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Erythrocytes with T-State–Stabilized Hemoglobin as a Therapeutic Tool for Postischemic Liver Dysfunction

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ABSTRACT

This study aimed to examine if T-state stabilization of hemoglobin in erythrocytes could protect against postischemic organ injury. Human erythrocytes containing three different states of Hb allosterity were prepared: control Hb (hRBC), CO–Hb that is stabilized under R-state with the 6-coordinated prosthetic heme (CO–hRBC), and α -NO-deoxyHb stabilized under T-state (α -NO–hRBC). To prepare α -NO–RBC, deoxygenated RBC was treated with FK409, a thiol-free NO donor, at its half molar concentration to that of Hb; this procedure resulted in the 5-coordinated NO binding on the α -subunit heme, as judged by electron spin resonance spectrometry. Rats were subject to 20 min systemic hemorrhage to maintain mean arterial pressure at 40 mm Hg, and reperfused with one of hRBCs. This protocol for ischemia, followed by 60 min reperfusion with physiological saline, caused modest metabolic acidosis and cholestasis. Administration of hRBC or CO–hRBC significantly attenuated cholestasis and improved acidosis. Rats treated with α -NO–hRBC exhibited greater recovery of metabolic acidosis and bile excretion than those treated with hRBC or CO–hRBC, displaying the best outcome of local oxygen utilization in hepatic lobules. Half-life time of α -NO–RBC administered *in vivo* was approximately 60 min. These results suggest that T-state Hb stabilization by NO serves as a stratum to treat postischemic organ dysfunction. *Antioxid. Redox Signal.* 8, 1847–1855.

INTRODUCTION

THERE ARE SEVERAL LINES of evidence that allosterity of hemoglobin (Hb) does not only determine the oxygen-carrying capacity of red blood cells (RBC) but also dictates an oxygen-sensing ability to regulate microvascular function. RBC can sense a reduction of PO_2 and an increase in PCO_2 by utilizing Hb: such alterations in local gas tension have been shown to cause conformational stabilization of T-state Hb, which is characterized by a 5-coordinated structure of the prosthetic heme, and thereby facilitate the release of molecular oxygen from Hb in the peripheral tissue, known as Bohr's effect (31). On the other hand, an elevation of PO_2 or partial

binding of carbon monoxide (CO) stabilizes R-state Hb, which is characterized by the 6-coordinated heme structure. The latter mechanism renders Hb ineffective in its delivery of O_2 to peripheral tissues, which is the primary cause of CO poisoning. The Hb allosterity of this kind has been reported to regulate microvascular function through multiple mechanisms. First, alterations in the Hb allosterity play a key role in regulation of the delivery of NO released from the cells (19, 24). Under the low oxygen tension where T-state Hb is stabilized, the release of RBC-derived NO is brought to the microvascular endothelium and helps vasorelaxation to guarantee an ample blood supply in hypoxic regions (10). RBC also have the ability to release ATP in response to hypoxia (3). As

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previously shown, extracellularly released ATP activates purinergic receptors on endothelial cells to trigger intracellular calcium ion and NO generation (6, 7). Such hypoxic responses of RBC appear to result from allosteric changes in Hb, so far as judged from the observation that CO, the R-state stabilizer, significantly diminished hypoxia-induced ATP release from the cells (6, 9).

Although neither mechanisms *in vivo* by which NO is degraded in and around RBC nor those resulting in hypoxia-triggered conductance of NO or ATP across RBC membrane have been well described yet (5, 14), these results tempted us to investigate if stationary stabilization of the T-state Hb allostery could serve as a therapeutic tool for improving hypoxic organ damages, since RBC with T-state stabilized Hb might have a greater ability to release oxygen and to trigger vasorelaxation for maintenance of ample blood supply than normal RBC. Recent studies revealed that T-state Hb can be stabilized without destroying RBC by treating the cells with membrane-diffusible NO under deoxygenated conditions (30, 31). In these studies, α -nitrosyl Hb, α -(Fe-NO)₂(Fe)₂ has been synthetically prepared. Despite the halved O₂-carrying capacity, α -nitrosyl Hb could represent a versatile low affinity O₂ carrier with improved features that might deliver O₂ to tissues effectively even after NO is sequestered at the heme site of Hb. However, solid evidence for effectiveness of RBC with α -nitrosyl Hb to treat hypoxic organ dysfunction *in vivo* and its pathophysiologic link to local O₂ metabolism have not fully been investigated *in vivo*. This study was designed to examine the protective effects of RBC with α -nitrosyl Hb on microvascular function, O₂ delivery, and organ function in the postischemic rat liver where the O₂ delivery is limited even under physiologic conditions.

