

## Brief Periods of Nitric Oxide Inhalation Protect against Myocardial Ischemia-Reperfusion Injury

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**Background:** Prolonged breathing of nitric oxide reduces myocardial ischemia-reperfusion injury, but the precise mechanisms responsible for the cardioprotective effects of inhaled nitric oxide are incompletely understood.

**Methods:** The authors investigated the fate of inhaled nitric oxide (80 parts per million) in mice and quantified the formation of nitric oxide metabolites in blood and tissues. The authors tested whether the accumulation of nitric oxide metabolites correlated with the ability of inhaled nitric oxide to protect against cardiac ischemia-reperfusion injury.

**Results:** Mice absorbed nitric oxide in a nearly linear fashion ( $0.19 \pm 0.02 \mu\text{mol/g} \cdot \text{h}$ ). Breathing nitric oxide rapidly increased a broad spectrum of nitric oxide metabolites. Levels of erythrocytic S-nitrosothiols, N-nitrosamines, and nitrosyl-hemes increased dramatically within 30 s of commencing nitric oxide inhalation. Marked increases of lung S-nitrosothiol and liver N-nitrosamine levels were measured, as well as elevated cardiac and brain nitric oxide metabolite levels. Breathing low oxygen concentrations potentiated the ability of inhaled nitric oxide to

increase cardiac nitric oxide metabolite levels. Concentrations of each nitric oxide metabolite, except nitrate, rapidly reached a plateau and were similar after 5 and 60 min. In a murine cardiac ischemia-reperfusion injury model, breathing nitric oxide for either 5 or 60 min before reperfusion decreased myocardial infarction size as a fraction of myocardial area at risk by 31% or 32%, respectively.

**Conclusions:** Breathing nitric oxide leads to the rapid accumulation of a variety of nitric oxide metabolites in blood and tissues, contributing to the ability of brief periods of nitric oxide inhalation to provide cardioprotection against ischemia-reperfusion injury. The nitric oxide metabolite concentrations achieved in a target tissue may be more important than the absolute amounts of nitric oxide absorbed.

*INHALED nitric oxide* (NO) is a selective pulmonary vasodilator that does not produce systemic hypotension when breathed at concentrations up to 80 parts per million (ppm). Inhaled nitric oxide is widely used to treat neonatal hypoxemia and acute pulmonary hypertension.<sup>1</sup> The selectivity of inhaled nitric oxide for the pulmonary vasculature is attributed to its high affinity for the heme moiety of hemoglobin and its rapid conversion, in the presence of oxygenated hemoglobin, to nitrate and methemoglobin. However, as early as 1993, it was appreciated that breathing nitric oxide had systemic effects and could prolong the bleeding time (rabbits and humans).<sup>2</sup> Subsequent reports demonstrated that breathing nitric oxide could decrease neointima formation after carotid artery injury (rats),<sup>3</sup> decrease thrombosis after thrombolysis (dogs),<sup>4</sup> and reduce reperfusion injury after mesenteric artery ischemia (cats)<sup>5</sup> or cardiac ischemia-reperfusion (mice<sup>6</sup> and pigs<sup>7</sup>). Moreover, recent human studies show that inhaled nitric oxide decreased reperfusion-associated inflammatory responses in ischemic limbs<sup>8</sup> and decreased hepatic injury after liver transplantation.<sup>9</sup>

Because nitric oxide has a short half-life in biologic fluids, it is unlikely that nitric oxide molecules absorbed into the bloodstream during inhalation reach the periphery in an unmodified form.<sup>10</sup> A variety of nitric oxide adducts and nitric oxide-derived metabolites have been identified in animal and human blood during nitric oxide inhalation. Formation of these nitric oxide products can be direct<sup>11</sup> *via* binding to heme-containing proteins through the transition metal center forming nitrosyl-heme species (nitric oxide-heme)<sup>12</sup> such as nitric oxide-hemoglobin.<sup>13</sup> Alternatively, nitric oxide metabolites may be generated indirectly<sup>14</sup> *via* secondary reactions with oxygen that form nitrosating species, such as N<sub>2</sub>O<sub>3</sub>. These nitrosating species, in turn, react with thiols to form S-nitrosothiols

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(RSNO)<sup>15</sup> or with amine groups to form *N*-nitrosamines (RNNO). In addition, breathing nitric oxide increases the blood levels of the end products of nitric oxide oxidation, nitrite and nitrate.<sup>16</sup> Despite early recognition that nitric oxide metabolites were formed during inhalation of nitric oxide,<sup>16</sup> a quantitative evaluation of the array of nitric oxide metabolites generated over time by breathing nitric oxide has not been reported.

To investigate the fate of inhaled nitric oxide, the first objective of this study was to determine the rate of nitric oxide absorption through inhalation and quantify the formation of nitric oxide metabolites in blood and various body tissues. The second objective was to determine whether levels of nitric oxide metabolites in blood or heart correlate with the duration of nitric oxide inhalation required to protect against cardiac ischemia-reperfusion injury. We report that nitric oxide inhalation leads to rapid accumulation of a broad spectrum of nitric oxide metabolites in the blood and tissues, and that even a brief period of nitric oxide inhalation (< 15 min) produces elevation of nitric oxide metabolites and is associated with a reduction of cardiac ischemia-reperfusion injury.

