Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction

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Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction. Am J Physiol Heart Circ Physiol 289: H2616–H2623, 2005. First published July 29, 2005; doi:10.1152/ajpheart.00546.2005.—Using inducible nitric oxide (NO) synthase (iNOS) knockout mice (iNOS−/−), we tested the hypotheses that 1) lack of iNOS attenuates cardiac remodeling and dysfunction and improves cardiac reserve postmyocardial infarction (MI), an effect that is partially mediated by reduction of oxidative stress due to reduced interaction between NO and reactive oxygen species (ROS); and 2) the cardioprotection afforded by iNOS deletion is eliminated by Nω-nitro-L-arginine methyl ester (l-NAME) due to inhibition of endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS). MI was induced by ligation of the left anterior descending coronary artery. Male iNOS−/− mice and wild-type controls (WT, C57BL/6J) were divided into sham MI, MI + vehicle, and MI + l-NAME (100 mg·kg−1·day−1) in drinking water for 8 wk. Cardiac function was evaluated by echocardiography. Left ventricular (LV) maximum rate of rise of ventricular pressure divided by pressure at maximum (dP/dt max) improvement was evaluated by echocardiography. Left ventricular (LV) was determined by histopathological and immunohistochemical staining. We found that the MI-induced increase in LV chamber dimension and the decrease in ejection fraction, an index of systolic function, were less severe in iNOS−/− compared with WT mice. l-NAME worsened LV remodeling and dysfunction further, and these detrimental effects were also attenuated in iNOS−/− mice, associated with better preservation of cardiac function. Lack of iNOS also reduced nitrotyrosine and 4-hydroxy-2-nonenal (4-HNE), markers for ROS, after MI, whereas Feng et al. (11) found a significant increase in survival and improved cardiac function after MI in iNOS−/− mice.

In the present study, we tested the hypothesis that a lack of iNOS limits or ameliorates progression of cardiac dysfunction and remodeling as well as oxidative stress post-MI. To further study whether this protective effect is due to an upregulation of constitutive NO in iNOS−/− mice, we used the NOS inhibitor Nω-nitro-L-arginine methyl ester (l-NAME) and tested whether the beneficial effect of iNOS deficiency is offset by l-NAME. Because NO reportedly plays an important role in β-adrenergic responsiveness in HF (3, 8, 9), we also examined the effects of l-NAME on the myocardial contractile response to isoproterenol (ISO) in both strains of mice with HF.

METHODS

Mice

Male iNOS−/− and WT controls (C57BL/6J) were purchased from Jackson Laboratories. Animals were housed in an air-conditioned

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ROLE OF iNOS IN CARDIAC FUNCTION AND REMODELING POST-MI
H2617

room with a 12-h:12-h light-dark cycle, received standard mouse chow, and drank tap water. This study was approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Induction of MI

Male mice 10–12 wk of age were anesthetized with pentobarbital sodium (50 mg/kg ip), intubated, and ventilated with room air using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space, the heart was exposed, and the pericardium was opened as described previously (41). The left anterior descending coronary artery (LAD) was ligated with an 8-0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was deemed successful when the anterior wall of the left ventricle (LV) became cyanotic and the ECG showed obvious ST segment elevation. The lungs were inflated by increasing positive end-expiratory pressure, and the thoracotomy site was closed. Sham-operated mice were subjected to the same procedure, except that the suture around the LAD was not tied. Animals were kept on a heating pad until they awoke.

Experimental Protocol

Four weeks after sham or coronary ligation, when mice with MI developed HF, iNOS−/− and WT mice were divided into sham ligation, MI+vehicle, and MI+t-L-NAME (100 mg·kg−1·day−1 in drinking water). This dose was sufficient to inhibit NO production when measuring urinary nitrate and nitrite (NOx) (data not shown). Treatment was maintained for 8 wk.

Systolic blood pressure. Systolic blood pressure (SBP) was measured in conscious mice using a noninvasive computerized tail-cuff system (BP-2000; Visitech Systems; Apex, NC) as described previously (23). Mice were trained for 7 days by daily measurements of SBP, which was recorded before coronary artery ligation and weekly thereafter. Three sets of ten measurements were made for each recording.