MATERIALS AND METHODS

Preparation and characterization of α -nitrosyl Hb in human erythrocytes

Human erythrocytes (RBC) were isolated from heparinized peripheral blood samples collected from healthy male volunteers who gave informed consents. The blood samples were centrifuged at 2,000 *g* for 10 min and washed twice with modified SAGM buffer solution. Hb in the RBCs was fully deoxygenated by gentle purge for 30 min with highly purified argon gas under acidic pH conditions, as described previously (30). A portion of the RBC-suspended solution was sampled and hemolyzed to determine Hb concentrations by measuring visible absorption spectra at 540 nm. Under maintenance of the temperature at 4°C, an NO donor, FK409 (a generous gift from Fujisama Pharmaceutical Inc., Osaka) was added to the RBC suspension at a half concentration of Hb in the suspension (30). This NO-donating reagent has the ability to release equimolar amounts of NO in the aqueous solution. Distinct from other NO donors such as *S*-nitroso glutathione, FK409 does not contain a sulfhydryl group in its chemical structure; such an advantage allowed us to avoid a possible contamination of exogenous *S*-nitrosothiols in experiments.

Structural characterization of the prosthetic heme of Hb by binding of NO was carried out by electron spin resonance

(EPR) spectrometry, according to the previous method (31). To this end, a portion of the NO-treated RBC were frozen at 77°K with liquid nitrogen to serve as a sample to detect triplet peaks, indicating a 5-coordinated structure of the heme of two α -subunits of Hb in the whole cell suspension (30). The 5-coordinated structure of the prosthetic heme of Hb was demonstrated by an EPR spectrometer (JEOL, Tokyo, Japan), as described elsewhere with minor modifications (30, 31). Briefly, the power and frequency of microwave were 1.0 mW and 9.1 GHz, respectively. Time duration and a range for scanning the EPR signal were 0.5 min and 326 \pm 25 mT, respectively. Unless mentioned, the NO-treated RBC sample was designated as α -NO-hRBC, unless mentioned otherwise. Separately, the control and CO-saturated RBC samples were prepared at 4°C and designated as hRBC and CO-hRBC, respectively. The Hb concentration of these RBC samples was prepared at 10 g/dl by adding the physiological saline and stored at 4°C until the animal experiments. The RBC samples were incubated at 37°C at 30 min prior to the start of the experiments *in vivo*.

In vivo experimental protocols for hemorrhage-induced liver dysfunction

The experimental protocols herein described were approved by our institutional guidelines provided by the Animal Care Committee of Keio University School of Medicine. Male Wistar rats weighing 240–280 g (CLEA Japan, Tokyo) were allowed free access to laboratory chow and tap water, and were fasted for 24 h prior to experiments. As described elsewhere (21, 23), rats were anesthetized with an intramuscular injection of pentobarbital sodium at 50 mg/kg, and their common bile duct and carotid and femoral arteries were cannulated to collect bile and blood samples, respectively. Bile output was monitored *in vivo* according to our previous method (4, 13). The femoral arterial cannulation was used to monitor systemic blood pressure and heart rate (HR). The carotid cannulation was to withdraw blood from circulation and to collect blood samples for blood-gas analyses. The arterial PO₂, pH, and concentrations of HCO₃⁻ and Hb were determined in the arterial blood samples, using the gas analyzer as described elsewhere (8, 18, 25). Based on determination of these parameters, base excess (BE) was also calculated as an index of metabolic acidosis. When necessary, pulse-Doppler flowmetry was conducted to measure visceral blood flow in the abdominal aorta.

Rats were exposed to acute hemorrhage by letting them bleed from a carotid arterial catheter, according to the protocol described previously (29). The mean arterial pressure (MAP) was maintained at 40 mm Hg for 20 min. The shed blood was collected in heparinized syringes and the volume was measured. The blood was kept at 37°C to be reinfused as autologous blood samples, when necessary. After completing the 20-min hypovolemic ischemia, one of hRBC was infused at 1 ml/min at a total volume equivalent to that of shed blood.

In vivo monitoring of hepatic microcirculation and local oxygen tension

In separate sets of experiments, rats used to observe hepatic microcirculation were prepared with tracheotomy to

establish the airway for inhalational anesthesia with sevoflurane, as described in our previous method (16). The anesthetized rats underwent midline and pericostal incisions to expose the left lobe of the liver. The current choice of anesthesia allowed us to guarantee stability of systemic blood pressure during 4 h observation of the left lobular portion of the liver surface. The exteriorized portion of the liver was set on the cover glass mounted in the plastic stage and observed through an inverted-type fluorescence microscope equipped with an high-speed video analyzing device combined with a photomultiplier to measure partial oxygen tension (PO_2) in microvessels by analyzing phosphorescence decay (20). When necessary, erythrocytes suspended in physiological saline were prepared by whole blood samples taken from donor rats and labeled with fluorescein isothiocyanate (FITC), according to our previous method (8, 29). Each rat received 100 μ l of FITC-labeled RBC suspension through an intravenous injection.

