Inhaled Nitric Oxide Enables Artificial Blood Transfusion Without Hypertension

Binglan Yu, PhD; Michael J. Raher, BS; Gian Paolo Volpato, MD; Kenneth D. Bloch, MD; Fumito Ichinose, MD; Warren M. Zapol, MD

Background—One of the major obstacles hindering the clinical development of a cell-free, hemoglobin-based oxygen carrier (HBOC) is systemic vasoconstriction.

Methods and Results—Experiments were performed in healthy mice and lambs by infusion of either murine tetrameric hemoglobin (0.48 g/kg) or glutaraldehyde-polymerized bovine hemoglobin (HBOC-201, 1.44 g/kg). We observed that intravenous infusion of either murine tetrameric hemoglobin or HBOC-201 induced prolonged systemic vasoconstriction in wild-type mice but not in mice congenitally deficient in endothelial nitric oxide (NO) synthase (NOS3). Treatment of wild-type mice by breathing NO at 80 ppm in air for 15 or 60 minutes or with 200 ppm NO for 7 minutes prevented the systemic hypertension induced by subsequent intravenous administration of murine tetrameric hemoglobin or HBOC-201 and did not result in conversion of plasma hemoglobin to methemoglobin. Intravenous administration of sodium nitrite (48 nmol) 5 minutes before infusion of murine tetrameric hemoglobin also prevented the development of systemic hypertension. In awake lambs, breathing NO at 80 ppm for 1 hour prevented the systemic hypertension caused by subsequent infusion of HBOC-201.

Conclusions—These findings demonstrate that HBOC can cause systemic vasoconstriction by scavenging NO produced by NOS3. Moreover, in 2 species, inhaled NO administered before the intravenous infusion of HBOC can prevent systemic vasoconstriction without causing methemoglobinemia. (Circulation. 2008;117:1982-1990.)

Key Words: endothelium  ■  hemoglobin  ■  hypertension  ■  nitric oxide  ■  vasoconstriction

Hemoglobin-based oxygen carriers (HBOC) have been investigated for clinical use as blood substitutes. These agents offer the potential to treat patients with anemia or hemorrhage in situations in which standard blood transfusions are not readily available (eg, traumatic injuries), the safety of the blood supply is not assured (eg, in countries with a high prevalence of HIV infections and/or insufficient safeguards), or when religious beliefs preclude standard transfusions.

Historically, the major problems associated with infusion of HBOC include a relatively brief circulating half-life, renal toxicity, and, most importantly, diffuse vasoconstriction potentially leading to coronary and cerebral vasospasm. The first 2 problems have been addressed by producing highly purified and chemically cross-linked hemoglobin molecules. However, the problem of HBOC-induced vasoconstriction remains unsolved. In human clinical trials, it has been suggested that the gastrointestinal side effects (nausea, vomiting, and loss of appetite) and the chest and abdominal pain associated with HBOC administration are the direct results of vasoconstriction.

The mechanisms responsible for HBOC-induced vasoconstriction are incompletely understood. Winslow has proposed an “autoregulation theory” suggesting that enhanced plasma O2 delivery by cell-free hemoglobin may trigger arteriolar vasoconstriction. Alternatively, it has been proposed that the scavenging of endothelium-derived nitric oxide (NO) by cell-free hemoglobin is responsible for the HBOC-induced vasoconstriction. Reiter and coworkers have suggested that, in patients with sickle cell disease, consumption of NO by high plasma concentrations of cell-free hemoglobin can predispose these patients to vasoocclusive crises. A free hemoglobin–induced “NO deficiency” has also been implicated in the pathogenesis of other human disorders such as hemolysis-associated smooth muscle dystonia, vasculopathy, and endothelial dysfunction. Administration of NO donor compounds, such as nitroglycerin or sodium nitroprusside, can attenuate HBOC-induced vasoconstriction but may also cause systemic hypotension.
Inhaled NO is a selective pulmonary vasodilator that has been used to treat pulmonary hypertension and to increase systemic oxygenation in babies and adults, as well as to prevent chronic lung disease associated with prematurity. Recent evidence suggests that inhaled NO may affect the systemic vasculature, leading to vasodilation when endogenous NO synthesis is inhibited (although this is not evident in mice). Moreover, inhaled NO can ameliorate ischemia/reperfusion injury of peripheral organs. Inhaled NO may exert systemic effects via interaction with circulating cells as they transit the lungs. Alternatively, some NO, once inhaled, may escape scavenging by hemoglobin and be converted to relatively stable products that can regenerate NO in the systemic circulation via interaction with circulating cells as they transit the lungs. Therefore, some NO, once inhaled, may escape scavenging by hemoglobin and be converted to relatively stable products that can regenerate NO in the systemic circulation via interaction with circulating cells as they transit the lungs.

