O-Raffinose Cross-Linking Markedly Reduces Systemic and Renal Vasoconstrictor Effects of Unmodified Human Hemoglobin

WILFRED LIEBERTHAL, ROBERT FUHRO, JANE E. FREEDMAN, GEORGE TOOLAN, JOSEPH LOSCALZO, and C. ROBERT VALERI

Renal (W.L., R.F.) and Cardiology (J.E.F., G.T., J.L., C.R.V.) Divisions, Evans Department of Medicine, and Naval Blood Research Laboratory, Boston University Medical Center, Boston, Massachusetts

Accepted for publication October 19, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

The hemodynamic effects of a 20% exchange-transfusion with different solutions of highly purified human hemoglobin A-zero (A₀) were evaluated. We compared unmodified hemoglobin with hemoglobin cross-linked with O-raffinose. Unmodified hemoglobin increased systemic vascular resistance and mean arterial pressure more than the O-raffinose cross-linked hemoglobin solution (by ~45% and ~14%, respectively). Unmodified hemoglobin markedly reduced cardiac output (CO) by ~21%, whereas CO was unaffected by the O-raffinose cross-linked hemoglobin solution. Unmodified and O-raffinose cross-linked hemoglobin solutions increased mean arterial pressure to comparable extents (~14% and ~9%, respectively). Unmodified hemoglobin increased renal vascular resistance 2-fold and reduced the glomerular filtration rate by 58%. In marked contrast, the O-raffinose cross-linked hemoglobin had no deleterious effect on the glomerular filtration rate, renal blood flow, or renal vascular resistance. The extents to which unmodified and O-raffinose cross-linked hemoglobin solutions inactivated nitric oxide also were compared using three separate in vitro assays: platelet nitric oxide release, nitric oxide-stimulated platelet cGMP production, and endothelium-derived relaxing factor-mediated inhibition of platelet aggregation. Unmodified hemoglobin inactivated or oxidized nitric oxide to a greater extent than the O-raffinose cross-linked hemoglobin solutions in all three assays. In summary, O-raffinose cross-linking substantially reduced the systemic vasoconstriction and the decrease in CO induced by unmodified hemoglobin and eliminated the deleterious effects of unmodified hemoglobin on renal hemodynamics and function. We hypothesize that O-raffinose cross-linking reduces the degree of oxidation of nitric oxide and that this contributes to the reduced vasoactivity of this modified hemoglobin.

Hemoglobin-based oxygen-carrying solutions (HBOCs) have a number of potential advantages as blood substitutes. They can be produced in large volumes, stored for prolonged periods, and administered rapidly, without the need for typing and cross-matching. These solutions can also be sterilized by pasteurization, reducing the risk of transmission of viruses and other infectious agents (Chang, 1997; Lieberthal, 1997). A major challenge to the development of a safe red cell-free HBOC has been the well recognized nephrotoxicity of crude hemolysate (Jaenike, 1967; Paller, 1988; Lieberthal, 1997). However, three fundamental observations made during the past few decades have markedly reduced the toxicity and increased the efficacy of hemoglobin solutions (Lieberthal, 1997).

First, in 1967, Rabiner et al. demonstrated that the presence of red blood cell membrane fragments (red cell “stroma”) contributed substantially to the toxicity and procoagulant activity of crude red cell hemolysates. This observation spurred the development of effective techniques to purify hemoglobin, a process that reduced, but did not eliminate, the toxicity of hemoglobin solutions (Savitsky et al., 1978, Lieberthal et al., 1987). More recently, further improvements

ABBREVIATIONS: HBOC, hemoglobin-based oxygen carrier; HAS, human serum albumin; BAEC, bovine aortic endothelial cell; MAP, mean arterial pressure; CO, cardiac output; EDRF, endothelium-derived relaxing factor; ECB, bovine endothelial cell on bead; SVR, systematic vascular resistance; GFR, glomerular filtration rate; RPF, renal plasma flow; RBF, renal blood flow; FF, filtration fraction; RVR, renal vascular resistance; UV, urine flow rate; UNaV, absolute excretion of sodium; UKV, absolute excretion of potassium; FeNa, fractional excretion of sodium; FeK, fractional excretion of potassium; SNO-GSH, S-nitrosoglutathione; GFP, gel-filtered platelets; NO, nitric oxide.
in the purification of hemoglobin has made possible the preparation of large volumes of hemoglobin of greater than 99% purity (Adamson and Moore, 1998).

A second important observation was that hemoglobin molecules could be chemically modified to prevent the rapid excretion of dimeric hemoglobin in the urine. Native hemoglobin (~64,500 kDa), when released from red cells, dissociated into dimers (~32,250 kDa) that were readily filtered at the glomerulus (Bunn et al., 1969). The presence of hemoglobin within the tubular lumen contributed substantially to hemoglobin-associated nephrotoxicity by causing intratubular obstruction and oxidant injury to renal epithelial cells (Jaenike, 1967; Puller, 1988; Zager and Gamelin, 1989). Chemical cross-linking of dimers into tetramers and/or oligomers is one effective form of modification that reduces the ability of hemoglobin to cross the glomerular basement membrane and gain access to the renal tubules and substantially reduces nephrotoxicity (Lieberthal, 1995, 1997). An added advantage of chemical modification is that it prolongs the half-life of the hemoglobin in the circulation by preventing elimination in the urine and slowing the rate of diffusion of hemoglobin from the circulation into the interstitial space (Lieberthal, 1995, 1997).

A third discovery, essential for the efficacy of HBOC solutions as effective oxygen carriers, was the use of chemical modification to optimize the oxygen affinity of the hemoglobin solution. Within the human red cell, the presence of 2,3 diphosphoglycerate (2,3-DPG) normally maintains the normal affinity of human hemoglobin for oxygen. Red cell-free hemoglobin loses this interaction with 2,3-DPG, so unmodified human HBOC solutions have a very high oxygen affinity. Chemical methods have been developed to reduce the oxygen affinity of the hemoglobin for oxygen, resulting in an oxygen carrier that effectively releases oxygen at the physiological pO$_2$ present in tissues (Benesch and Benesch, 1961; Lieberthal, 1997; Adamson and Moore, 1998).

