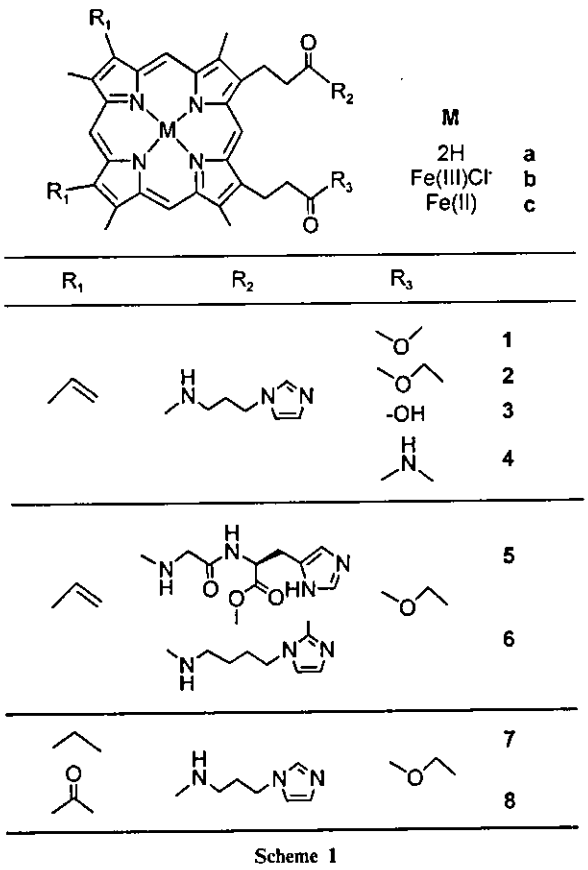


Scheme 1

of the crown ether-dithionite as reducing agent.[8] The UV-vis absorption spectrum of **2c** [Fe(II) complex] under a nitrogen (N_2) atmosphere showed a single broad band in the α,β region around 520–580 nm. This indicates the formation of a typical five-*N*-coordinate high-spin complex,[7] in which the proximal imidazole group intramolecularly coordinates to the central Fe(II) ion in the non-coordinating solvent (DMF) (Fig. 1). Because 2-methyl-imidazole significantly inhibits a sixth ligand binding to the *trans*-position, **6c** also demonstrated a similar 5-coordinated spectrum in DMF solution. Upon bubbling of the O_2 gas through the solution of **2c**, the spectral pattern immediately changed to that of the O_2 adduct complex. After adding carbon monoxide (CO) gas, the heme changed to a very stable carbonyl complex. Similar absorption changes were observed for all the heme derivatives, **1c–8c**. The absorption maxima (λ_{max}) of compounds **1c–8c** in DMF solution under N_2, O_2 and CO atmospheres are summarized in Table 1.

The positions and the relative intensities of all peaks were independent of the temperature changes from 5 to 25 °C. In general, the electron density of the porphyrin ring systematically changes the λ_{max} of the B-band and Q-band.[9] The replacement of the vinyl groups at the 3,8-positions of protoheme IX with ethyl groups (from **2c** to **7c**) produced a hypsochromic shift. In contrast, changing the vinyl groups to electron withdrawing acetyl groups (from **2c** to **8c**) produced a bathochromic shift.

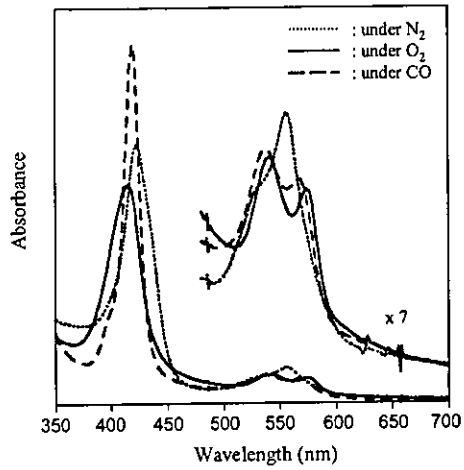


Fig. 1 UV-vis spectra of **2c** in DMF at 25 °C.

Table 1 Absorption maxima (λ_{max}) of heme derivatives in DMF under various conditions

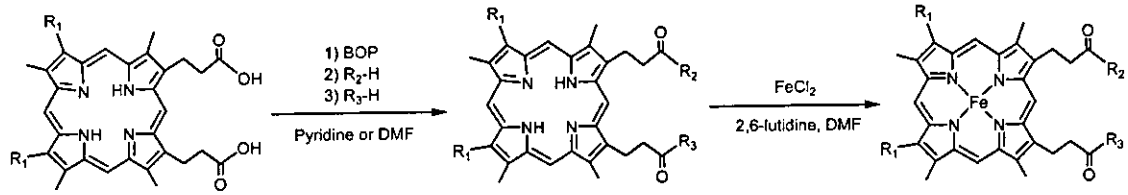
	λ_{max}/nm		
Compound	Under N_2	Under O_2	Under CO
1c (15 °C)	427, 530, 558	414, 543, 575	420, 540, 569
1c (25 °C)	424, 532, 559	412, 542, 575	420, 539, 567
2c (5 °C)	422, 531, 556	415, 541, 574	419, 537, 567
2c (25 °C)	421, 533, 557	409, 539, 571	418, 537, 565
3c (25 °C)	426, 537, 559	415, 543, 575	420, 539, 567
4c (5 °C)	421, 527, 555	413, 540, 572	417, 536, 564
5c (5 °C)	419, 529, 551	406, 537, 569	412, 534, 562
5c (25 °C)	423, 533, 557	408, 539, 573	419, 538, 567
6c (5 °C)	430, 555	413, 547, 576	418, 538, 561
7c (25 °C)	414, 523, 548	407, 531, 563	409, 529, 556
8c (5 °C)	440, 541, 571	432, 552, 579	434, 549, 576
8c (25 °C)	439, 545, 569	431, 552, 580	433, 548, 577

We could not find any significant difference in the absorption maxima of **1c–6c**, because modification of the propionic acids did not affect the electron density of the porphyrin macrocycle.

Preparation of rHSA–heme

Aqueous solutions of rHSA–heme were prepared by injecting an ethanol solution of the carbonylated heme into an aqueous solution of rHSA. The inclusion of heme into rHSA was confirmed by the following results: (1) Sepharose gel column chromatography showed the elution peaks of heme and rHSA coincided at the same position, (2) during dialysis of the rHSA–heme solution against phosphate buffer, the outer aqueous phase did not contain the heme component. The UV-vis absorption spectra of the obtained solution showed that the heme was retained as a CO adduct complex.

The binding number of heme in one rHSA was determined to be 0.9–1.1 (mol/mol) by assaying the iron and rHSA concentrations. The binding constant of **1b** for rHSA was estimated to be *ca.* 4×10^6 M^{-1}, which is approximately 1/25 of that for protohemin IX itself to albumin (*ca.* 1×10^8 M^{-1}).[10] Polar heme derivatives **3c** with monopropionic acid and **4c** with a methyl-



BOP: benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate

Scheme 2 Synthesis of protoheme IX derivatives.

Table 2 Absorption maxima (λ_{max}) of rHSA–hemes in phosphate buffer solution (pH 7.3) at 25 °C

Compounds	λ_{max}/nm		
	Under N_2	Under O_2	Under CO
rHSA–**1c**	420, 536, 561	414, 540, 567	419, 541, 566
rHSA–**2c**	420, 538, 561	416, 540, 567	421, 543, 567
rHSA–**5c**	422, 539, 561	418, 540, 571	422, 541, 569
rHSA–**8c**	444, 549, 571	432, 551, 580	440, 555, 578

amide capping group at the porphyrin periphery were partially oxidized to the Fe(III) state during the inclusion process. Since the binding force of the heme derivative to rHSA is a hydrophobic interaction,[11] relatively polar porphyrins may not be incorporated into a certain domain of rHSA and easily oxidized compared to more apolar ones.

The circular dichroism spectra of the rHSA–hemes (rHSA–**1c**, –**2c**, –**5c**, –**7c** and –**8c**) are almost identical to that of rHSA itself (not shown). This suggests that the secondary structure of the albumin host molecule did not change after incorporation of the hemes. Furthermore, the isoelectric points of these rHSA–hemes were all 4.8, which is the original value of rHSA. The surface net charges of rHSA remained unaltered after heme incorporation.

Dioxygenation of rHSA–heme in aqueous solution

Light irradiation of the CO adduct complex of rHSA–heme (rHSA–**1c**, –**2c**, –**5c**, –**6c**, –**7c** and –**8c**) under an N_2 atmosphere led to CO dissociation and demonstrated new spectral patterns with well-defined α and β bands. For example, the typical absorption spectral changes of rHSA–**2c** are shown in Fig. 2.

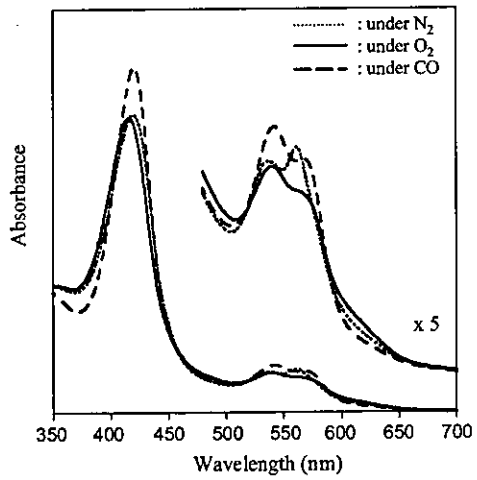


Fig. 2 UV-vis spectra of rHSA–**2c** in phosphate buffer solution (pH 7.3) at 25 °C.

From the nature of these spectra, we concluded that the obtained Fe(II) complexes are a mixture of Fe(II) 5-coordinated (high-spin) and 6-coordinated (low spin) species. It implies that the sixth coordinate position of the heme might be partially occupied by some amino acid residue of the protein scaffold. Upon exposure of O_2 to the Fe(II) complex of rHSA–**1c**, the spectrum changed to that of the O_2 adduct species. Although the aqueous micelle solution of **1c** with 5% surfactant (cetyltrimethylammonium bromide) forms a CO adduct complex, dioxygenation was not stable enough to measure the spectrum at 25 °C.[7e] In contrast, rHSA–**1c**, –**2c**, –**5c**, and –**8c** formed O_2 adduct complexes at 25 °C (pH 7.3) except for rHSA–**6c** and –**7c** (Table 2). The introduction of a methyl group to the 2-position of the imidazole ring is widely recognized to reduce the O_2 and CO binding affinities.[1] In this case, the strength of the imidazole coordination to the Fe(II) center is too weak to produce a stable O_2 adduct complex.

Table 3 Half-life ($\tau_{1/2}$) and O_2 binding affinity ($P_{1/2}$) of rHSA–hemes in phosphate buffer solution (pH 7.3) at 25 °C

Compounds	$\tau_{1/2}$/min	$P_{1/2}$/Torr
rHSA–**1c**	20	0.1
rHSA–**2c**	50	0.1
rHSA–**5c**	90	0.1
rHSA–**8c**	50	0.4

The oxidation process of dioxygenated rHSA–heme to the inactive Fe(III) state obeyed first-order kinetics. This indicates that the μ-oxo dimer formation was prevented by the immobilization of heme in the albumin structure. The half-life of the O_2 adduct complexes ($\tau_{1/2}$) and the O_2 binding affinities ($P_{1/2}$) of rHSA–hemes are summarized in Table 3. The histidylglycyl tail coordinated protoheme (**5c**) in rHSA showed the most stable O_2 adduct complex ($\tau_{1/2}$: 90 min) with respect to the imidazole bound ones. The more hydrophobic ethylpropionate (**2c**) also contributed to prolong the stability of the O_2 adduct complex relative to the methylpropionate protoheme (**1c**).

The $P_{1/2}$ values of rHSA–**1c**, –**2c** and –**5c** are 0.1 Torr at 25 °C. On the other hand, rHSA–**8c** showed a higher $P_{1/2}$ value (low O_2-binding affinity) compared to the others. The acetyl groups at the 3,8-positions of **8c** decrease the electron density of the porphyrin macrocycle, therefore $P_{1/2}$ could be significantly reduced. Traylor and co-workers found that the O_2 binding affinity of the chelated heme was sensitive to the electron density at Fe(II) and thus to the substituents at the heme periphery. The O_2 binding constant decreased by 1/6 upon changing the substituent from a vinyl to an acetyl group.[12] Our experimental data of hemes in rHSA are quite consistent with their observations.

Conclusion

A convenient one-pot synthesis of protoporphyrin IX derivatives with a covalently linked proximal base has been described. rHSA successfully incorporates the protoheme derivatives, providing an artificial hemoprotein, which can form an O_2 adduct complex at 25 °C. The rHSA–heme, in which the histidylglycil tail intramolecularly coordinates to the Fe(II) center, showed the most stable O_2 adduct complex with the relatively high O_2 binding affinity of 0.1 Torr.

Experimental

Materials and apparatus

All reagents were used as supplied commercially unless otherwise noted. All solvents were normally purified by distillation before use. DMF was distilled under reduced pressure in N_2. Pyridine was refluxed over and distilled from P_2O_5. The water was deionized using an ADVANTEC GS-200 system. The rHSA (Albrec®, 25 wt%) was obtained from NIPRO Corp. (Osaka).

