ABSTRACT

T-state stabilization of hemoglobin (Hb) has been thought to stimulate the ability of erythrocytes to release NO under hypoxic regions. This study aimed to examine if the stabilization could protect against post-ischemic organ injury. Washed human erythrocytes containing three different states of the Hb allostery were suspended in physiological saline at 10 g/dl in Hb concentration: control Hb (hRBC), CO-Hb that is stabilized under R-state with the 6-coodinated prosthetic heme (CO-hRBC), and α-NO-deoxyHb stabilized under T-state (αNO-hRBC). To prepare αNO-RBC, fully deoxygenated RBC was prepared 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 systemic blood pressure at 40 mmHg, and were reperfused with one of hRBCs. This protocol for ischemia followed by 60-min reperfusion with the physiological saline alone caused modest metabolic acidosis and cholestasis. Administration of hRBC or CO-hRBC significantly attenuated cholestasis and improved the acidosis. Rats treated with αNO-hRBC further exhibited greater recovery of visceral blood supply, metabolic acidosis and bile excretion than those treated with hRBC or CO-hRBC, and displayed the best outcome of local oxygen utilization in the 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 stratagem to treat post-ischemic organ dysfunction.

Key Words: hemoglobin allostery, hemorrhagic shock, liver microcirculation, nitric oxide, oxygen

INTRODUCTION

There are several lines of evidence that allostery of hemoglobin (Hb) does not only determine 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 P_{O_2} and an increase in P_{CO_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 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 P_{O_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 allostery of this kind has been reported to regulate microvascular function through multiple mechanisms: First, alterations in the Hb allostery 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 has been thought to be brought to microvascular endothelium and helps vasorelaxation to guarantee ample blood supply in hypoxic regions (10). RBC also has the ability to release ATP in response to hypoxia (3). As 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 appears to result from allosteric changes in Hb, so far as judged from 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 well been uncovered 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 the 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-diffusable NO under deoxygenated conditions (30)(31). In these studies, α-nitrosyl Hb,

$\alpha(Fe\text{-}NO)_2\beta(Fe)_2$ has been synthetically prepared; despite the halved O_2-carrying capacity, α-nitrosyl Hb could represent a versatile low affinity O_2 carrier with improved features that might deliver O_2 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 O_2 metabolism have not fully been investigated in vivo. This study was designed to examine protective effects of RBC with α-nitrosyl Hb on microvascular function, O_2 delivery and organ function in the post-ischemic rat liver where the O_2 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 given informed consents. The blood samples were centrifuged at 2000 *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 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 77K with liquid nitrogen to serve as a sample to detect triplet peaks indicating 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 +/- 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 hrs 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 PO_2, pH and concentrations of HCO_3^- 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 mmHg 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-hr 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 (P_{O_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 μl of FITC-labelled RBC suspension through an intravenous injection. For microvascular P_{O_2} measurements, Pd-meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-TCPP, Porphyrin Products, Eugine, 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. P_{O_2} values were calculated based on the Stern-Volmer equation as described elsewhere (20)(29). To measure the RBC velocity and P_{O_2} at 1 Hz, we used 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 post-ischemic 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 αNOHb in circulation.

In order to determine half-life time of α-NO-hRBC, 2 ml of the peripheral blood samples were collected from 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 77K 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 post-ischemic rats reperfused with autologous blood samples, indicating that the amount of endogenous αNOHb-containing rat erythrocytes is little in circulation, if any. In these experiments, the putative concentrations of circulating αNOHb 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 αNO-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 less than 0.05 were considered statistically significant.

RESULTS

Characterization of αNO-hRBC in vitro and in vivo

Figure 1 illustrates characterization of αNO-hRBC in vitro and in vivo. 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 spectrum in the upper panel, the heights of the triplet signal for αNO-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 αNOHb versus fully nitrosyl Hb was 0.51, suggesting that halves of the prosthetic heme are occupied with NO in αNO-hRBC.

We next examined half lifetime of $\alpha(Fe\text{-}NO)_2\beta(Fe)_2Hb$ of exogenously administered hRBC in the post-ischemic rats. As seen in the upper panel of Figure 2, immediately after the αNO-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 3 separate experiments (Lower panel). In these experiments, the rate of blood replacement with αNO-Hb hRBC was approximately 32%, and the average Hb concentration was 16.3 g/dl; apparent concentrations of αNO-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 αNOHb is approximately 60 min in vivo.

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

We have examined if different modification of Hb allostery, that is, αNO-hRBC, hRBC and CO-hRBC, could cause any differences in blood supply at the level of macrocirculation in vivo. As seen in Figure 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 αNO- 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% of the pre-ischemic 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 αNO-hRBC indicated approximately 2-fold 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 αNO-hRBC-treated rats distinct from those treated with hRBC or with CO-hRBC was also evident in the recovery of bile output from the post-ischemic liver (Figure 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 the both groups exhibited a significant recovery of bile output as compared with the group treated with physiological saline.

Potent improving effects of αNO-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 Figure 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 αNO-hRBC than in the groups treated with hRBC or CO-hRBC, while the improvement of MAP was comparable among these three groups (Figure 5). As shown in Figure 3, the group treated with αNO-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 not show any notable differences in MAP and the visceral blood flow, while the αNO-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 αNO-hRBC- and hRBC-treated groups.

Distinct effects of αNO-hRBC on the recovery of hepatic microvascular PO_2.