However, despite the development of increasingly efficient and effective ways of removing red cell stroma and of cross-linking hemoglobin, many HBOC solutions have continued to exhibit vasoconstrictor activity with resultant hypertension and reduced blood flow to various organs, including the heart, lung, and kidney (Savitsky et al., 1978; Vogel et al., 1986; Hess et al., 1993; Thompson et al., 1994). There is substantial evidence that these vasoactive properties of purified and modified HBOC solutions are due, at least in part, to the well-established ability of hemoglobin to bind and inactivate nitric oxide (NO) (Martin et al., 1986; Wennmalm et al., 1992; Kilbourn et al., 1994; Motterlini et al., 1996). This study was designed to evaluate the systemic and renal hemodynamic effects of a highly purified hemoglobin solution that has been chemically cross-linked with O-raffinose to form a product consisting predominantly of oligomers of hemoglobin with molecular masses ranging from 128 to 600 kDa (Baines et al., 1995; Adamson and Moore, 1998). We directly compared the hemodynamic effects of modified O-raffinose cross-linked hemoglobin with the highly purified unmodified human hemoglobin from which the cross-linked hemoglobin was made. We demonstrated that the unmodified hemoglobin caused a severe increase in systemic vascular resistance (SVR) and intrarenal vasoconstriction and markedly impaired CO as well as renal function. In striking contrast, the O-raffinose cross-linked hemoglobin had no deleterious effect on cardiac function, glomerular filtration rate (GFR), or renal vascular resistance (RVR) and only modestly increased SVR. We also provide in vitro evidence that O-raffinose cross-linking reduced the degree to which hemoglobin solutions inactivated NO.

**Materials and Methods**

**Reagents**

Human serum albumin (HSA), sodium nitrite, trichloroacetic acid (TCA), trypan blue, sepharose 2B, ADP, glutathione (GSH), and N-nitro-l-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO). Microcarrier beads (Cytodex 3) were obtained from Pharmacia (Upsala, Sweden). GMP enzyme immunoassay kits were purchased from Cayman Chemical Co. (Ann Arbor, MI). Inactin (thiobarbitual sodium) was purchased from Research Biochemicals International (RBI) (Natick, MA).

**Methods Used to Produce Hemoglobin Solutions**

**Unmodified Human Hemoglobin (A0).** Outdated human red cells were pooled and washed to remove plasma proteins, and the red cells were lysed by constant volume diafiltration against 50 mM Tris-Cl, pH 8.9. The crude hemoglobin was separated from red cell ghosts by membrane microfiltration. The hemoglobin was converted to carbon monoxhemoglobin and pasteurized for 10 h at 62°C. This process results in the removal of both phospholipid and nonhemoglobin protein. Further purification was achieved using both anion- and cation-exchange chromatography to remove basic and acidic protein contaminants and to further reduce lipid and encapsulated virus contamination (Haia, 1991; Adamson and Moore, 1998). The purity of the HbA0, confirmed by several methods, was greater than 99%. No red cell membrane proteins were detectable. The combined phosphatidylethanolamine and phosphatidylserine content of purified HbA0 was <2 μg/g hemoglobin (Adamson and Moore, 1998).

**Modification of Hemoglobin by O-Raffinose Cross-Linking.** For O-raffinose cross-linked hemoglobin solution 1, hemoglobin A0 was deoxygenated by contact with nitrogen gas and then cross-linked by reaction with the periodate-oxidized form of raffinose (O-raffinose). The multiple aldehyde groups of O-raffinose initially form imine, or Schiff base, linkages with amino groups within and between individual hemoglobin molecules. These reversible bonds were stabilized by chemical reduction using dimethylamine borane (Adamson and Moore, 1998). This reductive alkylation is irreversible and results in the covalent cross-linking of the two αβ hemoglobin dimers within amino groups within the 2,3-DPG binding pocket, thereby forming stable tetramers. In addition, O-raffinose reacts with surface amino groups of these tetramers to create intermolecular linkages, producing a stabilized oligomeric hemoglobin. During reductive alkylation, methemoglobin is reduced (from Fe$^{3+}$ to Fe$^{2+}$). Unreacted aldehydes are reduced to alcohols, thus preventing further cross-linking.

Low-molecular-weight hemoglobin molecules were removed by diafiltration against a buffered high salt solution (25 mM Tris, pH 7.4, containing 750 mM MgCl$_2$) using a Filtron Omega 50-kDa molecular mass-cutoff polyether sulfone diafiltration membrane. The high salt solution used for filtration results in complete dissociation of native (non-cross-linked) hemoglobin dimers, thereby allowing their efficient removal. Diafiltration was performed until the level of non-cross-linked hemoglobin was below 5% of total hemoglobin. The proportion of different molecular weight forms of hemoglobin in the solution was determined by size exclusion chromatography on an analytical Superose 12 HR10/30 FPLC column. The O-raffinose solution that results from the above procedure consists predominantly of polymers of hemoglobin (63.8% of total) with a molecular mass ranging from 128 to 600 kDa. However, a substantial proportion of hemoglobin in O-raffinose cross-linked hemoglobin solution 1 (31.8% of total) is a stable covalently cross-linked...
tetramer with a molecular mass of 64 kDa. A small proportion (2.6% of total) of hemoglobin in O-raffinose cross-linked hemoglobin solution 1 is non-cross-linked hemoglobin (Table 1). This preparation of hemoglobin has been called Hemolink (Adamson and Moore, 1998) but in this report is referred to as O-raffinose cross-linked hemoglobin solution 1.

To determine the extent to which the residual tetrameric (64 kDa) and non-cross-linked hemoglobin influenced the hemodynamic effects of the O-raffinose cross-linked hemoglobin solution 1, the solution was further processed to reduce the proportion of non-cross-linked and tetrameric hemoglobin.

O-Raffinose cross-linked solution 2 was prepared by further dialfiltration of solution 1 with a PLTK cellulose membrane with a molecular mass cutoff of 30 kDa against the same dissociating buffer used to dialyze O-raffinose cross-linked solution 1. O-Raffinose cross-linked hemoglobin solution 2 contained an increased proportion of oligomerized hemoglobin (81.1%) and reduced amounts of tetrameric hemoglobin (18.1%) and non-cross-linked hemoglobin (0.6%) (Table 1).

O-Raffinose hemoglobin cross-linked solution 3 was made by further dialysing O-raffinose hemoglobin solution 2 against a Filtron Omega polyether sulfone membrane (molecular mass cutoff of 70 kDa) in the presence of the same dissociating buffer used to dialyze O-raffinose cross-linked solutions 1 and 2. The O-raffinose cross-linked solution 3 contained substantially less tetrameric hemoglobin (1.8% of total) and of non-cross-linked hemoglobin (0.3%) than the other two cross-linked solutions; most of the hemoglobin (97.9% of total) was in the oligomerized form (Table 1).

All final O-raffinose cross-linked hemoglobin solutions were formulated in the oxygenated state in Ringer’s lactate (USP) for injection (Adamson and Moore, 1998).

### Characteristics of Resuscitation Fluids

HSA, unmodified (A,) SFII, and the three O-raffinose cross-linked hemoglobin solutions had comparable oncotic pressures, ranging from 20 to 24 mm Hg. Oncotic pressure was determined using a Wescor oncometer (model 4400; Logan, UT).

