Published in final edited form as:

J Appl Physiol. 2006 May ; 100(5): 1688–1691.

Salvage of focal cerebral ischemic damage by transfusion of high O₂-affinity recombinant hemoglobin polymers in mouse

Masaaki Nemoto¹, Toshiaki Mito¹, William S Brinigar², Clara Fronticelli¹, and Raymond C. Koehler¹

¹ Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins University, Baltimore, Maryland; and

² Department of Chemistry, Temple University, Philadelphia, Pennsylvania

Abstract

Cell-free hemoglobin solutions with high oxygen affinity might be beneficial for selectively delivering oxygen to ischemic tissue. A recombinant hybrid hemoglobin molecule was designed using the human α -subunit and the bovine β -subunit, with placement of surface cysteines to permit disulfide bond polymerization of the tetramers. The resulting protein generated from an *Escherichia coli* expression system had a molecular mass >1 MDa, a P₅₀ of ~3 Torr, and a cooperativity of $n = 1.0$. Anesthetized mice were transfused during 2-h occlusion of the middle cerebral artery. Compared with transfusion with 5% albumin, cerebral infarct volume was reduced by 41% with transfusion of a 3% solution of the high oxygen-affinity hemoglobin polymer and by 50% with transfusion of a 6% solution of the polymer. Transfusion of a 6% solution of a 500-kDa polymer possessing a P₅₀ of 17 Torr and a cooperativity of $n = 2.0$ resulted in a 66% reduction of infarct volume. These results indicate that cell-free Hb polymers with P₅₀ values much lower than that of red blood cell hemoglobin are highly capable of salvaging ischemic brain. The assumption that the P₅₀ of blood substitutes should be similar to that of blood might not be warranted when used during ischemic conditions.

Keywords

blood substitute; cerebral ischemia; middle cerebral artery; oxygen carrier

THE OXYGEN AFFINITY and cooperativity of Hb in red blood cells help to maximize O₂ loading in the lungs and unloading in peripheral tissue. Various cell-free Hb solutions have been developed as O₂-carrier transfusion fluids, but the optimal O₂ affinity for these modified Hbs is unclear. Initially, O₂ affinity that is similar to that of red cell-based Hb was thought to be best for O₂ transport. However, the first generation of cross-linked Hb tetramers with a P₅₀ (PO₂ at 50% oxyhemoglobin saturation) of 32 Torr was ineffective in clinical trials of hemorrhagic shock and stroke (13,14). Although poor efficacy is probably attributed to scavenging of nitric oxide by extravasated tetrameric Hb and consequent vasoconstriction (8, 12), increased precapillary O₂ loss might also contribute to peripheral vasoconstriction (9,16, 17). Thus a P₅₀ that is intermediate between that of red cell Hb and mitochondrial cytochromes might be optimal for minimizing precapillary O₂ loss and maximizing O₂ unloading in capillaries. This effect of unwanted precapillary O₂ loss can become more critical in a focal ischemic injury with limited O₂ supply via collateral blood vessels and with low ischemic tissue

Address for reprint requests and other correspondence: R. C. Koehler, Dept. of Anesthesiology and Critical Care Medicine, The Johns Hopkins Medical Institutions, 600 North Wolfe St., Blalock 1404, Baltimore, MD 21287 (e-mail: rkoehler@jhmi.edu).

GRANTS

This work was supported by a grant from the National Institute of Neurological Disorders and Stroke (NS-38684) and by the Eugene and Mary B. Meyer Center for Advanced Transfusion Practice and Blood Research at the Johns Hopkins University School of Medicine.

PO₂. Here, a low P₅₀ could be effective in delivering most of its O₂ into hypoxic tissue. In addition, the plasma represents a site of some resistance to O₂ flux between red blood cells and parenchyma, and the increase in O₂ solubility in plasma with cell-free Hb offers a way to reduce this diffusion resistance (9,10,17).

We previously reported the characterization of a polymer of human hemoglobin, Hb Prisca (6), and of a recombinant hybrid Hb with the α -subunit of human Hb_A and the β -subunit of bovine Hb (2). The latter has, in *Escherichia coli*, a higher expression level, which allowed us to produce this recombinant Hb in an amount sufficient for transfusion in mice. Appropriate amino acid substitutions were introduced to replace the naturally occurring cysteine residues while introducing a novel Cys residue at position β 9 in the β -chains on the protein surface. These substitutions resulted in the formation of intermolecular disulfide bonds that generate a nondissociable polymer that consists of ~7–8 Hb tetramers (2). This polymer, designated Hb Polytaur, has a P₅₀ of 17 Torr and a cooperativity of $n = 2.0$. Transfusion of Hb Polytaur in mice produced small increases in arterial blood pressure that were similar to those obtained with transfusion of albumin. However, when transfusion occurred after middle cerebral artery occlusion (MCAO), mice transfused with a 3% solution of Hb Polytaur had a 22% reduction in infarct volume (2). Thus Hb Polytaur appears to be capable of delivering O₂ to ischemic tissue, despite a low P₅₀ of 17 Torr. This result raised the possibility of whether Hb molecules possessing much lower P₅₀ than that of red blood cell Hb can be an effective O₂ carrier in ischemic tissue.

