Rate of NO scavenging alters effects of recombinant hemoglobin solutions on pulmonary vasoreactivity

THOMAS C. RESTA,1 BENJIMEN R. WALKER,1 MARK R. EICHINGER,2 AND MICHAEL P. DOYLE3
1Departments of Cell Biology and Physiology, and 2Pediatrics, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131-5218; and 3Baxter Healthcare Corporation, Boulder, Colorado 80301

Received 4 March 2002; accepted in final form 24 June 2002

Resta, Thomas C., Benjimen R. Walker, Mark R. Eichinger, and Michael P. Doyle. Rate of NO scavenging alters effects of recombinant hemoglobin solutions on pulmonary vasoreactivity. J Appl Physiol 93: 1327–1336, 2002.—Many hemoglobin-based oxygen carriers (HBOCs) produce systemic and pulmonary hypertension and may increase microvascular permeability as a consequence of nitric oxide (NO) scavenging. In this study, we examined the effects of two recombinant human hemoglobin solutions, rHb1.1 and rHb2.0 for injection (rHb2.0), with different rates of NO scavenging on vasoconstrictor reactivity and vascular permeability in isolated, saline-perfused rat lungs. We hypothesized that rHb1.1, a first-generation HBOC with an NO scavenging rate similar to that of native human hemoglobin, would exacerbate pulmonary vasoconstriction and permeability and that rHb2.0, a second-generation HBOC with an NO scavenging rate ~20- to 30-fold lower than that of rHb1.1, would minimally influence these responses. Consistent with this hypothesis, rHb1.1 enhanced pulmonary vasoconstrictor reactivity to both hypoxia and thromboxane mimetic U-46619 in a dose-dependent fashion. In contrast, rHb2.0 produced little or no change in reactivity to these stimuli. Furthermore, whereas rHb1.1 abrogated pulmonary vasodilation to the NO-donor S-nitroso-N-acetyl-penicillamine (SNAP), dose-dependent responses to SNAP were preserved, albeit attenuated, in lungs treated with rHb2.0. Finally, the capillary filtration coefficient was unaltered by either rHb1.1 or rHb2.0. We conclude that pulmonary hemodynamic responses to rHb2.0 are greatly reduced compared with those observed with rHb1.1, consistent with rHb2.0 having a diminished capacity to scavenge NO. In addition, neither hemoglobin solution measurably altered microvascular permeability in this preparation.

rHb1.1; rHb2.0 for injection; hypoxia; U-46619; nitric oxide; vascular permeability; isolated rat lung

Address for reprint requests and other correspondence: T. C. Resta, Vascular Physiology Group, Dept. of Cell Biology and Physiology, Univ. of New Mexico, Health Sciences Center, 915 Camino de Salud, N.E. Albuquerque, NM 87131-5218 (E-mail: tresta@salud.unm.edu).

HEMOGLOBIN-BASED OXYGEN CARRIERS (HBOCs) have been pursued for many years as therapeutic agents to be used in the treatment of existing or anticipated reductions in oxygen supply, e.g., hemorrhagic shock or surgical blood loss (46, 48). Although various modifications have eliminated the marked toxicities observed with the early solutions (23), both preclinical and clinical studies have demonstrated that many HBOCs produce systemic and pulmonary vasoconstriction (6, 17, 18, 24, 26, 42). HBOCs that have been most extensively tested in clinical trials include first generation intramolecularly cross-linked tetrameric hemoglobin solutions and polyhemoglobin solutions, created by intermolecular cross-linking of tetramers (8). Whereas vasoconstriction is observed after intravenous infusion of many tetrameric hemoglobin solutions (6, 8, 14, 17, 18, 24, 26, 42), polyhemoglobin solutions with relatively low concentrations of unreacted tetramer appear to exhibit reduced vasoconstrictor activity (8, 14). However, it is unclear whether polymerization of hemoglobin alone is sufficient to minimize vasoconstriction. Although polymerization of hemoglobin presumably reduces the extent of extravasation, the reported effects on vasoconstriction have been variable. Some studies have demonstrated that solutions of polymerized hemoglobins appear to cause minimal vasoconstriction (20, 38), whereas others have shown systemic vasoconstriction after infusion of hemoglobin polymerized with a variety of agents (1, 10, 43).

