Inhibition of NOS II prevents cardiac dysfunction in myocardial infarction and congestive heart failure

TAKAYUKI SAITO,1,2 FU HU,1 LARA TAYARA,1 LINDA FAHAS,1 HANI SHENNIB,2 AND ADEL GIAID1

Departments of Pathology, 1Medicine, and 2Cardiothoracic Surgery, The Montreal General Hospital and McGill University, Montreal, Quebec, Canada H3G 1A4 and 3Department of Cardiovascular Surgery, Nagoya City University Medical School, Nagoya, Japan 467-8601

Received 10 July 2001; accepted in final form 1 February 2002

Saito, Takayuki, Fu Hu, Lara Tayara, Linda Fahas, Hani Shennib, and Adel Giaid. Inhibition of NOS II prevents cardiac dysfunction in myocardial infarction and congestive heart failure. Am J Physiol Heart Circ Physiol 283: H339–H345, 2002. First published February 14, 2002; 10.1152/ajpheart.00596.2001.—Strong expression of the inducible form of nitric oxide synthase (NOS II) has been shown in the myocardium of patients with myocardial infarction (MI). We hypothesized that NOS II plays an important role in the development of MI and subsequent heart failure and that inhibition of NOS II may beneficially alter the course of the disease. Long-term administration (2 mo) of the selective NOS II inhibitor S-methylisothiourea (SMT) to rats with MI significantly improved cardiac function. A significant drop in mortality, lung water content, infarct size, and cardiomyocyte hypertrophy was also associated with the use of SMT. Plasma concentration of nitrite and nitrate was also reduced by SMT. Short-term administration of SMT (first 2 wk only) significantly reduced infarct size; however, it did not improve cardiac dysfunction measured 2 mo after MI. These findings demonstrate that induction of NOS II during MI exerts negative effects on cardiac function and structure. Long-term administration of a selective NOS II inhibitor may prove to be beneficial in the treatment of MI and congestive heart failure. S-methylisothiourea

NITRIC OXIDE (NO) is synthesized from L-arginine by different isoforms of nitric oxide synthase (NOS), including NOS I (neuronal), NOS II (inducible), and NOS III (endothelial) (19). In most cell types, including myocardial tissue, NOS II is induced after stimulation with lipopolysaccharide, cytokines, ischemia-reperfusion, and hypoxia (7, 18, 39, 40). Infiltinating macrophages and cardiac myocytes were identified as the major source of increased NOS II activity in the infarcted myocardium (40). Increased expression of NOS II is accompanied by increased level of nitrite and nitrate (NOx) after myocardial ischemia in humans (1) and animals (2, 42). Enzyme activity is sustained for a prolonged period and a high concentration of NO is released on induction of NOS II (19). Earlier studies (4, 17) have shown that excessive amounts of NO produced by local induction of NOS II may contribute to myocardial dysfunction and injury. Evidently, low doses of NO induce positive inotropic effects, whereas higher doses produce negative inotropic effects (3, 21, 26). The role of NOS II in myocardial infarction (MI) and congestive heart failure remains controversial because there are data to suggest protective (16, 33, 45) and maladaptive (11, 39, 41, 43, 44) effects for NOS II in myocardial injury and failure. We (10) have recently shown abundant expression of NOS II in the myocardium of patients with end-stage heart failure and also showed a significant correlation between myocardial NOS II expression and presence of infiltrating macrophages. Whether or not induction of NOS II contributes to the myocardial remodeling and dysfunction associated with MI and congestive heart failure remains to be elucidated. We hypothesize that sustained induction of myocardial NOS II contributes to myocardial remodeling and the development of heart failure. The aim of the present study was therefore to address the effect of short- and long-term inhibition of NOS II on the function and structure of the myocardium and mortality in a rat model of chronic left coronary artery ligation.

MATERIALS AND METHODS

Experimental MI. All animal work was performed in accordance with institutional guidelines and in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). Lewis male rats weighing 270–320 g were used for this study. Left ventricular (LV) free wall MI was induced as described previously (23). Study groups. A selective NOS II inhibitor, S-methylisothiourea (SMT), was dissolved in water and administered to rats by gavage. Rats were divided into five groups: 1) rats with MI and receiving SMT (5 mg·kg⁻¹·day⁻¹) 30 min before ligation and continued for 8 wk (SMT group, n = 19), 2) rats
with MI and receiving SMT (5 mg·kg⁻¹·day⁻¹) 30 min before ligation and continued for the first 2 wk (SMT2 group, n = 7), 3) rats with MI and receiving saline for 8 wk (MI group, n = 14), 4) sham-operated rats (sham group, n = 14), and 5) sham-operated rats treated with SMT (5 mg·kg⁻¹·day⁻¹) for 8 wk (sham-SMT group, n = 10). In the SMT group, hemodynamic measurement was recorded for only one-half of the surviving animals (n = 8). The remaining animals were used for the survival study only.

