Inhaled Nitrite Reverses Hemolysis-Induced Pulmonary Vasoconstriction in Newborn Lambs Without Blood Participation

Arlin B. Blood, PhD; Hobe J. Schroeder, MD, PhD; Michael H. Terry, RRT; Jeanette Merrill-Henry, RRT; Shannon L. Bragg, BS; Kurt Vrancken, MS; Taiming Liu, PhD; Jason L. Herring, PhD; Lawrence C. Sowers, PhD; Sean M. Wilson, PhD; Gordon G. Power, MD

Methods and Results — Pulmonary vascular pressures and resistances to flow were measured in anesthetized newborn lambs. Plasma hemoglobin concentrations were then elevated, resulting in marked pulmonary hypertension. This effect was attenuated if infused hemoglobin was first oxidized to methemoglobin, which does not scavenge NO. These results further implicate NO as a tonic pulmonary vasodilator. Next, while free hemoglobin continued to be infused, the lambs were given inhaled NO gas (20 ppm), inhaled sodium nitrite aerosol (0.87 mol/L), or an intravascular nitrite infusion (3 mg/h bolus, 5 mg · kg⁻¹ · h⁻¹ infusion). Inhaled NO and inhaled nitrite aerosol both resulted in pulmonary vasodilation. Intravascular infusion of nitrite, however, did not. Increases in exhaled NO gas were observed in lambs while breathing the nitrite aerosol (~20 ppb NO) but not during intravascular infusion of nitrite.

Conclusions — We conclude that the pulmonary vasodilating effect of inhaled nitrite results from its conversion to NO in airway and parenchymal lung tissue and is not dependent on reactions with deoxyhemoglobin in the pulmonary circulation. Inhaled nitrite aerosol remains a promising candidate to reduce pulmonary hypertension in clinical application. (Circulation. 2011;123:605-612.)

Key Words: hemoglobin • hypertension, pulmonary • nitric oxide • nitrite • vasodilation

Nitrite (NO₂⁻), an anion present in the plasma at mid-nanomolar concentrations, is derived from the diet and is a byproduct of nitric oxide (NO) metabolism.¹² Nitrite is converted into biologically relevant concentrations of NO by a number of biochemical pathways (see the review by Lundberg et al³). The production of NO from the reaction between nitrite and deoxyhemoglobin has received special attention because of its potential for regulating blood flow in hypoxic tissues: NO₂⁻ + DeoxyHb + H⁺ → MetHb + NO + OH⁻ (reaction 1). This reaction would be promoted in acidic hypoxic/ischemic regions where the deoxy form of hemoglobin prevails, providing local NO production and tending to match O₂ supply with O₂ requirements. Evidence supporting a role for reaction 1 includes the observation that nitrite infusion to the brachial artery decreases forearm vascular resistance¹⁵ and that inhaled nitrite reduces pulmonary hypertension in hypoxic newborn lambs.⁸ Despite these studies, attempts to measure vasoactive concentrations of NO generated by reaction 1 have been unsuccessful,⁷⁻⁹ possibly because the rate of NO production by reaction 1 appears to be at least 6 orders of magnitude slower than the rate at which NO would be scavenged by adjacent hemoglobin molecules in the red blood cell.

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A major pathway of NO inactivation within erythrocytes is its reactions with both oxyhemoglobin and deoxyhemoglobin. Either reaction is so rapid that only immeasurably small concentrations of NO can persist for more than milliseconds in the presence of excess hemoglobin.¹⁰ Therefore, when hemoglobin is circulating freely in plasma, it can be predicted to be a highly effective scavenger of NO. In fact, the increased incidence of pulmonary hypertension in patients with hemolytic disorders has been ascribed to depletion of...
NO by reaction with plasma hemoglobin,\textsuperscript{11} although this idea is not without controversy.\textsuperscript{12,13}

Nebulized inhaled nitrite has been shown to reduce pulmonary hypertension and to increase exhaled NO in hypoxic newborn lambs.\textsuperscript{6} The pulmonary resistance vessels are arterioles and precapillary sphincters that are in contact with both the bloodstream and the alveoli.\textsuperscript{14} This anatomic arrangement suggests that inhaled nitrite may cause pulmonary vasodilation by entering the blood at the point of the resistance vessels where it could be converted to NO by reaction 1. Alternatively, nitrite may be converted to NO by other reactions within the airways and adjacent tissues, resulting in vasodilation by a blood-independent mechanism. We hypothesized that scavenging NO in the blood brought about by infusing hemolyzed blood into the circulation would reveal a blood-independent mechanism for pulmonary vasodilation after nitrite inhalation. To test this hypothesis, we measured the effects of infusion of hemolyzed red cells followed by nitrite infusion, nitrite inhalation, or NO gas inhalation on pulmonary and systemic arterial vascular resistances in newborn lambs.