The proportion of hemoglobin in the form of methemoglobin was comparable in all hemoglobin solutions (4–5%). Oxyhemoglobin and methemoglobin were measured with a CO-oximeter (model 282; Instrumentation Laboratory, Lexington, MA). The p50 value was measured with a hemoxanalyzer using TES [2-[(tris(hydroxymethyl)methylamino)-1-ethane-silfonic acid] as the buffer (Asakura and Reilly, 1986)]. The p50 value of the unmodified hemoglobin was very low (14.6 mm Hg) (Table 1). The cross-linking procedure substantially reduced the oxygen affinity of all O-raffinose solutions to a comparable extent (p50 values ~44 mm Hg) (Table 1).

### Methods Used for In Vivo Rat Studies

#### General Surgical Procedures.

Male Sprague-Dawley rats (weight 275–350 g) were used for all experiments. Rats were fed regular Purina rat chow (Purina Mills, Chicago, IL) and allowed free access to water. Anesthesia was induced with an i.p. injection of thiobarbital sodium (Inactin; 110 mg/kg). Rats were placed on a thermostatically controlled heated table, and body temperature (monitored via a temperature probe in the carotid artery) was maintained between 36°C and 38°C. A tracheotomy was performed with polyethylene (PE-240) tubing, and both femoral arteries were cannulated with PE-50 tubing; one for blood pressure monitoring and the other for blood sampling. A bladder catheter (PE-90) was placed via a suprapubic incision for urine sampling. The right internal jugular vein was cannulated with two PE-50 catheters. Inulin and para-aminohippuric acid were infused via one catheter. The second catheter was advanced into the superior vena cava. This catheter was used for infusion of the resuscitation fluids (blood, hemoglobin solutions, or HSA) and for CO measurements as described below.

#### Exchange Transfusion.

The rats were subjected to a 20% exchange-transfusion by removing 4 ml of whole blood per 300 g b.wt. from a femoral artery over 15 min. Then animals were divided into separate groups to receive 4 ml/300 g b.wt. of one of the resuscitation fluids (described in Experimental Protocols) via a jugular venous line over a 10-min period.

#### Hemodynamic Measurements and Assays.

Cardiac output was measured with a Cardiomax II-R (Columbus Instruments Corp., Columbus, OH) using the thermodilution technique as previously described in detail (Thompson et al., 1995).

GFR and Renal Plasma Flow. GFR and effective renal plasma flow (RPF) were determined by the renal clearance of carboxyl-14C-labeled inulin and glycy1-2-3H-labeled para-aminohippuric acid, respectively (New England Nuclear, Boston, MA) as previously described in detail (Thompson et al., 1995). Urinary and plasma electrolyte concentrations were measured using ion-specific electrodes.

Renal blood flow (RBF), filtration fraction (FF), systemic vascular resistance (SVR), renal vascular resistance (RVR), and fractional excretion of sodium (FeNa) and potassium (FeK) were calculated using standard formulae (Thompson et al., 1995).

#### Experimental Protocols

##### Comparison of Hemodynamic and Renal Effects of O-Raffinose Cross-Linked Hemoglobin Solutions with Those of Whole Blood, Unmodified Hemoglobin, and HSA.

Animals were subjected to hemorrhage as described above and then randomly assigned to receive one of six different resuscitation fluids: 1) whole blood (n = 16), 2) HSA (n = 10), 3) unmodified hemoglobin (n = 15), 4) O-raffinose cross-linked hemoglobin solution 1 (n = 25), 5) O-raffinose cross-linked hemoglobin solution 2 (n = 10), and 6) O-raffinose cross-linked hemoglobin solution 3 (n = 10). In animals exchange-transfused with whole blood, the blood removed during the exchange was anticoagulated with heparin and administered back to the same animal. A separate group of rats (n = 5) was subjected to sham surgery. These “sham” rats were anesthetized and exposed to the same operative maneuvers as the other rats, except that no blood was removed and no resuscitative fluid was administered.

After an equilibration period of 30 min, four 20-min clearance periods were obtained in all groups for measurement of MAP, CO, and  

### Table 1

Characteristics of HSA and HBOC solutions

<table>
<thead>
<tr>
<th></th>
<th>HSA</th>
<th>Ae HBOC</th>
<th>O-Raffinose HBOC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Solution 1</td>
</tr>
<tr>
<td>Concentration (g/dl)</td>
<td>6.0</td>
<td>6.9</td>
<td>9.8</td>
</tr>
<tr>
<td>Colloid oncotic pressure (mm Hg)</td>
<td>20</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>Methemoglobin (%)</td>
<td>NM</td>
<td>4.7</td>
<td>4.1</td>
</tr>
<tr>
<td>P50 (mm Hg)</td>
<td>NM</td>
<td>14.6</td>
<td>45.5</td>
</tr>
<tr>
<td>P50 slope (n)</td>
<td>NM</td>
<td>2.24</td>
<td>1.32</td>
</tr>
<tr>
<td>Dimeric hemoglobin content (% of total)</td>
<td>NA</td>
<td>100</td>
<td>2.6</td>
</tr>
<tr>
<td>Tetrameric hemoglobin content (% of total)</td>
<td>NA</td>
<td>0</td>
<td>31.8</td>
</tr>
<tr>
<td>Oligomerized hemoglobin content (% of total)</td>
<td>NA</td>
<td>0</td>
<td>63.8</td>
</tr>
</tbody>
</table>

HBOC: hemoglobin-based oxygen-carrying solutions; NM, not measured; NA, not applicable.
groups of rats were also exchange-transfused with either unmodified hemoglobin (n = 4) or O-raffinose cross-linked hemoglobin solution 1 (n = 5) using the same methods described above. Heparinized blood was obtained by heart puncture 10 min after infusion. The blood was centrifuged, and the plasma was frozen at 70°C for measurement of hemoglobin levels. Two separate groups of rats were also exchange-transfused with either unmodified hemoglobin (n = 8) or O-raffinose cross-linked hemoglobin solution 1 (n = 5), and urine was collected for 110 min starting immediately after completion of the infusion of SFH. At the end of the urine collection, heparinized blood was obtained by heart puncture for hemoglobin measurement. The volume of urine excreted over the 110 min was measured, and the urine was frozen for measurement of hemoglobin levels. Total free hemoglobin in plasma and urine was measured using a dual-beam spectrophotometric assay as previously described. The volume of urine excreted over the 110 min was measured, and the urine was frozen for measurement of hemoglobin levels. Total free hemoglobin in plasma and urine was measured using a dual-beam spectrophotometric assay as previously described (Blakney and Dinwoodie, 1975). Using this assay, the lowest level of detection of hemoglobin is 1 mg/dl.