Echocardiography. Cardiac geometry and function were evaluated with a Doppler echocardiograph equipped with a 15-MHz linear transducer (Acuson c256; Mountain View, CA) as described previously (42). All studies were performed on conscious mice before MI and monthly thereafter. Ejection fraction (EF) was obtained from the short-axis view and calculated as [(LVDA − LVSA)/LVDA] × 100, where LVDA is LV diastolic area and LVSA is LV systolic area. Measurements were traced manually and digitized by goal-directed, diagnostically driven software installed within the echocardiograph. Three beats were averaged for each measurement.

LV function response to isoproterenol challenge. After 8 wk of vehicle or t-L-NAME treatment, mice were anesthetized, placed on a warm pad (37°C), and ventilated. A 1.4-Fr. micromanometer pressure catheter (Millar Instruments, Houston, TX) was advanced into the LV as described previously (7). The maximum rate of rise of ventricular pressure divided by the pressure at the moment such maximum occurs ([dP/dt]max/instant pressure [P]) as an index of isovolumic contraction was measured at baseline and in response to the β-adrenergic agonist isoproterenol (Iso; 100 ng/min for 5 min iv).

Histopathological Study

Heart weight and infarct size. At the end of the study, the heart was stopped during diastole by injecting 15% potassium chloride solution, then excised, and weighed. The LV was sectioned transversely into three slices from apex to base, rapidly frozen in isopentane precooled in liquid nitrogen, and stored at −70°C. To measure infarct size, a section from each slice was stained with Gomori trichrome. The infarcted portion of the LV was measured as described previously (40).

Myocyte cross-sectional area and interstitial collagen fraction. Six-micrometer sections from each slice were double-stained with fluorescein-labeled peanut agglutinin [to delineate the myocyte cross-sectional area (MCSA) and the interstitial space] and rhodamine-labeled Griffonia simplicifolia lectin I (to show the capillaries). Four radially oriented microscopic fields from each section of the noninfarcted area were photographed at a magnification of ×400. MCSA was measured by computer-based planimetry and averaged by using data obtained from all photographs. Interstitial collagen fraction (ICF) was measured with computer-assisted videodensitometry (Jandel; Corte Madera, CA) (26).

Expression of nitrotyrosine and 4-hydroxy-2-nonenal. Expression of nitrotyrosine (a marker for OONO−) and 4-hydroxy-2-nonenal (4-HNE, a by-product of lipid peroxidation and an indicator for oxidative stress) was determined by immunohistochemical staining. Briefly, 6-μm frozen sections were fixed with cool acetone. Endogenous peroxidase activity was blocked by 0.3% H2O2, and nonspecific binding was blocked by 5% normal goat serum. For OONO− detection, the sections were incubated with polyclonal anti-nitrotyrosine antibody (Sigma) at a 1:200 dilution overnight at 4°C. They were then incubated with biotinylated anti-rabbit IgG (secondary antibody) for 30 min, followed by avidin-biotin complex (ABC) reagent for 30 min and finally 3-amin-9-ethycarbazol (AEC) substrate (Vector) to detect immunoreactivity. Sections were counterstained with hematoxylin. For 4-HNE, monoclonal mouse anti-monoclonal anti-4-HNE antibody (Oxis) was used to detect lipid peroxidation as a result of ROS generation. The sections were fixed with cool acetone for 30 min and boiled in 10 mM citric acid buffer for 10 min for antigen retrieval. They were incubated in 0.3% H2O2 in PBS for 5 min and then with mouse IgG blocking reagent for 1 h and 5% normal goat serum for 30 min to block nonspecific binding. Afterward, they were incubated with primary antibody (1 μg/ml) at room temperature for 30 min, followed by biotinylated secondary antibody for 10 min and ABC reagent for 5 min. The immunoreaction was visualized by using AEC (Vector). Negative controls were incubated with the matching IgG isotype at the same concentration. Twelve color images of nitrotyrosine and 4-HNE staining were selected from three sections of the heart and viewed at ×400 magnification. For semiquantification, three blinded observers scored the area and intensity of staining. The scoring range was the following: 0, no visible staining; 1, faint staining; 2, moderate staining; and 3, strong staining.