**Methods**

**Surgical Instrumentation**

Procedures involving animals were preapproved by the Loma Linda University Institutional Animal Care and Use Committee. Under general anesthesia, 10- to 20-day-old lambs were surgically instrumented with arterial and venous catheters including a Swan-Ganz catheter in and a transonic blood flow probe on the main pulmonary artery. After surgery, anesthesia was maintained with intravenous ketamine (1 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}) and vecuronium (0.1 mg·kg\textsuperscript{-1}·h\textsuperscript{-1}). See the online-only Data Supplement for details.

**Study Protocol**

After baseline measurements, plasma hemoglobin concentrations were elevated by infusion of a loading bolus of hemolysate split 1:2 between the pulmonary artery and aorta, followed by a continuous intravenous infusion. After 90 minutes of hemolysate infusion, the lambs were given 1 of 3 treatments: inhaled NO gas (iNO) with 20 ppm (Ikaria, Clinton, NJ) introduced into the inspiratory limb of the ventilator circuit, inhalation of an aerosol produced from a 0.87 mol/L sodium nitrite solution via jet nebulizer (DeVilbiss, Healthcare, Somerset, PA) as previously described,\textsuperscript{6} or intravenous sodium nitrite to produce plasma nitrite concentrations of \(\approx\)100 μmol/L.

**Vascular Resistances**

Cardiac output and systemic arterial pressure, central venous pressure, and pulmonary artery pressure (PAP) were recorded continuously at 200 Hz (Powerlab 16 and Chart version 5.2, ADInstruments, Colorado Springs, CO). Systemic vascular resistance and pulmonary vascular resistance (PVR) were calculated as described in the online-only Data Supplement.

**Hemolyzed Blood Preparation**

Hemolyzed blood was prepared from heparinized adult sheep blood by 3 cycles of freeze-thawing. The hemolysate was centrifuged for 10 minutes at 5000 rpm at 4°C to remove any unlysed cells. Hemolyzed methemoglobin was prepared by first exposing oxygenated whole blood to NO gas, followed by multiple washes to remove nitrite, and then freeze-thaw cycling.

**Analytic Methodology**

Previously described methods were used to measure exhaled NO, arterial blood gases, and for hemoximetry,\textsuperscript{15} as well as plasma hemoglobin concentrations,\textsuperscript{16} nitrate,\textsuperscript{15} and L-arginine concentrations\textsuperscript{17} (see the online-only Data Supplement).

**Results**

**Hemolysis Causes Pulmonary Hypertension**

Hemolysate (resulting in plasma hemoglobin concentration \(\approx\)100 μmol/L, heme basis; Figure 1A) was infused and the
cardiovascular changes were noted, as shown in Figure 2. Mean PAP and PVR increased by 48% to 71% to reach nearly steady state with little or no effect on systemic pressure or vascular resistance. These changes were absent or markedly attenuated if the hemoglobin in the infused hemolysate was first oxidized to methemoglobin (Fe³⁺ state). This demonstrates that hemolysis results in selective pulmonary hypertensive response and suggests that the mechanism involves the scavenging of NO by plasma Fe²⁺-hemoglobin because methemoglobin, which does not scavenge NO, elicited only a small hypertensive response (Figure 2) to hemolysate infusion.

To further confirm that the selective pulmonary vasoconstrictive effects of hemolysate were due to hemoglobin and not some other constituent of hemolyzed blood, purified hemoglobin (Sigma Aldrich, St Louis, MO) was infused into 2 additional lambs with comparable selective increases in PAP and PVR noted (see Figure I in the online-only Data Supplement).

**Effect of Hemolysis on Plasma l-Arginine**

In addition to NO scavenging by plasma hemoglobin, the vasoconstrictive effects of hemolyzed blood could also be caused by depletion of plasma l-arginine resulting from the release of intraerythrocytic arginase. To assess this possibility, we measured l-arginine concentrations in the plasma of the lambs (Figure 1B). Plasma l-arginine concentrations decreased from baseline levels within 60 minutes after initiation of hemolysate infusion (from baseline of 127±11 to 93±7 μmol/L at 60 minutes).

