Carbon Monoxide–Bound Red Blood Cells Protect Red Blood Cell Transfusion-Induced Hepatic Cytochrome P450 Impairment in Hemorrhagic-Shock Rats

Shigeru Ogaki, Kazuaki Taguchi, Hiroshi Watanabe, Masaki Otagiri, and Toru Maruyama

Department of Biopharmaceutics (S.O., K.T., H.W., M.O., T.M.) and Center for Clinical Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences (H.W., T.M.), Kumamoto University; and Faculty of Pharmaceutical Sciences (M.O.) and DDS Research Institute, Sojo University (M.O.), Kumamoto, Japan

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ABSTRACT

Red blood cell (RBC) transfusions for massive hemorrhage induce systemic ischemia-reperfusion and influence the disposition and pharmacological activity of drugs as a result of a reduction in the level of expression and activity of cytochrome P450s (P450). It was reported that, when organ-preserving solutions are exposed to carbon monoxide (CO), the treatment was effective in suppressing the postreperfusion reduction in renal P450 levels in cases of kidney transplantation. Therefore, we hypothesized that transfusions with RBC that contain bound CO (CO-RBC) would protect the hepatic level of rat P450 during a massive hemorrhage, compared with plasma expanders and RBC resuscitation. To achieve this, we created 40% hemorrhagic-shock model rats, followed by resuscitation, with use of recombinant human serum albumin, RBCs, and CO-RBCs. At 1 hour after resuscitation, the expressions of hepatic P450 isoforms (1A2, 2C11, 2E1, and 3A2) were significantly decreased in the RBC resuscitation group, compared with the sham group. Such alterations in hepatic P450 significantly resulted in an increase in the plasma concentrations of substrate drugs (caffeine [1A2], tolbutamide [2C11], chlorzoxazone [2E1], and midazolam [3A2]) for each P450 isoform, and thus, the hypnotic action of midazolam could be significantly prolonged. Of interest, the reductions in hepatic P450 activity observed in the RBC group were significantly suppressed by CO-RBC resuscitation, and consequently, the pharmacokinetics of substrate drugs and the pharmacological action of midazolam remained at levels similar to those under sham conditions. These results indicate that CO-RBC resuscitation has considerable potential in terms of achieving safe and useful drug therapy during massive hemorrhages.

Introduction

Red blood cell (RBC) transfusions are the first administered care in cases of patients who are in a state of hemorrhagic shock and have greatly contributed to human health and welfare. However (and unfortunately), resuscitation from a massive hemorrhage by RBC transfusion causes systemic ischemia-reperfusion (Allan et al., 2011), a major deleterious factor that affects the maintenance of homeostasis. Several recent investigations have clearly shown that a warm hepatic ischemia-reperfusion induced a decrease in hepatic cytochrome P450 (P450) levels and metabolic activity (Izuishi et al., 2000; Eum and Lee, 2004). P450 is a heme protein that has a broad range of functions, including the metabolism and detoxification of endogenous and exogenous substances (Morgan, 2001). Because the majority of P450 is located in the liver, any alteration in hepatic P450 levels or activity would constitute a major influence on the disposition and pharmacological activities of drugs. In emergencies in which RBC transfusions are needed, P450-metabolized drugs, such as corticosteroid hormones, cardiac stimulants, and anesthetics, are frequently used to maintain general status; therefore, a decrease in P450 levels and activity because of ischemia-reperfusion as the result of an RBC transfusion can result in altered blood levels of these drugs and, consequently, could have an effect on their pharmacological characteristics. In fact, Harbrecht et al., 2005 reported that patients undergoing RBC transfusion experienced elevated blood concentrations of P450-metabolized drugs, such as flurbiprofen, mephenytin, and chlorzoxazone, probably as a result of suppressed metabolism. Therefore, maintaining reasonable levels of P450 during RBC transfusions is generally believed to be critical in minimizing changes in the disposition of P450 substrate drugs and their pharmacological effects, but drugs or treatments designed to achieve these conditions are not currently available.