The purpose of the present study was to determine whether a recombinant Hb polymer, possessing functional characteristics similar to myoglobin with very low P₅₀ and absent cooperativity, would be capable of reducing infarct volume from focal cerebral ischemia. High molecular weight polymers were obtained when the surface Cys were introduced without the substitution of the naturally occurring Cys. By retaining naturally occurring Cys residues, a large heterogeneous polymer, designated Hb (Polytaur)_n, was derived. Because it has been suggested that high concentrations of plasma Hb might deliver too much O₂ to precapillary arterioles and cause constriction, we tested the effect of two concentrations of Hb (Polytaur)_n, 3 and 6%. In addition, the effect of transfusing a 6% solution of Hb Polytaur was compared with previous results with a 3% solution of Hb Polytaur (2).

METHODS

Recombinant hemoglobin

The design of DNA plasmids used for expression of the human α -subunit and bovine β -subunit of the hybrid Hb in *E. coli* grown in a 5-liter fermentor as well as the steps for protein purification of the tetramers and polymerization of the tetramers have been described (2). All mutations were made using the pAlter system (Promega, Madison, WI). The β -globin gene carrying the desired mutations was excised from the pAlter plasmid with *Nco*I and *Bss*HI and inserted into pNFBovine (2) that had been digested with the same two enzymes. The β -globin gene of pDL α _H β _{BV} (2) was replaced with the mutated β -globin gene, using *Nco*I and *Hind*III to yield the plasmids expressing Hb Polytaur and Hb (Polytaur)_n. To construct Hb Polytaur, a cysteine residue was substituted for the β 9Ser. This substitution permitted spontaneous formation of stable disulfide bonds among the tetramers. In addition, the α 104Cys was replaced by Ser, and the β 93Cys was replaced by Ala to prevent spurious disulfide bond formation during protein refolding and to permit the formation of a relatively homogeneous polymer of ~500 kDa. To obtain Hb (Polytaur)_n, the α 104Cys and β 93Cys were left intact, resulting in polymers of heterogeneous sizes >1 MDa. The purified Hb proteins were dialyzed against lactated Ringer solution. The solution was filtered (0.45 μ m), and endotoxin was removed using Detoxigel (Pierce, Rockford, IL).

Experimental protocol

Because of the limited capacity of the fermentor to produce recombinant protein sufficient for physiological experiments, studies were performed on mice. All procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Male C57Bl/6 mice were anesthetized with ~1.5% halothane via face mask. Rectal temperature was maintained with a heating lamp. A femoral artery was catheterized. Focal cerebral ischemia was produced by advancing a single monofilament through the internal carotid artery to the origin of the middle cerebral artery (7). The tip of a 6-O monofilament was blunted by heat and coated with cyanoacrylic glue. An incision was made in the neck for isolating the right carotid artery. The filament was inserted into the internal carotid artery through a cut stump of the external carotid artery, and the tip was advanced 6 mm past the junction of the pterygopalantine artery to produce MCAO. Starting at 10 min after MCAO, a hypervolumetric exchange transfusion was performed to reduce hematocrit from ~44 to 36% over a 35-min period by infusing 0.95 ml in four-step increments, while alternating with the withdrawal of 0.7 ml in three-step increments from the femoral artery. In preliminary experiments, the increase in mean arterial pressure with transfusion of the Hb (Polytaur)_n solution (17 ± 3 mmHg) was similar to that with Hb Polytaur (16 ± 9 mmHg) and albumin (11 ± 5 mmHg) solutions. After 45 min of MCAO, the catheter was removed, incisions were closed, and halothane administration was discontinued. Neurologic deficit was evaluated, and mice without circling behavior were excluded. At 2 h of MCAO, the mouse was briefly anesthetized, and the monofilament was withdrawn from the internal carotid artery to permit reperfusion. At 24 h, the brain was harvested and cut into 2-mm coronal slabs for vital dye staining with a 1% solution of 2,3,5-triphenyltetrazolium chloride. The volume of the infarct was integrated over five slabs and corrected for swelling (7).

