Vascular response to infusions of a nonextravasating hemoglobin polymer

BARBARA MATHESON,1 HERMAN E. KWANSA,2 ENRICO BUCCI,2 ANNETTE REBEL,3 AND RAYMOND C. KOEHLER3
1Department of Physiology, Dental School, and 2Department of Biochemistry and Molecular Biology, Medical School, University of Maryland, Baltimore 21201; and 3Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland 21205

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Matheson, Barbara, Herman E. Kwansa, Enrico Bucci, Annette Rebel, and Raymond C. Koehler. Vascular response to infusions of a nonextravasating hemoglobin polymer. J Appl Physiol 93: 1479–1486, 2002.—The clinical utility of cross-linked tetrameric hemoglobin solutions is limited by peripheral vasoconstriction thought to be due to scavenging of nitric oxide. In addition, transfusion of crude preparations of hemoglobin polymers can cause arterial hypertension. We tested the hypothesis that eliminating low-molecular-weight components from the polymer solution would prevent extravasation and its associated pressor response. A zero-link polymer of bovine hemoglobin was developed without chemical linkers left between the tetramers. Transfusion of unprocessed preparations of these polymers in rats resulted in appearance of the polymer in the renal hilar lymph. However, eliminating the low-molecular-weight components with a 300-kDa diafiltration resulted in an average hydrodynamic radius of 250 Å and in undetectable levels of polymer in hilar lymph. Exchange transfusion in anesthetized rats and cats and in awake cats produced no increase in arterial pressure. In anesthetized cats, exchange transfusion with an albumin solution reduced hematocrit from 30 to 18%, increased cerebral blood flow, and diluted pial arterioles. In contrast, reducing hematocrit by transfusion of the diafiltered polymer did not increase cerebral blood flow as pial arterioles constricted. These results are consistent with the hypothesis that the increase in arterial pressure associated with cell-free hemoglobin transfusion depends on hemoglobin extravasation. Constriction observed in the cerebrovascular bed with a nonextravasating hemoglobin polymer at low hematocrit is presumably a regulatory response to prevent overoxygenation at low blood viscosity.

Two major problems hinder their development: 1) the intravascular retention of infused hemoglobin is very short, with half time in circulation much <1 h (27); 2) more importantly, vascular constriction occurs, which results in increased mean arterial pressure (MAP) (pressor response). This latter action decreases regional blood flow, thereby negating the increase in O2 delivery that was the original intent for using cell-free hemoglobins. Moreover, after infusion with a stabilized tetrameric hemoglobin, the reduction of blood flow is severe in the renal and splanchnic beds, whereas cerebral and coronary beds appear to be spared from reduction in blood flow (9, 26). Whereas well-hydrated experimental animals survive without evidence of altered kidney function (27), the decrease in renal blood flow would increase the risk of kidney failure in hypovolemic, dehydrated subjects, discouraging the use of hemoglobins in clinical settings.

The short half-life in circulation is a consequence of the rapid elimination in the urine and extravasation into tissues (27). The hemoglobinuria is due to the dissociability of tetrameric hemoglobin into dimers, which are small enough to pass the glomerular filter (15). Stabilized tetrameric hemoglobin, i.e., hemoglobin intramolecularly cross-linked between subunits, does not dissociate into dimers, cannot pass the glomerular filter, and has a longer half time of retention, which is near 3–4 h in the rat (5, 7, 12). However, in postglomerular and other capillary beds, extravasation remains a problem.

The permeability and permselectivity of endothelial capillary walls is a very complex subject (14). In a simplified view, as reported in the review by Rippe and Haraldsson (20), it can be considered a bimodal phenomenon, because of the presence of two major categories of pores, namely small pores with a radius near 40 Å and large pores with a radius of 250–300 Å. In addition (20), the endothelial wall carries a negative charge, which adds to the permselectivity of the pores. In the glomerulus, the negative charge reduces the size of the small pores to an effective radius of 30–35 Å. For

HEMOGLOBIN SOLUTIONS ARE INVESTIGATED with the goal of obtaining cell-free oxygen carriers as red cell substitutes for clinical infusional fluids. Conveniently, hemoglobin solutions can be easily sterilized, stored, and used without need for testing immunological compatibility.

