

PRECLINICAL STUDY

Nitric Oxide Inhalation Improves Microvascular Flow and Decreases Infarction Size After Myocardial Ischemia and Reperfusion

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- Objectives** The purpose of this study was to test if nitric oxide (NO) could improve microvascular perfusion and decrease tissue injury in a porcine model of myocardial ischemia and reperfusion (I/R).
- Background** Inhaled NO is a selective pulmonary vasodilator with biologic effects in remote vascular beds.
- Methods** In 37 pigs, the midportion of the left anterior descending coronary artery was occluded for 50 min followed by 4 h of reperfusion. Pigs were treated with a saline infusion (control; n = 14), intravenous nitroglycerin (IV-NTG) at 2 $\mu\text{g}/\text{kg}/\text{min}$ (n = 11), or inhaled nitric oxide (iNO) at 80 parts per million (n = 12) beginning 10 min before balloon deflation and continuing throughout reperfusion.
- Results** Total myocardial oxidized NO species in the infarct core was greater in the iNO pigs than in the control or IV-NTG pigs (0.60 ± 0.05 nmol/mg tissue vs. 0.40 ± 0.03 nmol/mg tissue and 0.40 ± 0.02 nmol/mg tissue, respectively; $p < 0.01$ for both). Infarct size, expressed as percentage of left ventricle area at risk (AAR), was smaller in the iNO pigs than in the control or IV-NTG pigs ($31 \pm 6\%$ AAR vs. $58 \pm 7\%$ AAR and $46 \pm 7\%$ AAR, respectively; $p < 0.05$ for both) and was associated with less creatine phosphokinase-MB release. Inhaled NO improved endocardial and epicardial blood flow in the infarct zone, as measured using colored microspheres ($p < 0.001$ vs. control and IV-NTG). Moreover, NO inhalation reduced leukocyte infiltration, as reflected by decreased cardiac myeloperoxidase activity (0.8 ± 0.2 U/mg tissue vs. 2.3 ± 0.8 U/mg tissue in control and 1.4 ± 0.4 U/mg tissue in IV-NTG; $p < 0.05$ for both) and decreased cardiomyocyte apoptosis in the infarct border zone.
- Conclusions** Inhalation of NO just before and during coronary reperfusion significantly improves microvascular perfusion, reduces infarct size, and may offer an attractive and novel treatment of myocardial infarction. (J Am Coll Cardiol 2007;50:808–17) © 2007 by the American College of Cardiology Foundation

Reperfusion of ischemic myocardium after percutaneous coronary intervention, bypass surgery, and heart transplantation may result in paradoxical myocardial injury compromising left ventricular (LV) function recovery (1,2). Increased production of reactive oxygen species, platelet aggregation, complement activation, neutrophil infiltration,

myocardial calcium overload, and cardiomyocyte apoptosis all contribute to reperfusion injury (3–6). Reperfusion injury is frequently associated with impaired myocardial tissue perfusion despite restoration of epicardial coronary flow, and microvascular obstruction (MVO) represents an important obstacle hindering recovery of LV systolic function and compromising long-term clinical outcome (7). Although experimental studies using antineutrophil antibodies and small early clinical trials using adenosine and nicorandil showed a beneficial effect on reperfusion injury (8,9), larger randomized clinical trials have, thus far, not identified an effective therapy for preventing MVO or salvaging heart muscle at risk of necrosis after reperfusion therapy for acute myocardial infarction (2,9–11).

Nitric oxide (NO) modulates many of the processes contributing to ischemia-reperfusion (I/R) injury. Nitric

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Manuscript received October 3, 2006; revised manuscript received April 2, 2007, accepted April 10, 2007.

oxide regulates vasomotor tone, platelet activation, interaction of platelets and leukocytes with the vessel wall, immune and inflammatory responses, and apoptosis. Administration of subvasodilator concentrations of NO-donor compounds in dogs (12) preserved coronary vasodilation, inhibited neutrophil accumulation, and reduced myocardial necrosis associated with transient coronary artery occlusion. In contrast, systematic administration of transdermal glyceryl trinitrate did not improve survival or left ventricular function in patients with acute myocardial infarction (13). However, systemic hypotension associated with the administration of NO-donor compounds remains a major shortcoming in the setting of reperfusion therapy for acute myocardial infarction (14).

Although initially best known for its selective pulmonary vasodilator action, inhaled nitric oxide (iNO) has more recently been shown to have effects in the systemic circulation. For example, breathing 80 parts per million (ppm) NO increased brachial artery blood flow during blockade of regional NO synthesis (15). Inhaled NO also increased blood flow in cat mesenteric venules subjected to I/R (16) and decreased infarction size in a murine myocardial I/R injury model (17). Whether or not iNO can improve myocardial microvascular perfusion after recanalization of an occluded coronary artery and thereby limit myocardial I/R injury remains to be determined. We report here that in a clinically representative porcine model of transient coronary artery occlusion, iNO increased microvascular flow in postischemic myocardium and decreased infarct size, suggesting a potential new strategy to target myocardial I/R in patients.

