Mixed S-Nitrosylated Polymerized Bovine Hemoglobin Species Moderate Hemodynamic Effects in Acutely Hypoxic Rats

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Hemoglobin (Hb)-based oxygen carriers (HBOCs) are being developed as a potential therapy for increasing tissue oxygenation, yet they have not reached their full potential because of unwanted hemodynamic side effects (vasoconstriction, low cardiac output, and oxygen delivery) due in part to nitric oxide (NO) scavenging by cell-free Hb. It may be possible to overcome the NO scavenging effect by coinfusing S-nitrosylated (SNO) HBOC along with unmodified HBOC. SNO-HBOC, like free Hb, may act as an NO donor in low-oxygen conditions. We hypothesized that an unaltered HBOC, polymerized bovine Hb (PBvHb), coinfused with an SNO-PBvHb, would improve hemodynamics and oxygen delivery during hypoxia. Vascular oxygen content and hemodynamics were determined after euvoletic rats were infused (3 ml) with lactated Ringer's solution, PBvHb, SNO-PBvHb, or PBvHb plus SNO-PBvHb (1:10) during normoxia or acute hypoxia (fraction of inspired oxygen = 10%, 120 min). Hemodynamic side effects resulting from PBvHb infusion (vasoconstriction, elevated pulmonary blood pressure, and reduced cardiac output) were offset by SNO-PBvHb in acute hypoxic, but not normoxic, conditions. These data support the potential use of HBOC mixed with SNO-HBOC for the treatment of conditions in which acute hypoxia is present, such as tumor oxygenation, wound healing, hemorrhagic trauma, and sickle cell and hemolytic anemia.

Keywords: S-nitrosylation; hemoglobin-based oxygen carrier; oxygen delivery; cardiac output; blood pressure

Cell-free hemoglobin (Hb)-based oxygen carriers (HBOCs) are currently being developed as therapies for a wide range of applications, including their use to enhance oxygen delivery to tissues during conditions of ischemia or hypoxia (1, 2). Thus, besides oxygen-carrying properties, a variety of therapies have been proposed for cell free Hbs, such as management of pedicled flaps, improving oxygen delivery in ischemic hearts, and treatment of vasopressor-refractory septic shock (2, 3). The hemodynamic effects of HBOC infusion, including vasoconstriction

CLINICAL RELEVANCE

Hemoglobin-based oxygen carriers (HBOCs) are being developed for blood substitutes, as well as for a wide variety of therapies for other conditions. However, due to unwanted hemodynamic side effects, HBOCs have not yet reached their full potential. We show here that infusing a mixture of S-nitrosylated and normal HBOCs mitigates these adverse hemodynamic side effects.

tion and decreased cardiac output (4–8), have hindered them from reaching their full potential as oxygen delivery agents (9–12). Although several potential mechanisms may explain the vasoconstrictive properties, it is generally recognized that most polymerized HBOCs produce vasoconstriction (including pulmonary) and hypertension in humans and animals due to the nitric oxide (NO) scavenging by luminal or extravasated cell-free Hb (12–14).

Due to the unwanted hemodynamic side effects of these polymerized HBOCs, significant efforts have been directed toward the engineering of novel biochemical prototypes of HBOCs to reduce vasoactivity. This has been met with some success (11, 15, 16), but, overall, the problem of HBOC-induced vasoconstriction remains unresolved (9). Surprisingly, although the suggestion has been made that S-nitrosylation of Hb could be a beneficial adjuvant to HBOCs, aside from biochemical modifications to the actual Hb, few studies have explored other means of managing NO scavenging by HBOCs. One straightforward approach would be replenishing the body with exogenous NO or an NO donor during HBOC administration. Two such studies have recently suggested that NO “augmentation” with HBOC administration is a viable approach to countering the NO depletion by polymerized bovine HBOC. Yu and colleagues (12) recently demonstrated that pretreatment of animals with NO inhalation and coinfusion of nitrite as source of NO moderated the vasoconstrictive and hemodynamic effects of glutaraldehyde-polymerized bovine Hb (PBvHb) infusion. Adopting a similar approach, our laboratory recently demonstrated that increasing *endogenous* NO activity using simultaneous treatment of HBOC with a phosphodiesterase enzyme (PDE)-5 inhibitor also ameliorated the negative hemodynamic effects of PBvHb infusion (17). These data suggest that elevating NO availability during treatment with HBOC may be a viable approach to negating the NO scavenging effects of polymerized HBOCs.

Pretreatment with inhaled NO and dual drug administration (HBOC with sildenafil/PDE-5 inhibitor) may be considered complicated therapies. However, investigations have shown that S-nitrosylated (SNO) Hb allows it to act as an NO donor rather than a scavenger (18–20). The highly conserved cysteines on the

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β chains of Hb in red blood cells (RBCs) (Cys β 93) can react with NO or S-nitrosothiols to form SNO Hb (20–22), and may act as an NO donor at low oxygen concentrations (18, 19, 22). SNO Hb created *ex vivo*, cell free, or in RBC, and (re)introduced into the vasculature, have induced vasodilation in conditions of low partial pressure of oxygen (PO₂). For example, infusion of SNO PEGylated HBOC increased perfusion and oxygen delivery in rat ischemic hearts (18) and tumors (23).

Notably, to date, an SNO-HBOC compound has not been confused with an HBOC for the purpose of compensating for the scavenged NO by the HBOC itself. Presently, just one HBOC product has passed regulatory requirements and is commercially available for use in dogs with anemia. This product, limited to veterinary use, and made from bovine Hb, is cross-linked after its isolation from RBCs (PBvHb). However, if PBvHb was ever approved for clinical use, there would be a readily available supply. Thus, it was the purpose of the present study to combine SNO-PBvHb with PBvHb infusion into awake hypoxic rats to determine the potential hemodynamic and oxygen delivery benefits that may result from this combined therapy. It was hypothesized that, under acute hypoxic conditions, infusion of SNO-HBOC would counter the systemic and pulmonary vasoconstrictor effects of HBOC, improving hemodynamics output and oxygen delivery.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (280–350 g and 10–12 wk of age) were obtained from a commercial vendor (Charles River, Wilmington, MA) and housed in the University of Colorado Health Sciences Center's Center for Laboratory Animal Care (elevation, 1,500 m). Animals were allowed *ad libitum* access to food and water, and kept on a 12-hour day–night cycle. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at University of Colorado Health Sciences Center.

Instrumentation

Rats were allowed to acclimate to Denver altitude (1,500 m; barometric pressure [P_B] $\sim$ 630 mm Hg) for at least 7 days before instrumentation. At 48 hours before surgery, rats were provided water supplemented with acetaminophen/codeine (0.5 mg/ml and 0.05 mg/ml, respectively) for postoperative analgesia. The animals were weighed and hematocrits were determined. Rats were anesthetized with a mixture of ketamine:Rompon (xylazine) (75:6 mg/kg, intraperitoneal). Under aseptic conditions, the left carotid artery was cannulated with a PE-50 (0.58-mm inside diameter [ID]; Becton Dickinson, Franklin Lakes, NJ) catheter. A PV-1 (0.28-mm ID; Becton Dickinson) catheter with a shallow bend at its tip was inserted into the right ventricle via the right jugular vein, and guided into the main pulmonary artery. Pressure tracings confirmed placement in the pulmonary artery. Next, two PE-50 (0.58-mm ID; Becton Dickinson) catheters were placed in the superior vena cava via the right jugular vein for venous blood collection, and to obtain cardiac output values. All catheters were flushed with heparinized saline, tied off, tunneled subcutaneously to the dorsal neck region, and exteriorized at the back of the neck. Animals were allowed at least 48 hours to recover before any treatments. Animals demonstrating signs of infection, diarrhea, or distress were excluded from study.