**Hemodynamic measurements.** Eight weeks after MI or sham surgery, animals were anesthetized with 2% isoflurane. A fluid catheter connected to a transducer was inserted into the right carotid artery. After arterial blood pressure and heart rate were measured, the catheter was advanced into the left ventricle (LV) and LV systolic pressure, LV end-diastolic pressure (LVEDP), and first derivative of LV pressure (±dP/dt) were determined. The chest was then opened and an 18-gauge catheter was inserted into the right atrium. After measuring central venous pressure (CVP), the catheter was advanced to the right ventricle and measured right ventricular systolic pressure (RVSP). Mean arterial pressure was calculated according to the following formula: diastolic arterial pressure + pulse pressure/3.

**Assay of plasma NOx.** NOx plasma levels were assessed as nitrite concentration. Blood was collected from right atrium after completion of hemodynamic measurement. Heparinized blood samples were centrifuged at 1,500 rpm for 15 min at 4°C. Supernatant was then collected and preserved at −80°C. Plasma NOx concentrations were determined colorimetrically with a commercially available kit (OXIS International; Portland, OR) based on the Griess reaction, after conversion of nitrate to nitrite with nitrate reductase. NOx concentrations were determined at an optical density of 540 nm by comparison with a standard curve with sodium nitrite and control baseline plasma as a blank (28).

**Morphometric analysis.** After hemodynamic measurements were taken, the rats were euthanized. The hearts were excised after perfusion and fixed with 4% phosphate-buffered paraformaldehyde, and the ventricles were weighed. The ventricles were then cut into three transversal sections of approximately identical thickness. Tissues were dehydrated and embedded in paraffin. From these sections, 5-μm-thick histological slices were obtained and stained with hematoxylin and eosin or Masson trichrome. Slides were examined by light microscope coupled to a computerized morphometry system (Image Pro Plus, Media Cybernetics). The ratio of scar length to LV circumpferences of the endocardium and epicardium was expressed as a percentage to define infarct size (25). Excised lung tissues from all animals were dried at 37°C for 2 wk, followed by determination of lung water content using the following formula: (wet weight-dry weight)/wet weight 100 (%). One hundred myocytes from 10 high-power views of each animal were randomly selected and analyzed for myocyte diameter.

**Immunohistochemistry.** Paraffin sections were immunostained with antisera to NOS II with a modification of the avidin-biotin-peroxidase methods (10). Briefly, sections were incubated serially with the following solutions: 1) 2% hydrogen peroxide for 30 min to block endogenous peroxide activity, 2) 0.3% Triton X-100 for 15 min to permeabilize the cell membrane, 3) 10% normal goat serum for 60 min to reduce nonspecific binding of the antiserum, 4) primary antiserum for 16 h at 4°C, 5) biotinylated goat anti-mouse or goat antirabbit IgG for 45 min, and 6) avidin-biotinylated horseradish peroxidase complex (Vectastain, Vector Laboratories; Burlingame, CA) for 45 min. Immunoreactive sites were visualized by incubation with 0.025% 3,3-diaminobenzidine and 0.01% hydrogen peroxide for 3 min. Phosphate-buffered saline (pH 7.4) was used to dilute each solution and to wash the sections three times between each step.

**Western blotting.** Infarcted and noninfarcted LV were separated and homogenized as described previously (22). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using 7% Tris-HCl gel (Bio-Rad). Protein blots were transferred onto a polyvinylidene difluoride membrane, and the membrane was incubated with a 1:750 dilution of anti-inducible NOS (1131–1144, Calbiochem) and then incubated with a 1:3,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad). Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as the substrates for visualization of the reaction product. To determine the amount of NOS II protein after immunoblotting, the membrane was scanned (ScanJet, Hewlett-Packard). Quantitative analysis of the bands was performed, and the intensity of the bands was expressed as a percentage of the sham group (22).

**Statistical analysis.** All results, except for mortality, are presented as means ± SE. One-way analysis of variance, followed by Fisher’s test, was used to compare differences among the groups. An unpaired t-test was used for comparing the values obtained from infarct size. Mortality was evaluated with the use of a χ²-test. Significant differences among the groups were defined by a value of P < 0.05.

