Inhaled nitric oxide decreases infarction size and improves left ventricular function in a murine model of myocardial ischemia-reperfusion injury

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Hataishi, Ryuji, Ana Clara Rodrigues, Tomas G. Neilan, John G. Morgan, Emmanuel Buys, Sruti Shiva, Rosemary Tambouret, Davinder S. Jassal, Michael J. Raher, Elissa Furutani, Fumito Ichinose, Mark T. Gladwin, Anthony Rosenzweig, Warren M. Zapol, Michael H. Picard, Kenneth D. Bloch, and Marielle Scherrer-Crosbie. Inhaled nitric oxide decreases infarction size and improves left ventricular function in a murine model of myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 290: H379–H384, 2006. First published January 27, 2005; doi:10.1152/ajpheart.01172.2005.—To learn whether nitric oxide (NO) inhalation can decrease myocardial ischemia-reperfusion (I/R) injury, we studied a murine model of myocardial infarction (MI). Anesthetized mice underwent left anterior descending coronary artery ligation for 30, 60, or 120 min followed by reperfusion. Mice breathed NO beginning 20 min before reperfusion and continuing thereafter for 24 h. MI size and area at risk were measured, and left ventricular (LV) function was evaluated using echocardiography and invasive hemodynamic measurements. Inhalation of 40 or 80 ppm, but not 20 ppm, NO decreased the ratio of MI size to area at risk. NO inhalation improved LV systolic function, as assessed by echocardiography 24 h after reperfusion, and systolic and diastolic function, as evaluated by hemodynamic measurements 72 h after reperfusion. Myocardial neutrophil infiltration was reduced in mice breathing NO, and neutrophil depletion prevented inhaled NO from reducing myocardial I/R injury. NO inhalation increased arterial nitrite levels but did not change myocardial cGMP levels. Breathing 40 or 80 ppm NO markedly and significantly decreased MI size and improved LV function after ischemia and reperfusion in mice. NO inhalation may represent a novel method to salvage myocardium at risk of I/R injury.

myocardial infarction; cardiac injury; murine model

AFTER ACUTE CORONARY OCCLUSION, the extent of myocardial injury is a crucial determinant of functional recovery and survival. Early restoration of myocardial blood flow decreases the magnitude of injury and improves the prognosis of myocardial infarction (MI). However, restoration of blood flow itself can increase myocardial damage (2). Reperfusion is accompanied by formation of reactive oxygen species (ROS), which disrupt cell membranes and mitochondrial metabolism, resulting in cellular necrosis and apoptosis (2). ROS also inactivate nitric oxide (NO) (18) and activate neutrophils (15) and platelets (28). Activated neutrophils can play an important role in reperfusion-induced damage, generating ROS, proteolytic enzymes, and proinflammatory mediators. Neutrophils and platelets are also involved in plugging capillaries, thereby limiting adequate flow after reperfusion and contributing to the “no-reflow” phenomenon (36).

NO, a free radical gas, is a potent vasodilator that inhibits platelet and leukocyte activation and adhesion (17, 24). Extensive study of the impact of NO donor compounds on myocardial ischemia-reperfusion injury in animal models has revealed varying effects on MI size and left ventricular (LV) function (for review see Ref. 41). The therapeutic use of NO donor compounds after MI has been hindered by their systemic vasodilator effects, potentially leading to hypotension and further compromise of myocardial perfusion.

Over the last decade, NO inhalation has proven valuable for treatment of hypoxic pulmonary hypertension in newborns. Because NO is rapidly bound to hemoglobin in vivo, it was initially suggested that the actions of inhaled NO would be limited to the lungs. More recently, however, inhaled NO has been shown to increase coronary artery patency after thrombolysis by decreasing platelet adhesion and aggregation (1) and to improve blood flow and decrease leukocyte adhesion in a feline model of intestinal I/R (13), effects that may favorably impact cardiac injury in myocardial I/R. The present study reports that inhaled NO can decrease cardiac injury and improve the recovery of LV function in a murine model of myocardial I/R without causing systemic hypotension.