Methods

Animal preparation. The study was approved by the Animal Care and Use Committee of the University of Leuven. Juvenile domestic pigs of both genders weighing 25 to 30 kg were used. Pigs were pretreated for 10 days with amiodarone to reduce life-threatening arrhythmias upon acute vessel occlusion (600 mg/day). Clopidogrel (150 mg/day) and aspirin (300 mg/day) were administered 1 day before and on the day of the procedure. Pigs were sedated using 3 mg/kg IM azaperone (Stresnil, Janssen Pharmaceuticals, Beerse, Belgium) and anaesthetized using an IV bolus of ketamine (1 mg/kg Anesketin, Eurovet, Heusden-Zolder, Belgium) followed by a 10 mg/kg/h continuous infusion of 2% propofol (AstraZeneca, Brussels, Belgium). Pigs were mechanically ventilated using a 50% oxygen gas mixture. Ventilation was adjusted to maintain physiologic PaCO₂ and pH. Continuous electrocardiographic monitoring of heart rate, rhythm, and ST-segment changes was performed.

An 8-F catheter was introduced into the right carotid artery to measure blood pressure and to access the coronary arteries for angiography. At selected time points, a 6-F Mikro-Tip pressure transducer catheter (Millar Instruments, Houston, Texas) or a pigtail catheter was inserted via the left carotid artery into the LV to measure maximum and

minimum rates of LV pressure development or to inject colored microspheres, respectively. All hemodynamic recordings were made for 1 minute at a sampling rate of 2,000/s before ischemia, after 40 min of cardiac ischemia, and at 30, 60, and 240 min of reperfusion. Data were processed using PowerLab recording and analysis software (AD Instruments, Oxfordshire, United Kingdom). A third 8-F catheter was inserted into the descending aorta via the left femoral artery and was used for blood sampling and reference blood flow measurements (using microspheres).

Transient ischemia of the anterior wall was induced by inflating a properly sized balloon-mounted stent for 50 min in the left anterior descending coronary artery (LAD) distal to the first diagonal branch. Coronary artery occlusion was confirmed by contrast injection and by electrocardiographic ST-segment elevation. After 50 min, the LAD balloon was deflated and restoration of normal coronary flow was documented by angiography.

After reperfusion for 240 min, the pigs were killed using an overdose of pentobarbital, and the hearts were excised. The LV was sectioned into 5 slices perpendicular to the heart base-apex axis, tissue sections were prepared for triphenyl tetrazolium chloride staining and histology, and biopsy specimens were obtained for myeloperoxidase (MPO) activity, total oxidized nitric oxide species (NO_x), and microsphere analysis.

Experimental protocols. Pigs were randomly assigned to receive saline (control; n = 14), iNO (n = 12), or intravenous nitroglycerin (IV-NTG) (n = 11) beginning 10 min before reperfusion. For the iNO group, NO (80 ppm) was added to the gas mixture used for ventilation using the INOvent delivery system (INO Therapeutics, Clinton, New Jersey). For the IV-NTG group, nitroglycerin was administered intravenously at the rate of 2 μg/kg/min. All therapies were continued throughout the reperfusion period.

Determination of myocardial area at risk and infarct size. After euthanasia, the LAD was reoccluded by inflation of a balloon in the stent, and 2% Evan's blue (2%) was injected into the left atrium to outline the area at risk (AAR). A transversely sectioned midventricular slice of the explanted heart was then incubated in 2,3,5-triphenyltetrazolium chloride (1.4%) at 37°C to evaluate viability. The extent of the infarct size (percentage of AAR) was determined by planimetry on a Zeiss KS300 microscope using National Institutes of Health image software independently by 3 experienced investigators blinded to the treatment group.

Myocardial myeloperoxidase activity. Myocardial samples taken from the infarct zone, border zone, and remote areas

Abbreviations and Acronyms

AAR	= area at risk
iNO	= inhaled nitric oxide
LV	= left ventricle/ventricular
MPO	= myeloperoxidase
MVO	= microvascular obstruction
NO	= nitric oxide
NO_x	= oxidized nitric oxide species
NTG	= nitroglycerin

were frozen in liquid nitrogen. The MPO activity was determined as described previously (18). The MPO activity in the supernatant was determined by measuring the changes in absorbance (450 nm) at 0.5, 1.5, 5, 7, 10, 20, 30, and 40 min (ELx 808 Ulytra microplate reader, Bio-Tek Instruments, Winooski, Vermont). MPO activity is expressed as units/mg protein. Myeloperoxidase activity in plasma at baseline and at the end of reperfusion was quantified by measuring the oxidation of guaiacol at A_{470} at 25°C. One unit is defined as the amount that consumes 1 $\mu\text{mol H}_2\text{O}_2$ per min at 25°C, and results are expressed as U/ml plasma.

Cardiac necrosis markers. Arterial blood samples were collected at baseline, after 15 and 40 min of ischemia, and every half hour during reperfusion for cardiac creatine phosphokinase (CK)-MB measurements, and all samples were analyzed at a central core clinical chemistry laboratory (University of Leuven). To quantitate the overall release of CK-MB over the 4-h reperfusion period, CK-MB versus time curves were plot fitted, and area under the curve (AUC) was derived, using the method reported by Vollmer et al. (19)

Regional blood flow measurements. To measure regional myocardial blood flow, 2 million colored microspheres (15- μm diameter; Triton Technologies, San Diego, California) were diluted in 10 ml saline, and different colors were injected into the LV at baseline, 40 min after occlusion, and at 4 h after reperfusion in 24 pigs (8 per group) (20). After killing, myocardial samples were obtained from endocardial and epicardial layers of the infarct and border zones, digested in 4 mol/l KOH with 1% Tween-80, and filtered. Microspheres were eluted using di-(ethylene glycol) ethyl ether acetate and analyzed using a luminescence spectrophotometer (8453E UV-visible spectroscopy system, Agilent, Santa Clara, California).