**RESULTS**

**Mortality of animals.** The overall mortality of animals with coronary artery ligation was significantly lower in the SMT group than the MI group (P < 0.05) (Table 1). SMT treatment for only the first 2 wk postligation (SMT2 group) also resulted in a better survival rate when compared with the MI group; however, the difference did not reach statistical significance (Table 1). Regardless of the type of group, mortality occurred mainly during the first 2 wk after coronary artery ligation. When we grouped all the SMT-treatment rats (SMT + SMT2), there was a significant drop in mortality during the first 2 wk postsurgery compared with the saline-treated MI group (11.5 vs. 42.9%, P < 0.05). There was zero mortality in the sham-operated animals (sham and sham-SMT groups).

**Cardiac function.** Ligation of left coronary artery resulted in a significant decrease in mean arterial pressure and ±dP/dt and a significant increase in LVEDP, CVP, and RVSP compared with sham-operated animals (Table 2). Long-term administration of SMT significantly reduced LVEDP compared with the saline-treated animals (P < 0.05) (Table 2). CVP and RVSP were also significantly decreased by the long-

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Overall Mortality, %</th>
<th>&lt;2 Wk Mortality, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI-saline</td>
<td>14</td>
<td>50</td>
<td>42.9</td>
</tr>
<tr>
<td>MI-SMT</td>
<td>19</td>
<td>15.8*</td>
<td></td>
</tr>
<tr>
<td>MI-SMT2</td>
<td>7</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>Combined MI-SMT group</td>
<td>26</td>
<td>11.5*</td>
<td></td>
</tr>
</tbody>
</table>

MI, myocardial infarction; SMT, S-methylisothiourea; SMT2, SMT treatment group for first 2-wk postligation. *P < 0.05.
Fig. 1. Myocardial inducible nitric oxide synthase (NOS II) immunoreactivity. NOS II immunoreactivity in heart sections of myocardial infarction (MI) (A–D and F) and sham (E) rats. A: strong immunostaining for NOS II in the heart of a rat with MI. B: higher magnification of the same heart as in A, showing strong immunostaining for NOS II in the myocytes, blood vessels (arrowheads), and macrophages (arrows) within the infarcted myocardium. C: weak immunostaining for NOS II in myofibroblasts within the infarcted myocardium. D: section of the noninfarcted myocardium of MI rat, showing moderate NOS II immunoreactivity in the myocytes and blood vessels (arrow). E: myocardium of sham rat showing weak immunostaining in the myocytes and blood vessels. F: negative control section showing no immunoreaction in the infarcted myocardium. A was magnified ×100, and B–F were magnified ×400.

Table 2. Hemodynamic measurements at 8 wk after left coronary artery ligation or sham surgery

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 12)</th>
<th>Sham-SMT (n = 13)</th>
<th>MI (n = 7)</th>
<th>SMT (n = 8)</th>
<th>SMT2 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>320 ± 11</td>
<td>310 ± 10</td>
<td>276 ± 17</td>
<td>309 ± 10</td>
<td>307 ± 7</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>101 ± 7</td>
<td>98 ± 6</td>
<td>79 ± 7</td>
<td>83 ± 5</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>117 ± 7</td>
<td>107 ± 6</td>
<td>96 ± 8</td>
<td>97 ± 7</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.0 ± 0.5</td>
<td>3.7 ± 0.4</td>
<td>18.8 ± 3.0</td>
<td>7.4 ± 1.1*</td>
<td>15.6 ± 4.5</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>4.638 ± 300</td>
<td>4.089 ± 246</td>
<td>3.189 ± 351</td>
<td>3.738 ± 288</td>
<td>3.210 ± 282</td>
</tr>
<tr>
<td>–dP/dt, mmHg/s</td>
<td>4.623 ± 243</td>
<td>4.281 ± 189</td>
<td>2.991 ± 363</td>
<td>3.666 ± 228</td>
<td>3.180 ± 384</td>
</tr>
<tr>
<td>CVP, mmHg</td>
<td>4.3 ± 0.4</td>
<td>4.0 ± 0.5</td>
<td>14.0 ± 2.2</td>
<td>5.5 ± 0.8**</td>
<td>12.0 ± 3.0</td>
</tr>
<tr>
<td>RVSP, mmHg</td>
<td>27 ± 1</td>
<td>24 ± 1</td>
<td>37 ± 4</td>
<td>25 ± 2**</td>
<td>28 ± 3*</td>
</tr>
</tbody>
</table>

HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dt, first derivatives of LV pressure; CVP, central venous pressure; RVSP, right ventricular systolic pressure. *P < 0.05, **P < 0.01 compared with MI.
term SMT treatment compared with saline treatment ($P < 0.01$). SMT treatment for the first 2 wk after surgery resulted in a significant decrease in RVSP ($P < 0.05$), but it did not significantly reduce LVEDP and CVP. Cardiac contractility represented by $±dP/dt$ was higher in the animals with long-term SMT treatment than in those both of the saline and short-term SMT treatment MI groups; however, these values did not reach statistical significance. Administration of SMT to sham animals did not significantly alter hemodynamic parameters.