Address for reprint requests and other correspondence: E. Bucci, Univ. of Maryland Medical School, Dept. of Biochemistry and Molecular Biology, 108 N. Greene St., Baltimore MD 21201 (E-mail: ebucci@umaryland.edu).

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this reason, despite a large surface available for the diffusion and elimination of catabolic products, glomeruli constitute a very selective barrier to macromolecular filtration. Similar to albumin, nondissociable, intramolecularly cross-linked hemoglobin tetramers, with a radius near 30 Å and a distinct negative charge, are not filtered through the glomerulus, whereas the dimeric form of hemoglobin with a radius near 24 Å and a decreased negative charge go readily across. Presumably because of the large pores, stabilized hemoglobin tetramers extravasate across postglomerular capillary beds and appear in the hilar lymph (16). It is likely that extravasation across the large pores of capillary beds accounts for the short half time of nondissociable tetramers [near 4 h vs. the 6 h for equal-size serum albumin (3, 17)].

The mechanism for the pressor response is thought to be the scavenging of the vasodilating agent nitric oxide (NO) by cell-free hemoglobin (17). It has also been suggested that decreased blood viscosity would decrease the shear-induced production of NO (28). In the former case, extravasation of hemoglobin across the endothelium into the area in which NO is utilized for the relaxation of the smooth muscles is important. In the other case, a large viscous hemoglobin polymer may contribute the necessary shear to stimulate production of NO by the endothelium.

In essence, there seems to be a relationship among extravasation, pressor response, and retention time, which could be investigated by infusions of a nonextravasating hemoglobin molecule. If size is the critical parameter to prevent extravasation, it is necessary to find a hemoglobin polymer with a size comparable to that of the large pores. A globular polymer including 30 hemoglobin tetramers would be necessary to obtain a diameter near 200 Å, with a mass near 2.0 MDa.

Polymerized hemoglobin solutions are commonly obtained by treatment with either glutaraldehyde or raffinose (6, 22). Glutaraldehyde is a bifunctional reagent that produces polymers by forming Schiff bases with the amino groups of lysyl residues in adjacent molecules. The Schiff base is not a stable covalent bond and must be stabilized by reduction with borohydrides. This latter reaction develops gaseous hydrogen, which, in hemoglobin solutions, leads to excessive foaming, which is difficult to control. In addition, the residual highly toxic glutaraldehyde impurities are difficult to completely eliminate by methods available in nonindustrial scientific laboratories. Polymerization with raffinose is performed in the presence of carbon monoxide, which is difficult to remove from the polymer in nonindustrial settings. Moreover, the products of these polymerization are highly heterogeneous, with molecular sizes distributed over a wide range, even including residual tetrameric molecules.

Here we describe the production of a large hemoglobin polymer with several advantages over the commonly used polymeric hemoglobin mentioned above. The novelty of the process is the “zero-link” reaction, so defined, because the final product contains none of the linking agent, thereby avoiding potential toxicity and the unloading after infusion of substances with difficult catabolism or toxic properties. Also, this process can be performed in nonindustrial laboratories. The final polymer has an average radius of 250 Å and an average mass in excess of 20 MDa.

Extravasation was assessed by observing the appearance or absence, after infusion, of hemoglobin material in renal hilar lymph in the rat. Vasoactivity was assessed by monitoring changes in MAP before and after polymer infusions and by direct observation of the diameters of brain pial arterioles via a cranial window in the cat. We also compared data obtained with the new polymer, after removal of low-molecular-weight material, with previous data obtained with tetrameric cross-linked hemoglobins and nonpurified preparations of the polymer.