Several mechanisms for the hemodynamic responses to HBOCs have been proposed, yet much of the available evidence indicates that scavenging of nitric oxide (NO) by extravascular tetrameric hemoglobin is the primary mechanism responsible for inducing the observed systemic vasoconstriction (6, 8, 9, 14, 42). Similar conclusions have been drawn from studies examining the effects of hemoglobin solutions on the pulmonary circulation (12, 17, 28). If hemoglobin solutions indeed elicit pulmonary hypertension or exacerbate existing pulmonary hypertension via NO scavenging, the resulting changes in microvascular pressure could potentially lead to increased capillary filtration and subsequent pulmonary edema. Pulmonary hypertension resulting from administration of hemoglobin could additionally result in right heart failure and associated complications. Recently, Doherty and col-
leagues (9) used genetic manipulation of the distal heme pockets of recombinant hemoglobins to reduce NO scavenging and demonstrated that the systemic hypertensive response in conscious rats is proportional to the rate of NO scavenging by hemoglobin (9). Although these results indicated that the systemic hemodynamic effects of recombinant hemoglobin could be ameliorated by altering its NO reactivity, the effects of similar alterations on the pulmonary responses to hemoglobin are unknown. In this study, we examined the influences of two recombinant hemoglobin solutions with different reaction rates with NO on vasoconstrictor reactivity in isolated, saline-perfused rat lungs. rHb1.1 is a first-generation recombinant human hemoglobin solution that has a NO scavenging rate similar to that of native human hemoglobin, whereas rHb2.0 for injection (rHb2.0) is a second-generation recombinant human hemoglobin solution that, through genetic manipulation of the distal heme pocket, has a rate of NO scavenging ~20- to 30-fold lower than that of rHb1.1. We hypothesized that rHb1.1 would augment pulmonary vasoconstrictor responsiveness, whereas rHb2.0 would have a minimal influence on vasoactivity.

Although HBOCs with presumably wild-type rates of NO scavenging have been shown to increase microvascular permeability within the rat mesenteric circulation (4), it is unclear whether HBOCs exert similar influences on permeability in the lung. Because inhibition of NO synthesis increases permeability in both pulmonary (29) and mesenteric circulations (5), we further hypothesized that rHb1.1 would increase pulmonary microvascular permeability to a greater extent than rHb2.0 as a consequence of greater NO scavenging capacity. Therefore, we additionally examined effects of each recombinant hemoglobin on vascular permeability.

Methods

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine (Albuquerque, NM). Male Sprague-Dawley rats (250–400 g; Harlan Industries) were used for all experiments.

Materials

The hemoglobin and human serum albumin (HSA) solutions used in this study were provided by Baxter Healthcare (Boulder, CO). rHb1.1 is a recombinant human hemoglobin expressed in Escherichia coli and extensively purified (25). Fusion of the α chains into a single di-α globin prevents dissociation into αβ dimers, and the oxygen affinity is decreased by incorporation of an amino acid substitution found in the Presbyterian variant of human hemoglobin (substitution of Lys for Asn at the β108 position). The resultant hemoglobin molecule is a stabilized pseudotetramer with a molecular weight (MW) of 64 and a P₅₀ of 32 Torr (25). rHb1.1 is formulated at a concentration of ~100 g/l in phosphate-buffered saline and was stored at ~80°C before use. At this hemoglobin concentration, rHb1.1 has a viscosity of ~1.9 cP and a colloid osmotic pressure of ~40 Torr.

rHb2.0 is a second-generation recombinant human hemoglobin in which the reaction rate with NO has been reduced by 20- to 30-fold. Like rHb1.1, rHb2.0 is expressed in E. coli and extensively purified, and the α chains are also fused to prevent dissociation. Amino acid substitutions were made in the distal heme pocket by site-directed mutagenesis to reduce the rate of NO scavenging. rHb2.0 is also polyethylene glycol-polymerized and derivatized and is formulated at a concentration of ~100 g/l in a gluconated electrolyte solution. At this hemoglobin concentration, rHb2.0 has a viscosity of ~2.3 cP and a colloid osmotic pressure of ~62 Torr. P₅₀ of rHb2.0 is ~34 Torr. rHb2.0 was stored at 4°C before use.

Two HSA solutions, HSA-1 and HSA-2, were used in this study. They were oncotically matched to rHb1.1 and rHb2.0, respectively, by adjusting the HSA concentrations to 100 and 134 g/l. Both HSA solutions were stored at 4°C before use. Angiotensin II (Sigma Chemical, St. Louis, MO) was dissolved in normal saline and stored at ~80°C. 9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F₂₀ (U-46619; Cayman) and S-nitroso-N-acetyl-penicillamine (SNAP; Sigma Chemical) were dissolved in 95% ethanol and stored at ~80°C. N⁶-nitro-L-arginine (L-NNA; Sigma Chemical) was dissolved directly in physiological saline solution (PSS) before perfusion of lungs.