In Vitro Assays of Hemoglobin-Nitric Oxide Interactions

Preparation of Endothelium-Derived Relaxing Factor Conjugate S-Nitrosothioglutathione. S-Nitrosothioglutathione (SNO-GSH) was prepared by reacting freshly prepared solutions of GSH with sodium nitrite at acidic pH, as previously described (Losoalzo, 1985; Mendelsohn et al., 1990). SNO-GSH was prepared within 10 min of use, kept at 4°C, and diluted as necessary into aqueous buffer immediately before addition to assay systems.

Preparation of Platelet-Rich Plasma. Peripheral blood was drawn from healthy adult human volunteers who had not consumed acetylsalicylic acid or any other platelet inhibitor for at least 7 days. The first 2 ml of blood drawn was discarded. Blood was then drawn into sodium citrate anticoagulant. The citrated blood was centrifuged (150g for 15 min at 22°C), and the supernatant (platelet-rich plasma) was removed for further processing.

Preparation of Gel-Filtered Platelets. Gel-filtered platelets (GFP) were obtained by passing platelet-rich plasma over a sepharose-2B column in Tyrode-HEPES-buffered saline. Tyrode-HEPES-buffered saline consisted of 140 mM NaCl, 6 mM HEPES, 2 mM NaHPO₄, 2 mM MgSO₄, 0.1% dextrose, and 0.4% bovine serum albumin (pH 7.4). Platelet counts were determined using a Coulter Counter (model ZM; Coulter Electronics, Hialeah, FL). Platelets were adjusted to 1.5 × 10⁶ platelets/ml by the addition of Tyrode-HEPES-buffered saline.

Measurement of Platelet Aggregation. Aggregation of GFP (0.2 ml) was monitored using a standard nephelometric technique in which changes in light transmission were recorded as a function of time (Born and Cross, 1963). Platelet aggregation was induced by adding 5 M ADP, and the absolute change of light transmission was recorded in a four-chamber aggregometer (Biodata, Hatboro, PA). GFP were incubated for 1 min at 37°C with either 1) SNO-GSH, 2) bovine endothelial cells on beads (ECBs), 3) SNO-GSH plus 1 µM hemoglobin or 4) ECBs plus 1 µM hemoglobin before the addition of ADP.

Measurement of Platelet cGMP Production. Measurements of platelet cGMP were made using previously published methods (Pradelles et al., 1989). Briefly, TCA (final concentration, 5% vol/vol) was added to GFP. Samples were vortexed, placed on ice, and centrifuged at 1500g for 10 min at 4°C. The supernatant was extracted with diethyl ether five times and assayed for cGMP by an enzyme-linked immunosorbent assay using cGMP antiserum (Cayman Chemical Co.).

Measurement of Platelet NO Production. We adapted a NO-selective microelectrode (Inter Medical Co., Ltd., Nagoya, Japan) (Ichimori et al., 1994) for use in a standard platelet aggregometer (Payton Associates, Buffalo, NY) to monitor platelet NO production and aggregation simultaneously. The NO monitoring device consisted of a headstage amplifier with a built-in power supply and two electrodes. The working electrode (0.2-mm diameter, made from Pt/Ir alloy) and counterelectrode were inserted using a micromanipulator into an aggregometer microcuvette containing a stir bar. The aggregometer, electrode, and headstage amplifier were housed in a Faraday cage to reduce electrical interference. The electrode current increased linearly from 10 to 350 pA over a range of NO concentrations. All experiments were conducted at constant temperature (37°C). Production of NO was determined by integrating the area under the response curve. Diethyl amine nonoate was used to derive a standard curve that was linear over the range of 10 to 500 nM with a typical correlation coefficient of 0.95.

Determination of Endothelium-Derived Relaxing Factor-Induced Inhibition of Platelet Aggregation. For the preparation of endothelial cell cultures on microcarrier beads (ECBs), bovine aortic endothelial cells (BAECs) were isolated and then grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum using established techniques (Freedman et al., 1995). Cells were grown to confluence on a microcarrier system of collagen-coated spherical beads (Cytodex) as previously described (Freedman et al., 1995). The ECBs were exposed to 30 M acetylsalicylic acid for 1 h at 37°C to inhibit cyclooxygenase activity and prostaglandin production and then washed three times immediately before use. Endothelial cells were checked for viability by trypan blue exclusion. ECBs were drawn up into a 1-ml Eppendorf pipette tip to deliver a fixed number of cell-coated beads as standardized in prior experiments (Freedman et al., 1995). Because a comparable number of beads was delivered to each test tube, we could assume that a comparable number of cells were present as well. The cell-coated beads were then dispersed into suspensions of GFP (Freedman et al., 1995) with or without 1 µM hemoglobin.

Measurement of Effect of Unmodified and Modified Hemoglobin on ECB-Induced Inhibition of Platelet Aggregation. BAECs grown on Cytodex beads (ECBs) were incubated with or without 1 µM unmodified or O-raffinose cross-linked hemoglobin solutions for 5 min. Then, GFP was added, and the suspension was stirred in an aggregometer for 5 min. The ECBs were allowed to settle, the GFP was removed, aggregation was induced with 5 µM ADP, and the extent of platelet aggregation was determined as described above. Cytodex beads without endothelial cells served as the control and had no effect on platelet aggregation. Also, preincubation of ECBs with the NO synthase inhibitor L-NAME before the addition of GPs abrogated inhibition of platelet aggregation, clearly demonstrating that endothelium-derived relaxing factor (EDRF) is the platelet-inhibitory factor in this assay.

Statistical Analyses

All data are expressed as mean ± S.E. Measurements of hemodynamic and renal function were obtained during four separate 20-min periods (see Materials and Methods). Because there was no statistical difference (as determined by ANOVA for repeated measures) among the four periods for any of the variables measured, the data from all four periods were averaged. Therefore, all the data presented represent the averaged data obtained over 80 min after exchange-transfusion. All variables of the seven groups of rats were compared by ANOVA. In those variables in which ANOVA demonstrated a difference among the groups, Duncan's or the Newman-Keuls test was performed. Statistics were done using SAS 6.12 statistical software. Values for individual clearance periods for SVR were compared using the Student’s t test with the Bonferroni correction. A p value of <.05 was considered significant.
Results

Comparison of Exchange Transfusion with Whole Blood, Unmodified Hemoglobin, O-Raffinose SFH Solution 1, and Iso-oncotic Albumin

**Hematocrit.** Hematocrit values, measured at the midpoint of each of the four periods of observation, were averaged. There was no difference between the hematocrit of sham-operated rats (46.7 ± 2%) and rats exchange-transfused with whole blood (47.0 ± 5%). The hematocrits of animals exchange-transfused with HSA (34.0 ± 5%), unmodified hemoglobin (38.2 ± 6%), O-raffinose cross-linked hemoglobin solution 1 (35.4 ± 5%), O-raffinose cross-linked hemoglobin solution 2 (34.0 ± 6%), and O-raffinose cross-linked hemoglobin solution 3 (33.2 ± 1%) were lower than those of sham-operated rats and rats exchange-transfused with whole blood (p < .01) (Fig. 1). The hematocrit of the rats exchange-transfused with unmodified hemoglobin was higher than the hematocrit of rats exchanged with HSA or O-raffinose cross-linked hemoglobin solutions 1, 2, and 3 (p < .01) (Fig. 1).