METHODS

Surgical procedures. After approval by the Massachusetts General Hospital Subcommittee on Research Animal Care, we studied 2- to 4-mo-old male C57BL/6J wild-type mice. The mice were anesthetized with intraperitoneal administration of ketamine (100 mg/kg) and xylazine (5 mg/kg) and ventilated at inspired O2 fraction of 1.0. Myocardial ischemia was induced by ligation of the left anterior descending coronary artery (LAD), as previously described (38). Mice were subjected to 30, 60, or 120 min of ischemia and then reperfused. After 24 h, the artery was religated, and 15-μm fluorescent microspheres (n = 7.5 × 105; Fluospheres, Molecular Probes, Eugene, OR) were injected into the LV for determination of the area at risk (AAR).
The heart was harvested, cut into 1-mm slices, and stained with 2,3,5 triphenyltetrazolium chloride (TTC) for measurement of MI size (38).

NO delivery. At 20 min before reperfusion, NO (80 ppm in N2) was added to the gas entering the ventilator of the treated mice. After recovery from anesthesia and extubation, the mice were placed in a chamber, as described previously (37), and breathed NO in air (inspired O2 fraction = 0.19) for the duration of the experiment. NO and nitrogen dioxide levels were constantly monitored in the ventilator and chamber outlet gas (INOVent delivery system, Datek-Ohmeda, Madison, WI). An NO dose-response curve was obtained from mice subjected to 60 min of ischemia: 0 ppm NO (n = 7), 20 ppm NO (n = 5), 40 ppm NO (n = 7), and 80 ppm NO (n = 6).

Measurement of AAR and MI size. After they were stained with TTC, the heart slices were preserved in 10% formalin for 24 h, and digital images were obtained via fluorescent microscopy for measurement of AAR and via light microscopy for measurement of MI size. The area without microspheres (AAR) and the non-TTC-stained region (MI) were measured on each slice (NIH Image software) and summed. We calculated the ratios of AAR to total myocardial area, MI to total myocardial area, and MI to AAR (38). For six animals, microsphere or TTC analysis could not be interpreted, and these data were excluded from the study.

Invasive hemodynamics. Hemodynamic measurements were obtained in mice 24 h and 3 days after reperfusion, as described previously (40), and interpreted by an echocardiographer blinded to the treatment group. HR, LV end-diastolic internal diameter, LV posterior wall thickness, and LV anterior wall thickness were measured, and LV fractional shortening was calculated using an M-mode echocardiogram obtained at the midpapillary level. LV end-diastolic and end-systolic volumes were measured on the two-dimensional parasternal long-axis view, and LV ejection fraction was calculated. One animal had poor echocardiographic windows and was excluded from the study.

Measurement of LV neutrophils. Hearts were harvested and embedded in paraffin. Sections (5 μm thick) were cut at 1-mm intervals from apex to base. Neutrophils were detected using an anti-mouse neutrophil monoclonal antibody (Cedarlane, Toronto, ON, Canada), counted on each slice, and summed.

Neutrophil depletion. To assess the importance of circulating neutrophils in the effect of NO breathing on MI size, mice were injected with 250 μg of anti-Gr-1 antibody (PharMingen, San Diego, CA) 72 and 24 h before 120 min of LAD ligation to ensure maximal neutrophil depletion (33). The efficacy of neutrophil depletion was determined by counting blood neutrophil concentrations after 24 h of reperfusion in mice with and without antibody treatment (n = 5 in each group).

Nitrite measurements. Nitrite levels in plasma and whole blood were determined in mice without ischemia and in mice subjected to 60 min of ischemia and 1 min reperfusion and breathing room air with (n = 5 nonischemic and 6 ischemic) or without (n = 5 nonischemic and 6 ischemic) 80 ppm NO for 20 min. Nitrite levels were measured with the reductive triiodide-based chemiluminescence assay with and without 2 min of pretreatment with a specific scavenger of nitrite (10% acid sulfanilamide) (49). Nitrite was measured in whole blood as previously described (10).

cGMP measurements. Myocardial cGMP concentrations were measured in mice, as described previously (47), 1 min after intraperitoneal injection of sodium nitroprusside (1.5 mg/kg, n = 3) or saline (n = 6). Plasma and myocardial cGMP levels were also measured in control mice after 30 min of air breathing without or with 80 ppm NO (n = 5 each). A subsequent group of mice in which the LAD was ligated for 30 min breathed room air (n = 3) or room air to which 80 ppm NO was added 20 min before reperfusion (n = 6). To inhibit endogenous NO production, all ischemic mice were injected with nitro-L-arginine methyl ester (100 mg/kg ip) 15 min before surgery. In ischemic mice, the blood and heart were harvested 1 min after reperfusion. In all the mice, blood methemoglobin levels were measured 30 min and 24 h after reperfusion during inhalation of 80 ppm NO (n = 5 mice at each time point).