**Effect of iNO or Intravenous or Inhaled Nitrite on PVR**

Ninety minutes after beginning the infusion of hemolyzed blood, the lambs were treated for another 90 minutes with iNO (n=5), inhaled nitrite aerosol (n=6), or intravenous nitrite (n=6). Both iNO and inhaled nitrite led to a significant decrease in PAP (26% to 31%) and PVR (28% to 34%; Figure 3). The finding that iNO could effectively diminish hemolysis-induced pulmonary hypertension was in accordance with the established selective vasodilating effects of iNO in the lungs. Likewise, the observation that inhaled nitrite aerosol was an effective pulmonary vasodilator was also consistent with previous work but is contrary to the idea that the underlying mechanism involves reaction 1 because this would require the movement of NO from the erythrocyte through the plasma where it would be scavenged by plasma hemoglobin. That this indeed happens is demonstrated by the
observation that the intravenous infusion of nitrite had minimal effect on PVR. This combination of results provides strong evidence that reaction 1 does not mediate the vasodilating effects of inhaled nitrite aerosol.

Consistent with previous reports, inhalation of nitrite aerosol resulted in a significant increase in exhaled NO (Figure 4A). In contrast, the intravenous infusion of nitrite did not increase exhaled NO. These differing responses to inhaled and intravenous nitrite cannot be explained by differences between circulating nitrite concentrations, which were at least as high in the lambs that received intravenous nitrite as in those that received inhaled nitrite (Figure 4B). The data indicate that a mechanism exists for the conversion of nitrite to NO within the airway and lung tissues, independently of the blood, with subsequent effects of NO on pulmonary resistance vessels.

One possible blood-independent mechanism for the conversion of nitrite to NO in the airways and alveoli is the nonenzymatic chemical disproportionation of nitrite. This reaction, first proposed to contribute significantly to gastric NO concentrations and later demonstrated to occur in ischemic tissues, involves the acidification of nitrite to produce nitrous acid (HNO₂), NO₂⁻ + H⁺ ↔ HNO₂ (reaction 2), which then rapidly decomposes via complex chemical reactions to produce NO and other nitrogen oxides. Reaction 2 is pH-dependent with a pKₐ of ~3.3. Although this is well below the pH of the nitrite solution nebulized in the current experiments (pH 7.0), we reasoned that, given the high concentration of nitrite in the aerosolized solution (0.87 mol/L), disproportionation of even a very small fraction of the nitrite would result in the elevations in exhaled NO concentrations observed in these studies. To test this possibility, the newborn lamb was replaced with a test “lung,” a 500-mL neoprene balloon (Ventlab, Mocksville, NC), that was mechanically ventilated with room air using the same ventilator, air flow, and nebulization rates as those used for the lambs. Nitrite solutions identical to those used in the lamb studies were prepared in PBS of varying pH (11.0, 8.5, 7.0, or 4.5) and then nebulized one at a time into the test lung while NO concentrations in the exhaled gas were measured. As shown in Figure 5, inhaled nitrite resulted in measurable increases in exhaled NO from the balloon, an effect that was inversely proportional to the pH of the nitrite solution. At pH 7.0, increases in NO exhaled from the balloon were similar in magnitude to the current and previously reported lamb experiments. These data show that increases in exhaled NO in lambs during inhalation of nitrite aerosol can be accounted for by nitrite disproportionation in the ventilator circuit and pulmonary airway gas space.

Sensitivity of Pulmonary Arteries to NO

It is generally accepted that for vasodilating effects, NO concentrations have to rise at least to the 5- to 10-nmol/L level. To determine the response curves in newborn lambs, isolated pulmonary arteries were studied by wire myography. Exposure of precontracted arteries to increasing concentrations of NO in the vessel bath resulted in measurable relaxation of the vascular smooth muscle at concentrations of ~1 nmol/L and higher with an EC₅₀ of 953±120 nmol/L (Figure 6), suggesting that NO concentrations in the nanomolar range are needed to produce a significant decrease in PVR.

Discussion

These experiments in the newborn lamb demonstrate that hemolyzed blood induces selective pulmonary vasoconstric-
tion mostly as a result of the scavenging of NO by hemoglobin free in plasma. This pulmonary hypertension is effectively reduced by the administration of iNO and nitrite aerosol but not by intravenous nitrite infusion. The data also show that the vasodilating effects of inhaled nitrite aerosol are not mediated by the reaction of nitrite with deoxyhemoglobin in red blood cells. Instead, the experiments suggest that inhaled nitrite aerosol produces vasoactive amounts of NO in the airways, alveoli, and other pulmonary tissues, which then relaxes vascular smooth muscle cells. Much of the NO is likely produced by nonenzymatic disproportionation.