For microvascular PO_2 measurements, Pd-meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-TCPP, Porphyrin Products, Eugene, OR) was used as a phosphorescence probe indicating O_2 -dependent quenching of the light emission (20, 29). The reagent was dissolved at 20 mg/ml in physiological saline containing bovine serum albumin and buffered at pH 7.4 with phosphate buffer. Rats received a slow bolus of Pd-TCPP at 30 mg/kg through an intravenous injection. Pd-TCPP circulating in hepatic central venules was excited by using the second harmonic of a Q-switched Nd/YAG pulse laser at 532 nm with 6-nsec pulse width, according to the previous method (29). Phosphorescence passing through a long-pass filter (>620 nm) was detected with a photomultiplier tube (R1894, Hamamatsu Photonics, Hamamatsu City, Shizuoka). The voltage signal was fed into a computer via an analog-to-digital converter (NR-2000, Keyence, Tokyo) with a sampling frequency of 200 kHz and sampling number of 500 points. PO_2 values were calculated based on the Stern–Volmer equation, as described elsewhere (20, 29).

To measure the RBC velocity and PO_2 at 1 Hz, we used a mechanical shutter to control the output of a mercury lamp and used the signal from a limit switch to trigger the Nd/YAG laser when the shutter was closed. The irradiation times of the laser and mercury lamp were 6 nsec and 300 msec, respectively. PO_2 values in central venules were measured as a function of time before and after exposure to the 20-min hypovolemic ischemia. Alterations in sinusoidal diameter, functional sinusoidal density, and RBC velocity and local intravascular PO_2 in central venules were compared between postischemic livers treated with hRBC and those treated with α -NO-hRBC. These measurements were performed before and 5, 10, 15, 30, and 60 min after the start of reperfusion.

Determination of half lifetime of exogenously administered α -NOHb in circulation

To determine the half-life time of α -NO-hRBC, 2 ml of the peripheral blood samples were collected from the carotid artery of rats, which were exposed to the 20-min hypovolemic ischemia, followed by desired duration of time for reperfusion of one of the hRBC samples. The collected blood samples were immediately frozen by liquid nitrogen at 77°K to serve as samples for EPR spectrometry. As described later in Results, α -nitrosyl heme was

undetectable in peripheral blood samples collected from the control and the postischemic rats reperfused with autologous blood samples, indicating that the amount of endogenous α -NOHb-containing rat erythrocytes is little in circulation, if any. In these experiments, the putative concentrations of circulating α -NOHb were calculated as a function of time for reperfusion. To this end, the circulating concentration of the Hb derived from hRBC administered exogenously was calculated, assuming that the total blood volume of the rat is 7% of the body weight. The α -NO-Hb concentrations were thus estimated by multiplying the concentrations of hRBC-derived Hb in the circulation and the percentage magnitude of the EPR triplet signal versus the initial magnitude measured at 5 min after reperfusion. This value of the nitrosyl Hb in circulation was designated as estimated nitrosyl-Hb concentrations, assuming that NO initially bound to α -heme of hRBC Hb does not move to rat RBC-derived Hb in circulation.

Statistical analyses

Differences in mean values among groups were analyzed by ANOVA with Fisher's multiple comparison test. *P* values <0.05 were considered statistically significant.

RESULTS

Characterization of α -NO-hRBC in vitro and in vivo

Figure 1 illustrates the characterization of α -NO-hRBC *in vitro*. In these experiments, hRBC was fully deoxygenated with pretreatment with O_2 -free Ar gas prior to the addition of the NO donor FK409. As seen in a representative EPR spec-

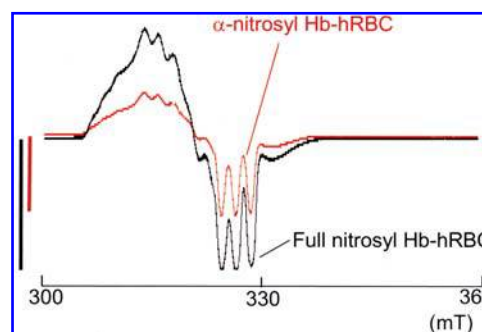


FIG. 1. Characterization of α -NO-human erythrocytes (α -NO-hRBC) prepared *in vitro* by electron paramagnetic resonance (EPR) spectrometry. A representative EPR signal of α -NO-hRBC with typical triplet signals indicating the 5-coordinated heme-NO complex. When FK409, a thiol-free NO donor, was added to the hRBC suspension at a half concentration versus Hb-associated heme concentration, the height of the triplet signal became about 50% versus that for hRBC treated with saturated concentrations of the NO donor (tetranitrosyl Hb). Considering an extremely greater affinity of NO to the α -subunit heme than to β -subunit one, these results suggest that exogenously applied NO is bound to the heme of α -subunits, forming 5-coordinated structure. *Black and red bars*: intensities of triplet signals in full nitrosyl and α -nitrosyl Hbs, respectively.

trum in the upper panel, the heights of the triplet signal for α -NO-hRBC prepared by the current protocol and that for hRBC fully saturated with NO were 0.78 and 1.53, respectively. These results indicate that the ratio of α -NOHb versus fully nitrosyl Hb was 0.51, suggesting that halves of the prosthetic heme are occupied with NO in α -NO-hRBC.