S-Nitrosylation of Polymerized Glutaraldehyde Hb (SNO + HBOC)

PBvHb (oxyglobin; Biopure, Cambridge, MA) is the product of a reaction with bovine Hb and glutaraldehyde performed under deoxygenation, and is thus partially locked in a T-state conformation (24). Bovine Hb only contains two Cys residues, which are located in the highly conserved β -globin chain 93 positions, and we have previously shown that, unlike other chemically modified Hbs, this Cys remains unmodified and free for S-nitrosylation (25).

PBvHb was SNO, as previously described (18). Briefly, equal volumes of 0.5 M reduced glutathione/100 μ M diethylene triamine pentaacetic acid (DTPA) and 0.5 M NaNO₂/100 μ M DTPA were reacted to yield SNO glutathione (GSNO). The pH of the PBvHb solution was adjusted to pH 9.2 using a KH₂PO₄/K₂HPO₄ buffer system. Then, a 10-fold molar excess of GSNO was reacted with the basic PBvHb in a dark reaction vessel at 4°C for 30 minutes. The reaction solution was dialyzed overnight against lactated Ringer's solution (R) to terminate the reaction and purify the sample.

S-Nitrosylation of β 93 Cysteine: Confirmation, NO Quantification, and Dose

Mass spectrometry (MS) (matrix-assisted laser desorption/ionization-MS and liquid chromatography–MS) was performed to: (1) confirm the presence of NO on the β chain of the PBvHb (2); determine if the S-nitrosylation process may have weakened or damaged the PBvHb structure before infusion; and (3) determine if the S-nitrosylation process altered *in vivo* stability of PBvHb in rat plasma samples (Figure 1A–1D). For a visual representation of SNO bond, the reader can refer to Reference 22 and Figure 2. MS was performed on PBvHb from starting material and in plasma samples as previously described (24, 26). The data are consistent with the addition of NO to PBvHb, as well as unaltered pre- and postinfusion stability of SNO-PBvHb. An additional globin chain ion, denoted α -globin (m/z at $\sim$ 15,260 [M+H]), is apparent in both PBvHb and SNO-PBvHb end-treatment plasma samples. This ion was determined by liquid chromatography/MS/MS to be of rat red cell Hb origin (data not shown), and not consistent with the bovine Hb amino acid sequence.

The Saville reaction was used to quantify the molar amount of NO bound to PBvHb, as previously described (27). The amount of S-nitrosothiol was quantified as the difference in absorbance between samples containing solution C (1% sulfanilamide and 0.2% HgCl₂ in 0.5 M HCl) and solution B (1% sulfanilamide in 0.5 M HCl). Standard curves were calculated from known concentrations of S-nitrosoglutathione stock solutions plotted against absorbance at 540 nm.

A whole-blood concentration of 1 μ M SNO cell-free Hb has been shown to elicit a vasodilatory response under acute hypoxic conditions (22). We confirmed this observation by infusion of SNO-PBvHb to achieve a final whole-blood concentration of roughly 2 μ M SNO-PBvHb (shown in figures, tables, and in the RESULTS). For all studies, SNO-PBvHb ($\sim$ 0.3 ml) was added to the lactated R or PBvHb solution (2.7 ml, $\sim$ 1:10 mixture) immediately before a study began and infused as a total 3 ml bolus.

Stopped-Flow Kinetic Studies

Reactions of ferric Hbs with NO were measured in the stopped flow, as previously described (28). Hb solutions (1 μ M in heme) were mixed with increasing concentration of NO (up to 100 μ M) to start the reaction, and the absorbance changes were monitored at 420 nm. Multiple kinetic traces were averaged, and nonlinear least-squares curves fitted to exponential equations to obtain reaction rate constants.

Oxygen Equilibrium Studies

Oxygen binding studies for Hb SNO-PBvHb were performed in a Hemox Analyzer (TCS Scientific, New Hope, PA). The samples were equilibrated with pure nitrogen gas and reoxygenated with air. The oxygen tension was measured using a Clark oxygen electrode (Model 5331 oxygen probe; Yellow Springs Instruments, Yellow Springs, OH). The oxygen saturation of Hb was monitored via a built-in, dual-wavelength spectrophotometer. A typical experiment was conducted with an Hb concentration between 60 and 75 μ M (heme) at 37°C, and each experiment was repeated three times. The final solution (4 ml) contained 4 μ L of the Hayashi enzymatic reduction system to maintain the metHb content to a minimum level (29). Oxygen equilibrium curves were obtained and analyzed yielding p50 (the partial pressure of oxygen at which Hb is 50% saturated), and n50, the Hill coefficient for oxygen binding. The data analysis was performed by nonlinear least-squares curve fitting of the Adair equations derived from the Hemox Analyzer software P50 Plus version 1.2.

Figure 1. Matrix-assisted laser desorption/ionization-mass spectrometry (MS) of polymerized bovine hemoglobin (PBvHb) and S-nitrosylated (SNO) PBvHb *in vitro* and *in vivo* are shown as (A) prenitrosylation PBvHb, (B) postnitrosylation PBvHb (SNO-PBvHb), (C) end-study plasma PBvHb, and (D) end-study plasma SNO-PBvHb plus PBvHb. Single mass ions [M+H] are shown for chemically modified bovine Hb's β , cross-linked α - α , and polymer fragments before and after S-nitrosylation and in plasma obtained at the end-of-experiment blood sampling. * α -Globin chain consistent with rat red blood cell Hb based on liquid chromatography/MS/MS analysis.

Model and Experimental Design

Before either normoxic or acute hypoxic exposure (10% O₂, 4 h), animals were randomized into one of four groups, each with a sample size of 8–10 animals ($n = 8$ –10): (1) lactated R (Henery Schein, Melville, NY) infused; (2) PBvHb; (3) SNO-PBvHb; and (4) SNO-PBvHb plus PBvHb. Additionally, as a comparison to the hypoxia-exposed SNO-PBvHb-treated animals, a separate group of animals ($n = 3$) received an infusion of GSNO (1 mM; 3 ml bolus).

Hemodynamic Measurements

Rats were placed in custom-designed, small, rectangular, Plexiglas chambers with a portal through which catheters could be passed. Catheters were flushed with heparinized saline and then connected to fluid-filled pressure transducers. All animals were exposed to hypoxia by flushing the chamber with 10% O₂ gas (fraction of inspired oxygen = 10% O₂). Once breathing hypoxic gas, hypoxic animals were not re-exposed to room air. Blood pressures, pulmonary artery blood pressures, heart rates, cardiac outputs, and blood gases were collected

after 30 minutes of hypoxic exposure, before any treatment or infusion.