**Mechanism and Treatment of Pulmonary Hypertension Caused by Hemolysis**

NO reacts with oxyhemoglobin at a nearly diffusion-limited rate,25 resulting in the conversion of NO to biologically inert nitrate. The diffusion of endothelium-derived NO into the erythrocyte is slowed nearly 1000-fold as a result of an unstirred layer of plasma surrounding the erythrocyte26 and a cell-free zone of plasma lining the endothelial layer of vessels.27 Accordingly, in the presence of free hemoglobin in plasma, NO scavenging is accelerated by 2 to 3 orders of magnitude, and the amount of NO from the endothelium available for vasodilation is decreased, even at plasma hemoglobin concentrations in the low-micromolar range.28 This NO scavenging is thought to cause hypertension associated with hemolysis or after administration of blood substitutes containing cell-free hemoglobin.29 The present observation that the pulmonary vasoconstricting effects of hemolysate are diminished by first oxidizing the hemoglobin to methemoglobin (Fe3+/H11001), which does not effectively scavenge NO, further suggests that the vasoconstrictive effects are due mostly to NO scavenging as opposed to the action of other hemolysate components. Similar pulmonary hypertension after infusion of reconstituted hemoglobin in solution without other hemolysate products further supports this conclusion (see the online-only Data Supplement).

Hemolysis also results in the release of erythrocytic arginine into the plasma,19 which might have limited endothelial NO production. Indeed, hypoargininemia, elevated plasma arginase, and endothelial impairment have been associated with hypertension in hemolytic patients,30 and in the present experiments, plasma arginine concentrations did decrease measurably, as shown in Figure 1B. The degree of hypoargininemia, however, seems unlikely to have caused pulmonary hypertension because similar decreases of arginine were observed in lambs treated with methemoglobin hemolysate, with no significant associated pulmonary hypertension.

Although systemic vasoconstriction is a hallmark response to millimolar concentrations of intravascular plasma hemoglobin and hemoglobin substitutes,29 the present studies demonstrate that micromolar concentrations of plasma hemoglobin result in selective pulmonary vasoconstriction. Our observations are consistent with recent results from a murine model of hemolysis-induced pulmonary hypertension31 and a
number of clinical associations between pulmonary hypertension and hemolytic conditions such as sickle cell disease, thalassemia, and autoimmune hemolytic anemia (see the review by Morris et al.12) although the true incidence of pulmonary hypertension and its proposed pathogenesis have been debated.12-13 The present findings support the idea that the pulmonary vasculature is particularly dependent on tonic vasodilation caused by NO.

iNO potently dilates the pulmonary vasculature but is scavenged by hemoglobin in the blood so rapidly that its systemic effects are minimal.20 In the case of pulmonary hypertension caused by hemolysis, iNO may have the added benefit of oxidizing cell-free hemoglobin to methemoglobin, thereby reducing the NO scavenging of plasma hemoglobin. This effect of iNO has been demonstrated previously in canine13 and murine34 hemolysis models and in sickle cell patients.35 The rapid reappearance of pulmonary hypertension after discontinuation of iNO in the present studies suggests that the duration of treatment may not have been sufficient to fully oxidize all plasma free hemoglobin. Alternatively, iNO may have inhibited pulmonary endothelial NO synthase activity36 so that, even after fully oxidizing the plasma free hemoglobin, there was insufficient endogenous NO production to allow pulmonary vasodilation.

Mechanism of Nitrite Reduction to NO and Pulmonary Vasodilation

The previous observation that administration of inhaled nitrite aerosol to newborn lambs resulted in pulmonary vasodilation and exhaled NO and that these effects were enhanced by systemic hypoxia46 was interpreted as support for the idea that the reaction of nitrite with deoxyhemoglobin produces vasoactive NO. The present study demonstrates in the same animal model, however, that the pulmonary vasodilation and exhaled NO observed during nitrite inhalation are not prevented by the presence of cell-free hemoglobin to scavenge NO in plasma. Furthermore, unlike inhaled nitrite, intravenous nitrite infusion resulted in neither pulmonary vasodilation nor exhaled NO. Together, these observations demonstrate that the mechanism for conversion of inhaled nitrite to NO does not occur in the blood in a newborn lamb model of pulmonary hypertension induced by hemolysis.

