Oxygen release from low and normal \(P_{50}\) Hb vesicles in transiently occluded arterioles of the hamster window model

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Sakai, Hiromi, Pedro Cabrales, Amy G. Tsai, Eishun Tsuchida, and Marcos Intaglietta. Oxygen release from low and normal \(P_{50}\) Hb vesicles in transiently occluded arterioles of the hamster window model. Am J Physiol Heart Circ Physiol 288: H2897–H2903, 2005. First published January 28, 2005; doi:10.1152/ajpheart.01184.2004.—A phospholipid vesicle encapsulating Hb [Hb vesicle (HbV)] has been developed as a transfusion alternative. One characteristic of HbV is that the O2 affinity \(P_{50}\) at which Hb is 50% saturated \(P_{50}\) of Hb can be easily regulated by the amount of the coencapsulated allosteric effector pyridoxal 5′-phosphate. In this study, we prepared two HbVs with different \(P_{50}\)s (8 and 29 mmHg, termed HbV8 and HbV29, respectively) and observed their O2-releasing behavior from an occluded arteriole in a hamster skinfold window model. Conscious hamsters received HbV8 or HbV29 at a dose rate of 7 ml/kg. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator, and the reduction of the intra-arteriolar PO2 100 μm down from the occlusion was measured by the phosphorescence quenching of preinfused Pd-porphyrin. The baseline arteriolar PO2 (maximum fraction, 0.55) was reduced to about 5 mmHg for all the groups. Occlusion after HbV8 infusion showed a slightly slower rate of PO2 reduction compared with that after HbV29 infusion. The arteriolar O2 content was calculated at each reducing PO2 in combination with the O2 equilibrium curves of HbVs, and it was clarified that HbV8 showed a significantly slower rate of O2 release compared with HbV29 and was a primary source of O2 (maximum fraction, 0.55) overwhelming red blood cells when the PO2 was reduced (e.g., <10 mmHg) despite a small dosage of HbV8. This result supports the possible utilization of Hb-based O2 carriers with lower \(P_{50}\) for oxygenation of ischemic tissues.

blood substitutes; artificial red blood cells; occlusion; microhemodynamics; liposome

PHOSPHOLIPID VESICLES encapsulating concentrated human Hb [Hb vesicles (HbV)] or liposome-encapsulated Hb can serve as a transfusion alternative whose O2 carrying capacity can be formulated to be comparable to that of blood (1, 5, 8, 16, 24, 30). The capsular structure of HbV (particle diameter ~250 nm) has characteristics similar to those of natural red blood cells (RBCs), because both have membranes that prevent direct contact of Hb with the components of blood and the endothelial lining, mitigating cellular injury due to Hb-mediated prooxidative species (4, 38). Furthermore, Hb encapsulation in vesicles prevents a hypertensive response induced by free Hbs that scavenge the endogenous vasorelaxation factors nitric oxide (NO) and carbon monoxide (12, 18, 26). The safety of HbV has been confirmed in rodent models in terms of the prompt metabolism of the components of HbV in the reticuloendothelial system, which was demonstrated by histopathological analysis and plasma biochemical analysis (28, 29).

One of the characteristics of the capsular HbV is that its physicochemical characteristics such as O2 affinity \(P_{50}\) are similar to that of RBCs or about 25–30 mmHg, which theoretically allows sufficient O2 unloading as blood transits the peripheral blood circulation as shown by the enhanced O2 release and improved exercise capacity in mutant mice that carry high \(P_{50}\) RBCs (36). Hemoglobin-based O2 carriers (HBOCs) of molecular dimensions as well as HbV could be effective for the targeted oxygenation of ischemic tissues (6, 43) because the small particle dimension would allow their passage through constricted or partially occluded vessels that do not allow the passage of RBCs (19). Blood flow in these vessels and in collateral vessels is usually slow, thus increasing RBC transit times (7, 11). As a result, tissue \(P_{02}\) is low and RBCs release most of their O2 before reaching the capillary circulation. As an example, if tissue \(P_{02}\) is below 5 mmHg, O2 saturation \(S_{aO2}\) of RBCs would be around 5%, and RBCs will have released most of their O2 before they reach the ischemic tissue. Thus an HBOC with a normal \(P_{50}\) similar to RBCs would not be effective for carrying O2 to the ischemic tissue.

