Selective Pulmonary Vasodilation by Inhaled Nitric Oxide Is Due to Hemoglobin Inactivation

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Background. Inhaled nitric oxide gas selectively decreases pulmonary artery pressure without affecting systemic arterial blood pressure. To determine if the selective pulmonary vasodilating effect of inhaled nitric oxide gas is due to inactivation by hemoglobin, we studied the ability of whole blood to inhibit the vasodilator activity of effluent from isolated lungs exposed to inhaled nitric oxide.

Methods and Results. The effluent from ventilated, Krebs-perfused rabbit lungs was passed directly over 3- to 4-mm rabbit aortic rings. Inhaled nitric oxide (150 ppm for 3 minutes) reduced pulmonary perfusion pressure, elevated by a continuous infusion of U46619, by 35±7% (x±SEM, n=5). Lung effluent from this series of experiments caused 40±13% relaxation of phenylephrine-preconstricted aortic rings. When blood was added to the combined lung/ring perfusion cascade (final hemoglobin concentration, 1 g/dL), inhaled nitric oxide again significantly reduced pulmonary perfusion pressure, but the effluent now failed to relax the aortic rings (30±6% [control] versus 1.5±1% [blood]). Both reduction in pulmonary perfusion pressure and relaxation of the rings during nitric oxide exposure were unchanged from control values after discontinuing the blood infusion.

Conclusions. The presence of hemoglobin, even in extremely small amounts, restricts the vasodilating effect of inhaled nitric oxide gas to the pulmonary circulation. (Circulation. 1993;88:2884-2887.)

Key Words: • nitric oxide • circulation • hemoglobin

Inhaled nitric oxide (NO) gas has been shown to be a selective pulmonary vasodilator that reverses pulmonary hypertension without affecting the systemic arterial blood pressure in vivo.1,2 The proposed mechanism of this selective pulmonary vasodilation is rapid binding to hemoglobin in red blood cells, which inactivates inhaled NO, restricting the vasodilating effects to the lungs and preventing systemic vasodilation.1 The interaction between NO and hemoglobin has been studied extensively in vitro;3,4 however, the effect of hemoglobin on the pulmonary selectivity of inhaled NO has not been determined. The purpose of this study, therefore, was to determine whether selective pulmonary vasodilation by inhaled NO gas is due to hemoglobin inactivation of NO by red blood cells in the vascular lumen of the lung.

Methods

Isolated Lung Preparation

Male New Zealand White rabbits (2.5 to 3.5 kg) were given heparin (300 U/kg) intravenously and euthanized with pentobarbital (50 mg/kg). The thoracic cavity was exposed via a median sternotomy, and the pulmonary artery and left atrium were cannulated. The heart and lungs were then removed en bloc, the lungs were suspended by securing the trachea to a cannula, and the isolated lung was enclosed in a water jacket (37°C). The lungs were ventilated with a mixture of oxygen and nitrogen at a rate of 20 breaths per minute and a tidal volume of 30 mL. Carbon dioxide (2% to 3%) was added to the inhaled gas mixture to maintain the pH of the lung effluent at 7.35 to 7.45. NO gas mixed with nitrogen was substituted for pure nitrogen to obtain the desired inspired NO concentration (150 ppm for 3 minutes) using volumetrically calibrated flowmeters as previously described5 and expired gases scavenged through an evacuation system. Inhaled NO concentration was determined by chemiluminescence (Thermo Environmental Instruments, Franklin, Mass), and NO2 concentration was <1% of total inspired NO.

The lungs were perfused via the pulmonary artery at 20 mL/min with Krebs solution (37°C) containing (mmol/L): 118.2 NaCl, 4.74 KCl, 2.54 CaCl2, 1.19 KH2PO4, 1.19 MgSO4, 26.2 NaHCO3, 11.1 dextrose, with 3% (wt/vol) dextran (60 to 90 kd) to maintain iso-oncotic pressure and 30 µmol/L indomethacin to inhibit cyclooxygenase activity.6 Pulmonary artery perfusion pressure (PaP) and airway pressures were continuously monitored by Gould P23 pressure transducers and recorded on a Gilson 5-6H polygraph. The left atrial catheter was positioned such that the effluent from the lung was directed over the aortic ring preparation. Concentrated drug solutions were delivered by microperistaltic pump to a sidearm of the pulmonary artery catheter at a flow rate of 0.1 mL/min. After each experiment, the lungs were blotted lightly, weighed, dried for 72 hours, and weighed again. Wet/dry weight ratios were then calculated, and lungs that had significantly increased wet/dry ratios were excluded from further consideration. Lungs were perfused for 15 min-

Received January 25, 1993; revision accepted August 15, 1993.

