Polymerized Bovine Hemoglobin Infusion Does Not Induce Lung Damage in a Rat Model

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Abstract: Establishing a hemoglobin-based oxygen carrier (HBOC) has potential to improve transfusion medicine and provide a novel intervention to minimize tissue damage during hypoxic or ischemic insults. However, *in vitro* studies suggest that HBOCs have toxic effects on endothelial cell barrier function. The purpose of this study was to determine if the HBOC polymerized bovine hemoglobin (PBvHb) alters pulmonary endothelial barrier function in rats during normoxia or hypoxia. Conscious male Sprague-Dawley rats were treated with lactated Ringer's (LR) or PBvHb (3 ml of 1.3 mg PBvHb/ml in lactated Ringer's) and exposed to 4hr of normoxia (F_iO_2=21%) or hypoxia (F_iO_2=10%). Evans blue dye (EBD) extravasation (estimate of pulmonary vascular leak), pulmonary artery (Pa) pressure, inflammatory mediators, vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (sFlt-1), and hypoxia inducible factor-1 (HIF-1α) were measured in the lung and/or plasma. As expected with hypoxia, Pa pressure increased ($p<0.05$). PBvHb also resulted in a significant increase in Pa pressure independent of hypoxia. While a significant main effect of PBvHb on EBD extravasation was observed, no differences in inflammatory cytokines or pulmonary white blood cells existed among groups. No pulmonary edema was present upon assessment of wet:dry lung weights or histological examination. Hypoxia did not influence plasma VEGF, but resulted in a small but significant increase in sFlt-1. Irrespective of hypoxia, PBvHb was associated with increased unbound VEGF and its soluble receptor sFlt-1. However, no differences in lung VEGF or HIF-1α were observed. We conclude that in this model, acute administration of PBvHb does not have severe overall effects on pulmonary endothelial barrier function or inflammation.

Keywords: Hypoxia, hemoglobin, pulmonary edema, inflammation.

INTRODUCTION

In the advancing field of transfusion medicine, the potential role of artificial blood and oxygen carriers is still unrealized. With the establishment of universal hemoglobin based oxygen carriers (HBOC), many obstacles involved with allogenic blood transfusions, including disease transmission, limited blood supply, short shelf life, and donor-recipient complications, could be avoided. Not only could use of artificial oxygen carriers reform transfusion therapy, it could improve tissue function in individuals suffering from various maladies affecting oxygen delivery such as vascular disease resulting in cardiac or cerebral ischemia [1-4]. Additionally, artificial oxygen carriers could provide a novel intervention to prevent tissue damage during extreme environmental exposure including hypobaric hypoxia. Thus, it is of clinical importance that a safe and effective blood substitute be developed.

Negative secondary effects that have been associated with HBOCs used in clinical settings include increased blood pressure and decreased cardiac output, likely from nitric oxide (NO) scavenging [5], and toxic effects on endothelial barrier function that may stem from oxidation-reduction reactions [6-9]. These negative secondary effects have, unfortunately, essentially brought human research into the safe and effective use of HBOCs to a standstill. However, previous research from our laboratory suggests that HBOCs can indeed increase oxygen content [10] in both normoxic and hypoxic conscious rat models. Importantly, we have recently shown that the negative hemodynamic effects [11] (hypertension, decreased cardiac output) associated with HBOC administration can be moderated or even overcome by concurrent use of phosphodiesterase-5 inhibitors or via nitrosylation of the hemoglobin [11,12]. Therefore, we believe that our conscious rat model employing normoxia and hypoxia may represent a viable model for studying the utility of HBOC administration in a variety of clinically relevant conditions associated with inadequate oxygen supply/delivery, unless the secondary toxic effects on endothelial barrier function remain problematic.

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As mentioned previously, it is thought that secondary toxic effects of HBOCs on endothelial barrier function may stem from oxidation-reduction reactions [6-9]. Specifically, the autoxidation of oxyhemoglobin (Fe^{2+}) leads to ferrihemoglobin (Fe^{3+}) and a superoxide anion, which dismutates into hydrogen peroxide. It has been suggested that the reactive oxygen species formed during the autoxidation of HBOC can lead to activation of transcription factors NF-κB [9], hypoxia inducible factor-1α (HIF-1α) [13], stress fiber formation [7], and induction of apoptotic cell death [8], all of which can subsequently alter endothelium barrier function properties. Investigations of the effects of HBOC on the vascular endothelium have primarily been conducted in cell culture models in which conditions are tightly controlled. For example, previous investigations have the specific endothelial cell type (i.e., aorta, pulmonary, etc.), molar concentrations of Fe^{2+} and Fe^{3+}, time of incubation, and additional stressors such as hypoxia or hydrogen peroxide rigorously defined [7-9,13]. In addition, HBOC that are known to readily auto-oxidize [14] are often used. However, *in vitro* studies of HBOC-induced effects cannot take into account the extra- and intracellular enzymes that are produced by endogenous antioxidant defense systems (glutathione, superoxide dismutase, etc.) and/or other factors in the blood and tissue, which may attenuate or exacerbate the effects observed in cultured cells. Further, many investigations of HBOC physiological effects have employed hemorrhagic, hypovolemic and exchange-transfusion models [15-17]. To our knowledge, no investigation to date has focused on pulmonary vascular endothelial barrier function after HBOC-infusion in a euvolemic animal model during both normoxia and hypoxia.