MATERIALS AND METHODS

Animal Studies

Lymph studies. Experiments were done on male Sprague-Dawley rats weighing between 300 and 380 g and anesthetized with Inactin (120 mg/kg). Cannulas were placed in the trachea, right femoral vein and artery, and urinary bladder, as previously described (16). Each animal was given a saline (0.95% NaCl) load of 1 ml/100 g body wt at 0.1 ml/100 g body wt/min followed by a sustaining infusion at 0.01 ml/100 g body wt/min. The left kidney was exposed via a flank incision, and the renal lymphatics were identified and cannulated as previously described (16). After a control lymph sample was collected, an isovolemic exchange transfusion of zero-link polymerized adipoyl-cross-linked bovine hemoglobin (ZL-HbBv) was started. Exchange transfusion was accomplished via the femoral vein by using a syringe pump and simultaneous withdrawal of an equivalent volume of blood via the right femoral artery, as previously described (16).

Cardiovascular response in cats. All procedures were approved by the institutional animal care and use committee. Anesthesia was induced in laboratory-bred cats with 5% halothane. After oral intubation of the trachea, anesthesia was maintained on 1.5% halothane with mechanical ventilation while a femoral artery and vein were catheterized. For the remainder of the experiment, anesthesia was maintained by intravenous infusion of pentobarbital (10–12 mg·kg⁻¹·h⁻¹) plus fentanyl (60 µg·kg⁻¹·h⁻¹). A closed cranial window was constructed over the parietal cortex for monitoring pial arteriolar diameter by intravital microscopy (2). An isovolumetric exchange transfusion with a 10–12% ZL-HbBv solution was performed over ~30 min to reduce hematocrit (Hct) from ~30 to 21%. Other groups received either no transfusion or an exchange transfusion with a 5% albumin solution.

In a separate group of cats, the effect of ZL-HbBv transfusion on arterial pressure was assessed in the awake state. A femoral artery and vein were catheterized under sterile conditions during halothane anesthesia. One day later, an exchange transfusion of ZL-HbBv was performed to reduce Hct to ~23%. Arterial blood pressure was monitored with the awake cats lying unrestrained in a cage. Intermittent monitoring was performed over a 2-day period.

Physicochemical Methods

Static light scattering was measured with a DAWN multiangle light-scattering apparatus (Wyatt Technology, Santa Barbara, CA), according to the procedure recommended by...
the manufacturer (29), including continuous monitoring of the scattering of the elution profile emerging from a gel filtration column.

Dynamic light-scattering measurements were performed by using a DynaPro-801 instrument (Protein Solution, Charlotte, NC), following the instruction and software recommended by the company.

Oxygen binding isotherms were measured in 0.1 M Tris-Cl at pH 7.4 at 37°C by using a hemoxanalyzer apparatus (TCS, New Hope, PA).

The oncotic activity of the polymers was measured with a WESCOR 4000 oncometer. Solutions were made up in lactated Ringer and tested against lactated Ringer titrated to pH 7.4 with 0.08 M dibasic phosphate.

The reaction was stopped by adding of ethylene-diamine to a final concentration of 0.1 M, followed by dialysis against 50 mM phosphate at pH 7.4. In this buffer, the compound was pasteurized by heating at 70°C for 2 h, under anaerobic conditions obtained by flushing with nitrogen and adding 1 mg/ml of NaCl.

Viscosity was measured by using an Ostwald cuvette, following classic procedures.

Adipoyl cross-linked bovine hemoglobin (adipoyl-XL-HbBv) was obtained as described by reacting deoxy-bovine hemoglobin with bis(3,5-dibromosalicyl)adipate. No attempt was made to separate the two major components of the reaction cross-linked between the 81 lysines and 81-xe, respectively (13), across the opposite dimeric partners α₁β₁ and α₂β₂.