## Materials and Methods

### *Experimental Animals*

Male C57BL/6J mice fed a standard diet (RMH 3000, Prolab; PMI International, St. Louis, MO) were studied. All animal experimental protocols were approved by both the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, Massachusetts, and the Institutional Animal Care and Use Committee at Boston University School of Medicine, Boston, Massachusetts.

### *Measurement of Nitric Oxide Absorption*

Mice ( $n = 4$ ) were placed in a chamber (PLY3211; Buxco Research Systems, Wilmington, NC) and exposed to air supplemented with 80 ppm NO for 60 min. The quantity of nitric oxide absorption was calculated from the difference between inlet and outlet nitric oxide concentrations (parts per million), multiplied by the gas flow rate (3 l/min), and divided by the molar volume of nitric oxide at standard temperature and pressure (22.4 l/mol). To account for the dilution of nitric oxide with ambient air when the chamber was opened to insert the mouse (3 s), as well as the generation of nitrogen dioxide in the chamber, "sham absorption of nitric oxide" was measured under the same conditions without inserting a mouse ( $3.3 \pm 0.1 \mu\text{mol/h}$ ,  $n = 4$ ). Nitric oxide absorbed (total absorption of nitric oxide,  $7.9 \pm 0.6 \mu\text{mol/mouse} \cdot \text{h}$ , minus sham absorption of nitric oxide,  $3.3 \mu\text{mol}$ ), was divided by body weight and was expressed as  $\mu\text{mol NO/g}$  body weight per hour (Additional information regarding our detailed description of nitric oxide absorption measurements is available on the ANESTHESIOLOGY

Web site at <http://www.anesthesiology.org>, in Web Enhancement 1-Materials and Methods.)

### *Whole Body, Tissue, Blood, and Urine Sampling for Measurement of Nitric Oxide Metabolites*

For measurement of whole body nitric oxide metabolite levels, mice that breathed air without ( $n = 5$ ) or with ( $n = 4$ ) 80 ppm NO for 1 h in the chamber were subsequently anesthetized with diethyl ether and killed by cervical dislocation. Rapid full body homogenization was achieved with a Waring blender (Waring Products, Torrington, CT) using a mixture of frozen and chilled phosphate-buffered saline (at 1:5, wt/vol) containing *N*-ethylmaleimide (10 mM) and EDTA (2.5 mM), and the resulting homogenate was immediately analyzed.

Additional mice were placed in the chamber and exposed to air or 80 ppm NO in air for 0.5, 5, 15, and 60 min ( $n = 4-7$  per time point). After the exposure, mice were anesthetized with diethyl ether. Blood was withdrawn from the left ventricle (LV) and immediately centrifuged at 16,000g for 3-5 min at room temperature (22°C) to separate erythrocytes from plasma. Erythrocytes were subjected to hypotonic lysis in water containing *N*-ethylmaleimide (10 mM) and EDTA (2.5 mM). To remove blood, tissues were perfused *via* the LV with room air-equilibrated phosphate-buffered saline supplemented with *N*-ethylmaleimide (10 mM) and EDTA (2.5 mM) for 1 min. Brain, heart, liver, kidney, lung, and fat were harvested, homogenized, and subjected to immediate analysis. Urine was obtained *via* direct puncture of the bladder. Nitrite, nitrate, RSNO, RNNO, and nitric oxide-heme species were quantified in plasma, erythrocytes, tissues, and whole body homogenates. Because of volume limitations, urine analysis was restricted to nitrite and nitrate only.

To investigate the effect of reduced oxygen availability as occurring during ischemia on nitric oxide metabolite levels in the heart, mice were placed in the chamber and breathed either 8% oxygen in nitrogen (Hypoxia,  $n = 5$ ) or 80 ppm NO in 8% oxygen for 60 min (Hypoxia + nitric oxide,  $n = 5$ ). After the exposure, mice were anesthetized with diethyl ether, and cardiac nitric oxide metabolite concentrations were measured immediately after the tissue perfusion with phosphate-buffered saline containing *N*-ethylmaleimide (10 mM) and EDTA (2.5 mM) and homogenizing.

### *Quantitation of Nitroso/Nitrosyl Species and Oxidation Products of Nitric Oxide*

Methods for the detection of nitroso (RSNO and RNNO) and nitrosyl (nitric oxide-heme) compounds, as well as the oxidation products of nitric oxide (nitrite and nitrate), in blood and tissues have been detailed previously.<sup>17</sup> Quantification was achieved by group-specific denitrosation after injection of biologic samples into either a triiodide-containing reaction mixture (nitroso spe-

cies) or a potassium ferricyanide solution (nitrosyl species) constantly purged with nitrogen, and oxidation products of nitric oxide were measured with the aid of a gas phase chemiluminescence detector (CLD 77am sp; EcoPhysics, Ann Arbor, MI). Nitrate and nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer; Eicom, San Diego, CA).