Results

Effect of Sodium Nitrite on the Hypertensive Response to HBOC

Sodium nitrite (Sigma-Aldrich, St Louis, Mo) was dissolved in PBS, and the pH was adjusted to 7.4. A final volume of 50 μL PBS solution containing 48 mmol sodium nitrite was administered via a tail vein and followed 5 minutes later by infusion of murine tetrameric hemoglobin solution (0.48 g/kg).

Effect of Inhaled NO on Systemic Blood Pressure After Challenge With HBOC-201 in Awake Lambs

Awake, spontaneously breathing lambs were studied. Lactated Ringer solution was administered at 10 mL/kg per hour. All measurements and samples were obtained at baseline and before and at the end of each treatment. In all 11 lambs, venous blood was withdrawn into a heparinized syringe and stored at 4°C for 2 days before reinfusion. Three groups of lambs were studied. One group (n=3) received an infusion of autologous whole blood (warmed at 37°C, 1.44 g hemoglobin/kg over 20 minutes) while breathing an FIO2 of 0.3. A second group (n=3) received an infusion of HBOC-201 (1.44 g/kg over 20 minutes) while breathing at FIO2=0.3. A third group (n=5) breathed 80 ppm NO at FIO2=0.3 for 1 hour, followed by discontinuation of NO gas breathing and infusion of HBOC-201 (1.44 g/kg over 20 minutes) while breathing at FIO2=0.3.

Statistical Analysis

All values are expressed as mean±SEM. Data were analyzed by repeated-measures ANOVA with interaction. A paired t test with a Holm-Sidak adjustment was used to compare the changes in clearance of murine tetrameric hemoglobin or HBOC-201. A multilinear regression model analysis was tested in the invasive hemodynamic measurements in anesthetized mice. Detailed explanations of the statistical methods are provided in the online-only Data Supplement. Probability values <0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Invasive Hemodynamic Measurements in Anesthetized Mice
To further explore the mechanisms by which infusion of tetrameric hemoglobin causes systemic hypertension in awake WT mice, we performed invasive hemodynamic measurements in anesthetized WT (n=15) and NOS3−/− mice (n=13) before and 3 minutes after infusion of murine tetrameric hemoglobin or whole blood, as a control. At baseline, left ventricular (LV) end-diastolic pressure (LVEDP), maximum rate of developed LV pressure (dP/dtmax), minimum rate of developed LV pressure (dP/dtmin), time constant of isovolumic relaxation (τ), and central venous pressure (CVP) were similar between genotypes (Table). LV end-systolic pressure (LVESP), arterial elastance (Ea), and systemic vascular resistance (SVR) were greater at baseline in NOS3−/− than in WT mice (P<0.05 for all 3). Infusion of murine whole blood did not change the heart rate, LVESP, LVEDP, cardiac output, dP/dtmax, dP/dtmin, SVR, Ea, τ, or CVP in either genotype. However, infusion of murine tetrameric hemoglobin in WT mice increased LVESP, LVEDP, SVR, Ea, and τ and decreased cardiac output without affecting dP/dtmax, dP/dtmin, or CVP. In contrast, the infusion of murine tetrameric hemoglobin into NOS3−/− mice did not alter heart rate, LVESP, LVEDP, cardiac output, dP/dtmax, dP/dtmin, SVR, Ea, τ, or CVP. These results suggest that infusion of tetrameric hemoglobin causes systemic vasoconstriction and impairs cardiac diastolic function via a mechanism that depends on NOS3.