Carbon monoxide (CO) possesses anti-inflammatory, antiapoptotic, and antioxidant activities, suggesting that it could be used to prevent organ damage caused by ischemia-reperfusion injury (Otterbein et al., 2000; Brouard et al., 2002; Zuckerbraun et al., 2005; Stec et al., 2008; Motterlini and Otterbein, 2010). Nakao et al., 2008 recently reported an interesting observation, in which the exposure of organ preservation solutions to CO was effective in suppressing the postreperfusion reduction in renal P450 levels in a rat model of kidney transplantation. This suggests that the use of CO might be useful in reducing P450 levels, even in the case of resuscitation from hemorrhagic shock via an RBC transfusion. However, of note, a carrier for controlling the

ABBREVIATIONS: AUC, area under the blood concentration–time curve; CO, carbon monoxide; CO-RBC, carbon monoxide–bound red blood cell; P450, cytochrome P450s; HPLC, high-performance liquid chromatography; IL-6, interleukin-6; RBC, red blood cell; rHSA, recombinant human serum albumin; ROS, reactive oxygen species; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α.
disposition of CO would be essential in successful clinical applications of CO. Fortunately, because CO binds easily and stably to hemoglobin in RBCs, RBCs represent a leading candidate for use as a carrier for delivering CO. In fact, Cabrales et al., 2007 recently reported that, when CO-bound RBCs (CO-RBCs) are injected into hemorrhaging hamsters, they are as effective as the systemic or microhemodynamic administration of RBCs, and the cytoprotective effect in the subcutaneous microcirculation is higher than that of RBCs. In addition, Sakai et al., 2009 reported that CO-RBCs showed a resuscitative effect in hemorrhagic-shock rats and that oxidative damage to organs was reduced in comparison with RBCs. These findings led us to the hypothesis that CO-RBCs have the potential for use as a novel RBC transfusion preparation with a P450 protein protective effect.

The objective of this study was to determine (1) whether CO-RBC transfusion represents a promising resuscitative fluid and (2) to what extent would CO-RBC transfusions influence the hepatic levels of various rat P450 isoforms (1A2, 2C11, 2E1, and 3A2) and the disposition and pharmacological action of P450-metabolized drugs, compared with recombinant human serum albumin (rHSA) and a RBC transfusion using 40% exsanguinated model rats.

Materials and Methods

Preparation of Resuscitative Fluids. The three resuscitative fluids used for this study were prepared as follows. First, to prepare the rHSA resuscitative fluid, a 25% rHSA (Nipro Co, Osaka, Japan) solution was diluted to 5% with saline solution. Second, the RBC resuscitative fluid was prepared as described in a previous report (Taguchi et al., 2011b); blood samples from donor male Sprague-Dawley rats were withdrawn into heparinized syringes (ca. 0.15 ml of 10,000 IU/ml heparin to 10 ml of blood) and centrifuged. The pellet was then washed three times to remove plasma components by resuspension in saline followed by centrifugation (3000g, 10 min, 4°C). The hemoglobin concentration of a washed RBC suspension was determined using a hemoglobin test kit (Wako Chemicals, Saitama, Japan) and was adjusted to 10 g/dl. A washed RBC suspension (8.6 ml) was mixed with a solution of rHSA (25%, 1.4 ml) to regulate (rHSA) in the suspending medium to 5 g/dl and the colloid osmotic pressure to -20 torr. Third, CO gas was gently bubbled through the RBC resuscitative fluid, prepared as described above, for 5 minutes. This solution was designated as the CO-RBC resuscitative fluid (Sakai et al., 2009).

Preparation of Hemorrhagic-Shock Model Rats and Resuscitation from Hemorrhagic Shock. All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University. Eighty-eight male Sprague-Dawley rats (180–210 g; Kyoudo Co, Kumamoto, Japan) were anesthetized and polyethylene catheters (PE 50 tubing, outer diameter equal to 0.965 mm and inner diameter equal to 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were then introduced into the left femoral artery. Hemorrhagic shock was induced by removing 40% of the total blood volume according to a previous report (Taguchi et al., 2009, 2011a). Systemic blood volume was estimated to be 56 ml/kg (Sakai et al., 2004). Under this experimental condition, the mean arterial pressure remained at less than 40 mm Hg for 30 minutes. The hemorrhagic-shock rats were resuscitated by an infusion of rHSA (n = 20), RBC (n = 20), or CO-RBC (n = 20) resuscitative fluid at a rate of 1 ml/min. The volume of the infused resuscitative fluids was equal to 40% of total blood volume at baseline. After resuscitation from shock, the femoral artery was ligated, and the skin was sutured with a stitch. In addition, thirteen hemorrhagic-shock rats were not resuscitated with any fluid, and 15 rats were subjected to a sham operation. All animals were maintained in a temperature-controlled room with a 12-hour dark/light cycle and ad libitum access to food and water. The resuscitated rats were selected at random from each group and were used at 1 and 24 hours after resuscitation in the experiments outlined below.