RESULTS

Inhaled NO and MI size. The AAR was similar in untreated and treated animals for all durations of ischemia; MI size increased in all groups with longer periods of ischemia (P < 0.0005). Compared with untreated animals, the ratio of MI area to LV area and the ratio of MI area to AAR decreased in animals inhaling 80 ppm NO (Table 1, Fig. 1A). A decrease in the MI-to-AAR ratio with NO was observed for all durations of ischemia: inhalation of 80 ppm NO decreased MI area-to-AAR ratio by 53, 51, and 45% after 30, 60, and 120 min of ischemia, respectively, and 24 h of reperfusion (Table 1). Inhalation of 40 ppm NO decreased MI area-to-AAR ratio after 60 min of ischemia and 24 h of reperfusion, whereas inhalation of 20 ppm NO did not (Fig. 1B).

| Table 1. Effects of 80 ppm NO inhalation size after ischemia and reperfusion |
|------------------------------|---------|----------|---------|
|               | AAR/LV, % | MI/LV, % | MI/AAR, % |
| 30 min of ischemia  |         |         |         |
| Air               | 13      | 64±3    | 20±3    | 32±4    |
| NO                | 13      | 58±5    | 9±1*    | 15±2*   |
| 60 min of ischemia |         |         |         |
| Air               | 7       | 73±8    | 28±5    | 37±6    |
| NO                | 6       | 78±5    | 14±2    | 18±3*   |
| 120 min of ischemia|        |         |         |
| Air               | 11      | 71±4    | 35±3    | 51±5    |
| NO                | 9       | 71±3    | 20±2*   | 28±3*   |

Values are means ± SE. AAR/LV, ratio of area at risk (AAR) to total left ventricular (LV) area; MI/LV, ratio of myocardial infarction (MI) area to total LV area; MI/AAR, ratio of MI area to AAR; NO, mice breathing air with 80 ppm nitric oxide (NO). *P < 0.05 vs. air.
Inhaled NO and LV size and function.

LV size and function did not differ between mice subjected to 30 min of ischemia and 24 h of reperfusion and normal controls (data not shown).

LV end-diastolic internal diameter and LV end-diastolic volume increased similarly in mice subjected to 60 min of myocardial ischemia and 24 h of reperfusion whether they breathed room air without or with 80 ppm NO (Table 2, Fig. 2). After 120 min of ischemia and 24 h of reperfusion, there was a trend toward less increase in LV end-diastolic volume in animals breathing room air with 80 ppm NO ($P < 0.05$; Table 2).

Fractional shortening and LV ejection fraction decreased less in mice breathing room air with 80 ppm NO than in mice breathing room air after 60 and 120 min of ischemia (Table 2, Fig. 2).

Hemodynamic parameters obtained after 60 min of ischemia and 24 h of reperfusion with or without inhalation of 80 ppm NO for 24 h did not differ from normal control values (data not shown). After 120 min of ischemia and 24 h of reperfusion, LV systolic and diastolic indexes were similarly decreased in both groups. However, after 3 days of reperfusion, LV end-systolic pressure and maximum and minimum rates of LV pressure development were significantly higher in mice breathing 80 ppm NO in air than in mice breathing room air after 60 and 120 min of ischemia (Table 2, Fig. 2).

Blood pressure and metabolic effects of NO inhalation.

Inhalation of 80 ppm of NO in air did not lower systemic blood pressure: 89/64 mmHg before vs. 89/64 at 30 s and 86/62 mmHg at 5 min of 80 ppm NO inhalation, respectively. NO breathing did not change HR (data not shown). Methemoglobin levels were 0.9$±$0.1 and 3.5$±$1% after 30 min and 24 h of 80 ppm NO inhalation, respectively.

