Deficiency of iNOS does not attenuate severe congestive heart failure in mice

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ALTHOUGH SOME ADVANCES have been made in the treatment of heart failure, the prognosis of congestive heart failure (CHF) patients remains ominous. Despite such prevalence and severity, many of the pathogenic mechanisms are unappreciated or entirely misunderstood. Nevertheless, several lines of evidence indicate that certain genes may be upregulated during this process and consequently contribute to the pathogenesis of heart failure. The ability to understand the individual contributions of each of these genes may energize the development of targeted therapeutics and decrease the widespread nature of CHF.

One potential contributor to the progression of CHF is nitric oxide (NO) derived from the inducible form of NO synthase (iNOS). Since its identification as an endothelium-derived relaxing factor (EDRF) in 1980 (11), NO has been implicated in numerous pathological sequelae as both a beneficial and deleterious moiety. NO is constitutively synthesized at low levels by the vascular endothelium (endothelial NOS) with the general effect of maintaining vascular homeostasis (15). However, iNOS-derived NO has specifically been implicated as a contributor to the development of heart failure (6, 20, 22). Indeed, biopsies from human hearts with dilated cardiomyopathy (4, 5, 13) and ischemic cardiomyopathy (13) demonstrate robust iNOS expression. The upregulation of iNOS in heart failure is thought to contribute to the pathophysiology of CHF, and iNOS upregulation is thought to be the result of the generation of several proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interferon-γ (10). These inflammatory cytokines impair vascular endothelial function and exacerbate the pathophysiology of CHF by limiting coronary blood flow. It has been suggested that overproduction of NO by iNOS during CHF aggravates the severity of CHF (6–8). However, the potential pathological targets of iNOS in CHF are numerous.

We hypothesized that genetic ablation of iNOS would attenuate the severity of CHF and improve survival in mice. We subjected iNOS-deficient (iNOS−/−) mice to CHF and assessed CHF pathophysiology in terms of ventricular morphology, cardiac function, and survival to ascertain the role of iNOS in severe CHF.

METHODS

Mice. Male, C57BL/6 (wild type, n = 73) and iNOS−/− (n = 56) mice were purchased from Jackson Laboratory (Bar Harbor, ME). The wild-type mice were randomized to sham occlusion (n = 17) of the coronary artery or permanent occlusion (n = 56) of the coronary artery. The iNOS−/− mice were also randomized to sham (n = 8) or permanent occlusion (n = 48) of the coronary artery. Mice were entered into the CHF protocol at 3 mo of age. The individual performing the experiments was blinded to the mouse genotype until all data analyses were completed.

All experimental procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, Revised 1985), approved by the Council of the American Physiological Society, and with federal and state regulations. All experimental procedures were approved by the Louisiana State University Medical Center Animal Care and Use Committee.

Myocardial infarction protocol. Ligation of the left anterior descending coronary artery (LAD) was performed as described previously (16, 17). Mice were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg). Body temperature was maintained at 37°C using a rectal thermometer and infrared heating lamp. With the use of direct visualization through a fiber-optic ring light, the mice were orally intubated with polyethylene-90 tubing and connected via loose junction to a rodent ventilator (model 683, Harvard Apparatus). The ventilator was set at a tidal volume of 1.5 ml and a rate of 120 breaths/min and supplemented with

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100% oxygen. A median sternotomy was performed, and the proximal left anterior descending (LAD) coronary artery was visualized and ligated with a 7-0 silk suture mounted on a tapered needle (BV-1, Ethicon). The LAD coronary artery was ligated at a proximal location under the left atrial appendage. Ischemia was confirmed by the appearance of hypokinesis, pallor distal to the occlusion, and depressed fractional shortening according to echocardiography. The occlusion remained intact throughout the 4-wk protocol. In several experiments, ischemia was also confirmed by profound electrocardiographic changes (e.g., ST segment elevation). The chest wall was closed, and the mice were given subcutaneous butorphanol tartrate (0.1 mg/kg sc) were administered every 12 h for 3 days after the acute myocardial infarction (MI) procedure. Butorphanol tartrate (0.1 mg/kg sc) was administered every 12 h post MI for 3 days after the acute myocardial infarction (MI) procedure.

Assessment of iNOS protein. Wild-type and iNOS−/− mice (n = 3 mice/group) were subjected to the MI protocol. Three days after coronary ligation, hearts were harvested, rinsed, and snap frozen in liquid nitrogen. Whole hearts were homogenized, and proteins were extracted. Western blot analysis was performed according to conventional protocols. Briefly, 50 μg of total protein were loaded per sample. Even loading and transfer were confirmed by Ponceau and Coomassie stains. Primary iNOS antibody was used at a concentration of 1:4,000. Membranes were exposed to chemiluminescent (ECL) reagents, documented on ECL film, and scanned into a personal computer.