**Systemic Hemodynamics.** MAP in rats exchange-transfused with unmodified hemoglobin was increased by ~14% compared with whole blood-exchanged animals (Table 2). O-Raffinose cross-linked hemoglobin solution 1 increased the MAP to a comparable extent (~10%) (Table 2). In contrast, in rats exchange-transfused with O-raffinose cross-linked hemoglobin solutions 2 and 3, MAP increased by ~3 to 5%, a change that was not statistically significant (Table 2).

The unmodified hemoglobin markedly reduced CO by ~21% compared with rats exchange-transfused with whole blood. In contrast, the CO of rats exchange-transfused with O-raffinose cross-linked hemoglobin solutions 1, 2, and 3 was comparable to the CO of blood-exchanged and sham rats. HSA solution increased CO compared with whole blood-exchanged animals by ~30% (Table 2).

Unmodified hemoglobin solution produced a marked increase in SVR (~45%) compared with animals exchange-transfused with whole blood (Table 2, Fig. 2). O-Raffinose cross-linked hemoglobin solutions 1, 2, and 3 increased SVR to a substantially lesser extent (~10–14%) compared with unmodified hemoglobin (Table 2, Fig. 2). HSA produced a fall in SVR of ~26% (Table 2). SVR was comparable among the four observation periods in rats exchange-transfused with whole blood, unmodified hemoglobin, and O-raffinose cross-linked hemoglobin solution 1 (Fig. 2).

**Renal Hemodynamics.** Unmodified hemoglobin markedly reduced GFR, RPF, and RBF and increased RVR 2-fold compared with rats exchange-transfused with whole blood (Table 3). In contrast, O-raffinose cross-linked hemoglobin solutions 1, 2, and 3 had no effect on GFR, RBF, or RVR compared with rats exchange-transfused with whole blood (Table 3). However, rats exchange-transfused with O-raffinose cross-linked hemoglobin solutions 2 and 3 had a greater GFR (by ~22%) and RBF (by ~10%) and a lower RVR (by ~25%) than rats exchange-transfused with O-raffinose cross-linked hemoglobin solution 1. There was no difference between O-raffinose cross-linked hemoglobin solutions 2 and 3 on GFR, RPF, or RVR. The filtration fraction (whole blood, 35 ± 1%; sham operated, 34 ± 1%; HSA, 31 ± 1%; unmodified hemoglobin solution, 32 ± 2%; O-raffinose cross-linked hemoglobin solution 1, 32 ± 1%; O-raffinose cross-linked hemoglobin solution 2, 33 ± 1%; O-raffinose cross-linked hemoglobin solution 3, 33 ± 1%; HSA solution, 33 ± 1%) was comparable among the four observation periods (Table 3).

---

**Fig. 1.** Effect of exchange-transfusion on hematocrit. Rats were subjected to sham surgery (n = 5) or exchange-transfused with whole blood (n = 16), HSA (n = 10), unmodified hemoglobin (n = 15), O-raffinose cross-linked solution 1 (n = 25), O-raffinose cross-linked solution 2 (n = 10), or O-raffinose cross-linked solution 3 (n = 10). *p < .01 versus blood group. †p < .01 versus O-raffinose cross-linked solutions 1, 2, and 3, and HSA.
hemoglobin solution 3, 32 ± 1%) was not statistically different between any of the groups.

**Urinary Electrolyte Excretion.** The absolute and fractional excretion of sodium was higher in animals exchange-transfused with HSA, unmodified hemoglobin, and all three O-raffinose cross-linked hemoglobin solutions compared with the whole blood-exchanged and sham groups (Table 4). However, sodium excretion was comparable in rats exchanged

---

**TABLE 2**

Comparison of effects of sham surgery and exchange transfusion with whole blood, HSA, unmodified hemoglobin, and O-raffinose cross-linked hemoglobin solutions 1, 2, and 3 on MAP, CO, and SVR

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>MAP (mm Hg)</th>
<th>CO (μl/min/300 g)</th>
<th>SVR (mm Hg/μl/min/300 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood (n = 16)</td>
<td>109 ± 3</td>
<td>112 ± 3</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>Sham (n = 5)</td>
<td>111 ± 2</td>
<td>108 ± 3</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>HSA (n = 10)</td>
<td>104 ± 2</td>
<td>144 ± 5</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>Unmodified hemoglobin solution (n = 15)</td>
<td>124 ± 2, a,b,c</td>
<td>88 ± 4, a,b,c</td>
<td>1.43 ± 0.06, a,b,c</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 1 (n = 25)</td>
<td>119 ± 2, a,b,c</td>
<td>108 ± 2, d</td>
<td>1.13 ± 0.03, a,b,c</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 2 (n = 10)</td>
<td>113 ± 5</td>
<td>102 ± 3, d</td>
<td>1.10 ± 0.04, a,b,c</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 3 (n = 10)</td>
<td>114 ± 4</td>
<td>106 ± 4, d</td>
<td>1.09 ± 0.05, a,b,c</td>
</tr>
</tbody>
</table>

* p < .01 compared with rats exchange-transfused with whole blood.
* p < .01 compared with sham-operated rats.
* p < .01 compared with group exchange-transfused with HSA.
* p < .01 compared with group exchange-transfused with unmodified hemoglobin.

**Fig. 2.** Effect on SVR during each of four periods of observation obtained after exchange-transfusion. Rats exchanged-transfused with blood (stippled columns), O-raffinose cross-linked solution 1 (cross-hatched columns), and unmodified hemoglobin (filled columns). *p < .01 versus blood group.
† p < .01 versus O-raffinose cross-linked solution 1.

