Oxygen release from low and normal P$_{50}$ Hb vesicles in transiently occluded arterioles of the hamster window model

Hiromi Sakai,¹ Pedro Cabrales,²,³ Amy G. Tsai,²,³ Eishun Tsuchida,¹ and Marcos Intaglietta²,³

¹Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, Japan; and ²Department of Bioengineering, University of California-San Diego, and ³La Jolla Bioengineering Institute, La Jolla, California

Submitted 27 November 2004; accepted in final form 24 January 2005

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 O$_2$ affinity [P$_{O_2}$ 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 O$_2$-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 P$_{O_2}$ 100 μm down from the occlusion was measured by the phosphorescence quenching of preinfused Pd-porphyrin. The baseline arteriolar P$_{O_2}$ (50–52 mmHg) decreased to about 5 mmHg for all the groups. Occlusion after HbV8 infusion showed a slightly slower rate of P$_{O_2}$ reduction compared with that after HbV29 infusion. The arteriolar O$_2$ content was calculated at each reducing P$_{O_2}$ in combination with the O$_2$ equilibrium curves of HbVs, and it was clarified that HbV8 showed a significantly slower rate of O$_2$ release compared with HbV29 and was a primary source of O$_2$ (maximum fraction, 0.55) overwhelming red blood cells when the P$_{O_2}$ was reduced (e.g., <10 mmHg) despite a small dosage of HbV. This result supports the possible utilization of Hb-based O$_2$ 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 O$_2$ 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 O$_2$ affinity [O$_2$ tension at which Hb is half-saturated with O$_2$ (P$_{50}$)] can be easily regulated by manipulating the amount of an allosteric effector coencapsulated in HbV. This property provides additional flexibility in formulating the O$_2$ transport properties of HbV by comparison with the chemically modified Hbs whose P$_{50}$ is modified and fixed by chemical reactions such as cross-linking or polymer conjugation (34). We use pyridoxal 5′-phosphate (PLP) as the allosteric effector (33, 45). For example, coencapsulation of PLP at the molar ratio of PLP to Hb of 2.5:1 yields a P$_{50}$ of about 29 mmHg. On the other hand, HbVs without PLP have a P$_{50}$ of 8 mmHg. Historically, P$_{50}$ was set similar to that of RBCs or about 25–30 mmHg, which theoretically allows sufficient O$_2$ unloading as blood transits the microcirculation. Decreasing O$_2$ affinity (increasing P$_{50}$) increases O$_2$ unloading in the peripheral blood circulation as shown by the enhanced O$_2$ release and improved exercise capacity in mutant mice that carry high P$_{50}$ RBCs (36).

Hemoglobin-based O$_2$ 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$_{O_2}$ is low and RBCs release most of their O$_2$ before reaching the capillary circulation. As an example, if tissue P$_{O_2}$ is below 5 mmHg, O$_2$ saturation (SaO$_2$) of RBCs would be around 5%, and RBCs will have released most of their O$_2$ before they reach the ischemic tissue. Thus an HBOC with a normal P$_{50}$ similar to RBCs would not be effective for carrying O$_2$ to the ischemic tissue.

In this study, we evaluate the rate of O$_2$ 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 O$_2$ in hypoxic conditions and establish their suitability for oxygenating ischemic tissues.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
MATERIALS AND METHODS

Preparation of HbVs. HbVs with different P_{SO_2} 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_{SO_2} = 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_{SO_2} = 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-phosphatidylethanolamine, cholesterol, and 1,5-di-O-octadecyl-N-succinyl-1-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 membrane) and the particle size of HbVs was regulated by an extrusion method. The sample of HbVs was stored at 4 degrees C until use. 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 Hemox 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 window. The hamster was placed in a perforated plastic tube from which the window chamber protruded to minimize 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 HbVs 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. Table 1. Physicochemical properties of HbV_{8} and HbV_{29} compared with hamster blood

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HbV_{8}</th>
<th>HbV_{29}</th>
<th>Hamster Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb concentration, g/dl</td>
<td>10</td>
<td>10</td>
<td>14.8 ± 0.5</td>
</tr>
<tr>
<td>Particle diameter, nm</td>
<td>250 ± 64</td>
<td>247 ± 44</td>
<td>5,000–7,000*</td>
</tr>
<tr>
<td>P_{SO_2}, mmHg</td>
<td>8</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>Molar ratio of PLP to Hb</td>
<td>0</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>MetHb, %</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>HbCO, %</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td></td>
</tr>
</tbody>
</table>

HbVs and HbV_{29}, Hb vesicles (HbVs) at 8- and 29-mmHg PO_2 at which Hb is 50% saturated (P_{SO_2}) PLP, pyridoxal 5'-phosphate. *Size of hamster red blood cells (RBCs) (39).