### Table 2. Effects of 80 ppm NO inhalation on echocardiographic parameters after 60 or 120 min of myocardial ischemia and 24 h of reperfusion

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>$\Delta$ LVIDd</th>
<th>$\Delta$ FS</th>
<th>$\Delta$ LVEDV</th>
<th>$\Delta$ LVEF</th>
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</thead>
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<tr>
<td>60 min, ischemia-reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Air</td>
<td>9</td>
<td>13$±$3</td>
<td>$-33±5$</td>
<td>83$±$28</td>
<td>$-29±6$</td>
</tr>
<tr>
<td>NO</td>
<td>8</td>
<td>23$±$4</td>
<td>$-12±6^*$</td>
<td>57$±$18</td>
<td>$-10±5^*$</td>
</tr>
<tr>
<td>120 min, ischemia-reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air</td>
<td>25</td>
<td>29$±$3</td>
<td>$-57±5$</td>
<td>115$±$16</td>
<td>$-53±4$</td>
</tr>
<tr>
<td>NO</td>
<td>27</td>
<td>23$±$2</td>
<td>$-32±4^*$</td>
<td>80$±$9†</td>
<td>$-33±3^*$</td>
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</tbody>
</table>

Values are means $±$ SE, expressed as percent change between baseline and 24 h of reperfusion. $\Delta$ LVIDd, percent change of LV end-diastolic diameter; $\Delta$FS, percent change of shortening fraction; $\Delta$LVEDV, percent change of LV end-diastolic volume; $\Delta$LVEF, percent change of LV ejection fraction. *$P < 0.05$ vs. air. †$P = 0.05$ vs. air.
Inhaled NO reduced myocardial injury induced by 30 min to 2 h of ischemia. Invasive hemodynamic techniques revealed that, at 72 h after 2 h of ischemia, LV systolic and diastolic function was better preserved in mice that breathed 80 ppm NO for the first 24 h than in those that breathed only air. These findings strongly suggest that the beneficial effects of NO inhalation on LV function after I/R can persist long after NO inhalation ceases.

In a variety of animal myocardial I/R models, systemic administration of NO or NO donor compounds during ischemia or at reperfusion decreased myocardial tissue injury (21, 22, 27). Several authors, however, reported that NO donor compounds can have deleterious effects on infarct size (29) and LV function (8, 29, 32) in cardiac I/R injury. In studies of patients presenting with an MI, nitroglycerin has been reported to improve LV function and prevent adverse LV remodeling (23, 31). However, in large randomized clinical trials, nitrates did not alter survival rates after MI (3, 4). One possible explanation for these discrepancies is that NO donor compounds appear to have the greatest impact when they are administered early after MI (23); however, early therapy is not always feasible, because infusion of these agents can cause systemic hypotension. In

**DISCUSSION**

In the present study, we report that, in a murine model of myocardial I/R, inhalation of 40 or 80 ppm NO for 20 min before and 24 h after reperfusion decreased MI size and improved LV function.

**Role of neutrophils.** Immunohistochemical staining was used to detect neutrophils in myocardial tissue obtained from mice subjected to 60 min of ischemia and 24 h of reperfusion. The number of neutrophils in mice breathing air with 80 ppm NO was markedly reduced compared with mice breathing air without NO: 4 ± 1 vs. 18 ± 4 mm⁻² (P < 0.02).

Injection of an antineutrophil antibody resulted in a marked decrease in the neutrophil concentration measured in circulating blood: 43 ± 13 mm⁻³ in animals injected with anti-Gr-1 antibody vs. 4.5 ± 2.2 × 10³ mm⁻³ in untreated animals. After neutrophil depletion, NO inhalation did not alter MI area-to-AAR ratio in mice subjected to 120 min of ischemia followed by 24 h of reperfusion: MI area/LV area = 24 ± 4 and 25 ± 4% in mice breathing air with and without 80 ppm NO, respectively, and MI area/AAR = 35 ± 6 and 36 ± 7% in mice breathing air with and without 80 ppm NO, respectively.

**Nitrite measurements.** In healthy mice, breathing 80 ppm NO in air for 20 min increased plasma nitrite levels by 0.80 μM (4.5-fold, P < 0.0005 vs. baseline; Fig. 3). Similarly, after 30 min of ischemia and 1 min of reperfusion, plasma nitrite levels were increased by 0.91 μM in mice that breathed 80 ppm NO for 20 min (7-fold, P < 0.0005; Fig. 3A). The level of nitrite in whole blood increased by 0.66 and 0.43 μM after 20 min of NO inhalation in healthy and ischemic mice, respectively (P < 0.0005 and P < 0.05; Fig. 3B).