Effect of Inhaled NO on Systemic Blood Pressure After Challenge With Murine Tetrameric Hemoglobin or HBOC-201 in Awake Mice
To examine whether inhaled NO can prevent the systemic vasoconstriction induced by tetrameric hemoglobin infusion in mice, we investigated the impact of breathing 80 ppm NO on the vasoconstrictor response to murine tetrameric hemoglobin. Inhalation of 80 ppm NO in air, beginning 1 hour before and continuing during and after administration of murine tetrameric hemoglobin, completely prevented the increase of SBP (117±1 versus 141±4 mm Hg at 10 minutes; P=0.004; n=5; Figure 2A).
Blood samples were taken 10 minutes after infusion of murine tetrameric hemoglobin into mice breathing air supplemented with or without NO, and plasma methemoglobin levels were measured. In mice continuously breathing 80 ppm NO for 1 hour after administration of murine tetrameric hemoglobin, plasma methemoglobin levels were much greater than those detected in mice breathing air without NO (74±10% versus 4±1%; P<0.001). These observations suggest that inhaled NO prevents the vasocostructor effects of murine tetrameric hemoglobin by oxidizing it to methemoglobin, which appears not to scavenge endothelium-derived NO.16

To investigate whether methemoglobin can produce systemic hypertension, SBP was measured after infusing various concentrations of methemoglobin (from 3% to 100%) in mice (n=5). Infusion of murine tetrameric hemoglobin with 100% methemoglobin did not alter SBP, suggesting that methemoglobin does not scavenge NO. However, injecting murine tetrameric hemoglobin containing lower concentrations of methemoglobin (3%, 6%, and 14.5%) increased SBP (Figure 2B).

Because breathing NO during administration of murine tetrameric hemoglobin oxidized the infused hemoglobin to methemoglobin (impairing its ability to carry oxygen), we investigated whether pretreatment with inhaled NO could attenuate or prevent the systemic hypertension associated with administration of murine tetrameric hemoglobin or HBOC-201 without causing plasma methemoglobinemia. Mice breathed NO in air (80 ppm for 1 hour, 80 ppm for 15 minutes, or 200 ppm for 7 minutes), immediately followed by intravenous administration of murine tetrameric hemoglobin, and SBP was measured serially. Blood samples were withdrawn every 15 minutes after murine tetrameric hemoglobin infusion to monitor plasma hemoglobin and methemoglobin levels. Breathing NO at 80 ppm for 1 hour blocked the systemic vasoconstriction induced by subsequent infusion of murine tetrameric hemoglobin (n=5; Figure 3A). The tetrameric hemoglobin that we prepared from murine blood contained 2±0% methemoglobin. Pretreatment with inhaled NO did not increase plasma methemoglobin levels (2±1% at 15 minutes and 3±1% at 60 minutes; Figure 3B). When the duration of NO inhalation (80 ppm) was decreased from 1 hour to 15 minutes, NO pretreatment was still able to prevent the systemic hypertension induced by the subsequent infusion of murine tetrameric hemoglobin or HBOC-201 (Figure 3C and 3D; n=5). Similarly, pretreatment with 200 ppm NO breathing for 7 minutes prevented the systemic hypertension after infusion of murine tetrameric hemoglobin (Figure 3E; n=5). However, pretreatment by breathing 80 ppm NO for 5 minutes was unable to prevent the systemic hypertension induced by the subsequent infusion of murine tetrameric hemoglobin (data not shown). Invasive hemodynamic measurements revealed that pretreatment of WT mice with inhaled NO at 80 ppm for 15 minutes abolished the increase of LVESP, LVEDP, SVR, Ea, and τ induced by murine tetrameric hemoglobin infusion (n=12; Table). These observations demonstrate that pretreatment with inhaled NO for short periods can prevent the systemic vasoconstriction and diastolic dysfunction produced by HBOC infusion without causing its oxidation to methemoglobin.

