, we estimate the flux of NO generated from NOC-22 under the experimental conditions to be \(\approx 10\) nmol \(\cdot\) min\(^{-1}\) \(\cdot\) mg\(^{-1}\) protein, which is comparable to the estimated steady-state concentration of NO in cardiac myocytes (between 0.1 and 0.2 \(\mu\)mol/L). No differences in Ca\(^{2+}\)-induced MPT were observed, however, in mitochondria isolated from WT and iNOS-transgenic hearts (data not shown). These observations suggest that overexpression of iNOS does not alter the intrinsic sensitivity of the mitochondria to undergo permeability transition; however, physiological levels of NO inhibit permeability transition, in part by dissipating the membrane potential required for Ca\(^{2+}\) uptake.

**Figure 3.** Constitutive expression of iNOS decreases I/R injury in isolated perfused hearts. Hearts from WT and iNOS-transgenic mice were subjected to 30 minutes of ischemia followed by reperfusion, and the coronary effluent was collected at the indicated times for measurement of lactate dehydrogenase (A; LDH) or creatine kinase (B; CK). Reperefusion was initiated at time 0. Measurements of total lactate dehydrogenase and creatine kinase release during the entire 40 minutes of reperfusion are shown in C and D, respectively. *P<0.05 vs WT (n=4 to 9). E, Representative images of WT (i) and transgenic (ii) hearts after triphenyltetrazolium chloride staining. F, Infarct size, delineated by triphenyltetrazolium chloride staining, is expressed as percent of the LV. *P<0.05 vs WT (n=7 to 12).

**NO Prevents Reperfusion-Induced Oxygen Free Radical Generation**

Reperfusion injury has been linked to the generation of oxygen-derived free radicals.\(^{2,27}\) Hence, to further elucidate the cardioprotective mechanisms elicited by NO, we studied free radical generation in WT and iNOS-transgenic hearts. Oxygen radicals generated on reperfusion were trapped with DMPO and quantified by electron paramagnetic resonance spectroscopy. As shown in Figure 6A, reperfusion was associated with a burst of free radical production. The levels of spin-trapped radicals returned to baseline after 120 seconds of reperfusion. The amount of radicals trapped from reperfused hearts was significantly attenuated in iNOS-transgenic hearts compared with WT hearts. In contrast, treatment with the MPT inhibitor cyclosporin A increased radical production (Figure 6B). Although cyclosporin A was found to promote functional recovery of WT hearts subjected to 30 minutes of ischemia and 40 minutes of reperfusion (supplemental Figure
induced oxygen radical production. NO maintains the mitochondrial pore in the closed state, so its protection cannot be abolished by stabilizing the open state of the pore.

**Discussion**

The major finding of this study is that a cardiomyocyte-specific increase in iNOS leads to sustained cardioprotection without causing overt myocyte injury or dysfunction. Transgenic overexpression of iNOS in cardiac myocytes reduced infarct size and decreased I/R-induced free radical production and MPT, indicating that overexpression of iNOS significantly enhances the ability of the heart to withstand oxidative and mitochondrial stress. Significantly, our observations demonstrate that manipulating a single myocyte gene can establish a chronically cardioprotected phenotype. This mode of cardioprotection differs in its time course from both early (which lasts 1 to 2 hours) and late (48 to 72 hours) preconditioning, both of which offer only transient protection. The permanent cardioprotection described here is due to the activity of iNOS, which is now widely recognized as an obligatory mediator of late preconditioning, and relates to favorable changes in 2 major determinants of I/R injury: free radical generation and MPT. These observations offer new insights into the mechanism of NO-mediated protection and have significant clinical implications for developing long-term prophylactic antiischemic interventions.

The results of this study show that a transgenic increase in iNOS is associated with a decrease in both I/R injury in isolated perfused hearts and infarct size after coronary occlusion in situ. These observations imply that the chronic cardioprotection afforded by iNOS is independent of the experimental model used to assess its efficacy. The concordance between in situ and ex vivo models also indicates that iNOS-mediated protection is not related to systemic changes in blood-borne responses, favorable neutrophil-endothelium interactions, or alterations in autonomic regulation but instead can be attributed to changes in the response of cardiac myocytes to ischemia.