Measurement of Systemic Responses. Blood gases were evaluated before hemorrhage (baseline), after hemorrhage, and 1 hour after resuscitation. Blood samples were collected from the right femoral artery. A blood gas analyzer (i-STAT; Fuso Pharmaceuticals Industries, Tokyo, Japan) was used for analyses of PaO2, PaCO2, pH, base excess, and lactate.

Western Blot Analysis. At 1 or 24 hours after resuscitation from hemorrhage, the vena cava of the rats was cannulated with a polyethylene catheter (diameter, 0.9 mm), and the liver was perfused with 1.15% KCl. The liver was homogenized (1000 rpm, 5 minutes, on ice) using a homogenizer (Iwamoto Scientific Technology, Tokyo, Japan) in lysis buffer (0.23 M sucrose, 5 mM Tris-HCl, 2 mM EDTA, 50 mM protease inhibitor cocktail; pH, 7.4). After centrifuging (550g, 5 minutes, 4°C) the homogenized liver preparation, the supernatant was used as the liver extract. After measurement of the protein content using a Bradford assay, each sample was suspended in loading buffer (2% SDS, 125 mM Tris-HCl, pH 7.4, 20% glycerol, and 5% 2-mercaptoethanol) and heated at 100°C for 2 minutes. The samples were separated by 12.5% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) by wet blotting. The blots were blocked with 5% ECL advance blocking agents (GE Healthcare Bio-sciences Corp., Piscataway, NJ) in Tris-buffered saline (TBS) containing 0.3% Tween (TBS-T). The blots were washed once with TBS-T and incubated for 2 hours at room temperature with primary antibodies specific for CYP1A2, CYP2C11, CYP2E1, and CYP3A2 (polycyonal goat anti-rat CYP1A2, CYP2C11, and CYP2E1 and polycyonal rabbit anti-rat CYP3A2; Nozan, Kanagawa, Japan) in TBS-T. The blots were washed three times with TBS-T and incubated with secondary antibodies (horseradish peroxidase-linked anti-goat and anti-rabbit IgG; Invitrogen, Eugene, OR) for 1 hour at room temperature. The blots were washed three times with TBS-T, and the immunoblots were visualized using an ECL system (ECL Advance Western Blotting Detection Kit; GE Healthcare Bio-sciences Corp.) with LAS-3000mini (Fujifilm, Tokyo, Japan). The band intensities were quantified using the National Institutes of Health Image analysis software (http://rsbweb.nih.gov/ij/).

The Pharmacokinetic Experiment Protocol with P450 Cocktail. The pharmacokinetic experiments using the P450 cocktail were performed as described in a previous report (Liu et al., 2009). The P450 cocktail consisted of four probe drugs: caffeine (Nacalai tesque Co., Kyoto, Japan) for CYP1A2, tolbutamide (Alexis Co., Lausen, Switzerland) for CYP2C11, chlorozoxazine (Oxford Biomedical Research Inc., Cherrybrook, New South Wales, Australia) for CYP2E1, and midazolam (Sigma Chemicals Inc, St. Louis, MO) for CYP3A2. At 1 hour after resuscitation from hemorrhage, the P450 cocktail (2.5 mg/kg each drug) was administered to each rat via the tail vein. Blood samples were collected at multiple time points after injection of the P450 cocktail (5, 15, and 30 minutes and 1, 2, 4, 6, 12, and 24 hours), and the plasma was separated by centrifugation (3000g, 5 minutes, at 4°C). The concentration of drugs in plasma was determined by high-performance liquid chromatography (HPLC) according to a previous report (Liu et al., 2009). The HPLC system consisted of a Hitachi L-6000 pump, a Hitachi L-4000 UV detector operated at 230 nm, and Hitachi D-2500 Chromato integrator. LC analyses were performed on a Cadenza-CD-C18 column (25 × 3.0 mm inner diameter; particle size, 3 μm).

The Pharmacodynamic Experiments Protocol. The pharmacodynamic experiments for midazolam involved the method developed by Wang et al., 2010, with minor modifications. The hemorrhagic-shock rat model was prepared under ether anesthesia and resuscitated as described above. All rats were awake within 30 minutes after resuscitation. At 1 hour after resuscitation from hemorrhage, midazolam (5 mg/kg) was administered to each rat via the tail vein. After the administration of midazolam, the hypnotic effect index was recorded. The experiments were performed in a quiet room at room temperature. The time lapse between the administration of midazolam and the onset of losing the righting reflex was recorded as sleep latency. The interval between the loss and recovery of the righting reflex was recorded as the sleeping time.