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utes before the beginning of each experiment to allow for stabilization of pressures and washout of blood cells from the preparation.

**Aortic Ring Preparation**

A separate group of male New Zealand White rabbits (2.5 to 3.5 kg) were given heparin (300 U/kg) intravenously and euthanized with pentobarbital (50 mg/kg). The thoracic cavity was exposed via a median sternotomy, and the thoracic aorta was removed. A 3- to 4-mm ring segment of aorta was then prepared with care taken to keep the endothelium intact. The ring was mounted between an adjustable support and a fixed isometric force transducer (Gilson T-1030) by which changes in ring tension were recorded. Rings were superfused with Krebs bicarbonate solution (37°C, aerated with 95% oxygen and 5% CO₂, with 5 μmol/L disodium EDTA and 20 U/mL SOD) delivered at 5 mL/min by peristaltic pump. The resting tension of the ring was increased to 4 g over 1 hour and allowed to equilibrate for an additional 90 minutes before the addition of 0.2 μmol/L phenylephrine to the ring perfusate. The constricted ring was then allowed to equilibrate for an additional 30 minutes before each experiment.

**Experimental Protocol**

Before starting each experiment, the entire effluent from the lung was directed over the aortic ring such that only lung effluent would superfuse the ring within 15 seconds. A continuous infusion of the thromboxane analog U46619 (0.5 to 1.25 nmol/min) was begun until the pulmonary artery infusion pressure (Pₚₐ) was approximately 50 mm Hg. NO was then added to the inhaled gas mixture (150 ppm) for 3 minutes, and the effects on Pₚₐ and aortic ring tension were noted. When Pₚₐ returned to control levels after stopping of NO inhalation, an infusion of whole blood was delivered to the pulmonary artery catheter such that the blood containing Krebs solution perfused both the isolated lung and the aortic ring. The final concentration of hemoglobin in the effluent fluid was 1 g/dL (1.5 μmol/L). After 15 minutes, the effect of inhaled NO on Pₚₐ and aortic ring tension was again tested. The blood infusion was then discontinued, and after a 15-minute perfusion period to allow blood to be washed from the lung and the ring, the response to inhaled NO was again determined. The effects of inhaled NO on Pₚₐ and aortic ring tension were determined in a similar fashion in the presence of albumin or hemoglobin (1 μmol/L) infusions.

**Reagents**

Whole rabbit blood was obtained from the lung donor animal before each experiment with heparin (20 U/mL) added and stored at room temperature for not more than 2 hours before use. This was added to the Krebs perfusate via a microperistaltic pump to a sidearm of the pulmonary artery catheter at a flow rate of 0.1 mL/min. The final hemoglobin concentration in the effluent fluid perfusing both lung and aortic ring was 1 g/dL (1.5 μmol/L) during the infusion (Nova Biomedical Analyzer, Waltham, Mass). To determine if hemoglobin or the nonheme-containing portion of blood was responsible for inactivation of inhaled NO, separate infusions of free hemoglobin or albumin were added to the perfusate, and inhaled NO exposure was repeated. Final concentrations of hemoglobin and albumin were, therefore, chosen to approximate those in the perfusate during the whole blood/Krebs infusion. Bovine hemoglobin (MW, 64 500; Sigma Chemical Co, St Louis, Mo) was prepared as previously described and added to the Krebs such that the final hemoglobin concentration of perfusate entering the lung was 1 μmol/L (similar to the 1.5 μmol/L measured in the perfusate effluent above). Bovine serum albumin (Sigma Chemical Co) was delivered in a similar manner such that the final concentration in the perfusate (5 mg/mL) was comparable to that during whole blood/Krebs infusion (3.6 mg/mL).  

**Nitric Oxide Measurement in Lung Effluent**

To determine the qualitative nature of the vasodilator exiting the lung in the pulmonary effluent during inhaled NO administration, samples of effluent were analyzed for NO and NO₂ content using the chemiluminescence technique of Archer. Briefly, 0.5-mL aliquots of lung effluent were injected into a glass purge chamber that was evacuated to a pressure of 3 mm Hg by a vacuum pump, and NO was stripped from the liquid to gas phase by bubbling it with nitrogen gas. The gas specimen was then drawn into a chemiluminescent NO analyzer (model 14A, Thermo Environmental Instruments, Franklin, Mass), and NO and NO₂ concentrations (ppm) were determined. The NO analyzer was calibrated by measuring the signal produced by known amounts of NO in saline, which was linear between NO doses of 3 to 300 pmol/L. Effluent samples were taken during baseline Krebs infusion, U46619 infusion, U46619 and inhaled NO administration, U46619 and blood, and U46619, blood, and inhaled NO administration in three experiments.