All animals then underwent a 30-minute (10 min/ml) infusion of lactated R (3 ml), PBvHb (1.3 g/kg in a 3 ml vol), SNO (2 μ M whole-blood concentration; 0.3 ml SNO:2.7 ml lactated R), or SNO plus PBvHb (2 μ M whole-blood concentration; 0.3 ml SNO:2.7 ml PBvHb) through a venous catheter. Hemodynamic variables were measured in all groups at 60 and 120 minutes after infusion. Blood gases were measured at baseline and 120 minutes. Cardiac output was measured using Cardiogreen (catalog no. I2633; Sigma-Aldrich, St. Louis, MO) with the dye-dilution method. Between data collection points, the rats were monitored for any signs of distress, but were otherwise left undisturbed. Animals were killed with an overdose of sodium pentobarbital (100 mg/kg) via a jugular catheter after final measurements.

Measurement of Plasma Ferrous and Ferric Hb

Plasma from the end-of-study blood samples were used to determine content of ferrous PBvHb (Fe²⁺, oxy/deoxy) and ferric PBvHb (Fe³⁺,

Figure 2. End-study plasma Hb content represented as heme concentration (μM). (A) The 2-hour postinfusion ferrous and ferric content in plasma. (B) Black lines are spectra of three samples from PBvHb infused animals gray lines are spectra from Ringer's solution (R) lactate-treated animals. (C) Black lines are spectra of three samples from SNO-PBvHb-infused animals; gray lines are spectra from R lactate-treated animals. RL, Ringers lactate.

deoxy) using a photodiode array spectrophotometer (Model 8453; Hewlett Packard, Palo Alto, CA). Blank rat plasma was used to correct for background interference and turbidity. Molar concentrations of ferrous and ferric heme in PBvHb were determined using a multicomponent analysis based on the extinction coefficients for each Hb species.

Measurement of SNO-PBvHb Solution and Plasma Nitrite Levels

Nitrite levels were determined in the SNO-PBvHb starting solution and in the end-point plasma samples collected from six ($n = 6$) animals in each treatment group, unless otherwise indicated, using a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI). The assay was performed per manufacturer's instructions. Plasma samples were evaluated in duplicate using a dilution, as recommended by the manufacturer for analysis of plasma samples, whereas the noninfused SNO-PBvHb was evaluated without dilution.

Blood Gas and Co-Oximetry Measurements in Whole Blood and Plasma

Blood samples were collected immediately after hemodynamic measurements had been obtained at baseline and 120 minutes. Arterial (0.2 ml) and venous (0.2 ml) blood was withdrawn via carotid and venous catheters, respectively, into blood gas syringes, and analyzed (ABL5 and co-oximetry via OSM3; Radiometer, Copenhagen, Denmark) with algorithms specific to rat and bovine Hb.

Plasma-phase co-oximetry. Heparinized microhematocrit capillary tubes (100 μl, catalog no. 22362566; Fisherbrand, Pittsburgh, PA) were immediately filled from each 1-ml blood gas syringe (*see above*), sealed from room air with a removable cap (catalog no. 8889212000; Kendall Healthcare, Mansfield, MA), and spun on a capillary centrifuge to separate cell and plasma fractions. The plasma portion of the capillary tube was aspirated into the co-oximeter. Measurements were excluded if any portion of the cell fraction was inadvertently aspirated into the analyzer. Care was taken to avoid exposing blood samples to room air.

Calculation for Oxygen Delivery

Oxygen delivery was calculated for Hb in whole blood and PBvHb in the plasma phase (Eq.1 below). No measurable endogenous Hb was present in the plasma:

$$DO_2 = (CaO_2) \times CI,$$

where DO_2 is oxygen delivery, CI is cardiac index, and CaO_2 and CvO_2 are arterial and venous content, respectively (CaO_2 and $CvO_2 = [HbO_2\%/100] \times \text{total hemoglobin (tHb)} \times \gamma$ [where γ is oxygen capacity for rat or bovine Hb; OSM3 programmed algorithms]).

TABLE 1. BLOOD GAS RESPONSES IN TREATED AND UNTREATED RATS EXPOSED TO EITHER NORMOXIC OR ACUTE HYPOXIC CONDITIONS

	Normoxia		Acute Hypoxia*	
	Baseline	120 min	Baseline	120 min
Mean pH				
R	7.51 ± 0.01	7.49 ± 0.01	7.58 ± 0.01†	7.57 ± 0.01†
PBvHb	7.48 ± 0.01	7.52 ± 0.01	7.60 ± 0.02†	7.56 ± 0.01†
SNO-PBvHb	7.47 ± 0.02	7.45 ± 0.01	7.57 ± 0.01†	7.54 ± 0.01†
SNO-PBvHb + PBvHb	7.48 ± 0.01	7.50 ± 0.02	7.58 ± 0.01†	7.52 ± 0.01†
Mean PCO ₂ , mm hg				
R	31 ± 1	32 ± 1	22 ± 1†	21 ± 1†
PBvHb	33 ± 1	30 ± 2	23 ± 1†	20 ± 1†
SNO-PBvHb	32 ± 2	29 ± 2	22 ± 1†	19 ± 1†
SNO-PBvHb + PBvHb	31 ± 1	30 ± 2	22 ± 1†	20 ± 1†
Mean PO ₂ , mm hg				
R	76 ± 2	80 ± 4	34 ± 2†	41 ± 4†
PBvHb	75 ± 4	80 ± 6	33 ± 2†	38 ± 2†
SNO-PBvHb	76 ± 5	79 ± 4	34 ± 2†	35 ± 4†
SNO-PBvHb + PBvHb	75 ± 4	76 ± 4	32 ± 2†	38 ± 4†

Definition of abbreviations: PBvHb, polymerized bovine hemoglobin; PCO₂, partial pressure of carbon dioxide; R, lactated Ringer's solution; SNO, S-nitrosylated; SNO-PBvHb + PBvHb, 1:10 mixture of SNO-PBvHb and PBvHb.

* Acute hypoxia defined as 10% O₂. Values are means ± SEM.

† $P < 0.001$; compared to normoxia, all hypoxia values were significantly different.