We next examined the half lifetime of α -(Fe-NO)₂(Fe)₂Hb of exogenously administered hRBC in the postischemic rats. As seen in the upper panel of Fig. 2, immediately after the α -NO-hRBC administration (T5) the triplet signal was detectable in systemic circulation. The height of the signal became weakened as a function of time after reperfusion. Such a decay of the triplet signal as a function of time for reperfusion was analyzed in three separate experiments (lower panel). In these experiments, the rate of blood replacement with α -NO-Hb hRBC was approximately 32%, and the aver-

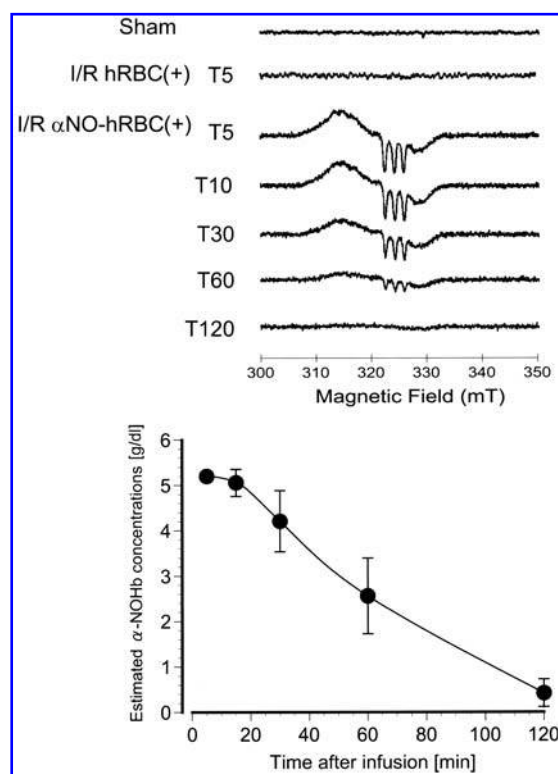


FIG. 2. Determination of exogenously administered α -NO-hRBC by EPR spectrometry in systemic blood samples in rats exposed to ischemia-reperfusion. *Upper panel:* Representative time history of the decay of α -NOHb-associated triplet signals in the blood samples. Sham and I/R hRBC(+); EPR signals of peripheral blood samples collected from sham-operated rats and from rats exposed to ischemia followed by 5-min reperfusion with hRBC, respectively. I/R α -NO-hRBC(+); temporal alterations in the EPR signals from rats reperused with α -NO-hRBC. Measurements were carried out at 5, 10, 30, 60, and 120 min after the onset of reperfusion. *Lower panel:* Decay of the magnitude of the triplet signal as a function of time for reperfusion. Data denote the relative intensities versus the maximum value measured at 5 min and indicate mean \pm SE of three separate experiments. Note that half-life time of α -NOHb is approximately 60 min in systemic circulation.

age Hb concentration was 16.3 g/dl; apparent concentration of α -NO-Hb at 5 min after the start of reperfusion was approximately 5.0 g/dl. The concentrations of the circulating nitrosyl Hb were decreased as a function of time for reperfusion, indicating that the half life time of α -NOHb is approximately 60 min *in vivo*.

α -NO-hRBC improves recovery of visceral blood flow and bile output

We examined whether different modification of Hb allostery (α -NO-hRBC, hRBC, and CO-hRBC) could cause any differences in blood supply at the level of macrocirculation *in vivo*. As seen in Fig. 3, temporal alterations in MAP showed no significant differences and displayed a comparable recovery to the baseline during resuscitation among the three groups. HR values in the α -NO-hRBC-treated group were also comparable to those in the hRBC-treated group, while those treated with CO-hRBC group exhibited an incomplete recovery with no statistical significance versus other two groups (middle panel). During the 20-min period of hypovolemic hypoxia, visceral blood flow decreased by 50%

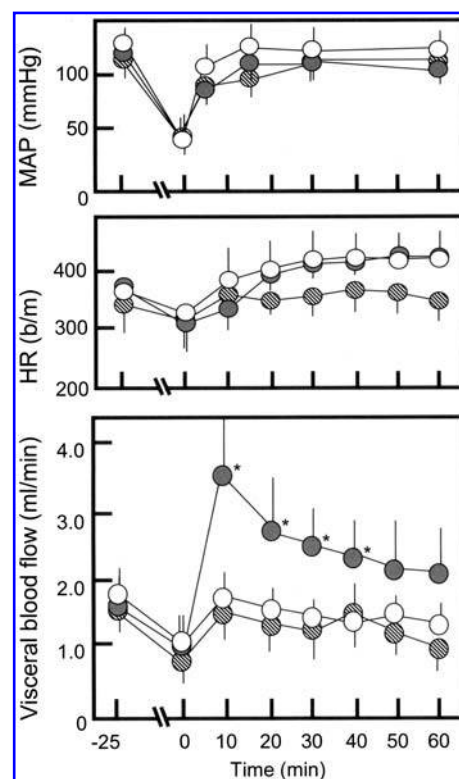


FIG. 3. Temporal alterations in mean arterial pressure (MAP), heart rate (HR), and visceral blood flow of rats exposed to the 20-min hypovolemic ischemia, followed by reperfusion with hRBC (open circles), CO-hRBC (striped circles), and α -NO-hRBC (shaded circles). Data indicate mean \pm SE of five to seven separate experiments for each group. * p < 0.05 as compared with sham-operated controls. Note that α -NO-hRBC significantly increases splanchnic blood flow without altering MAP, suggesting a decrease in systemic vascular resistance.