Histologic markers of neutrophil infiltration and cardiomyocyte apoptosis. Myocardial 5- μm sections from paraffin-embedded biopsy specimens of the infarct, border, and remote zones were stained with hematoxylin-eosin and an antiserum specific for MPO. Hemorrhage and the number of infiltrating neutrophils were semiquantitatively evaluated by an experienced pathologist (E.V.), blinded to the treatment groups, using a scoring system where 0 indicated reaction absent, 0.5 minimal reaction, 1 mild reaction observed only at high-power magnification, 2 moderate reaction observed at low power, and 3 severe reaction.

Apoptosis in the border zone was evaluated using terminal dUTP nick-end labeling (TUNEL) with an in situ cell death detection kit (Roche Diagnostics, Vilvoorde, Belgium) according to the manufacturer's instructions. Apoptotic rate was expressed as the percentage of TUNEL-positive nuclei divided by total number of nuclei, calculated in 10 randomly selected high-power fields of the border zone.

Tissue and plasma NOx and nitrite measurements. Myocardial transmural biopsies taken from the infarct, border, and remote zones were weighted and homogenized in 400 μl 0.5 mol/l NaOH using a Ribolyzer (Hybaid, Ashford, United Kingdom). Samples kept on ice for 15 min were deproteinized with an equal volume of 10% zinc sulfate, precipitates were centrifuged at 14,000 g, and total NOx was determined in the supernatant using ozone-based chemiluminescence after injection in vanadium (III)-chloride reductants in line with the Sievers Model 280 NO analyzer (Boulder, Colorado) (21,22). Arterial blood samples were collected at baseline, 45 min after occlusion, and at 30, 120, and 240 min of reperfusion into lithium-heparin tubes and processed immediately to determine plasma total NOx and plasma nitrite levels using triiodide-based gas phase chemiluminescence (Sievers 280) (22,23).

Statistical analysis. Statistical analyses were performed using SAS statistical software (version 9.12, SAS Institute, Cary, North Carolina). Data are expressed as mean \pm SEM. Analysis of variance followed by a Bonferroni or Fisher correction was used to analyze differences between groups with normally distributed data. Repeated measurement analysis of variance was used to test serial hemodynamic values obtained at different time points during the experimental protocol. Because MPO activity in myocardial tissue did not follow a normal distribution, Kruskal-Wallis nonparametric statistics were reported, and differences between groups were identified using a Mann-Whitney or Wilcoxon test. A p value of <0.05 was considered to be statistically significant.

Results

Clinical course and ischemic complications. Of the 37 pigs subjected to coronary artery occlusion and randomized, 1 animal in the control group and 1 in the IV-NTG group died because of ventricular fibrillation during the ischemia. Ventricular fibrillation also occurred during reperfusion in 3 control, 2 IV-NTG, and 1 iNO pig, but all were successfully cardioverted. Blood gas analysis performed at baseline and during reperfusion confirmed normal oxygenation and ventilatory parameters, and no increase in methemoglobin levels was observed with NO inhalation (data not shown), suggesting effective methemoglobin reductase activity (24).

Hemodynamic measurements and left ventricular function. There were no differences between the groups at baseline in heart rate, mean arterial blood pressure (MAP), or maximal and minimal rates of pressure development (dP/dt_{max} and dP/dt_{min} , respectively). Heart rate increased modestly in all groups during I/R (Table 1), and MAP decreased 40 min after ischemia in all 3 groups. The MAP further decreased during the early reperfusion period only in the IV-NTG group. The LV dP/dt_{max} and dP/dt_{max} corrected for instantaneous pressure ($dP/dt_{\text{max}}/IP$) decreased 40 min after balloon occlusion in all groups, but by the end of reperfusion only IV-NTG pigs showed a further reduc-

Table 1 Hemodynamic Data at Baseline, End of Ischemia, and at Reperfusion

	Baseline	Ischemia 40 min	Reperfusion		
			30 min	60 min	240 min
HR (beats/min)					
Control	95 ± 9	115 ± 7*	110 ± 8	116 ± 9*	100 ± 7
iNO	108 ± 13	113 ± 10	126 ± 10	115 ± 11	120 ± 13
IV-NTG	91 ± 10	100 ± 10	99 ± 8	100 ± 12	71 ± 7†
MAP (mm Hg)					
Control	80 ± 4	75 ± 6	75 ± 6	76 ± 6	69 ± 5
iNO	79 ± 5	69 ± 4	71 ± 3	69 ± 4	72 ± 4
IV-NTG	84 ± 5	75 ± 3	68 ± 4*	75 ± 5	71 ± 8
dP/dt_{max} (mm Hg/s)					
Control	2,089 ± 170	1,791 ± 179*	1,802 ± 160*	1,774 ± 127*	1,431 ± 106*
iNO	2,045 ± 154	1,546 ± 131*	1,708 ± 113	1,603 ± 156*	1,583 ± 173*
IV-NTG	2,219 ± 226	1,726 ± 216*	1,678 ± 224*	1,767 ± 220*	1,327 ± 138*†
dP/dt_{min} (mm Hg/s)					
Control	-1,254 ± 153	-1,105 ± 145	-1,013 ± 134	-1,041 ± 127	-903 ± 118*
iNO	-1,530 ± 206	-933 ± 121*	-1,067 ± 91*	-1,030 ± 113*	-1,089 ± 145*
IV-NTG	-1,417 ± 141	-962 ± 81*	-792 ± 85*	-894 ± 100*	-607 ± 73*†
dP/dt_{max}/IP					
Control	45.2 ± 3.8	40.2 ± 3.3	40.3 ± 3.0	41.3 ± 2.4	37.7 ± 2.6*
iNO	44.7 ± 4.6	34.1 ± 3.0*	40.4 ± 3.0	40.0 ± 4	38.7 ± 4.3*
IV-NTG	44.1 ± 5.4	34.8 ± 4.6*	36.0 ± 5.3*	37.4 ± 4.9*	29.5 ± 2.9*†