Effect of Infusion of Sodium Nitrite on the Hypertensive Response to HBOC

Breathing NO leads to the accumulation of NO metabolites including nitrite,11,14 and nitrite may be converted back to NO via nitrite reductases including deoxyhemoglobin.18 To examine whether nitrite administration could prevent the systemic hypertension caused by challenge with murine tetrameric hemoglobin, sodium nitrite (48 nmol, 0.13 mg/kg) was administered intravenously 5 minutes before murine tetrameric hemoglobin was infused. The dose of sodium nitrite chosen was based on the findings of Duranski et al,19 who reported that intraventricular injection of nitrite (48 nmol) reduced myocardial infarct size in mice subjected to cardiac ischemia and reperfusion.19 Five minutes after nitrite (48 nmol) was infused into mice, plasma nitrite levels were 1.9-fold greater than baseline values (0.58±0.09 versus 0.31±0.06 μmol/L; P<0.05; n=7). Nitrite administration did not alter blood pressure before murine tetrameric hemoglobin was infused. Administration of nitrite blocked the systemic hypertension induced by challenge with HBOC.

Table. Comparison of Cardiac Function and Systemic Hemodynamic Measurements in WT and NOS3−/− Mice Before and After Infusion of Murine Tetrameric Hemoglobin Solution

<table>
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<tr>
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<th>WT Baseline (n=7)</th>
<th>Whole Blood</th>
<th>WT Baseline (n=8)</th>
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<td>623±15</td>
<td>608±13</td>
<td>602±12</td>
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<td>LVEDP, mm Hg</td>
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<td>13 480±1340</td>
<td>12 060±1170</td>
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<tr>
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<td>−12 800±980</td>
<td>−11 730±540</td>
<td>−10 110±640</td>
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<tr>
<td>CVP, mm Hg</td>
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<td>3±0</td>
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</table>

Values are mean±SEM. WT indicates infusion of whole blood or murine tetrameric hemoglobin with breathing air in WT mice; NOS3−/−, infusion of whole blood or murine tetrameric hemoglobin with breathing air in NOS3−/− mice; and WT+iNO, breathing 80 ppm NO in air for 15 minutes, followed by discontinuation of NO gas breathing and infusion of murine tetrameric hemoglobin solution in WT mice.

*P<0.001 vs baseline; †P<0.05 vs WT baseline; ‡P<0.001 vs infusion of tetrameric hemoglobin in WT mice without inhaled NO.
hypertension caused by the subsequent infusion of murine tetrameric hemoglobin (n=5; P<0.05 versus both the whole blood group and the nitrite plus murine tetrameric hemoglobin group; Figure 4A). However, 10 minutes after murine tetrameric hemoglobin infusion, the plasma methemoglobin level increased to 10±2% (P=0.001 versus baseline, P=0.002 versus pretreatment with inhaled NO; Figure 4B). This concentration of methemoglobin (10±2%) is insufficient to account for the ability of nitrite to block HBOC-induced hypertension because infusion of murine tetrameric hemoglobin solution containing 14.5% methemoglobin caused systemic hypertension (Figure 2B).

**Effect of Inhaled NO on Systemic Blood Pressure After Challenge With HBOC-201 in Awake Lambs**

Because, in mice, pretreatment with inhaled NO at 80 ppm for 1 hour prevented the systemic hypertension induced by subsequent administration of murine tetrameric hemoglobin or HBOC-201, we investigated whether this effect could be reproduced in a larger species, the awake normovolemic, nonanemic lamb. Mean arterial pressure was continuously monitored in each group (Figure 5A). Infusion of autologous whole blood (1.44 g hemoglobin/kg) did not significantly alter mean arterial pressure. Mean arterial pressure increased immediately after infusion of HBOC-201 (1.44 g/kg) in the group breathing at FIO2 0.3 without NO (P<0.05 versus autologous whole blood group). In contrast, pretreatment with inhaled NO blocked the systemic hypertensive effects of HBOC-201 challenge (P<0.05 versus HBOC-201 without inhaled NO). After administration of HBOC-201, plasma methemoglobin levels in the group pretreated with inhaled NO were not different from those in the group that was not pretreated with inhaled NO (Figure 5B).

