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.

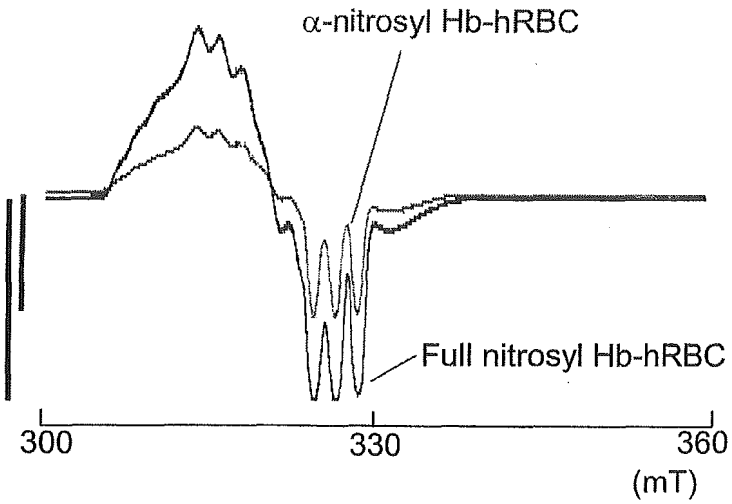
Figure 3 Temporal alterations in mean arterial pressure (MAP), heart rate (HR) and visceral blood flow of rats exposed to the 20-min hypovolemic ischemia followed by reperfusion with hRBC (open circles), CO-hRBC (striped circles) and αNO-hRBC (shaded circles). Data indicate mean +/- SE of 5-7 separate 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.

Figure 7 Differences in oxygen saturation profiles between hRBC (solid line) and αNO-hRBC (broken line). Solid and broken arrows indicate estimations of differences in SaO_2 for hRBC and αNO-RBC during transition across hepatic microcirculation, respectively. The estimation is based on observations that mean PO_2 values in periportal regions are 70 mmHg in both groups and those in central venules are 28 mmHg and 33 mmHg in the groups treated with αNO-hRBC and normal hRBC, respectively (See Figure 6E).



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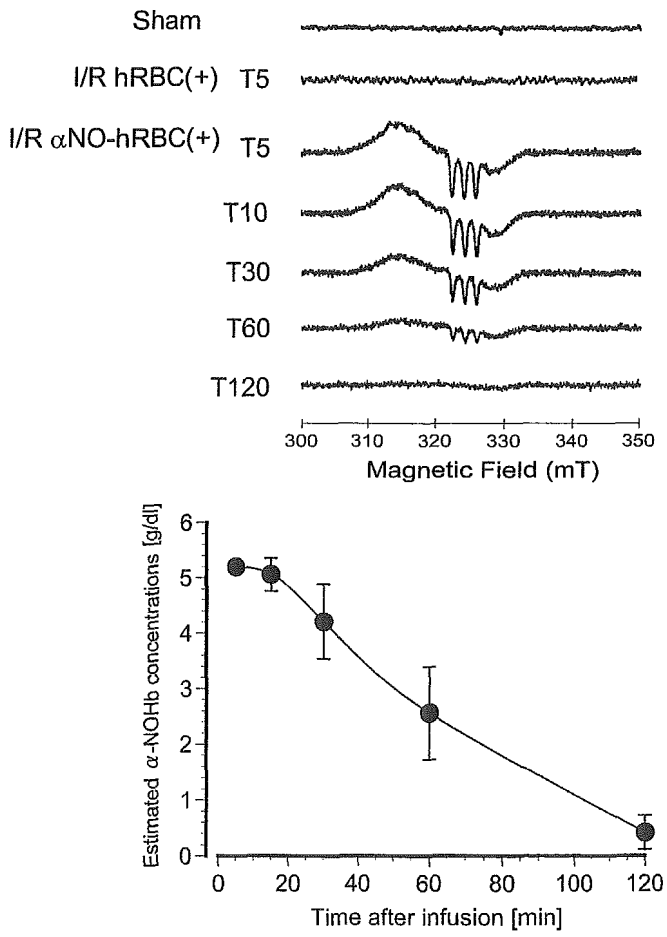