iNOS protein expression. Hearts were homogenized in lysis buffer and protease inhibitors and incubated on ice for 30 min. After centrifugation, protein content was detected by using a Bio-Rad protein assay kit. Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in TBS-T (20 mM Tris, 137 mM NaCl, and 0.1% Tween 20) and incubated on ice for 30 min to block nonspecific binding. Afterward, they were incubated with primary antibody (1 μg/ml at room temperature for 30 min, followed by biotinylated secondary antibody for 10 min and ABC reagent for 5 min. The immunoreaction was visualized by using AEC (Vector). Negative controls were incubated with the matching IgG isotype at the same concentration. Twelve color images of nitrotyrosine and 4-HNE staining were selected from three sections of the heart and viewed at ×400 magnification. For semiquantification, three blinded observers scored the area and intensity of staining. The scoring range was the following: 0, no visible staining; 1, faint staining; 2, moderate staining; and 3, strong staining.

Data analysis. Data are means ± SE. Two strains of mice (iNOS−/− and WT mice) were used to compare the effect of treatments (sham, HF+vehicle, and MI+t-L-NAME) on SBP, heart rate (HR), cardiac function, heart weight (HW), and morphological changes. Student’s two-sample t-test was used to compare differences between groups, either between strains or within a given strain. Hochberg’s step-up procedure was used to adjust the P values. The type one error rate was set at α = 0.05. The difference is considered statistically significant when P < α.

RESULTS

Mortality

Of the 30 WT mice that underwent LAD ligation, 2 mice (6.7%) died after surgery and 5 mice died of cardiac rupture...
within a week, for a rupture rate of 17.9%. Of the 28 iNOS−/− mice that underwent coronary ligation, none died after surgery, whereas 2 mice died of cardiac rupture, for a rupture rate of 7.1%. None of the sham-ligated mice died.

**Body weight, HW, and Infarct Size**

There were no significant differences in body weight, LV weight (LVW), right ventricle weight (RVW), or total HW between strains in the sham-ligated groups. Neither coronary artery ligation nor L-NAME had any effect on body weight. MI caused a significant increase in LVW, RVW, or HW in both WT and iNOS−/− mice, and no difference was detected between strains. L-NAME increased all these parameters further, particularly in WT mice (LVW and HW, P < 0.01; RVW, P < 0.05 vs. MI+ vehicle); this effect was not statistically significant in iNOS−/− mice (P < 0.05, WT vs. iNOS−/−). Infarct size was similar among groups or between strains in mice with or without L-NAME (Table 1).

**SBP and HR**

There were no significant differences in basal SBP and HR among groups. SBP fell significantly after MI and more so in iNOS−/− than in WT mice. L-NAME increased SBP in both strains compared with vehicle. No difference between strains was detected. HR was similar among groups (Table 1).

### Cardiac Function and Remodeling

EF and LV diastolic dimension (LVDd) were similar for sham WT and iNOS−/− mice. MI decreased EF and increased LVDd in both iNOS−/− and WT mice compared with sham mice, and the decrease in EF tended to be greater in WT than in iNOS−/− mice, though it did not reach statistical significance. L-NAME decreased EF and increased LVDd further in both strains; however, the decreases in LV function and chamber dilatation were less severe in iNOS−/− mice compared with WT mice (Fig. 1). The dp/dt/iP response to Iso was similar in both strains with and without MI; however, in the presence of L-NAME, this β-adrenergic response was significantly blunted in WT with MI mice (P < 0.001 vs. HF+ vehicle) but well preserved in iNOS−/− mice (Fig. 2).