Thin-layer chromatography was carried out on 0.2 mm precoated plates of silica gel 60 F254 (Merck). Purification was performed by silica gel 60 (Merck) column chromatography. The infrared spectra were measured with a JASCO FT/IR-410 spectrometer. The UV-vis absorption spectra were recorded by a JASCO V-570 spectrophotometer. The ^{1}H-NMR spectra were recorded using a JEOL Lambda 500 spectrometer. Chemical shifts were expressed in parts per million downfield from Me_4Si as the internal standard. The FAB-MS spectra were obtained using a JEOL JMS-SX102A spectrometer.

Synthesis of porphyrin derivatives

O-Methyl-L-histidyl-glycine[13] and 4-(*N*-(2-methylimidazolyl))-butylamine[14] were synthesized according to the reported procedures.

3,18-Divinyl-8-(3-methoxycarbonyl)ethyl-12-(3-(*N*-imidazolyl)propylamido)ethyl-2,7,13,17-tetramethylporphyrin (1a). A pyridine (7 mL) solution of 3-(*N*-imidazolyl)propylamine (35 μL, 0.29 mmol) was added dropwise to protoporphyrin IX (200 mg, 0.36 mmol) and benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate (411 mg, 0.93 mmol) in pyridine (20 mL) and stirred for 30 min at room temperature. The mixture was reacted for 4 h at 40 °C. After the addition of methanol (10 mL), the solution was stirred for another 12 h at 40 °C. The mixture was then poured into a 10% NaCl solution (1 L, 4 °C) and the suspension was centrifuged for 30 min at 7000*g*. The supernatant was discarded and the precipitate was collected and dried *in vacuo*. The residue was chromatographed on a silica gel column using $CHCl_3/CH_3OH$ = 8/1 (v/v) as the eluent. The main band was collected and dried at room temperature for several hours *in vacuo*, giving compound **1a** as a purple solid (75 mg, 20%). R_f = 0.3 ($CHCl_3/CH_3OH$ = 8/1 (v/v)); IR (NaCl) ν = 1731 (C=O, ester), 1646 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 506, 542, 575, 630 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.0 (s, 2H, inner), 1.8–2.4 (m, 4H, –(CH_2)$_2$–Im), 2.7 (m, 4H, –CH_2–COO–, NH–CH_2–), 3.2 (t, 2H, –CONH–CH_2–), 3.3–3.7 (m, 18H, por–CH_3, –CH_2–CO–, –$COOCH_3$), 4.2 (d, 4H, por–CH_2–), 5.4 (s, 1H, Im), 6.0–6.3 (m, 4H, =CH_2 (vinyl)), 6.4 (d, 1H, Im), 6.6 (d, 1H, Im), 8.0–8.4 (m, 2H, –CH= (vinyl)), 9.7 (m, 4H, *meso*); MS *m/z*: 681.67.

Fe(III) complex of 1a (1b). Iron(II) chloride tetrahydrate (106 mg, 0.53 mmol) was added to a dry DMF (10 mL) solution of **1a** (36 mg, 53 μmol) and 2,6-lutidine (30 μL, 0.27 mmol) under an N_2 atmosphere. The reaction mixture was stirred at 70 °C for 3 h. After confirming the disappearance of the porphyrin's fluorescence (600–800 nm, ex. 400 nm), the solution was cooled to room temperature and poured into 10% NaCl solution (1 L, 4 °C). The suspension was centrifuged for 30 min at 7000*g* and the supernatant was discarded. The precipitate was dried *in vacuo* and chromatographed on a silica gel column using $CHCl_3/CH_3OH$ = 8/1 (v/v) as the eluent. The main band was collected and dried at room temperature for several hours *in vacuo* to give compound **1b** as a brown solid (27 mg, 68%). R_f = 0.3 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1728 (C=O, ester), 1646 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 389, 513, 641 nm; HR-MS *m/z*: calcd for $C_{41}H_{43}O_3N_7Fe$: 737.2777, found: 737.2778 [M^+].

3,18-Divinyl-8-(3-ethoxycarbonyl)ethyl-12-(3-(*N*-imidazolyl)-propylamido)ethyl-2,7,13,17-tetramethylporphyrin (2a). The synthetic procedure of compound **2a** was the same as that used for **1a** except for using ethanol instead of methanol. Yield 30%; R_f = 0.4 ($CHCl_3/CH_3OH$ = 10/1); IR (NaCl) ν = 1650 (C=O, amide), 1732 (C=O, ester) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 409, 544, 580, 633 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.1 (s, 2H, inner-NH), 0.8–0.9 (t, 3H, –COO–CH_2–CH_3), 1.3–1.5 (t, 2H, –CONH–CH_2–CH_2–), 3.0–3.1 (t, 2H, –CH_2–Im), 3.1–3.3 (m, 4H, –CH_2–COO), 3.5–3.7 (m, 12H, por–CH_3), 3.8–3.9 (m, 2H, –COO–CH_2–CH_3), 4.2–4.4 (d, 4H, por–CH_2–), 6.1 (s, 1H, Im), 6.1–6.4 (q, 5H, =CH_2 (vinyl), Im), 6.6–6.7 (d, 1H, Im), 6.9–7.0 (d, 1H, Im), 8.1–8.3 (m, 2H, –CH= (vinyl)), 9.8–10.2 (m, 4H, *meso*); MS *m/z*: 695.29.

Fe(III) complex of 2a (2b). Iron insertion to **2a** was carried out by the same procedure as in the **1b** preparation. Yield 80%; R_f = 0.3 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1651 (C=O, amide), 1725 (C=O, ester) cm^{-1}, UV-vis ($CHCl_3$) λ_{max} = 406, 520, 578 nm; HR-MS *m/z*: calcd. for $C_{42}H_{45}O_3N_7Fe$: 751.2933, found: 751.2953 [M^+].

3,18-Divinyl-8-(3-carboxy)ethyl-12-(3-(*N*-imidazolyl)propyl-amido)ethyl-2,7,13,17-tetramethylporphyrin (3a). Sodium hydroxide (2 N, 4.5 mL) was added to the methanol (10 mL) solution of **2a** (266 mg, 0.38 mmol) and the mixture was stirred for 12 h at room temperature. It was brought to dryness *in vacuo*. Methanol was added to the residue and the mixture was added dropwise to 10% NaCl solution (pH 2, 4 °C). It was centrifuged for 30 min at 7000*g* and the precipitate was collected and dried *in vacuo*, affording compound **3a** as a brown solid (187 mg, 78%), IR (KBr) ν = 1652 (C=O, amide), 1707 (C=O, –COOH) cm^{-1}; UV-vis (DMSO) λ_{max} = 409, 508, 543, 578, 631 nm; ^{1}H-NMR (d_6-DMSO) δ: −3.5 (s, 2H, inner-NH), 1.6–1.7 (t, 2H, –CONH–CH_2–CH_2–), 2.8–2.9 (t, 2H, –CH_2–Im), 3.1–3.3 (m, 2H, –CONH–CH_2–), 3.5–3.9 (m, 12H, por–CH_3), 4.2–4.4 (d, 4H, por–CH_2–), 6.1 (s, 1H, Im), 6.1–6.4 (q, 5H, =CH_2 (vinyl), Im), 6.6–6.7 (d, 1H, Im), 6.9–7.0 (d, 1H, Im), 8.5–8.6 (m, 2H, –CH= (vinyl)), 10.2–10.4 (m, 4H, *meso*); MS *m/z*: 670.41.

Fe(III) complex of 3a (3b). Iron insertion to **3a** was carried out by the same procedure as in the **1b** preparation. Yield 80%; IR (KBr) ν = 1646 (C=O, amide), 1707 (C=O, –COOH) cm^{-1}; UV-vis (DMSO) λ_{max} = 403, 508, 631 nm; HR-MS *m/z*: calcd. for $C_{40}H_{41}O_3N_7Fe$: 723.2620, found: 724.2668 [M + H^+].

3,18-Divinyl-8-(3-methylamido)ethyl-12-(3-(*N*-imidazolyl)-propylamido)ethyl-2,7,13,17-tetramethylporphyrin (4a). Compound **4a** was synthesized according to the same procedure as for **1a** except for using methyl amine instead of methanol. Yield 20%; R_f = 0.5 ($CHCl_3/CH_3OH$ = 3/1); IR (NaCl) ν = 1631 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 409, 509, 543, 579, 632 nm; ^{1}H-NMR (CD_3OD, $CDCl_3$) δ: −4.0 (s, 2H, inner), 1.8–2.4 (m, 4H, –(CH_2)$_2$–Im), 2.5 (t, 3H, –CONH–CH_3), 2.9 (m, 2H, –CONH–CH_2–), 3.3 (m, 4H, –CH_2–CONH–), 3.4–3.6 (m,12H, por–CH_3), 5.5 (s, 1H, Im), 6.0 (s, 1H, Im), 6.1–6.4 (m, 4H, =CH_2 (vinyl)), 6.8 (m, 1H, Im), 8.1–8.3 (m, 2H, –CH= (vinyl)), 9.7–9.9 (q, 4H, *meso*); MS *m/z*: 680.69.

Fe(III) complex of 4a (4b). Iron insertion to **4a** was carried out by the same procedure as in the **1b** preparation. Yield 67%; R_f = 0.3 ($CHCl_3/CH_3OH$ = 5/1); IR (NaCl) ν = 1646 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 521, 565 nm; HR-MS *m/z*: calcd. for $C_{41}H_{44}O_3N_7Fe$: 736.2937, found: 736.2938 [M^+].

3,18-Divinyl-8-(3-ethoxycarbonyl)ethyl-12-(((3-*N*-glycyl-L-histidinyl)-9-oxymethyl)carbonyl)ethyl-2,7,13,17-tetramethyl-porphyrin (5a). The synthetic procedure of compound **5a** was same as that used for **1a**. DMF was used instead of pyridine, because it dissolves *O*-methyl-L-histidyl-glycine. Yield 15%; R_f = 0.4 ($CHCl_3/CH_3OH$ = 15/1); IR (NaCl) ν = 1635 (C=O, amide), 1725 (C=O, ester) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 405, 505, 541, 577, 627 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.6 (s, 2H, inner-NH), 2.7–2.9 (m, 2H, Im–CH_2–), 3.0–3.5 (m, 18H, por–CH_3, –CH_2–CH_2–CO–NH–, –CH_2–CH_2–COO–CH_2–CH_3), 3.6 (s, 2H, –CONH–CH_2–CONH–), 3.8 (s, 3H,–OCH_3), 4.0–4.3 (d, 4H, por–CH_2–), 4.3–4.5 (m, 1H, α-CH), 6.0–6.4 (m, 4H, =CH_2 (vinyl)), 7.4 (s, 1H, Im–H), 8.0–8.3 (m, 5H, –CH= (vinyl), Im–H), 9.8–10.0 (m, 4H, *meso*-H); MS *m/z*: 782.68.

Fe(III) complex of 5a (5b). Iron insertion to **5a** was carried out by the same procedure as in the **1b** preparation. Yield 75%; R_f = 0.5 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1660 (C=O, amide), 1734 (C=O, ester) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 388, 508, 637 nm; HR-MS *m/z*: calcd. for $C_{44}H_{46}O_6N_8Fe$: 838.2890, found: 839.2929 [M + H^+].

3,18-Divinyl-8-(3-ethoxycarbonyl)ethyl-12-(4-(*N*-(2-methyl-imidazolyl))butylamido)ethyl-2,7,13,17-tetramethylporphyrin (6a). Compound **6a** was synthesized by the same procedure as for **1a** except for using 4-(*N*-(2-methylimidazolyl))butylamine instead of 3-imidazolylpropylamine. Yield 20%; R_f: 0.1 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1732 (C=O, ester), 1651 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 506, 542, 576, 630 nm; ^{1}H-NMR ($CDCl_3$) δ: −4.2 (s, 2H, inner-H), 0.4–0.6 (m, 4H, CONH–CH_2–(CH_2)$_2$–), 1.4–1.5 (d, 3H, Im–CH_3), 2.2–2.4 (m,

2H, –CONH–CH_2–), 2.8–3.1 (m, 4H, por–CH_2–CH_2–), 3.2–3.3 (t, 2H, –CH_2–Im), 3.4 (s, 3H, –COO–CH_3), 3.5–3.8 (m, 12H, por–CH_3), 4.2–4.4 (t, 4H, por–CH_2–), 5.6–5.7 (d, 1H, Im), 5.8 (m, 1H, Im), 6.1–6.4 (q, 4H, $=CH_2$ (vinyl)), 8.1–8.2 (m, 2H, –CH= (vinyl)), 9.8–10.1 (m, 4H, *meso*); MS *m/z*: 709.72.

Fe(III) complex of 6a (6b). Iron insertion to **6a** was carried out by the same procedure as in the **1b** preparation. Yield 64%; R_f = 0.2 ($CHCl_3/CH_3OH$ = 8/1); IR (NaCl) ν = 1732 (C=O, ester), 1652 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 401, 580, 630 nm; HR-MS *m/z*: calcd. for $C_{43}H_{47}O_3N_7Fe$: 765.3090, found: 766.3184 [M + H$^+$].