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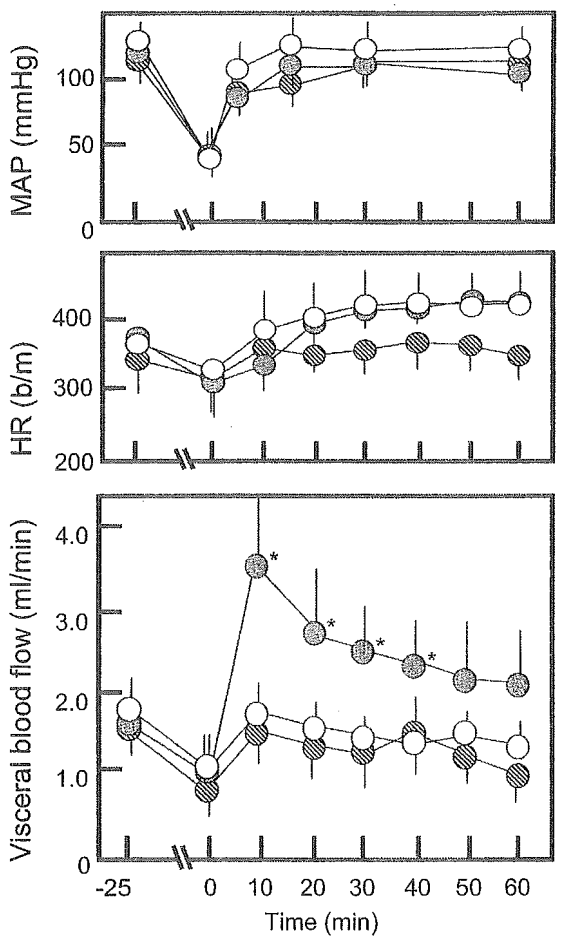

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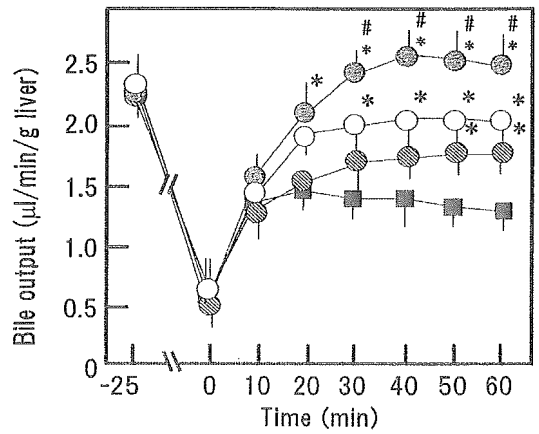

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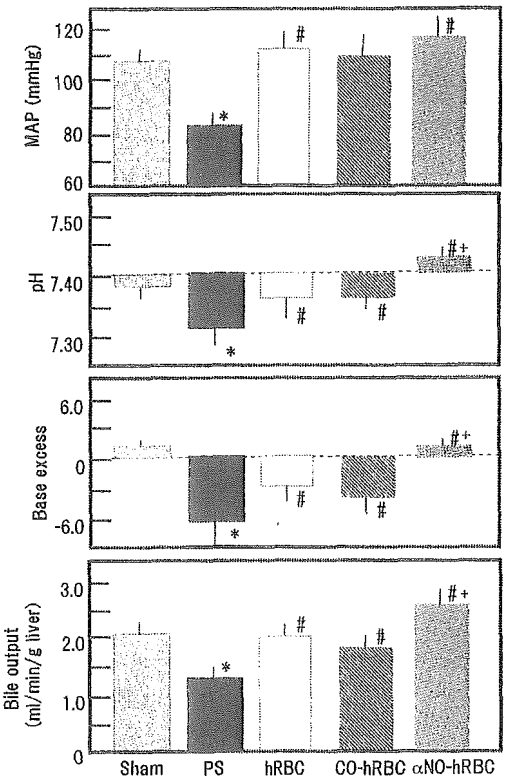