Our primary goal for this study was to examine the effect of polymerized bovine hemoglobin (PBvHb) administration, the HBOC employed in our previous investigations of HBOCs and hemodynamics [10-12], on the pulmonary vasculature of conscious euvolemic animals, in both ambient and hypoxic environments. We chose to investigate PBvHb effects in hypoxic conditions as it is well-established that hypoxia imposes an increase in pulmonary artery pressure that can, in turn impose a stress capable of increasing vascular permeability. Therefore, we were interested in not only observing the effects of PBvHb on the pulmonary vascular barrier in normoxic, euvolemic conditions, but also in a setting in which pulmonary vascular barrier function is already at risk. Based on previously published findings [6-9,18,19], we hypothesized that PBvHb infusion would induce an inflammatory response and increase vascular endothelial growth factor (VEGF), a known permeability factor. Therefore, studies were designed to measure pulmonary vascular leak via extravasation of Evans Blue dye (EBD), plasma and lung inflammatory markers, and VEGF concentrations. Secondarily, the experiments were designed to determine the PBvHb effect on the upstream transcription factor of VEGF, HIF-1α, and, finally, to determine if changes in circulating VEGF are reflections of changes in the soluble VEGF receptor, fms-like tyrosine kinase-1 (sFlt-1).

MATERIALS AND METHODOLOGY

Ethical Approval

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees at Colorado State University and/or University of Colorado Denver Health Sciences Center. Protocols were consistent with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Animals

Male Sprague-Dawley rats (280-350g and 10-12 weeks of age) were obtained from Charles River (Wilmington, MA) and housed at either Colorado State University Laboratory Animal Research facility or the University of Colorado Denver Health Sciences Center for Laboratory Animal Care. All animals were allowed to acclimate to ambient conditions for seven days prior to any experimentation. Animals were allowed *ad libitum* access to food and water and kept on a 12 hr day-night cycle. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees at Colorado State University and/or University of Colorado Denver Health Sciences Center.

Experimental Design

Rats were assigned to one of four groups: **(1)** normoxic conditions (F_iO_2 =21% O_2) treated with lactated Ringer's solution (Henery Schein, Melville NY) (NX-LR); **(2)** normoxic treated with polymerized bovine hemoglobin (Biopure™, Cambridge, MA) (NX-PBvHb); **(3)** hypoxic conditions (via exposure to hypoxic gas or hypobaric hypoxic equivalent of F_iO_2=10%) treated with lactated Ringer's solution (HX-LR);or **(4)** hypoxic treated with polymerized bovine hemoglobin infused (HX-PBvHb). Two sets of experiments (n=6-10/group for each set of experiments) were carried out due to inability to utilize tissues from the rats assessed for vascular leak, for evaluation of the other dependent variables. The first experiments examined the effect of acute PBvHb treatment on pulmonary vascular leak and pulmonary artery pressure in rats exposed to either normoxia or hypoxia for four hours. This duration of hypoxia was based on previous experiments identifying clearance of PBvHb in our rat model [10]. The second study focused on examining the effects of PBvHb infusion on inflammation and circulating inflammatory mediators and on permeability factors including vascular endothelial growth factor (VEGF) and fms-like tyrosine kinase-1 (sFlt-1), known to induce pulmonary vascular leak [17-19].

Effects of PBvHB on Pulmonary Vascular Leak and Pulmonary Artery Pressure

Instrumentation

As previously described [10-12], indwelling catheters were placed into the jugular vein and pulmonary artery 48 h prior to any treatments. Briefly, rats were anesthetized with a mixture of ketamine: xylazine (75 mg/kg: 6 mg/kg I.P.) and under aseptic conditions a PV-1 (0.28 mm ID, BD Diagnostics, Franklin Lakes, NJ) catheter 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, a PE-50 (0.58 mm ID, BD Diagnostics) catheter was placed in the superior vena cava via the right jugular vein for treatment infusions. 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 h to recover prior to any treatments. Animals were monitored for signs of infection, diarrhea, or distress and were excluded from the study if any such signs were apparent (n=1 excluded due to apparent infection).

Treatments and Pulmonary Artery Pressures

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. Animals were exposed to normoxic and hypoxic conditions by flushing the chamber with appropriate gases (room air or 10% oxygen with balance nitrogen). Once breathing hypoxic gas, hypoxic animals were not re-exposed to room air. Baseline pulmonary artery pressures of normoxic and hypoxic animals were collected prior to lactated Ringer's or PBvHb infusion. Animals then underwent a 30 min (1ml/10 min) venous infusion of either lactated Ringer's or PBvHb (3 ml of 1.3 mg PBvHb/ml in lactated Ringer's). Immediately after the infusion, the groups were split, with half remaining in room air and half being subjected to hypoxia. Throughout exposure to hypoxia, rats were monitored for any signs of distress but were otherwise left undisturbed. Four hours after infusion and exposure to hypoxia or normoxia, pulmonary artery pressures were measured.