3,18-Diethyl-8-(3-carboxy)ethyl-12-(3-(*N*-imidazolyl)propyl-amido)ethyl-2,7,13,17-tetramethylporphyrin (7a). Compound **7a** was synthesized by the same procedure as for **1a** except for using mesoporphyrin IX instead of protoporphyrin IX. Yield 10%; R_f: 0.4 ($CHCl_3/CH_3OH$ = 20/1); IR (NaCl) ν = 1732 (C=O, ester), 1651 (C=O, amide) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 408, 506, 542, 576, 630 nm; ^{1}H-NMR ($CDCl_3$) δ: 0.8 (m, 3H, CH_3–CH_2–O–), 1.6 (m, 2H,–CH_2–CH_2–Im), 1.8 (t, 6H, CH_3–CH_2–Por), 2.9 (m, 2H, CH_3–CH_2–O–), 3.1 (m, 4H, –CH_2–COO–), 3.2 (m, 2H, –NH–CH_2–), 3.6 (m, 12H, CH_3–Por), 3.8 (m, 2H,–CH_2–Im), 4.1 (m, 4H,CH_3–CH_2–Por), 4.4 (m, 4H, Por–CH_2–), 6.6 (s, 1H, –NHCO–), 6.0–6.8 (d, 3H, Im), 10.0 (m, 4H, *meso*); MS *m/z*: 699.32.

Fe(III) complex of 7a (7b). Iron insertion to **7a** was carried out by the same procedure as in the **1b** preparation. Yield 62%; R_f: 0.2 ($CHCl_3/CH_3OH$ = 6/1); IR (NaCl) ν = 1732 (C=O, ester), 1668 (C=O, amide) cm^{-1}; UV-vis (DMF) λ_{max} = 394, 566, 591 nm; MS *m/z*: calcd for $C_{42}H_{49}O_3N_7Fe$: 755.3292, found 755.3246 [M$^+$].

3,18-Diacetyl-8-(3-carboxy)ethyl-12-(3-(*N*-imidazolyl)propyl-amido)ethyl-2,7,13,17-tetramethylporphyrin (8a). Compound **8a** was synthesized by the same procedure as for **1a** except for using diacetyldeuteroporphyrin IX instead of protoporphyrin IX. Yield 27%; R_f: 0.1 ($CHCl_3/CH_3OH$ = 6/1); IR (NaCl) ν = 1735 (C=O, ester), 1651 (C=O, amide, ketone) cm^{-1}; UV-vis ($CHCl_3$) λ_{max} = 423, 516, 551, 586, 640 nm; ^{1}H-NMR ($CDCl_3$) δ: 1.5 (m, 2H, –CH_2–CH_2–Im), 2.9–3.1 (M, 4H, –CH_2–Im, –NH–CH_2–), 3.2–3.3 (m, 16H, –CH_2–COO, CH_3–Por), 3.4 (m, 6H, CH_3–CO–), 3.6 (m, 3H, CH_3–OCO–), 4.1 (m, 4H, Por–CH_2–), 6.0 (d, 1H, Im), 6.6 (m, 1H, Im), 6.9 (m, 1H, Im), 10 (m, 4H, *meso*); MS *m/z*: 712.

Fe(III) complex of 8a (8b). Iron insertion to **8a** was carried out by the same procedure as in the **1b** preparation. Yield 64%; R_f: 0.1 ($CHCl_3/CH_3OH$ = 6/1); IR (NaCl) ν = 1735 (C=O, ester), 1651 (C=O, amide, ketone) cm^{-1}; UV-vis (DMF) λ_{max} = 418, 550, 578 nm; HR-MS *m/z*: calcd. for $C_{41}H_{43}O_5N_7Fe$: 769.2675, found 769.2697 [M$^+$].

Preparation of ferrous complex in DMF solution

The central Fe(III) ion of the porphyrin derivatives were reduced to the Fe(II) state using the complex of 18-crown-6 ether with $Na_2S_2O_4$ in DMF under aerobic conditions as previously reported.[8]

Preparation of rHSA–heme

Aqueous ascorbic acid (0.2 M, 10 μL) was added to an ethanol solution of the hemin derivative (2 mM, 1 mL) under a CO atmosphere. After complete reduction of the central Fe(III) ion, the ethanol solution (2 mM, 25 μL) was injected into the phosphate buffer solution (1 mM, pH 7.3, 2.5 mL) of rHSA (20 μM) under an Ar atmosphere. The formation of carbonyl rHSA–heme was confirmed by its UV-vis spectrum. The binding ratio of heme to rHSA was estimated by each concentration. The heme concentration was measured by the assay of iron ion using inductively coupled plasma spectrometry (Seiko, SPS7000A). The rHSA concentration was determined by bromocresol green along with the Albumin Test Wako kit (Wako Pure Chemical Industries).

Measurement of O_2 binding ability

The half-life of the O_2 adduct complex was determined by the time course of spectral changes, and the O_2 binding affinity ($P_{1/2}$) was determined by spectral changes at various partial pressures of O_2 according to previous reports.[2,15] rHSA–heme concentrations of 20 μM were normally used for UV-vis absorption spectroscopy. The spectra were recorded within the range of 350–700 nm.

Acknowledgements

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Exchange transfusion with entirely synthetic red-cell substitute albumin-heme into rats: Physiological responses and blood biochemical tests

Yubin Huang,[1] Teruyuki Komatsu,[1] Hisashi Yamamoto,[2] Hirohisa Horinouchi,[3] Koichi Kobayashi,[3] Eishun Tsuchida[1]

[1]*Advanced Research Institute for Science and Engineering, Waseda University,3-4-1 Okubo, Shinjuku, Tokyo 169-8555, Japan*

[2]*Pharmaceutical Research Center, NIPRO Corporation, 3023 Nojimachi, Kusatsu, Shiga 525-0055, Japan*

[3]*Department of General Thoracic Surgery, School of Medicine, Keio University,35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan*

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Abstract: Recombinant human serum albumin (rHSA) incorporating 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-[tetrakis{α,α,α,α-*o*-(1-methylcyclohexanoyl)amino}-phenyl]porphinatoiron(II) [albumin-heme (rHSA-heme)] is an artificial hemoprotein which has the capability to transport O_2 *in vitro* and *in vivo*. A 20% exchange transfusion with rHSA-heme into anesthetized rats has been performed to evaluate its clinical safety by monitoring the circulation parameters and blood parameters for 6 h after the infusion. Time course changes in all parameters essentially showed the same features as those of the control group (without infusion) and rHSA group (with administration of the same amount of rHSA). Blood biochemical tests of the withdrawn plasma at 6 h after the exchange transfusion have also been carried out. No significant difference was found between the rHSA-heme and rHSA groups, suggesting the initial clinical safety of this entirely synthetic O_2-carrier as a red-cell substitute. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 63–69, 2004

Key words: exchange transfusion; entirely synthetic red-cell substitute; albumin-heme; blood biochemical tests; O_2 carrier.

INTRODUCTION

Although hemoglobin (Hb)-based O_2 carriers are currently undergoing clinical trials as red-cell substitutes or oxygen therapeutics, there are still some concerns about new infectious pathogens in Hb and unresolved side effects such as vasoconstriction.[1–4] Recombinant human serum albumin (rHSA) incorporating the synthetic heme albumin-heme is an artificial hemoprotein that has the potential to bind and release O_2 under physiological conditions in the same manner as Hb and myoglobin.[5–7] In fact, the albumin-heme can transport O_2 through the body and release O_2 to tissues as a red-cell substitute without any acute side effects.[8,9] For example, rHSA including four molecules of 2-[8-{*N*-(2-methylimidazolyl)}-octanoyloxymethyl]-5,10,15,20-[tetrakis{α,α,α,α-*o*-(1-methylcyclohexanoyl)amino}phenyl]porphinatoiron(II) (Scheme 1) is one of the promising materials.[7] Recent study on the 30% exchange transfusion with rHSA-heme after 70% hemodilution with 5 wt % rHSA with the use of anesthetized rats demonstrated that the administration of this material improved the circulatory volume and resuscitated the hemorrhagic shock state.[10] The declined MAP and the mixed venous partial O_2 pressure immediately recovered, and the lowered renal cortical O_2-pressure also significantly increased.

In order to evaluate the initial clinical safety of this albumin-based O_2-carrier, a 20% exchange transfusion with rHSA-heme into anesthetized rats was performed, and the time courses of the circulation parameters (MAP, HR, respiration rate) and blood parameters (paO_2, pvO_2, pH, blood cell numbers) were measured for 6 h, which is adequate time to determine acute toxicity. Blood biochemical tests of the withdrawn plasma were also been carried out.

Correspondence to: E. Tsuchida; e-mail: eishun@waseda.jp
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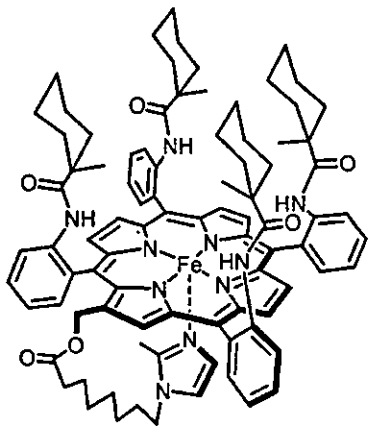
MATERIALS AND METHODS

Preparation of rHSA-heme

Recombinant human serum albumin (rHSA, Albrec®, 25 wt %) was obtained from the NIPRO Corp. (Osaka). The 5



Scheme 1

g/dL rHSA was made by diluting Albrec® with saline solution (Otsuka Pharmaceutical Co., Ltd.). The rHSA-heme solution ([rHSA]: 4.9 g/dL, pH 7.45, [heme]: 2.8 m*M*, O_2-binding affinity ($p_{1/2}O_2$): 37 Torr) used for the experiments was prepared according to a previously reported procedure.[11] The red-colored rHSA-heme solution was filtered with the use of DISMIC 25CS045AS just before use.

Exchange transfusion

The investigations were carried out with 18 male Wister rats (305±3.6 g). The animals were placed on the heating pad under an inhalation anesthesia with sevofluran; its concentration was kept 2.0% for the operations and 1.5% for the experiments. After incision was made in the neck, the heparinized catheter (Natsume Seisakusho SP-55) was introduced into the right common carotid artery for blood withdrawal. Other catheters (SP-31) were inserted into the left femoral artery for a continuous MAP monitoring, and the right femoral vein for sample injection.

After stabilization of the animal condition, the 20% exchange transfusion (total blood volume of rat was estimated to be 64 mL/kg weight) was performed by 1 mL blood withdrawal via the common carotid artery and 1 mL rHSA-heme infusion from the femoral vein (each 1 mL/min) with four repeating cycles (n = 6, rHSA-heme group). Blood was taken from the artery (0.3 mL) and vein (0.2 mL) at the following five points; (i) before, (ii) immediately after, (iii) 1 h after, (iv) 3 h after, and (v) 6 h after the exchange transfusion. MAP and HR were recorded with the use of a polygraph system (NIHON KODEN LEG-1000 Ver. 01-02 or PEG-1000 Ver. 01-01) at the same time point as stated above. Withdrawn blood was rapidly applied to a blood gas system (Radio Meter Trading ABL555) to obtain the O_2-pressure (paO_2) and pH for the arterial blood, and the O_2-pressure (pvO_2) for the venous blood. The blood cell numbers were counted by a multisystem automatic blood cell counter (Sysmex KX-21). After 6 h, 4 mL of the venous blood was taken for each animal before sacrifice by sodium pentobarbital overdose. The blood samples were centrifuged at 4°C (Beckman Coulter Co., Optima LE-80K for 3500 × rpm, 10 min), and the plasma phase was frozen (−20°C) for blood biochemical tests. As a reference group, the 5 g/dL rHSA solution was administered similarly into rats (n = 6, rHSA group). Furthermore, six rats without infusion (operation only) were also set as a control group.

All animal handling and care was in accordance with the NIH guidelines. The protocol details were approved by the Animal Care and Use Committee of Keio University.

Blood biochemical tests

A total of 30 analytes, that is, total protein (TP), albumin (Alb), albumin/globulin ratio (A/G), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-glutamyltransferase (γ-GTP), leucine aminopeptidase (LAP), choline esterase (ChE), total bilirubin (TBil), direct bililubin (DBil), creatinine (CRN), blood urea nitrogen (BUN), uric acid (UA), amylase, lipase, creatine phosphokinase (CPK), total cholesterol (TCho), free cholesterol (FCho), cholesterol ester (ECho), β-lipoprotein (β-LP), high-density lipoprotein (HDL)-cholesterol, neutral fat (triglyceride, TG), total lipid, free fatty acid (FFA), phospholipids (PhL), K^+, Ca^{2+} and Fe^{3+}, were measured by Kyoto Microorganism Institute (Kyoto).

Data analysis

MAP, HR, respiration rate, paO_2 and pvO_2 were represented as percent ratios of the basal values with mean ± standard error of mean (SEM). Body temperature, pH, blood cell numbers and the data of blood biochemical tests were shown as mean ± SEM.