TABLE 2. NORMOXIC RATS SYSTEMIC HEMODYNAMIC RESPONSES TO S-NITROSYLATED POLYMERIZED BOVINE HEMOGLOBIN

	Baseline	60 min	120 min
Mean arterial pressure, mm Hg			
R	125 ± 5 ^a	124 ± 9 ^a	124 ± 7 ^a
PBvHb	130 ± 4 ^a	158 ± 2 ^{*,b}	154 ± 2 ^{*,b}
SNO-PBvHb	125 ± 3 ^a	137 ± 5 ^a	134 ± 5 ^a
SNO-PBvHb + PBvHb	120 ± 4 ^a	140 ± 6 ^{*,a,b}	135 ± 8 ^{*,a,b}
Total peripheral resistance, mm Hg · L ⁻¹ · min ⁻¹			
R	2072 ± 181 ^a	2684 ± 442 ^a	2833 ± 401 ^a
PBvHb	2078 ± 311 ^a	3342 ± 470 ^{*,a}	5532 ± 466 ^{*,a,b}
SNO-PBvHb	3230 ± 332 ^a	3219 ± 302 ^a	3634 ± 342 ^a
SNO-PBvHb + PBvHb	2138 ± 254 ^a	4385 ± 625 ^{*,a}	6460 ± 840 ^{*,b}
Cardiac index, L · min ⁻¹ · kg ⁻¹			
R	182 ± 22 ^a	168 ± 33 ^a	153 ± 31 ^a
PBvHb	194 ± 26 ^a	136 ± 14 ^{*,a}	83 ± 7 ^{*,b}
SNO-PBvHb	135 ± 12 ^a	138 ± 19 ^a	108 ± 11 ^{a,b}
SNO-PBvHb + PBvHb	165 ± 17 ^a	117 ± 11 ^{*,a}	74 ± 10 ^{*,b}
Heart rate, beats · min ⁻¹			
R	414 ± 16 ^a	373 ± 09 ^{a,b}	412 ± 14 ^{a,b}
PBvHb	421 ± 18 ^a	339 ± 14 ^{*,b}	353 ± 09 ^{b,c}
SNO-PBvHb	427 ± 18 ^a	406 ± 18 ^a	423 ± 16 ^a
SNO-PBvHb + PBvHb	395 ± 14 ^a	353 ± 20 ^{*,a,b}	355 ± 14 ^{*,c}
Stroke volume index, ml · kg ⁻¹			
R	0.14 ± 0.02 ^a	0.13 ± 0.01 ^a	0.11 ± 0.01 ^a
PBvHb	0.15 ± 0.02 ^a	0.13 ± 0.02 ^a	0.08 ± 0.01 ^{*,a}
SNO-PBvHb	0.11 ± 0.01 ^a	0.11 ± 0.02 ^a	0.08 ± 0.02 ^a
SNO-PBvHb + PBvHb	0.16 ± 0.02 ^a	0.11 ± 0.02 ^{*,a}	0.07 ± 0.01 ^{*,a}

For definition of abbreviations see Table 1; values are means ± SEM.

* $P < 0.05$, compared to baseline.

^{a,b} For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

Statistical Analysis

For all groups, means (±SEM) are reported. Statistical comparisons between groups were initially analyzed with a multifactorial (fraction of inspired oxygen, treatment) with repeated measures (time) ANOVA (Tables 1–4). If this analysis revealed differences among groups for baseline values, an analysis of covariance was performed, and adjusted means for 60 and 120 minutes were generated and evaluated. The Tukey-Kramer multiple comparison was used to identify differences between group means. Percent changes from baseline were calculated for key variables, and a single-factor ANOVA with a Tukey-Kramer multiple comparison test was used to determine statistical differences among groups. Statistical analyses were performed using JMP version 5 statistical software package (SAS Institute, Cary, North Carolina) with statistical significance set at a P value of 0.05 or less.

RESULTS

Stopped-Flow Kinetic Studies

The representative time traces of NO binding with ferric oxyglobin and SNO-oxyglobin are typically biphasic processes, as indicated by the nonlinear least-squares regression analysis. The observed fast and slow rate constants under pseudo-first-order conditions were plotted versus NO concentration for both Hbs (Figure 3). The fast and slow rate constants of the bimolecular reaction of NO and ferric oxyglobin were 0.023 and 0.017 $\mu\text{M}^{-1} \text{s}^{-1}$, respectively. Similarly, the fast and slow rate constants were obtained for ferric SNO oxyglobin as 0.025 and 0.015 $\mu\text{M}^{-1} \text{s}^{-1}$.

Oxygen Equilibrium Studies

Comparison of the oxygen equilibrium curves for oxyglobin and SNO-oxyglobin demonstrated nearly superimposable oxygen association and dissociation curves (Figure 4). The mean (±SEM) p_{50} for three separate evaluations were 25.73 (±0.179) (mm Hg) and 28.29 (±2.57) for oxyglobin and SNO-

oxyglobin, respectively. The Hill numbers were 1.34 (±0.02) and 1.15 (±0.04) for oxyglobin and SNO-oxyglobin, respectively.

Plasma Ferrous and Ferric Hb

At 2 hours after treatment, infusion plasma samples showed no significant oxidation of Fe^{2+} to Fe^{3+} in the R, PBvHb, or SNO-PBvHb plus PBvHb groups (Figure 2).

Nitrite Levels in SNO-PBvHb Solution and Plasma

The amount of nitrite present in the preinfusion starting material (SNO-PBvHb) was below the limit of detection and equivalent to

TABLE 3. NORMOXIC RATS PULMONARY VASCULAR HEMODYNAMIC RESPONSES TO S-NITROSYLATED POLYMERIZED BOVINE HEMOGLOBIN

	Baseline	60 min	120 min
Mean pulmonary artery pressure, mm Hg			
R	23 ± 1 ^a	23 ± 1 ^{a,b}	23 ± 2 ^{a,b}
PBvHb	23 ± 1 ^a	29 ± 1 ^{*,a}	26 ± 2 ^{*,a}
SNO-PBvHb	21 ± 1 ^a	20 ± 1 ^b	20 ± 1 ^b
SNO-PBvHb + PBvHb	21 ± 1 ^a	25 ± 2 ^{*,a,b}	25 ± 2 ^{*,b}
Pulmonary vascular resistance, mm Hg · L ⁻¹ · min ⁻¹			
R	355 ± 23 ^{a,b}	483 ± 106 ^a	498 ± 84 ^a
PBvHb	390 ± 63 ^{a,b}	529 ± 42 ^{*,a}	920 ± 75 ^{*,b}
SNO-PBvHb	557 ± 55 ^b	512 ± 66 ^b	574 ± 74 ^b
SNO-PBvHb + PBvHb	374 ± 49 ^a	650 ± 91 ^{*,a}	1072 ± 249 ^{*,b}

For definition of abbreviations see Table 1; values are means ± SEM.

* $P < 0.05$, compared to baseline.