### Myocyte Size and Interstitial Collagen Deposition

ICF and MCSD were similar between strains in the sham groups and increased significantly and similarly after MI in both strains. L-NAME increased ICF and MCSD further in WT mice (ICF, P < 0.01; MCSA, P < 0.05 vs. MI+ vehicle), but

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**Table 1. Body weight, BP, HR, HW, and IS 2 mo after MI or sham MI**

<table>
<thead>
<tr>
<th>Genotype Treatment</th>
<th>WT</th>
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<tr>
<td></td>
<td>Sham</td>
<td>MI+ Vehicle</td>
<td>MI+L-NAME</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Body wt, g</td>
<td>28.9 ± 0.6</td>
<td>29.5 ± 0.4</td>
<td>28.9 ± 0.5</td>
<td></td>
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<tr>
<td>BP, mmHg</td>
<td>106 ± 5</td>
<td>101 ± 2</td>
<td>113 ± 2e</td>
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<tr>
<td>HR, beats/min</td>
<td>666 ± 18</td>
<td>651 ± 18</td>
<td>632 ± 12</td>
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<tr>
<td>LVW, mg/10 g body wt</td>
<td>30.6 ± 0.6</td>
<td>36.7 ± 1.6b</td>
<td>44.1 ± 1.6d</td>
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<tr>
<td>RVW, mg/10 g body wt</td>
<td>7.7 ± 0.2</td>
<td>8.6 ± 0.4c</td>
<td>10.5 ± 0.6b</td>
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<tr>
<td>HW, mg/10 g body wt</td>
<td>42.3 ± 0.9</td>
<td>50.5 ± 2.3b</td>
<td>61.9 ± 2.6d</td>
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<tr>
<td>IS, %</td>
<td>28.5 ± 3.9</td>
<td>30.9 ± 4.0</td>
<td>30.1 ± 2.9</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>12</td>
<td>14</td>
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<tr>
<td>Body wt, g</td>
<td>27.9 ± 0.4</td>
<td>28.3 ± 0.4</td>
<td>28.7 ± 0.5</td>
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<tr>
<td>BP, mmHg</td>
<td>113 ± 3</td>
<td>104 ± 2e</td>
<td>120 ± 4c</td>
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<tr>
<td>HR, beats/min</td>
<td>717 ± 19</td>
<td>677 ± 10</td>
<td>664 ± 8</td>
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<tr>
<td>LVW, mg/10 g body wt</td>
<td>31.1 ± 0.6</td>
<td>37.3 ± 1.7b</td>
<td>40.1 ± 0.9e</td>
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<td>RVW, mg/10 g body wt</td>
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<tr>
<td>HW, mg/10 g body wt</td>
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<td>51.4 ± 2.3b</td>
<td>55.0 ± 1.2d</td>
<td></td>
</tr>
<tr>
<td>IS, %</td>
<td>31.9 ± 2.1</td>
<td>30.1 ± 2.9</td>
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</tr>
</tbody>
</table>

Values are means ± SE; n, number of mice. WT, wild-type control mice; iNOS−/−, inducible nitric oxide synthase knockout mice; MI, myocardial infarction; L-NAME, N-nitro-l-arginine methyl ester; BP, blood pressure; HR, heart rate; LVW, left ventricle weight; RVW, right ventricle weight; HW, heart weight; IS, infarct size. *P < 0.05; †P < 0.01 vs. sham within strains; ‡P < 0.05; ‡‡P < 0.01; ‡‡‡P < 0.001 vs. MI+ vehicle within strain; ††P < 0.05 between strains given the same treatment.

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**Fig. 1.** Comparison of left ventricular (LV) ejection fraction (EF, left) and diastolic dimension (LVDd, right) in inducible nitric oxide synthase (iNOS) knockout mice (iNOS−/−) and their wild-type controls (WT) with sham myocardial infarction (sham MI) or MI-treated with vehicle (MI+Veh) or N-nitro-l-arginine methyl ester (MI+L-NAME) mice. *P < 0.05; ††P < 0.001.

**Fig. 2.** Changes in the maximum rate of rise of ventricular pressure divided by the pressure at the moment such maximum occurs (dp/dt/iP) after isoproterenol challenge in iNOS−/− mice and WT controls subjected to sham MI or MI+Veh or MI+L-NAME. *P < 0.05.
this effect was absent in iNOS−/− mice (P < 0.01 vs. WT) (Fig. 3).