**Statistical Analysis**

Inhaled NO-induced relaxation is expressed as percent decrease in either Pₚₐ in the lung or grams of tension in the aortic ring during inhaled NO exposure. Statistical significance was at the P < .05 level and was determined by single-factor ANOVA and Newman-Keuls test.

**Results**

Fig 1 shows the simultaneous Pₚₐ in the lung and aortic ring tension in a representative experiment. After the response to U46619 reached a plateau, NO was added to the inspired gas mixture for 3 minutes, and Pₚₐ decreased from 32 to 22 mm Hg (Fig 1, left). The tension on the aortic ring, which was continuously exposed to the effluent from the lung, similarly decreased from 3.5 to 2.1 g, beginning within 15 seconds of the lung response. On stopping NO inhalation, Pₚₐ returned to 35 mm Hg and aortic ring tension to 3.5 g. A continuous infusion of blood was then begun such that the blood perfused both the lung and the aortic ring. In the presence of blood, however, inhaled NO lowered Pₚₐ from 38 to 24 mm Hg, but the aortic ring tension was unchanged (3.3 g; Fig 1, right).

In a series of five separate lung/aorta preparations, inhaled NO–induced relaxation of the preconstricted isolated lung was unaffected by the addition of blood to the combined lung/aortic ring perfusate (35±7% versus 30±6%, NS) (Fig 2). The ability of the lung effluent to
relax the aortic ring, however, was eliminated in the presence of blood (40±13% versus 1.5±1% relaxation, \(P<.01\)). After discontinuation of the blood infusion, inhaled NO relaxed both the lung and the aortic ring (27±8% and 35±8%, respectively).

To determine whether another blood protein or free hemoglobin affects inhaled NO selectivity, similar experiments were performed by adding either albumin or hemoglobin to the Krebs/dextran perfusate (Fig 3). Unlike whole blood, albumin had no effect on either inhaled NO–induced lung (26±4% versus 21±4%, NS) or aortic ring relaxation (37±9% versus 38±10%, NS) compared with control (Krebs only) values. Hemoglobin (1 \(\mu\)mol/L) had no effect on inhaled NO–induced lung relaxation compared with Krebs controls (22±3% versus 21±4%, NS) but eliminated the aortic ring relaxation induced by the lung effluent (6.5±4% versus 37.5±10%, \(P<.01\)). This inhibition of aortic ring relaxation by free hemoglobin was not significantly different from that of whole blood (6.5±3.8% versus 1.5±0.7%, NS).

Lung effluent contained 3 pmol/mL NO during inhaled NO exposure in the presence of a blood-free perfusate by chemiluminescence analysis. During the blood infusion, however, lung effluent contained no measurable NO during inhaled NO administration. NO content in effluent samples obtained during baseline

Fig 1. Simultaneous tracings of pulmonary perfusion pressure \(P_{PA}\) in the isolated lung (upper record) and tension (grams, g) in the aortic ring (lower record). Inhaled nitric oxide (NO) gas caused significant relaxation in both the lung and in the aortic ring that was exposed to effluent from the inhaled NO–treated lung (left). In the presence of a whole blood infusion (hemoglobin 1 g/dL in the lung effluent), however, inhaled NO lowered the \(P_{PA}\) in the lung but had no effect on aortic ring tension (right).

Fig 2. Bar graph shows effect of inhaled nitric oxide (NO) on both pulmonary perfusion pressure (lung) and aortic ring tone before (Krebs), during (Blood), and after (Krebs) addition of a whole blood infusion to the combined lung/ring system. Data are mean±SEM (n=5). The ability of inhaled NO to decrease the pulmonary perfusion pressure in the isolated lung was unaffected by the presence of blood (35±7% versus 30±6% versus 27±8% relaxation). The vasodilator ability of the effluent from these lungs, however, was eliminated in the presence of blood (40±13% versus 1.5±1% versus 35±8% relaxation). *\(P<.01\).