**TABLE 3**

Comparison of effects of sham surgery and exchange transfusion with whole blood, HSA, unmodified hemoglobin, and O-raffinose cross-linked hemoglobin solutions 1, 2, and 3 on GFR, RPF, RBF, and RVR

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>GFR (ml/min/300 g)</th>
<th>RPF (ml/min/300 g)</th>
<th>RBF (ml/min/300 g)</th>
<th>RVR (mm Hg/ml/min/300 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood (n = 16)</td>
<td>2.8 ± 0.1</td>
<td>8.1 ± 0.3</td>
<td>15.4 ± 0.6</td>
<td>8.0 ± 0.6</td>
</tr>
<tr>
<td>Sham (n = 5)</td>
<td>3.2 ± 0.2</td>
<td>9.4 ± 0.5</td>
<td>17.6 ± 0.9</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>HSA (n = 10)</td>
<td>3.2 ± 0.1</td>
<td>10.5 ± 0.5, *</td>
<td>16.0 ± 0.8</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>Unmodified hemoglobin solution (n = 15)</td>
<td>3.8 ± 0.2, a,b,c</td>
<td>5.9 ± 0.5, a,b,c</td>
<td>9.6 ± 0.9, a,b,c</td>
<td>15.9 ± 1.9, a,b,c</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 1 (n = 25)</td>
<td>2.7 ± 0.1, d</td>
<td>9.0 ± 0.3, d</td>
<td>14.0 ± 0.5, a,b,c,d</td>
<td>9.2 ± 0.4, a,b,c,d</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 2 (n = 10)</td>
<td>3.4 ± 0.1, e,e</td>
<td>10.4 ± 0.5, d, e</td>
<td>15.8 ± 0.6, e,e</td>
<td>7.3 ± 0.2, e,e</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 3 (n = 10)</td>
<td>3.2 ± 0.1, d, e,e</td>
<td>10.4 ± 0.4, d, e,e</td>
<td>15.6 ± 0.6, d, e,e</td>
<td>7.6 ± 0.4, d, e,e</td>
</tr>
</tbody>
</table>

* p < .01 compared with rats exchange-transfused with whole blood.
* p < .01 compared with sham-operated rats.
* p < .01 compared with group exchange-transfused with HSA.
* p < .01 compared with group exchange-transfused with unmodified hemoglobin.
* p < .01 compared with group exchange-transfused with O-raffinose cross-linked solution 1.
with HSA, unmodified hemoglobin, and all three O-raffinose cross-linked hemoglobin solutions (Table 4). The effects of HSA, unmodified hemoglobin, and O-raffinose cross-linked hemoglobin solution 1 are likely due, at least in part, to the consequence of infusing a large volume of red cell-free solution containing both colloid and sodium.

**Comparison of Plasma Hemoglobin Levels and Urinary Excretion Rates of Unmodified Hemoglobin and O-Raffinose Cross-Linked Hemoglobin Solution 1**

Both unmodified hemoglobin and O-raffinose cross-linked hemoglobin solution 1 had comparable plasma hemoglobin levels 10 min after exchange-transfusion (Table 5). The concentration of both hemoglobin solutions decreased over the 110-min period of observation, but the plasma level of the unmodified hemoglobin fell to a far greater extent than that of the O-raffinose cross-linked hemoglobin (Table 5). A substantial amount (282 ± 0.1 μg/min) of the unmodified hemoglobin was excreted in the urine. The total amount of unmodified hemoglobin eliminated by the kidney represented at least 35% of the administered load. In contrast, no hemoglobin was detectable in the urine in rats exchange-transfused with O-raffinose cross-linked hemoglobin solution 1.

**Interactions Between Hemoglobin Solutions and NO in Vitro**

Effect of Hemoglobin Solutions on Platelet Release of NO. Platelets activated with 5 μM ADP produce NO, which diffuses from the platelets and inhibits the recruitment of additional platelets to the growing platelet thrombus (Freedman et al., 1997). We have adapted a microelectrode methodology to measure platelet NO production in real time during the platelet aggregation response. NO bound by hemoglobin is not detected by this method.

Gel-filtered platelet aggregation was induced with 5 μM ADP in the absence or presence of 1 μM heme in the form of unmodified hemoglobin or O-raffinose cross-linked hemoglobin solutions 1, 2, or 3. As shown in Table 6, the unmodified hemoglobin inhibited NO release by aggregating platelets almost completely (by ~99%). The O-raffinose cross-linked hemoglobin solutions also reduced NO release substantially but did so to a significantly less extent than the unmodified hemoglobin (by ~80%) (Table 6). NO release in the presence of the three O-raffinose cross-linked hemoglobin solutions was comparable (Table 7).

**Effect of Hemoglobin Solutions on Platelet cGMP Production Induced by SNO-GSH.** Gel-filtered platelets were incubated with 10 μM SNO-GSH for 5 min. Platelet proteins were then precipitated with TCA, and cGMP content was determined in the supernatant. Unmodified hemoglobin markedly suppressed platelet cGMP below control values (Table 7). In contrast, all the O-raffinose cross-linked hemoglobin solutions had only a modest effect on cGMP production, which was not statistically different from control values (Table 7).

**Effect of Hemoglobin Solutions on EDRF Inhibition of Platelet Aggregation.** BAECs grown on Cytodex beads (ECBs) were incubated with or without 1 μM unmodified or O-raffinose cross-linked SFH solutions for 5 min. Then, GFP was added, and the suspension was stirred in an aggregometer for 5 min. The ECBs were allowed to settle, the GFP was removed, and aggregation was induced with 5 μM ADP. As shown in Fig. 3, unmodified hemoglobin reversed the inhibition of platelet aggregation by EDRF released from ECBs, with the extent of aggregation increasing 230% above control (Fig. 3). In contrast, O-raffinose cross-linked hemoglobin solutions had a far more modest effect on EDRF inhibition of platelet aggregation, increasing the extent of aggregation by only 130 to 160%. There was no significant difference among

### TABLE 5

<table>
<thead>
<tr>
<th>Plasma concentration of unmodified hemoglobin and O-raffinose cross-linked hemoglobin solution 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min Postinfusion</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>mg/dl</td>
</tr>
<tr>
<td>Unmodified hemoglobin (n = 12)</td>
</tr>
<tr>
<td>Cross-linked hemoglobin solution 1 (n = 10)</td>
</tr>
</tbody>
</table>

a p < .01 compared with 110 min.

b p < .01 compared with unmodified hemoglobin.
As a result, the increase in MAP induced by O-raffinose cross-linked hemoglobin solution 1 was proportionately comparable to the increase in SVR induced by this modified hemoglobin solution. Also, unlike the unmodified hemoglobin, O-raffinose cross-linked hemoglobin solution 1 had no effect on the GFR, RBF, or RVR compared with rats exchanged with whole blood (Tables 3 and 4). Thus, our data demonstrate that the process of O-raffinose cross-linking markedly alters the vasoactive properties of the unmodified hemoglobin solution. Furthermore, the adverse effects of unmodified hemoglobin on cardiac and renal function were completely eliminated by O-raffinose cross-linking.