Evaluation of arterial and left ventricular hemodynamics. To assess the closed-chest hemodynamic status, a 1.4-Fr Millar (SPR-671, Millar; Houston, TX) pressure transduction catheter was inserted similar to methods described previously (16, 18). Mice were anesthetized with ketamine (50 mg/kg ip) and pentobarbital (50 mg/kg ip) and supplemented with oxygen via a nasal cone. The right common carotid artery was isolated, the catheter was inserted, and the catheter was advanced to the aorta. Approximately 10 s of data were recorded. Off-line assessment of these data yielded systolic blood pressure, diastolic blood pressure, mean arterial blood pressure, and heart rate.

The catheter was then advanced through the aortic valve into the left ventricle (LV). Approximately 10 s of data were recorded. Subsequent off-line evaluation provided LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), LV developed pressure, and the first derivative of the LV pressure curve (minimum and maximum dP/dt). Data for each animal were calculated from at least 10 s of chart recording (arithmetic mean).

Echocardiographic assessment of LV function. In vivo transthoracic echocardiography of the LV using a 15-MHz linear array transducer (15L8) interfaced with a Sequoia C256 (Acuson) was performed as described previously (16). The cardiac output values were corrected for the animals’ weights (in μL·min−1·g−1). All data were calculated from 10 independent cardiac cycles/experiment.

Pulmonary edema. Accumulation of pulmonary fluid was assessed by weighing the (wet) lungs from mice subjected to MI (or sham). The lungs were then placed in a drying oven (Econotherm Laboratory Oven, Precision) for 7 days at 40°C. The lungs were weighed, and the dry weights were recorded. The difference between the wet and dry weights yielded the pulmonary fluid accumulation value.

Statistical analyses. Data were analyzed by Student’s unpaired t-test or ANOVA with post hoc analysis (Bonferroni) using StatView (SAS Institute; Cary, NC) software. Data are reported as means ± SE with differences accepted as significant when P < 0.05.

**RESULTS**

**Myocardial infarct size in wild-type and iNOS−/− mice.** In a separate group of mice (n = 8 mice/group), myocardial infarct size was determined at 24 h after permanent proximal coronary artery ligation (Fig. 1). The area at risk per LV was similar [P = not significant (NS)] in the wild-type (48.0 ± 2.2%) and iNOS−/− mice (50.8 ± 1.9%). The infarct size per LV was 41.5 ± 2.2% in the wild-type group and 42.7 ± 1.5% in the iNOS−/− group (P = NS between groups). Finally, infarct size per area at risk was 86.5 ± 1.4% in wild-type mice and 84.5 ± 1.1% in the iNOS−/− mice (P = NS between groups).

**Assessment of iNOS protein.** Wild-type mice exposed to the MI protocol demonstrated expression of iNOS protein by 3 days after coronary occlusion. However, no iNOS protein was detectable in hearts from the iNOS−/− mice (Fig. 2, inset).

**Survival during CHF.** CHF induced a profound (P < 0.01) decrement in survival (Fig. 2) in the wild-type (32%) and iNOS−/− (35%) mice compared with sham wild-type (100%) and sham iNOS−/− (100%) mice. Deficiency of iNOS did not significantly (P = NS) affect survival compared with wild-type mice after 1 mo of CHF. The survival of wild-type mice at 1 mo did not differ from the survival at 6 mo (unpublished data, S. P. Jones and D. J. Lefer). Therefore, we would not expect any differences in survival at extended time points in our model, unlike findings from other groups using less severe models of heart failure (21).

**Hemodynamics during CHF.** Systemic and ventricular hemodynamics were measured after 1 mo of coronary artery occlusion (Table 1). Systemic hemodynamics were not significantly (P = NS) different among the sham groups, wild-type, and iNOS−/− mice subjected to the CHF protocol. Although LVSP was not significantly different among the three experimental groups, LVEDP was significantly (P < 0.05) higher in the wild-type and iNOS−/− hearts compared with sham hearts. In addition, LV developed pressure was significantly (P < 0.05) lower in the wild-type and iNOS−/− hearts compared with sham hearts.