In this study, we evaluate the rate of O2 release from HbVs with high and low \(P_{50}\)s from arterioles immediately after their occlusion. We selected arterioles with diameters of about 50 μm because this size of arterioles contributes significantly to tissue oxygenation in normal conditions (13). This model was selected to determine the ability of HbVs to retain or release O2 in hypooxic conditions and establish their suitability for oxygenating ischemic tissues.

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MATERIALS AND METHODS

Preparation of HbVs. HbVs with different P_50 values were prepared under sterile conditions as previously reported (32, 34, 37). Hb was purified from outdated donated human blood provided by the Japanese Red Cross Society (Tokyo, Japan). HbVs with a P_50 = 29 mmHg (HbV_{29}) was prepared by adding the allosteric effector pyridoxal 5'-phosphate (PLP; 14.7 mM, Sigma Chemical; St. Louis, MO) to Hb (38 g/dl) at a molar ratio of PLP to Hb = 2.5. HbVs with a P_50 = 8 mmHg (HbV_{8}) were prepared by adding no allosteric effector to the Hb solution. The Hb solution was encapsulated within vesicles composed of Presome PPG-I [a mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylycholine, cholesterol, and 1,5-di-O-octadecyl-N-succinyl]-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemicals; Osaka, Japan), and the particle size of HbVs was regulated by an extrusion method. The surface of the HbVs was modified with polyethylene glycol (molecular mass: 5 kDa, 0.3 mol% of the lipids in the outer surface of vesicles) using 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-polyethylene glycol (Sunbright DSPE-50H, H-form, NOF; Tokyo, Japan). HbVs were suspended in a physiological salt solution and sterilized with filters (Dismic, Toyoo Roshi; Tokyo, Japan; pore size: 0.45 μm) and deoxygenated with N₂ bubbling for storage. The endotoxin content was measured with a modified Limulus amebocyte lysate assay, and the level was less than 0.2 EU/ml (27). The O_2 equilibrium curves (OECs) of HbV_{29} and HbV_{8} were obtained by a Hemos Analyzer (TCS-Medical Products; Philadelphia, PA), as shown in Fig. 1. The physicochemical parameters of the HbVs are listed in Table 1.

Animal model and preparation. Experiments were carried out in 12 male Syrian golden hamsters (59 ± 12 g body wt, Charles Rivers; Worcester, MA). The dorsal skinfold consisting of two layers of skin and muscle was fitted with two titanium frames with a 15-mm circular opening and surgically installed under intraperitoneal pentobarbital sodium anesthesia (50 mg/kg body wt, Abbott Laboratory; North Chicago, IL). After the hair on the back skin of the hamster was removed, layers of skin muscle were separated from the subcutaneous tissue and removed until a thin monolayer of muscle including the small artery and vein and one layer of intact skin remained. A coverglass (diameter 12 mm) held by one frame covered the exposed tissue allowing intravital observation of the microcirculation (20, 22, 25).

Polyethylene (PE) tubes (PE-10, Becton-Dickinson; Parsippany, NJ; ~1 cm) were connected to PE-50 tubing (~25 cm) via silicone elastomer medical tubes (~4 cm, Technical Products; Decatur, GA) and were implanted in the jugular vein and the carotid artery. They were passed from the ventral to the dorsal side of the neck and exteriorized through the skin at the base of the chamber. Patency of the catheters was ensured by filling them with heparinized saline (40 IU/ml). Microvascular observations of the awake and unanesthetized hamsters were performed 5 days after chamber implantation to mitigate the effects of surgery. The hamster was placed in a perforated plastic tube from which the window chamber protruded to minimize animal movement without impeding respiration. All animal studies were approved by the Animal Care and Use Committee of University of California-San Diego and performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996).