Isolated Lung Preparation

Lungs were isolated from rats by using established procedures (11, 32–35). Animals were anesthetized with pentobarbital sodium (25 mg ip). After the trachea was cannulated with a 17-gauge needle stub, the lungs were ventilated by using a Harvard positive-pressure rodent ventilator (model 683) at a frequency of 55 breaths/min and a tidal volume of 2.5 ml with a warmed and humidified gas mixture (6% CO₂ in room air). Peak inspiratory pressure was set at 9 cmH₂O, and positive end-expiratory pressure was maintained at 3 cmH₂O. After a median sternotomy, heparin (100 U in 0.1 ml) was injected directly into the right ventricle, and the pulmonary artery was cannulated with a 13-gauge needle stub. The preparation was immediately perfused at 0.8 ml/min by a Masterflex microprocessor pump drive (model 7524-10) with PSS containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose with 4% bovine serum albumin (wt/vol) (all from Sigma Chemical). The left ventricle was cannulated with a plastic tube (4-mm outer diameter), and the heart and lungs were removed en bloc and suspended in a humidified chamber maintained at 38°C. Perfusion rate was gradually increased to 30 ml/min·kg body wt⁻¹. Thirty milliliters of perfusate were washed through the lungs and discarded before recirculation was initiated with the remaining 30 ml. Per fusate was pumped through a water-jacketed Radnoti bubble trap maintained at 38°C before entering the pulmonary circulation. Experiments were performed with lungs in zone 3 conditions achieved by elevating the perfusate reservoir until venous pressure (Pᵥ) was ~3.5 mmHg. For those experiments assessing capillary filtration coefficient (Kᵥ; see Assessment of vascular permeability), the perfusate reservoir was suspended from a Grass model FT03 force-displacement transducer for continuous measurement of reservoir weight. Because the perfusion system is a closed circuit, monitoring reservoir weight (and thus volume) provides a means of assessing fluid flux across the pulmonary microvasculature (34). Lungs were allowed 30 min to equilibrate before protocols were begun. Sodium bicarbonate was added to the perfusate reservoir as needed to maintain physiological pH (~7.4). Perfusate PO₂, PCO₂, and pH were measured by using
an ABL5 blood-gas analyzer. Pulmonary arterial pressure (P{\(a\)) and P{\(v\)} were measured via side ports in the arterial and venous lines by using Spectramed model P23 XL pressure transducers and recorded on a Gould RS 3400 chart recorder. Output signals from the P{\(a\)}, P{\(v\)}, and reservoir weight channels of the chart recorder were continuously displayed on a computer screen by using a data acquisition and analysis system (AT-CODAS, Dataq Instruments).

Isolated Lung Protocols

Responses to hypoxia. The following methods were used to contrast the effects of rHb1.1 and rHb2.0 on segmental constrictor responses to the physiologically relevant stimulus hypoxia, which primarily produces arterial constriction in the pulmonary circulation (21). At the beginning of the 30-min equilibration period, rHb1.1 or rHb2.0 was added to the perfusate reservoir to obtain concentrations of 5 or 20 mg/ml. These concentrations were chosen to approximate the estimated plasma hemoglobin concentrations obtained from 250 and 1,000 mg/kg iv doses in intact animals in preliminary experiments. Separate sets of lungs were treated with equivalent volumes of the control solutions HSA-1 and HSA-2. After equilibration, lungs were challenged with a 500-ng bolus of angiotensin II (100 {\(\mu\)l} injected directly upstream of the lung to assess vascular reactivity under normoxic conditions. After return of perfusion pressure to baseline values, capillary pressure (P{\(c\)}) was estimated by a double-occlusion procedure to allow calculation of segmental resistances (see Calculations and Statistics) as described previously (32–35). Hypoxic vasoconstriction was then initiated by ventilating lungs with a hypoxic gas mixture (0% O{\(2\})-6% CO{\(2\})-balance N{\(2\})). Preliminary experiments demonstrated that ventilation with this gas mixture generated perfusate O{\(2\}) levels within the pathophysiological range (~25 Torr). P{\(c\)} was determined during the peak pressor response to hypoxia to assess effects of the hemoglobin solutions on segmental vascular responses to hypoxia.