Values are presented as mean ± SEM; n = 13 in control; n = 12 in iNO; and n = 10 in IV-NTG groups. *p < 0.05 compared with baseline; †p < 0.05 compared with ischemia.

dP/dt_{max} and dP/dt_{min} = maximum and minimum rates of left ventricular pressure development, respectively; HR = heart rate; iNO = inhaled nitric oxide; IP = instantaneous pressure; IV-NTG = intravenous nitroglycerin; MAP = mean arterial blood pressure.

tion in both systolic and diastolic function (Table 1). In contrast, this progressive decline in LV function was not observed in iNO pigs, suggesting that NO inhalation is safe and may have a favorable effect on LV contractile function (Table 1).

Evaluation of myocardial infarction. To investigate whether or not IV-NTG or iNO reduces cardiac injury upon reperfusion, infarct size as a fraction of AAR was measured using planimetry (Fig. 1). In addition, release of CK-MB in serum was followed over time. The IV-NTG treatment did not significantly decrease myocardial infarction (MI) size as a fraction of AAR. Compared with control pigs, iNO reduced MI size by 47% (Fig. 1). Serum CK-MB levels were less in iNO pigs than in control and IV-NTG pigs beginning 90 min after reperfusion (p < 0.05) (Fig. 2), and the AUC for CK-MB showed a trend in favor of iNO-treated pigs (p = 0.06 vs. control; p = 0.07 vs. IV-NTG).

Regional myocardial blood flow during I/R. To investigate whether or not NTG infusion or NO breathing during reperfusion was associated with improved microvascular flow, we compared colored microsphere distribution in endocardial and epicardial regions at baseline and at the end of reperfusion in the 3 groups of pigs (Fig. 3). In control pigs after I/R, endocardial blood flow was absent in the infarct core and severely reduced in the border zone (7 ± 11% of baseline blood flow), and epicardial flow was markedly reduced in the infarct core and in the border zone (6 ± 12% and 8 ± 10%, respectively). In contrast, in iNO

pigs, regional blood flow was better preserved in the endocardial infarct and border zone (18 ± 9% and 43 ± 12% respectively; p < 0.05 vs. control for both), as well as in the epicardial infarct and border zones (35 ± 11 and 44 ± 9%, respectively; p < 0.05 vs. control for both). The IV-NTG did not significantly increase endocardial and epicardial blood flow in the infarct area or border zone (Fig. 3).

Histologic analysis and myeloperoxidase activity in myocardium and plasma. After ischemia and reperfusion, severe hemorrhage was detected predominantly in the infarct area of control pigs and was less prominent in the IV-NTG and iNO pigs (Figs. 4A to 4C). The semiquantitative hemorrhage score was less in iNO than in control pigs (0.1 ± 0.1 vs. 0.9 ± 0.3; p < 0.05) (Fig. 5A). To investigate whether or not differences in reperfusion injury were associated with altered infiltration of leukocytes, we counted the number of infiltrating neutrophils and measured MPO activity in the reperfused myocardium. Neutrophil infiltration in the infarct zone of control and IV-NTG pigs was significantly greater than in iNO pigs (Figs. 4D to 4F). The neutrophil infiltration score was less in iNO than in control pigs (0.9 ± 0.2 vs. 1.8 ± 0.2 and 1.0 ± 0.2 vs. 1.7 ± 0.2 in the infarct and border areas, respectively; p < 0.05, Kruskal-Wallis test) but was unaffected by IV-NTG (1.7 ± 0.2 and 1.2 ± 0.2 in the infarct and border areas, respectively) (Fig. 5B). The MPO activity was significantly greater in the infarct and border areas than in the remote area in all of the groups (data not shown). There was no difference in MPO