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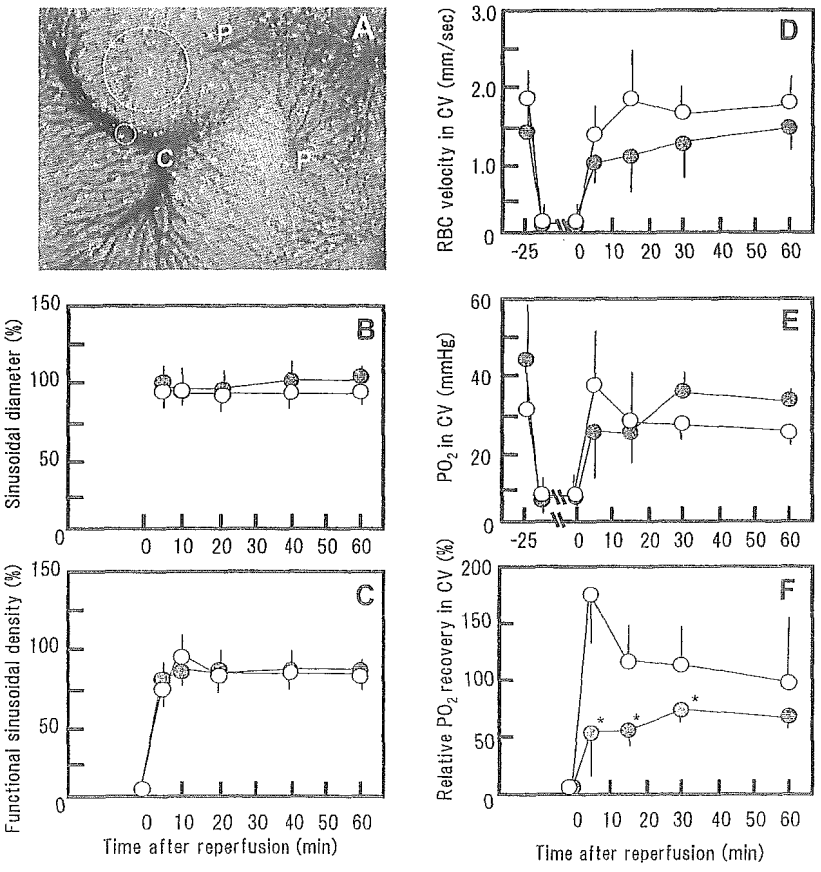

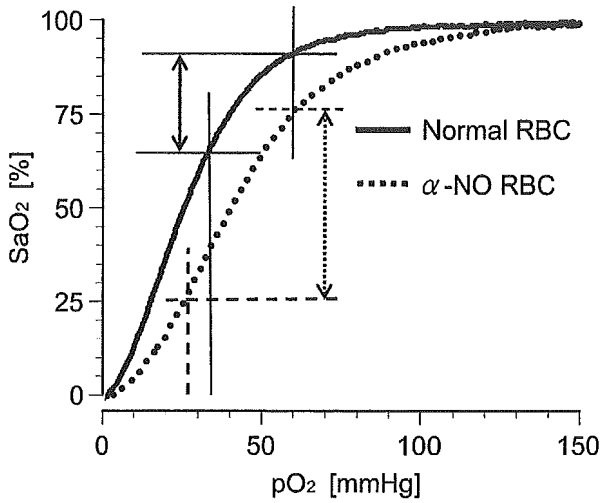

Figure 7

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Carbon Monoxide as a Guardian against Hepatobiliary Dysfunction