Dose response to U-46619. Additional protocols assessed the effects of the hemoglobin solutions on segmental pulmonary vasoconstrictor responses to the thromboxane mimetic U-46619. U-46619 provides consistent and stable pressor responses in this preparation and, unlike hypoxia, constricts both arterial and venous segments of the pulmonary vasculature (32–35). After assessment of K{\(e\}) (see Assessment of vascular permeability) in separate sets of lungs perfused with rHb1.1, rHb2.0 (5 or 20 mg/ml), or their respective control HSA solutions, baseline P{\(c\)} was determined by double occlusion, and a cumulative concentration-response curve to U-46619 (50, 100, 200 nM) was generated. Stable pressor responses to each dose of U-46619 were allowed to develop before administration of subsequent doses. P{\(c\)} was assessed at the plateau of the pressor response by double occlusion, and a cumulative concentration-response relationship to SNAP (1 {\(\mu\}M, 10 {\(\mu\}M) was generated. P{\(c\)} was again assessed at the point of maximal vasodilation to each dose of SNAP. These concentrations of SNAP were demonstrated to provide concentration-dependent responses in preliminary experiments. Furthermore, we have previously shown that N-acetylpenicillamine is without vasoactive effects in this preparation (32), which suggests that responses to SNAP are NO mediated.

Assessment of vascular permeability. To examine potential effects of the hemoglobin solutions on pulmonary vascular permeability, K{\(e\}) and fluid flux were assessed by using methodology similar to that previously described (2, 11, 34). Lungs used for subsequent determination of responses to U-46619 (see Dose-response to U-46619) were perfused with PSS containing 5 or 20 mg/ml of either rHb1.1, rHb2.0, or equivalent volumes of their HSA control solutions. After the 30-min equilibration period, flow was adjusted from the normal 30 ml/min kg body wt{\(^{-1}\}) until perfusate reservoir weight was near isogravimetric (26 ± 2 {\(\mu\}g/min), and baseline P{\(c\)} was determined by double occlusion. P{\(c\)} was then rapidly increased by 5 mmHg for a period of 15 min. Perfusion reservoir weight rapidly decreased with this change in P{\(v\)}, followed by a slower, linear decrease in weight for the remainder of the 15 min. The initial rapid decrease in reservoir weight occurs primarily because of vascular filling associated with recruitment and distension, whereas the slower loss of fluid represents transcapillary filtration (2). At the end of the 15-min period, P{\(c\)} was again determined, and P{\(v\)} and flow returned to normal values (3.5 mmHg and 30 ml/min kg body wt{\(^{-1}\}) respectively). It was assumed that changes in interstitial oncotic and hydrostatic pressures were minimal during the measurement period. The rate of fluid flux under baseline conditions was subtracted from that after elevation of P{\(v\}). K{\(e\}) was calculated by dividing the corrected linear rate of fluid flux between the 5- and 15-min time points by the change in P{\(c\)} that occurred with the elevation in P{\(v\}). K{\(e\}) was normalized to lung tissue dry weight obtained after 3 wk in a 60°C oven and was expressed in ml/min kg body wt{\(^{-1}\}) dry lung tissue{\(^{-1}\}). As an index of edema formation, lung wet weight-to-dry weight ratios were calculated after completion of all experimental protocols examining responses to hypoxia and U-46619.

Effects of endothelial injury on vascular permeability. The following protocols were designed to demonstrate increases in K{\(e\}) and lung wet weight-to-dry weight ratios in response to proteamine sulfate, a compound previously demonstrated to increase pulmonary vascular permeability through microvascular endothelial damage (7, 11). Lungs were isolated and perfused with PSS containing proteamine sulfate (1 mg/ml; Sigma Chemical, Grade X) and papaverine (100 {\(\mu\}M; Sigma Chemical). After a 60-min equilibration period, K{\(e\}) was calculated as described in Assessment of vascular permeability. Papaverine was included at a dose previously demonstrated to inhibit the pulmonary vasoconstrictor actions of proteamine in this preparation (7). Parallel control experiments were conducted in a separate set of lungs equilibrated with papaverine alone before assessment of K{\(e\}). Lung wet weight-to-dry weight ratios were calculated for each group as an index of edema formation.
Calculations and Statistics

Total pulmonary vascular resistance was calculated as the difference between Pa and Pv divided by flow (30 ml·min⁻¹·kg body wt⁻¹). Pulmonary arterial resistance was calculated as the difference between Pa and Pc divided by flow. Similarly, pulmonary venous resistance was calculated as the difference between Pc and Pv divided by flow. Vasodilatory responses to SNAP were calculated as a percent reversal of U-46619-induced vasoconstriction for the total pulmonary vasculature as well as for arterial and venous segments.