Pulmonary Vascular Protein Extravasation

As previously described, pulmonary vascular leak was assessed by lung protein extravasation determined by the Evans blue dye (EBD) method [20]. Briefly, EBD (20 mg/kg) was injected via the venous catheter following lactated Ringer's or PBvHb treatments and exposure to hypoxia or normoxia, but 15 minutes prior to euthanasia with an overdose of sodium pentobarbital (100 mg/kg) via the jugular catheter. Animals were then transcardially perfused with phosphate buffered saline. Each animal's left caudal lobe was removed. After weighing, EBD was extracted from by formamide (100%) incubation (18 h, 37°C). The other half of the lung was immediately weighed, oven dried (65°C, 48 h), and weighed again. Wet weight:dry weight ratio in the left lung was used to estimate the dry weight of the other half of the lung. The extracted dye was quantified in a spectro-photometer by measuring the absorbance at 600 nm against standards of EBD dissolved in formamide. EBD extravasation is expressed as nanograms of EBD per milligram of dried tissue.

Effects of PBvHB on Inflammation, VEGF, and sFlt-1

Immediately following venous infusion of either lactated Ringer's or PBvHb (3 ml, 1.3 mg PBvHb/ml, 1.3 g PBvHb /kg body weight), the groups were split with half remaining in room air and half being subjected to hypoxia. Throughout exposure to hypoxia, rats were monitored for any signs of distress but were otherwise left undisturbed and were not re-exposed to room air. Four hours following infusion and exposure to hypoxic (or normoxic) conditions, animals were anesthetized with a mixture of ketamine: Rompon (xylazine) (75 mg/kg: 6 mg/kg I.P.) until a surgical plane of anesthesia was reached. Blood was withdrawn via cardiac puncture and collected into EDTA Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) and placed on ice until centrifuged at 4°C for 15 minutes at 2800 rpm. Plasma was withdrawn and

stored at -80°C until analyzed. A thoracotomy was performed, the left main bronchus ligated, and the left lung was removed and immediately frozen in liquid nitrogen. The right lung was perfused via the trachea with 10% formalin for histological analyses.

Histology

Lungs from 3 rats per treatment group were fixed with 10% buffered formalin by airway inflation at constant pressure (~25 cm of H_2O pressure), paraffin embedded and sectioned to 4 μm and stained with hematoxylin and eosin. The slides were examined in a blinded fashion for neutrophils / macrophages and/or red blood cells in the alveolar spaces, thickened alveolar septa, hemorrhage or blebs, and interstitial pneumonia.

Cytokine Microarray Analyses

Plasma samples were analyzed for inflammatory markers using the Meso Scale Discovery Sector Imager 6000 (Meso Scale Discovery, Gaithersburg, MD). The Meso Scale Imager uses electrochemiluminescence to quantify serum protein levels. Interleukins (IL)- 1,4,5, and 13; TNFα, interferon-γ (IFN-γ); and growth related oncogene (GRO-kc) were determined from a commercially available Meso Scale rat inflammatory marker plate following manufacturer's instructions. Samples were run in triplicate and quantified against standards provided by the manufacturer.

Plasma VEGF and sFlt-1 Analyses

Concentrations for vascular endothelial growth factor (VEGF) and the VEGF receptor fms-like tyrosine kinase-1 (sFlt-1) were analyzed from plasma samples by commercially available ELISA kits (R&D Systems, Minneapolis, MN; catalog # DY564 and MVR100, respectively). The ELISA kit specific for VEGF detected only free VEGF, whereas the ELISA kit for sFlt-1 detected total sFlt-1, bound and unbound to VEGF.

Lung HIF and VEGF Analyses:

HIF1-α DNA Binding

Nuclear extracts from lung tissue were prepared using the Panomics nuclear extraction kit following manufacturer's instructions (Panomics, Fremont, CA). Briefly, frozen tissues were homogenized by mortar and pestle, and lysis buffers provided by Panomics were used to separate cytosolic and nuclear fractions. HIF1-α DNA binding activity in nuclear extracts was detected using the commercially available enzyme-linked immunoassay (ELISA) kit (Panomics). In this assay HIF1-α binds to oligodeoxynucleotides with a HIF specific binding site and this binding is detectable at a wavelength of 450 nm. Samples were analyzed in 96 well plates on a spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA).