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 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 HbVs 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. Microvascular 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 HbVs 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 hemorrhilation, HbVs were suspended in an albumin solution to regulate colloid 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 mm) 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 substage 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 micrometer 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...
OXYGEN RELEASE FROM Hb VESICLES

Light was gathered from an optical window of 20 × 5 μm placed longitudinally along the blood vessels. Measurements in the blood compartment were made every second using a single flash.

The PO2 decay curves induced by the occlusion were obtained before the infusion of HbVs and 20 min after the infusion of HbVs. The \( \text{Sao}_2 \), of HbVs at every PO2 were obtained from the OECs (Fig. 1), and the total O2 content in blood (ml O2 in 1 dl blood) can be estimated using the following equation:

\[
\text{O2 content} = 23.6 \times \frac{[\text{Sao}_2(\text{RBC}) + 0.0667 \times \text{Sao}_2(\text{HbV})]}{100} + 2.42 \times \frac{\text{PO2}}{713}
\]  

In this calculation, we used 15 g/dl as the average Hb concentration in arterial blood (14.8 ± 0.5 g/dl, hemecion concentration 9.3 mM), which was measured with a handheld photometer (B-Hemoglobin Photometer, Hemocyte). One hundred milliliters of blood contain 23.6 ml O2 bound to Hb when \( \text{Sao}_2 \), is 100% (volume of an ideal gas at 37°C) according to Boyle-Charlé’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 (ml) = 9.3 × 10⁻³ (mol) × 0.082 × (273 + 37) × 1000]. The physically dissolved O2 content at 1 atm O2 (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. \( \text{Sao}_2 \), of RBCs and \( \text{Sao}_2 \), of HbVs were estimated as 18.61 and 79.6%, respectively. The HbV8 group showed higher O2 content than the \( \text{Sao}_2 \), of RBCs, which in turn was higher than the \( \text{Sao}_2 \), of HbVs. The \( \text{Sao}_2 \), of HbVs at every PO2 were obtained from the OECs (Fig. 1).

Changes in PO2 in arterioles after occlusion in the presence of HbVs. Arteriolar PO2 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, PO2 fell to about 10 and 5 mmHg after 10- and...
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).

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>18.6 ± 1.23</td>
<td>20.3 ± 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.

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.

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).

Fig. 5. A: time course of the arteriolar O2 content in whole blood of an occluded arteriole before and after infusion of 7 ml/kg HbV8 or HbV29 into hamsters. The O2 contents were calculated using Eq. 2 and the data of OECs (Fig. 1) and Po2 changes (Fig. 3). B: time course of the O2 content derived from HbVs in the blood. The contributions of HbVs are derived from the data in A and magnified in scale. C: rate of O2 loss dO2/dt from HbVs. The graphs in B were differentiated and plotted.
during the occlusion. Immediately after occlusion, the O$_2$ content decreased rapidly. The HbVs group showed a slower rate of reduction compared with the HbV$_{29}$ group and the group before HbV infusion. To demonstrate the contribution of HbVs clearly, only the O$_2$ content of HbVs is shown in Fig. 5B. HbV$_8$ showed a very slow rate of O$_2$ release. After 30 s of occlusion, the arteriolar PO$_2$ decreased to 5.2 $\pm$ 0.7 mmHg. However, SaO$_2$(HbV$_8$) was 26.1 $\pm$ 7.3% and did not reach steady state but continued O$_2$ release. HbV$_{29}$ showed almost no change after 15 s, and SaO$_2$(HbV$_{29}$) was 7.4 $\pm$ 1.0% after 30 s. Figure 5C shows the rate of O$_2$ loss from HbVs obtained by the differentiation of the graphs in Fig. 5B. HbV$_{29}$ showed the fastest O$_2$ loss with the maximum of 0.18 ml O$_2$/dl blood sec after only 2 s of occlusion and did not supply O$_2$ after 17 s. On the other hand, HbV$_8$ showed a moderate O$_2$ loss and showed the maximum of 0.08 ml O$_2$/dl blood after 10 s of occlusion and continued to release O$_2$ until 30 s.

Figure 6 shows the fraction of O$_2$ in blood originating from HbVs. Before occlusion of the arterioles, the fractions of HbV$_8$ and HbV$_{29}$ are very small and similar because of the small dosage compared with the originally present RBCs. However, after occlusion, the fraction of O$_2$ from HbV$_8$ increased significantly and was about 0.55 after 10 s. This indicated that HbV$_8$, and not RBCs, was the main source of the O$_2$ carrier when PO$_2$ attained very low values.

**DISCUSSION**

The principal finding of this study is that HbV$_8$ (P$_{50}$ = 8 mmHg) with a high O$_2$ affinity (low P$_{50}$) releases O$_2$ at a slower rate than does HbV$_{29}$ in occluded arterioles of the hamster dorsal skinfold model. Furthermore, we found that HbV$_8$, and not HbV$_{29}$, is the main O$_2$ source in ischemic conditions.

