Mice Lacking Inducible Nitric Oxide Synthase Have Improved Left Ventricular Contractile Function and Reduced Apoptotic Cell Death Late After Myocardial Infarction

Flora Sam, Douglas B. Sawyer, Zhonglin Xie, Donny L.F. Chang, Soeun Ngoy, Daniel A. Brenner, Deborah A. Siwik, Krishna Singh, Carl S. Apstein, Wilson S. Colucci

Abstract—Nitric oxide produced by inducible nitric oxide synthase (NOS2) has been implicated in the pathophysiology of chronic myocardial remodeling and failure. We tested the role of NOS2 in left ventricular (LV) remodeling early (1 month) and late (4 months) after myocardial infarction (MI) in mice lacking NOS2. MI size measured 7 days, 1 month, and 4 months after MI was the same in NOS2 knockout (KO) and wild-type (WT) mice. The LV end-diastolic pressure-volume relationship measured by the isovolumic Langendorff technique showed a progressive rightward shift from 1 to 4 months after MI in WT mice. LV developed pressure measured over a range of LV volumes was reduced at 1 and 4 months after MI in WT mice ($P<0.05$ and $P<0.01$ versus shams, respectively). In KO mice, the rightward shift was similar to that in WT mice at 1 and 4 months after MI, as was peak LV developed pressure at 1 month after MI. In contrast, at 4 months after MI, peak LV developed pressure in KO mice was higher than in WT mice ($P<0.05$ versus WT) and similar to that in sham-operated mice. At 1 month after MI, the frequency of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive myocytes in the remote myocardium was increased to a similar extent in WT and KO mice. At 4 months after MI, the frequency of apoptotic myocytes was increased in WT mice but not in KO mice ($P<0.05$ versus WT). Improved contractile function and reduced apoptosis were associated with reduced mortality rate in KO mice at 4 months after MI. Thus, NOS2 does not play an important role in determining infarct size or early LV remodeling during the first month after MI. In contrast, during late (ie, 4 months after MI) remodeling, NOS2 in remote myocardium contributes to decreased contractile function, increased myocyte apoptosis in remote myocardium, and reduced survival. (Circ Res. 2001;89:351-356.)

Key Words: myocardial remodeling • nitric oxide synthase • myocardial infarction • mouse • apoptosis

Nitric oxide (NO), alone or in combination with other reactive oxygen species, can depress myocyte contractile function,1 attenuate myocyte hypertrophy,2 and cause myocyte death3,4 and/or apoptosis.5,6 Both inducible nitric oxide synthase (NOS2)7-9 and inflammatory cytokines that induce NOS2, such as tumor necrosis factor-$\alpha$10,11 may be increased chronically in failing myocardium. These observations have led to the thesis that expression of NOS2 in the myocardium contributes to abnormal function and pathological remodeling in chronic myocardial failure.12

To test the role of NOS2 in chronic myocardial remodeling, transgenic mice lacking NOS2 were subjected to myocardial infarction (MI) by coronary ligation.13 At 1 and 4 months after MI, left ventricular (LV) chamber volume and contractile function were assessed using the isovolumic (balloon-in-LV) Langendorff technique, and myocyte apoptosis was assessed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method.

Materials and Methods

Experimental Model

NOS2 knockout (KO) mice in the F6 generation on a C57Bl6/J background were obtained from Dr Victor Laubach (University of North Carolina).14 Age-matched wild type (WT) C57Bl6/J mice were obtained from Charles River (Cambridge, Mass). MI was caused by ligation of the left anterior descending artery as previously described in detail.13,15,16 A total of 112 WT and 79 KO mice underwent surgery. Of these, 141 (WT, 82; KO, 59) had coronary ligation and 50 (WT, 30; KO, 20) had sham ligation. For coronary ligation, the perioperative mortality rate during the first 24 hours was similar in WT (45%) and KO (44%) mice. For the sham operation, the perioperative mortality rate during the first 24 hours was 10% and 0% in WT and KO mice, respectively. Mice were given food and water ad libitum. One and 4 months after MI, the hearts were

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removed for study by the isovolumic Langendorff technique. The protocol was approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Assessment of LV Function
LV function was assessed using an isolated, isovolumic, red cell-perfused, Langendorff heart preparation as previously described. Briefly, after anesthesia, hearts were excised, a short cannula was inserted into the aortic root, and the coronary arteries were perfused with red cell–containing perfusate. Perfusion pressure was monitored via a sidearm of the aortic cannula and LV pressure via a collapsed polyvinyl chloride balloon placed in the LV via the left atrium and secured in place connected to a pressure transducer (Gould-Statham P23dB).