During embryonic development, the pulmonary arterial vasculature forms alongside the airways, resulting in the pulmonary resistance vessels being in close proximity to the smaller airways.37 The closeness is particularly true at the level of the small precapillary arterioles and sphincters, which are juxtaposed directly with the terminal airways without an intervening basal lamina.14 Functional evidence of the ability of the airway to influence pulmonary vascular tone is demonstrated by the observation that hypoxic pulmonary vasoconstriction is caused by decreases in airway PO2 more so than decreased PO2 of the pulmonary arterial circulation.38 The present experiments with the neoprene test lung demonstrate that measurable amounts of inhaled nitrite can be converted to NO independently of pulmonary tissues. The pH dependence of this reaction is suggestive of disproportionation (reaction 2). The comparable level of exhaled NO in the test lung and lambs at pH 7.0 shows that disproportionation is quantitatively sufficient to explain the source of exhaled NO in the lambs during nitrite inhalation. Whether gas-phase NO concentrations during nitrite inhalation were adequate to cause pulmonary vasodilation is unclear. Dyar et al.39 have reported the ED50 for iNO to be 39 ppm (78 nmol/L in aqueous solution), which does not contradict our dose-response curve (Figure 6). This gas-phase NO concentration is 3 orders of magnitude higher than those measured in the exhaled air of the lambs receiving inhaled nitrite in the current study. This discrepancy may indicate that other pathways are also involved in the vasodilating effects of inhaled nitrite, which could be converted to NO inside the walls of the resistance vessels or in their proximity by reacting with a number of metal-containing proteins such as xanthine oxidase,40 aldehyde oxidase,41 NO synthase,42 cytochrome c43; the reactions of these proteins with nitrite in pulmonary tissue do not appear to have been characterized to date.

It is worth noting that the use of hemolyzed blood in the present experiments confounds assessment of the contribution of the reaction of deoxyhemoglobin and nitrite to the control of pulmonary vascular tone. In previous studies, intravenous nitrite has been reported to produce significant pulmonary vasodilation during hypoxia, but the effects were transient and the nitrite dose was ~5-fold higher than in the present study.15 In experiments with isolated rat lungs ventilated with hypoxic gas, perfusion with buffer containing 20 μmol/L nitrite resulted in vasodilation, but the effect was abolished by the addition of whole blood to the perfusate.46 Thus, the role of the conversion of nitrite to NO by reaction with deoxyhemoglobin in the regulation of pulmonary vascular tone has yet to be fully characterized at physiological nitrite concentrations.

Clinical Implications

The present study, in agreement with previous work,6 demonstrates that inhaled nitrite aerosol is an effective treatment for pulmonary hypertension in the newborn lamb. In a clinical setting, the relative simplicity, safety, prolonged action,3 and cost-effectiveness of handling and administering a nitrite aerosol compared with compressed NO gas may make inhaled nitrite an attractive alternative for many intensive care settings. However, the disproportionation reaction implicated in the present studies would also likely include intermediates such as N2O3, N2O4, and NO2,23 all three of which are reactive in biological tissues and may lead to nitrosative and oxidative damage. Although no significant signs of methemoglobin accumulation, deterioration in pulmonary function, or other adverse effects were observed in the present or prior6 lamb studies, toxicity was not a primary focus and should be studied before clinical application of this treatment.

Conclusions

The present experiments provided nitrite as an aerosol in the inspired air to newborn lambs under normoxic conditions. The inhaled nitrite was converted to vasodilating concentrations of NO and led to distinct increases in plasma nitrite concentrations. However, in contrast to previous experiments...
that proposed that the underlying mechanism involved the conversion of nitrite to NO by reaction with deoxyhemoglobin, the present studies demonstrate that inhaled nitrite aerosol is converted to NO by reactions that occur outside the blood and that include nonenzymatic, pH-dependent disproportionation.

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This work was supported by a grant from the American Thoracic Society and Pulmonary Hypertension Association (Dr Blood) and National Institutes of Health grant HL095973 (Dr Blood).

Disclosures
Dr Power is listed as a coinventor on a patent application for the use of inhaled nitrite. The other authors report no conflicts.