All data are expressed as means ± SE. Values of n refer to the number of animals in each group. Where appropriate, a one- or two-way ANOVA was used to make comparisons between treatment groups. If differences were detected by ANOVA, multiple-comparison procedures were performed by using the Student-Newman-Keuls test. A probability of P < 0.05 was accepted as statistically significant for all comparisons.

RESULTS

Baseline Segmental Vascular Resistances

Total and segmental baseline resistances were not different between lungs treated with rHb1.1 or rHb2.0 compared with their respective control solutions (HSA-1 and HSA-2) for either 5 or 20 mg/ml concentrations (Table 1). However, total and arterial resistances were significantly less in the 20 mg/ml rHb2.0 group compared with rHb1.1 at the same concentration. Furthermore, arterial resistances were slightly, but significantly, greater for lungs treated with 20 mg/ml rHb1.1 or HSA-1 compared with their respective 5 mg/ml groups. No differences in baseline venous resistances were observed between treatment groups.

Responses to Hypoxia

Total and arterial hypoxic vasoconstriction was greater in the presence of 5 mg/ml rHb1.1 compared with HSA-1 (Fig. 1A). In contrast, hypoxic responses were not different between the rHb2.0 and HSA-2 groups and were significantly less in lungs treated with rHb2.0 compared with those treated with rHb1.1. Hypoxia had little effect on venous resistances, which did not differ between groups. Similar findings were ob-

Table 1. Baseline segmental vascular resistances

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total (mmHg)</th>
<th>Arterial (mmHg)</th>
<th>Venous (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSA-1</td>
<td>11</td>
<td>0.064 ± 0.005</td>
<td>0.028 ± 0.004</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>rHb1.1</td>
<td>11</td>
<td>0.091 ± 0.007</td>
<td>0.043 ± 0.005</td>
<td>0.048 ± 0.005</td>
</tr>
<tr>
<td>HSA-2</td>
<td>12</td>
<td>0.075 ± 0.009</td>
<td>0.031 ± 0.006</td>
<td>0.044 ± 0.003</td>
</tr>
<tr>
<td>rHb2.0</td>
<td>11</td>
<td>0.073 ± 0.005</td>
<td>0.034 ± 0.004</td>
<td>0.039 ± 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSA-1</td>
<td>16</td>
<td>0.089 ± 0.008</td>
<td>0.048 ± 0.006†</td>
<td>0.041 ± 0.004</td>
</tr>
<tr>
<td>rHb1.1</td>
<td>16</td>
<td>0.101 ± 0.009</td>
<td>0.062 ± 0.008†</td>
<td>0.040 ± 0.003</td>
</tr>
<tr>
<td>HSA-2</td>
<td>15</td>
<td>0.069 ± 0.005</td>
<td>0.032 ± 0.004</td>
<td>0.037 ± 0.003</td>
</tr>
<tr>
<td>rHb2.0</td>
<td>20</td>
<td>0.075 ± 0.006*</td>
<td>0.036 ± 0.005*</td>
<td>0.039 ± 0.003</td>
</tr>
</tbody>
</table>

Values are means ± SE. Resistances are in mmHg·ml⁻¹·min⁻¹·kg body wt. HSA, human serum albumin; n, no. of rats; rHb, hemoglobin solution. †P < 0.05 vs. rHb1.1 at the same concentration. *P < 0.05 vs. corresponding 5 mg/ml value.

Fig. 1. Segmental resistance changes (Δ) to hypoxia in lungs treated with 5 mg/ml of hemoglobin solution rHb1.1 or rHb2.0 (A), 20 mg/ml rHb1.1 or rHb2.0 (B), or their respective control solutions [human serum albumin (HSA)-1 and -2]. Data are means ± SE of n = 5–7 lungs per group. *P < 0.05 vs. HSA-1. †P < 0.05 vs. corresponding 5 mg/ml value. #P < 0.05 vs. rHb1.1.

Dose-Response to U-46619

Total vasoconstrictor responses to 50 and 100 nM U-46619 were greater in lungs treated with 5 mg/ml rHb1.1 compared with the HSA-1 control group (Fig. 2A). In addition, total vasoconstrictor responses to 50 nM U-46619 were greater in lungs treated with 5 mg/ml rHb1.1 compared with lungs that received a similar concentration of rHb2.0. In contrast, total changes in resistance to U-46619 were not different between lungs treated with 5 mg/ml rHb2.0 and HSA-2. No significant differences were observed between groups for either arterial or venous responses to U-46619 (Fig. 2, B and C).