^{a,b} For the group comparisons, same letter designations are not significant; different letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

TABLE 4. HYPOXIC RATS SYSTEMIC HEMODYNAMIC RESPONSES TO S-NITROSYLATED POLYMERIZED BOVINE HEMOGLOBIN

	Baseline	60 min	120 min
Mean arterial pressure, mm Hg			
R	111 ± 3 ^a	100 ± 4 ^a	108 ± 4 ^a
PBvHb	108 ± 2 ^a	114 ± 4 ^a	125 ± 5 ^{a,b}
SNO-PBvHb	115 ± 4 ^a	98 ± 3 ^a	110 ± 3 ^a
SNO-PBvHb + PBvHb	109 ± 3 ^a	107 ± 5 ^a	123 ± 4 ^{a,b}
Total peripheral resistance, mm Hg · L ⁻¹ · min ⁻¹			
R	2179 ± 217 ^a	1868 ± 120 ^a	2690 ± 231 ^a
PBvHb	2194 ± 114 ^a	2343 ± 212 ^a	4899 ± 506 ^{a,b}
SNO-PBvHb	2485 ± 130 ^a	1690 ± 306 ^a	2544 ± 191 ^a
SNO-PBvHb + PBvHb	2121 ± 247 ^a	2336 ± 379 ^a	3190 ± 440 ^a
Cardiac index, L · min ⁻¹ · kg ⁻¹			
R	147 ± 14 ^a	164 ± 7 ^a	124 ± 17 ^a
PBvHb	137 ± 13 ^a	142 ± 19 ^a	65 ± 13 ^{a,b}
SNO-PBvHb	154 ± 15 ^a	249 ± 23 ^{a,b}	168 ± 20 ^a
SNO-PBvHb + PBvHb	164 ± 13 ^a	164 ± 24 ^{a,b}	135 ± 19 ^a
Heart rate, beats · min ⁻¹			
R	425 ± 25 ^a	440 ± 12 ^a	433 ± 12 ^a
PBvHb	421 ± 18 ^a	380 ± 20 ^a	362 ± 21 ^a
SNO-PBvHb	437 ± 15 ^a	428 ± 12 ^a	420 ± 10 ^a
SNO-PBvHb + PBvHb	415 ± 18 ^a	400 ± 7 ^a	384 ± 13 ^a
Stroke volume index, ml · kg ⁻¹			
R	0.112 ± 0.01 ^a	0.123 ± 0.01 ^a	0.091 ± 0.01 ^{a,b}
PBvHb	0.110 ± 0.01 ^a	0.125 ± 0.01 ^a	0.062 ± 0.01 ^a
SNO-PBvHb	0.108 ± 0.01 ^a	0.166 ± 0.02 ^a	0.118 ± 0.01 ^{a,b}
SNO-PBvHb + PBvHb	0.150 ± 0.02 ^a	0.136 ± 0.02 ^a	0.123 ± 0.02 ^b

For definition of abbreviations see Table 1; values are means ± SEM.

* $P < 0.05$, compared to baseline.

^{a,b} For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

background absorbance. The end-study nitrite levels in plasma showed no difference between groups: (1) R, 5.55 (±1.24 μM); (2) PBvHb, 5.11 (±1.44 μM); (3) SNO-PBvHb, 6.46 (±1.77 μM); and (4) SNO-Hb plus PBvHb, 3.17 (±2.09 μM). These data suggest that neither infused nitrite nor *in vivo*-generated nitrite dictated the study findings.

Blood Gases

Arterial PO₂, partial pressure of carbon dioxide, and pH. R, PBvHb, SNO-PBvHb, or SNO-PBvHb + PBvHb did not alter PO₂, partial pressure of carbon dioxide, or pH during normoxia or hypoxia. Hypoxia produced expected decreases in PO₂ and partial pressure of carbon dioxide, and increases in pH ($P < 0.001$, Table 1).

Hemodynamics

Normoxia results. Under normoxic conditions, PBvHb produced expected changes in hemodynamics, elevated blood pressures (systemic and pulmonary), and resistances, and lowered cardiac output (Table 2–3). SNO-PBvHb infusion had no effect on hemodynamics (blood pressures, cardiac output, and resistances) when infused alone or in combination with PBvHb (Table 2–3). However, SNO-PBvHb infusion decreased vascular variables relative to PBvHb infusion alone (Table 2).

Hypoxia results. MEAN ARTERIAL PRESSURE AND TOTAL PERIPHERAL RESISTANCE. Mean arterial pressure and peripheral resistance were increased (15 and 123%; $P = 0.01$ and $P < 0.001$, respectively) at 120 minutes after PBvHb treatment. SNO-PBvHb infusion alone decreased these variables (–15 and –32%; $P \leq 0.03$) at 60 minutes. A combined infusion of SNO-PBvHb and PBvHb increased mean arterial pressure at 120 minutes, but showed no change in peripheral resistances at any

time point (Figure 5, Table 4). Infusion of GSNO alone caused an immediate and significant decrease (–50%; $P \leq 0.006$) in mean arterial pressure (MAP), which returned to normal within 20 minutes of infusion in both normoxia- and hypoxia-exposed rats (Figure 5A, inset).

MEAN PULMONARY ARTERY PRESSURE AND TOTAL PULMONARY VASCULAR RESISTANCE. Mean pulmonary artery pressures increased from untreated values to a maximum (37%; $P = 0.01$) at 60 minutes after PBvHb infusion, and remained elevated at 120 minutes, whereas total pulmonary vascular resistance in-

Figure 3. Stopped-flow kinetic studies to determine nitric oxide (NO) binding of ferric PBvHb and SNO-PBvHb. The fast (k_1) and slow (k_2) rate constants plotted against NO concentration are shown as circles and squares for oxyglobin (open symbols) and SNO-oxyglobin (closed symbols), respectively.

Figure 4. Oxygen equilibrium curves. Oxygen equilibrium obtained at 37° using a Hemox analyzer show the processes of oxygen association (left) and oxygen dissociation (right) for oxyglobin (OG; black circles) and SNO-oxyglobin (gray circles). PO_2 , partial pressure of oxygen.

creased from baseline throughout the study time course, showing the highest value (121%; $P < 0.001$) at 120 minutes (Figure 6, Table 5). Infusion of SNO-PBvHb alone showed no effect on pulmonary artery pressures and total pulmonary vascular resistance. SNO-PBvHb infused with PBvHb did not prevent the increase seen with infusion of PBvHb alone in pulmonary artery pressure or total pulmonary vascular resistance from baseline values (Figure 6, Table 5).

CARDIAC INDEX, STROKE VOLUME, AND HEART RATE. Treatment with PBvHb induced a dramatic decline (−54%; $P = 0.001$) in cardiac index between 60 and 120 minutes (Figure 7, Table 4). This was associated with decreased stroke volume and heart rate (Table 4). Treatment with SNO-PBvHb increased cardiac index and stroke volume at 60 minutes (Figure 7, Table 4). Infusion with the combination of SNO-PBvHb plus PBvHb showed no changes in cardiac index, stroke volume, or heart rate from pretreatment values (Figure 7, Table 4).

OXYGEN DELIVERY. The delivery of oxygen was compromised by PBvHb infusion, despite increased oxygen carrying capacity (Figure 7, Table 6). Hb concentration and arterial oxygen content increased (~12%; $P \leq 0.02$) with PBvHb and SNO-PBvHb plus PBvHb infusion, and was reduced with R and SNO infusion ($P \leq 0.03$). Infusion of SNO-PBvHb alone or in combination with PBvHb did not change oxygen delivery from

Figure 5. Systemic vascular effects of coinfusion of SNO polymerized bovine Hb (SNO-PBvHb) with PBvHb in rats exposed to acute hypoxia. * $P < 0.05$ versus baseline values. (A). Mean arterial pressure. (B) Total peripheral resistance. Inset: acute effects of SNO glutathione (GSNO) infusion (* $P < 0.05$) versus baseline values. ^{a,b}For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

baseline values at any time point. This is demonstrated in Figure 7, where the percent changes are graphed.