#### Myocardial Ischemia-Reperfusion Injury

Mice were anesthetized by intraperitoneal administration of ketamine (120 mg/kg) and xylazine (5 mg/kg) and ventilated (MiniVent 845; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) at a fraction of inspired oxygen ( $F_{iO_2}$ ) of 0.99–1.0. Myocardial ischemia was induced by ligation of the left coronary artery for 60 min, followed by reperfusion for 24 h.<sup>6</sup> Nitric oxide was administered during ischemia for 60, 5, or 0.5 min, just before reperfusion. During the surgical procedures, an  $F_{iO_2}$  of 0.99–1.0 without or with 80 ppm NO was applied using two separate mechanical ventilators. After 24 h, the artery was re-ligated, and either fluorescent microspheres (0.25 ml, 10- $\mu$ m diameter, FluoSpheres; Invitrogen Corporation, Carlsbad, CA; for 60- and 5-min nitric oxide inhalation studies) were injected into the LV, or tissue-marking dye (0.25 ml, TMD-BL; Triangle Biomedical Science Inc., Durham, NC; for 0.5-min nitric oxide inhalation study) was injected into the right carotid artery to determine the area at risk (AAR). The heart was excised, and four consecutive 1-mm cardiac slices were stained with 2,3,5-triphenyl-tetrazolium chloride (1% wt/vol; Sigma-Aldrich, St. Louis, MO) for the measurement of myocardial infarction (MI) size. LV, AAR, and MI area were measured by computer-assisted planimetry (NIH Image J 1.34; Bethesda, MD), and AAR/LV and MI/AAR ratios were calculated.<sup>6</sup>

#### Data Acquisition and Statistical Analysis

All data are presented as mean  $\pm$  SEM. Data were analyzed using one-way analysis of variance with means comparison using the Bonferroni test (Origin 7.0; OriginLab Corporation, Northampton, MA). To compare the changes of nitric oxide metabolites in blood, tissues, and urine, each time point was compared with the corresponding baseline value. For the comparison of cardiac nitric oxide metabolite levels in mice breathing low oxygen concentrations, a separate one-way analysis of variance with a means comparison using the Bonferroni test was performed for the comparison of the four groups; *i.e.*, baseline, breathing nitric oxide in air for 60 min, breathing 8% oxygen for 60 min, and breathing nitric oxide in 8% oxygen for 60 min. To analyze the effects of breathing nitric oxide on ischemia-reperfusion injury, analyses were performed for 60-, 5-, and 0.5-min inhalations of nitric oxide and compared with each con-

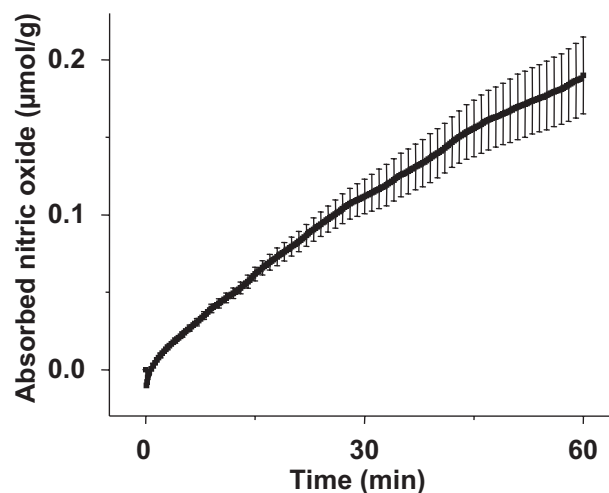


Fig. 1. The absorption of nitric oxide (NO) by mice breathing 80 parts per million NO for 60 min. Mice breathed air supplemented with 80 parts per million NO for 60 min in a vented chamber ( $n = 4$ ). Nitric oxide concentrations were recorded continuously at the chamber outlet during the first 30 s, and thereafter, inlet and outlet were sampled every 15 s. The difference between inlet and outlet concentrations multiplied by the gas flow rate and time provides an accurate measurement of total nitric oxide gas absorbed by each mouse. The *line* represents the mean absorption of nitric oxide, and SEM is shown at 1-min intervals.

trol group. *P* values less than 0.05 were considered significant.

## Results

#### Uptake of Inhaled Nitric Oxide

To investigate the metabolic fate of inhaled nitric oxide, we first measured the absorption of nitric oxide from ambient gas during spontaneous ventilation in awake mice. The rate of nitric oxide uptake was nearly linear with time ( $0.19 \pm 0.02 \mu\text{mol NO/g body weight} \cdot \text{h}$ ; fig. 1).

#### Inhaled Nitric Oxide Is Converted into Longer-lived Metabolites

To further characterize the pharmacokinetics of absorbed nitric oxide, we examined how much of the absorbed nitric oxide could be recovered as nitric oxide metabolites. To measure the accumulation of nitric oxide metabolites during nitric oxide inhalation (80 ppm, 1 h), mice were killed and homogenized, and individual nitric oxide metabolites in whole body extracts were quantified. Nitric oxide inhalation led to an increase in the total body concentrations of all the nitric oxide metabolites we examined (table 1). Nitrate concentrations increased 18-fold and represented 97% of the total nitric oxide metabolites measured. Levels of nitric oxide-heme increased 13-fold, RSNO 8-fold, RNNO 5-fold, and nitrite 2-fold (table 1). Fifty-three percent ( $0.10 \pm 0.02 \mu\text{mol/g}$ ) of the nitric oxide absorbed from the gas phase during inhalation for 1 h was recovered as nitric oxide metabolites in the whole body extracts.