**Discussion**

In the present study, we report that the vasoconstriction caused by administration of murine tetrameric hemoglobin or HBOC-201 depends on NOS3 in mice. Pretreatment with inhaled NO prevented the systemic hypertension induced by administration of murine tetrameric hemoglobin or HBOC-201 without causing methemoglobinemia. Invasive hemodynamic measurements confirmed that pretreatment by breathing 80 ppm NO for 15 minutes blocked the systemic hypertension and diastolic dysfunction induced by subsequent infusion of murine tetrameric hemoglobin. We also report that in awake lambs, pretreatment with inhaled NO (80 ppm for 1 hour) prevented the systemic hypertension caused by subsequent infusion of HBOC-201.
Figure 3. A, Tail-cuff SBP (mm Hg) was measured after pretreatment by breathing 80 ppm NO for 1 hour and subsequent infusion of murine tetrameric hemoglobin (Hb) solution (n=5). Additional mice received the murine tetrameric hemoglobin solution without NO pretreatment (n=7). B, Plasma methemoglobin (MetHb) concentration (%) at various times after infusion of murine tetrameric hemoglobin after pretreatment by breathing 80 ppm NO in air for 1 hour (n=5). C, SBP was measured after infusion of murine tetrameric hemoglobin solution in mice pretreated without or with breathing 80 ppm NO for 15 minutes (n=5 in each group). D, SBP was measured after infusion of HBOC-201 in mice pretreated without or with breathing 80 ppm NO for 15 minutes (n=5 in each group). E, SBP was measured after infusion of murine tetrameric hemoglobin solution in mice pretreated without or with breathing 200 ppm NO for 7 minutes (n=5 in each group). iNO indicates inhaled NO. *P<0.05 vs group breathing air without NO.
The autoregulation theory suggests that enhanced plasma O2 delivery by cell-free hemoglobin triggers vasoconstriction.6,20 Our study casts doubt on the autoregulation theory because tetrameric hemoglobin delivered oxygen similarly to the arterioles of NOS3−/−/H11002−/−/H11002 mice yet did not produce systemic vasoconstriction in the former. In NOS3-deficient mice, infusion of phenylephrine increased the systemic blood pressure, confirming the ability of NOS3−/− mice to vasoconstrict. Thus, our results provide evidence that the scavenging of endothelium-derived NO (synthesized by NOS3) by cell-free tetrameric hemoglobin is the primary mechanism responsible for the vasoconstriction observed after administration of HBOC-containing tetramer. Our results support and extend prior studies that demonstrated that chemical inhibition of all 3 NOS isoforms with N-nitro-L-arginine methyl ester prevented the vasoconstriction induced by tetrameric hemoglobin transfusion in cats.21

A major limitation to the clinical application of artificial blood transfusion has been the concern that some HBOC have caused coronary vasoconstriction that would reduce coronary perfusion and result in myocardial ischemia.22–25 Several hemoglobin modifications have been attempted to limit vasoconstriction after HBOC administration. One strategy has been to genetically engineer the heme pocket of hemoglobin to reduce its NO affinity.26,27 Another strategy has been to attenuate extravasation of HBOC through endothelial junctions by producing larger hemoglobin molecules, such as polyhemoglobin or conjugated hemoglobin.1

We evaluated an alternative strategy to prevent HBOC-induced vasoconstriction by pretreatment with inhaled NO. Similar to the observations of Minneci et al.,16 we observed that although breathing 80 ppm NO during and after the administration of tetrameric hemoglobin prevented systemic hypertension, the plasma ferrous hemoglobin was rapidly oxidized, resulting in high plasma methemoglobin levels.
Methemoglobin is unable to bind and transport oxygen to tissues. In contrast, NO breathing did not increase methemoglobin levels inside the red cell, apparently because of the ample methemoglobin reductase activity in this compartment. Intravenous administration of murine tetrameric hemoglobin containing 100% methemoglobin did not produce a systemic vasopressor response, providing in vivo evidence that methemoglobin in plasma does not significantly scavenge NO produced by the endothelium. However, concurrent inhalation of high levels of NO with HBOC administration will not enable HBOC-based therapies because the oxygen-carrying capacity of the plasma hemoglobin is largely abrogated.