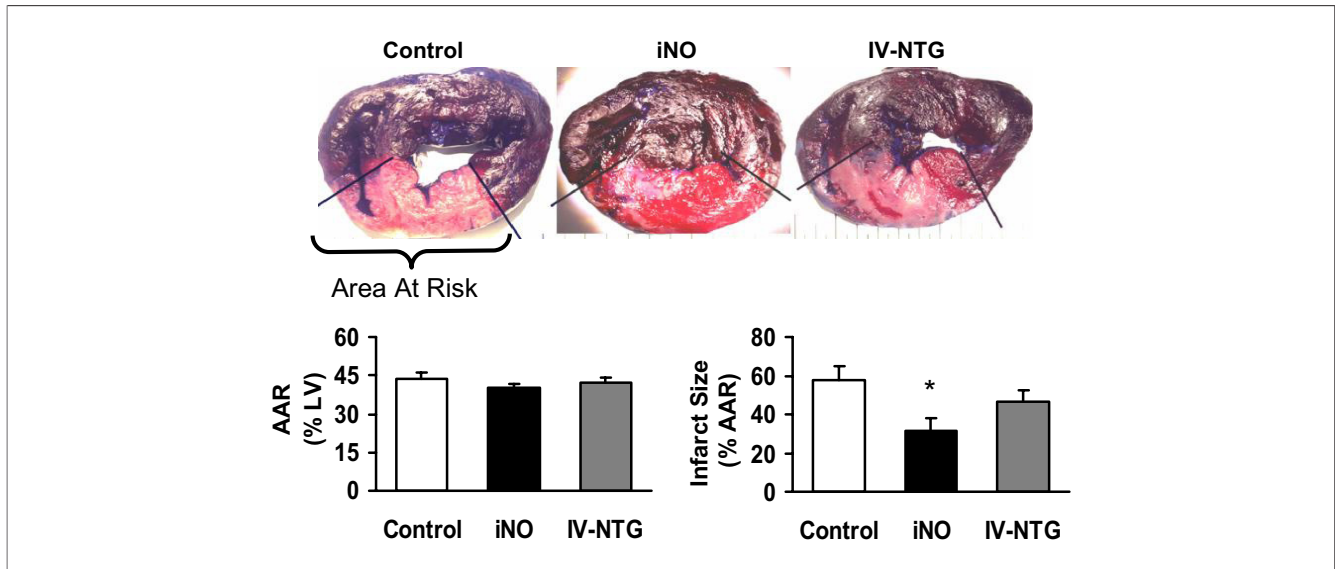


Figure 1 iNO Reduces Infarct Size After Ischemia-Reperfusion Injury

(Top) Evan's blue and triphenyl tetrazolium chloride staining of representative transverse midventricle sections from pigs subjected to left anterior descending coronary artery occlusion (50 min) and reperfusion (4 h) treated with saline (control), inhaled nitric oxide (iNO), or intravenous nitroglycerin (IV-NTG). The area at risk (AAR) is delineated by the absence of dark blue staining (indicated by oblique lines); viable myocardium within this region is stained red, and infarcted tissue remains white. (Bottom) Quantitative measurement of left ventricular area at risk (% LV, left) and infarct size (% of AAR, right) in control (n = 13), iNO (n = 12), and IV-NTG (n = 10) pigs; *p < 0.05 versus control.

activity in the remote myocardium between the groups. However, MPO activity in the infarct area was significantly less in the iNO group than in the control group (0.8 ± 0.2 U/mg vs. 2.3 ± 0.8 U/mg protein; $p < 0.05$), whereas IV-NTG did not reduce MPO (1.4 ± 0.4 U/mg protein; $p = 0.2$ vs. control) (Fig. 5B). In additional animals we also measured the MPO activity in plasma, as a marker of

neutrophil activation, and observed a significant increase in the control pigs (from 0.27 ± 0.04 U/ml at baseline to 2.14 ± 0.62 U/ml at the end of reperfusion) but not after

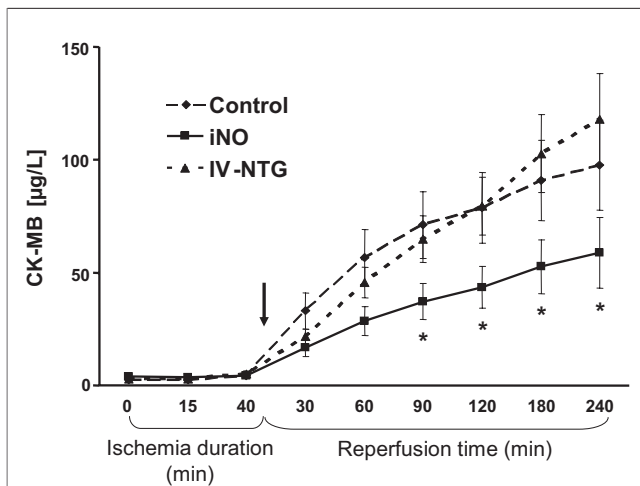


Figure 2 CK-MB Isoform Release During Ischemia and Reperfusion

Creatine phosphokinase-MB isoform (CK-MB) levels measured in plasma samples obtained at baseline (time 0), during 50 min of ischemia, and 30, 60, 90, 120, 180, and 240 min after reperfusion. Arrow indicates time of reperfusion. Data are shown as mean \pm SEM; *p < 0.05 versus control and IV-NTG. Abbreviations as in Figure 1.

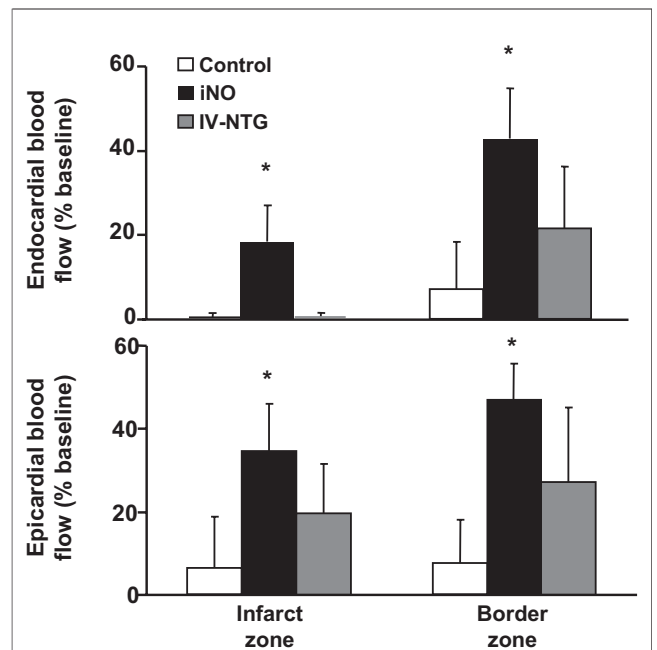


Figure 3 Regional Myocardial Blood Flow After 4 h of Reperfusion

Endocardial and epicardial regional myocardial blood flow in the infarct area and border zone of pigs receiving saline infusion (control), iNO, or IV-NTG. Data are shown as mean \pm SEM; *p < 0.05 versus control. Abbreviations as in Figure 1.