**Synthesis of ZL-HbBv**

The ZL-HbBv was obtained, starting with oxy-adipoyl-XL-HbBv at 6 g/dl, in the presence of 5 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1 mg/ml of 1-ethyl-3-(3-hydroxysulfosuccinimide in 0.1 M MOPS, pH 7.4 at 37°C. The reaction was stopped by the addition of ethylene-diamine to a final concentration of 0.1 M, followed by dialysis against 50 mM phosphate at pH 7.4. In this buffer, the compound was pasteurized by heating at 70°C for 2 h, under anaerobic conditions obtained by flushing with nitrogen and adding 1 mg/ml of NaCl.

The ZL-HbBv was obtained, starting with oxy-adipoyl-XL-HbBv at 6 g/dl, in the presence of 5 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1 mg/ml N-hydroxysulfosuccinimide in 0.1 M MOPS, pH = 6.7, at room temperature for 3 h at 37°C. The reaction was stopped by the addition of ethylene-diamine to a final concentration of 0.1 M, followed by dialysis against 50 mM phosphate at pH 7.4. In this buffer, the compound was pasteurized by heating at 70°C for 2 h, under anaerobic conditions obtained by flushing with nitrogen and adding 1 mg/ml of NaCl.

The difference between the molecular sieve elution patterns of ZL-HbBv before and after diafiltration over a 300-kDa NMW membrane is shown in Fig. 2. Before filtration (Fig. 2A), there is an overabundance of material with molecular weight near and below 500 kDa, including a distinct peak of residual tetrameric molecules. After diafiltration, the low-molecular-weight material is eliminated, and the retentate has an average molecular weight near 2 MDa. The heterogeneity of the molecular size can be estimated to range from R₅₀ = 120 Å (1.5 MDa, 92%) to R₅₀ = 290 Å (86 MDa, 8%).

![Fig. 1. Zero-link polymerizing reaction by activation of carboxyl groups with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). XL-HbBv, cross-linked bovine hemoglobin.](image-url)

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Chemistry of the Polymerizing Reaction

Water-soluble EDC was used to activate the side chain carboxylate groups of the surface of adipoyl-XL-HbBv, forming a highly reactive ester bond. These activated carboxyls react with the side chains of the lysyl residues (primary amines) of adjacent hemoglobin molecules to form stable amide bonds, similar to peptide bonds. Although the activated carboxyls can react also with sulfhydryl groups to generate thiol ester linkages, or with hydroxyl groups forming additional ester linkages, adjacent hemoglobin tetramers are polymerized mainly by pseudopeptide CO-NH bonds among adjacent tetramers. Thus no chemical linkers are left in the polymers, and the reaction can be defined as “zero link” (10). N-hydroxysulfosuccinimide or plain N-hydroxysuccinimide can be used to increase the yield of the reaction. The extent of polymerization can be modulated by changing the amounts of either the additives or EDC in the reaction mixture. The final product is indicated as ZL-HbBv. A schematic of the reaction is shown in Fig. 1.
Static light scattering indicated that the small components following the main peak in the Fig. 2B are only washouts of absorbed material with high molecular weight. This is the polymer used in the present experiments.

As expected, the colloid osmotic pressure per unit weight of ZL-HbBv is very low, ~10 times less than that of equal weights of bovine serum albumin and of a tetrameric hemoglobin intramolecularly cross-linked with sebacoyl residue (sebacoyl-HbA) (5) (Fig. 3). The concentration-dependent viscosity is shown in Fig. 4. Despite the vastly different molecular size, the viscosity of ZL-HbBv at 6 g/dl appears to match that of plasma. This and the linearity of the concentration dependence suggest that the polymer is a collection of very compact, rigid molecules, as if they had globular structures.

The intravascular retention time in the cat was 10.0 ± 2.4 h (n = 5), similar to that monitored in the rat (7.0 ± 1.5 h; n = 4).