Infusion of HbV_{8} and HbV_{29} and occlusion of an arteriole. The unanesthetized animal was placed in a perforated plastic tube and stabilized under the microscope. Animals were suitable for the experiments if systemic variables were within normal range, namely, heart rate >340 beats/min, mean arterial pressure >80 mmHg, systemic hematocrit >45%, and arterial PO_2 >50 mmHg, and microscopic examination of the tissue in the chamber did not reveal signs of edema or bleeding. Baseline measurements of microvascular parameters and PO_2 (see below) were performed before the infusion of HbV_{8} or HbV_{29} suspended in physiological saline solution into the venous line at 7 ml/kg. Systemic blood volume was estimated as 70 ml/kg. In our previous reports of resuscitation from hemorrhagic shock or hemodilution, HbVs were suspended in an albumin solution to regulate colloidal osmotic pressure (30, 33). However, in the present study, we did not use albumin to minimize the hypervolemic effect. For the same reason, the infusion amount was minimized to equal 10% blood volume (7 ml/kg).

After we stabilized the condition and measured the systemic parameters for 20 min, diameter and blood flow of the selected arterioles were measured. Large feeding arterioles or small arcading arterioles (diameter 53.0 ± 6.6 μm) were selected for observation. The arterioles were occluded by means of a glass micropipette whose end was drawn into a long fiber by a pipette puller (Fig. 2). The fiber was bent over a flame, and the knee of the bend was used to press on the intact skin of the preparation mounted in an inverted microscope that allowed observation of the opposite side, i.e., the intact microcirculation. Once an arteriole was selected for measurement, the microoccluder is moved to the skin side, between the intact skin and the optics of the stage illumination. The tip of the occluder was placed near the center of the optical field of view of the microscope, and the vessel was similarly placed using the stage micrometric position control. This arrangement allowed for direct microscopic observation of the occluded vessel and the stopped flow as shown in Fig. 2. The duration of occlusion was 30 s.

Measurement of microhemodynamic parameters. Microvessels were observed by transillumination with an inverted microscope (IMT-2, Olympus; Tokyo, Japan). Microscopic images were video recorded (Cohu 4815-2000; San Diego, CA) and transferred to a television videocassette recorder (Sony Trinitron PVM-1271Q monitor; Tokyo, Japan) and Panasonic AG-7355 video recorder (Tokyo, Japan). Arterioles were classified according to their position within the microvascular network according to the previously reported scheme (33). Microvascular diameter and RBC velocity before occlusion.
sion were analyzed on-line in the arterioles (14, 15). Vessel diameter was measured with an image-shearing system (Digital Video Image Shearing Monitor 908, I.P.M.; San Diego, CA), whereas RBC velocity was analyzed by photodiodes and the cross-correlation technique (Velocity Tracker Mod-102 B, I.P.M.). The blood flow rate (Q) was calculated using the following equation:

\[ Q = \frac{(\text{RBC velocity})}{R_e} \times \left( \frac{\text{diameter}}{2} \right)^3 \]  

where \( R_e = 1.6 \) and is the ratio of the centerline velocity to average blood velocity according to data from glass tubes (20).

Palladium-porphyrin bound to bovine albumin solution (7.6 wt%, 0.1 ml) was injected intravenously 20 min before the infusion of HbVs. Arteriolar blood \( PO_2 \) was noninvasively determined by measuring the rate of decay of phosphorescence emitted by the metalloporphyrin complex after pulsed light excitation, which is a function of the local \( O_2 \) concentration (17, 40, 44). The relationship between phosphorescence lifetime and \( PO_2 \) is given by the following Stern-Volmer equation:

\[ \tau^{-1} = \frac{1}{\tau_0} + k_q \times \tau_0 \times PO_2 \]  

where \( \tau_0 \) and \( \tau \) are the phosphorescence lifetimes in the absence of molecular \( O_2 \) and at a given \( PO_2 \), respectively, and \( k_q \) is the quenching constant, with both factors being pH and temperature dependent.