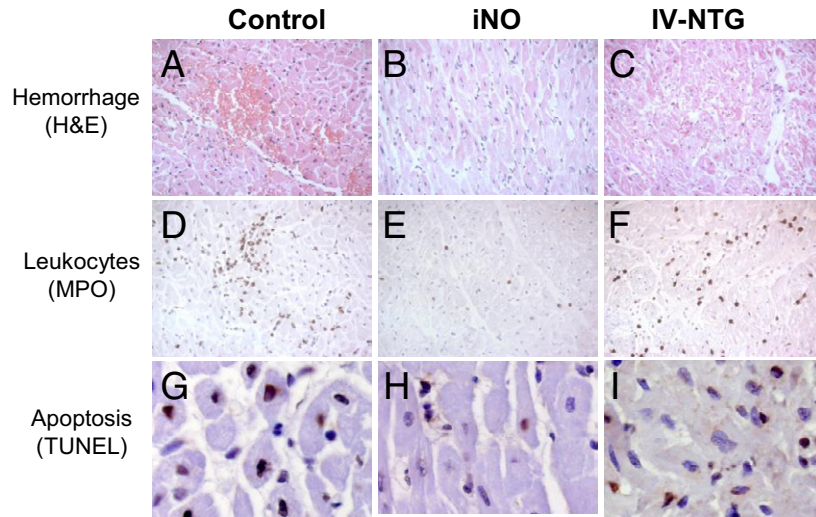


Figure 4 Histologic Analysis of Representative Myocardial Sections After Ischemia-Reperfusion Injury

Hematoxylin and eosin (H&E) staining (**A to C**) shows severe hemorrhage in the infarct zone of control pigs but not in IV-NTG and iNO-treated pigs. Myeloperoxidase (MPO) immunostaining in the border zone (**D to F**) indicates neutrophil infiltration in control pigs which was less in iNO pigs but not in IV-NTG pigs. Terminal dUTP nick-end labeling (TUNEL) staining for apoptosis (**G to I**) indicates less apoptosis in the border zone of the iNO pigs. Original magnifications: $\times 200$ (**A to F**) and $\times 400$ (**G to I**). Abbreviations as in Figure 1.

iNO (from 0.33 ± 0.01 at baseline to 0.36 ± 0.01 U/ml at the end of reperfusion).

Cell death rate, expressed as the percentage of nuclei which were TUNEL positive, was significantly less in the border zone of iNO pigs compared with control and IV-NTG pigs (16 ± 2 vs. 28 ± 1 and 26 ± 2 , respectively; $p < 0.01$ for both) (Figs. 4G to 4I, Fig. 5C).

Oxidized NO species in myocardial tissue and plasma. To determine whether or not administration of iNO or IV nitroglycerin was associated with accumulation of NO metabolites in the heart, we measured NOx in infarct, border, and remote zones. Inhaled NO increased tissue NOx levels in the infarct core of reperfused myocardium but not in the border and remote zones (Fig. 6). The NOx levels

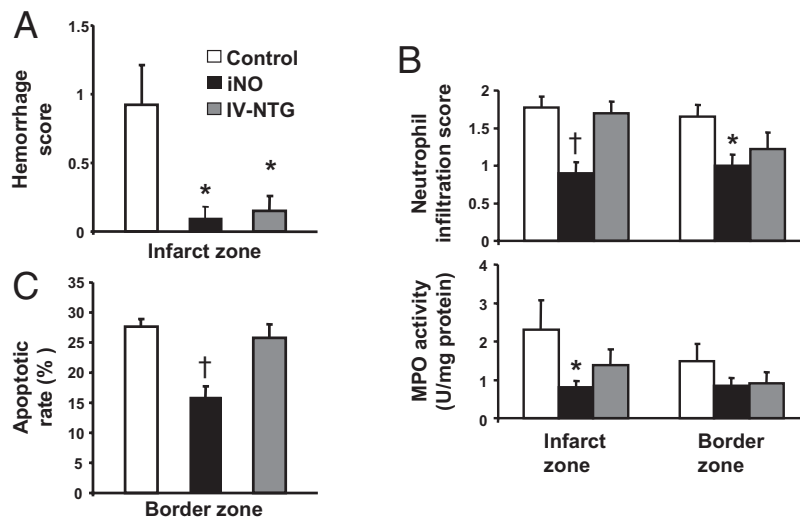
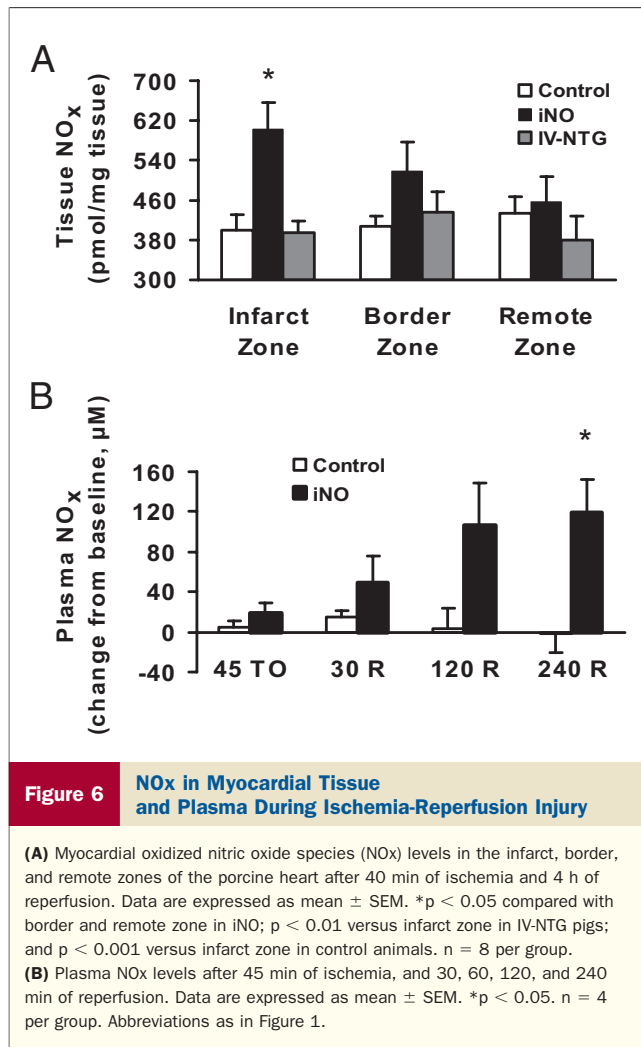