Available data suggest that the beneficial effects of O-raffinose cross-linking of hemoglobin on CO may not be shared by other some other methods used to modify hemoglobin solutions. In phase II clinical trials, a polymerized form of bovine hemoglobin (HBOC-201; Biopure Corp. Inc., Cambridge, MA) was reported to reduce CO in patients, an effect that resulted in a net reduction in oxygen delivery (Kasper et al., 1996). It remains to be determined whether the beneficial effects of O-raffinose cross-linking on CO observed in this study in rats occur in humans as well.

We also examined the extent to which the residual vasoactive effects of the O-raffinose cross-linked hemoglobin solution 1 are due to the presence of non-cross-linked hemoglobin and tetramers of hemoglobin in this solution (Table 1). We compared the effects of O-raffinose hemoglobin in which the concentration of the non-cross-linked hemoglobin (O-raffinose cross-linked hemoglobin solution 2) or non-cross-linked as well as cross-linked tetrameric hemoglobin (O-raffinose cross-linked hemoglobin solution 3) was almost completely eliminated (Table 1). The effects of exchange-transfusion with these two hemoglobin solutions on systemic and intrarenal hemodynamics were no different from those of whole blood (Table 6). Thus, the removal of non-cross-linked and tetrameric hemoglobin from O-raffinose cross-linked hemoglobin 1 did not provide any substantive advantages in these studies (Table 2).

Urinary excretion of sodium was increased in rats exchange-transfused with all the hemoglobin solution, whether unmodified or O-raffinose cross-linked (Table 4). The effect of the hemoglobin solutions on sodium excretion was comparable to that observed in rats exchange-transfused with the HSA. The cause of this increase in sodium excretion in the HSA and hemoglobin-exchanged rats is probably multifactorial. However, rats exchanged with either the hemoglobin solutions or HSA received about twice the volume of red cell-free electrolyte solution as rats exchanged with whole blood. This augmented sodium load likely contributed to the natriuresis associated with all these solutions.

We also demonstrated that as expected, the plasma retention time of the O-raffinose cross-linked hemoglobin solution is prolonged compared with that of unmodified hemoglobin (Table 5), an effect that was likely due to the increased size of the O-raffinose cross-linked hemoglobin, which prevented the loss of hemoglobin in the urine (Table 5) and slowed the diffusion of hemoglobin across capillary walls.

Additional studies were done to examine the mechanisms responsible for the striking reduction in vasoactivity associated with O-raffinose cross-linking of hemoglobin. One mechanism by which the unmodified hemoglobin molecule may potentially induce systemic and intrarenal vasoconstriction

### TABLE 6

<table>
<thead>
<tr>
<th>Effect of hemoglobin solutions on platelet NO release</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>nM</strong></td>
</tr>
<tr>
<td><strong>GFP</strong></td>
</tr>
<tr>
<td><strong>GFP + unmodified hemoglobin</strong></td>
</tr>
<tr>
<td><strong>GFP + O-raffinose cross-linked hemoglobin solution 1</strong></td>
</tr>
<tr>
<td><strong>GFP + O-raffinose cross-linked hemoglobin solution 2</strong></td>
</tr>
<tr>
<td><strong>GFP + O-raffinose cross-linked hemoglobin solution 3</strong></td>
</tr>
</tbody>
</table>

GFP, gel-filtered platelets stimulated with 5 μM ADP (n = 3).

*p < .05 compared with GFP.

**p < .05 compared with GFP + unmodified hemoglobin.

#### TABLE 7

<table>
<thead>
<tr>
<th>Effect of hemoglobin solutions on SNO-GSH-induced platelet cGMP production</th>
<th>pmol/108 platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP</strong></td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td><strong>GFP + SNO-GSH</strong></td>
<td>0.35 ± 0.05a</td>
</tr>
<tr>
<td><strong>GFP + SNO-GSH + unmodified hemoglobin</strong></td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td><strong>GFP + SNO-GSH + O-raffinose cross-linked hemoglobin solution 1</strong></td>
<td>0.21 ± 0.14abc</td>
</tr>
<tr>
<td><strong>GFP + SNO-GSH + O-raffinose cross-linked hemoglobin solution 2</strong></td>
<td>0.16 ± 0.06abc</td>
</tr>
<tr>
<td><strong>GFP + SNO-GSH + O-raffinose cross-linked hemoglobin solution 3</strong></td>
<td>0.17 ± 0.05abc</td>
</tr>
</tbody>
</table>

SNO-GSH, S-nitrosothioglutathione.

(a = 3).

*p < .05 compared with all other samples.

**p < .05 compared with GFP + SNO-GSH.

***p < .05 compared with unmodified hemoglobin.

the three O-raffinose cross-linked hemoglobin solutions (Fig. 3).

**Discussion**

We compared the systemic and renal hemodynamic effects of O-raffinose cross-linked hemoglobin solutions with the highly purified unmodified hemoglobin used to make the cross-linked hemoglobin. Because both the unmodified and modified solutions are of comparable purity (Adamson and Moore, 1998), any differences in cardiac, renal, or vascular effects of these solutions cannot be ascribed to differences in the amount of nonhemoglobin contaminants. This represents the first report in which the hemodynamic and renal effects of cross-linked hemoglobin solutions have been compared with those of the its unmodified precursor.

We demonstrated marked differences in the systemic and intrarenal effects between modified and unmodified hemoglobin solutions. The unmodified hemoglobin solution resulted in a marked elevation in SVR (of ~45%) (Table 2). MAP was also increased by the unmodified hemoglobin, but this increase was relatively modest (~14%) (Table 2). The marked proportional difference in the severity of the increase in MAP and SVR induced by unmodified hemoglobin can be accounted for by the profound depressant effect of unmodified hemoglobin on CO (Table 2).

The effects of O-raffinose cross-linked hemoglobin solution 1 on systemic and renal hemodynamics were strikingly different from those of the unmodified hemoglobin. The increase in SVR induced by O-raffinose cross-linked hemoglobin solution 1 (~14%) was relatively modest compared with unmodified hemoglobin (Table 2). Furthermore, O-raffinose cross-linked hemoglobin solution 1 had no deleterious effect on CO.

As a result, the increase in MAP induced by O-raffinose cross-linked hemoglobin solution 1 was proportionately comparable to the increase in SVR induced by this modified hemoglobin solution. Also, unlike the unmodified hemoglobin, O-raffinose cross-linked hemoglobin solution 1 had no effect on the GFR, RBF, or RVR compared with rats exchanged with whole blood (Tables 3 and 4). Thus, our data demonstrate that the process of O-raffinose cross-linking markedly alters the vasoactive properties of the unmodified hemoglobin solution. Furthermore, the adverse effects of unmodified hemoglobin on cardiac and renal function were completely eliminated by O-raffinose cross-linking.