DISCUSSION

Overview of Principal Findings

The implications of our main finding was that, during an acute hypoxic insult (10% O_2), the hemodynamic side effects (vaso-

Figure 6. Pulmonary vascular effects of coinfusion of SNO-PBvHb with PBvHb in rats exposed to acute hypoxia. $P < 0.05$ versus baseline values. ^{a,b}For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

constriction, elevated pulmonary blood pressure, and reduced cardiac output) of PBvHb infusion can be offset by a simultaneous infusion of small amounts of the SNO-PBvHb). SNO infusion had no effect in normoxic conditions, confirming previous work (22). However, it is unknown whether the SNO-glutaraldehyde cross-linked Hb retained some ability for relaxed to tense (T) transition states that presumably allow for NO unloading in a hypoxic environment, or this phenomenon occurred via some other as-yet undetermined mechanism. Generally, low-oxygen-affinity HBOCs tend to retain the T-state conformation due to cross-linking and/or polymeriza-

TABLE 5. HYPOXIC RATS PULMONARY HEMODYNAMIC RESPONSES TO S-NITROSYLATED POLYMERIZED BOVINE HEMOGLOBIN

	Baseline	60 min	120 min
Mean pulmonary artery pressure, mm Hg			
R	28 ± 2 ^a	31 ± 2 ^a	31 ± 3 ^a
PBvHb	27 ± 1 ^a	37 ± 2 ^{a,b}	35 ± 3 ^{a,b}
SNO-PBvHb	26 ± 1 ^a	25 ± 1 ^a	28 ± 1 ^a
SNO-PBvHb + PBvHb	31 ± 1 ^a	31 ± 2 ^{a,b}	32 ± 2 ^a
Pulmonary vascular resistance, mm Hg · L ⁻¹ · min ⁻¹			
R	551 ± 79 ^a	551 ± 33 ^{a,b}	689 ± 46 ^a
PBvHb	583 ± 56 ^a	769 ± 80 ^{a,b}	1291 ± 107 ^{a,b}
SNO-PBvHb	568 ± 41 ^a	437 ± 85 ^a	698 ± 37 ^a
SNO-PBvHb + PBvHb	633 ± 67 ^a	687 ± 131 ^a	820 ± 150 ^{a,b}

For definition of abbreviations see Table 1; values are means ± SEM.

* $P < 0.05$, compared to baseline.

^{a,b} For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

Figure 7. Cardiac and oxygen delivery effects of coinfusion of SNO-PBvHb with PBvHb in rats exposed to acute hypoxia. * $P < 0.05$ versus baseline values. ^{a,b}For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

tion (30). However, our data suggest a more pronounced and sustained effect on hemodynamics with SNO-PBvHb versus SNO-PBvHb plus PBvHb, indicating that lowered cooperativity (T↔relaxed state transition) may be sufficient to facilitate Cys93 NO release in a concentration-dependent fashion. Future studies will need to resolve this issue, and the implications for S-nitrosylation of HBOCs. However, we know that the small amount of SNO-PBvHb added is not sufficient to have any significant effect on the overall blood oxygen binding properties.

NO Augmentation Improves Effectiveness of HBOCs

Our data are novel, and support the suggestion that S-nitrosylation of an HBOC may serve as an agent to deliver NO in conditions of low PO_2 , overcoming the adverse hemodynamic side effects induced by PBvHb. Our data indirectly suggest that: (1) neither infused nitrite nor *in vivo*-generated nitrite dictated the study findings; (2) NO availability in the presence of SNO Hb appears to occur in low- PO_2 environments (3); bovine polymerized HBOC scavenges NO, resulting in vasoconstriction, thereby elevating blood pressures and reducing cardiac output; and (4) combined therapy of SNO with PBvHb results in improved hemodynamics and oxygen delivery in hypoxic conditions, likely by supplying NO.

This study adds support to the rationale that NO augmentation is a viable method to manage the NO-scavenging effects of certain HBOCs. Previous experiments have indicated that NO augmentation/enhancement from either an exogenous or endogenous source (i.e., inhalant [12]) or treatment with a PDE-5 inhibitor (17) offset the negative hemodynamic side effects of bovine polymerized HBOC infusion. The current study supports these observations, and extends the treatment options of NO augmentation to include the S-nitrosylated bovine polymerized HBOC as an effective cotreatment with bovine polymerized Hb. One advantage of S-nitrosylation over intravenous sodium nitrite administration is the lack of heme

TABLE 6. OXYGEN DELIVERY, HEMOGLOBIN, AND OXYGEN CONTENT WITH LACTED RINGER'S SOLUTION, POLYMERIZED BOVINE HEMOGLOBIN, S-NITROSYLATED PBvHb, AND A MIXTURE OF S-NITROSYLATED POLYMERIZED BOVINE HEMOGLOBIN AND POLYMERIZED BOVINE HEMOGLOBIN

	Baseline, before Infusion	120 min after Infusion
Oxygen Delivery, ml O ₂ · min ⁻¹ · kg ⁻¹		
R	14 ± 2 ^a	12 ± 1 ^a
PBvHb	14 ± 1 ^a	8 ± 2 ^{*,a}
SNO-PBvHb	17 ± 2 ^a	19 ± 2 ^b
SNO-PBvHb + PBvHb	17 ± 1 ^a	15 ± 2 ^{a,b}
Hemoglobin, gm · 100 ml ⁻¹		
R	14.6 ± 0.4 ^a	13.1 ± 0.4 _{WB} ^{*,a} NA _{PL}
PBvHb	13.5 ± 0.5 ^a	15.3 ± 0.3 _{WB} ^{*,a} 2.1 ± 0.2 _{PL} ^b
SNO-PBvHb	14.5 ± 0.5 ^a	13.6 ± 0.8 _{WB} ^a NA _{PL}
SNO-PBvHb + PBvHb	13.7 ± 0.6 ^a	15.0 ± 0.3 _{WB} ^{*,a} 1.95 ± 0.2 _{PL} ^b
Methemoglobin, % tHb		
R	0.08 ± 0.01 ^a	0.00 _{WB} ^a 0.00 _{PL} ^a
PBvHb	0.05 ± 0.04 ^a	0.28 ± 0.01 _{WB} ^b 1.6 ± 0.2 _{PL} ^c
SNO-PBvHb	0.03 ± 0.01 ^a	0.00 _{WB} ^a 0.21 ± 0.2 _{PL} ^b
SNO-PBvHb + PBvHb	0.03 ± 0.01 ^a	0.00 _{WB} ^a 1.31 ± 0.2 _{PL} ^c
Oxygen content, ml O ₂ · 100 ml ⁻¹		
R	9.8 ± 0.62 ^a	9.9 ± 0.9 ^a
PBvHb	9.9 ± 0.31 ^a	12.4 ± 0.7 ^{*,a}
SNO-PBvHb	10.1 ± 0.42 ^a	10.77 ± 0.1 ^a
SNO-PBvHb + PBvHb	9.9 ± 0.5 ^a	11.1 ± 0.3 ^{*,a}

Definition of abbreviations: NA, not applicable; PBvHb, polymerized bovine hemoglobin; PL, plasma layer; tHb, total hemoglobin; R, lactated Ringer's solution; SNO, S-nitrosylated; SNO-PBvHb + PBvHb, 1:10 mixture of SNO-PBvHb and PBvHb; WB, whole blood.