Statistical analysis were performed by repeated-analysis measures of variance (ANOVA) followed by the paired t-test for comparison with a basal value (body temperature), by the Bartlett test followed by the Tukey-Kramer multiple comparison test for pH, blood cell numbers, and the results of the blood biochemical tests, and by the Kruskal-Wallis test followed by the Tukey-Kramer multiple comparison test for more than three groups (MAP, HR, respiration rate, paO_2, and pvO_2). Values of $p < 0.05$ were considered significant. The statistical analytical software used was StatView (SAS Institute, Inc.).

RESULTS

Circulation parameters

The basal values of some measurements, the data values of which are represented by percent ratios, are summarized in Table I. There are no significant differences between the three groups (control, rHSA, and rHSA-heme groups).

The body temperature of each group was constantly

TABLE I
Basal Values of Each Group

	Control	rHSA	rHSA-heme
MAP (mmHg)	99 ± 2.8	101 ± 2.8	100 ± 6.1
HR (beats/min)	404 ± 19	435 ± 19	420 ± 13
Respiration rate (breaths/min)	66 ± 0.7	75 ± 4.0	70 ± 2.1
paO_2 (mmHg)	84.7 ± 3.1	84.9 ± 3.7	80.8 ± 2.2
pvO_2 (mmHg)	51.0 ± 1.5	45.7 ± 1.1	51.0 ± 1.7
Body weight (g)	305 ± 4.0	304 ± 2.8	305 ± 4.1

maintained within 36.9–37.4°C during the experiments [Fig. 1(a)].

The MAP time course in the control group demonstrated only a small deviation within 83.8–100.0% for 6 h. In the rHSA and rHSA-heme groups, the observed changes in MAP were almost the same as those of the control group. They ranged within 93.2–100 and 89.3–100%, respectively. It is remarkable that no vasoactive reaction was seen after the infusion of rHSA-heme [Fig. 1(b)].

The HR of the control, rHSA, and rHSA-heme groups remained unaltered for 6 h. The values of the control, rHSA, and rHSA-heme groups were in the range of 98.3–103.9, 96.9–108.8, and 85.7–100.0%, respectively [Fig. 1(c)].

The respiration rates also remained stable during the measurements. No significant difference was recognized among the three groups [Fig. 1(d)].

Blood-gas parameters

No difference in the pH changes was observed in the three groups. The pH values of the control, rHSA, and rHSA-heme groups were constant in the narrow ranges of 7.42–7.45, 7.42–7.44, and 7.42–7.44, respectively [Fig. 2(a)].

The paO_2 values of the control, rHSA, and rHSA-heme groups were also constant in the range of 100–108.1, 100–109.1, and 100–110.8%, respectively, by the end of measurements [Fig. 2(b)].

The pvO_2 of the control, rHSA and rHSA-heme groups demonstrated only small changes within 81.5–100.0, 84.9–108.4, and 81.9–100.0%, respectively [Fig. 2(c)].

Blood cell numbers

The Hct values of the control group were unchanged from 34.8–37.0% during the experiment. On the other hand, the 20% exchange transfusion with the

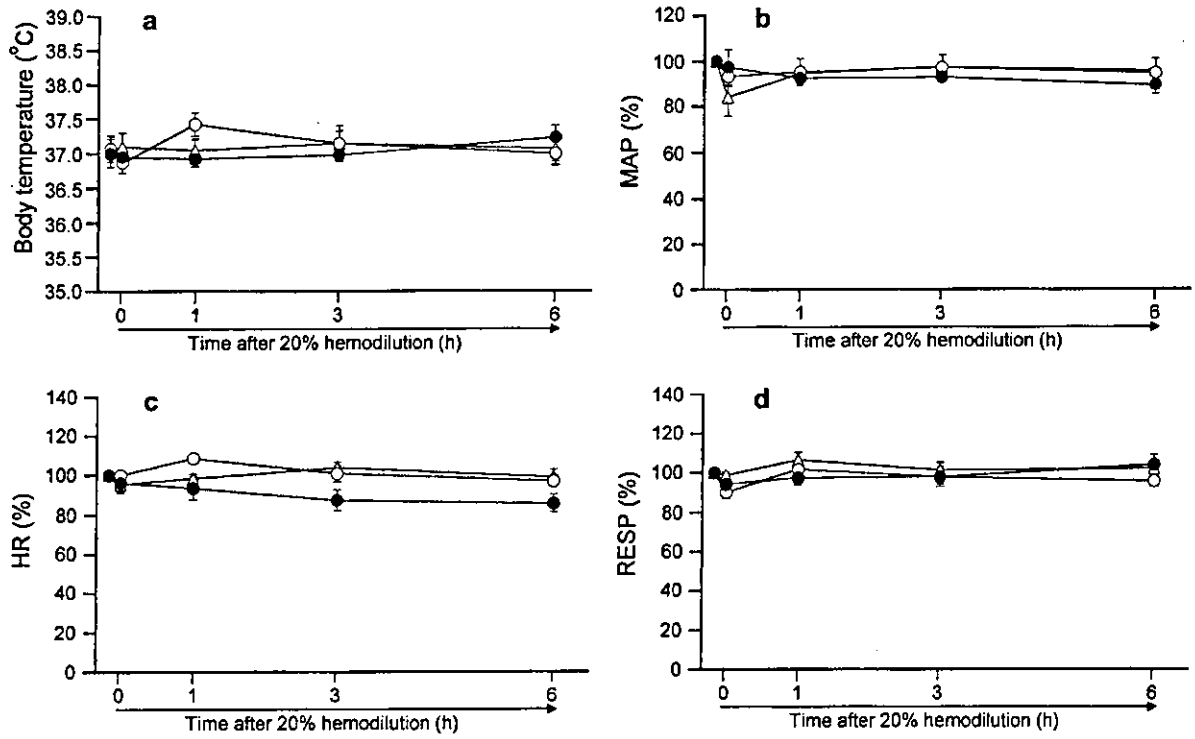


Figure 1. (a) Time courses of body temperature, (b) mean arterial pressure (MAP), (c) heart rate (HR), and (d) respiration rate (RESP) in anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of six rats (triangles, control group without infusion; open circles, rHSA group; solid circles, rHSA-heme group).

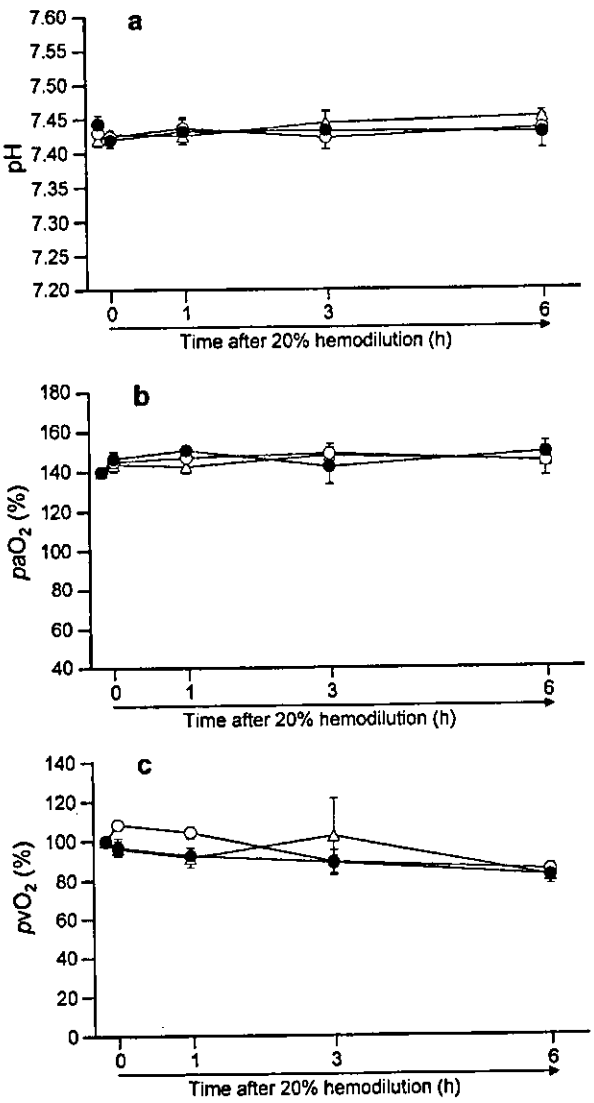


Figure 2. Time courses of (a) blood pH, (b) arterial blood O_2 pressure (paO_2), and (c) venous blood O_2-pressure (pvO_2) in anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of six rats (triangles, control group without infusion; open circles, rHSA group; solid circles, rHSA-heme group).

rHSA or rHSA-heme solution led to rapid decreases in the Hct from 39.0 to 31.7% or from 42.2 to 33.8%, respectively. These declined values were constant for 6 h [Fig. 3(a)]. Concomitantly, the RBC numbers in the rHSA and rHSA-heme groups decreased from 634.8×10^4 to $512.8\times10^4/\mu L$ and from 620.3×10^4 to $497.7\times10^4/\mu L$ by the exchange transfusion. They did not recover for 6 h [Fig. 3(b)].

The WBC numbers in the rHSA and rHSA-heme groups also showed similar declines; however, they appeared to be relatively slow with some deviations [Fig. 3(c)]. The PLT numbers of the rHSA and rHSA-heme groups changed in the range of 57.1–76.9 × $10^4/\mu L$ and 58.4–65.6 × $10^4/\mu L$, respectively [Fig. 3(d)].

Blood biochemical tests

In order to evaluate liver function, kidney function and electrolyte balance after the infusion of rHSA-heme, total 30 analytes of the blood biochemical tests were selected for rat plasma (Fig. 4). In the rHSA group, the A/G ratio increased, and TCho, Echo, and HDL-C decreased compared to the control group.

In the rHSA-heme group, the A/G ratio and fatty acid increased, and LDH, ALP, TCho, Echo, HDL-C, and PhL decreased in comparison to the control group. However, the other analytes showed almost the same values as those of the control group. With respect to the rHSA group, no significant difference was found except for the increases in the amylase, free fatty acid, and iron concentration, and the decrease of ALP and ECho.

DISCUSSION

After the 20% exchange transfusion with the 5 g/dL rHSA solution, the Hct and RBC numbers decreased approximately 80% of their basal values. This was only because of the 20% dilution and did not imply any acute toxicity of rHSA. The significant increase in the A/G ratio was caused by the slightly high rHSA concentration of 5 g/dL. Because the albumin concentration in rat plasma is generally 3–4 g/dL, the exchange transfusion with this sample led to the increase in the albumin concentration and decrease in the globulin concentration, thus resulting in the elevated A/G ratio. The hemodilution by the 20% replacement of the animal's blood volume could reduce the level of the analytes immediately after the infusion; however, the majority of the data in the rHSA group recovered within 6 h; only LDH, TCho, Echo, and HDL-C showed values that were about 80% of the corresponding ones in the control group.

Because FCho was almost the same as that in the control group, the decreases in TCho, ECho, and HDL-C were presumably caused by the decreasing of ECho, which might come from the depressed synthetic function in the livers by administration of the external protein. The circulation parameters and blood gas parameters in the rHSA group moved within the narrow range for 6 h—almost the same as those observed in the control group. Based on these data, the authors are certain that the administration of rHSA

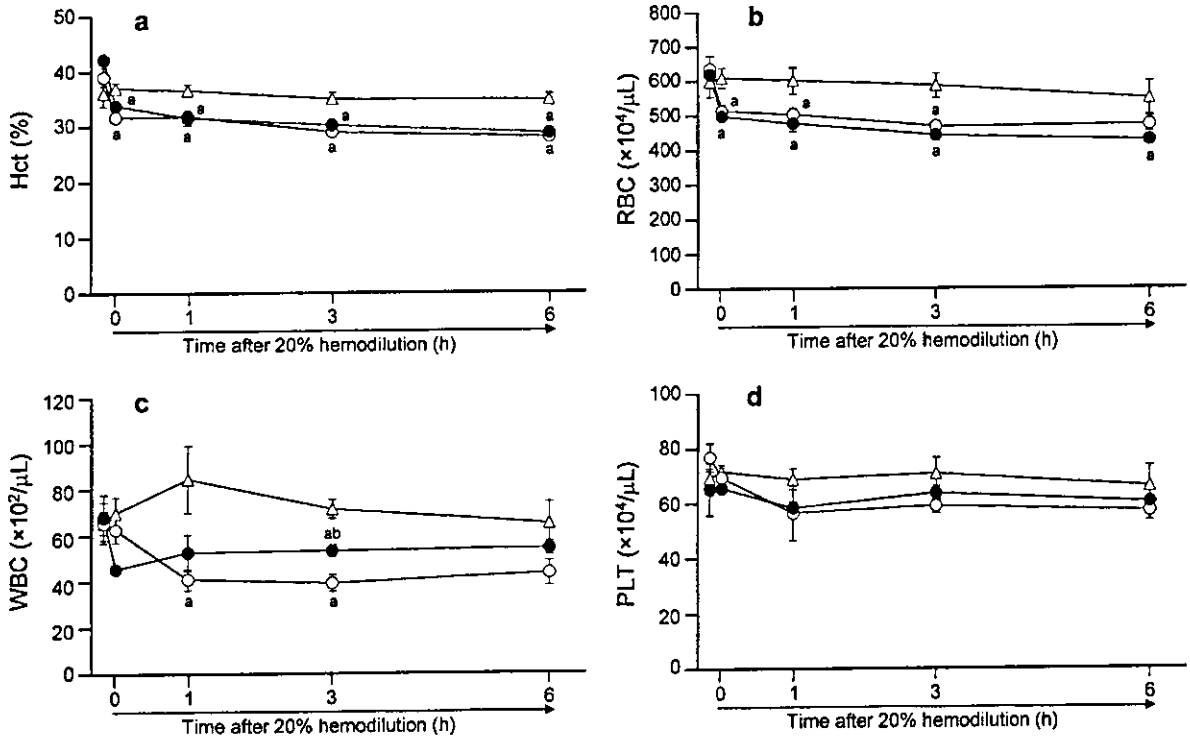


Figure 3. Time courses of hematocrit (Hct) value (a), red blood cell (RBC) numbers (b), white blood cell (WBC) numbers (c) and platelet (PLT) numbers (d) in whole blood of anesthetized rats after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of 6 rats (triangles, control group without infusion; open circles, rHSA group; solid circles, rHSA-heme group). $^{a}p < 0.05$ versus control group (Tukey-Kramer test).

into anesthetized rats did not induce any toxic reaction under the present experimental conditions.