**cGMP measurements.** In healthy mice, injection of sodium nitroprusside increased cGMP levels in the heart: 10.7 ± 2 vs. 4.3 ± 0.7 pmol GMP/mg protein (P < 0.01). After 30 min of NO inhalation, plasma cGMP levels were increased in healthy mice and mice subjected to cardiac I/R (data not shown). The myocardial concentration of cGMP, however, was unchanged by NO inhalation (80 ppm) in healthy and ischemic mice (Fig. 3C).

**Table 3. Hemodynamic measurements after 120 min of ischemia and 72 h of reperfusion in mice breathing air with or without 80 ppm NO**

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
<th>80 ppm NO</th>
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<tbody>
<tr>
<td>HR, beats/min</td>
<td>628±13</td>
<td>676±24</td>
</tr>
<tr>
<td>LVESP, mmHg</td>
<td>86±3</td>
<td>101±3*</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>7±1</td>
<td>8±1</td>
</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
<td>10.579±518</td>
<td>14.732±875*</td>
</tr>
<tr>
<td>dP/dt min, mmHg/s</td>
<td>8.232±762</td>
<td>12.015±1.481*</td>
</tr>
<tr>
<td>LVEDP, ms</td>
<td>5.6±0.2</td>
<td>5.2±0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6). Treatment was started 20 min before reperfusion and continued for 24 h. HR, heart rate; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt max, maximum rate of developed LV pressure; dP/dt min, minimum rate of developed LV pressure; τ, time constant of isovolumic relaxation. *P < 0.05 vs. room air.

**Fig. 3.** A: plasma nitrite levels. In control (healthy) mice and mice subjected to 60 min of ischemia and 1 min of reperfusion (ischemic), plasma nitrite levels increased after 20 min of 80 ppm NO inhalation. B: whole blood nitrite levels. In healthy mice and mice subjected to 60 min of ischemia and 1 min of reperfusion, whole blood nitrite levels increased after 20 min of 80 ppm NO inhalation. In A and B, values are means ± SE of 5 healthy room air- and NO-breathing mice and 6 ischemic room air- and NO-breathing mice. C: myocardial cGMP levels. Inhalation of 80 ppm NO did not increase myocardial cGMP levels in healthy mice (n = 5) and mice subjected to 30 min of ischemia and 1 min of reperfusion (n = 3). Values are means ± SE of 5 healthy and room air- and NO-breathing, 3 ischemic room air-breathing, and 6 ischemic NO-breathing mice. *P < 0.05 vs. room air.
contrast to NO donor compounds, we found that NO inhalation decreased MI size in mice subjected to cardiac I/R without altering blood pressure; this lack of systemic vasodilation potentially allows administration early in patients presenting with MI.

Over the past decade, inhaled NO has been extensively employed as a selective pulmonary vasodilator (for review see Ref. 20). It was initially hypothesized that NO was rapidly scavenged and inactivated by hemoglobin, precluding any systemic effects, and, indeed, systemic hypotension does not occur when humans breathe NO gas. However, since this initial hypothesis, investigators have reported that inhaled NO increases the bleeding time of rabbits (19) and urinary flow in anesthetized pigs with phenylephrine-induced hypertension (46), whereas it decreases neointimal formation in balloon-injured rat carotid arteries (25), platelet-mediated thrombosis after thrombolysis in canine coronary arteries (1), and leukocyte adhesion to activated endothelium in a feline model of mesenteric I/R (13). NO inhalation also reduces leukocyte accumulation and improves contractile function in hearts isolated from endotoxin-challenged rats (34).

Neutrophils adhere to the coronary vascular endothelium after a few minutes of reperfusion (43) and become activated, leading to release of proteolytic enzymes and ROS (11). NO inhibits the adhesion of neutrophils (30) and their release of ROS (7, 14). In cardiac I/R, administration of NO donor compounds decreases leukocyte accumulation (26, 27). In the present study, inhaled NO markedly decreased accumulation of neutrophils in myocardial tissues after I/R. Moreover, depletion of circulating leukocytes by antibody treatment blocked neutrophil infiltration and improved LV function without decreasing systemic blood pressure. Clinical trials are required to ascertain whether NO inhalation may be an effective therapy to improve outcomes of patients presenting with MI.

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