After 20 minutes of baseline perfusion at a pressure of 80 to 100 mm Hg and a pacing rate of 420 bpm (7 Hz), the intracardiac balloon volume was set to the lowest possible volume at which minimal LV pressure tracings could be recorded. Systolic and diastolic pressure-volume relationships were determined by increasing the LV balloon volume in 5-μL increments using an airtight Hamilton syringe. The LV balloon volume was increased until peak LV developed pressure was reached and a further increase led to a decrease in LV developed pressure, as previously described.

Perfusion Fixation and Histological Assessment
After pressure-volume curves were obtained, the heart was perfused with saline to clear the red cells. With the LV balloon volume set to give an end-diastolic pressure of 5 mm Hg, the heart was arrested in diastole with KCl and perfusion-fixed with 10% buffered formalin at a pressure of 80 mm Hg. After 3 days of fixation, the balloon was removed, excess formalin was blotted, and the heart was weighed.

Infarct Size
Fixed hearts were embedded in paraffin and sections from apex, mid-LV, and base were stained with Trichrome Masson. Infarct size was determined as the mean percent of epicardial and endocardial circumferential occupancy by scar in the three sections, as described by Pfeffer et al. In a subset of mice killed at 7 days after MI, the atria and right ventricle were removed, and the LV and infarct (along with a small rim of normal-appearing peri-infarct tissue) were carefully dissected, weighed, and frozen in liquid nitrogen. For these hearts, MI size was estimated from the ratio of the infarct to the whole LV weights.

TUNEL and Hoechst 33258 Staining
Sections that were 5 μm thick from the LV apex, midcavity, and base were stained with fluorescent TUNEL using a cell death detection assay (Boehringer), as previously described. Cosing staining with Hoechst 33258 (10 μM for 30 minutes) was performed for visualization of all nuclei. Sections were visualized by fluorescence microscopy using a Nikon Diaphot 300 microscope, and images were acquired with a Bioquant image acquisition and analysis system using an Optronics camera.

For each heart, the total number of TUNEL-positive myocyte nuclei in the noninfarcted region was counted in 6 sections (2 sections each from the apex, midcavity, and base) and normalized to the estimated total number of nuclei in the sections, as previously described. Apoptotic myocytes in the infarct and immediate peri-infarct regions were excluded from counting.

Immunohistochemistry
Sections (4 μm thick) were deparaffinized, rehydrated, and blocked with 1.5% normal horse serum. NOS2 expression was detected using a polyclonal rabbit anti-mouse NOS2 antibody (1:200, Calbiochem) and Vectastain avidin-biotin peroxidase and biotinylated secondary antibody (Vector Laboratories). The sections were visualized under bright-field microscopy and images were recorded using an Optronics camera with Bioquant hardware and software.

Statistical Analysis
Pressure-volume relationships were compared by two-way ANOVA for repeated measures. Individual variables (developed pressure, apoptotic myocytes) were tested by one-way ANOVA, followed by post hoc analysis with the Bonferroni correction for multiple observations or unpaired t test. Overall survival of all animals was assessed by Kaplan-Meier analysis. Survival in individual experimental groups at 1 and 4 months was also tested by χ² test. A P value of ≤0.05 was considered significant. All data are presented as mean±SEM.

Morphometric Measurements

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<th>Seven Days</th>
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<th>Four Months</th>
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<td>WT</td>
<td>NOS2-KO</td>
<td>WT</td>
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<td>MI (n=5)</td>
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<td>Sham (n=6)</td>
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<td></td>
<td>MI (n=5)</td>
<td>MI (n=5)</td>
<td>Sham (n=6)</td>
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<tr>
<td>Body weight, g</td>
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<td>23.2±0.3</td>
<td>29.5±0.7</td>
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<td>Heart weight, mg</td>
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<td>138.2±4.2</td>
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<td>HW/BW ratio, mg · g⁻¹</td>
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<tr>
<td>MI size, %</td>
<td>28±5</td>
<td>27±4</td>
<td>36±4</td>
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*P<0.05 vs age-matched sham.
†P<0.01 vs age-matched sham.
Results

NOS2 Expression Late After Myocardial Infarction
Myocardial NOS2 protein expression was assessed by immunohistochemistry in remote myocardium obtained 4 months after MI in WT mice. NOS2 staining was absent in sham-operated mice but was readily visible in the remote myocardium from the infarcted mice, where its location was diffuse and primarily in myocytes (Figure 1).