Western Blot VEGF Analyses

The cytosolic fraction remaining from the Panomics lung tissue nuclear extraction was used to identify VEGF via standard immunoblotting techniques. Protein concentrations were determined with a bicinchonic acid protein assay (Pierce Biotechnology, Rockford, IL). Samples were stored at -80°C until analyzed and 35 μg of protein was separated by standard SDS-polyacrylamide gel electrophoresis using 4-

15% precast gradient gels (Bio-Rad Laboratories, Hercules, CA) followed by transfer to pure nitrocellulose membranes. Following transfer, blots were blocked overnight at 4°C with 5% bovine serum albumin in Tris buffered saline with 0.1% Tween 20 followed by room temperature incubations in primary antibodies for VEGF (VEGF A-20 – rabbit polyclonal IgG, sc-152; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 for 90 min and secondary antibodies conjugated to horseradish peroxidase (HRP) (stabilized goat anti-rabbit HRP-conjugated, #1858415; Pierce Biotechnology) at a dilution of 1:5000 for 60 min. To account for any differences in total protein loading, β-actin was detected by incubation in primary antibodies against β-actin conjugated to HRP (Actin C-2 HRP, sc-8432; Santa Cruz Biotechnology) at a dilution of 1:1000 for 60 min. Bands were detected via chemiluminescence (West Dura, Pierce Biotechnology) with subsequent imaging (UVP BioSpectrum, Upland, CA) and densitometric analyses (UVP Visionworks). Results were normalized to β-actin and expressed relative to control (NX-LR) values.

Statistical Analyses

Significance level was established *a priori* at p < 0.05 with 95% confidence interval. All data were analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL) using a general linear model analysis of variance and expressed as means ± SEM unless otherwise specified. When appropriate, a Tukey HSD was employed *post hoc* to compare between treatment means. Data from the plasma concentrations for GRO-kc, IL-5, VEGF, sFlt-1 and from wet weight-to-dry weight ratios were normalized by a log transformation. Data sets (IFN-γ, IL-1β, IL-4, IL-13, and TNFα) that did not satisfy normal distribution criteria and were analyzed using a non-parametric analysis of variance (Kruskal-Wallis Test).

RESULTS

Pulmonary Effects

Pulmonary Artery Pressure

As expected, acute hypoxia exposure increased pulmonary artery pressure. Independent of the effects of hypoxia, PBvHb treatment also resulted in a small but significant increase in pulmonary artery pressure (Fig. **1**, inset), yet the effects of hypoxia and PBvHb were not additive (Fig. **1**).

Evans Blue Dye Protein Extravasation

Compared to normoxia, EBD extravasation was unchanged after 4 hr of acute hypoxia (main effects data not shown). However, compared to lactated Ringer (LR) treated animals, EBD extravasation was increased in PBvHb treated animals (Fig. **2**, inset). Lung wet weight:dry weight ratios, however, did not differ between LR and PBvHb groups (data not shown, p = 0.84). When comparing EBD extravasation across all groups, no significant differences were observed (Fig. **2**). Pearson's correlation revealed that there was no relationship between pulmonary artery pressure and EBD extravasation (data not shown).