**Table 1. Whole Body Analysis of Nitric Oxide (NO) Metabolites in Mice Breathing Air or Air Supplemented with 80 ppm NO for 60 min**

	Control (n = 5)	Inhaled NO (n = 4)
Nitrate	5.6 ± 1.4	98 ± 22†
Nitrite	0.96 ± 0.17	2.2 ± 0.5*
RSNO	0.06 ± 0.01	0.50 ± 0.08*
RNNO	0.05 ± 0.00	0.27 ± 0.05*
NO-heme	0.02 ± 0.00	0.26 ± 0.06*

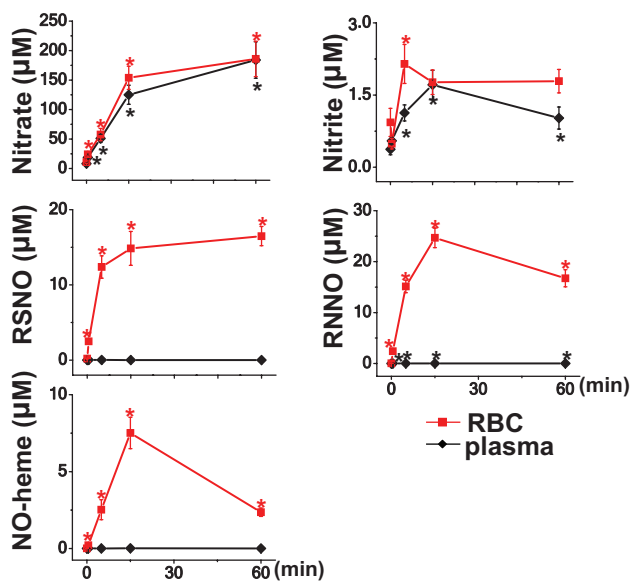
Data are expressed in micromolars.

\*  $P < 0.05$ , †  $P < 0.01$  vs. control.

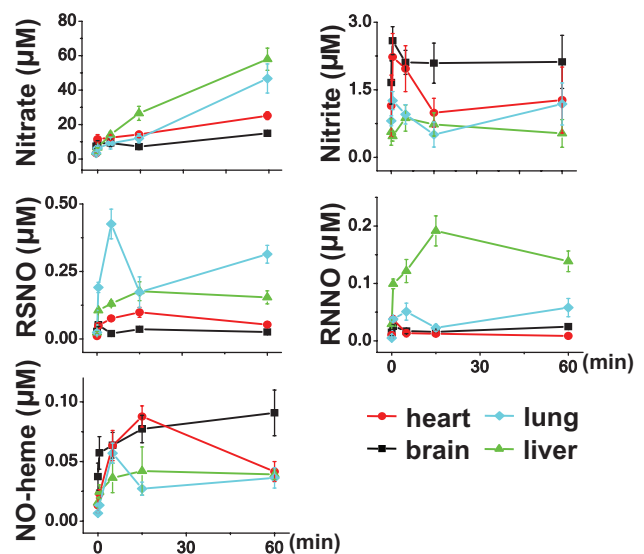
NO-heme = nitrosyl-heme species; ppm = parts per million; RNNO = *N*-nitrosamines; RSNO = *S*-nitrosothiols.

### Nitric Oxide Inhalation Increases Nitric Oxide Metabolite Concentrations in Blood and Tissues

To gain detailed insight into the dynamics of uptake, distribution, and secondary metabolism of the nitric oxide absorbed during inhalation, the concentrations of nitric oxide metabolites were measured in blood (both erythrocytes and plasma; fig. 2) and tissues (heart, lung, brain, liver, kidney, and fat; fig. 3) of mice breathing air with or without 80 ppm NO for 0, 0.5, 5, 15, and 60 min (additional information regarding each concentration, the number of animals studied, and the  $P$  value are available on the ANESTHESIOLOGY Web site at <http://www.anesthesiology.org>, in Web Enhancement 2-table 1 and Web Enhancement 3-table 2). Breathing air without nitric



**Fig. 2. Distribution and kinetics of accumulation of nitric oxide metabolites in mice breathing nitric oxide (plasma and erythrocytes).** Concentrations of nitric oxide metabolites were measured in blood of mice breathing air supplemented with nitric oxide for 0, 0.5, 5, 15, and 60 min ( $n = 5-7$ ). \*  $P < 0.05$  versus mice not breathing nitric oxide. NO-heme = nitrosyl-heme species; RBC = erythrocytes; RNNO = *N*-nitrosamines; RSNO = *S*-nitrosothiols. Additional information regarding each concentration, the number of animals studied, and the  $P$  value are available on the ANESTHESIOLOGY Web site at <http://www.anesthesiology.org>, in Web Enhancement 2-table 1.



**Fig. 3. Distribution and kinetics of accumulation of nitric oxide metabolites in mice breathing nitric oxide (heart, lung, brain, and liver).** Concentrations of nitric oxide metabolites were measured in tissues of mice breathing air supplemented with nitric oxide for 0, 0.5, 5, 15, and 60 min ( $n = 4-7$ ). NO-heme = nitrosyl-heme species; RNNO = *N*-nitrosamines; RSNO = *S*-nitrosothiols. Additional information regarding each concentration, the number of animals studied, and the  $P$  value are available on the ANESTHESIOLOGY Web site at <http://www.anesthesiology.org>, in Web Enhancement 3-table 2.

oxide for varying periods of time (5, 15, and 60 min) did not alter nitric oxide metabolite concentrations in blood or any of the tissues we studied (data not shown).