of the preischemic control period in all groups. Upon reperfusion, rats administered with hRBC or with CO-hRBC displayed a recovery towards the control levels, while those treated with α -NO-hRBC indicated approximately twofold elevation of the flow at the early phase of reperfusion, which was followed by a gradual decay in the later period. Such a feature of the α -NO-hRBC-treated rats distinct from those treated with hRBC or with CO-hRBC was also evident in the recovery of bile output from the postischemic liver (Fig. 4). To be noted, no significant difference in the recovery of visceral blood supply and bile output during reperfusion was seen between CO-hRBC- and hRBC-treated groups, although both groups exhibited a significant recovery of bile output as compared with the group treated with physiological saline.

Potent improving effects of α -NO-hRBC on metabolic acidosis

In the current experimental model, the replacement of the shed blood with reperfusion with physiological saline induced significant decreases in arterial blood pH and base excess, when the data were compared among the groups at 60 min after the start of reperfusion (solid bars in Fig. 5). These results indicated that the protocol for hypovolemia followed by the 60-min reperfusion of the saline caused metabolic acidosis modestly but evidently. On the other hand, reperfusion with hRBC or with CO-hRBC significantly improved alterations in these parameters; the recovery of MAP is almost comparable to the sham-operated baseline level, while that of pH and base excess was partial so far. The recovery of these parameters for metabolic acidosis became more evident in the group treated with α -NO-hRBC than in the groups treated with hRBC or CO-hRBC, while the improvement of MAP was comparable among these three groups (Fig. 5). As shown in Fig. 3, the group treated with α -NO-hRBC displayed a greater increase in visceral blood flow than that treated with hRBC transiently at the early phase of reperfusion. However, at 60 min after the onset of reperfusion, the two groups did

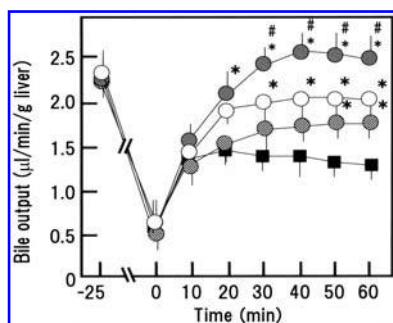


FIG. 4. Temporal alterations in bile output of rats exposed to the 20-min hypovolemic ischemia, followed by reperfusion with hRBC (open circles), CO-hRBC (striped circles), and α -NO-hRBC (shaded circles). Closed squares; rats exposed to the ischemia followed by reperfusion with physiological saline (PS). Data indicate mean \pm SE of six to eight separate experiments for each group. * $p < 0.05$ as compared with the PS-treated group. # $p < 0.05$ as compared with the values for the hRBC-treated group.

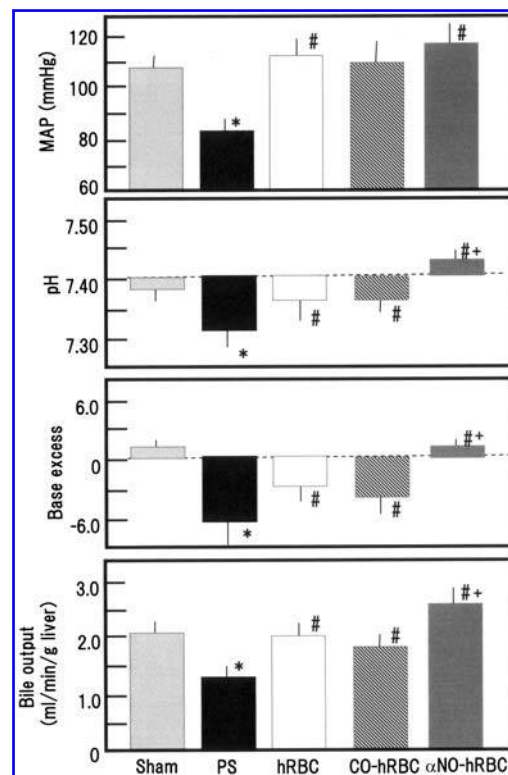


FIG. 5. Effects of postischemic administration of hRBC, CO-hRBC, and α -NO-hRBC on alterations in mean arterial pressure (MAP), pH, and base excess in arterial blood, and bile output of rats exposed to the 20-min hypovolemic ischemia, followed by 60-min reperfusion. The administration of one of these hRBCs or physiological saline (PS) was completed at 5 min after the start of reperfusion. Sham: data collected from sham-operated controls. Data indicate mean \pm SE of 10–12 separate experiments for each group. * $p < 0.05$ as compared with sham-operated controls. # $p < 0.05$ as compared with the values for the PS-treated group. + $p < 0.05$ as compared with the values for the hRBC-treated group. Note that rats treated with α -NO-hRBC displays the best outcomes of recovery from metabolic acidosis and cholestasis among the groups examined.

not show any notable differences in MAP and the visceral blood flow, while the α -NO-hRBC-treated group indicated the greatest recovery of bile output. This observation tempted us to examine if the time history of the recovery of local microvascular hemodynamics and oxygen utilization was different during the reperfusion period between the α -NO-hRBC- and hRBC-treated groups.