**Nitrotyrosine and 4-HNE Expression**

Immunohistochemical studies showed little nitrotyrosine expression in the myocardium in any of the sham groups. There was a significant increase after MI in both strains, particularly in WT mice (P < 0.001 vs. iNOS−/−). L-NAME significantly decreased nitrotyrosine expression in WT mice (P < 0.01) (Fig. 4). 4-HNE was also significantly increased post-MI in both strains, though it was marginally greater in WT mice (P = 0.073). L-NAME did not affect 4-HNE expression in either strain, though once again it was marginally greater in WT mice (P = 0.075) (Fig. 5).

**iNOS Protein Expression in Hearts**

Western blot analysis showed that iNOS was weakly expressed in the sham WT group but increased significantly after MI. iNOS expression was absent in iNOS−/− mice with or without MI (Fig. 6, left). eNOS expression did not differ

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**Fig. 3.** Comparison of LV interstitial collagen fraction (ICF, left) and myocyte cross-sectional area (MCSA, right) in iNOS−/− mice and WT controls subjected to sham MI or MI+Veh or MI+L-NAME. *P < 0.05; **P < 0.01; ***P < 0.001.

**Fig. 4.** Representative nitrotyrosine staining (A, positive staining shown in brown) and scoring of expression (B) in noninfarcted LV myocardium from WT controls and iNOS−/− 3 mo after sham MI or MI+Veh or MI+L-NAME. **P < 0.01; ***P < 0.001.
between strains with sham MI. MI increased eNOS protein expression similarly in both strains (Fig. 6, right).

**DISCUSSION**

We found that 1) under normal conditions, SBP and cardiac morphology and function in iNOS**/-** mice were not different from their WT controls, suggesting that iNOS may not play an important role in regulating basal cardiac hemodynamics and function; 2) although cardiac dysfunction and remodeling post-MI tended to be less severe in iNOS**/-** mice, differences in LVEF, chamber dilatation, myocyte hypertrophy, and interstitial fibrosis between strains did not reach statistical significance; 3) MI induced profound expression of nitrotyrosine and 4-HNE, markers for oxidative stress, which were attenuated in iNOS**/-** mice; and 4) with L-NAME treatment, iNOS**/-** mice showed less severe cardiac hypertrophy, chamber dilatation, and LV dysfunction as well as a greater contractile response to Iso.

**Effect of iNOS Deficiency on Cardiac Remodeling and Dysfunction Post-MI**

Evidence indicates that after MI, increased iNOS mRNA expression peaks at 3 days and then declines in the infarct region, whereas in the noninfarcted myocardium it peaks at 14–28 days and remains elevated, suggesting that iNOS is involved in both early and late remodeling post-MI (35). Increased iNOS activity has also been reported in various forms of HF (17, 19, 31, 35). We found that iNOS protein expression in the noninfarcted myocardium was significantly increased 3 mo after MI in the WT controls. However, the pathophysiological significance of iNOS induction in HF is not clear. We and others have shown that pharmacological inhibition of iNOS significantly reduced infarct size in rats with acute myocardial ischemia (38) and prevented cardiac dysfunction and heart failure post-MI (31). In the present study, we found that mice lacking iNOS had better preserved cardiac function and tended to have less severe chamber dilatation and myocyte hypertrophy post-MI. Feng et al. (11) and Sam et al. (32) reported that iNOS knockout mice had decreased mortality, improved LV function, lower plasma nitrate, and reduced programmed cell death during both the acute and chronic phase of MI. Furthermore, using transgenic mice overexpressing iNOS, Mungrue et al. (29) found that overexpression of iNOS led to increased ONOO⁻ production and sudden death due to heart block. Taken together, these data indicate that increased
NO production from iNOS promotes myocyte death, myocardial remodeling, and dysfunction (2, 22, 33). However, there are conflicting reports showing that deficiency of iNOS neither reduces mortality nor attenuates severity of HF in mice with MI (20), nor does an overexpression of iNOS have a detrimental effect on the heart (18). Although we have no definitive explanation for the different findings regarding the role of iNOS in HF, variable infarct size, genetic background of transgenic lines, and stage of HF should all be taken into consideration when interpreting the data.