During inhalation of nitric oxide, nitrate concentrations in plasma and erythrocytes increased linearly over the first 15 min and then tended to reach a plateau level (fig. 2). Nitrate concentrations were almost identical in plasma and erythrocytes at all time points. Nitrite concentrations in plasma reached a plateau within 15 min, whereas erythrocytic nitrite peaked as early as 5 min. Nitrite levels were lower than nitrate levels in plasma and erythrocytes by two orders of magnitude. The concentrations of RSNO, RNNO, and nitric oxide-heme increased markedly in erythrocytes (610-fold for RNNO, 535-fold for nitric oxide-heme, and 85-fold for RSNO;  $P < 0.001$  for all) and greatly exceeded the erythrocytic or plasma nitrite concentration. In contrast, breathing nitric oxide did not significantly increase RSNO or nitric oxide-heme concentrations in plasma, and plasma RNNO levels increased only 3-fold ( $P < 0.001$  vs. baseline). These results suggest that plasma nitrite, nitrate, and RNNO, as well as erythrocytic RSNO, RNNO, nitric oxide-heme, nitrite, and nitrate, may all contribute to the transport of bioavailable nitric oxide from the lung to the periphery.

In the heart, increased RSNO and nitric oxide-heme levels were detected at 0.5 and 5 min, respectively, and maximum levels were attained after 15 min of nitric oxide inhalation (9- and 7-fold increases, respectively;  $P < 0.001$  vs. baseline for both; fig. 3). Cardiac RNNO concentrations were maximal at 0.5 min ( $P < 0.05$  vs. baseline)

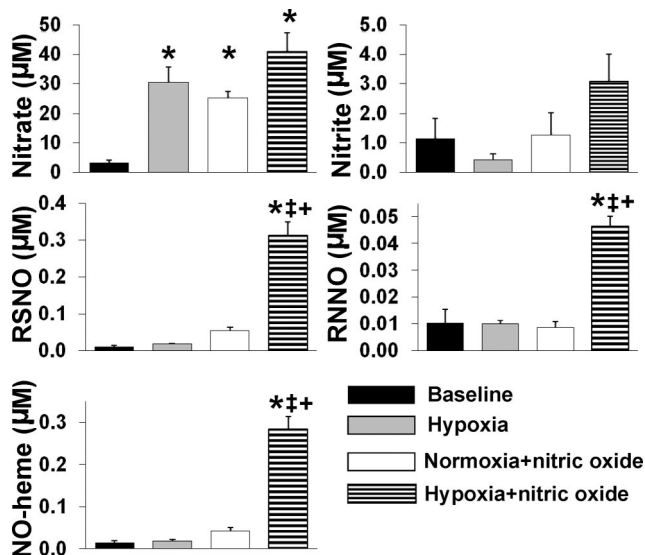


Fig. 4. Effects of hypoxia on the cardiac levels of nitric oxide metabolites. Concentrations of cardiac nitric oxide metabolites were measured in cardiac tissue of mice breathing air (Baseline), 8% oxygen (Hypoxia), air supplemented with nitric oxide (Normoxia + nitric oxide), or 8% oxygen balance nitrogen supplemented with nitric oxide (Hypoxia + nitric oxide) for 60 min. \*  $P < 0.05$  versus Baseline. ‡  $P < 0.05$  versus Hypoxia. †  $P < 0.05$  versus Normoxia + nitric oxide. NO-heme = nitrosyl-heme species; RNO = *N*-nitrosamines; RSNO = *S*-nitrosothiols.

and returned to baseline thereafter despite continued inhalation of nitric oxide (fig. 3). The concentration of nitrate increased as early as 0.5 min and remained elevated thereafter. In contrast, cardiac nitrite levels were not elevated significantly during the inhalation of nitric oxide.

To study the effect of hypoxia on the heart, cardiac nitric oxide metabolite levels were measured in mice breathing a low oxygen concentration, with and without 80 ppm NO. Cardiac nitrate levels markedly increased after awake mice breathed 8% oxygen for 60 min (Hypoxia; a 9-fold increase *vs.* baseline;  $P < 0.05$ ; fig. 4). After 80 ppm NO was breathed in 8% oxygen for 60 min (Hypoxia + nitric oxide), the levels of nitric oxide-heme, RSNO, and RNO were markedly elevated over those of Normoxia + nitric oxide (7-, 6-, and 5-fold increases, respectively;  $P < 0.05$ ; fig. 4).

Inhalation of nitric oxide led also to rapid increases in nitric oxide metabolite concentrations in the lung, brain, and liver (fig. 3) but not in kidney or fat (additional information regarding the concentrations of nitric oxide metabolites in kidney and fat are available on the ANESTHESIOLOGY Web site at <http://www.anesthesiology.org>, in Web Enhancement 3-table 2). The highest RSNO levels were achieved in the lung with peak concentrations attained within 5 min. During inhalation of nitric oxide, RNO concentrations increased markedly in the liver and less so in the lung. In contrast, in the brain, inhalation of nitric oxide led to the accumulation of nitric oxide-heme, but not RSNO or RNO. Breathing nitric oxide did not significantly increase nitrite levels in any of the tissues that we studied. Taken together,