NO has been shown to play an obligatory role not only in the delayed cardioprotection elicited by ischemic preconditioning but also in that provided by physical exercise, NO donors, adenosine A1 receptor agonists, opioid δ1 receptor agonists, and endotoxin derivatives. Thus, generation of NO by iNOS appears to be a final common pathway that mediates the late phase of ischemic preconditioning, pharmacological preconditioning, and preconditioning induced by physical stimuli. Indeed, late preconditioning could be viewed as a state of increased NO availability. However, to the best of our knowledge, this is the first report that constitutive overexpression of iNOS in the heart is associated with sustained cardioprotection associated with a decrease in the production of oxygen-derived free radicals and alleviation of detrimental changes in the mitochondria. These observations reveal a new mechanism whereby iNOS imparts protection against I/R injury, i.e., protection of the mitochondria (without increasing mitochondrial biogenesis or inducing permanent changes in the mitochondrial structure and function), and advance our understanding of the cardiovascular function of this protein.

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**Figure 4.** Inhibition of MT in iNOS-transgenic hearts. A, Isolated perfused WT or iNOS-transgenic hearts were loaded with [3H]-DOG, and mitochondrial entrapment of the label was measured to assess MT. The radioactivity associated with the mitochondrial fraction was normalized to total radioactivity in the homogenate. Bars represent fractional radioactivity retained in the mitochondrial. *P<0.05 vs WT control (n=3 to 6); **P<0.001 vs WT I/R (n=4 to 6). B, I/R-induced NAD+ depletion in WT and iNOS-transgenic hearts. Bars represent NAD+ levels in I/R hearts expressed as a percentage to the appropriate per-
fusion controls (4<0.005 vs WT; n=8). C, Cytochrome c was detected by Western blot using anti-cytochrome c antibodies in postmitochondrial fractions prepared from WT and iNOS-transgenic hearts after I/R and normalized to total protein. *P<0.005 vs WT (n=5).

VI) and to decrease the release of cytochrome c in the cytosol, it did not affect mitochondrial retention of [3H]-DOG (supplemental Figure VII). Furthermore, iNOS-mediated cardioprotection was not abolished by the MPT opener atractyloside, and iNOS-transgenic hearts treated with atractyloside displayed better recovery of function and lower levels of lactate dehydrogenase and creatine kinase release than did the WT hearts (supplemental Figure VIII). Collectively, these observations indicate that iNOS decreases reperfusion-
NO could protect the heart from ischemic injury by several mechanisms. Previous studies have shown that NO stimulates the opening of the mitochondrial K<sub>ATP</sub> channel and inhibits apoptosis by nitrosylating caspases. In addition, an increase in iNOS can enhance the expression of antioxidant proteins. Our results suggest that the cardioprotection afforded by iNOS is not due to permanent changes in mitochondria because no difference in MPT was observed in mitochondria isolated from WT and iNOS-transgenic hearts. This observation is in agreement with results of other studies showing that mitochondria isolated from naïve or preconditioned hearts show no difference in their ability to undergo MPT. Thus, inhibition of MPT in iNOS-transgenic hearts, similar to that in hearts preconditioned by ischemia, does not appear to be due to a change in the intrinsic sensitivity of the mitochondria to undergo permeability transition per se. This view is consistent with our previous observation that NO must be generated during I/R; ie, NO is not only a trigger but also a mediator of preconditioning. On the basis of these considerations, we propose that the cardioprotective networks stimulated by iNOS converge on the mitochondria, strengthening their defense against ischemic changes and preventing them from triggering cell death pathways. The protective effects of iNOS on the mitochondria may be facilitated by its localization. In iNOS-transgenic hearts, significant levels of iNOS were associated with the mitochondria (supplemental Figure IX). Mitochondrial association of iNOS also was observed in WT hearts preconditioned by ischemia (supplemental Figure X). Although further experiments are required to understand the mechanism by which iNOS associates with the mitochondria and to assess the significance of these findings fully, these data suggest that mitochondrial protection by iNOS may be facilitated by the generation of NO near the mitochondria.

Although MPT induction has been proposed as the key event that triggers myocyte cell death during reperfusion, the mechanisms that trigger MPT during I/R are poorly understood. Increased generation of free radicals could be a trigger, which could increase mitochondrial calcium overload and trigger pore opening. Hence, our observation that cardiomyocyte-specific expression of iNOS prevents radical gener-
serves complex I activity and propose that because cyclosporin A prevents MPT, it pre-
decreased I/R injury (this study and others\textsuperscript{15,34–36}) in WT
Although cyclosporin A prevented cytochrome c release and further clarified by the data obtained with cyclosporin A. The relationship between MPT and free radical generation is which in turn could prevent MPT and decrease I/R injury. This also is consistent with the observation that mitochondria isolated from WT and iNOS-transgenic hearts were equally susceptible to MPT. It follows from these considerations that clinical interventions to directly inhibit MPT are likely to be less successful than those that increase iNOS in the heart (eg, exercise, pharmacological preconditioning, or iNOS gene therapy) because NO, in addition to inhibiting MPT, prevents free radical generation. Therefore, the dual protection provided by iNOS, which could be chronically induced in the heart, may be a therapeutically accessible pathway for establishing sustained resistance to myocardial I/R injury.