References

Nitric oxide (NO) is a tonic vasodilator in the normal circulation. Free hemoglobin in the plasma, as occurs during hemolysis, rapidly scavenges NO, resulting in decreased perivascular NO concentrations and vasoconstriction. Recent reports suggest that the pulmonary vasculature is especially sensitive to hemolysis as demonstrated by the increased incidence of pulmonary hypertension in patients with hemolytic disorders. In the present studies in newborn sheep, we found that infusion of hemolyzed blood increased resistance to pulmonary blood flow with little or no systemic effect, indicating that the pulmonary vasculature is particularly sensitive to the effects of hemolysis. Oxidation of plasma hemoglobin to methemoglobin, which does not scavenge NO, prevented the pulmonary hypertension caused by hemolysis. We have shown previously that inhaling an aerosol containing nitrite reverses pulmonary hypertension in newborn lambs, presumably by conversion to NO. The present studies now show that the transformation of nitrite to NO does not depend on nitrite reacting with hemoglobin in red cells as previously proposed but rather must occur in pulmonary airways and tissues. Free NO released within lung parenchymal tissues would then reach smooth muscle of lung resistance vessels and escape scavenging by erythrocytic or plasma hemoglobin. The studies demonstrate again that inhaled nitrite is an effective pulmonary vasodilator and point toward the existence of reactions that produce NO from nitrite in the lung.
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Inhaled nitrite mediates pulmonary vasodilation without blood participation.


Supplemental Material

Supplemental Methods-

*Surgical Instrumentation*- All procedures involving animals were pre-approved by the Loma Linda University Institutional Animal Care and Use Committee. Lambs at 10 to 20 days of age were anesthetized with intravenous pentothal (10 mg/kg) followed by intubation and mechanical ventilation with 1-2% isoflurane in oxygen. Catheters were placed in the common carotid artery with the tip advanced to the aortic arch for infusion of hemolysate, in the brachial artery for measurement of arterial blood gases, plasma nitrite, and arginine and in the femoral artery for measurement of systemic blood pressures. Venous catheters were placed for administration of nitrite and drugs and measurement of central venous pressure and plasma hemoglobin and nitrite concentrations. A Swan-Ganz catheter was inserted via a femoral vein and advanced to position the tip in the pulmonary artery for measurement of pulmonary artery pressure (PAP, with the balloon deflated) and pulmonary venous “wedge” pressure (PVP, with the balloon inflated). After removal of the left third rib an 8 mm Transonic® blood flow probe was placed around the main pulmonary artery where it provided a measure of total pulmonary blood flow and cardiac output. The lamb ductus arteriosus is functionally closed within two or three days after birth.
After surgical instrumentation was complete isoflurane was discontinued and the lambs were given intravenous anesthesia (ketamine \(1\text{ mg kg}^{-1}\text{.hr}^{-1}\) and vecuronium \(0.1\text{ mg.kg}^{-1}\text{.hr}^{-1}\)). The lambs were connected to a pressure-driven infant ventilator (Sechrist IV-100B) and the settings were adjusted to achieve normal arterial blood gases. Body temperature was maintained using a warming pad and heat lamp.

**Study Protocol-** After baseline measurements, hemolyzed blood was injected by simultaneous infusion over 30 sec of \(0.023\text{ g of hemoglobin per kg body weight into the pulmonary artery via the distal port of the Swan Ganz catheter and of 0.07 g of hemoglobin per kg into the arteries via the carotid artery catheter, followed by a continuous infusion of 0.1 g of hemoglobin-kg^{-1}.hr^{-1}\) into the pulmonary artery. Following 90 min of hemolysate infusion, the lambs were given one of three treatments: inhaled NO (iNO) with 20 ppm NO gas (Ikaria, Clinton, NJ) introduced into the inspiratory limb of the ventilator circuit, a dose found to provide effective pulmonary vasodilation with few or no adverse effects (54); inhalation of an aerosol produced from a 0.87 M nitrite solution via jet nebulizer (DeVilbiss, Healthcare, Somerset, PA) at a rate of 15 mg of nitrite per min as previously described \(^1\); and intravenous sodium nitrite at a rate of \(3\text{ mg.kg}^{-1}\) over 30 sec followed by \(5\text{ mg.kg}^{-1}.hr^{-1}\) continuous infusion, a dose calculated to provide plasma nitrite concentrations of \(\sim 100\text{ μM}\) based on previous studies of the pharmacokinetics of nitrite in newborn lambs \(^2\). Nitrite solutions were prepared by dissolving sodium nitrite (Sigma-Aldrich) in 0.1 M phosphate buffered saline (PBS) solution (pH 7.4, Corning pH meter 430 with Thermo Scientific Orion probe, Fischer Scientific). For preparation of nitrite solutions for inhalation in the artificial lung, immediately prior to the experiment nitrite was dissolved into 0.1 M PBS in which the pH had already been adjusted to the predetermined levels. pH of the solutions was verified following the experiment and did not vary by more than 5% from the predetermined level.
Blood samples were collected from the brachial artery for measurement of plasma hemoglobin, nitrite, L-arginine, and blood gases. Samples for hemoglobin, nitrite, and L-arginine measurements were immediately centrifuged at 10,000 rpm for 30 sec. The resulting plasma supernatant was divided into three 180-μL aliquots. Aliquots were immediately frozen in liquid nitrogen for subsequent assay.