In one experiment, mice were transfused with either a solution containing 5% albumin ($n = 6$), 3% Hb (Polytaur)_n ($n = 7$), or 6% Hb (Polytaur)_n ($n = 5$). In a second experiment, mice were transfused with either a solution containing 5% albumin ($n = 7$), 3% Hb Polytaur ($n = 7$), or 6% Hb Polytaur ($n = 5$). For each polymer, infarct volume was compared among the three groups that were transfused with albumin and the 3 and 6% Hb solutions by analysis of variance and the Newman-Keuls multiple range procedure, with significance set at the $P < 0.05$ level. Data are presented as means $\pm$ SD.

RESULTS

Recombinant Hb (Polytaur)_n possessed a P_{50} of ~3 mmHg and a Hill coefficient of $n = 1$ (Fig. 1). Exchange transfusion with a 3% solution of Hb (Polytaur)_n during MCAO markedly reduced infarct volume to 59% of that in the albumin-transfused group (Fig. 2). When the concentration of Hb (Polytaur)_n was increased to 6%, infarct size was reduced to 50% of that in the albumin-transfused group. Infarct volume as a percent of the volume of the cerebral hemisphere was $58 \pm 12\%$ in the albumin-transfused group, $35 \pm 17\%$ in the group transfused with 3% Hb (Polytaur)_n, and $28 \pm 6\%$ in the group transfused with 6% Hb (Polytaur)_n. The values in the Hb-treated groups were significantly different from the value in the albumin-treated group whether absolute infarct volume or percentage of hemisphere values was analyzed. The value in the albumin-transfused group was not different from that in a group without transfusion ($58 \pm 5\%$).

Exchange transfusion with a 3% solution of Hb Polytaur after the onset of MCAO decreased infarct volume to 78% of that obtained in the albumin-transfused group, as previously reported (2). When the concentration of Hb Polytaur was increased to 6%, further reduction in infarct volume was observed (Fig. 2). Infarct volume was 34% of that measured in the albumin-transfused group. As a percent of the hemisphere volume, infarct volume was $61 \pm 4\%$ in the albumin-transfused group, $49 \pm 13\%$ in the group transfused with 3% Hb Polytaur, and $22 \pm$

8% in the group transfused with 6% Hb Polytaur. Infarct size with 3% Hb (Polytaur)_n was significantly less than with 3% Hb Polytaur, whereas infarct size with 6% Hb (Polytaur)_n was not significantly different from that with 6% Hb Polytaur.

DISCUSSION

The major finding of this study is that exchange transfusion of cell-free recombinant Hb polymers with high O₂ affinity during focal cerebral ischemia is capable of reducing infarct size. It has generally been assumed that cell-free Hb should have an O₂ affinity similar to red blood cell-based Hb to provide optimal O₂ delivery. However, the reduction in infarct volume with high-affinity Hb polymers implies that these polymers are capable of delivering O₂ to ischemic tissue. In models of severe anemia and hemorrhagic shock, transfusion of high O₂ affinity Hb was observed to produce better capillary perfusion and improved oxygenation in the hamster skin-fold microcirculation (16,19), due in part to less arteriolar constriction and less precapillary O₂ loss. A similar mechanism might be operative in metabolically active brain tissue. High O₂ affinity might limit O₂ loss across pial arterioles and conserve O₂ delivery to tissue with low PO₂.

Experimental studies of MCAO in rat demonstrated a beneficial effect of transfusion of a fumaryl cross-linked tetrameric Hb possessing a P₅₀ of 32 Torr (1,4,5). However, this product was not efficacious in a clinical trial of stroke (13). Transfusion during MCAO of a sebacyl cross-linked tetrameric Hb possessing a P₅₀ of 34 Torr failed to reduce acute injury volume in cats, possibly because improved intraischemic blood flow at reduced hematocrit was delayed by over 2 h after the transfusion (11). Polymerization of Hb tetramers reduces peripheral extravasation and the associated vasoconstriction (8). However, in many cases, polymerization can decrease cooperativity and P₅₀, thereby resulting in an O₂ carrier with properties analogous to myoglobin. With large-scale polymerization resulting in heterogeneous polymers >1 MDa, a P₅₀ of ~3 Torr and a cooperativity $n = 1.0$, Hb (Polytaur)_n decreased infarct volume by 41% when transfused at a 3% concentration. This reduction was significantly greater than the 22% reduction seen with the 3% solution of Hb Polytaur possessing a P₅₀ of 17 Torr and a cooperativity of $n = 2.0$. Thus transfusing a relatively dilute solution of a cell-free Hb with very low P₅₀ unexpectedly produced a large reduction in infarct volume.