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None.

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CLINICAL PERSPECTIVE

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B  

Suppl. Fig. 1  

WT  

TG  

Citrate synthase activity (nmol/min/mg protein)  

WT  

iNOS-TG  

N.S.
Suppl. Fig. 3

A) Graph showing the oxygen consumption and state transitions (State 3: +ADP, State 4: +Respiratory Substrate) in WT and TG lines.

B) Bar graph comparing the ratio of State 3: State 4 and ADP:O between WT and iNOS TG lines.

C) Western blot image showing band intensities for WT and TG lines with KDa markers.

D) Bar graph showing the band intensity (A.U.) for different bands (I, II, III, IV, V) in WT and iNOS TG lines.

E) Bar graph comparing the cytochrome c oxidase activity (umoles/min/mg protein) between WT and iNOS TG lines.
Suppl. Fig. 4A

Mitochondrial ROS

pmol H2O2/ml/mg protein

<table>
<thead>
<tr>
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<th>WT</th>
<th>iNOS TG</th>
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<td>+</td>
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<tr>
<td>malate/glutamate</td>
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<td>+</td>
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<tr>
<td>rotenone</td>
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Suppl. Fig. 4B

Mitochondrial ROS

pmol H2O2/ml/mg protein

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<td>+</td>
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<tr>
<td>succinate/rotenone</td>
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<tr>
<td>antimycin A</td>
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Suppl. Fig. 5

A

NAD⁺ Concentration (% of control)

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<th>Vehicle</th>
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**CONTROL**              **I/R**  
**DETA/NO**              **I/R**

B

Band Density (A.U.)

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<th>DETA/NO</th>
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<td>DETA/NO I/R</td>
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* * #
Suppl. Fig. 7

A. Cytochrome c/GAPDH

B. Mitochondrial 3H-Deoxyglucose

C. NAD+ Concentration

* indicates statistical significance.
Suppl. Fig. 8

**A**

Ischemia

- **WT+ Atrac.**
- **TG + Atrac.**

**B**

Developed pressure (mm Hg)

- WT+ Atrac.
- TG + Atrac.

**C**

End-diastolic pressure (mm Hg)

- WT+ Atrac.
- TG + Atrac.

**D**

Total LDH release

- WT
- TG

+ Atractyloside

**E**

Total CK release

- WT
- TG

+ Atractyloside

* indicates significant difference
A. Myocardial NOX (nmol/mg protein)

B. Mitochondrial iNOS Protein

C. iNOS activity (pmol L-citrulline/min/mg protein)
MATERIALS AND METHODS

**Measurement of NOS activity and NO\textsubscript{x}:** NOS activity was determined by measuring the conversion of \[^{14}\text{C}\]-L-arginine to \[^{14}\text{C}\]-L-citrulline as described by Bredt and Snyder\textsuperscript{1}. For measuring NO\textsubscript{x}, tissue samples were homogenized in a buffer containing 25 mmol/L Tris·HCl (pH 7.5), 0.5 µmol/l EDTA, and 0.5 µmol/l EGTA and centrifuged at 14,000x\textit{g} for 15 min, and the resulting supernatants were collected as cytosolic fractions. The supernatants were loaded onto a Centricon-30 filter and centrifuged to remove molecules smaller than 30 kDa. Nitrite was assayed using the Griess reaction\textsuperscript{2}. Nitrate content was determined after conversion of nitrate to nitrite with \textit{Aspergillus} nitrate reductase\textsuperscript{2}.