**Immunohistochemistry for NOS II.** In the MI group, strong NOS II immunoreactivity was seen in the myocytes, endocardium, and endothelium of intramyocardial vessels in both infarcted and noninfarcted myocardium and in macrophages in the infarcted myocardium. The expression of NOS II was more prominent in the border area between the infarcted and the noninfarcted myocardium (Fig. 1A). The surviving myocytes, endothelial cells, and macrophages in the border area showed the most intense expression for NOS II (Fig. 1B). No immunostaining was seen in the negative control sections immunostained with the nonimmune serum or immunoabsorbed with the NOS II antigen (Fig. 1C).

**Morphological data.** Lung water content was significantly lower in the MI animals treated with SMT for 2 mo compared with those treated with saline (78.3 ± 0.5% vs. 80.4 ± 0.4%, $P < 0.05$). Short-term treatment with SMT did not affect lung water content (80.1 ± 0.7%). MI size was significantly smaller in the animals treated with SMT either long or short term, compared with those treated with saline (SMT, 44.3 ± 3.6%; SMT2, 43.5 ± 3.3%; MI, 58.9 ± 2.1%; $P < 0.05$) (Fig. 2A and B). There was no significant difference in the infarct size between the long-term-treated and short-term-treated animals with SMT. The diameter of myocytes in the noninfarcted myocardium was significantly smaller in the animals in the chronic SMT treatment group compared with that in the SMT2 or saline treatment groups (SMT, 15.2 ± 0.3 μm; SMT2, 16.6 ± 0.8 μm; MI, 17.8 ± 1.0 μm; $P < 0.01$ SMT compared with MI, $P < 0.05$ SMT compared with SMT2) (Fig. 2C).

**Plasma concentration of NOx.** When compared with sham (32.2 ± 2.0 μM), saline-treated MI group showed significantly higher concentrations of NOx ($P < 0.05$). Long-term administration of SMT to the MI rats resulted in a significant decrease in the plasma concentration of NOx compared with the saline-treated MI group (MI, 43.5 ± 7.3 μM vs. SMT, 22.6 ± 2.5 μM, $P < 0.01$) (Fig. 4). Because administration of SMT in the SMT2 group was stopped at 6 wk before death, the plasma concentration of NOx in this group was comparable to that of the saline-treated MI group and was significantly higher than that of the long-term SMT-treated group (39.7 ± 7.1 μM, $P < 0.01$). SMT did not significantly affect NOx concentration in sham-operated animals (sham-SMT: 28.2 ± 2.2 μM).

**Western blotting.** The noninfarcted myocardium of animals with experimental MI had significantly increased level of NOS II protein compared with the infarcted myocardium or the myocardium of the sham group (Fig. 5). SMT treatment had no effect on the level of NOS II protein in sham or MI animals.

**DISCUSSION**

Congestive heart failure due to MI is associated with induction of numerous neurohumoral factors and inflammatory mediators. NO is one of the most important inflammatory mediators that has been shown to exert negative inotropic effect on the myocardium (3, 21, 26). Increased expression of local inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, and interleukin-6 induces myocardial production of NO through activation of NOS II expression (9, 34–36, 38). We have previously shown strong expression of NOS II in the myocardium of patients with end-stage heart failure secondary to ischemic heart disease (10). In this study, we show that left coronary artery ligation induced strong immunoreactivity for...
NOS II in the surviving cardiomyocytes and in the macrophages within the infraction zone. Endothelial cells throughout the myocardium exhibited strong immunoreactivity for NOS II. Myocytes and vascular endothelial cells in the noninfarcted area also had abundant immunoreactivity for NOS II. In addition, protein level of NOS II was higher in the noninfarcted myocardium compared with the scar or with the normal myocardium of sham rats. Selective inhibition of NOS II significantly reduced mortality and improved diastolic dysfunction and myocardial remodeling in rats with MI and congestive heart failure, suggesting a pathological role for NOS II-derived NO in this disease process.

The efficacy of selective NOS II inhibition in experimental acute MI has been extensively investigated (11, 39, 42, 43). The principal idea behind these studies was that a reduction in NOS II-derived NO by infiltrating inflammatory cells and cardiomyocytes would reduce nonspecific tissue injury and contractile dysfunction while preserving low concentrations of NO released by the endothelial constitutive NOS, which accounts for the physiological vasomotor response and antithrombogenic properties. Unlike nonselective NOS inhibitors, selective NOS II inhibition has been reported not to affect myocardial blood flow in the noninfarcted zone of the LV (43).