Data Analysis. A two-compartment model was used to determine the pharmacokinetic parameters after administering the P450 cocktail. Pharmacokinetic parameters were estimated by curve-fitting using MULTL, a normal least-squares program (Yamaoka et al., 1981). Data are shown as the means ± S.D. for the indicated number of animals. Significant differences among each group were examined using Student’s t test. The Spearman test was used for the correlation analysis. A probability value of P < 0.05 was considered to be statistically significant.

Results

Systemic Response to Hemorrhagic Shock and Resuscitation by RBCs and CO-RBCs. All rats that received RBCs or CO-RBCs as a resuscitative fluid survived until their planned death. In contrast, all
correlations between the AUC and the expression of CYP2E1 were midazolam: caffeine: tolbutamide, and midazolam were negatively correlated with the expression of CYP1A2, CYP2C11, and 3A2 protein, respectively (Table 2). RBC resuscitation group were similar to those for the sham group were significantly increased, and the results for the rHSA and CO-RBC resuscitation group showed a similar profile to that for the sham group. Compared with the sham group, the protein expressions of all P450 isoforms in the RBC resuscitation group were significantly decreased, compared with the sham group, whereas those in the rHSA and CO-RBC resuscitation group were maintained at constant levels (Fig. 2). Furthermore, these changes were maintained in all groups during the entire period of the experiment (24 hours after resuscitation). These data suggest that CO-RBC resuscitation protects against at least four hepatic P450 isoforms.

Expression of Isoforms of the Hepatic P450 Protein. To evaluate the effect of resuscitative fluids on hepatic P450 levels, the expression levels of the hepatic protein P450 isoforms CYP1A2, 2C11, 2E1, and 3A2 were determined using Western blot analysis. At 1 hour after resuscitation, the protein expressions of all P450 isoforms in the RBC resuscitation group were significantly decreased, compared with the sham group, whereas those in the rHSA and CO-RBC resuscitation group were maintained at constant levels (Fig. 2). Furthermore, these changes were maintained in all groups during the entire period of the experiment (24 hours after resuscitation). These data indicate that CO-RBC resuscitation protects against at least four hepatic P450 isoforms.

Plasma Concentration of P450 Cocktail. We next addressed the issue of whether changes in the levels of P450 isoform proteins directly affected the pharmacokinetics of the process using four probe drugs, namely, caffeine (1A2), tolbutamide (2C11), chlorzoxazone (2E1), and midazolam (3A2), which are specifically metabolized by the P450 isoforms. Fig. 3 shows the time course for the plasma concentration of the four probe drugs in the sham, rHSA, RBC, and CO-RBC resuscitation groups. The plasma concentrations of the four probe drugs were substantially elevated in the RBC resuscitation group, and the concentrations in the rHSA and CO-RBC resuscitation group showed a similar profile to that for the sham group. Compared with the sham group, the area under the blood concentration-time curve (AUC) for the four probe drugs in the RBC resuscitation group were significantly increased, and the results for the rHSA and CO-RBC resuscitation group were similar to those for the sham group (Table 2).

Moreover, as shown in Fig. 4, A, B, and D, the AUCs for caffeine, tolbutamide, and midazolam were negatively correlated with the expression of CYP1A2, CYP2C11, and 3A2 protein, respectively (caffeine: $r=0.99 \ (P = 0.042)$; tolbutamide: $r=0.99 \ (P = 0.046)$; midazolam: $r=0.99 \ (P = 0.028)$). Similarly, tendencies for negative correlations between the AUC and the expression of CYP2E1 were observed for chlorzoxazone (Fig. 4C; $r=0.98, \ P = 0.086$). In addition, considering all of the data shown in Fig. 4A–D, a significantly negative correlation exists between them (Fig. 4E; $r = 0.93, \ P = 0.017$), indicating that such a relation was common among the P450 isoforms. These data confirm that the significant alternations in hepatic P450 expressions by hemorrhaging and resuscitation were major contributors to the changes in the AUC for the P450 metabolized drugs.

Pharmacological Effect of Midazolam. To evaluate whether the alteration in P450 protein expression has an effect on pharmacological parameters, sleep latency and sleeping time induced by the administration of midazolam were measured. As shown in Fig. 5A, sleeping latency remained essentially unchanged among all groups. However, sleeping time in the RBC resuscitation group was significantly increased, compared with that in the sham group, whereas sleeping time for the rHSA and CO-RBC resuscitation group was similar to that for the sham group (Fig. 5B).