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Carbon monoxide (CO) generated through the reaction of heme oxygenase (HO) has attracted great interest in regulation of hepatobiliary homeostasis. The gas generated by HO-2 in the hepatic parenchyma can modestly activate soluble guanylate cyclase (sGC) expressed in hepatic stellate cells in a paracrine manner and thereby constitutively relax sinusoids. Kupffer cells express HO-1, the inducible isozyme, even under normal unstimulated conditions and constitutes approximately 30% of the total HO activity in this organ. Upon exposure to a variety of stressors such as cytokines, endotoxin, hypoxia and oxidative stress, the liver induces HO-1 and overproduces CO. The stress-inducible CO has been shown to guarantee ample blood supply during detoxification of heme and thus to play a protective role in the liver. However, molecular mechanisms by which CO serves as a protectant for hepatocytes, the cells expressing little sGC, remain to be solved. Previous observation suggested that CO modulates intracellular calcium mobilization through inhibiting cytochrome P-450 activities and thereby maintain stroke volume of bile canalicular contraction in cultured hepatocytes. CO also stimulates mrp2-dependent excretion of bilirubin-IXα and helps heme catabolism. Although a direct molecular target responsible for the latter event remains unknown, such properties of CO could support xenobiotic metabolism through its actions on sinusoidal hemodynamics and hepatobiliary systems.

Key Words: Carbon monoxide, Heme Oxygenase, Soluble Guanylate Cyclase, Gas Biology, Xenobiotic Metabolism, Metabolome.

GASEOUS SIGNAL TRANSDUCTION is an event where gaseous molecules produced or utilized in the body transfer biological signals to their receptors. Such signal transducing systems occur among a wide range of life including microorganisms and mammals. Molecular oxygen (O_2) is not only necessary to maintain aerobic ATP synthesis in mitochondria but also serves as a primary substrate to synthesize the signaling gases such as nitric oxide (NO) and carbon monoxide (CO) (Kashiwagi et al., 2002; Suematsu et al. 2000). These gaseous monoxides are generated by oxygenases such as NO synthase (NOS) and heme oxygenase (HO), respectively. On the other hand, 3-phosphoglyceric acid, a product of glycolysis, serves as a substrate for serine which interacts with homocysteine to generate cysteine. Besides its roles for synthesis of glutathione, taurine and sulfate ion, cysteine serves as a substrate for cystathionine β-synthase and/or cystathionine γ-lyase to generate hydrogen sulfide (H_2S) (Abe and Kimura, 1996. Zhao et al., 2001. Eto et al., 2002. Fujii et al., 2005, Sugiura et al., 2005). The liver is one of the major organs where these multiple gases appear to be abundantly generated to be utilized for maintenance of the organ homeostasis. Among these gases, this review focused on recent advances in understanding of pathophysiologic roles of CO in maintenance of the hepatobiliary function, and its effect on generation systems of other gases will be discussed.

HEPATIC CO GENERATION UNDER PHYSIOLOGICAL AND PATHOPHYSIOLOGIC CONDITIONS

CO is a gaseous product of the HO reaction that utilizes molecular oxygen to oxidatively degrade protoheme IX into biliverdin-IXα, ferrous iron and the gas. CO has been considered a gaseous mediator analogous to NO that activates soluble guanylate cyclase (sGC) as a common transducer to relax vascular systems (Suematsu et al., 1994, 1995; Motterlini et al., 1998), or to trigger neural transmission (Verma et al., 1993). However, as described later, these two gases possess biochemical features distinct from each other in terms of the ability to modify function of heme proteins. In mammals, HO exists in two forms: HO-1 and HO-2. HO-1 is induced by varied stressors such as cytokines, heavy metals, ROS and hypoxia. Microvascular actions of endogenously generated CO was first demonstrated in the liver (Suematsu et al., 1994, 1995). The liver constitutes a major organ responsible for detoxification of the

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