At a 6% concentration, both polymers reduced infarct size similarly by 50–66%. The large magnitude of this reduction with 2 h of MCAO suggests that transfusion of these O₂ carriers during the first 45 min of MCAO maintains a viable state in a significant amount of tissue that would normally be in the core and penumbra regions of ischemia. The ability to salvage tissue with more delayed treatment was not currently examined. Because tissue mitochondrial PO₂ is presumed to drop to critically low levels during cerebral ischemia, elevating O₂ delivery by even modest amounts could have profound effects in ischemic regions with marginal levels of perfusion. Although regional blood flow was not measured autoradiographically in the present study, cell-free Hb may improve oxygenation independent of any increased perfusion that might occur at reduced hematocrit and blood viscosity. By increasing the effective solubility of O₂ in the plasma, cell-free Hb can facilitate the diffusion of O₂ from the red blood cell to the endothelium (9,10). Furthermore, because of the corpuscular nature of red blood cells in a single line with interspaced plasma in capillaries, the addition of an O₂ carrier in the plasma increases the effective surface area for O₂ diffusion across the endothelium (17). In addition, the low perfusion pressure across the microcirculation during ischemia is thought to increase the heterogeneity of red blood cell flux among capillaries, although plasma flow is maintained in most capillaries (15,18). Capillaries perfused with plasma and low red blood cell flux would have disproportionately improved O₂ flux after cell-free Hb transfusion and more homogeneous O₂ delivery. Thus cell-free Hb with low P₅₀ theoretically can improve oxygenation in ischemic tissue by multiple mechanisms.

Increasing the concentration of the infused Hb from 3 to 6% salvaged additional tissue in the case of Hb Polytaur. However, the additional salvage with a 6% solution of Hb (Polytaur)_n was not significantly greater than the 3% solution, presumably because the 3% solution was already salvaging most of the tissue outside the ischemic core, where perfusion is too low to benefit from an increase in O₂ carrying capacity. Increasing the concentration of the fumaryl cross-linked tetrameric Hb to 20% was noted to produce an additional benefit (4). Higher concentrations were not tested in the present study because the production yield is reduced and our fermentor system has limited capacity for producing large quantities of the protein. Higher concentrations could be beneficial by 1) increasing the O₂ delivery during MCAO, 2) extending the duration of elevated plasma of these Hbs, which have a circulating half-life of ~3 h in rodents (2,3), and 3) by increasing the oncotic pressure. The concern that a high concentration of transfused Hb might cause unwanted vasoconstriction and limit O₂ delivery was not borne out by the present data on infarct volume in ischemic brain.

Therefore, the present data indicate that cell-free Hb polymers with P₅₀ values much lower than that of red blood cell Hb are highly capable of salvaging ischemic brain. The assumption that P₅₀ of blood substitutes should be similar to that of blood might not be warranted when used during ischemic conditions.

Acknowledgements

The authors are grateful to Sarah Texel for technical assistance and to Tzipora Sofare for editorial assistance.