Cardiac Morphology
To examine the role of NOS2 in post-MI remodeling, MI was caused in WT and NOS2 KO mice, which were studied 1 or 4 months after MI. Infarct size averaged between 36% and 40% in the 1- and 4-month post-MI groups and was similar in WT and KO mice at both times (Table). Likewise, in a separate group of animals killed 7 days after MI, MI size was similar in WT and KO mice (Table). Body weight (BW) was similar in WT and KO mice. In WT mice, heart weight (HW) and the HW/BW ratio were increased 1 and 4 months after MI. HW and the HW/BW ratio were increased to a similar degree in the WT and KO mice at 1 and 4 months after MI (Table). Neither ascites nor pleural effusion was observed at sacrifice in any group, and the lung and liver wet/dry weight ratios were similar in all groups (data not shown).

LV End-Diastolic Pressure-Volume Relationships
At 1 month after MI, the LV end-diastolic pressure-volume relationship was shifted rightward in WT mice (P<0.05 versus shams) (Figure 2). The rightward shift after MI was of similar magnitude in KO mice (P=0.31 versus WT). At 4 months after MI, there was a further rightward shift in this relationship (P<0.01 versus sham), which was of similar magnitude in WT and KO mice (Figure 2).

LV Developed Pressure
The relationship between LV developed pressure and volume was shifted to the right at 1 and 4 months after MI in both WT and KO hearts (Figure 3). To correct for heart-to-heart variations in LV end-diastolic volume, LV developed pressure for each heart was normalized to the volume at which the maximum developed pressure (LV DevP$_{\text{max}}$) was observed in that heart, as previously described. At 1 month after MI, LV DevP$_{\text{max}}$ was similar in WT (86±6 mm Hg) and KO (72±6 mm Hg) mice (Figure 4). At 4 months after MI, LV DevP$_{\text{max}}$ in WT mice was depressed, whereas in KO mice the LV DevP$_{\text{max}}$ (122±6 mm Hg) was greater than that in WT hearts (P<0.05) and was similar to that in sham hearts (131±4 mm Hg) (Figure 4).

LV Volume (% maximum volume)

Myocyte Apoptosis
At 1 month after MI, the frequency of TUNEL-positive nuclei was increased in WT (20±3 per 10$^5$ nuclei) versus sham-operated (5±1 per 10$^5$ nuclei) mice and was increased to a similar extent in KO mice (21±8 per 10$^5$ nuclei) (Figure 5). At 4 months after MI, the frequency of TUNEL-positive nuclei in hearts from KO mice (12±3 per 10$^5$ nuclei) was decreased com-
Survival
Separate cohorts of mice were studied at 1 and 4 month after MI. In the 1-month cohort, survival after MI was similar in the WT and KO mice (WT, 18 of 24; KO, 13 of 18; *P = 1.0). In the 4-month cohort, survival was increased in KO mice (WT, 10 of 21; KO, 12 of 15; *P < 0.05). Kaplan-Meier analysis of the combined cohorts likewise showed a separation that appeared between 1 and 4 months after MI (Figure 6).

Discussion
The major new findings of this study are that (1) NOS2 protein expression is expressed in remote myocardium late (4 months) after myocardial infarction and (2) contractile dysfunction, myocyte apoptosis, and mortality rate are reduced in NOS2-deficient mice late, but not early, after myocardial infarction.