**Hemolyzed blood preparation**- Hemolyzed blood was prepared from adult heparinized sheep blood. The erythrocytes were lysed by three cycles of freeze-thawing. The hemolysate was centrifuged for 10 min at 5000 rpm at 4ºC to remove any unlysed cells and debris, and the hemoglobin concentration was determined (OSM3, Radiometer Copenhagen, Denmark).

**Preparation of methemoglobin**- Whole blood was oxygenated by equilibration with room air in a syringe tonometer, and then equilibrated to a mixture of NO gas (Mathison Tri-Gas, Newark, CA) in nitrogen at a concentration of 10 moles of NO per mole of heme. To remove any nitrite formed during equilibration with NO, the cells were centrifuged and washed with four volumes of normal saline a total of five times. Following the final centrifugation, the cells were re-suspended in a volume of plasma equivalent to that present in the original sample. The blood was then hemolyzed by freeze-thaw cycling as described above.

**Exhaled nitric oxide**- Exhaled NO was measured throughout the experiments by continuous sampling gas from the external opening of the endotracheal tube with a NO chemiluminescence analyzer (Sievers 280i, Boulder, CO) as previously described. A hydrophilic filter (Whatman “Solvent IFD”, Fischer Scientific) was placed in-line between the lamb and the nitric oxide analyzer to collect condensation. Filters were replaced for each lamb.

**Arterial blood gases**- Arterial blood gases were measured by blood gas analyzer (ABL5, Radiometer, Copenhagen, Denmark) and total hemoglobin concentration and oxy-
methemoglobin fractions were measured by hemoximetry (OSM3, Radiometer, Copenhagen, Denmark) calibrated for ovine blood.

*Plasma nitrite*- Plasma nitrite concentrations were determined by triiodide chemiluminescence measurements (280i, Sievers, Boulder, CO) as previously described ².

*Plasma L-arginine assay*- Plasma arginine concentrations were measured using an isotope-dilution gas chromatograph/mass spectrometry (GC/MS) method ³. Plasma samples obtained as as described above were snap-frozen in liquid nitrogen, and stored at -70 °C until assay. $^{13}$C₆-arginine (Arg⁺⁶) (Cambridge Isotope Laboratories, Andover, MA) was used as an internal standard. Following purification by trichloroacetic acid extraction ⁴, samples were dried in siliated vials in a SpeedVac and stored at -70 °C until analysis by GC/MS. Five hundred μL trifluoracetic anhydride (TFAA) was added to the dried samples that were then purged with nitrogen. Samples were sonicated for 2-3 min and heated for 5 min at 100 °C. Next the samples were cooled, uncapped, and dried under a gentle stream of nitrogen, resuspended in 200 μl acetonitrile with 25% TFAA and capped.

Samples (1 μL) were injected into an Agilent 6890N gas chromatograph equipped with an Agilent 5973 inert Mass Selective Detector. Using a 20:1 split injection, samples were separated on the GC using a 10-degrees/min oven ramp from 100 to 180 and 20 degrees/min from 180 to 280 on a HP-5MS column (0.25 mm, 30m, 0.25 μm). The mass spectrometer was operated in selective ion mode monitoring 375 to 386 m/z. Using chromatographic peak areas for the 375 (arginine) and 381 (Arg⁺⁶) ions, arginine concentration was calculated using the labeled arginine (Arg⁺⁶) as an internal standard.

*Plasma hemoglobin*- Plasma hemoglobin concentrations were determined using the Drabkin method ⁵.
**Vascular resistances**- Cardiac output (CO), systemic arterial (AP), central venous (CVP), and pulmonary artery (PAP) pressures were recorded continuously at 200Hz (Powerlab 16 analog-to-digital converter and Chart v 5.2 for Mac software). Pulmonary venous pressure (PVP) equals the "wedge pressure" (see above). Systemic vascular resistance (SVR) was calculated as (mean AP – mean CVP) / mean CO and pulmonary vascular resistance (PVR) was calculated as (mean PAP – mean PVP) / mean CO.