Light was gathered from an optical window of \( 20 \times 5 \mu m \) placed longitudinally along the blood vessels. Measurements in the blood compartment were made every second using a single flash.

The \( PO_2 \) decay curves induced by the occlusion were obtained before the infusion of HbVs and 20 min after the infusion of HbVs. The \( SaO_2 \) of HbVs at every \( PO_2 \) were obtained from the OECs (Fig. 1), and the total \( O_2 \) content in blood (ml \( O_2 \) in 1 dl blood) can be estimated using the following equation:

\[ O_2 \text{ content} = 23.6 \times \frac{[SaO_2(\text{RBC}) + 0.0667 \times SaO_2(\text{HbV})]}{100} + 2.42 \times \frac{PO_2}{713} \]  

In this calculation, we used 15 g/dl as the average Hb concentration in arterial blood (14.8 ± 0.5 g/dl, hemec concentration 9.3 mM), which was measured with a handheld photometer (B-Hemoglobin Photometer, Hemocue). One hundred milliliters of blood contain 23.6 ml \( O_2 \) bound to Hb when \( SaO_2 \) is 100% (volume of an ideal gas at 37°C) according to Boyle-Charle’s gas law, \( PV = nRT \), where \( P \) (in atm) is atmospheric pressure, \( V \) (in liters) is gas volume, \( n \) is mole number, \( R \) is the gas constant (0.082 atm·l·K⁻¹·mol⁻¹), and \( T \) is absolute temperature \((23.6 \text{ (ml)} = 9.3 \times 10^{-3} \text{ (mol)} \times 0.082 \times (273 + 37) \times 1,000) \). The physically dissolved \( O_2 \) content at 1 atm \( O_2 \) (713 mmHg) after subtracting the vapor pressure of water = 47 mmHg at 37°C was calculated to be 2.42 ml in 100 ml water. \( SaO_2 \) (RBC) and \( SaO_2 \) (HbV) are \( SaO_2 \)'s of RBCs and HbVs, respectively, at each arteriolar \( PO_2 \) during the experiments.

HbVs were suspended in physiological saline solution ([Hb] = 10 g/dl); therefore, their infusion lowered colloid osmotic pressure, causing the extravasation of plasma fluid. To account for this, we carried out our measurements 20 min after HbV infusion and assumed that this interval was sufficient for normalizing blood volume through the release of extra fluid to the interstitium, thus increasing plasma Hb concentration by 6.7%.

**Data analysis.** Data are given as means ± SD for the indicated number of animals. Data were analyzed using ANOVA followed by Fisher’s protected least-significant difference test between groups according to the previous studies. Student’s \( t \)-test was used for comparisons within each group. All statistics were calculated using GraphPad Prism 4.01 (Graph Pad Software; San Diego, CA). Changes were considered statistically significant if \( P < 0.05 \).

**RESULTS**

**Hemodynamic properties of arterioles.** The profiles of the selected arterioles, diameters, centerline RBC velocities, blood flow rates, and intra-arteriolar \( PO_2 \) values before and after infusion of HbVs are listed in Table 2. There was no significant difference between the groups. The \( O_2 \) content in blood attributed to hamster RBCs and physically dissolved \( O_2 \) at the observed arteriolar \( PO_2 \) was estimated as 18.61 ± 0.07 ml O2/dl blood according to Eq. 3. After the infusion of HbV8 and HbV29, the \( O_2 \) content increased to 20.30 ± 1.18 and 20.17 ± 1.54 ml O2/dl blood, respectively, due to the \( PO_2 \) bound to HbVs. The contributions of HbV8 and HbV29 to whole \( O_2 \) content were 1.51 ± 0.01 and 1.25 ± 0.07 ml O2/dl blood, respectively. The HbV8 group showed higher \( O_2 \) content than the HbV29 group due to the higher \( SaO_2 \) (HbV8), which was 95.9 ± 0.6% compared with the \( SaO_2 \) (HbV29) of 79.6 ± 4.7%.