Figure 5 Quantitative Analysis of Myocardial Hemorrhage, Neutrophil Infiltration, and Cardiomyocyte Apoptosis After Ischemia-Reperfusion Injury

(**A**) Hemorrhage scores in the infarct zone of control pigs and pigs treated with iNO or IV-NTG. (**B**) Score of neutrophil infiltration (**top**) and MPO activity (**bottom**) in the infarct and border zones of animals receiving saline, iNO, or IV-NTG. (**C**) Percentage of TUNEL-positive cardiomyocytes in the infarct border zone. Data are expressed as mean \pm SEM; * $p < 0.05$ versus control; † $p < 0.05$ versus control and IV-NTG. Abbreviations as in Figures 1 and 4.



in the infarcted area of iNO pigs were greater than in control or IV-NTG pigs. Moreover, IV-NTG did not alter NOx levels in infarct, border, and remote zones. To investigate whether or not the cytoprotective effect of iNO is associated with increased myocardial cyclic guanosine monophosphate (cGMP) levels, we measured myocardial cGMP concentrations in 7 iNO, 8 IV-NTG, and 7 control pigs. The cGMP concentrations in the infarcted myocardium of iNO-treated animals were significantly higher than in IV-NTG-treated or control animals (0.41 ± 0.28 pmol/mg protein vs. 0.18 ± 0.11 and 0.07 ± 0.06 ; $p = 0.02$ and 0.001 , respectively).

In additional animals, plasma total NOx and nitrite levels were measured at baseline, at 45 min after occlusion, and at 30, 120, and 240 min into reperfusion with and without iNO ($n = 4$ per group). In the iNO group, but not in control pigs, plasma NOx levels increased after I/R in a time-dependent manner ($p < 0.05$) (Fig. 6B). In addition, we did not see a consistent increase in plasma nitrite levels with iNO (from 0.57 ± 0.25 μ mol/l to 0.87 ± 0.46 μ mol/l vs. from 0.29 ± 0.11 μ mol/l to 0.15 ± 0.05 μ mol/l in control animals).

Discussion

In this study, we report that inhalation of 80 ppm NO starting just 10 min before restoration of blood flow to an ischemic myocardial territory protects against cardiac reperfusion injury in pigs. The NO breathing was safe, and the protection observed with iNO compared favorably to the effects of IV administration of the pharmacologic NO donor NTG. Inhalation of NO did not increase the frequency of arrhythmias and did not reduce systemic blood pressure, whereas IV-NTG infusion reduced blood pressure, as well as systolic and diastolic function, after I/R. Inhalation of NO, but not infusion of NTG, significantly reduced myocardial infarct size and decreased the release of CK-MB, a marker of cardiac necrosis, while augmenting microvascular perfusion in the infarct core and border zone. Protection from reperfusion injury by iNO was associated with a reduction in infiltrating neutrophils and MPO activity in the infarct and border area and by a cytoprotective effect on cardiomyocytes in the ischemic border region. Inhaled NO, but not IV-NTG, significantly increased myocardial NOx levels and cGMP concentrations in the infarct area during reperfusion.

Inhaled NO has traditionally been considered to be a selective pulmonary vasodilator devoid of significant systemic hemodynamic effects, presumably because NO reaching the bloodstream immediately interacts with oxyhemoglobin to form methemoglobin and bioinactive nitrate (25). Yet, systemic effects of breathing NO have been observed in animal models, including reduction of neointimal formation in balloon-injured rat carotid arteries (26), platelet-mediated cyclic flow variations in canine coronary arteries (27), platelet activation and aggregation in rats (28), vasoconstriction and leukocyte recruitment in feline mesenteric microvessels subjected to I/R (29), and myocardial dysfunction in endotoxemic rats (30). Taken together with our studies on cardiac I/R injury in mice (17) and, in the present study, pigs, these data strongly support the concept that iNO can importantly influence systemic vascular beds.

There are several possible mechanisms by which iNO can elicit systemic effects. One possibility is that iNO may modify circulating cells (including leukocytes and platelets) as they pass through the lungs, inhibiting their ability to elicit I/R injury. In this respect, we have observed that iNO can fully prevent the increase in both plasma and tissue myeloperoxidase levels following I/R injury. Alternatively, iNO may form S-nitrosothiols or NO-heme complexes in plasma proteins or in circulating cells with regeneration of NO in systemic vascular beds. A third possibility is that iNO may react with superoxide to form peroxynitrite, which in turn may have dose-dependent cardioprotective effects against I/R (31,32). Finally, iNO may be converted to nitrite, likely via a plasma NO oxidase such as ceruloplasmin (33). Nitrite may be converted back to NO in an acidic/hypoxic environment present in reperfused myocardium by xanthine oxidase (34,35) or deoxyhemoglobin (36,37). Al-

ternatively, it may protect myocardium from I/R-induced injury via NO-independent signaling (38).