Inflammation, VEGF, and sFlt-1

Plasma Inflammatory Markers

PBvHb did not affect inflammatory markers, GR0-kc, IFN-γ, IL-1β, 4, 5, or TNF-α during normoxia or hypoxia (Table **1**). There was no histological evidence of alveolar
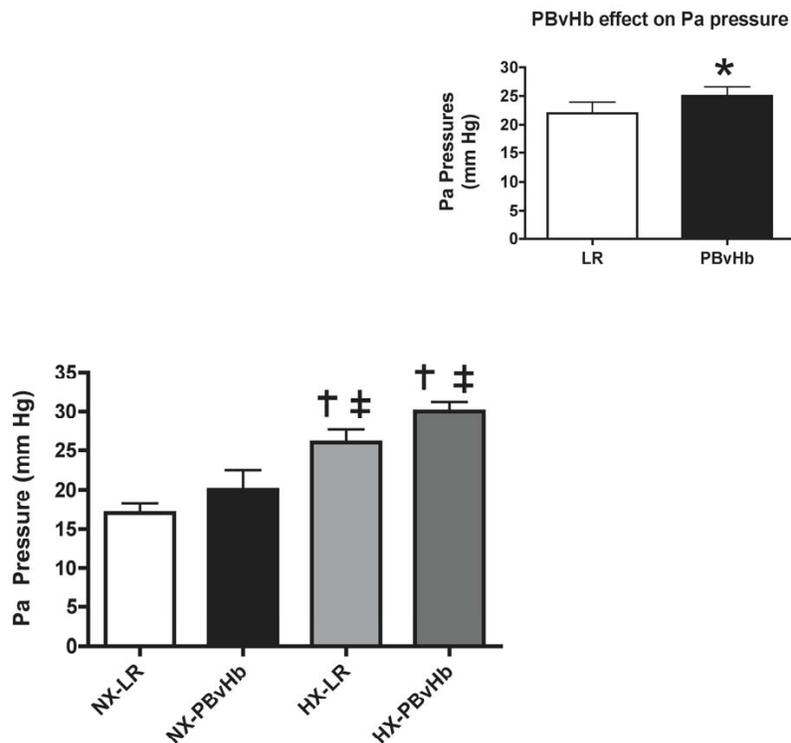
Fig. (1). Pulmonary artery (Pa) pressures across all experimental groups (mean±SEM). Inset: PBvHb treatment effect on pulmonary artery pressure. LR=lactated Ringer's, PBvHb=polymerized bovine cell free hemoglobin, NX=normoxia, HX=hypoxia; * significantly greater than LR (p < 0.05); † significantly greater than NX-LR (p < 0.001); ‡ significantly greater than NX- PBvHb (p < 0.05); (n=6-9/group).
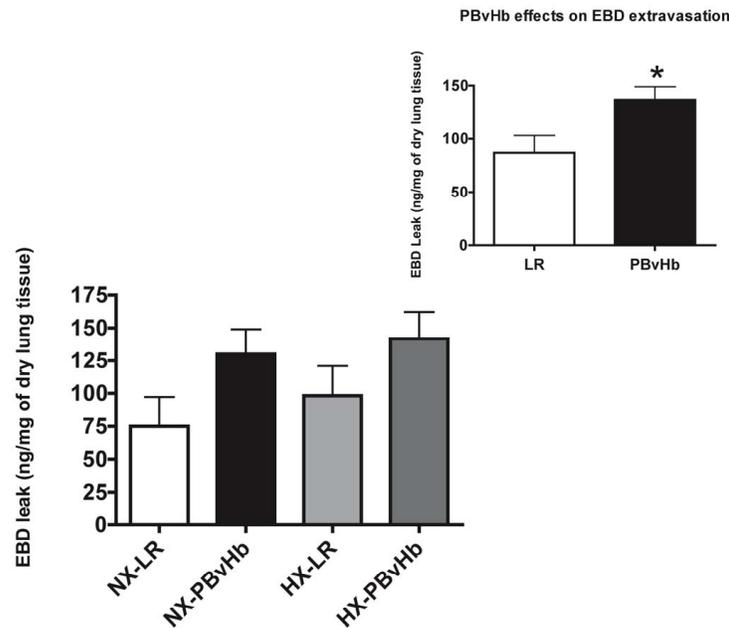
Fig. (2). Pulmonary vascular leak as assessed by Evan's blue dye (EBD) extravasation (mean±SEM). Inset: PBvHb treatment effect on pulmonary vascular leak. LR=lactated Ringer's, PBvHb=polymerized bovine cell free hemoglobin, NX=normoxia, HX=hypoxia; * significantly greater than LR ($p < 0.05$); (n=6-9/group)

Table 1.   **Plasma inflammatory cytokines (pg/ml) 4 hours following treatment**

	IFN-γ	IL-1β	IL-4	IL-5*	IL-13	GRO-kc*	TNFα
NX-LR (n=6)	ND	79.9±23.8	0.1±0.1	1.1±0.3	10.6±9.1†	2.5±0.2	2.9±2.7
NX-PBvHb (n=6)	0.7±0.4	85.1±28.3	0.07±0.07	1.2±0.3	ND	2.7±0.3	17.3±13.5
HX-LR (n=6)	ND	41.7±25.2	0.05±0.05	0.3±0.3	ND	2.2±0.2	ND
HX-PBvHb (n=5)	ND	141.6±85.6	0.04±0.03	1.4±0.3	ND	2.7±0.3	12.1±9.6

*Indicates data were log transformed to meet the assumptions of equal variance prior to analysis. † indicates different from all other groups for that cytokine. ND = not detected.

WBC, thickened septa, hemorrhage or blebs and interstitial pneumonia. Therefore, for quantification purposes, summations of alveolar neutrophils and macrophages were used for comparison between groups. Histological analyses showed no increases in lung macrophages or neutrophils in PBvHb infused animals, suggesting no local inflammatory response to PBvHb in the lung (Fig. **3**).

Plasma VEGF and sFlt-1

VEGF, a protein known to induce pulmonary vascular permeability, was determined in the plasma of each animal. Plasma VEGF concentration was significantly increased in PBvHb treated animals compared to untreated cohorts (Fig. **4**, inset). The form of plasma VEGF measured was that not bound to its inhibitory soluble receptor, fms-like tyrosine kinase-1 (sFlt-1). Therefore, the concentration of sFlt-1 was also determined. We observed greater ($p<0.05$) plasma sFlt-1 concentrations in PBvHb infused animals compared to LR infused animals (Fig. **4**, inset). However, a Pearson correlation

analyses showed no relationship between VEGF and sFlt-1 concentrations (data not shown).

Lung HIF-1α and VEGF

To investigate whether the lung contributed to increased plasma VEGF concentrations, nuclear and cytosolic protein in the lung was analyzed for HIF-1α and VEGF, respectively. There was no effect of hypoxia or PBvHb on lung or VEGF (Fig. **5**) HIF-1α DNA binding (Fig. **6**).