Oxygen binding isotherms showed a partial pressure of oxygen at half saturation of hemoglobin near 4 mmHg and the absence of oxygen binding cooperativity at pH 7.4, 37°C, in 0.1 M Tris-HCl buffer. There is practically no pH dependence of the oxygen affinity and no sensitivity to polyphosphates and CO₂.

Lymph Data

As already reported (16), unmodified hemoglobin, nondissociable tetramers like sebacoyl-HbA (5), and the ZL-HbBv, when it contained low-molecular-weight material as shown in Fig. 2A, extravasate in the hilar lymph of the kidney. Instead, the 300-kDa retentate ZL-HbBv currently in use failed to appear in the lymph, as shown in Table 1. As an internal control for the permeability of the capillaries, Evans blue-labeled albumin was administered after the ZL-HbBv (11). It appeared in the lymph within a time of 8 min.

Vasoactivity

Transfusion of ZL-HbBv did not produce significant changes in MAP in anesthetized rats or anesthetized or awake cats. In contrast, transfusion of sebacoyl-HbA and other cross-linked tetrameric hemoglobins in the cat produces 20- to 30-mmHg increases in MAP (2, 25, 26). Figure 5 shows the raw data obtained in separate sets of anesthetized and awake cats. In both cases, there was no consistent increase in MAP over time.
after transfusion. The scattering of the data in the awake cats reflects their unrestrained activity.

Observation through a cranial window showed that the diameter of the pial arterioles on the brain surface of anesthetized cats is affected by infusion of ZL-HbBv. A 40% exchange transfusion was performed with either a 5% albumin or a 6% ZL-HbBv solution, to an equivalent Hct value. As shown in Fig. 6, albumin transfusion produced vasodilation and increased the blood flow, as measured with radiolabeled microspheres, from $52 \pm 9$ to $85 \pm 10$ ml·min$^{-1}$·100 g$^{-1}$ ($n = 5$). This result was expected from the reduced oxygen content. Instead, exchange transfusion with ZL-HbBv resulted in vasoconstriction. However, cerebral blood flow was unchanged in this case ($44 \pm 5$ to $43 \pm 2$ ml·min$^{-1}$·100 g$^{-1}$; means $\pm$ SE, $n = 5$).

**DISCUSSION**

In designing ZL-HbBv, we tried to avoid many of the previously mentioned production problems. To reduce heterogeneity, we adopted the strategy of polymerizing hemoglobins that were previously cross-linked intramolecularly so as to avoid the presence of dimeric components. Furthermore, the zero-link reaction (10), by directly linking together amino and carboxyl groups of the surface of adjacent molecules, avoided the presence inside the polymer of residual chemicals of unknown pharmacological activity and produced extensive polymerization, forming compact quasi-globular molecules. An important aspect of the polymerization reaction is that it can be controlled by calibrating the amount of the reagents, so that very little low-molecular-weight components are produced, and the diafiltration over a 300-kDa NMW membrane does not substantially decrease the yield of the preparation. Moreover, the reaction is not species specific and, as reported (19), can be used both on human and bovine hemoglobins or, presumably, any other mammalian hemoglobin. For convenience, in this report, we focused on the polymeric form of bovine hemoglobin.

The passage of molecules through the “large pores” of the capillaries is regulated by the size and charge of the molecules (20). In previous experiments, our laboratory has found that cross-linked hemoglobins that do not filter across the glomerular capillaries pass across the postglomerular capillaries and enter the renal lymph (16). In the present studies, we find that the 300-kDa retentate of ZL-HbBv, with an average radius of 240 Å, does not appear in renal hilar lymph. The heterogeneity of the polymer implies radii distributed between 120 and 330 Å. These are approximate esti-