Lack of Effect on MI Size
During the first several days after MI, NOS2 expression is increased in infarcted myocardium. In rabbits subjected to coronary ligation, NOS2 activity in the infarct region increased 3 days after MI and returned to baseline by 2 weeks.21 In the rat, NOS2 mRNA expression in the infarcted region peaked 6 hours after coronary ligation and returned to baseline by 1 week.22 Likewise, in the mouse, we found that NOS2 mRNA was increased in the infarct region at 3 days after MI, peaked at 7 days, and returned to baseline by 14 days.23

NOS2-derived NO can cause myocyte apoptosis.5,6 Because infarct size is in part related to the number of apoptotic myocytes,24 it is possible that NOS2 would contribute to MI size. However, in the present study, MI size in NOS2 KO mice was not different from that in WT mice at 7 days, 1 or 4 months after MI. Thus, NOS2 is not a major determinant of MI size in the setting of a persistent coronary occlusion. This conclusion is similar to that of Guo et al,25 who also found no difference in infarct size in NOS2 KO (versus WT) mice. In contrast, pharmacological inhibition of NOS2 with 5-methylisothiourea sulfate in the rat was associated with a 40% reduction in infarct size.22 This discrepancy may reflect nonsselective actions of this agent. NOS2 has been shown to contribute to late ischemic preconditioning26 and may thus play an important protective role in reducing infarct size in the setting of repetitive episodes of ischemia followed by reperfusion.

Lack of Effect on Early Remodeling or Mortality Rate
LV volume, contractile function, and mortality rate were the same in KO and WT mice 1 month after MI. Thus, as with MI size, NOS2 does not appear to play a role in the early post-MI remodeling of myocardium remote from the infarction. The frequency of apoptosis in remote myocardium at 1 month was the same in WT and KO mice, suggesting that early apoptosis is mediated via an NOS2-independent mechanism.

Improved Contractile Function, Decreased Apoptosis, and Improved Survival Late After Myocardial Infarction
LV contractile function, myocyte apoptosis in the remote myocardium, and survival were all improved in NOS2 KO mice 4 months after MI. Thus, in contrast to the apparent lack of involvement of NOS2 at 1 month after MI, NOS2 contributes to late remodeling and survival. It is noteworthy that although survival was similar in NOS2 KO and WT mice 1 month after MI, reduced apoptosis and improved contractile function at 4 months were associated with improved survival in the KO mice. The improvement in LV developed pressure observed in the NOS2 KO animals is modest and seen only at the highest LV volumes. However, because it is possible that the animals that died had worse LV function, these observations made in the surviving animals may underestimate the beneficial effect of NOS2 KO.

These data demonstrate an association between improved contractile function and survival but do not establish a cause-and-effect relationship. Likewise, although the data demonstrate that NOS2 contributes to apoptosis in remote myocardium late after MI, the data do not allow a conclusion about the relationship between the decrease in apoptosis and improved contractile function. NOS2 KO was not associated.
with a decrease in LV dilation, suggesting that the beneficial effects observed were not due to improved structural remodeling. Given the well-documented ability of NOS2-derived NO to cause myocyte apoptosis in vitro, it is possible that improved contractile function reflects a decrease in myocyte loss. In this regard, it is noteworthy that acute rejection of a transplanted heart in the mouse is reduced in NOS2-deficient mice and is associated with a reduction in apoptosis.36,27 However, we cannot exclude the possibility that the reduction in apoptosis in the KO mice is an indirect response to another beneficial effect of NOS2 KO. For example, elimination of NOS2 might have allowed for more myocyte hypertrophy.28 Although we did not measure myocyte hypertrophy directly, this possibility seems unlikely because heart weights were increased to a similar degree in WT and KO mice after MI. Alternatively, NO might cause alterations in the expression of proteins involved in the regulation of myocyte contractility. Such an effect on myocyte contractile phenotype is suggested by the observation that endotoxin-induced myocardial dysfunction is reduced in NOS2 KO mice.29 In summary, these findings extend the pathophysiological role of NOS2 to chronic myocardial remodeling. There is now evidence that oxidative stress is increased in chronically failing myocardium10–33 and an increase in reactive oxygen species has been demonstrated in the mouse heart late after MI.34 It will thus be important to determine the interaction between NOS2-derived NO and other reactive oxygen species.35 It is also interesting that, in contrast to the detrimental effects of NOS2-derived NO, it appears that NOS3-derived NO to cause myocyte apoptosis in vitro,36,37 it is possible that improved contractile function reflects a decrease in myocyte loss. 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