DISCUSSION

Principle Findings

The goal of this study was to determine if infusion of PBvHb, in conscious, euvolemic animals, altered pulmonary vascular endothelial barrier function four hours post infusion in normoxic and hypoxic environments. Hypoxic models were chosen as a means of imposing a clinically and environmentally relevant stress for which artificial oxygen
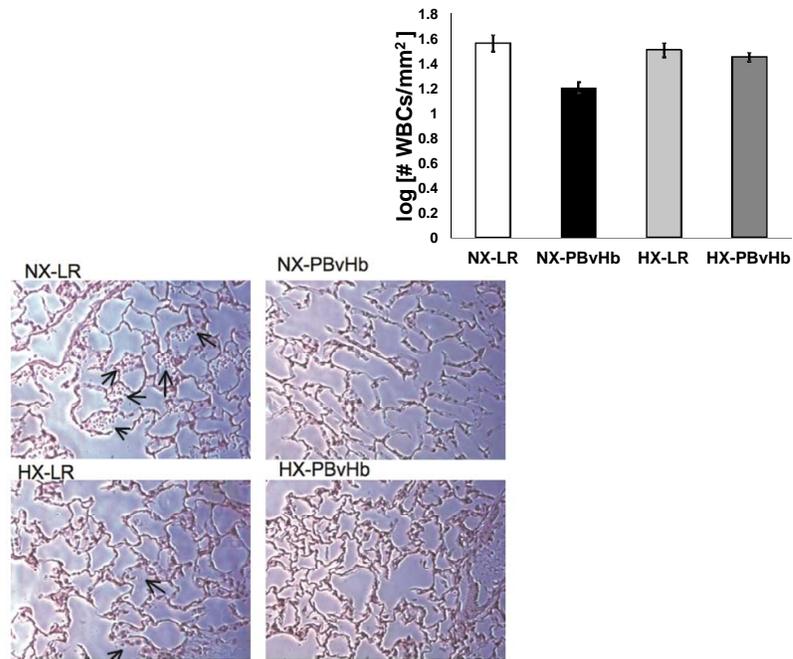
Fig. (3). Inflammatory response to PBvHb. Representative images from hematoxylin and eosin staining in paraffin embedded lung tissue. Arrows indicate areas with observe WBC infiltration. Inset: Quantification of intra-alveolar white blood cells (WBC) per mm^2 observed in fixed lung sections (mean of 6 fields/section/sample±SEM; n=3/group;). These data were log transformed to meet the assumption of equal variance. LR=lactated Ringer's, PBvHb=polymerized bovine cell free hemoglobin, NX=normoxia, HX=hypoxia.
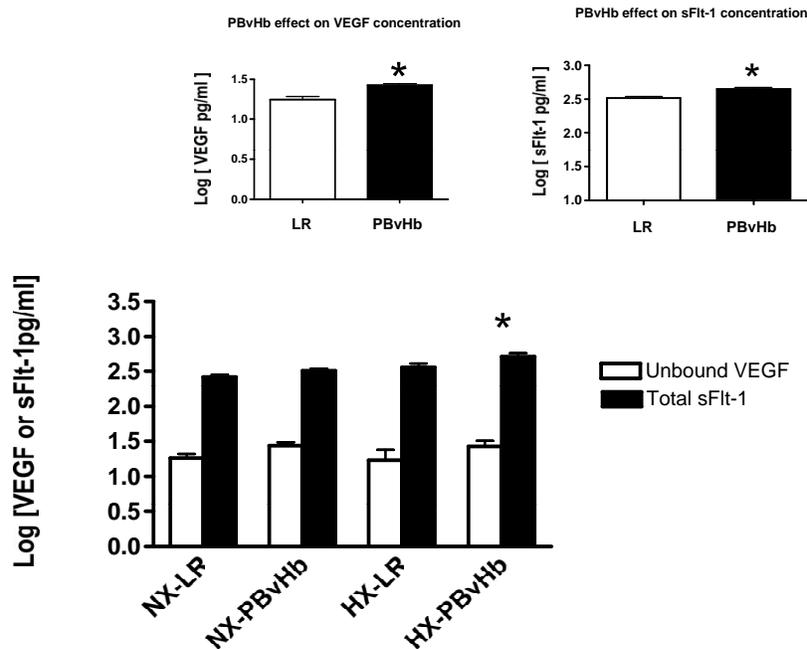
Fig. (4). Comparison of plasma concentrations of vascular endothelial growth factor (VEGF) and fms-like tyrosine kinase 1 (sFlt-1) across experimental groups (mean±SEM). Insets: Effect of treatment with PBvHb on unbound plasma VEGF and total plasma sFlt-1 concentrations. These data were log transformed to meet the assumption of equal variance. * significantly greater than LR ($p < 0.05$). LR=lactated Ringer's, PBvHb=polymerized bovine cell free hemoglobin, NX=normoxia, HX=hypoxia. (n=6-9/group)

carriers could provide an effective intervention to prevent tissue damage and improve oxygen delivery. The data revealed that although PBvHb elicited a small increase in EBD extravasation, no frank pulmonary edema was evident and no lung or systemic inflammation was detectable in any group. An increase in plasma VEGF concentration, accompanied by an increase in soluble inhibitory receptor

sFlt-1, was observed with PBvHb treatment during normoxia and hypoxia.