Values are means ± SEM.

* $P < 0.05$, compared to baseline.

^{a,b} For the group comparisons, same-letter designations are not significantly different; different-letter designations represent significant differences ($P < 0.05$) among treatments, within time period (baseline, 60, or 120 min).

oxidation and ferric Hb accumulation in circulation. We observed little or no oxidation in plasma HBOCs or the animals' red blood cells. This situation can be contrasted with levels as high as 10–12% ferric Hb only 10 minutes after infusion of HBOCs and sodium nitrite (12).

The present experiment was not designed to determine the exact fate of NO released from SNO-PBvHb. Thus, we cannot state for certain or whether NO delivered from SNO-Hb exerted its effect directly on the vasculature, or if the NO was immediately scavenged from PBvHb, allowing other endogenous pools of NO to become available. Additionally, in contrast to most other studies where HBOC has been infused within the setting of low volume and/or low blood pressure (31–33), it should also be noted that the current model involved adding to vascular volume with HBOCs in a hypoxic, euvoletic animal. In models of hemorrhagic shock, various versions of HBOC, including PBvHb infused for resuscitation, have also demonstrated vasoconstriction and reduced cardiac output (34). It would be important to identify whether a combination therapy of SNO-PBvHb and PBvHb, as used in the current study, would be an effective supportive therapy in a hypovolemic model. It is possible that it would be less effective in that circumstance, because of normal arterial blood PO₂ that could preclude the unloading of NO. Further research is warranted to answer these questions, not only with PBvHb, but also with other HBOCs.

S-Nitrosylation of HBOC and Vascular Pressure

The current study is in agreement with others that have reported systemic and pulmonary vasoconstriction with polymerized HBOC infusion (5, 6, 8, 35). It should be noted that not all polymerized HBOC versions cause vasoconstriction. Tsai and colleagues (16) have demonstrated that a polyethylene glycol-decorated and polymerized HBOC did not cause vasoconstriction, despite NO binding similar to PBvHb and αα-cross-linked HBOC. They speculated that the polyethylene glycol-decorated HBOC had a higher oncotic pressure than the other HBOCs and increased blood volume, thereby, increasing microvascular blood flow. Notably, cardiac index did not fall with polyethylene glycol-decorated and polymerized HBOC treatment. However, the mechanisms remain undetermined, but of interest.

As expected, we noted that SNO-PBvHb had an effect only in animals exposed to acute hypoxia. SNO-PBvHb moderated the rise in mean arterial pressure caused by PBvHb at 60 minutes, but not 120 minutes. This is likely a consequence of diminished NO from SNO-PBvHb, and increased peripheral resistance caused by the PBvHb, along with the fact that cardiac output was maintained with combined therapy. Furthermore, these data suggest that SNO-PBvHb either does not form naturally *in vivo* or, if it does, not in high enough concentrations to offset its own NO-scavenging effects. Thus, for this situation to be an effective NO donor HBOC, PBvHb must be S-nitrosylated to a supraphysiological concentration. Our study did not address potential toxicological effects, an issue that clearly needs to be investigated.

We observed no effect on the mean pulmonary artery pressure from SNO-PBvHb in hypoxic animals. This is contrary to previous reports of SNO Hb infusions in the isolated, perfused rat lung. In these studies SNO-Hb augmented the hypoxic pulmonary vasoconstrictive response (36, 37). Notably, though, those studies were performed with HBOCs that differ markedly from PBvHb. In the current study, PBvHb was infused, whereas, in the isolated lung study (36, 37), acellular human Hb that was not cross-linked was used. It is well known that un-cross-linked Hb tetramers readily and rapidly dissociate when infused into the blood stream (10, 14). As mentioned earlier, observations from others demonstrate that there are differences in the hemodynamic effects between the different biochemical versions of HBOC (10, 11). Our results would extend those observations to suggest that this will hold true for the S-nitrosylation effect of different HBOCs.

In the present study, cardiac index was increased at 60 minutes in hypoxic animals that received only SNO polymerized bovine HBOC treatment. This is in agreement with others that observed improved cardiac function after an SNO Hb infusion. Recently, Asanuma and colleagues (18) reported that human SNO PEGylated Hb infusion improved contractile and metabolic function in a canine ischemic myocardium. Asanuma and colleagues concluded that low PO₂ in the ischemic myocardium allowed for preferential NO release and increased coronary blood flow and cardiac function. However, in the current study, coronary blood flow was not measured as a means to assess a direct cardiac effect of SNO bovine Hb. Likely, the increased cardiac index in our model was from the combination of a systemic and coronary arteriolar vasorelaxation after SNO-PBvHb infusion. We are currently broadening the scope of our studies so that we can learn more of the general applicability of what we have shown with PBvHb.

Conclusions

Data from the current study indicate that S-nitrosylation of PBvHb was effective in restoring hemodynamics and oxygen