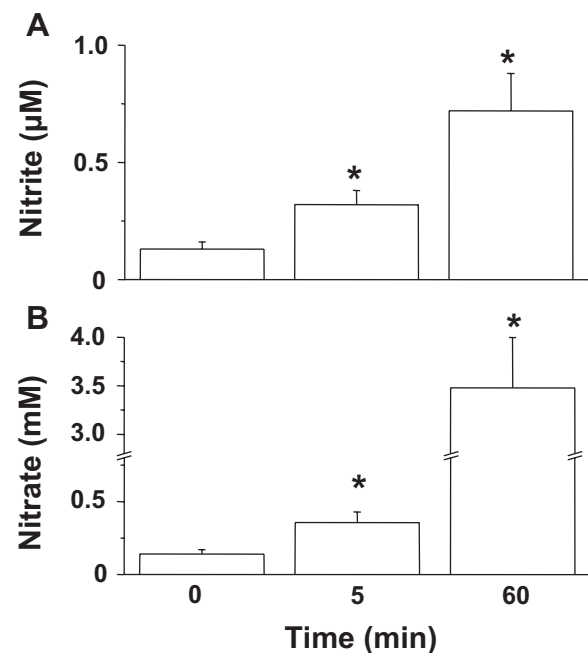


Fig. 5. Measurement of nitrite (A) and nitrate (B) in urine from mice breathing nitric oxide. Mice received air supplemented with nitric oxide for 0, 5, and 60 min ( $n = 8, 7,$  and  $9,$  respectively). \*  $P < 0.05$  versus mice not breathing nitric oxide.

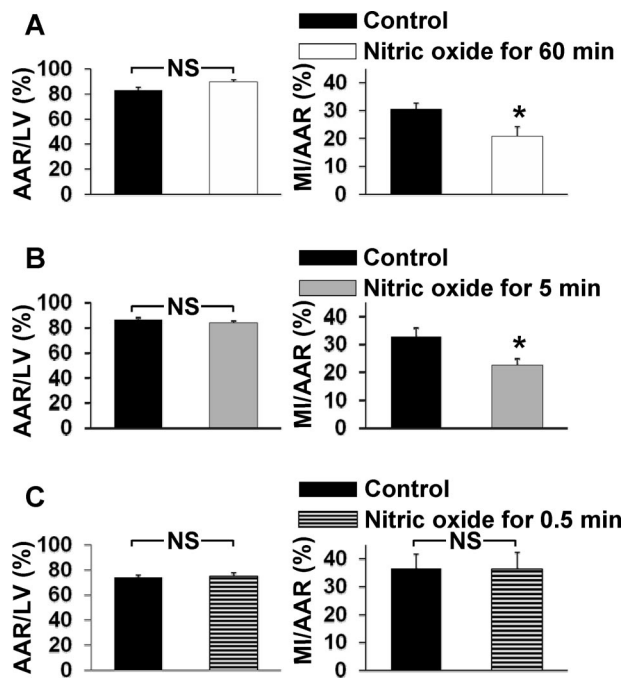
marked differences in nitric oxide metabolite regulation exist between the blood, heart, and other tissues, suggesting that generation or metabolism of nitric oxide metabolites or both are quite tissue specific.

#### Urinary Excretion of Nitrite and Nitrate

During nitric oxide inhalation, nitrite and nitrate began to accumulate in the urine as early as 0.5 min (data not shown). After 60 min of nitric oxide inhalation, concentrations of nitrite in the urine were  $0.7 \pm 0.2 \mu\text{M}$  ( $P < 0.01$  *vs.* baseline level; fig. 5A) and were similar to those detected in plasma ( $1.0 \pm 0.3 \mu\text{M}$ ). In contrast, in mice breathing nitric oxide for 60 min, urinary nitrate concentrations ( $3.5 \pm 0.5 \text{ mM}$ ) were 19-fold greater than those of plasma ( $P < 0.0001$  *vs.* baseline; fig. 5B). As an estimate of the quantity of absorbed nitric oxide that was excreted in the urine, the average concentration of nitrate after 60 min of nitric oxide breathing ( $3.5 \text{ mM}$ ) was multiplied by the volume of urine collected ( $119 \pm 16 \mu\text{l}$ ,  $n = 9$ ) at the same time point. We estimate that approximately 9% of the nitric oxide absorbed over 1 h is excreted in the urine.

#### Short-term Inhalation of Nitric Oxide Protects against Myocardial Ischemia-Reperfusion Injury

In a previous study of mice subjected to 60 min of cardiac ischemia and 24 h of reperfusion, we learned that continuous breathing of 80 ppm NO for 24 h decreased MI size as a fraction of myocardial area at risk (MI/AAR).<sup>6</sup> In the current study, the observation that blood levels of nearly all nitric oxide metabolites detected after breathing nitric oxide for 5 min were similar



**Fig. 6.** Inhalation of nitric oxide for short durations limits myocardial ischemia–reperfusion injury. All mice underwent left coronary artery occlusion for 60 min followed by 24 h of reperfusion. Mice received nitric oxide during ischemia for 60 min ( $n = 10$  and  $9$  for control and inhaled nitric oxide mice, respectively; *A*), 5 min immediately before reperfusion ( $n = 9$  and  $8$  for control and inhaled nitric oxide mice, respectively; *B*), or 0.5 min immediately before reperfusion ( $n = 7$  and  $6$  for control and inhaled nitric oxide mice, respectively; *C*). Control mice did not receive nitric oxide. \*  $P < 0.05$  versus control. AAR = area at risk; LV = left ventricle; MI = myocardial infarction; NS = not significant.

to those detected at 60 min (fig. 2) led us to determine whether a shorter duration of nitric oxide inhalation ( $\leq 60$  min) could modify MI/AAR at 24 h after reperfusion. The overall 24-h mortality rate of mice in our study after ischemia–reperfusion was 8%. In mice breathing nitric oxide for 60 or 5 min before reperfusion, MI/AAR was decreased by 32% ( $P < 0.05$ ; fig. 6A) or 31% ( $P < 0.05$ ; fig. 6B), respectively. In contrast, breathing nitric oxide for only 30 s just before reperfusion did not alter the degree of cardiac ischemia–reperfusion injury (fig. 6C).