In the present study, plasma NOx was significantly elevated in the animals with MI even 8 wk after ligation, and long-term SMT treatment effectively reduced NO production. However, SMT treatment for only the first 2 wk did not reduce the NOx level when measured at death because administration of SMT had already been terminated 6 wk earlier. This finding can strongly support the existence of prolonged NOS II activity even in the congestive heart failure, which is consistent with our previously published data in patients with congestive heart failure (10). In the present study, because we observed little expression of NOS II in cardiac myocytes and endothelial cells of sham control rats, we added another sham group treated with SMT to examine the effect of selective inhibition of NOS II on cardiac function and structure in normal animals. How-
ever, administration of SMT to these animals did not cause any significant changes in cardiac function or morphology. In addition, SMT did not significantly affect the plasma concentration of NOx in sham animals. These findings are consistent with previous reports (11) demonstrating that inhibition of NOS II by aminoguanidine had no effect on the steady-state hemodynamics in sham rats, suggesting that NOS II does not significantly contribute to normal cardiovascular function.

Although there was no difference in infarct size between long-term and short-term treatment with SMT, long-term treatment provided better cardiac performance. This could be attributed to the sustained expression of NOS II in congestive heart failure. Indeed, though it needs to be addressed in future studies, one can postulate how inhibition of NOS II contribute to better cardiac function. First, high concentration of NO attenuates myocyte contraction and catecholamine responses (3, 5). Inhibition of NOS II may therefore enhance the cellular sensitivity to adrenergic stimuli, leading to potentiation of the inotropic responsiveness of the preserved myocardium (14). Second, NO may contribute to LV dysfunction, inhibition of Na+/K+/ATPase activity, or inactivation of sodium membrane channels (6, 20, 30). Third, NO donor has been reported to affect cardiac remodeling by increasing collagen deposition (34). Moreover, expression of NOS II has been reported to be associated with apoptotic cell death, which may contribute to organ dysfunction due to a progressive decline in the number of functional cardiomyocytes (29, 32). Taken together, excessive amount of NO derived from myocardial NOS II appears to contribute to the development of myocytes death, myocardial dysfunction and remodeling, and increased mortality associated with MI and congestive heart failure. This is supported by the recent report that selective modulation of increased NOS II activity by SMT exerted beneficial effects and improved survival in a rodent model of septic shock (31), and the findings of significant improvement in survival rate and post-MI cardiac function in NOS II mutant compare with NOS II wild-type mice (8).

One of the significant findings of the present study is the reduction in infarct size in the SMT treatment groups. Numerous studies have examined the effect of selective or nonselective NOS inhibition on the development of MI after short- or long-term coronary artery ligation. Some of these studies (42, 43) showed no significant effect on infarct size, whereas others have shown a significant reduction in infarct size (39, 44). However, in all of the previous pharmacological studies, infarct size was determined only up to 3 days after short- or long-term coronary artery ligation. Previously published studies (39, 43) as well as our current study show induction of NOS II well beyond 3 days after coronary artery ligation. As such, this may explain the discrepancy between our findings and that of previous studies. On the other hand, reduction of infarct size has not been demonstrated in studies using mice lacking the NOS II gene (8, 13, 24, 46). It is important to recognize that there are potential limitations in the use of genetically engineered mice, and a direct comparison to pharmacological studies cannot be made. For example, the deletion of a single gene can alter the cellular physiology of transgenic mice. This can potentially lead to compensatory/adaptive changes that may explain some of the differences in the present study compared with those using the NOS II knockout mice. In addition to infarct size, SMT treatment resulted in a significant reduction in mortality. These findings are supported by previous studies (8, 24) demonstrating a role for NOS II in the increased mortality after chronic ligation of coronary artery.

In conclusion, the data presented here clearly showed that myocardial NOS II plays an important role in the development of MI and congestive heart failure. Prolonged expression of NOS II in the failing myocardium appears to significantly contribute to myocardial dysfunction in the setting of experimental congestive heart failure. Inhibition of NOS II could beneficially alter the course of the disease. Thus intervention of NOS II pathway may provide new therapeutic strategies for the treatment of heart failure secondary to MI.

This work was supported in part by the Canadian Institute for Health Research and the Heart and Stroke Foundation of Canada. A. Giaid is supported by a scholarship from Fonds de la Recherche en Santé du Québec.

REFERENCES

NOS II INHIBITION IN MYOCARDIAL INFARCTION

H345