References

1. Aronowski J, Strong R, Grotta JC. Combined neuroprotection and reperfusion therapy for stroke. Effect of lubeluzole and diaspirin cross-linked hemoglobin in experimental focal ischemia. *Stroke* 1996;27:1571–1576. [PubMed: 8784132]
2. Bobofchak KM, Mito T, Texel SJ, Bellelli A, Nemoto M, Traystman RJ, Koehler RC, Brinigar WS, Fronticelli C. A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O₂ transporter. *Am J Physiol Heart Circ Physiol* 2003;285:H549–H561. [PubMed: 12689854]
3. Bucci E, Razynska A, Kwansa H, Matheson-Urbaitis B, O'Hearne M, Ulatowski JA, Koehler RC. Production and characteristics of an infusible oxygen-carrying fluid based on hemoglobin intramolecularly cross-linked with sebacic acid. *J Lab Clin Med* 1996;128:146–153. [PubMed: 8765210]
4. Cole DJ, Drummond JC, Patel PM, Nary JC, Applegate RL. Effect of oncotic pressure of diaspirin cross-linked hemoglobin (DCLHb) on brain injury after temporary focal cerebral ischemia in rats. *Anesth Analg* 1996;83:342–347. [PubMed: 8694316]
5. Cole DJ, Drummond JC, Patel PM, Reynolds LR. Hypervolemic-hemodilution during cerebral ischemia in rats: effect of diaspirin cross-linked hemoglobin (DCLHb) on neurologic outcome and infarct volume. *J Neurosurg Anesthesiol* 1997;9:44–50. [PubMed: 9016440]
6. Fronticelli C, Arosio D, Bobofchak KM, Vasquez GB. Molecular engineering of a polymer of tetrameric hemoglobins. *Proteins* 2001;44:212–222. [PubMed: 11455594]
7. Goto S, Sampei K, Alkayed NJ, Doré S, Koehler RC. Characterization of a new double-filament model of focal cerebral ischemia in heme oxygenase-2-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 2003;285:R222–R230. [PubMed: 12663258]
8. Matheson B, Kwansa HE, Bucci E, Rebel A, Koehler RC. Vascular response to infusions of a nonextravasating hemoglobin polymer. *J Appl Physiol* 2002;93:1479–1486. [PubMed: 12235050]
9. McCarthy MR, Vandegriff KD, Winslow RM. The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen carriers. *Biophys Chem* 2001;92:103–117. [PubMed: 11527583]
10. Page TC, Light WR, McKay CB, Hellums JD. Oxygen transport by erythrocyte/hemoglobin solution mixtures in an in vitro capillary as a model of hemoglobin-based oxygen carrier performance. *Microvasc Res* 1998;55:54–64. [PubMed: 9473409]

11. Rebel A, Ulatowski JA, Joung K, Bucci E, Traystman RJ, Koehler RC. Regional cerebral blood flow in cats with cross-linked hemoglobin transfusion during focal cerebral ischemia. *Am J Physiol Heart Circ Physiol* 2002;282:H832–H841. [PubMed: 11834476]
12. Sampei K, Ulatowski JA, Asano Y, Kwansa H, Bucci E, Koehler RC. Role of nitric oxide scavenging in vascular response to cell-free hemoglobin transfusion. *Am J Physiol Heart Circ Physiol* 2005;289:H1191–H1201. [PubMed: 15894576]
13. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, Stern KN, Koudstaal PJ. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. *Stroke* 1999;30:993–996. [PubMed: 10229733]
14. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, Rodman G. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: a randomized controlled efficacy trial. *JAMA* 1999;282:1857–1864. [PubMed: 10573278]
15. Theilen H, Schrock H, Kuschinsky W. Gross persistence of capillary plasma perfusion after middle cerebral artery occlusion in the rat brain. *J Cereb Blood Flow Metab* 1994;14:1055–1061. [PubMed: 7929648]
16. Tsai AG, Vandegriff KD, Intaglietta M, Winslow RM. Targeted O₂ delivery by low-P50 hemoglobin: a new basis for O₂ therapeutics. *Am J Physiol Heart Circ Physiol* 2003;285:H1411–H1419. [PubMed: 12805024]
17. Vadapalli A, Goldman D, Popel AS. Calculations of oxygen transport by red blood cells and hemoglobin solutions in capillaries. *Artif Cells Blood Substit Immobil Biotechnol* 2002;30:157–188. [PubMed: 12066873]
18. Villringer A, Them A, Lindauer U, Einhaupl K, Dirnagl U. Capillary perfusion of the rat brain cortex. An in vivo confocal microscopy study. *Circ Res* 1994;75:55–62. [PubMed: 8013082]
19. Wettstein R, Tsai AG, Erni D, Winslow RM, Intaglietta M. Resuscitation with polyethylene glycol-modified human hemoglobin improves microcirculatory blood flow and tissue oxygenation after hemorrhagic shock in awake hamsters. *Crit Care Med* 2003;31:1824–1830. [PubMed: 12794426]

Fig. 1. Oxygen dissociation curve of Hb Polytaur (solid line) and Hb (Polytaur)_n (dashed line). Measurements were performed at 37°C in 0.1 M phosphate buffer at pH 7.4.

Fig. 2.

Infarct volume ($\pm$ SD) in mice subjected to 2 h of middle cerebral artery occlusion and to an exchange transfusion performed between 10 and 30 min of occlusion with a solution of either A: 5% albumin ($n = 6$), 3% Hb (Polytaur)_n ($n = 7$), or 6% Hb (Polytaur)_n ($n = 5$); or B: 5% albumin ($n = 7$), 3% Hb Polytaur ($n = 7$), or 6% Hb Polytaur ($n = 5$). * $P < 0.05$ from albumin group (0% infused Hb concentration). + $P < 0.05$ between 3 and 6% Hb concentrations.