After the exchange transfusion with the rHSA-heme solution, no significant difference was seen in the circulation parameters and blood gas parameters. Previous studies to elucidate the influence of albumin-heme on the MAP changes and microcirculation in the capillaries demonstrated that neither vasoconstriction nor hypertension occurred, because of the low permeability of the albumin vehicle through the vascular endothelium.[9] The latest exchange transfusion experiment with rHSA-heme after isovolemic hemodilution also supported this hypothesis.[10]

By careful inspection of the results from the blood biochemical tests, it was found that the rHSA-heme group showed higher values of amylase and free fatty acid, and lower values of ALP and ECho compared to those of the rHSA group. In general, amylase has two isozymes, which are secreted by the pancreatic parenchyma and salivary gland. When the concentrations of the amylase and lipase (pancreatic parenchyma enzyme) simultaneously increase, it may be a sign of pancreatitis and other pancreas disorders.[12-14] However, the slight increase in the lipase concentration in the current protocol was not significant. Therefore, the possibility of pancreas disorders caused by the rHSA-heme infusion is considered negligible. The high amylase value is probably due to the isozyme secreted by the salivary gland.

With the increase in the free fatty acid, the TG concentration also tended to elevate to some degree. The increase in TG may activate the lipase, which acts as its decomposition enzyme, and results in the increasing free fatty acid as a metabolite. The relation between rHSA-heme and the TG increase is still not clear.

CONCLUSIONS

The appearance of the all animals showed absolutely no change for 6 h after the 20% exchange transfusion with albumin-heme. The physiological responses of the blood circulation, gas equilibria, and blood cell numbers in the rHSA-heme group were almost the same as those of the control and rHSA groups. MAP and HR did remain constant after the injection of the rHSA-heme, suggesting again that the albumin-based O_2-carrier does not induce the vasoconstriction. The blood biochemical tests of the withdrawn plasma of rHSA-heme group showed results similar to those of the control and rHSA groups, implying no acute toxicity by the exchange transfusion

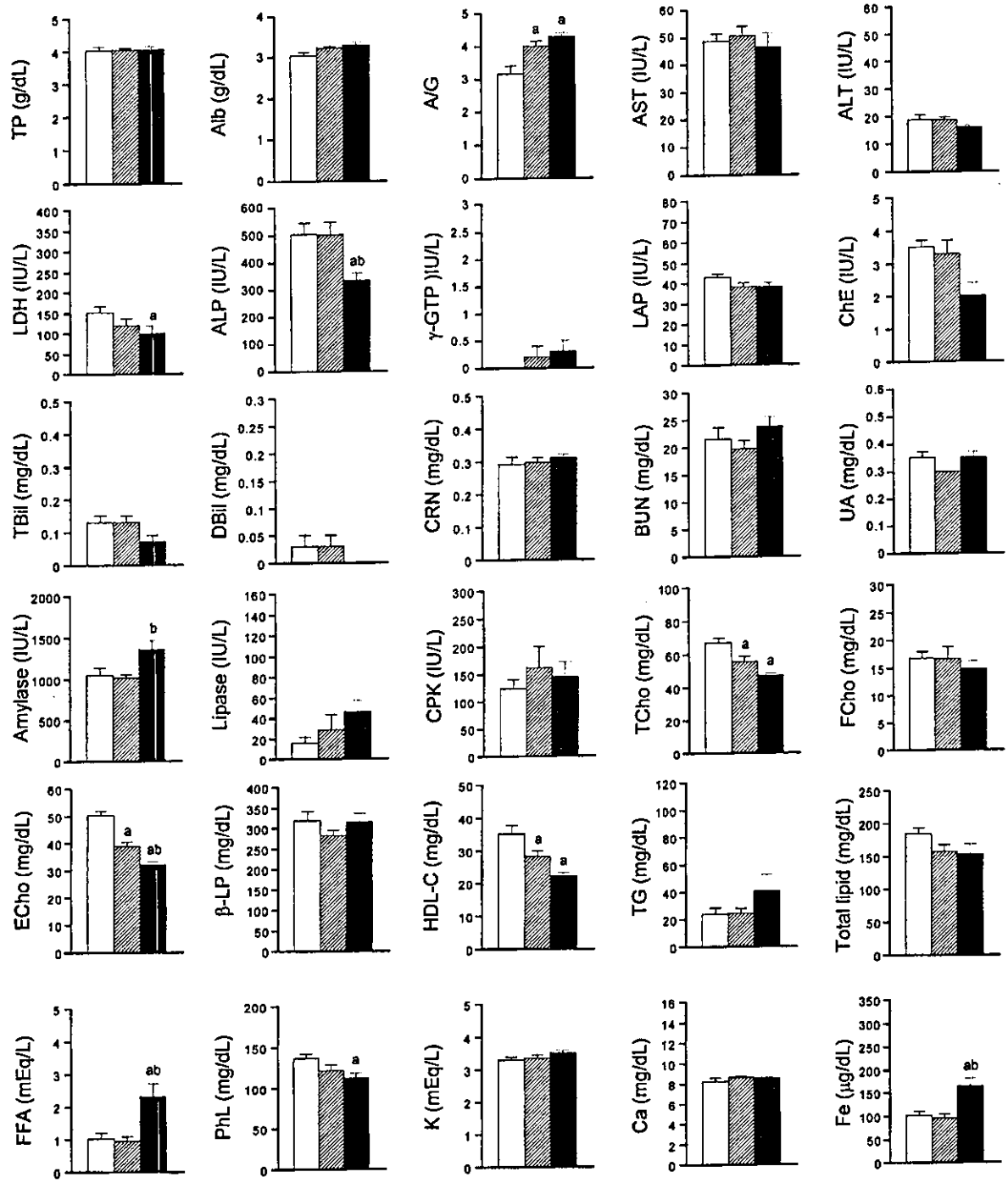


Figure 4. Blood biochemical tests of rat plasma after 20% exchange transfusion with rHSA-heme or rHSA solution. Each value represents the mean ± SEM of six rats (white bar, control group without infusion; diagonal bar, rHSA group; black bar, rHSA-heme group). [a]$p < 0.05$ versus control group (Tukey-Kramer test); [b]$p < 0.05$ versus rHSA group (Tukey-Kramer test).

with rHSA-heme. These results showed the initial clinical safety of the rHSA-heme solution, which allows us to undergo further advanced preclinical testing of this synthetic O_2-carrying hemoprotein as a new class of red-cell substitutes. Biochemical tests and histopathological observations for 7 days after the exchange transfusion with rHSA-heme will be reported in a forthcoming article.

The authors are grateful to Dr. Toshiya Kai (NIPRO Corp.) for preparation and characterizations of the albumin-heme solutions. They also thank Dr. Ichiro Hirotsu

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Dioxygenation of Human Serum Albumin Having a Prosthetic Heme Group in a Tailor-Made Heme Pocket

Teruyuki Komatsu,*,† Naomi Ohmichi,† Patricia A. Zunszain,‡ Stephen Curry,‡ and Eishun Tsuchida*,†

Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan, and Department of Biological Sciences, Imperial College London, Huxley Building, South Kensington Campus, London SW7 2AZ, United Kingdom

Received July 4, 2004; E-mail: eishun@waseda.jp

Human serum albumin (HSA, MW = 66.5 kD) is the most abundant plasma protein in our bloodstream and serves as a transporter for small hydrophobic molecules such as fatty acids, bilirubin, and steroids.[1,2] Hemin dissociated from methemoglobin is also bound within a narrow D-shaped cavity in subdomain IB of HSA with an axial coordination of Tyr-161 and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (Figure 1).[3,4] In terms of the general hydrophobicity of the α-helical pocket, HSA potentially has features similar to the heme-binding site of myoglobin (Mb) or hemoglobin (Hb). However, even if one reduces the ferric HSA−hemin to obtain a ferrous complex, it is immediately oxidized by O_2. This is due to the fact that HSA lacks a proximal histidine, which enables the heme group to bind O_2.[3−5] We have shown that HSA incorporating tetraphenylporphinatoiron derivatives having a covalently linked axial-base can absorb O_2 under physiological conditions with a O_2 binding affinity similar to that of Hb.[6]

In this paper, we report for the first time the introduction of a proximal histidine into the subdomain IB of HSA by site-directed mutagenesis to construct a tailor-made heme pocket, which allows a reversible O_2 binding to the prosthetic heme group. Laser flash photolysis experiments revealed that this artificial hemoprotein appears to have two different geometries of the axial-imidazole coordination and shows rather low O_2 binding affinity.

We designed two recombinant HSA (rHSA) mutants, in which single or double mutations were introduced into subdomain IB: I142H [rHSA(A)] and I142H/Y161L [rHSA(B)] (Figure 1). Replacement of Y161 by histidine was not done because modeling experiments indicated that the distance from N_ϵ(H142) to Fe(heme) would be too great (>4 Å). In our mutants, the N_ϵ(H142)−Fe distance was estimated to be 2.31 Å (compared to 2.18 Å in Mb). The specific mutations were introduced into the HSA coding region in a plasmid vector (pHIL-D2 HSA) using the QuikChange (Stratagene) mutagenesis kit, and the mutants were expressed in the yeast species *Pichia pastoris*.[7] The rHSA(wild-type or mutants)−hemin complexes were prepared essentially according to our previously reported procedures, except that myristate was not added.[4] The resulting hemoproteins exhibited only a single band in SDS-PAGE.

In the absorption spectrum of the rHSA(wt)−hemin solution, the distinct charge-transfer (CT) band of Fe^{3+}−phenolate appeared at 625 nm.[8] A magnetic circular dichroism (MCD) spectrum showed a W-shaped feature in the Soret-band region.[9] These results imply the formation of a high-spin Fe^{3+} complex with the phenolate oxygen ligand of Y161, which is quite consistent with that found in the crystal structure.[3,4]

rHSA(B)−hemin did not exhibit the CT band because of the Y161L mutation and was easily reduced to the corresponding ferrous complex by adding a small molar excess amount of aqueous $Na_2S_2O_4$ under an Ar atmosphere. A single broad absorption band (λ_{max} = 559 nm) in the α,β region was very similar to that of deoxy Mb and indicated the formation of a five-N-coordinate Fe^{2+} complex (Figure 2).[10] The spectral pattern was unaltered in the temperature range of 0−25 °C. The shape of the asymmetric MCD spectrum also resembled that of deoxy Mb.[11] This suggests that the heme is accommodated into the tailor-made heme pocket with an axial coordination involving His-142.

Upon exposure of the rHSA(B)−heme solution to O_2 gas, the UV−vis absorption changed to that of the dioxygenated complex (λ_{max} = 412, 537, 573 nm) at 0−25 °C[10] (lifetime of the O_2−adduct: ca. 10 min). After exposure to flowing CO, the heme produced a typical carbonyl complex (λ_{max} = 419, 538, 565 nm).

On the contrary, rHSA(A)−heme could not bind O_2 even at low temperature (~0 °C). It can be thought that the polar phenolate residue at the top of the porphyrin platform is likely to accelerate the proton-driven oxidation of the Fe^{2+} center. Replacing Y161 by

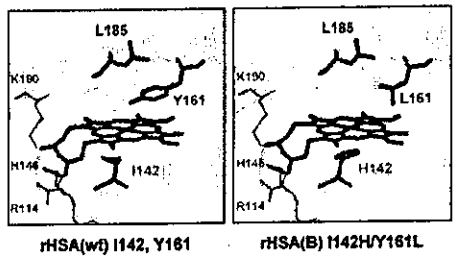

Figure 1. Prosthetic heme group complexed within the heme pocket in subdomain IB of rHSA(wt) and rHSA(B) mutant produced on the basis of the crystal structure coordinate of the rHSA−hemin complex (ref 4).

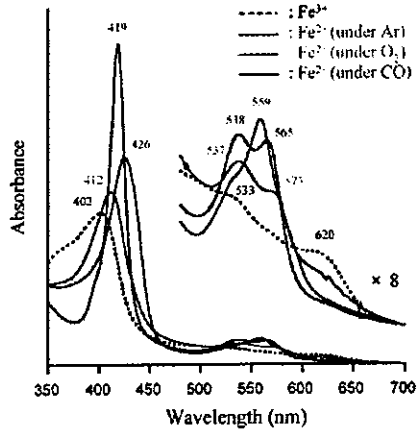

Figure 2. UV−vis absorption spectral changes of rHSA(B)−heme in phosphate buffered solution (pH 7.0, 50 mM) at 8 °C.