PBvHb and Pulmonary Vascular Leak

The novel data presented in the current study are contradictory to *in vitro* reports of cell free hemoglobin impairing pulmonary endothelial barrier function.
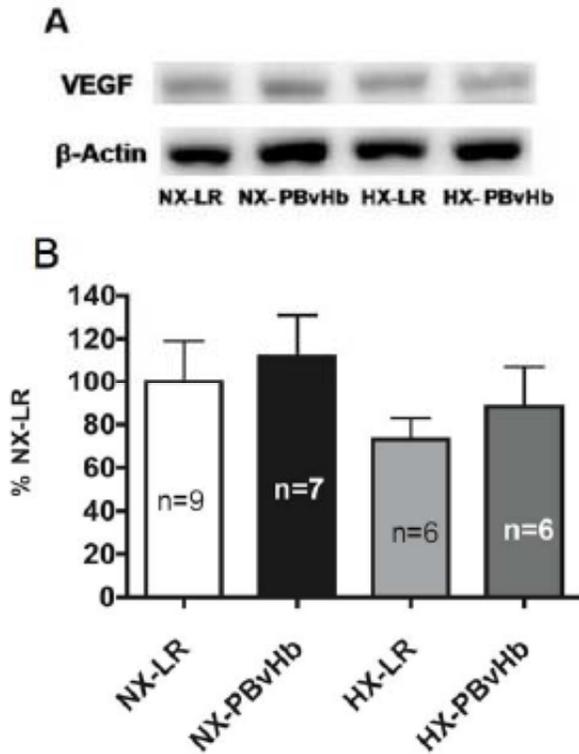
Fig. (5). Western blot analyses of lung VEGF (mean±SEM). (A) Representative images from western blot analyses; (B) Average densitometric analysis of VEGF western blots (n=6-9/group); normalized to β-actin and expressed as percent of mean NX-LR. LR=lactated Ringer's, PBvHb=polymerized bovine cell free hemoglobin, NX=normoxia, HX=hypoxia.

Endothelial cell culture models have been used previously to demonstrate that treatment with cell free or cross linked hemoglobin can promote increased permeability and stress fiber formation [7], increased apoptotic cell death [13], and activation of the transcription factors NF-κB [9] and HIF-1α [13], all of which are known to increase expression of permeability factors. It is generally believed that the effects of cell free hemoglobin on the vascular endothelium are due, at least in part, to reactive oxygen species formation. Oxidant formation associated with cell free hemoglobin treatment can occur as a result of the spontaneous autoxidation process of oxyhemoglobin ($HbFe^{2+}$) to ferrihemoglobin ($HbFe^{3+}$). However, it has been suggested that PBvHb is not as easily oxidized to met hemoglobin compared to other types of cell free hemoglobin such as DBBF (bis (3,5 dibromosalicy) fumarate) often used in other *in vitro* studies [13], and thus is less toxic on endothelial cells. We previously reported that four hours following PBvHb infusion, the metHb concentration in the plasma layer increased from 0 to 12% and 2.6% in normoxic and hypoxic conditions, respectively [10], suggesting that PBvHb-induced less reactive oxygen species formation during hypoxia. In the current study, an overall PBvHb treatment effect was associated with a small but significant increase in EBD extravasation. However, the mechanism(s) associated with this observation is unclear. Additionally, because we did not observe signs of pulmonary edema, we interpret the increase in pulmonary vascular leak that was

associated with PBvHb infusion as insufficient for overwhelming the fluid clearance mechanisms and, therefore, incapable of inducing alveolar flooding. No significant relationship between pulmonary artery pressure and EBD extravasation was found. Taken together, no signs of pulmonary edema, inflammation, or inflammatory cell infiltration suggested that, in this model, vascular toxicities typically attributed to cell free hemoglobin were not clinically relevant. Despite what appears to be a clinically insignificant finding, it seems prudent to continue to exercise caution in monitoring pulmonary health if using cell free hemoglobin for clinical applications.

PBvHb, Inflammation, VEGF, and sFlt-1

A paucity of published research exists investigating the potential effects of cell free hemoglobin on the inflammatory response. It was previously reported that cultured endothelial cells treated with cell free hemoglobin had greater induction of NF-κB, an important transcription factor for inflammatory cytokines (TNF-α, IL-1, 6, and 8) thought to increase vascular endothelial permeability [9]. However, we were unable to detect any increase in plasma inflammatory cytokines or alveolar neutrophils / macrophages 4 h following PBvHb infusion. Importantly, these data suggest that the small increase in EBD extravasation observed with PBvHb was not associated with dectable inflammatory mediators.