**Measurements of mitochondrial swelling:** Freshly prepared cardiac mitochondria from C57/BL6 mice were resuspended in MPT assay buffer containing, in mmol/l: 40, HEPES; 195, mannitol; 25, sucrose; 0.010, EGTA. MPT reactions were carried out in a total volume of 0.2 ml using 0.5 mg/ml mitochondrial protein in the presence of 1 µmol/L rotenone and 5 mmol/l succinate. Samples were pretreated with the indicated additives for 5 min. Changes in optical density at 520 nm were recorded to monitor mitochondrial swelling following pretreatment and the addition of 60 µmol/L of CaCl\textsubscript{2}. 
**Measurement of mitochondrial depolarization:** Isolated mitochondria in suspension were pretreated with 5 $\mu$mol/L Rhodamine 123 for 5 min. Cyanide or NOC-22 was then added as indicated and allowed to incubate for an additional 5 min. Calcium (60 $\mu$mol/l) was added at $t = 10$ min. Loss of membrane potential was measured by increased fluorescence intensity (excitation 490 nm/emission 535 nm).

**Enzyme activity measurements:** Activity of citrate synthase in whole heart homogenates and in isolated mitochondrial fractions was performed using the Citrate Synthase Assay Kit (Sigma, St. Louis, MO; #CS0720). Aldose reductase activity was measured as described3.

**Perfused heart studies:** Mice were treated with heparin (1000 IU/kg body weight i.p.) and anesthetized with sodium pentobarbital (50 mg/kg i.p.). The hearts were isolated and perfused in the retrograde Langendorff mode at constant (70 mmHg) pressure using a modified Krebs Henseleit (KH) buffer solution containing (in mmol/L): NaCl, 118; KCl, 4.7; MgSO$_4$, 1.2; KHPO$_4$,1.2; CaCl$_2$, 2.5; EDTA, 0.5; glucose, 11; and NaHCO$_3$, 22 at 37°C. Left ventricular (LV) pressure was measured using a water-filled balloon. Diastolic pressure was set at 5-10 mmHg. Hearts were allowed to equilibrate for 20 min and then subjected to global ischemia for 30 min while immersed in ungassed KH buffer at 37°C to maintain temperature. Lactate dehydrogenase (LDH) and creatine kinase (CK) activities were measured in the effluent using commercially available kits (from Roche and Pointe Scientific, respectively). After 40 min of reperfusion,
Infarct size was determined by triphenyltetrazolium chloride (TTC) staining. Hearts were perfused with 1% TTC in KH buffer, incubated for 15 min at 37°C, stored in 10% buffered formalin, and then cut into 1 mm-thick transverse sections. Infarct size was measured by videoplanimetry and expressed as a percentage of LV weight.

**Western blotting:** Protein expression was assessed by using standard SDS/PAGE Western immunoblotting techniques as previously described and total protein loading was normalized to Ponceau staining. iNOS antibodies were obtained from Upstate Biosciences, Inc. The antibody against cytochrome c oxidase was obtained from Invitrogen.

**Electron microscopy:** Wild-type and iNOS-TG mice were anesthetized with sodium pentobarbital (0.1 ml, 40 mg/kg, ip) and sacrificed by removal of the heart. The left ventricle was opened vertically with a razor blade and the papillary muscle was removed intact and fixed overnight in 2.5% glutaraldehyde. Tissue was embedded in epoxy resin and ultrathin tissue sections on copper grids were stained with osmium tetroxide and uranyl acetate and photomicrographs taken using TEM (1,000-28,000x magnification). Photomicrographs were scanned and analyzed using MetaMorph (Universal Imaging Corp.) to calculate mitochondrial size (arbitrary units, AU). Over 800 mitochondria per papillary section were analyzed and size class distribution per 50 AU was determined.
Measurements of mitochondrial DNA: Mitochondrial DNA (mtDNA) was isolated from iNOS and WT mice and quantified by both semiquantitative and quantitative PCR as described previously. Briefly, ~25 mg of frozen heart from iNOS TG mice and WT was processed for DNA extraction with DNeasy kit as per manufacturer’s instructions (Qiagen, Valencia CA). Total DNA content was determined fluorometrically. PCR was carried out at 94 °C for a hot start, a 94 °C heat denaturation for 40 seconds, a 52 °C annealing step for 40 °C seconds, a 72°C extension step for 1 minute for a total of 30 cycles with 7 minutes at 72°C after the final cycle before being held at 4°C, using primers for cytochrome b (Forward 5’GGATCAAAACACCCCAACGG3’, Reverse 3’GTATGCCTAAGATGCGAGT5’) and GAPDH (Forward 5’AACTTGCCATTGGAAGG3’, Reverse 3’TCTGTAGTAGGGACGTAGG3’). PCR products were electrophoresed on 2% agarose gel with 1μg/μl ethidium bromide. Bands were quantified using NIH Image J software. To determine mtDNA content, cytochrome b signal was normalized to GAPDH DNA. For quantitative PCR (qPCR), similar running conditions utilizing Sybergreen IQ supermix (Biorad, Hercules CA) were used to quantify relative mtDNA content via cytochrome b/gapdh signal. All assays were performed on a Biorad iCycler optical module with iCycler IQ version 3.1 interface software.