† Waseda University.
‡ Imperial College London.

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Table 1. O_2 and CO binding Parameters of rHSA(B)–heme in Phosphate Buffered Solution (pH 7.0, 50 mM) at 22 °C

hemoprotein	k_{on}^{CO} (M^{-1} s^{-1})	$k_{on}^{O_2}$ (M^{-1} s^{-1})	$k_{off}^{O_2}$ (s^{-1})	$P_{1/2}^{O_2}$ (Torr)
rHSA(B)–heme[a]	2.0×10^6 (I), 2.7×10^5 (II)	7.5×10^6 (I and II)	221 (I), 1.7×10^3 (II)	18 (I), 134 (II)
Mb[b]	5.1×10^5	1.4×10^7	12	0.51
Hb (*R*-state)[c]	4.6×10^6	3.3×10^7	13	0.24

[a] Number in parentheses (I or II) indicates species I or II. [b] At 20 °C; ref 17. [c] At 20–21.5 °C; ref 18.

hydrophobic leucine greatly enhanced the stabilization of the O_2–adduct complex.

We then employed laser flash photolysis (Nd:YAG SHG; λ = 532 nm; 6 ns pulse width) to evaluate the kinetics of O_2 and CO bindings to rHSA(B)–heme.[12-14] Interestingly, the time dependence of the absorption change accompanying the CO recombination showed double-exponential profiles; the ratio of the amplitude of the fast and slow phases was always 3:2. On the other hand, the rebinding process of O_2 obeyed monophasic decay. On the basis of studies on synthetic model hemes, it has been shown that the proximal-side steric effect is the only primary factor that influences the association rate for CO but not for O_2.[12-14] One possible explanation is that there may be two different geometries of the axial His-142 coordination and that each one shows the individual kinetics of the CO rebinding. Marden and co-workers also found a similar behavior in CO association with HSA–heme and interpreted it as indicating that there are two orientations of the heme plane in the albumin scaffold.[5] In our case, the alternative geometries may arise because the heme molecule binds into the pocket of subdomain IB in two orientations related by a 2-fold rotation about its center (180° rotational isomers). Asymmetric 3,8-divinyl groups at the porphyrin periphery, in particular, would occupy different positions that result in a small divergence of the porphyrin ring and its iron center. The crystal structure analyses could not resolve this ambiguity, because the two configurations exist as a mixture.[3,4]

Our hypothesis is consistent with infrared spectroscopy data. The CO coordinated with rHSA(B)–heme showed a broad ν_{CO} at 1962 cm^{-1} with a shoulder at 1942 cm^{-1}. We therefore suggest that there are two different modes of π-back-donation from the central Fe^{2+} to the bound CO. It is remarkable that the lower stretching frequency is very close to that of Mb (ν_{CO} = 1943 cm^{-1}).[15] Attempts to determine the ratio of the two hemin orientations by ^1H NMR spectroscopy unfortunately failed.[16] The downfield spectra of rHSA(B)–hemin in met and met-azido forms did not show sharp resonances of the four porphyrin CH_3 groups.

The two geometries of the His-142 ligation in rHSA(B)–heme should yield two different O_2 binding affinities. By analyzing CO/O_2 competitive binding following laser flash photolysis, we obtained the association and dissociation rate constants for O_2 ($k_{on}^{O_2}$, $k_{off}^{O_2}$), and the O_2 binding affinities ($P_{1/2}^{O_2}$) for these two species (I and II) (Table 1).[12-14] The $P_{1/2}^{O_2}$ values were determined to be 18 and 134 Torr, respectively; this means that the O_2 binding affinities were 2.8 and 0.4%, respectively, of that observed for Mb.[17,18] Kinetically, for species I with $P_{1/2}^{O_2}$ of 18 Torr, an 18-fold increase in the $k_{off}^{O_2}$ value leads to the low affinity for O_2. Repeated studies with synthetic model hemes and engineered mutants of Mb or Hb have shown that low polarity of the microenvironment around the heme site decreases the O_2-binding affinity by increasing the dissociation rate constant.[12-14,17] In this albumin-based hemoprotein, the porphyrin ring is buried in the core of the pocket entirely made of hydrophobic residues. Therefore, the O_2 binding affinity becomes significantly lower than those of Mb and Hb. In species II with $P_{1/2}^{O_2}$ of 134 Torr, the proximal pull effect could further increase the $k_{off}^{O_2}$ value and may cause a large decline in the O_2 binding affinity.

We have recently found that heme is accommodated into the different architecture of a tailor-made heme pocket in rHSA (Y161L/L185H) [rHSA(C)] and that the rHSA(C)–heme complex also binds O_2 as well. In this case, the proximal histidine coordinates with the heme plane from the roof side, and the O_2 molecule binds from the floor side (Figure 1). Our combined structural and mutagenic approach allows us to significantly enhance the O_2 binding properties of rHSA–heme complexes and thereby mimic the diverse biological reactivities of natural hemoproteins. From the viewpoint of clinical applications, "O_2-carrying plasma albumin" could be of extreme medical importance not only for red blood cell substitutes but also for O_2-therapeutic reagents. The crystal structure analysis of this new class of artificial hemoproteins is now underway.

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Supporting Information Available: Time dependence of the absorption change accompanying the CO or O_2 rebinding to rHSA(B)–heme after the laser flash photolysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Exchange transfusion with synthetic oxygen-carrying plasma protein "albumin-heme" into an acute anemia rat model after seventy-percent hemodilution

Teruyuki Komatsu,[1] Hisashi Yamamoto,[2] Yubin Huang,[1] Hirohisa Horinouchi,[3] Koichi Kobayashi,[3] Eishun Tsuchida[1]

[1]*Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan*

[2]*Pharmaceutical Research Center, NIPRO Corporation, 3023 Nojimachi, Kusatsu-shi, Shiga 525-0055, Japan*

[3]*Department of General Thoracic Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*

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Abstract: Recombinant human serum albumin (rHSA) incorporating the synthetic heme "albumin-heme" is an oxygen-carrying plasma protein that has the potential to be a red blood cell substitute. The physiological responses to a 30% exchange transfusion with two types of albumin-heme (rHSA-FecycP, rHSA-FepivP) solutions after 70% isovolemic hemodilution with 5 g/dL rHSA were investigated using anesthetized rats. The circulation parameters, blood parameters, renal cortical oxygen pressure (pO_2), and muscle tissue pO_2 were carefully monitored for 60 min after the injection. The declined mean arterial pressure and the mixed venous partial pO_2 significantly recovered to 70.8 and 91.9% of the basal values by intravenous infusion of albumin-hemes, respectively. The lowered renal cortical pO_2 also increased, indicating oxygen transport by this synthetic hemoprotein. The administration of albumin-heme into the acute anemia rat model after hemorrhage improved the circulatory volume and resuscitated the shock state. Both rHSA-FecycP and rHSA-FepivP transported oxygen through the body. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 644–651, 2004

Key words: exchange transfusion; entirely synthetic oxygen-carrier; hemoprotein; albumin-heme; red blood cell substitute

INTRODUCTION

Because the risk of transmission of viral illness by transfused blood has become extremely low, transfusion of donor blood has nowadays become a routine procedure. However, this level of safety has been achieved at great cost, and the hepatitis virus or unknown infectious pathogens cannot be completely excluded by the nucleic acid test system. Furthermore, (i) the transfusion of donor blood requires cross-matching and compatibility tests to avoid the consequences of a hemolytic reaction in the recipient, and (ii) the purified red blood cells (RBCs) should be stored at 4°C. These requirements substantially limit the availability of blood in a disaster or emergency. Under this background, several types of hemoglobin (Hb)-based oxygen carriers have been studied as an RBC substitute or oxygen therapeutic agent.[1–4] Nevertheless, these materials do not fulfill all the requirements of blood replacement compositions. The first concern is the source of human Hb, which is limited by the availability of outdated human blood. Animal blood will raise anxiety of transmission of animal pathogens; for instance, bovine Hb may harbor prions. The Hb products potentially carry risks because of the biological origin of the raw materials. Additional problems of Hb-based oxygen carriers (i.e., modified Hb) are the high colloid osmotic pressure[5] and its vasopressor effect, which increase blood pressure and decrease cardiac output.[6–8] Although diaspirin intramolecularly crosslinked Hb is no longer developed, half of the products in advanced clinical trials still showed vasoconstriction.[4] The precise mechanism of

Correspondence to: E. Tsuchida; e-mail: eishun@waseda.jp
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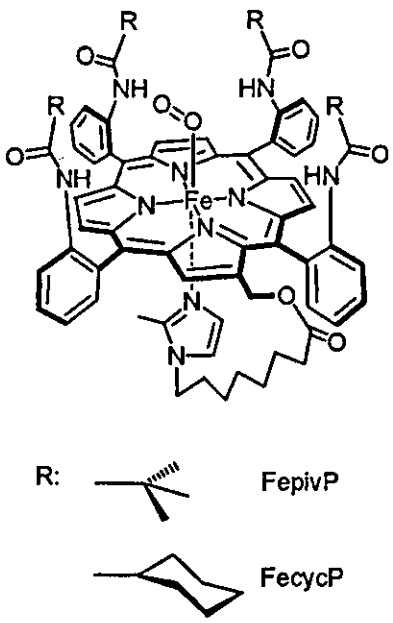


Chart 1

this hypertension is controversial, but many investigators suspect that the small Hb molecules penetrate the vascular endothelium and capture the endothelial-derived relaxing factor, namely, nitric oxide. In fact, glutaraldehyde crosslinked human polyHb does not show vasoactivity.[9] Others believe that the excessive delivery of oxygen to arteriolar vascular walls induces autoregulatory vasoconstriction.[10–13]

We have prepared entirely synthetic oxygen carriers without Hb. The incorporation of synthetic heme, 2-[8-{*N*-(2-methylimidazolyl)}octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamidophenyl)porphinatoiron(II) (FepivP, Chart 1), into recombinant human serum albumin (rHSA) provides an artificial hemoprotein (rHSA-heme; rHSA-FepivP) that can reversibly bind and release oxygen under physiological conditions (pH 7.3, 37°C) like Hb.[14–19] An FepivP analog, 2-[8-{*N*-(2-methylimidazolyl)}-octanoyloxymethyl]-5,10,15,20-tetrakis{α,α,α,α-*o*-(1-methylcyclohexanamido)phenyl}porphinatoiron(II) (FecycP, Chart 1), which contains more hydrophobic 1-methylcyclohexanoyl fences, is also included into rHSA as well and the obtained rHSA-FecycP hybrid showed the same oxygen-binding equilibrium and a longer lifetime ($\tau_{1/2}$) of the oxy species compared with that of rHSA-FepivP.[20] The physicochemical properties and oxygen-transporting ability of these rHSA-based oxygen carriers, which are free of infectious pathogens, satisfy the initial clinical requirements as an RBC substitute. It is remarkable that the colloid osmotic pressure of 5 g/dL rHSA, rHSA-FecycP, and rHSA-FepivP are all the same.[15] We herein report the systematic evaluations of the physiological responses to exchange transfusions with two types of albumin-heme solutions, rHSA-FecycP and rHSA-FepivP, in anesthetized rats. The animals were first placed into an acute anemia induced by 70% hemodilution and then underwent a 30% exchange transfusion with albumin-hemes. Circulation parameters and blood parameters were monitored for 60 min after the injection. The *in vivo* oxygen deliveries to the renal cortex and the muscle tissue were also measured.

MATERIALS AND METHODS

Preparation of rHSA-heme solutions

The rHSA (Albrec®, 25 wt %) was obtained from NIPRO Corp. (Osaka, Japan). The 5 g/dL rHSA was made by diluting Albrec® with saline solution (Otsuka Pharmaceutical Co., Ltd.). Two types of albumin-hemes (rHSA-FecycP and rHSA-FepivP [rHSA]: approximately 5 g/dL) used for the exchange transfusions were prepared according to our previously reported procedure with some modifications.[19] The characteristics of these oxygen-carrying fluids are almost identical (Table I) except for the stability of the oxygenated species; oxy rHSA-FecycP showed a 4.5-fold longer lifetime against autooxidation than that of oxy rHSA-FepivP at 37°C *in vitro*. These red-colored albumin-heme solutions were filtered using a DISMIC 25CS045AS just before use.