Distinct effects of α -NO-hRBC on the recovery of hepatic microvascular PO_2

Figure 6 illustrates the effects of the α -NO-hRBC administration on alterations in sinusoidal diameter, functional sinusoidal density, RBC velocity, and PO_2 in the central venules of postischemic liver lobules and a comparison of effects of the hRBC administration. At sinusoidal levels (a large circle in Panel A), temporal alterations in the diameter of microvessels (Panel B), and functional sinusoidal density

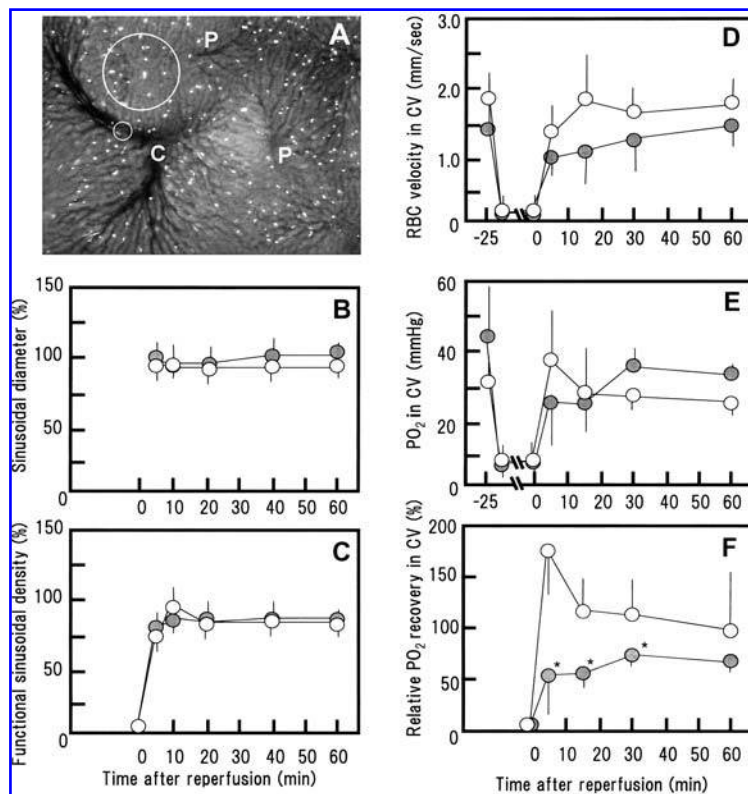


FIG. 6. Effects of the α -NO-hRBC administration on alterations in sinusoidal diameter, functional sinusoidal density, RBC velocity, and PO_2 in central venules of postischemic liver lobules. (A) A representative picture showing traffics of FITC-labeled RBC in the rat hepatic microcirculation. P and C; portal and central venules. Large and small circles indicate regions for determination of functional sinusoidal density and RBC velocity, respectively. Bar: 100 μm . (B) and (C) Relative changes in sinusoidal diameter and functional sinusoidal density. Data indicate mean \pm SE of five to seven separate experiments. (D, E, and F) Alterations in RBC velocity, PO_2 , and relative PO_2 recovery in central venules (CV). Data indicate mean \pm SE of four to five separate experiments. Open and closed circles indicate data from hRBC- and α -NO-hRBC-treated groups, respectively. * $p < 0.05$ as compared with the hRBC-treated control group.

(Panel C) were comparable between the two groups. As seen in Panel D, the recovery of RBC velocity measured at central venules appeared modestly greater in the hRBC-treated group than in the α -NO-hRBC-treated one, but without any statistical significance over the entire course of observation because of the large variation of measurements. Absolute values of PO_2 in central venules were also varied, not only among different rats, but also among individual venules in the same animal, and the measurements indicated no statistical significance (Panel E). However, when the net recovery of PO_2 values was plotted as a function of time after reperfusion, distinct features of local O_2 utilization became evident between the two groups. In the hRBC-treated group, PO_2 values in central venules were abruptly elevated during the initial 5-min period of reperfusion and gradually decreased to the end of observation. On the other hand, those in the α -NO-hRBC-treated group displayed rather slow recovery at the early reperfusion period and reached a plateau level. As a result, the PO_2 in central venules was significantly lower in the α -NO-hRBC-treated group than in the hRBC-treated group during the initial 30-min reperfusion period (Panel F).