Consistent with effects of rHb1.1 on hypoxic vasoconstriction (Fig. 1), changes in total and segmental
pulmonary vascular resistances to U-46619 were greater in lungs treated with 20 mg/ml rHb1.1 compared with those treated with rHb2.0 (Fig. 3). Total constriction to U-46619 at each concentration (Fig. 3A) and arterial responses to both 100 and 200 nM concentrations of U-46619 (Fig. 3B) were also greater in the rHb2.0 group compared with the HSA-2 control group. Also consistent with the concentration-dependent effects of rHb1.1 on hypoxic reactivity (Fig. 1), greater changes in total and segmental resistances were observed in lungs treated with 20 vs. 5 mg/ml rHb1.1 (Figs. 2 and 3). A similar concentration-dependent effect of rHb2.0 was apparent for total resistance at the 100 nM concentration of U-46619 (Figs. 2A and 3A) and for arterial resistance at both 100 and 200 nM concentrations (Figs. 2B and 3B).

Vasodilatory Responses to Exogenous NO

Table 2 illustrates U-46619-induced changes in total, arterial and venous resistances before assessment of vasodilatory responses to SNAP. Although U-46619 was administered to generate similar increases in resistance between groups, we observed a slightly greater arterial constriction in rHb1.1- and HSA-2-treated lungs compared with those administered HSA-1.

Table 2. Segmental vascular resistance changes in response to U-46619 for experiments examining vasodilatory responses to SNAP

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total</th>
<th>Arterial</th>
<th>Venous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 mg/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSA-1</td>
<td>5</td>
<td>0.235 ± 0.021</td>
<td>0.148 ± 0.018</td>
<td>0.087 ± 0.006</td>
</tr>
<tr>
<td>rHb1.1</td>
<td>5</td>
<td>0.300 ± 0.024</td>
<td>0.217 ± 0.025*</td>
<td>0.083 ± 0.013</td>
</tr>
<tr>
<td>HSA-2</td>
<td>5</td>
<td>0.328 ± 0.031</td>
<td>0.225 ± 0.014*</td>
<td>0.103 ± 0.028</td>
</tr>
<tr>
<td>rHb2.0</td>
<td>5</td>
<td>0.313 ± 0.026</td>
<td>0.197 ± 0.035</td>
<td>0.117 ± 0.011</td>
</tr>
</tbody>
</table>

Values are means ± SE. SNAP, S-nitro-N-acetyl-penicillamine.

*P < 0.05 vs. HSA-1.
SNAP elicited concentration-dependent vasodilation in lungs perfused with HSA-1, HSA-2, and 20 mg/ml rHb2.0 (Fig. 4). In contrast, total and segmental vasodilatory responses to 1 and 10 μM SNAP were abolished in the presence of 20 mg/ml rHb1.1. Although total vasodilation to 10 μM SNAP was slightly, but significantly, attenuated in the presence of rHb2.0 compared with HSA-2 (Fig. 4A), no significant differences were observed between these groups for arterial and venous segments of the vasculature (Fig. 4, B and C). Both total and segmental vasodilatory responses to 1 μM SNAP were significantly attenuated by rHb2.0. Except for a slightly lower total vasodilation to 10 μM SNAP in HSA-2- vs. HSA-1-treated lungs (Fig. 4A), no significant differences were observed in SNAP-induced vasodilation between HSA control groups.

Kfc and Lung Wet Weight-to-Dry Weight Ratios

No differences in Kfc were observed under baseline conditions between treatment groups for either 5 mg/ml (Fig. 5A) or 20 mg/ml (Fig. 5B) concentrations of rHb1.1 and rHb2.0. Wet weight-to-dry weight ratios of lungs subjected to HPV determinations were not different between 5 mg/ml rHb1.1 or rHb2.0 (A); 20 mg/ml rHb1.1 or rHb2.0 (B), or their respective control solutions (HSA-1, HSA-2). Values are means ± SE of n = 5–6 lungs per group. There are no significant differences.