**LV dimensions.** Occlusion of the LAD coronary artery produced a large anterior MI that led to significant changes in LV mass (Fig. 3A), chamber diameter (Fig. 3B), and fractional shortening (Fig. 3C). Despite large myocardial infarcts, both wild-type (6.5 ± 0.29 mg/g) and iNOS−/− (5.87 ± 0.40 mg/g) hearts demonstrated significant (P < 0.01) increases in heart-to-body weight ratios 1 mo after coronary artery occlusion compared with both sham groups. However, the extent of cardiac hypertrophy was not significantly (P = NS) different between the wild-type and iNOS−/− hearts.
With the use of Doppler flow analysis during echocardiography, cardiac output was measured in all groups of mice after 4 wk of MI (Fig. 5). Cardiac output was significantly ($P < 0.05$) diminished in wild-type (441 $\pm$ 20 $\mu$l min$^{-1}$.g$^{-1}$) but not iNOS$^{-/-}$ (471 $\pm$ 26 $\mu$l min$^{-1}$.g$^{-1}$) mice compared with sham groups. Cardiac output in wild-type and iNOS$^{-/-}$ was not significantly ($P = N S$) different between the two groups.

Pulmonary edema is associated with depressed cardiac function in the setting of infarct-induced LV failure. In Fig. 6, pulmonary edema is expressed as the absolute fluid weight in the lungs of sham and CHF mice. Wild-type (107 $\pm$ 2 mg) and iNOS$^{-/-}$ (104 $\pm$ 4 mg) mice exhibited significantly ($P < 0.05$) more pulmonary fluid accumulation than sham groups. However, there was no difference between wild-type and iNOS$^{-/-}$ groups.

**DISCUSSION**

The present study was designed to investigate the potential protective effects of genetic ablation of iNOS in a murine model of severe CHF after acute MI. The findings reported here are inconsistent with the idea that iNOS significantly contributes to the pathogenesis of severe CHF in mice. iNOS$^{-/-}$ mice did not exhibit improvements in survival, LV contractile performance, or pulmonary edema. Although these findings are in stark contrast to a sizeable body of evidence suggesting a deleterious role of iNOS in CHF, this difference may be due to the severity of the CHF model that we employed in the present study.

Numerous studies have demonstrated the presence and/or activity of iNOS in various forms of heart failure. However, the association between enhanced iNOS-derived NO production and CHF may merely have been the demonstration of a coincidental phenomenon. It is possible that iNOS becomes upregulated during CHF as a compensatory response to alterations in peripheral vascular resistance and/or tissue perfusion. Considering evidence suggesting iNOS is constitutively expressed during fetal life (1, 2), the myocardium may revert to a fetal gene program during heart failure and the iNOS gene may be upregulated merely as a consequence.

In a recent study (19), mice with targeted overexpression of iNOS exhibited a phenotype supportive of the idea that iNOS exerts negative cardiac contractile effects. In this study (19), overexpression of iNOS (without infarction) caused increased mortality, cell death, and conduction disturbances. Although this study provides support for the idea that iNOS exerts injurious cardiac effects, another (nearly simultaneous) study published by Heger et al. (14) found no significant negative effects in terms of cardiac function in iNOS-overexpressing mouse hearts. It is possible that there were significant differences in the amount of NO being produced in the two lines of

**Table 1. Systemic and LV hemodynamics in sham, WT, and iNOS$^{-/-}$ mice subjected to 4 wk of coronary artery occlusion**

<table>
<thead>
<tr>
<th></th>
<th>HR, beats/min</th>
<th>MABP, mmHg</th>
<th>SBP, mmHg</th>
<th>DBP, mmHg</th>
<th>LVSP, mmHg</th>
<th>LVEDP, mmHg</th>
<th>LVDP, mmHg</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>388 $\pm$ 10</td>
<td>78 $\pm$ 3</td>
<td>92 $\pm$ 4</td>
<td>64 $\pm$ 3</td>
<td>91 $\pm$ 3</td>
<td>0 $\pm$ 1</td>
<td>91 $\pm$ 3</td>
</tr>
<tr>
<td>WT</td>
<td>402 $\pm$ 12</td>
<td>71 $\pm$ 3</td>
<td>84 $\pm$ 3</td>
<td>60 $\pm$ 3</td>
<td>82 $\pm$ 4</td>
<td>10 $\pm$ 4*</td>
<td>72 $\pm$ 6*</td>
</tr>
<tr>
<td>iNOS$^{-/-}$</td>
<td>406 $\pm$ 18</td>
<td>76 $\pm$ 6</td>
<td>89 $\pm$ 6</td>
<td>65 $\pm$ 6</td>
<td>89 $\pm$ 4</td>
<td>14 $\pm$ 5*</td>
<td>75 $\pm$ 5*</td>
</tr>
</tbody>
</table>

Data are means $\pm$ SE; $n$ = 10–14 mice/group. Data were collected at 4 wk after coronary artery occlusion. HR, heart rate; MABP, mean arterial blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVSP, left ventricular (LV) systolic pressure; LVEDP, LV end-diastolic pressure; LVDP, LV developed pressure. *$P < 0.05$ vs. sham; otherwise, $P = N S$. 