Fig 3. Bar graph shows effect of albumin, blood, and hemoglobin (Hgb) on relaxation of aortic rings exposed to the perfusion effluent from lungs ventilated with inhaled nitric oxide (NO). Data are mean±SEM. Compared with control lung/ring experiments (Krebs, n=5), the presence of albumin (n=5) had no effect on inhaled NO–induced aortic ring relaxation (37±9% versus 38±10%). The presence of both blood (n=5) and hemoglobin (n=3), however, significantly reduced the effect of inhaled NO on aortic ring relaxation (1.5±1% and 6.5±4%, respectively). None of the infusions affected inhaled NO–induced reduction in pulmonary perfusion pressure. *\(P<.01\).
and U46619 infusions (with and without blood infusion) was undetectable. NO₂ content in lung effluent during inhaled NO administration was also undetectable.

Discussion

These results demonstrate that the presence of blood restricts the vasodilating effect of inhaled NO gas to the pulmonary circulation. This inactivation of inhaled NO-induced systemic dilation by blood is reversible and occurs even with extremely small amounts of blood. Furthermore, it is the hemoglobin component of whole blood that is specifically responsible for the selective pulmonary vasodilation observed during NO gas inhalation since albumin, the major nonheme-containing blood protein, failed to inhibit inhaled NO-induced dilation of the aortic ring.

Inhaled NO gas has been shown recently to produce significant pulmonary vasodilation without causing systemic vasodilation.¹,² The ability of inhaled NO gas to antagonize pulmonary vasoconstriction without reducing blood pressure or systemic vascular resistance makes it an ideal agent for the treatment of a variety of pulmonary vascular disorders. Preliminary reports confirm its effectiveness in treating persistent pulmonary hypertension of the newborn¹¹,¹² and primary pulmonary hypertension in adults.³ Although several investigators have proposed that the mechanism of selective pulmonary vasodilation by inhaled NO gas is inactivation by hemoglobin,¹²,¹¹,¹² our experiments are the first to prove this hypothesis.

The affinity of NO for hemoglobin was described by Hermann³ more than a century ago. Hemoglobin combines with NO to form nitrosyl hemoglobin with an affinity 1500 times greater than that for carbon monoxide.³ The ability of hemoglobin to block endothelium-dependent relaxation was one of the factors that led to the identification of endothelium-derived relaxing factor as NO.¹⁴ Whereas hemoglobin has been shown to prevent endothelium-derived NO-induced relaxation in the isolated lung,⁶ the effect of hemoglobin on inhaled NO-induced pulmonary vasodilation has not been examined.

It is interesting to note that the blood-free effluent from the lung exposed to inhaled NO contained measurable NO (by chemiluminescence), which demonstrated potent vasodilator properties after exiting the lung. The absence of detectable NO in the effluent either by chemiluminescence or aortic ring bioassay during the blood infusion clearly demonstrates that some component of whole blood completely binds inhaled NO once it enters the vascular space. Whereas blood contains numerous cellular and hormonal substances, the fact that free hemoglobin and not albumin inactivates inhaled NO to the same degree as whole blood argues strongly that hemoglobin in red blood cells is responsible for selective pulmonary vasodilation. In addition, experiments in blood-free isolated hearts have shown that more than 86% of infused NO is converted to NO₂ during passage through the coronary circulation.¹⁵ NO₂ was not detected in lung effluent during inhaled NO exposure, however, suggesting another difference between inhaled and perfused NO.

The observation that an extremely small amount of blood (1 g/dL hemoglobin) effectively restricts inhaled NO-induced vasodilation to the pulmonary circulation has direct bearing on the use of inhaled NO gas in the treatment of pulmonary vascular diseases. Whereas the effect of different hemoglobin concentrations on the pulmonary selectivity of inhaled NO vasodilation will need to be examined in vivo, the normal range of human hemoglobin values is at least 10 times the amount used in our experiments.¹⁶ This suggests that inhaled NO gas may be effective even in patients with anemia or hemoglobinopathies.

Summary

The presence of hemoglobin in red blood cells, even in extremely small amounts, restricts the vasodilating effect of inhaled NO gas to the pulmonary circulation. This suggests that selective pulmonary vasodilation from inhaled NO gas primarily is due to inactivation by hemoglobin in the pulmonary circulation.

Acknowledgments

This study was supported in part by National Heart, Lung, and Blood Institute grants HL-13315 and HL-40863, the Society of Cardiovascular Anesthesiologists, and the Foundation for Anesthesia Education and Research.

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*Circulation*. 1993;88:2884-2887
doi: 10.1161/01.CIR.88.6.2884

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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