The immediate occlusion of blood flow in the arterioles caused a rapid reduction of O$_2$ content. Similar phenomena have been observed by Richmond et al. (23) in rat spinotrapezius muscle tissue. There is substantial evidence that the arteriolar wall is a significant O$_2$ sink, consuming O$_2$ at a rate that is much greater than most tissues (9, 35, 42), which explains in part the significant and rapid drop of PO$_2$ found in our study. In our experiments, only one arteriole was occluded at a time in the intact subcutaneous tissue, and arteriolar PO$_2$ decreased to about 5 mmHg, which was higher than the critical PO$_2$ (2.9 $\pm$ 0.5 mmHg) in the rat spinotrapezius muscle tissue (23). Although the O$_2$ supply was significantly reduced, diffusion of O$_2$ from the other surrounding arterioles, venules, and capillaries near the occlusion should contribute to maintaining tissue PO$_2$ at a higher value than in the study of Richmond et al. (23), where the supply of blood to the tissue was stopped altogether. SaO$_2$(HbV$_8$) at 5 mmHg is estimated to be about 26% according to the OECs (Fig. 1), which is higher than that for HbV$_{29}$ (6%) and RBCs (2%); thus HbV$_8$ remains a source of O$_2$ for a longer period in a prolonged occlusion, because the fraction of O$_2$ from HbV$_8$ was 0.5 or higher, overwhelming the contribution from RBCs, as shown in Fig. 6.

A limitation of our experimental method is that SaO$_2$ is estimated under the assumption that conditions in the target arteriole are identical to that of the OEC measurement; however, the O$_2$ affinity of Hb changes as a function of temperature, pH, electrolyte concentration, and CO$_2$ content. Local ischemic conditions caused by the occlusion could affect pH and increase CO$_2$ tension, resulting in a slight decrease in the O$_2$ affinity (increased P$_{50}$); however, it is unlikely that this would introduce a significant error in the measurement of O$_2$ release considering the short duration of the occlusion (30 s).

We have previously demonstrated using an artificial narrow polymer tube (inner diameter: 28 $\mu$m) surrounded by a sodium dithionate solution to consume O$_2$ that a Hb solution under continuous flow conditions (1 mm/s) facilitates O$_2$ release when mixed with RBCs. Conversely, HbV did not show this phenomenon (31). This difference is due to the small size of O$_2$-bound acellular Hb molecules, which diffuse and therefore contribute to the facilitated O$_2$ transport (21, 31), whereas HbVs (diameter, about 250 nm) are too large to show sufficient diffusion for the facilitated O$_2$ transport. In these conditions, O$_2$ affinity (P$_{50}$) becomes the determining factor for the rate of O$_2$ release and transport to the vessels wall. Thus, in our present results, the presence of HbVs did not facilitate the reduction of PO$_2$ or O$_2$ content but retarded the reduction of PO$_2$ and O$_2$ content.

Our experimental model is designed to characterize the O$_2$ 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 HbV$_8$ could be a significant source of O$_2$ in an ischemic condition with significantly lowered tissue PO$_2$. Because of the small dosage of HbV$_8$ (7 ml/kg), the O$_2$ content in the blood after occlusion (5 ml O$_2$/dl blood at 5 s) is significantly smaller than the baseline value (20 ml O$_2$/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 (P$_{50}$ = 15 mmHg) suspended in dextran effectively oxygenated ischemic collateralized tissue in skin flaps. This phenomenon could be explained by low P$_{50}$ HbVs retaining O$_2$ 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,
OXYGEN RELEASE FROM Hb VESICLES

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 coencapsulated PLP (33, 45). Recent studies showed the effectiveness of HBOCs with a lower P50 (higher O2 affinity) as a means of implementing O2 delivery targeted to ischemic tissue (2, 3, 41, 43). Thus this experimental method provides data useful for the design and optimization of O2 carriers and suggests the possible utilization of HbVs for therapeutic approaches aimed at remediating ischemic conditions.

ACKNOWLEDGMENTS

The authors greatly acknowledge A. Barra and C. Walser (University of California-San Diego) for help with the animal preparations, Dr. S. Takeoka and Dr. K. Sou (Waseda University) for the preparation of the HbVs, and Dr. D. Erni (Inselspital University Hospital, Bern, Switzerland) for meaningful discussions.

GRANTS

This study was supported in part by Health Sciences Research grants (Regulatory Science, Artificial Blood Project); the Ministry of Health, Labour and Welfare, Japan (H16-YAKU-069, 071); Japan Society for the Promotion of Science Grant-In-Aid for Scientific Research B16300162; and National Health, Lung, and Blood Institute Bioengineering Partnership Grant R24 HL-64395 and Grants R01 HL-40696 and R01 HL-62354. H. Sakai was an overseas research fellow of the Society of Japanese Pharmacopoeia.

REFERENCES