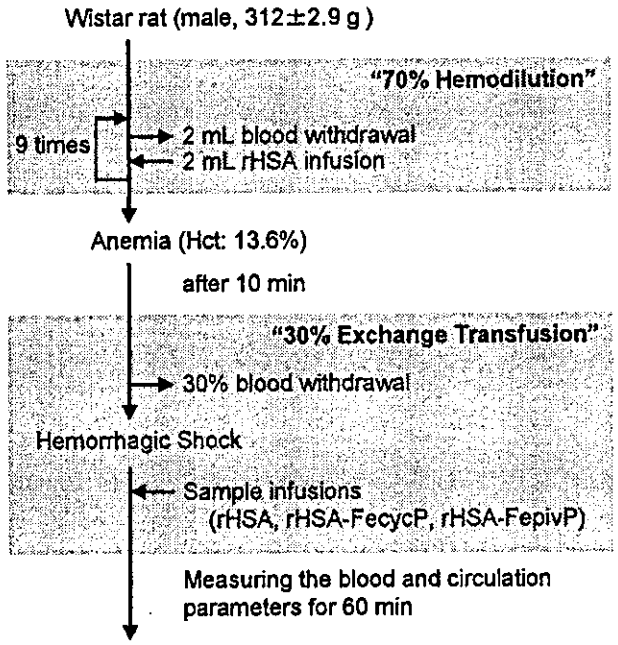
Hemodilution and exchange transfusion

The investigations were performed according to our previously reported experimental protocols with 24 male Wistar rats (312 ± 2.9 g) (Scheme 1).[21] All animal handling and care were in accordance with the National Institutes of Health guidelines. The protocol details were approved by the Animal Care and Use Committee of Keio University.

The animals were placed on a heating pad under an inhalation anesthesia with sevoflurane; its concentration was kept at 2.0% for the operations and 1.5% for the experiments. After an incision was made in the neck, the heparinized catheter (Natume Seisakusho SP-55) was introduced into the right common carotid artery for blood withdrawal. Other catheters (SP-31) were also inserted into the left femoral artery for continuous mean arterial pressure (MAP) monitoring, and the right femoral vein for sample injection.

TABLE I
Characteristics of the Albumin-Heme Solutions

	rHSA-FecycP	rHSA-FepivP
pH	7.45	7.46
Viscosity (cP)	1.1	1.1
Heme (m*M*)	2.8	2.7
Met-heme content (%)	0	0
rHSA (%)	4.9	4.9
*p*50 (mmHg) at 37°C	37	32



Scheme 1. Diagram illustrating the exchange, bleeding procedures, and materials.

Polarographic oxygen-electrodes (Intermedical POE-10N and POE-40PS) were inserted into the left renal cortex and muscle in the abdomen. Core temperature was measured with a rectal thermometer (Technol Seven D617-1).

After stabilization of the animal's condition, isovolemic 70% hemodilution was performed using 5 g/dL rHSA solution. Blood withdrawal via the common carotid artery (2 mL) and rHSA infusion from the femoral vein (2 mL) (each 1 mL/min) were repeated for nine cycles until the hematocrit (Hct) was reduced to 13.6% (32% of the initial Hct value: 42.6%). After 10 min, a 30% volume of the circulatory blood was withdrawn, producing a severe hemorrhagic shock state. The same volume of the samples (rHSA-FecycP and rHSA-FepivP) was then intravenously (iv) injected (n = 6 each). As negative- or positive-control groups, rats were infused with the 5 g/dL rHSA solution or the shed rat blood ([heme] = 5.3 m*M*) (rHSA group and whole blood group, n = 6 each). A blood-taking from the artery (0.3 mL) and vein (0.2 mL) was performed at the following six points: (i) before the 70% hemodilution, (ii) immediately after the hemodilution, and (iii) 10 min after the hemodilution, (iv) immediately after the 30% bleeding, (v) immediately after the sample infusion, and (vi) 60 min after the sample infusion. The animals were sacrificed after the experiments by sodium pentobarbital overdose. MAP was monitored through the femoral artery catheter connected to a transducer (NIHON KODEN TP-400 T or Becton Dickinson P23XL) with a pressure coupler and an amplifier (NIHON KODEN PP-101H and AP-100H). An electrocardiogram was measured using a bioelectric coupler and an amplifier (NIHON KODEN PC-101H and AC-100H), and heart rate (HR) was obtained from its R wave. MAP and HR were recorded by a Polygraph System (NIHON KODEN LEG-1000 version 01-02 or PEG-1000 version 01-01) at the following eight points: (i) before the 70% hemodilution, (ii) immediately after the hemodilution, (iii) 10 min after the hemodilution, (iv) immediately after the 30% bleeding, (v) immediately after the sample infusion, (vi) 5 min, (vii) 30 min, and (viii) 60 min after the sample infusion. The renal cortical oxygen-tension [ptO_2(R)] and muscle tissue oxygen-tension [ptO_2(M)] were simultaneously measured with a tissue oxygen-pressure monitor (Inter Medical PO_2-100DW) using an oxygen electrode (described above). Withdrawn blood was rapidly applied to a blood gas system (Radio Meter Trading ABL555) to measure the oxygen pressure (paO_2), pH and carbon dioxide pressure ($paCO_2$) of the arterial blood, and the oxygen pressure (pvO_2) of the venous blood. The RBC numbers were counted for the mixture of arterial blood (30 μL) and ACD-A solution using a multisystem automatic blood cell counter (Sysmex KX-21).

Data analysis

MAP, HR, respiration rate, paO_2, pvO_2, ptO_2(R), ptO_2(M), and $paCO_2$ are represented by percent ratios of the basal values as mean ± standard error of mean (SEM). Body temperature, pH, RBC numbers, and body weight are denoted by mean ± SEM.

Statistical analyses were performed using the Tukey-Kramer multiple comparison test for more than three groups [pH, RBC numbers, body weight, MAP, HR, respiration rate, paO_2, pvO_2, ptO_2(R), ptO_2(M), and $paCO_2$], and by repeated-measures analysis of variance followed by paired t test for comparison with a basal value (body temperature). Values of $p < 0.05$ were considered significant. The statistical analytic software used was StatView (SAS Institute Inc.).

RESULTS

Blood parameters

The basal values of several measurements, for which data are shown in percent ratios, are summarized in Table II. There are no significant differences in the four groups.

The 70% hemodilution decreased the Hct to 12.8–14.3% and RBC numbers to 202.2–223.2 × 10^4/μL, leading to acute anemia [Fig. 1(a,b)]. By a further 30% exchange transfusion with the whole blood of rat, both parameters slightly increased to 14.3% and 229.7 × 10^4/μL, respectively. They remained constant during the experimental period. In the rHSA-FecycP and rHSA-FepivP groups, both parameters decreased to 7.8–9.6% and 129.2–149.2 × 10^4/μL by the sample replacement. They corresponded well to the calculated values.

Survival time

After the exchange transfusion with the 5 g/dL rHSA solution (rHSA group), all animals died within 32 min;

TABLE II
Basal Values of Each Group

	rHSA	Whole blood	rHSA-FecycP	rHSA-FepivP
MAP (mmHg)	110 ± 2.9	115 ± 3.4	101 ± 7.3	107 ± 4.8
HR (beats/min)	371 ± 14	379 ± 17	380 ± 22	349 ± 10
Respiration rate (breaths/min)	66 ± 3.2	67 ± 4.4	60 ± 2.3	64 ± 1.7
ptO_2(R) (mmHg)	29.2 ± 2.8	38.5 ± 2.6	31.7 ± 3.6	30.2 ± 4.3
ptO_2(M) (mmHg)	39.8 ± 3.6	38.5 ± 3.7	44.2 ± 4.6	42.7 ± 3.9
paO_2 (mmHg)	83.8 ± 2.3	84.2 ± 2.3	87.8 ± 2.6	81.7 ± 2.2
pvO_2 (mmHg)	47.9 ± 1.6	46.8 ± 1.9	48.2 ± 3.1	47.4 ± 1.1
$paCO_2$ (mmHg)	42.0 ± 0.9	40.2 ± 0.8	39.6 ± 1.2	41.7 ± 1.0
Body weight (g)	312 ± 3.5	312 ± 3.9	311 ± 3.4	314 ± 0.8

the average survival time was 14.9 ± 4.3 min. However, all animals survived >60 min in the rHSA-FecycP, rHSA-FepivP, and whole blood groups. The body temperatures remained constant throughout the measurements (rHSA-FecycP group: 36.1°–36.6°C; rHSA-FepivP group: 36.3°–36.7°C; whole blood groups: 36.2°–36.8°C).

MAP, HR, and respiration rate

The 70% hemodilution decreased MAP to 77.4–81.9% of the basal value, and the further 30% bleeding decreased it to 24.9–37.7% of the baseline [Fig. 2(a)]. In contrast to the fact that no recovery was observed by the iv administration of 5 g/dL rHSA, the lowered MAP was increased to 85.6% of the baseline by the whole blood injection. In the rHSA-FecycP and rHSA-FepivP groups, the decreased MAP was elevated to 70.1 and 71.4% of the basal values (80.7 and 87.1% of the values before the bleedings).

The HR and respiration rate had decreased to 88.6–97.4 and 76.5–82.5% of the baselines by the 30% bleeding [Fig. 2(b,c)]. Both parameters returned to the initial levels by the injection of rHSA-FecycP, rHSA-FepivP, and whole blood within 30 min.

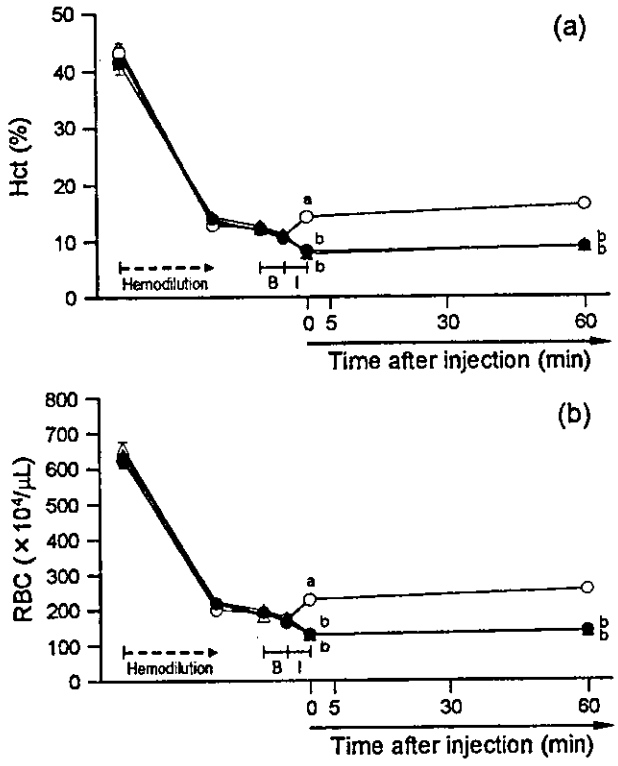


Figure 1. Effect of rHSA-heme solutions on Hct (a), and RBC numbers (b) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^{a}p < 0.05$ versus rHSA group. $^{b}p < 0.05$ versus whole blood group.

ptO_2(R) and ptO_2(M)

After the 30% bleeding, ptO_2(R) decreased to 59.9–77.3% of the baseline [Fig. 3(a)]. Whereas the iv administrations of rHSA solution did not show restoration, the injection of the rHSA-FecycP, rHSA-FepivP, or whole blood immediately increased the ptO_2(R) level to 84.1–94.8% of the baselines (102.1–112.3% of the values before the bleeding). They remained unaltered by the end of the measurements.

The ptO_2(M) had also decreased to 49.4–52.9% of the basal value after the isovolemic hemodilution, and further decreased to 12.1–26.7% because of the 30% bleeding [Fig. 3(b)]. The iv administration of the whole blood increased the ptO_2(M) to 62.8% of the baseline after 60 min. In the rHSA-FecycP and rHSA-FepivP groups, the increasing ratios were relatively small: 34.2 and 36.9% of the baselines (76.2 and 66.8% of the values before the bleedings).

Blood gas parameters

The paO_2 increased to 111.3–123.2% of the baseline after the isovolemic hemodilution, and reached 148.0–153.6% after the 30% bleeding [Fig. 4(a)]. The injection of the whole blood showed a significant effect for restoration. Nonetheless, the recoveries by the albu-

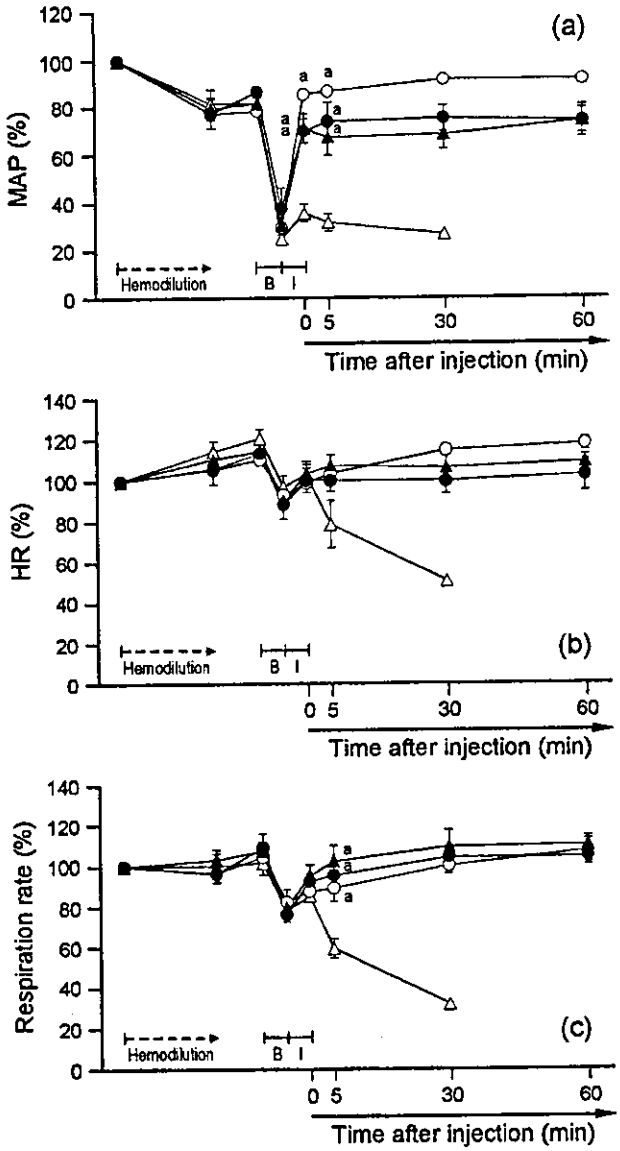


Figure 2. Effect of rHSA-heme solutions on MAP (a), HR (b), and respiration rate (c) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^{a}p < 0.05$ versus rHSA group.

min-heme solutions were almost to the same extent as that seen in the rHSA group.