We also attempted to measure PO_2 values in portal venules. As seen in Fig. 6A (denoted as P in the panel), it was difficult in rats to find a terminal inlet vessel of portal veins through the surface observation of the liver. Because of this technical difficulty, we were unable to perform accurate PO_2 measurements in the large terminal portal inlets. However, so far as judged from such measurements in periportal regions, including multiple sinusoids adjacent to the terminal portal venules, PO_2 measured at 60 min were approximately 60 mm Hg in the both groups (data not shown). These results collectively sug-

gest that the O_2 consumption occurring between portal and central venules appears to be greater in the α -NO-hRBC-groups than in the hRBC-treated one. Since the intravascular PO_2 determines O_2 -saturation rates (SaO_2) of Hb in RBCs, the data indicating periportal and pericentral PO_2 values allow us to estimate the net delivery of O_2 to the hepatic pa-

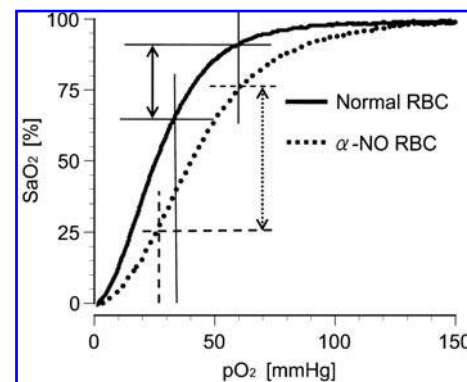


FIG. 7. Differences in oxygen saturation profiles between hRBC (solid line) and α -NO-hRBC (broken line). Solid and broken arrows indicate estimations of differences in SaO_2 for hRBC and α -NO-hRBC during transition across hepatic microcirculation, respectively. The estimation is based on observations that mean PO_2 values in periportal regions are 70 mm Hg in both groups and those in central venules are 28 and 33 mm Hg in the groups treated with α -NO-hRBC and normal hRBC, respectively (See Fig. 6E).

renchyma and to compare the values between the two groups. Figure 7 illustrates differences in SaO_2 values in periportal and pericentral microvessels between the groups treated with control and α -NO-hRBCs. As demonstrated in previous studies (30, 31), α -nitrosylation of Hb in human RBC caused the right shift of the oxygen saturation curve (dotted line). When mean periportal PO_2 values (60 mm Hg) and those in pericentral venules (33 mm Hg in the normal hRBC and 28 mm Hg in the α -NO-hRBC, as seen in Fig. 6E) of the postischemic livers at 60 min were superimposed on the saturation curves, the net differences in SaO_2 between the portal and central venules were estimated; as seen, the difference for α -NO-hRBC (dotted arrow) was twice greater than that for hRBC (solid arrow). To be noted is that halves of O_2 -binding pockets were occupied with NO in α -NO-hRBC. Considering that central RBC velocities did not differ significantly between the two groups (Fig. 6D), these results suggest that α -NO-hRBC and hRBC brings comparable amounts of O_2 to the tissue to the postischemic livers.

DISCUSSION

The present study is the first to examine if administration of RBC with T-state-stabilized Hb by NO pretreatment could improve postischemic dysfunction of peripheral organs such as the liver. Theoretically, T-state stabilization of the Hb allostery does not only enhance oxygen-carrying capacity of RBC to peripheral tissues, but is also believed to lower the threshold to trigger hypoxic vasodilation through delivering NO to microcirculation (19, 24). Such a possible role of Hb allostery in RBC-mediated regulation of organ microvascular function has been considered a mechanism for RBC-dependent delivery of NO under physiologic conditions. However, since R-to-T conversion of the Hb allostery in normal RBC occurs most prominently at specific oxygen tension around 25–30 mm Hg near the P_{50} value (Fig. 1), the theory of Hb-mediated microvascular regulation cannot be applied globally to various organs without actual measurements of local PO_2 and microvascular hemodynamics. A novel laser technology to collect quantitative information of microvascular oxygen delivery has herein allowed us to assess this issue: as seen in results shown in Figs. 1 and 6, the liver is such an organ where α -NO-hRBC could exert its distinct ability to deliver O_2 and to regulate microvascular blood flow.

The current protocols for hypovolemic ischemia induced modest but notable extents of metabolic acidosis concurrently with a significant reduction of the basal bile output, as seen in effects of reperfusion with physiological saline (Fig. 5). Under these circumstances, reperfusion of NO-free hRBC completely repressed the reduction of bile output and induced a recovery of oxygen consumption in the liver parenchyma (Fig. 6E), though a decrease in base excess was not completely recovered. Such effects of hRBC were able to be mimicked by CO-hRBC, suggesting that oxygen delivery by exogenous hRBC is unnecessary to restore the liver function in this particular model. In other words, after shedding 30%–35% of the blood, the rest of rat RBC could function for O_2 delivery in the presence of exogenous hRBC or CO-hRBC. The presence of RBC in circulation does not only con-

tribute to tissue oxygen delivery but also results in an increase in blood viscosity, the factor necessary to maintain wall shear stress to stimulate endothelium-derived vasodilatory mechanisms (1). Considering this fact, improving effects of reperfusion with hRBC on metabolic acidosis and reduced bile output is unlikely to result from its O_2 -carrying capacity.