Statistical analysis: Data are reported as mean ± SEM. Comparisons between two groups were performed with unpaired Student’s t-tests. Comparisons among multiple groups or between two groups at multiple time-points were performed by
either one-way or two-way ANOVA, as appropriate, followed by paired or unpaired Student's t-tests with the Bonferroni correction.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. 1: Levels of NOx and iNOS in wild-type (WT) and iNOS-transgenic (TG) mice. (A) Total myocardial NOx content in WT and iNOS-TG mice (n=6) and (B) Total iNOS protein expression in WT and transgenic hearts as determined by Western blot analysis (n=6; * P < 0.05 versus WT).

Supplemental Fig. 2: Measurement of mitochondrial morphology, size, and number in iNOS-TG and WT hearts. (A) Transmission electron micrographs of glutaraldehyde-fixed left ventricular papillary muscle from iNOS-TG and WT mouse hearts (x1,900). (Inset) Higher magnification TEM (x9,800) of mitochondria. (B) Quantification of the size and number of mitochondria visualized by EM. Mitochondria (800-1000 per heart) were measured by size class using MetaMorph software. Arbitrary units represent pixel number as estimations of mitochondrial area. The number of mitochondria in each size class was then calculated. (Inset) Citrate synthase activity was comparable in heart homogenates from WT and iNOS-TG mice and was used as an index of mitochondrial abundance (n=4; N.S. no significant difference).

Supplemental Fig. 3: Transgenic expression of iNOS does not stimulate mitochondrial biogenesis. (A) Representative Western blots using anti-iNOS, anti-cytochrome c, and anti-cytochrome c oxidase (CcO-IV) antibodies. Quantification of abundance of cytochrome c oxidase (B) and cytochrome c (C)
in iNOS versus WT hearts. (*P<0.05 versus WT; n=3-7 per group). (D) Changes in the abundance of cytochrome b transcript in WT and iNOS-TG hearts. The corresponding gel is shown in panel (E). The ratio of mitochondrial to nuclear DNA was calculated as the ratio of cytochrome b DNA to GAPDH DNA quantity. (N.S. – no significant difference).

Supplemental Fig. 4: Oxygen consumption and the abundance of mitochondrial complexes in WT and TG hearts. (A) Clarke electrode recordings from mitochondria isolated from WT and iNOS-TG hearts. State 4 respiration was measured by adding Complex I substrates (10 mmol/L pyruvate and 5 mmol/L malate) and State 3 respiration was measured following the addition of 0.3 mmol/L ADP. The rate of oxygen consumption was calculated from the slope of tracing as indicated. (B) Group data for state 3:state 4 ratio and ADP:O ratio of WT and TG mitochondria. (C) Representative native-PAGE gel of respiratory complexes of mitochondria isolated from WT and iNOS-TG hearts. The outer membrane of the mitochondria was disrupted prior to loading. The proteins were separated on a 4-12 % gradient gel and stained with Coomassie blue. The major respiratory complexes I-IV and V (ATP synthase) are indicated based on the expected molecular weight of individual macromolecular complexes. (D) Group data showing the abundance of individual complexes in WT and iNOS TG mitochondria. (E) cytochrome c oxidase (complex IV) activity in WT and iNOS-TG hearts determined using a kit from Sigma Chemical Co as per manufacturers instructions. * P < 0.05 (n = 4 separate hearts in each group).
Supplemental Fig. 5: The generation of reactive oxygen species (ROS) from mitochondria isolated from WT and iNOS-TG hearts. Mitochondria isolated from WT and TG hearts were incubated with Amplex Red (10 μmole/L) and H$_2$O$_2$ production was measured at excitation/emission wavelengths of 535 and 580 nm, respectively, for 10 min in the presence of (A) 1.25 mmol/L malate and 2.5 mmol/L glutamate or (B) 2.5 mmol/L succinate and 2.5 μmol/L rotenone. Rotenone (2.5 μmol/L) and antimycin A (0.5 μmol/L) were added to maximize ROS production and changes in fluorescence were measured for an additional 10 min. No significant difference was observed between iNOS-TG and WT mitochondria (n = 4 separate hearts in each group).