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**Table 1. Plasma and lymph concentrations of the respective hemoglobins and serum albumin 30 min after the exchange transfusion**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasma concentration, mg/ml</th>
<th>Half time, min</th>
<th>Lymph concentration, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin</td>
<td>$23 \pm 0.7$</td>
<td>$305 \pm 3$</td>
<td>$7.9 \pm 2.0$</td>
</tr>
<tr>
<td>Sebacoyl-HbA</td>
<td>$25 \pm 0.5$</td>
<td>$220 \pm 9$</td>
<td>$6.6 \pm 2.0$</td>
</tr>
<tr>
<td>ZL-HbBv</td>
<td>$11 \pm 0.8$</td>
<td>$404 \pm 87$</td>
<td>Nondetectable</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE; $n = 4$ for all compounds. Sebacoyl-HbA, human hemoglobin intramolecularly cross-linked with sebacoyl residue; ZL-HbBv, zero-linked polymerized adipoyl-cross-linked bovine hemoglobin.

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Fig. 5. Mean arterial pressure response after infusion of ZL-HbBv in either anesthetized (A) or awake (B) cats. Each line represents an individual cat.

Fig. 6. Percent change in pial arteriolar diameter in a time control group with no transfusion and after exchange transfusion with albumin or ZL-HbBv solutions to reduce hematocrit from ~30 to 18%. Values are means $\pm$ SE.
mates, because precise details of the distribution are not accessible at these large molecular weights. This implies that molecules with radii $<100\,\text{Å}$ are still a possibility. These dimensions are smaller, although comparable, to those of the large pores of the endothelial walls. The total failure of ZL-HbBv to appear in renal lymph is probably due to the charge selectivity of the endothelium. In fact, ZL-HbBv is endowed with a large negative charge, as evident in electrophoresis, where its anodic mobility is much faster than that of the starting hemoglobin. It should be stressed that the prompt extravasation into the lymph of Evans blue-labeled albumin, administered at the end of these experiments, indicated that the pores of the endothelium were not damaged by the polymer.

The origin of the pressor response to cell-free hemoglobin infusion has yet to be clearly identified. At present, the prevailing hypothesis is that vasoconstriction is produced because infused hemoglobin scavenge endothelial-derived NO, leading to decreased NO-dependent relaxation of arteriolar smooth muscle. Evidence supporting this hypothesis includes the observation that a very similar pressor response can be produced by inhibiting the NO synthase (17), that inhibiting NO synthase after tetrameric hemoglobin transfusion produces little additional peripheral vasoconstriction (26), and that administration of the NO synthase substrate L-arginine attenuates the peripheral vasoconstrictor response to tetrameric hemoglobin transfusion (23).

The present data show that elimination of low-molecular-weight material from the infused polymer produced a longer lifetime and did not appear in the hilar lymph, indicating absence of extravasation from the postglomerular capillaries. It completely inhibited pressor response. This suggests that pressor response is produced only when hemoglobin extravasates. These observations are consistent with the report of Sakai et al. (21) that the extent of the pressor response is inversely proportional to the size of infused hemoglobins and that encapsulated hemoglobins, with diameters near 1,000 nm, do not produce vasoconstriction. Similarly, Abassi et al. (1) observed that the pressor response of a cross-linked hemoglobin decreases after polymerization with glutaraldehyde. We propose that the small residual pressor response, produced by these other polymers, was due to the presence of low-molecular-weight material. Sakai et al. (21) and Abassi et al. (1) do not report diafiltration with a 300-kDa NMW membrane (or similar) as a preparative step for their polymers. Analogous results were obtained also in our laboratory with our earlier preparations of ZL-HbBv, which were not diafiltered with a 300-kDa NMW membrane (16).

It can be proposed that, as long as scavenging of NO occurs within the luminal side of the endothelium, no pressor response is produced, presumably because the amount of NO scavenging by cell-free hemoglobin is not substantially greater than that scavenged by red cell-based hemoglobin. It is when the scavenging occurs on the abluminal side of the endothelium adjacent to smooth muscle that vasoconstriction apparently occurs.