Consideration of the role of O_2 -carrying capacity is also useful to determine the superior effects of α -NO-hRBC versus hRBC on the recovery of metabolic acidosis and cholestatic changes in the postischemic liver in the current model. As far as judged from estimated differences in the net drop of SaO_2 versus hepatic microvascular O_2 gradient between portal and central venules (Fig. 7), α -NO-hRBC has the twice greater O_2 -carrying capacity than hRBC. However, since 50% of Hb-derived heme of α -NO-hRBC is occupied with NO, the actual amount of oxygen that could be released by this cell in response to the hepatic microvascular O_2 gradient is almost the same as that by NO-free hRBC. If the postischemic recovery of the tissue O_2 consumption is assumed to be identical between the two groups, central PO_2 values in the α -NO-hRBC group should be the same as those in the hRBC group. However, as seen in the data in the early period of reperfusion (Fig. 6), the recovery of central PO_2 in the α -NO-hRBC-treated group was relatively slow, being only 30%–50% of that in the hRBC-treated group. Such a discrepancy in central PO_2 recovery between the estimation and real measurements cannot simply be explained by an insignificant difference in central RBC velocity between the two groups (Panel D in Fig. 6). Assuming that the local oxygen delivery across microvascular beds is diffusion-limited but not flow-limited (28), the current observation allows us to hypothesize that the α -NO-hRBC administration leads to greater recovery of O_2 consumption than in the postischemic liver tissue. Since hepatocytes constitute a major cellular component of the O_2 consumption (23), such a hypothesis is fully supported by our observation that the α -NO-hRBC-treated liver displayed the most notable recovery of the basal bile output, which highly depends on energy-dependent transport of bile salts across hepatocytes (21, 22). Again, the improving effects of α -NO-hRBC seem to be attributable to its greater ability to stimulate O_2 consumption, and are unlikely to result from its distinct ability of O_2 -carrying capacity.

Although mechanisms by which the α -NO-hRBC administration results in improved O_2 consumption still remain unknown in the postischemic liver, several possibilities can be considered. First, α -NO-hRBC could deliver more NO to hepatic microcirculation, help cancel superoxide generated upon the early reperfusion period, and attenuate the postischemic damages of hepatocytes that mainly consume oxygen transferred from microcirculation. Such a cancellation of superoxide by endogenous NO could actually be shown to take place in the RBC-free isolated perfused rat liver exposed to anoxia–reperfusion and to help attenuate a reduction of bile output (26, 27). However, several lines of the current experimental data led us to exclude such a hypothesis. The hepatic sinusoids are known to exhibit vasodilation in response to NO or CO through mechanisms involving soluble guanylate cyclase in Ito cells, the liver-specific pericytes (12, 15, 25). However, in liver treated with α -NO-hRBC, central RBC velocity was lower than in that treated with hRBC, despite the absence of difference in sinusoidal diameter during the

whole course of observation (Fig. 6). Furthermore, the delivery of NO from circulation to parenchyma could suppress O₂ consumption in hepatocytes through its ability to inhibit cytochrome oxidase and reduce the basal output (23).

In this context, the second putative mechanism to be considered is involvement of ATP that could also be released from RBC in response to a reduction of local PO₂. Vasoactive responses to extracellularly released ATP are known to differ among different organs (7). In most organs, including intestine and skeletal muscle, extracellular ATP can stimulate purinergic receptors on endothelial cells to increase intracellular calcium ion and stimulate NO production (6). However, the hepatic sinusoid was previously shown to display vasoconstriction by ATP through mechanisms involving Ito cells (12). In addition, hepatocytes can respond to extracellular ATP to stimulate vesicular transport of bile salts and contractility of bile canaliculi and to thereby increase the basal bile output (2, 17, 22). Such tonic actions of ATP on hepatic microvasculature and hepatocytes are obviously supported by oxidative phosphorylation, being in good agreement with our results showing increased O₂ consumption by the α -NO-hRBC-treated postischemic liver. Thus, effects of T-state stabilization on the ability of RBC to excrete ATP in response to reduced PO₂ and identification of purinergic receptors involved in the mechanisms deserve further study and are now underway in our laboratory.

In conclusion, artificial stabilization of T-state Hb by NO is beneficial to protect postischemic liver dysfunction though stimulating O₂ consumption rather than through facilitating the gas delivery. Since the experimental model for hypovolemic hypotension used in the current study gives only modest dysfunction of the liver and never induces shock states, whether the same treatment with T-state-stabilizing RBC is beneficial for treating clinically relevant hemorrhagic shock or irreversible organ injury remains to be examined. However, use of modest severity of the hemorrhagic model seemed suitable to examine the effects of α -NOHb-hRBC on tissue oxygen delivery and consumption in a reliable manner, so far as the protocol for hypotension did not induce heterogeneity in lobular perfusion or any reflow phenomenon and irreversible cell damages that could cause a large variation of O₂ measurements *in vivo*. Biological actions of T-state stabilized RBC on O₂ delivery and consumption deserves further study, given the evidence for its ability to improve hemorrhagic shock or irreversible organ injury.

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ABBREVIATIONS

α -NO-hRBC, human red blood cells containing α -nitrosyl Hb; BE, base excess; CO-hRBC, human red blood cells containing COHb; EPR, electron spin resonance; FITC, fluorescein isothiocyanate; HR, heart rate; MAP, mean arterial pressure; Pd-TCPP, Pd-meso-tetra-(4-carboxyphenyl)-porphyrin.

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