## Discussion

In the current study, we provide the first quantitative and temporal characterization of the levels of nitric oxide metabolites that accumulate in the blood and peripheral tissues during nitric oxide inhalation. Moreover, we report that inhalation of nitric oxide for as little as 5 min before reperfusion can reduce infarct size in a murine model of myocardial ischemia–reperfusion injury, which supports the notion that increased levels of one or more nitric oxide metabolites in the blood contribute to the cardioprotective effects of breathing nitric oxide.

It is increasingly appreciated that breathing nitric oxide can elicit a wide spectrum of physiologic effects in

peripheral tissues<sup>18</sup>; however, the mechanisms responsible for these salutary effects are incompletely understood. One possibility is that exposure of leukocytes and platelets to high nitric oxide concentrations as they transit the lung may inhibit their activation in peripheral tissues. On the other hand, multiple research groups have observed that inhalation of nitric oxide leads to the formation of nitric oxide metabolites in the bloodstream<sup>12,16</sup> and tissues.<sup>19</sup> To better understand the mechanisms responsible for the extrapulmonary effects of inhaled nitric oxide, we quantitatively assessed the fate of inhaled nitric oxide in whole body extracts, as well as in blood and representative tissues.

In mice breathing nitric oxide (80 ppm), the rate of nitric oxide absorption was essentially linear, and approximately  $0.19 \mu\text{mol/g}$  body weight was absorbed within 1 h. Of the gaseous nitric oxide absorbed, we estimate that approximately 9% ( $0.017 \mu\text{mol/g}$  body weight) was excreted in the urine. RSNO, RNNO, nitric oxide–heme, nitrite, and nitrate recovered from whole body extracts accounted for 53% ( $0.10 \mu\text{mol/g}$  body weight) of the absorbed nitric oxide. The fate of the absorbed nitric oxide that was not detected as nitric oxide metabolites is currently unknown. Some of the absorbed nitric oxide may have been converted to metabolites not readily detected by the techniques we used (such as nitrotyrosine, nitrated fatty acids, and other nitrated species or stable *C*- or *N*-nitroso compounds). Alternatively, nitric oxide may have been reduced to nitrous oxide or nitrogen and exhaled. In whole body extracts, nitrate represented nearly 97% of the nitric oxide metabolites accumulating during nitric oxide inhalation, consistent with previous studies showing that conversion of absorbed nitric oxide into nitrate represents the major metabolic pathway for inhaled nitric oxide.<sup>16,20</sup>

Lecour *et al.*<sup>19</sup> reported that breathing 100 or 200 ppm NO increased nitric oxide concentrations in peripheral tissues, as detected by electron spin resonance spectroscopy combined with a spin-trapping technique. However, in contrast to our observations demonstrating that nitric oxide–heme levels increased as early as 5 min after the start of 80 ppm NO inhalation, Lecour *et al.* did not detect an increase in cardiac nitric oxide concentrations in rats breathing 100 ppm NO for 45 min. This discrepancy may be explained by the differing techniques for nitric oxide detection or trapping: Our chemiluminescence-based technique is sufficiently sensitive to quantify the steady state concentrations achieved in a given compartment at baseline while nitric oxide is bound to its natural ligands. In contrast, electron spin resonance spectroscopy-based techniques require the accumulation of nitric oxide over a period of time using a transition metal–thiol complex as the trapping agent. In the latter technique, nitric oxide is bound to an exogenous ligand rather than a natural ligand, and higher than normal tissue nitric oxide concentrations are achieved, facili-



tating detection. However, techniques that use exogenous nitric oxide trapping agents inevitably perturb endogenous equilibria involving nitric oxide and its metabolites, and the apparent nitric oxide concentrations achieved depend on the probe's distribution and saturation characteristics, the stability of the nitric oxide complex formed, and the duration of nitric oxide accumulation.

In a recent study, Lang *et al.*<sup>9</sup> reported that in patients undergoing liver transplantation, breathing 80 ppm NO increased plasma nitrate and nitrite concentrations, as well as erythrocytic nitrate and nitric oxide-heme levels, but did not increase erythrocytic RSNO and RNNO levels. In contrast, we observed that breathing nitric oxide markedly increased erythrocytic RNNO and RSNO levels (600- and 80-fold, respectively). The reasons for this discrepancy are unclear. It is possible that differences in thiol reactivity between rodent and human hemoglobin contribute to differences in the concentrations of nitroso products formed.<sup>21</sup>

During inhalation of nitric oxide, the rate of nitric oxide metabolite accumulation differed depending on the tissue we studied. Increased levels of RSNO, RNNO and nitric oxide-heme were measured in the heart of mice breathing nitric oxide, and importantly, the concentrations achieved were similar to those detected in mice carrying a transgene that directs systemic expression of nitric oxide synthase 3.<sup>22</sup> Of note, this strain of mice was shown to be protected from cardiac ischemia-reperfusion injury.<sup>22</sup> The accumulation of RSNO in the lung during inhalation of nitric oxide is consistent with the observations of Moya *et al.*<sup>23</sup> In brain, only nitric oxide-heme levels increased after breathing nitric oxide for 15 min. The constancy of increased nitric oxide-heme levels in the brain despite continued nitric oxide inhalation may reflect decreased import or increased export of nitric oxide metabolites and/or down-regulation of endogenous nitric oxide production. In comparison to the other tissues we studied, breathing nitric oxide led to the greatest accumulation of RNNO in the liver (a 6.6-fold increase by 15 min), and increased hepatic RSNO and nitric oxide-heme concentrations were observed. Differences in the distribution of nitric oxide metabolites detected in these tissues strongly suggest that detection of these metabolites is not attributable to blood contaminating the tissues. Moreover, these findings suggest that the uptake, metabolism, or excretion of nitric oxide metabolites is regulated in a tissue-specific manner.