The pvO_2 decreased to 56.5–69.8% of the basal value after the 30% bleeding. The low value was not improved by the iv administration of 5 g/dL rHSA [Fig. 4(b)]. In contrast, the infusions of rHSA-FecycP, rHSA-FepivP, and whole blood immediately increased pvO_2 to 88.9–94.8%.

The changes in pH showed the same profile in all groups until 60 min after injection [Fig. 4(c)]. The pH of 7.39–7.42 was slightly increased to 7.47–7.49 after the bleeding and reduced to 7.34–7.37 by the administration of the samples, which slowly returned close to the initial level except for the rHSA-FecycP group.

The $paCO_2$ decreased to 66.3–73.9% of the basal value after the 30% bleeding. The iv administration of whole blood immediately increased it to 95.1% [Fig. 4(d)]. The recovery by the infusion of the rHSA-FecycP or rHSA-FepivP solution was relatively small (80.9–88.0%).

DISCUSSION

In this study, animals were anesthetized with sevoflurane, which is widely used in clinical situations. We had evaluated the oxygen-transporting ability of rHSA-FepivP using a similar acute anemia model with pentobarbital as an anesthesia.[21] Most of the parameter changes showed the same extent as those observed in the former experiment, except for the small degree of $ptO_2(R)$ reduction after the bleeding (32%) (50% decrease in the former study). It is probably attributable to the difference in the blood exchanging ratio.

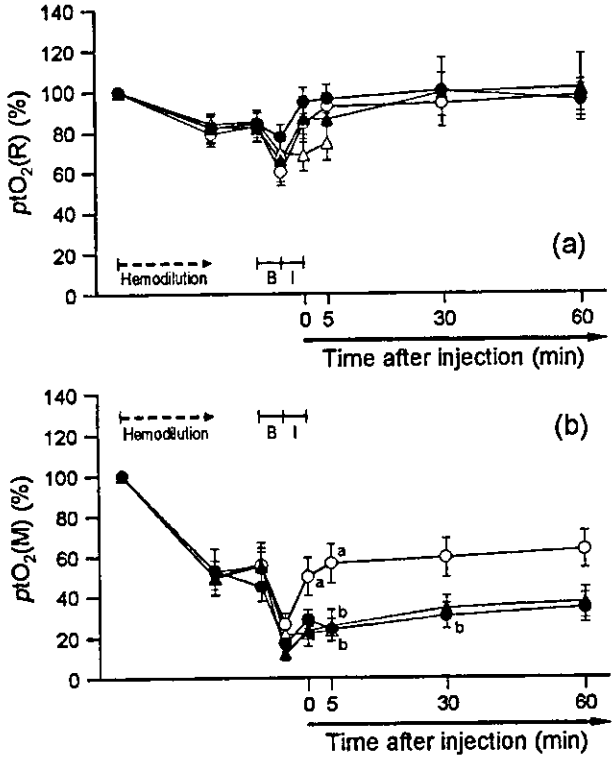


Figure 3. Effect of rHSA-heme solutions on the $ptO_2(R)$ (a) and $ptO_2(M)$ (b) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^{a}p < 0.05$ versus rHSA group. $^{b}p < 0.05$ versus whole blood group.

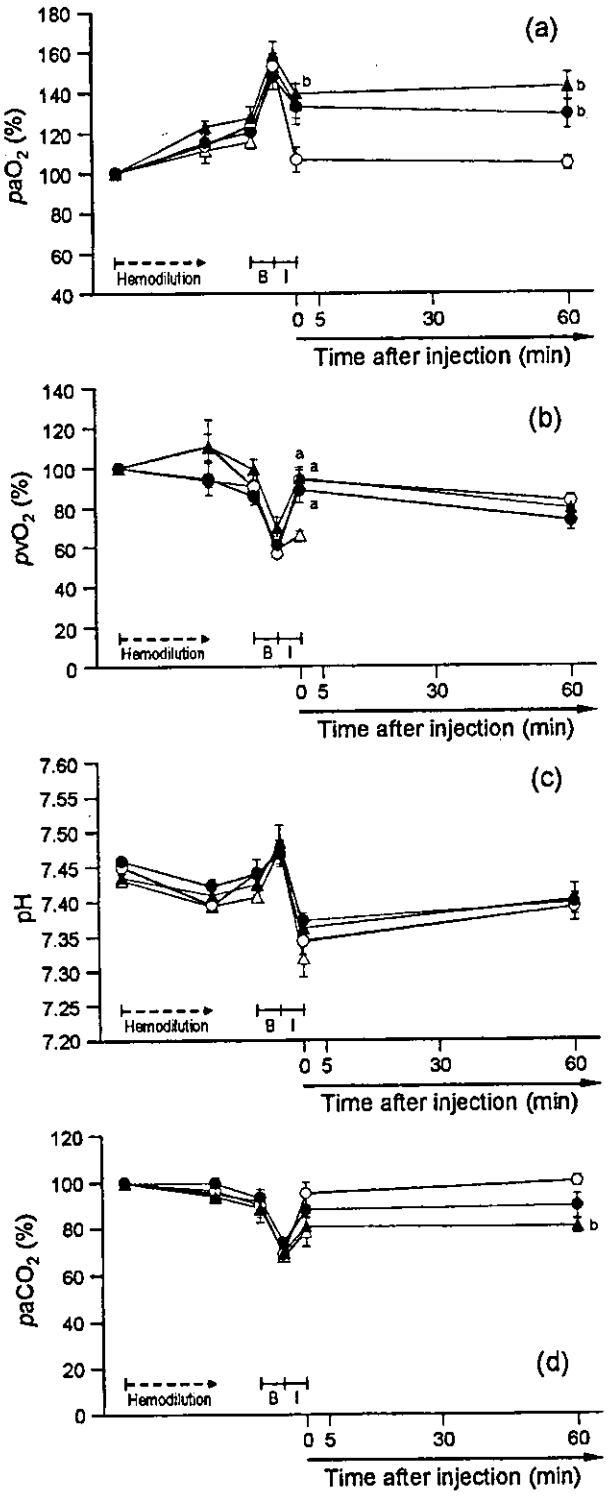


Figure 4. Effect of rHSA-heme solutions on paO_2 (a), pvO_2 (b), pH (c), and $paCO_2$ (d) in anesthetized rats subjected to hemodilution and hemorrhage. Each value represents the mean ± SEM of six rats (●, rHSA-FecycP group; ▲, rHSA-FepivP group; ○, whole blood group; and △, rHSA group). B, bleeding; I, sample injection. $^b p < 0.05$ versus whole blood group. $^a p < 0.05$ versus rHSA group.

In the glass capillaries for the Hct determinations, the supernatant after centrifugation (12 kG, 5 min) was colored red by the homogeneously dispersed albumin-heme molecules without any aggregation. This indicates that the albumin-heme fluids have a good solubility with the blood components.

Acute anemia after 70% hemodilution

The quantitative changes in Hct and RBC numbers showed that the 70% hemodilution and 30% exchange transfusion were precisely controlled. In acute anemia, the oxygen supply centralizes in vital organs, and the muscle tissues become hypoxic by peripheral vasoconstriction. In fact, the ratio of ptO_2 reduction in the muscle tissues after hemodilution was significant compared with that observed in the renal cortex.

Hemorrhagic shock by 30% bleeding

Decreasing the circulation blood volume induced a significant reduction of MAP, ptO_2(R), ptO_2(M), pvO_2, and $paCO_2$. Among the vital organs, renal perfusion is first impaired because of redistribution of the systemic blood flow; therefore, ptO_2(R) is sensitive to a subtle change in the blood circulation and oxygen delivery. The decreased pvO_2 after the 30% bleeding implies a low oxygen saturation of Hb and increased oxygen extraction. The HR and respiration rate had also decreased from the initial values. However, paO_2 was increased to 153.6% (129.6 mmHg) of the baseline, which could be attributable to hyperventilation. The $paCO_2$ decreased to 70.3% of the baseline and the pH was increased to 7.48. A respiratory alkalosis by hyperventilation might overcome the metabolic acidosis effect.

Responses to administration with albumin-heme

In all groups, the pH levels decreased to 7.35 just after sample injections. The rapid increase in blood volume could improve the circulatory flows and wash out the lactate in the tissues.

By administration of the 5 g/dL rHSA solution, the MAP, HR, respiration rate, ptO_2(R), ptO_2(M), paO_2, pvO_2, and $paCO_2$ did not recover, leading to death within 32 min. In contrast, the infusion of whole blood improved these values to their initial levels except for ptO_2(M). In the rHSA-FecycP and rHSA-FepivP groups, the animals survived >60 min after the infusion, and the HR, respiration rate, ptO_2(R), and pvO_2 showed similar recoveries as observed in the whole

blood group. MAP, ptO_2(M), paO_2, pH, and $paCO_2$ also returned, but not adequate relative to the whole blood group. We are certain that the albumin-heme solutions have the potential to (i) resuscitate the hemorrhagic shock, (ii) stabilize the blood circulation, and (iii) transport oxygen throughout the body. Nevertheless, the oxygen delivery to the peripheral tissues is still insufficient. The possible reasons were considered from the viewpoints of the physicochemical properties of albumin-heme.

Oxygen-binding affinity (p_{50}) (oxygen partial pressure in which 50% of Hb or albumin-heme is oxygenated)

It is known that an allosteric effect observed in the oxygen binding to Hb [the Hill coefficient (n) = 3.0] has implications for physiological use; the oxygen-transporting efficacy of RBCs between lungs [oxygen pressure (pO_2): 110 mmHg] and muscle tissues (pO_2: 40 mmHg) is estimated to be 22% at 37°C. The oxygen-equilibrium curve of albumin-heme does not show cooperativity; n is 1.0. However, the oxygen-binding affinity of albumin-hemes are adjusted somewhat low (rHSA-FecycP: p_{50} = 37 mmHg; rHSA-FepivP: p_{50} = 32 mmHg, respectively), so that they are able to show a similar oxygen-transporting efficacy of 22–23%.

Some investigators reported that a low p_{50} value is important to avoid the hypertensive effect; that is, the constrictive response is caused by excessive oxygen delivery to the arterioles.[10–13] McCarthy et al.[22] suggested that the increased oxygen delivery can be limited by increasing the molecular size, oxygen-binding affinity, and viscosity. Indeed, the PEG-conjugated Hb with a molecular weight of 90 kDa had no effect on the MAP.[23] However, Doherty et al.[24] denied this hypothesis based on their systematic experiments using recombinant Hbs (rHbs) with various p_{50} values; the small differences in the oxygen-equilibrium curves of the rHbs did not affect the magnitude of the pressor response. Our previous top-loading experiments using the albumin-heme solution (p_{50}: 32 mmHg) on the MAP changes and microcirculatory observations of the capillaries also showed that neither vasoconstriction nor hypertension occurred, because of its low permeability through the vascular endothelium.[18] Thus, the differences in p_{50} may not be essential.

Heme concentration

The heme concentration of the shed blood to use the exchange transfusion in the whole blood group was 5.3 m*M*. In contrast, the concentrations of the FecycP or FepivP in the rHSA-FecycP or rHSA-FepivP solutions were 2.7–2.8 m*M*. The low heme concentration of albumin-heme is probably related to insufficient oxygen delivery, which may result in the low recovery of ptO_2(M).

Viscosity

It has been reported that an endothelial-derived relaxing factor (nitric oxide) is generated in response to the shear stress on the capillary wall.[25] Recently, maintenance of the plasma viscosity has been proposed as a crucial mechanism to preserve the shear force during microcirculation.[26] The prompt flow by the injection of the albumin-heme solution with low viscosity (1.1 cP) compared with blood (4.4 cP) may reduce the peripheral resistance and induce partial vasoconstriction, which would reduce the blood flow. The correct measurement of the cardiac output is necessary to interpret the pO_2 data and to assay the oxygen delivery.

In conclusion, all physiological responses to the exchange transfusion with albumin-heme in acute anemia reveal that this synthetic RBC substitute has the capability to resuscitate the hemorrhagic shock. A significant difference could not be found between the two types of albumin-hemes, rHSA-FecycP and rHSA-FepivP. This rHSA-based oxygen-carrying plasma expander will become a promising material for a new class of RBC substitutes. The safety and oxygen delivery of albumin-heme will be reported in a forthcoming article.

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