The present data do not allow us to conclusively distinguish between these mechanisms, and several mechanisms may act in concert. We observed a trend toward increased plasma nitrite levels after NO inhalation in the pigs, which is in agreement with earlier observations in plasma and red blood cells of mice breathing NO (17) and with reports that nitrite administration attenuated cardiac I/R injury in mice (39). We also observed that iNO significantly augmented blood plasma NO_x levels and tissue NO_x levels, specifically in the infarct zone of pig hearts subjected to I/R. These latter findings may support the hypothesis that increased nitrite levels in NO-breathing pigs are converted to NO selectively in ischemic myocardium. However, we cannot exclude other explanations, including the possibility that breathing NO induces NO synthesis in ischemic myocardium.

The beneficial effects of iNO on reperfusion injury may be attributable to its ability to improve microvascular function. It has long been recognized that microvascular injury typically develops after ischemia (40). We started NO administration before restoring myocardial blood flow to test its efficacy as adjunctive therapy at the onset of reperfusion, a clinically attractive window for myocardial protection. Because porcine hearts lack collateral circulation, it is unlikely that NO reached the ischemic myocardium during coronary occlusion. Confirming earlier observations in mice (17), the present data clearly demonstrate that iNO reduces neutrophil infiltration and plasma and tissue MPO activity after myocardial I/R. Inhaled NO may attenuate adherence and activation of leukocytes to injured endothelium, decreasing production of reactive oxygen species in the heart. Leukocyte extravasation and degranulation, as reflected by increased MPO levels, can exacerbate vascular inflammation, impair endothelium-dependent relaxation, and obstruct coronary flow (41). Interestingly, it has recently been reported that MPO can aggravate this inflammatory response by acting as an NO oxidase and reducing local NO bioavailability in rodent models of endotoxemia (42) and myocardial I/R (43). Alternatively, iNO may exert its cardioprotective effects as a direct scavenger of superoxide radicals (44) or as downstream mediator of ischemic post-conditioning (45,46).

Inhaled NO reduced myocardial injury, at least in part, by decreasing cardiomyocyte apoptosis. We observed that TUNEL-positive cardiomyocytes were 50% less frequent in the border zone of iNO pigs than in control and IV-NTG pigs. Low levels of NO can directly prevent apoptosis in isolated cardiac myocytes (47,48). Alternatively, iNO may decrease apoptosis by attenuating injurious signals (e.g., activated neutrophils), scavenging superoxide radicals (49), or by improving myocardial perfusion.

Importantly, iNO, but not IV-NTG, dramatically decreased MVO in the infarct and border zones after cardiac I/R. Although direct infusion of a NO donor significantly

and potentially decreases platelet activation *in vivo* and has a cardioprotective effect (50,51), the greater efficacy of iNO may be attributable in part to enhanced bioavailability. We have calculated that the total amount of NO that is made bioavailable during 4-hour inhalation of 80 ppm NO is about 14-fold higher than during a continuous NTG infusion of 2 $\mu\text{g}/\text{kg}/\text{min}$ (data not shown). The greater NO bioavailability is also associated with increased plasma NO_x levels and higher cGMP levels in the reperfused postischemic territory.

Clinical implications. Despite restoration of brisk epicardial flow in the great majority of patients presenting with myocardial infarction and following extracorporeal circulation during bypass surgery, myocardial recovery often remains suboptimal, with considerable morbidity and mortality. Microvascular obstruction after successful coronary interventions is associated with a poor prognosis (52,53). Simple assessment of the myocardial microcirculation using myocardial blush grades allows stratifying the prognosis of high-risk coronary patients into excellent, intermediate, or poor survival (54). Thus far, randomized pharmacologic therapies targeting the MVO associated with I/R injury have failed to further improve myocardial perfusion and survival (9). The present findings strongly suggest that iNO can significantly improve myocardial blood flow upon reperfusion of occluded epicardial arteries and, thereby, has the potential to beneficially affect one of the most important determinants of clinical outcome and recovery of LV function in patients with acute coronary syndromes. Although L-arginine and NO-donor compounds may be considered for the treatment of I/R injury in peripheral vascular disease (where effects on systemic blood pressure are less critical), the present data in a porcine MI model suggest that iNO may be a particularly attractive and safe alternative strategy, because it does not compromise blood pressure.

Study limitations. We administered only a single dose of NO (80 ppm) for inhalation, based on previous studies in mice (17) and cannot exclude whether higher or lower doses would have been more effective (44). The advantage of lower doses would be decreased risk of methemoglobin and cardiac peroxynitrite formation. We also chose a single dose of IV-NTG to reflect routine clinical practice (55), and we cannot exclude different results with higher doses. The duration of therapy was maintained for 4 h, but shorter or longer treatment regimens may be equally or more effective and warrant future investigation. Finally, the effect of a reduction in infarct size observed in this study on the subsequent development of heart failure remains to be determined.

Conclusions

These results demonstrate that treatment with iNO at 80 ppm in pigs during myocardial ischemia and reperfusion is safe and confers protection against reperfusion injury. Inhaled NO represents an attractive novel strategy for the

treatment of post-MI reperfusion injury and microvascular dysfunction.

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