In the murine model of cardiac ischemia-reperfusion injury, nitric oxide was administered only during the ischemia period, in which nitric oxide metabolites can reach the ischemic tissue *via* the circulation only after subsequent coronary reperfusion. Using this model, breathing nitric oxide for 5 min (just before reperfusion) significantly decreased the cardiac injury. In contrast, breathing nitric oxide for 0.5 min just before reperfusion (a duration of breathing nitric oxide that resulted in

nitric oxide metabolite concentrations in blood that were consistently lower than those measured in mice breathing nitric oxide for 5 or 60 min) did not protect against cardiac ischemia-reperfusion injury. We acknowledge that, because the dead space and cardiac output differ between spontaneous breathing and mechanically ventilated animals, the uptake and distribution of nitric oxide in awake mice may differ from that in mice undergoing a thoracotomy and transient coronary artery occlusion (during which nitric oxide was administered through the animal's ventilator). Nevertheless, our findings correlated with the observation that brief periods of nitric oxide inhalation were capable of protecting against cardiac ischemia-reperfusion injury in mice. The substantial elevations of cardiac nitric oxide metabolites measured in mice breathing 8% oxygen supplemented with nitric oxide in this study suggests that myocardial ischemia is likely to alter the cardiac nitric oxide metabolite levels produced by nitric oxide inhalation. To our knowledge, this is the first report of the hypoxia-related effects of inhaled nitric oxide on nitric oxide metabolites in cardiac tissue.

Importantly, all of the nitric oxide metabolites that we found to be elevated in the blood are capable of producing nitric oxide-related effects in the periphery. For example, RSNOs (*e.g.*, SNO-hemoglobin and SNO-albumin) are known to dilate blood vessels,<sup>24,25</sup> and RNNOs can increase cyclic guanosine monophosphate concentrations<sup>26</sup> and induce relaxation of bovine coronary arteries.<sup>27</sup> Especially in the ischemic myocardium, where tissue pH may decrease below 5.5 after 30 min of ischemia, nitrite<sup>28</sup> or nitrate<sup>29</sup> may generate nitric oxide. Accordingly, although the half-life of nitric oxide is short, blood nitric oxide metabolites with longer lifetimes can deliver nitric oxide from the lung to distant organs and regenerate nitric oxide in the periphery. Moreover, it is also possible that the downstream effects of nitric oxide metabolites may themselves be longer lasting.

Because any one nitric oxide metabolite can be converted into many others, the determination of which single nitric oxide metabolite confers cardioprotection, either solely or in concert with other nitric oxide metabolites, remains a challenge. It has been previously reported that many nitric oxide donors and nitric oxide metabolites, including S-nitrosoglutathione and nitrite, can protect the heart from ischemia-reperfusion injury, perhaps *via* differing pathways. Sun *et al.*<sup>30</sup> reported an attenuation of cardiac ischemia-reperfusion injury by S-nitrosoglutathione, which was associated with an increase of S-nitrosylation of the mitochondrial L-type Ca<sup>2+</sup> channel. Nitrite protected the heart when injected into the left ventricle at 5 min before the reperfusion<sup>31</sup> and produced cardioprotection by attenuating mitochondrial respiration *via* inhibiting complex I *in vitro*.<sup>32</sup> Moreover, a recent study indicates that S-nitrosocysteine may protect the heart through nitric oxide-independent pathways.<sup>33</sup>

Therefore, although nitric oxide itself may be the active molecule in cardioprotection, RSNOs and other nitric oxide metabolites seem to have a similar capability of protecting the heart.

Although our results and those of others suggest that inhaled nitric oxide can decrease cardiac ischemia-reperfusion injury in animal models, it is not known whether breathing nitric oxide will decrease MI size in patients experiencing an acute coronary artery occlusion (ST-segment elevation MI). It is encouraging to note that breathing 80 ppm NO has shown to decrease ischemia-reperfusion injury in human studies.<sup>8,9</sup> If our observations in mice can be extrapolated to humans, the findings suggest the possibility that brief durations of nitric oxide inhalation may prove beneficial in patients at risk for cardiac ischemia-reperfusion injury.

In summary, we report that inhaled nitric oxide dynamically increases the levels of blood and tissue nitric oxide metabolites and that the degree of accumulation of each nitric oxide metabolite is tissue specific. Moreover, brief periods of nitric oxide inhalation can reduce infarct size in a murine model of cardiac ischemia-reperfusion injury with similar efficacy as much longer periods of nitric oxide inhalation, suggesting that the concentrations of nitric oxide metabolites achieved in the target tissue may be more important for protection than the absolute amounts of nitric oxide absorbed or delivered. The protective effects of breathing nitric oxide are likely to be attributable to nitric oxide metabolites that are rapidly transported in a bioactive form *via* the blood from the lung to the heart.

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