Vascular endothelial growth factor is a known permeability agent and, during acute hypoxia, has been associated with pulmonary vascular permeability [21-25]. VEGF protein concentrations are regulated through the transcription factor HIF-1 during hypoxia [26]. During hypoxia, cytosolic HIF-1α is stabilized by inhibition of oxygen dependent enzymatic hydroxylation of proline residues [3,4,7]. HIF-1α subsequently translocates to the nucleus where it binds the constitutively expressed β-subunit
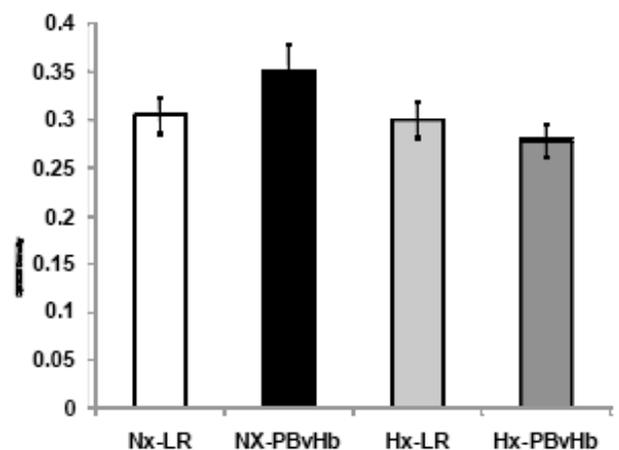
Fig. (6). DNA binding analyses of lung HIF1-α (mean optical density±SEM). HIF1-α DNA binding activity in nuclear extracts was detected using the commercially available enzyme-linked immunoassay (ELISA) kit (Panomics). HIF1-α binds to oligodeoxynucleotides with a HIF specific binding site and this binding is detectable spectrophotometrically at a wavelength of 450 nm. No significant differences (p>0.05) were detectable.

(HIF1-β), forming HIF-1. In addition to hypoxia, it has been suggested that HIF-1α stabilization can occur can occur via reactive oxygen species generation [27]. Recently, Yeh and Alayash [13] reported that HIF-1α expression was increased in bovine aortic endothelial cells incubated with the chemically modified hemoglobin DBBF (bis (3,5 dibromosalicy) fumarate). Further, their data showed that HIF-1α activation corresponded linearly with the decay of $HbFe^{2+}$ and accumulation of the ferric form ($HbFe^{3+}$). In the current study, four hours after PBvHb infusion, there was no difference between groups in either lung nuclear HIF-1α or cytosolic VEGF concentration. Though not assessed directly in the experiments reported here, previous experiments in our laboratory suggest that this may be a reflection of a small percentage of PBvHb that decayed to Fe^{3+} by four hours post treatment [10]. Indeed, both hypoxia and reactive species [28] can increase VEGF and sFlt-1 via activation of HIF-1α [28,29]. Our finding of greater unbound VEGF in presence of greater total (bound and unbound) sFlt-1 with PBvHb infusion during hypoxia, suggests that there was an increase in circulating VEGF rather than a decrease in VEGF soluble receptor binding.

CONCLUSIONS

In conclusion, in euvolemic rats exposed to either normoxia or hypoxia and treated with PBvHb, a small increase in EBD extravasation (an estimate of vascular leak) was observed. However, this occurred in the absence of a detectable systemic or local inflammatory response and in the absence of pulmonary edema. Modest increases in plasma VEGF and sFlt-1 concentrations were observed with no increase in lung HIF activation or VEGF expression. While it is important to consider species differences with respect to susceptibility to vascular leak and other factors including endogenous antioxidants when evaluating the potential usefulness of cell free hemoglobin, the current findings suggest that in a euvolemic rodent model under both normoxic and hypoxic conditions, there is an absence of an acute inflammatory response or lung damage following PBvHb treatment.

ACKNOWLEDGEMENTS

ABBREVIATIONS

EBD	=	Evans blue dye
Fe^{2+} and Fe^{3}	=	Ferrous and ferric iron
F_iO_2	=	Fraction of inspired oxygen
GRO-kc	=	Growth related oncogene; CXCL1
HBOC	=	Hemoglobin-based oxygen carrier
HIF-1α	=	Hypoxia inducible factor-1alpha
HX-LR	=	Hypoxic conditions treated with lactated Ringer's solution
HX-PbvHb	=	Hypoxic treated with polymerized bovine hemoglobin infused
IFN-γ	=	Interferon-gamma
IL	=	Interleukin
LR	=	Lactated Ringer's
NF-κB	=	Nuclear factor kappa B
NO	=	Nitric oxide
NX-LR	=	Normoxic conditions treated with lactated Ringer's solution
NX-PbvHb	=	Normoxic treated with polymerized bovine hemoglobin
Pa	=	Pulmonary artery pressure
PbvHb	=	Polymerized bovine hemoglobin
sFlt-1	=	Fms-like tyrosine kinase-1
TNFα	=	Tumor necrosis factor alpha
VEGF	=	Vascular endothelial growth factor

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