This hypothesis is supported by data of Asano et al. (2), who have shown that the pial arterioles of brain, as observed through a cranial window in the cat, respond to the addition of acetylcholine with a vasodilation, which is insensitive to the luminal presence of infused sebacoyl-HbA (Fig. 7). Instead, the vasodilation produced by acetylcholine is inhibited by even very small amounts of hemoglobin, $10^{-7}\,\text{M}$, when it is added on the abluminal side of the vessels through the cranial window (Fig. 7). The response to acetylcholine depends on the endothelial production of NO. Blood vessels in the brain have the tight endothelial junctions, which constitute the blood-brain barrier. This prevents extravasation of large molecules.
sation of tetrameric hemoglobin. Thus the presence of hemoglobin in the plasma of a vascular bed with tight junctions does not interfere with the endothelial-dependent vascular relaxation, mediated by NO. Extravasation into the abluminal side is required to elicit a pressor response.

This evidence indicates a strong correlation between extravasation and pressor response, suggesting that only polymers retained after ultrafiltration on a membrane with a 300-kDa NMW membrane (or similar treatment) should be used in infusional fluids.

As an alternative to the NO scavenging hypothesis, Vandegriff and Winslow (28) proposed that the reduced shear viscosity of anemic blood, resulting from exchange transfusion, reduces the tonic stimulation of the endothelial sensor (8) responsible for the production of NO. The rheological effects of altered viscosity involving vasodilation and modifications of vascular beds (24) is so complex that its role in the pressor response remains controversial at this time. The hypothesis is also inconsistent with the present data, because the exchange transfusion with ZL-HbBv decreases the viscosity of blood without any MAP increase. It should also be noted that, in our laboratory, top-load infusions of tetrameric hemoglobins, which do not produce anemia and do not modify the viscosity of blood, still resulted in a pressor response.

Although we did not observe an increase in arterial pressure after ZL-HbBv infusion, brain pial arterioles did constrict. However, this constriction does not appear to be related to plasma or whole blood viscosity, resulting from the exchange transfusion and consequent decrease in the Hct. In fact, exchange transfusion to a similar Hct with an albumin solution with a viscosity similar to that of ZL-HbBv resulted, as expected, in dilation of pial arterioles (Fig. 6). Also, whereas the blood flow increased after albumin infusion, it remained unchanged with ZL-HbBv. It should be stressed that, because of the similar Hct, the only difference in the circulating plasma in the two cases was the total oxygen content of the fluids, produced by the presence of ZL-HbBv.

Our laboratory has evidence that, in hypoxic anemia, hemodilution produces an increased blood flow inversely proportional to the total amount of oxygen present in the bloodstream (25). This, coupled with the reduced viscosity of hemodiluted blood, regulates the size of the arterioles. Thus the reduced diameter of the brain arterioles after infusion of ZL-HbBv, in the absence of a general increase in MAP, in the absence of extravasation, and with an unchanged blood flow, appears to be a regulatory phenomenon that keeps the delivery of oxygen constant and prevents excessive oxygenation of the tissues when blood viscosity is decreased.

The concept that, in the absence of cerebral vasoconstriction, ZL-HbBv would provide excess oxygen delivery was not expected, because the polymer has a high oxygen affinity with partial pressure of oxygen at half saturation of hemoglobin of only 3 mmHg. Moreover, the concentration of the polymer in the circulating plasma averaged 2 g/dl, replacing only in part the 4 g/dl of red cell hemoglobin eliminated by the 40% exchange transfusion. It is as if a distinct amount of oxygen were delivered by the cell-free carrier, despite its low concentration and very high oxygen affinity. Thus the proposal that there is a potential excess oxygen delivery in vivo by cell-free carriers, possibly due to facilitated oxygen diffusion (28), deserves scrutiny. The relevance of the oxygen affinity of the carrier to its efficacy in delivering oxygen in vivo needs investigation.

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