Supplemental Fig. 6: Inducible NOS is required for inhibition of MPT by DETA/NO preconditioning. (A) I/R-induced myocardial NAD$^+$ depletion in vehicle (PBS) or DETA/NO-preconditioned WT or iNOS-null mice. Mice were pretreated with PBS or DETA/NO and 24 h later their hearts were excised, perfused with aerobic buffer, and then subjected to 30 min ischemia and 30 min of reperfusion. Bars represent NAD$^+$ levels in I/R hearts expressed as a percentage of sham hearts. *P<0.05 versus WT-DETA/NO I/R; n=4; **P<0.001 versus WT sham. (B) Slot blot analysis of protein adducts of the lipid peroxidation product 4-hydroxy-trans-2-nonenal (HNE) from control (untreated), vehicle-treated, and DETA/NO-treated hearts subjected 24 h later to 30 min of ischemia, followed by 30 min of reperfusion (I/R). LV tissue was homogenized
and slot blots were developed using anti-protein-HNE antibodies\textsuperscript{8}. * P < 0.002 versus control and # P< 0.005 versus vehicle-treated hearts subjected to I/R.

**Supplemental Fig. 7: Effect of cyclosporin A on ischemia-reperfusion (I/R) injury.** Isolated WT hearts were subjected to 50 min perfusion only or 30 min of global ischemia and 20 min of reperfusion were treated without or with cyclosporin A (CsA) added before ischemia and changes in (A) developed pressure; (B) end-diastolic pressure; (C) left ventricular dP/dt; (D) total lactate dehydrogenase (LDH) and (E) total creatine kinase (CK) release were measured. Note that functional recovery was better in CsA-treated WT I/R hearts (n=3) vs WT I/R alone (n=3) hearts (* P < 0.05 vs I/R alone; #, P < 0.01 vs WT perfused only).

**Supplemental Fig. 8: Effect of cyclosporin A on ischemia-reperfusion (I/R) injury and mitochondrial permeability transition.** Isolated WT hearts were subjected to 30 min of global ischemia and 20 min of reperfusion without or with cyclosporin A (CsA) and changes in: (A) cytosolic levels of cytochrome c, (B) mitochondrial retention of [\textsuperscript{3}H]-deoxyglucose (DOG), and (C) NAD\textsuperscript{+} level were measured as described in the text. * P <0.05 (n = 3 hearts in each group). Note: Cyclosporin A decreased I/R-injury and prevented I/R-induced release of cytochrome c and NAD\textsuperscript{+} depletion from the mitochondria into the cytosol, although no change in mitochondrial retention of DOG was observed.


**Supplemental Fig. 9: Effect of atractyloside on ischemia-reperfusion (I/R)-induced injury.** Isolated WT and iNOS-TG hearts were subjected to 30 min of global ischemia and 20 min of reperfusion were treated with 1mmol/L atractyloside added before ischemia and changes in (A) developed pressure; (B) end-diastolic pressure; (C) left ventricular dP/dt; (D) total lactate dehydrogenase (LDH) and (E) creatine kinase (CK) release were measured. Note that functional recovery was better and the release of LDH and CK was less in iNOS-TG hearts (n= 5) vs WT (n=4) hearts (*, P < 0.05).

**Supplemental Fig. 10: Mitochondrial iNOS activity and sub-fractional association in iNOS-TG mice.** (A) Total myocardial NOx content in WT and iNOS-TG mice (n=6); (B) Mitochondrial iNOS protein expression in WT and transgenic hearts as determined by Western blot analysis (n=6); (C) Total and mitochondrial iNOS activity in WT and iNOS-TG hearts as determined by measuring the formation of L-citrulline (n=6).

**Supplemental Fig. 11: Ischemic preconditioning-induced mitochondrial association of iNOS.** Mitochondria were isolated from the anterior LV wall or the ischemic zone 24 h after sham operation (control) or ischemic PC. Isoform-specific NOS expression in the mitochondria was determined by immunoblotting using antibodies raised against iNOS (A), eNOS (B), or nNOS (C). Crude brain extracts were used as a positive control for nNOS. (D) Relative increase in iNOS protein in mitochondrial fractions from control and preconditioned hearts (*P<0.05
versus control, n=4). Ischemic preconditioning was induced as described before$^{4,5}$ and changes in NOS levels were measured 24 h after ischemia.
Reference List