A most important finding of our present study is that breathing high levels of NO before but not during administration of HBOC prevented the development of systemic hypertension in awake mice. The prevention of systemic hypertension by NO breathing did not come at the cost of oxidizing the cell-free hemoglobin to methemoglobin. Moreover, we report that in awake instrumented lambs, breathing 80 ppm NO for 1 hour prevented the systemic hypertension induced by subsequent challenge with HBOC-201. The pulmonary vasodilator effects of inhaled NO are rapidly dissipated after NO breathing is discontinued, and whether breathing NO can modulate systemic vascular tone remains controversial. One hypothesis is that during NO breathing, NO-exposed blood cells are responsible for the systemic effects of inhaled NO. Stamler and colleagues proposed that NO can react with Cys of the hemoglobin β-chain and can be converted back to NO in the periphery. Alternatively, accumulating evidence shows that breathing NO increases plasma levels of NO metabolites including nitrite. Nitrite has been implicated as a potential mediator of the systemic vasodilation induced by tissue hypoxia, and nitrite formed during NO inhalation could be responsible for the ability of inhaled NO pretreatment to prevent HBOC-induced systemic hypertension. We observed that sodium nitrite infusion increased plasma nitrite levels and prevented the systemic hypertension caused by the subsequent infusion of HBOC. However, nitrite infusion raised plasma methemoglobin levels, partially inactivating oxygen transport. Nonetheless, because infusion of tetrameric hemoglobin containing equivalent levels of methemoglobin (14.5%) still caused systemic hypertension, these findings suggest that nitrite can prevent HBOC-induced hypertension via a mechanism that does not require oxidation of hemoglobin.

The findings that pretreatment with inhaled NO prevents the systemic vasoconstriction induced by HBOC in 2 species suggest that this strategy may be applicable to humans. If the studies of pretreatment with inhaled NO in the mouse and sheep can be extrapolated to human beings, breathing NO may enable transfusion with artificial blood for the following reasons. First, pretreatment with inhaled NO prevented the subsequent systemic vasoconstriction noted after intravenous administration of hemoglobin solutions containing tetrameric hemoglobin. Second, inhaled NO pretreatment did not cause plasma methemoglobinemia, whereas continuing NO inhalation at 80 ppm during cell-free hemoglobin transfusion dramatically increased plasma methemoglobin levels. Third, because inhaling NO does not cause hypotension, it will likely be feasible to deliver the gas noninvasively while intravenous access is obtained, for example, in hypotensive trauma patients, before HBOC infusion and resuscitation.

In conclusion, we report that vasoconstriction induced by administration of HBOC is abolished in NOS3−/− mice. Pretreatment with inhaled NO prevents the systemic hypertension induced by subsequent intravenous administration of HBOC without causing plasma methemoglobinemia. Our data support a definitive link between cell-free hemoglobin and endothelial NO consumption. Pretreatment with NO inhalation may provide a novel strategy that can enable the transfusion of artificial blood without causing systemic hypertension.

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Disclosures
Dr Zapol receives royalties on patents licensed by Massachusetts General Hospital to Linde Corp and INO Therapeutics on inhaled NO. Dr Zapol is also head of the scientific advisory board of Ikaria Inc and chair of the Gemifund, a granting board of Linde Corp. Drs Zapol and Bloch serve on the Scientific Advisory Board of INO Therapeutics LLC. The remaining authors report no conflicts.

References
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Pretreatment by breathing NO prevents the systemic hypertension induced by subsequent intravenous administration of cell-free hemoglobin. Nat Biotechnol. 1998;16:672–676.


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