delivery during PBvHb infusion in rats subjected to acute hypoxia. These data support the potential use of combined HBOC with SNO-HBOC in the treatment of conditions in which acute hypoxia is present, such as tumor oxygenation, wound healing, hemorrhagic trauma, and sickle cell and hemolytic anemia.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Nemoto M, Mito T, Brinigar W, Fronticelli C, Koehler RC. Salvage of focal cerebral ischemic damage by transfusion of high O₂-affinity recombinant hemoglobin polymers in mouse. *J Appl Physiol* 2006; 100:1688–1691.
- Ortega DP, Davis MR, Sampson JB, Dick E, Kashyap V, Kerby J. Bovine hemoglobin-based oxygen-carrying solution (HBOC-201) improves flap survival in a rat model of epigastric flap failure. *Microsurgery* 2006;26:203–206.
- Isaac G, Yi G-H, Schulman AR, Morrow BT, Cheng Y, Gu A, Zhang G, Oz MC, Burkhoof D, Wang J. A polymerized bovine hemoglobin oxygen carrier preserves regional myocardial function and reduces infarct size after acute myocardial ischemia. *Am J Physiol Heart Circ Physiol* 2006;291:H1126–H1137.
- Bonegio RGB, Fuhro R, Ragno G, Valeri CR, Lieberthal W. A comparison of the acute hemodynamic and delayed effects of 50% exchange transfusion with two different cross-linked hemoglobin based oxygen carrying solutions and pentastarch. *Artif Cells Blood Substit Immobil Biotechnol* 2006;34:145–157.
- Hess JR, MacDonald VW, Brinkley WW. Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. *J Appl Physiol* 1993;74:1769–1778.
- Hess JR, MacDonald VW, Gomez C, Coppes V. Increased vascular resistance with hemoglobin-based oxygen carriers. *Artif Cells Blood Substit Immobil Biotechnol* 1994;22:361–372.
- Tsai AG, Friesenecker B, Cabrales P, Hangai-Hoger N, Intaglietta M. The vascular wall as a regulator of tissue oxygenation. *Curr Opin Nephrol Hypertens* 2006;15:67–71.
- Irwin DC, Foreman B, Morris K, White M, Sullivan T, Jacobs R, Monnet E, Hackett T, Tissotvanpatot MC, Hamilton KL, Gotshall RW. Polymerized bovine hemoglobin decreases oxygen delivery during normoxia and acute hypoxia in the rat. *Am J Physiol Heart Circ Physiol* 2008;295:H1090–H1099.
- Alayash AI, D'Agnillo F, Buehler PW. First-generation blood substitutes: what have we learned? Biochemical and physiological perspectives. *Expert Opin Biol Ther* 2007;7:665–675.
- Buehler PW, Alayash AI. All hemoglobin-based oxygen carriers are not created equally. *Biochim Biophys Acta* 2008;1784:1378–1381.
- Winslow RM. Current status of oxygen carriers ('blood substitutes'): 2006. *Vox Sang* 2006;91:102–110.
- Yu B, Raher MJ, Volpato GP, Bloch KD, Ichinose F, Zapol WM. Inhaled nitric oxide enables artificial blood transfusion without hypertension. *Circulation* 2008;117:1982–1990.
- Cabrales P, Tsai AG, Intaglietta M. Nitric oxide regulation of micro-vascular oxygen exchange during hypoxia and hyperoxia. *J Appl Physiol* 2006;100:1181–1187.
- Moore EE, Johnson JL, Cheng AM, Masuno T, Banerjee A. Insights from the studies of blood substitutes in trauma. *Shock* 2005;24:197–205.
- Alayash AI. Oxygen therapeutics: can we tame haemoglobin? *Nat Rev Drug Discov* 2004;3:152–159.
- Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. *Blood* 2006;108:3603–3610.
- Gotshall RW, Hamilton KL, Foreman B, Tissotvanpatot MC, Irwin DC. Hemoglobin-based oxygen carrier and phosphodiesterase-5 inhibition. *Crit Care Med* 2009;37:1988–1993.
- Asanuma H, Nakai K, Sanada S, Minamino T, Takashima S, Ogita H, Fujita M, Hirata A, Wakeno M, Takahama H, et al. S-nitrosylated and PEGylated hemoglobin, a newly developed artificial oxygen carrier, exerts cardioprotection against ischemic hearts. *J Mol Cell Cardiol* 2007;42:924–930.
- Buehler PW, Alayash AI. Oxygen sensing in the circulation: "cross talk" between red blood cells and the vasculature. *Antiox Redox Signal* 2004;6:1000–1010.
- Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature* 1996;380:221–226.
- Ricardo KFS, Shishido SM, de Oliveira MG, Krieger MH. Characterization of the hypotensive effect of S-nitroso-N-acetylcysteine in normotensive and hypertensive conscious rats. *Nitric Oxide* 2002;7: 57–66.
- Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 1997;276:2034–2037.
- Sonveaux P, Kaz AM, Snyder SA, Richardson RA, Cardenas-Navia LI, Braun RD, Pawloski JR, Tozer GM, Bonaventura J, McMahon TJ, Stamler JS, Dewhirst MW. Oxygen regulation of tumor perfusion by S-nitrosohemoglobin reveals a pressor activity of nitric oxide. *Circ Res* 2005;96:1119–1126.
- Buehler PW, Boykins RA, Jia Y, Norris S, Freedberg DI, Alayash AI. Structural and functional characterization of glutaraldehyde-polymerized bovine hemoglobin and its isolated fractions. *Anal Chem* 2005;77: 3466–3478.
- Boykins R, Buehler PW, Jia Y, Venable R, Alayash AI. O-raffinose crosslinked hemoglobin lacks site-specific chemistry in the central cavity: structural and functional consequences of beta93Cys modification. *Proteins* 2005;59:840–855.
- Buehler PW, D'Agnillo F, Hoffman V, Alayash AI. Effects of endogenous ascorbate on oxidation, oxygenation and toxicokinetics of cell-free modified hemoglobin after exchange transfusion in rat and guinea pig. *J Pharmacol Exp Ther* 2007;323:49–60.
- Herold S, Rock G. Reactions of deoxy-, oxy-, and methemoglobin with nitrogen monoxide: mechanistic studies of the S-nitrosothiol formation under different mixing conditions. *J Biol Chem* 2003;278:6623–6634.
- Alayash AI, Fratantoni JC, Bonaventura C, Bonaventura J, Cashon RE. Nitric oxide binding to human ferrihemoglobins cross-linked between either [alpha] or [beta] subunits. *Arch Biochem Biophys* 1993;303: 332–338.
- Hayashi A, Suzuki M, Shin M. An enzymatic reduction system for myoglobin and methemoglobin, and its application to functional studies of oxygen carriers. *Biochim Biophys Acta* 1973;310:309–316.
- Jia Y, Ramasamy S, Wood F, Alayash AI, Rifkind J. Cross-linking with O-raffinose lowers oxygen affinity and stabilizes haemoglobin in a non-cooperative T-state conformation. *Biochem J* 2004;384:367–375.
- Manly G, Hemphill J, Morabito D, Erickson V, Holcroft J, Derugin N, Knudson N. Small-volume resuscitation with the hemoglobin substitute HBOC-201: effect on brain tissue oxygenation. *Adv Exp Med Biol* 2003;530:311–317.
- Patel M, Feinstein A, Saenz A, Majetschak M, Proctor K. Prehospital HBOC-201 after traumatic brain injury and hemorrhagic shock in swine. *J Trauma* 2006;61:46–56.
- Rivera-Chvez F, Huerta S, Brown R, York G, Minei J. Resuscitation from hemorrhagic shock comparing standard hemoglobin-based carrier (HBOC) 201 versus 7.5% hypertonic HBOC 201. *J Trauma* 2007;63:1113–1119.
- Johnson T, Arnaud F, Dong F, Philbin N, Rice J, Asher L, Arrisueno M, Warndorf M, Gurney J, McGwin G, et al. Bovine polymerized hemoglobin (hemoglobin-based oxygen carrier-201) resuscitation in three swine models of hemorrhagic shock with militarily relevant delayed evacuation—effects on histopathology and organ function. *Crit Care Med* 2006;34:1464–1474.
- Olson JS, Foley EW, Rogge C, Tsai A-L, Doyle MP, Lemon DD. NO scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic Biol Med* 2004;36:685–697.
- Deem S, Kim J-U, Manjula BN, Acharya AS, Kerr ME, Patel RP, Gladwin MT, Swenson ER. Effects of S-nitrosation and cross-linking of hemoglobin on hypoxic pulmonary vasoconstriction in isolated rat lungs. *Circ Res* 2002;91:626–632.
- Deem S, Kim SS, Min J-H, Eveland R, Moulding J, Martyr S, Wang X, Swenson ER, Gladwin MT. Pulmonary vascular effects of red blood cells containing S-nitrosated hemoglobin. *Am J Physiol Heart Circ Physiol* 2004;287:H2561–H2568.