**Induction of iNOS and Oxidative Stress**

Oxidative stress has been implicated in structural abnormalities in HF post-MI, causing loss of contractile function (11, 29). These detrimental effects may be due to persistent high amounts of NO reacting with superoxide anion, generating the highly reactive oxidant OONO\(^{-}\). In vitro studies have shown that iNOS not only produces NO but is also capable of generating superoxide independently of NO (39). Moreover, not only is superoxide itself toxic to the heart, but it also leads to the formation of H\(_2\)O\(_2\), which may also be toxic (5, 16, 24, 29). Thus blockade or deletion of iNOS may decrease not only NO production but also formation of superoxide, in turn reducing generation of H\(_2\)O\(_2\). It is important to point out that H\(_2\)O\(_2\) and its oxidizing metabolites can promote lipid peroxidation. In the present study, we found that nitrotyrosine and 4-HNE, markers of OONO\(^{-}\)/H\(_2\)O\(_2\) production and lipid peroxidation, were significantly increased post-MI, associated with a worsening of cardiac function and remodeling. Moreover, the increase in nitrotyrosine was much less pronounced in iNOS\(^{-/-}\) mice and correlated with better cardiac function (LV shortening fraction). Interestingly, although NOS inhibition with L-NAME decreased nitrotyrosine expression in WT mice (as would be expected from inhibition of NO production), cardiac function and remodeling were even worse. In fact, L-NAME would be expected to inhibit NO and potentially superoxide coming from NOS. However, because we did not see reduced 4-HNE expression, a marker for lipid peroxidation and general oxidation, our data seem to suggest that in this model, superoxide and its attendant oxidative metabolites are derived from another enzymatic source than NOS. Furthermore, by lowering steady-state NO levels, L-NAME could potentially lengthen the half-life of tissue superoxide and promote its conversion to other deleterious oxidants. Indeed, many studies (15, 25, 27, 30) have suggested a role for NADPH oxidase and mitochondrial oxidases in cardiac ROS production and toxicity. The role of NADPH oxidases in this response requires further investigation.

**Mice With iNOS Deficiency Have Better Cardiac Reserve Under NOS Inhibition**

One characteristic of the failing heart is attenuated inotropic responsiveness to \(\beta\)-adrenergic stimulation, probably due to downregulation of \(\beta_1\)- and \(\beta_2\)-adrenergic receptors. Alteration of the balance between \(\beta_3\) and the cAMP-linked \(\beta_1\) and \(\beta_2\) receptors has also been implicated in the pathophysiology of HF (4, 10). Recent studies (14, 37) demonstrated that eNOS and nNOS are part of the signaling cascade for the \(\beta_3\)-adrenergic receptor, contributing to a negative inotropic effect. Studies have shown that increased nNOS-derived NO production in patients and experimental animals with HF is associated with decreased cardiac contractility and that inhibition of nNOS enhanced the LV contractile response to \(\beta\)-adrenergic stimulation in rats with HF (3, 8, 9), whereas disruption of iNOS improved \(\beta\)-adrenergic inotropic responsiveness (13). In the present study, we found that mice lacking iNOS have less severe cardiac hypertrophy, dilatation, and dysfunction and better preserved cardiac contractile function when constitutive NOS isoforms are inhibited. Although the precise mechanisms are not understood, it is possible that L-NAME could eliminate...
the decreased β-adrenergic response mediated by other NOS isoforms, thereby increasing cardiac contractility. This needs to be studied further.

In summary, our study provides experimental evidence that a lack of iNOS does not significantly reduce LV dilatation, dysfunction, myocyte hypertrophy, and fibrosis post-MI. However, these parameters tended to be less severe in iNOS−/− mice, associated with decreased oxidative stress as evidenced by decreased expression of nitrotyrosine and 4-HNE. In the presence of l-NAME, iNOS−/− mice had less severe cardiac remodeling and dysfunction and a greater contractile response to β-adrenergic stimulation. We conclude that iNOS may not play an important pathological role in HF; however, lack of iNOS improves cardiac reserve post-MI, particularly when constitutive NOS isoforms are blocked. Decreased oxidative stress and other adaptive mechanisms independent of NOS may be partially responsible for such an effect, which needs to be studied further.

REFERENCES