DISCUSSION

The present study examined effects of two recombinant hemoglobin solutions with relatively high, i.e., wild-type (rHb1.1), and low (rHb2.0) rates of NO scav-
enging on vasoconstrictor reactivity and permeability within the pulmonary circulation. The major findings from this study are as follows: 1) rHb1.1 augmented pulmonary vasoconstrictor reactivity to both hypoxia and U-46619 in a dose-dependent fashion. However, rHb2.0 produced little or no change in reactivity to these stimuli. 2) Whereas rHb1.1 abolished pulmonary vasodilation to the NO-donor SNAP, concentration-dependent responses to SNAP were preserved, although attenuated, in lungs treated with rHb2.0. 3) \(K_{FC}\) was unaltered by either rHb1.1 or rHb2.0, suggesting that neither hemoglobin solution measurably influences microvascular permeability in the isolated, perfused rat lung. These findings demonstrate that pulmonary vasoactive responses to the second generation HBOC, rHb2.0, are greatly reduced compared with those observed with rHb1.1, a first generation HBOC, and are consistent with rHb2.0 having a diminished capacity to scavenge NO in this preparation.

Hemoglobin-based blood substitutes that have been tested most extensively in clinical trials include first-generation intramolecularly cross-linked tetrameric hemoglobin solutions, and polyhemoglobin solutions, created by intermolecular cross-linking of tetramers (8). Intravenous infusion of many tetrameric hemoglobin solutions produces systemic and pulmonary vasoconstriction (8, 14, 24, 26, 42), whereas those polyhemoglobin solutions with low concentrations of unreacted tetramer appear to exhibit reduced vasoconstrictor activity. However, it is unclear whether polymerization of hemoglobin alone is sufficient to minimize vasoconstriction. Differences in experimental protocol (topload administration to normovolemic animals, exchange transfusion, resuscitation from hemorrhage, etc.) complicate the interpretation of the published data. Although polymerization of hemoglobin presumably reduces the extent of extravasation, the reported effects on vasoconstriction have been variable. Some studies have demonstrated that solutions of polymerized hemoglobins appear to cause minimal vasoconstriction (20, 38), whereas others have clearly shown systemic vasoconstriction after infusion of hemoglobin polymerized with a variety of agents (1, 10, 43). Doyle et al. (10) found that glutaraldehyde-treated tetrameric hemoglobin (i.e., hemoglobin monomer, MW = 64) and glutaraldehyde polymerized hemoglobin (average MW of \(~200–1,000\)) elicited similar changes in peripheral vascular resistance in conscious rats. Therefore, the reduction in vasoconstrictor activity reported for glutaraldehyde-polymerized hemoglobins may not be due solely to the increase in molecular size.

Several mechanisms for the hypertensive responses to hemoglobin solutions have been proposed, including scavenging of NO (22, 42, 44), increased endothelin synthesis (16, 39), sensitization of \(\alpha\)-adrenergic receptors (15, 41), and precapillary autoregulation (47). Of these proposed mechanisms, NO scavenging associated with extravasation of tetrameric hemoglobin appears to be the primary mechanism by which HBOCs cause...
vasoconstriction (8, 14). Supporting this conclusion are findings that inhibition of NO synthesis attenuated the hypertensive response to diaspirin-cross-linked hemoglobin (DCLHb), a first-generation HBOC with a wild-type NO scavenging rate (22), and that enhancement of NO synthesis by co-infusion of l-arginine diminished the hemodynamic responses to DCLHb (42). In addition, rHb1.1 attenuated NO-dependent relaxation in isolated aortic rings (36). Recently, Doherty and colleagues (9) used genetic manipulation of the distal heme pockets of recombinant tetrameric hemoglobin to reduce NO scavenging and demonstrated that the hypertensive response in conscious rats is proportional to the rate of NO scavenging by hemoglobin.