Figure 6 illustrates effects of the αNO-hRBC administration on alterations in sinusoidal diameter, functional sinusoidal density, RBC velocity and PO_2 in central venules of post-ischemic liver lobules and a comparison to 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 (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 αNO-hRBC-treated one but without any statistical significance over the entire course of observation because of 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 stiatistical 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 αNO-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 were significantly lower in the αNO-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 Figure 6A (denoted as P in the panel), it was difficult in rats to find 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 mmHg in the both groups (data not shown). These results collectively suggest that the O_2 consumption occurring between portal and central venules appears to be greater in the αNO-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 parenchyma 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 αNO-hRBCs. As demonstated in previous studies (30)(31), α-nytrosylation of Hb in human RBC caused the right shift of the oxygen saturation curve (dotted line). When mean periportal PO_2 values (60 mmHg) and those in pericentral venules (33 mmHg in the normal hRBC and 28mmHg in the αNO-hRBC as seen in Figure 6E) of the post-ischemic 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 αNO-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 αNO-hRBC. Considering that central RBC velocities did not differ significantly between the two groups (Figure 6D), these results suggest that αNO-hRBC and hRBC brings comparable amounts of O_2 to the tissue to each other in the post-ischemic livers.

DISCUSSION

The present study is the first to examine if administration of RBC with T-state-stabilized Hb by NO pretreatment could improve post-ischemic 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 mechanisms 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 mmHg near the P_{50} value (Figure 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 Figures 1 and 6, the liver is such an organ where αNO-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 (Figure 5). Under these circumstances, reperfusion of NO-free hRBC completely repressed the reduction of bile output and induce a recovery of oxygen consumption in the liver parenchyma (Figure 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 contribute 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 for the role of O_2-carrying capacity is also useful to figure out superior effects of αNO-hRBC versus hRBC on the recovery of metabolic acidosis and cholestatic changes in the post-ischemic 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 (Figure 7), αNO-hRBC has the twice greater O_2-carrying capacity than hRBC. However, since 50% of Hb-derived heme of αNO-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 post-ischemic recovery of the tissue O_2 consumption is assumed to be identical between the two groups, central PO_2 values in the αNO-hRBC group should be the same as those in the hRBC group. However, as seen in the data in the early period of reperfusion (Figure 6), the recovery of central PO_2 in the αNO-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 insignificant difference in central RBC velocity between the two groups (Panel D in Figure 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

αNO-hRBC administration leads to greater recovery of O_2 consumption than in the post-ischemic 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 αNO-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, improving effects of αNO-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 αNO-hRBC administration results in improved O_2 consumption still remain unknown in the post-ischemic liver, several possibilities can be considered. First, αNO-hRBC could deliver more NO to hepatic microcirculation, help cancel superoxide generated upon the early reperfusion period, and attenuate the post-ischemic 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 the liver treated with αNO-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 (Figure 6). Furthermore, the delivery of NO from circulation to parenchyma could suppress O_2 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 PO_2. Vasoactive responses to extracellularly released ATP are known to differ among different organs (7). In most organ 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 O_2 consumption by the αNO-hRBC-treated post-ischemic liver. Thus, effects of T-state stabilization on the ability of RBC to excrete ATP in response to reduced PO_2 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 post-ischemic liver dysfunction though stimulating O_2 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 induced heterogeneity in lobular perfusion or no reflow phenomenon and irreversible cell damages that could cause a large variation of O_2 measurements in vivo. Biological actions of T-state stabilized RBC on O_2 delivery and consumption deserves further studies given the evidence for its ability to improve hemorrhagic shock or irreversible organ injury.

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Abbreviation list

αNO-hRBC: human red blood cell containing α-nitrosyl Hb
BE: base excess
CO-hRBC: human red blood cell 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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Figure Legends

Figure 1 Charaterization of αNO-human erythrocytes (αNO-hRBC) prepared in vitro by electron paramagnetic resonance (EPR) spectrometry. A representative EPR signal of αNO-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 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 nytrosyl and α-nitrosyl Hbs, respectively.

Figure 2 Determination of exogenously administered αNO-hRBC by EPR spectrometry in systemic blood samples in rats exposed to ischemia-reperfusion. ***Upper panel***: Representative time history of the decay of αNOHb-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 αNO-hRBC(+); temporal alterations in the EPR signals from rats reperfused with αNO-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 +/- SE of 3 separate experiments. Note that half-life time of αNOHb is approximately 60 min in systemic circulation.

Figure 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 αNO-hRBC (shaded circles). Data indicate mean +/- SE of 5-7 separate experiments for each group. *P< 0.05 as compared with sham-operated controls. Note that αNO-hRBC significantly increases splanchnic blood flow without altering MAP, suggesting a decrease in systemic vascular resistance.

Figure 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 αNO-hRBC (shaded circles). Closed squares; rats exposed to the ischemia followed by reperfusion with physiological saline (PS). Data indicate mean +/- SE of 6-8 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.

Figure 5 Effects of post-ischemic administration of hRBC, CO-hRBC and αNO-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 +/- 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 αNO-hRBC displays the best outcomes of recovery from metabolic acidosis and cholestasis among the groups examined.

Figure 6 Effects of the αNO-hRBC administration on alterations in sinuoisdal diameter, functional sinusoidal density, RBC velocity and PO_2 in central venules of post-ischemic liver lobules. A: A representative picture showing traffics of FITC-labelled 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 sinuoisdal diameter and functional sinusoidal density. Data indicate mean +/- SE of 5-7 separate experiments. D, E and F: Alterations in RBC velocity, PO_2, and relative PO_2 recovery in central venules (CV). Data indicate mean +/- SE of 4-5 separate experiments. Open and closed circles indicate data from hRBC- and αNO-hRBC-treated groups, respectively. *P< 0.05 as compared with the hRBC-treated control group.