**Vessel Contractility Studies**-

Contractility studies were performed on 4th or 5th order pulmonary arteries that were dissected free of parenchyma and cut into 5 mm long rings in a phosphate-free balanced salt solution (BSS) of the following composition (mM): 126 NaCl; 5 KCl; 10 HEPES; 1 MgCl₂; 2 CaCl₂; 10 glucose; pH 7.4 (adjusted with NaOH). The arterial rings were mounted on a wire myograph and suspended in organ baths (Radnoti glass instruments, Inc. Monrovia, CA) that contained 5 ml of modified Krebs-Henseleit (K-H) solution at 4 – 5°C containing (in mM): 120 NaCl; 4.8 KCl; 1.2 K₂HPO₄; 25 NaHCO₃; 1.2 MgCl₂; 2.5 CaCl₂; 10 glucose maintained at 37°C and aerated with 21% O₂-5% CO₂ balanced with N₂ (pH=7.4) 

At the beginning of each experiment, vessels were equilibrated without tension for thirty minutes to one hour. Vessel rings were tensioned by stretching the vessels progressively to obtain a resting tension of 7.4 mN, as performed previously 

Following injection of 5-HT (final concentration 10 µM), NO-saturated K-H solution (2 mM NO) in various volumes was injected to achieve the final concentrations shown in Figure 7. The resulting tension at each NO concentration was normalized for comparison to the maximum response (=100%) obtained with 10 µM 5-HT (T₅-HTmax). Vessels rings achieved steady-state tension within 2 min following each NO injection. One vessel bath was equipped with an amperometric NO probe (ISO-NOP, Apollo 4000, World Precision Instruments, Sarasota, FL) for monitoring NO concentrations. EC₅₀ for NO
vasodilating the arteries was calculated by fitting the data to the Gaddum/Schild EC$_{50}$ equation in Prism, v5.0 (Graphpad Software, LaJolla, CA).

**Statistical analysis**- Average values are presented as mean ± SEM. Changes in measured parameters over time were determined by one-way ANOVA with repeated measures to assess significant changes from baseline, and two-way ANOVA to assess significant differences between study groups. If the overall ANOVA indicated statistical significance, Bonferroni’s post-hoc analysis was performed following both one- and two-way ANOVAs to identify significant differences at specific time points. All statistical analyses were carried out using Prism 5.0 (Graphpad Software Inc, La Jolla, CA). Statistical significance was accepted at P<0.05.

**Effect of purified hemoglobin infusion on pulmonary and systemic vascular resistance.**

To further confirm that the selective pulmonary vasoconstrictive effects of hemolysate were due to hemoglobin and not some other constituent of hemolyzed blood, purified hemoglobin (Sigma Aldrich, St Louis, MO) was infused into an additional 2 lambs. Purified hemoglobin, which was virtually all oxidized to methemoglobin upon receipt from Sigma, was dissolved in physiological saline solution to a final concentration of 0.1 g/ml and passed through a Whatman 0.20 μm filter. Within 30 minutes prior to injection into the lambs, sodium dithionite was added to achieve a final ratio of two moles dithionite per mole of heme to reduce the heme centers to the ferrous (Fe$^{2+}$) state. The mixture was then equilibrated with room air to oxidize any remaining dithionite, and the hemoglobin was scanned spectrophotometrically as previously described $^7$ to verify that it was >98% oxyhemoglobin. The purified hemoglobin was then injected into the pulmonary artery (0.023 g/kg) and systemic arteries (0.07 g/kg) simultaneously over a period of 30 seconds, a loading dose of hemoglobin similar to that of the
hemolyzed blood experiments. To ensure that changes in pulmonary arterial pressure were not caused by the dithionite and its metabolites rather than hemoglobin, aliquots of dithionite equal to those used to reduce methemoglobin were dissolved in oxygenated physiological saline and infused intravenously at the end of each experiment, with no detectable effect on pulmonary or systemic pressures (data not shown).

As shown in Supplemental Figure 1, both lambs responded to the purified hemoglobin boluses with an acute increase in pulmonary arterial pressure and vascular resistance. In contrast, increases in systemic pressures and vascular resistance were relatively modest or non-existent. These data are consistent with the idea that hemoglobin, as opposed to other constituents of hemolyzed blood, is the primary cause of selective pulmonary hypertension observed in our studies.

Supplemental Figure 1

Supplemental Figure 1- Effect of intravascular infusion of purified hemoglobin into two newborn lambs. Similar to the responses observed following infusion of lysed whole blood, a rapid increase in pulmonary artery pressures and vascular resistances was observed with minimal effect in the systemic vasculature. These data are consistent with the idea that the pulmonary hypertensive effects of hemolysis are mediated by hemoglobin as opposed to other components of lysed blood.
Supplemental References