Changes in \( PO_2 \) in arterioles after occlusion in the presence of HbVs. Arteriolar \( PO_2 \) before occlusion was about 50–52 mmHg in average for all groups and started to decrease significantly immediately after occlusion, as shown in Fig. 3. In all groups, \( PO_2 \) fell to about 10 and 5 mmHg after 10-
30-s occlusion, respectively. When the PO2 values were expressed as relative to the baseline values (before occlusion), infusion of HbV8 tended to show a slower rate of reduction of PO2 compared with the infusion of HbV29 and without infusion (Fig. 4). There was a significant difference between the HbV8 infusion and before infusion groups only at 7 s ($P < 0.035$).

SaO2(RBC) and SaO2(HbV) at every arteriolar PO2 value can be estimated using the OECs in Fig. 1 assuming that the conditions in the arteriole (such as temperature and pH) do not change significantly from the normal condition (37°C, pH 7.4).

### Table 2. Profiles of arterioles for occlusion before and after infusion of HbVs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before Infusion</th>
<th>HbV8</th>
<th>HbV29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteriolar diameter, μm</td>
<td>53.0 ± 6.6</td>
<td>56.2 ± 6.8</td>
<td>55.8 ± 6.9</td>
</tr>
<tr>
<td>Centerline flow velocity, mm/s</td>
<td>3.1 ± 0.5</td>
<td>3.4 ± 0.7</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Blood flow rate, nl/s</td>
<td>6.8 ± 1.6</td>
<td>8.7 ± 3.1</td>
<td>8.5 ± 2.1</td>
</tr>
<tr>
<td>Arteriolar PO2, mmHg</td>
<td>50.7 ± 4.7</td>
<td>51.4 ± 4.8</td>
<td>52.1 ± 5.3</td>
</tr>
<tr>
<td>SaO2(RBC), %</td>
<td>78.1 ± 5.1</td>
<td>76.0 ± 7.7</td>
<td>77.9 ± 6.5</td>
</tr>
<tr>
<td>SaO2(HbV), %</td>
<td>95.9 ± 0.6†</td>
<td>79.6 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>O2 content in whole blood, ml O2/dl blood</td>
<td>18.61 ± 1.23</td>
<td>20.30 ± 1.18*</td>
<td>20.17 ± 1.54*</td>
</tr>
<tr>
<td>O2 content in HbV, ml O2/dl blood</td>
<td>1.51 ± 0.01</td>
<td>1.25 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. Arteriolar PO2, O2 saturation (SaO2) and O2 contents were obtained during 6 s before occlusion. *$P < 0.05$ vs. before infusion; †$P < 0.05$ vs. RBCs and HbV29.

Fig. 3. Time course of PO2 in the blood of an occluded arteriole (diameter, 53.0 ± 6.6 μm) before and after infusion of 7 ml/kg HbV8 or HbV29 into hamsters. Measurements were made in blood at a distance of 50 μm from the point of occlusion. Most vessels equilibrate to intravascular partial pressure in the range of 4–6 mmHg about 15–20 s after occlusion.

Fig. 4. Changes in PO2 relative to before occlusion. The data in Fig. 3 were averaged. Baseline values before occlusion were obtained as the average of 6 values before occlusion and fixed as 1.0. There was a significant difference between the HbV8 infusion and before infusion groups only at 7 s ($P = 0.035$).