*P = 0.05*
transgenic mice. In addition, the transgenic system or locus of integration into the genome may also have influenced the deleterious effects of iNOS overexpression in the Mungrue et al. (19) study. Nevertheless, our present findings coupled with those Heger and co-workers (14) cast serious doubt on the obligate role of iNOS-derived NO in the pathogenesis of cardiac dysfunction and heart failure. The Schrader laboratory subsequently performed additional informative studies with iNOS-overexpressing mice (12, 24). These studies revealed that myoglobin acts as a physiological barrier against the potential pathological effects of excessive iNOS production. Such studies provide additional support against the idea that iNOS-derived NO has the capacity to exert deleterious effects on cardiac function.

Although difficult to directly compare with our study, data regarding the potential protective effects of NOS inhibition in acute cardiogenic shock have been presented by Cotter et al. (3). Unlike our present study, such studies by Cotter et al. (3) involved small human populations with varied genetic/risk.
factor backgrounds. More problematic is the use of the pan-
NOS inhibitor N\textsuperscript{-}nitro-L-arginine methyl ester (\textsuperscript{l}-NAME). Be-
cause \textsuperscript{l}-NAME can inhibit all NOS isoforms, any results
would be difficult to interpret. Previous murine studies of
infarct-induced heart failure found the loss (23) of endothelial
NOS to exacerbate CHF and the overexpression (16) of endo-
thelial NOS to improve CHF outcomes. On the basis of these
studies, we would hypothesize that the use of \textsuperscript{l}-NAME in our
model would exacerbate the extent of heart failure. However,
the data would be difficult to evaluate because of the lack of
selectivity of \textsuperscript{l}-NAME for a particular NOS isoform coupled
with the potentially divergent roles of inducible and endothelial
NOS isoforms.

More relevant to our present study, Feng et al. (9) subjected
\textsuperscript{iNOS}\textsuperscript{−/−} mice to coronary artery occlusion for 1 mo and found
protective effects compared with wild-type mice. Interestingly,
the authors demonstrated improved contractile function and
survival in the \textsuperscript{iNOS}\textsuperscript{−/−} mice (9). Such findings lend credence
to the idea of \textsuperscript{iNOS} (and excess NO production) as a negative
modulator of contractile function during heart failure. Another
study (21) of \textsuperscript{iNOS}\textsuperscript{−/−} mice in heart failure did not find any
significant differences between \textsuperscript{iNOS}\textsuperscript{−/−} and wild-type mice at
1 mo postinfarction. However, the same authors (21) observed
significant improvement in ventricular function and survival in
\textsuperscript{iNOS}\textsuperscript{−/−} mice at 4 mo after coronary artery ligation compared
with wild-type mice. The percentage of mice surviving in the
present study at 1 mo is about 32\% compared with 75\%
reported by Sam et al. (21) in the previous study. Furthermore,
the infarct size per LV is \textasciitilde 42\% in the present study in both
wild-type and \textsuperscript{iNOS}\textsuperscript{−/−} mice compared with about 30\%
in the study by Sam et al. (21). Thus our model of CHF is much
more severe (35–50\% larger infarcts) than that of the other authors
(21). The fact that our model of CHF is more severe than that
previously reported with \textsuperscript{iNOS}\textsuperscript{−/−} mice may help to reconcile
the differences that were observed. That is, our findings pro-
vide solid evidence that \textsuperscript{iNOS} is not involved in the pathogen-
esis of severe, acute CHF, whereas Sam et al. (21) demon-
strated the deleterious consequences of \textsuperscript{iNOS} in a more mod-
erate infarct-induced heart failure model. Although the present
model induces severe heart failure in mice, we have recently
demonstrated that it is indeed possible to observe significant
improvements in survival in this model of CHF (16). In this
regard, transgenic mice that overexpress endothelial NOS
within the endothelium are protected against CHF after acute
MI. Thus the contention that our model is too severe to observe
improvements is invalid.

As with any experimental study in laboratory animals, study
limitations exist. The populations used in the present study do
not have the characteristics of human patients at risk of
developing heart failure. The mice used are healthy, adult (not
old) mice without any of the known risk factors for heart
disease. Future studies should incorporate the investigation of
animal models exhibiting clinically relevant risk factors. Also,
the use of anesthesia may also affect the interpretation of the
results. Specifically, anesthesia may negatively affect cardiac
function and make subtle differences difficult to document.

Severe, acute MI without reperfusion induces profound
cardiac dysfunction and decompensated CHF. Despite the
preponderance of evidence suggesting the contrary, ablation of
\textsuperscript{iNOS} does not affect the extent of severe CHF in the present
model. It is possible that \textsuperscript{iNOS} is involved in models of
moderate CHF, but the present findings certainly cast doubt on
the causative role of \textsuperscript{iNOS} in severe CHF.

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