Available data suggest that the beneficial effects of O-raffinose cross-linking of hemoglobin on CO may not be shared by other some other methods used to modify hemoglobin solutions. In phase II clinical trials, a polymerized form of bovine hemoglobin (HBOC-201; Biopure Corp. Inc., Cambridge, MA) was reported to reduce CO in patients, an effect that resulted in a net reduction in oxygen delivery (Kasper et al., 1996). It remains to be determined whether the beneficial effects of O-raffinose cross-linking on CO observed in this study in rats occur in humans as well.

We also examined the extent to which the residual vasoactive effects of the O-raffinose cross-linked hemoglobin solution 1 are due to the presence of non-cross-linked hemoglobin and tetramers of hemoglobin in this solution (Table 1). We compared the effects of O-raffinose hemoglobin in which the concentration of the non-cross-linked hemoglobin (O-raffinose cross-linked hemoglobin solution 2) or non-cross-linked as well as cross-linked tetrameric hemoglobin (O-raffinose cross-linked hemoglobin solution 3) was almost completely eliminated (Table 1). The effects of exchange-transfusion with these two hemoglobin solutions on systemic and intrarenal hemodynamics were no different from those of whole blood (Table 6). Thus, the removal of non-cross-linked and tetrameric hemoglobin from O-raffinose cross-linked hemoglobin 1 did not provide any substantive advantages in these studies (Table 2).

Urinary excretion of sodium was increased in rats exchange-transfused with all the hemoglobin solution, whether unmodified or O-raffinose cross-linked (Table 4). The effect of the hemoglobin solutions on sodium excretion was comparable to that observed in rats exchange-transfused with the HSA. The cause of this increase in sodium excretion in the HSA and hemoglobin-exchanged rats is probably multifactorial. However, rats exchanged with either the hemoglobin solutions or HSA received about twice the volume of red cell-free electrolyte solution as rats exchanged with whole blood. This augmented sodium load likely contributed to the natriuresis associated with all these solutions.

We also demonstrated that as expected, the plasma retention time of the O-raffinose cross-linked hemoglobin solution is prolonged compared with that of unmodified hemoglobin (Table 5), an effect that was likely due to the increased size of the O-raffinose cross-linked hemoglobin, which prevented the loss of hemoglobin in the urine (Table 5) and slowed the diffusion of hemoglobin across capillary walls.

Additional studies were done to examine the mechanisms responsible for the striking reduction in vasoactivity associated with O-raffinose cross-linking of hemoglobin. One mechanism by which the unmodified hemoglobin molecule may potentially induce systemic and intrarenal vasoconstriction.
is by inactivating or binding NO (Martin et al., 1986; Baylis et al., 1990; Lieberthal et al., 1991, 1997; Wennmalm et al., 1992; Schultz et al., 1993; Kilbourn et al., 1994; Thompson et al., 1994). The ability of hemoglobin to inhibit endothelium-dependent vasodilation (EDRF) was demonstrated long before endothelial release of NO was shown to account for this activity (Palmer et al., 1987). Subsequently, the complex interactions between hemoglobin and NO have been elucidated (Wennmalm et al., 1992, Iwamoto and Morin, 1993; Kilbourn et al., 1994; Motterlini et al., 1996). Hemoglobin can also bind NO in at least two ways. A stable complex can be formed between the heme group of deoxyhemoglobin and NO (Motterlini et al., 1996). Also, binding of NO to sulfhydryl groups on the globin chain of hemoglobin has recently been described (Jia et al., 1996). In addition to binding NO, hemoglobin can inactivate NO by producing superoxide (Rifkind et al., 1988, Winterbourn, 1990), which oxidizes NO to peroxynitrite and, ultimately, nitrite and nitrate.

To determine whether the reduced vasoactivity of the O-raffinose cross-linked hemoglobin solutions was related to alterations in the interactions between hemoglobin and NO, we compared the effects of the unmodified and O-raffinose cross-linked hemoglobin solutions on NO activity in vitro. We used three different assay systems, including platelet NO production (Table 6), platelet cGMP production (Table 7), and EDRF-induced inhibition of platelet aggregation (Fig. 3). These in vitro assays all indicated that the three O-raffinose cross-linked hemoglobin solutions have substantially less NO binding and/or inactivating properties than the unmodified hemoglobin solution in vitro.

On the basis of these in vitro data, we hypothesize that O-raffinose cross-linking reduces the degree to which NO is scavenged by the hemoglobin molecule and that this contributes to the reduced vasoconstrictor effects of O-raffinose cross-linked hemoglobin. However, it is important to point out that the role of hemoglobin-NO interactions in mediating the vasoactive effects of cell-free hemoglobin remains highly controversial (Doherty et al., 1998; Rohlfis et al., 1998). Additional studies are necessary to elucidate the mechanism or mechanisms by which the process of O-raffinose cross-linking reduces NO scavenging by hemoglobin.

In summary, unmodified hemoglobin caused marked systemic and intrarenal vasoconstriction and a profound reduction in both CO and GFR. In marked contrast, O-raffinose cross-linked hemoglobin solutions increased SVR only modestly and had no effect on CO, GFR, or renal hemodynamics. Because this is the first in vivo study that has compared unmodified and modified hemoglobin solutions of identical purity, we were able to establish that the hemoglobin molecule itself has vasoconstrictor properties that cannot be attributed to the effects of nonhemoglobin contaminants. We have also shown that the process of O-raffinose cross-linking substantially ameliorates the vasoconstrictor effects of unmodified hemoglobin and eliminates the adverse effects of unmodified hemoglobin of cardiac and renal function. The mechanisms responsible for the beneficial effects of O-raffinose cross-linking have not been directly examined in vivo. However, our in vitro studies suggest that O-raffinose cross-linked hemoglobin inactivates NO to a lesser extent than unmodified hemoglobin. We therefore suggest that the differences in the hemodynamic effects of the unmodified and O-raffinose cross-linked hemoglobin solutions are related to differences in the extents to which NO is inactivated by modified and unmodified hemoglobin solutions in vivo. The development of a hemoglobin solution with no acute nephrotoxic or cardiotoxic effects is an encouraging advance in the development of a therapeutically useful HBOC.

Acknowledgments

We are grateful to Hemosol Inc. for providing us with all the hemoglobin solutions used in these studies.
References


Wilfred Lieberthal, M.D., Renal Section, Evans Building, Room 428, Boston Medical Center, 88 East Newton St., Boston, MA 02118. E-mail: wliebert@bu.edu