Although the hypertensive effects of HBOCs in the pulmonary circulation are not as well documented as those in the systemic circulation, several reports have described pulmonary vasoconstrictor/contractile responses secondary to exposure to hemoglobin. Freas et al. (12) observed that DCLHb caused porcine pulmonary arterial and venular rings to contract and that both endothelium denudation and NO synthesis inhibition abolished this effect. Similarly, Heller et al. (17) found that NO donors significantly blunted hypertensive responses to stroma-free hemoglobin in isolated rabbit lungs. These studies suggest that scavenging of NO by hemoglobin may be responsible for hemoglobin-induced pulmonary hypertension. Furthermore, these and other studies (9, 18) imply that HBOCs with reduced NO scavenging capacities may be advantageous in minimizing the potential for development of pulmonary hypertension and subsequent edema formation. To further clarify this issue, we compared effects of hemoglobin solutions with different NO scavenging rates on pulmonary vasoconstrictor reactivity in isolated rat lungs. rHb1.1 is a first-generation recombinant human hemoglobin solution that has a NO scavenging rate very similar to that of native human hemoglobin, whereas rHb2.0 is a second-generation recombinant human hemoglobin solution that, through genetic manipulation of the distal heme pocket, has a much lower rate of NO scavenging (~20- to 30-fold lower than that of rHb1.1). Consistent with greater NO scavenging capacity of rHb1.1, we found that, whereas rHb1.1 dose dependently enhanced pulmonary arterial and venous constriction to both hypoxia and U-46619, rHb2.0 did not alter hypoxic constriction and only slightly augmented arterial responsiveness to U-46619 at the higher concentration of rHb2.0. These findings are consistent with an effect of endogenous NO to attenuate both hypoxic and U-46619-induced pulmonary vasoconstriction, as previously reported (3, 33, 37). We further demonstrated that rHb1.1 abolished NO-dependent vasodilation to SNAP, whereas rHb2.0 only modestly attenuated responses to SNAP. Together, these results suggest that reduced hemodynamic responses to rHb2.0 vs. rHb1.1 are a function of a diminished capacity of rHb2.0 to scavenge NO.

Despite markedly enhanced reactivity to vasoconstrictor stimuli in isolated lungs treated with rHb1.1, we observed no apparent effect of either hemoglobin solution on baseline vascular resistances. These findings suggest that endogenous NO does not contribute to maintenance of basal vascular tone in this preparation and are consistent with previous observations from our laboratory that basal tone is similarly unaltered after NOS inhibition with L-NNA (32, 35). However, considering that L-NNA initiates constriction in lungs from pulmonary hypertensive rats (31) and in lungs from neonatal piglets (30) and lambs (13, 27), it is possible that recombinant hemoglobin with substantial NO reactivity could initiate pulmonary hypertension in similar preparations.

Although infusion of hemoglobin solutions could potentially lead to pulmonary edema as a consequence of elevated microvascular pressure, it is additionally possible that these HBOCs increase endothelial permeability secondary to NO bioinactivation. Evidence that some first-generation polyethylene glycol-conjugated HBOCs increase macromolecular permeability in the rat mesenteric circulation supports this possibility (4). However, discrepancies exist in the literature with respect to influences of NO on pulmonary vascular permeability. Whereas several studies have reported a lack of effect of either exogenous or endogenous NO on permeability in isolated perfused rabbit (45) and rat lungs (11) and in the pulmonary circulation of chronically instrumented sheep (19), others have demonstrated that endogenous NO may have protective influences in maintaining low permeability in isolated rabbit lungs (29). Our present findings suggest that neither rHb1.1 nor rHb2.0 alters Kf in isolated perfused rat lungs. In agreement with these data, we observed no differences in lung wet weight-to-dry weight ratios after either hypoxic or U-46619 constriction between lungs treated with the lower dose (5 mg/ml) of each hemoglobin solution or HSA control solution. The higher dose of rHb2.0 (20 mg/ml) similarly had no significant effect on lung wet weight-to-dry weight ratio. The greater wet weight-to-dry weight ratios observed for lungs treated with 20 mg/ml rHb1.1 compared with other groups are consistent with the augmented hypoxic and U-46619-induced vasoconstriction by rHb1.1 and, therefore, greater vascular hydrostatic pressure in that group. These results suggest that neither modified hemoglobin nor endogenous NO alters Kf in this preparation.

In summary, we have examined the influence of two recombinant hemoglobin solutions with different rates of NO scavenging on vasoconstrictor reactivity and permeability within the pulmonary circulation. Our results demonstrate that pulmonary hemodynamic responses to rHb2.0 are greatly reduced compared with those observed with the first generation hemoglobin, rHb1.1, and are consistent with rHb2.0 having a diminished capacity to scavenge NO in this preparation. Furthermore, these data do not support a role for these recombinant hemoglobin solutions in modulating pulmonary vascular permeability. Our findings therefore
lend promise for use of HBOCs with low NO reactivity as oxygen therapeutics.

The authors gratefully acknowledge the expert technical assistance of Minerva Murphy and Anna Holmes. The authors also thank Drs. Kenneth Burhop, Maura Matthews, Michael Saunders, and Timothy Estep for critical comments on the manuscript. This work was supported by a grant from Baxter Healthcare (to T. C. Resta). T. C. Resta is a Parker B. Francis Fellow in Pulmonary Research.

REFERENCES