SaO2(RBC) and SaO2(HbV) at every arteriolar PO2 value can be estimated using the OECs in Fig. 1 assuming that the conditions in the arteriole (such as temperature and pH) do not change significantly from the normal condition (37°C, pH 7.4). Figure 5A shows the changes in the whole arteriolar O2 content...
Although the O₂ supply was significantly reduced, diffusion of O₂ from the other surrounding arterioles, venules, and capillaries near the occlusion should contribute to maintaining tissue PO₂ at a higher value than in the study of Richmond et al. (23), where the supply of blood to the tissue was stopped altogether. SaO₂(HbV₈) at 5 mmHg is estimated to be about 26% according to the OECs (Fig. 1), which is higher than that for HbV₂₉ (6%) and RBCs (2%); thus HbV₈ remains a source of O₂ for a longer period in a prolonged occlusion, because the fraction of O₂ from HbV₈ was 0.5 or higher, overwhelming the contribution from RBCs, as shown in Fig. 6.

A limitation of our experimental method is that SaO₂ is estimated under the assumption that conditions in the target arteriole are identical to that of the OEC measurement; however, the O₂ affinity of Hb changes as a function of temperature, pH, electrolyte concentration, and CO₂ content. Local ischemic conditions caused by the occlusion could affect pH and increase CO₂ tension, resulting in a slight decrease in the O₂ affinity (increased P₅₀); however, it is unlikely that this would introduce a significant error in the measurement of O₂ release considering the short duration of the occlusion (30 s).

We have previously demonstrated using an artificial narrow polymer tube (inner diameter: 28 μm) surrounded by a sodium dithionate solution to consume O₂ that a Hb solution under continuous flow conditions (1 mm/s) facilitates O₂ release when mixed with RBCs. Conversely, HbV did not show this phenomenon (31). This difference is due to the small size of O₂-bound acellular Hb molecules, which diffuse and therefore contribute to the facilitated O₂ transport (21, 31), whereas HbVs (diameter, about 250 nm) are too large to show sufficient diffusion for the facilitated O₂ transport. In these conditions, O₂ affinity (P₅₀) becomes the determining factor for the rate of O₂ release and transport to the vessels wall. Thus, in our present results, the presence of HbVs did not facilitate the reduction of PO₂ or O₂ content but retarded the reduction of PO₂ and O₂ content.

Our experimental model is designed to characterize the O₂ release behavior of blood from an occluded microvessel and does not directly related to clinical ischemic conditions because the occlusion of the small arteriole for 30 s does not induce tissue ischemia other than the transient event in the proximity of the microvessel. However, our data suggest that HbV₈ could be a significant source of O₂ in an ischemic condition with significantly lowered tissue PO₂. Because of the small dosage of HbV₈ (7 ml/kg), the O₂ content in the blood after occlusion (5 ml O₂/dl blood at 5 s) is significantly smaller than the baseline value (20 ml O₂/dl blood at 0 s). To enhance the contribution of HbVs, a larger dosage and sustained blood flow would be required. Contaldo et al. (7) recently demonstrated that inducing hemodilution using up to 50% blood exchange with HbV (P₅₀ = 15 mmHg) suspended in dextran effectively oxygenated ischemic collateralized tissue in skin flaps. This phenomenon could be explained by low P₅₀ HbVs retaining O₂ in the upstream vessels and delivering it to the ischemic tissue via collateral arterioles, even when these may have significantly slower blood flow. It has been proposed that small-sized HBOCs oxygenate ischemic tissue by being able to pass through constricted or partially occluded vessels that do not allow the passage of RBCs; however, the results from Contaldo et al. (17) as well as those from our experimental model do not serve to support this concept, because arterioles were completely ligated or occluded. It should be noted, however, that an advantage of small HBOCs, including HbVs,
is that they are homogeneously dispersed in the plasma phase and therefore can deliver O2 more homogeneously to the periphery than RBCs because microvascular hematocrit is heterogeneous particularly in pathological states. In such conditions, HbVs with a higher O2 affinity should show a slower O2 unloading that would be effective for oxygenating ischemic tissues.

In conclusion, HbVs provide the unique feature of allowing for the regulation of P50 by modulating the amount of coen-

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