As shown in Fig. 5C, sleeping time induced by midazolam was negatively correlated with the levels of CYP3A2 expression ($r=0.99, \ P = 0.007$). These data indicate that the changes in the pharmacological effect of midazolam are largely dependent on the expression of the CYP3A2 protein, even under conditions of hemorrhaging and resuscitation.

Discussion

The clinical indications for RBC transfusion, the usefulness of which is unquestionable, include an emergency administration to combat massive bleeding. During such RBC transfusions for a massive hemorrhage, ischemia-reperfusion injury occurs systemically in many organs, especially in the liver, which, in turn, can change the dispositions of therapeutic drugs and their pharmacological effects as a result of a reduction in the level of expression and activity of P450. The present study focused on this issue using hemorrhagic-shock model rats and further examined the effect of CO-RBC on hepatic P450 protection and resuscitative action. The major findings of the present study are as follows: (1) CO-RBCs appear to be as effective as RBCs as a resuscitation fluid, (2) CO-RBCs significantly suppressed reductions in P450 isoform levels that occur during RBC resuscitation from a massive hemorrhage, and (3) consequently, such an inhibition in the alteration of P450 isoform levels by CO-RBCs significantly suppressed the changes in the pharmacokinetic-pharmacodynamic properties for P450 metabolizing drugs.

Harbrecht et al., 2005 examined the disposition of drugs metabolized by P450 isoforms in 23 critical ill trauma patients after nonresuscitated rats ($n = 13$) died within 1.5 hours after the induction of hemorrhagic shock, and 6 of the 20 rats that had been resuscitated by rHSA died during the experiment (Fig. 1). Changes in blood gas parameters were not significantly different among the rHSA, RBC, and CO-RBC resuscitative fluid groups (Table 1). Moreover, the CO-RBC resuscitative fluid group showed no signs of hypoxia or abnormal behavior after resuscitation. These data indicate that CO-RBC resuscitation did not present any toxic liability and would be expected to function as a resuscitation fluid, analogous to RBC resuscitation.
RBC transfusions and reported that their metabolic activities by P450 were decreased and were associated with ischemia-reperfusion injury. In this study, we also found marked reductions in the levels of hepatic P450 isoforms associated with RBC resuscitation, and of interest, this was significantly inhibited in the case of CO-RBC resuscitation close to the sham levels (Fig. 2). Correspondingly, after CO-RBC resuscitation, the dispositions of some P450 substrate drugs (caffeine, tolbutamide, chlorzoxazone, and midazolam) for the respective P450 isoforms and the hypnotic action of midazolam remained at levels similar to those found for the sham group (Figs. 3 and 5; Table 2). In those observations, changes in the AUC of the substrate drugs of each P450 isoform or the pharmacological effect of

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**TABLE 1**

Laboratory variables during hemorrhagic-shock resuscitation by rHSA, RBCs, or CO-RBCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>pH</th>
<th>PaO₂</th>
<th>PaCO₂</th>
<th>BE</th>
<th>Lac</th>
<th>HbCO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Torr</td>
<td>Torr</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Before hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHSA</td>
<td>7.35 ± 0.03</td>
<td>75.8 ± 4.4</td>
<td>39.1 ± 4.2</td>
<td>1.1 ± 1.3</td>
<td>0.7 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>RBC</td>
<td>7.35 ± 0.02</td>
<td>76.7 ± 3.7</td>
<td>39.3 ± 3.1</td>
<td>1.7 ± 0.7</td>
<td>0.7 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>CO-RBC</td>
<td>7.36 ± 0.05</td>
<td>74.3 ± 3.5</td>
<td>40.7 ± 7.8</td>
<td>1.0 ± 1.7</td>
<td>0.7 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>After hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHSA</td>
<td>7.22 ± 0.08*</td>
<td>102.1 ± 11.3**</td>
<td>24.8 ± 6.8**</td>
<td>-4.3 ± 1.6**</td>
<td>4.2 ± 1.1**</td>
<td>ND</td>
</tr>
<tr>
<td>RBC</td>
<td>7.21 ± 0.03*</td>
<td>101.8 ± 9.2**</td>
<td>24.7 ± 8.3**</td>
<td>-5.3 ± 2.1**</td>
<td>4.4 ± 1.3**</td>
<td>ND</td>
</tr>
<tr>
<td>CO-RBC</td>
<td>7.21 ± 0.08*</td>
<td>100.0 ± 10.1**</td>
<td>26.7 ± 4.5**</td>
<td>-3.7 ± 0.6**</td>
<td>4.1 ± 1.1**</td>
<td>26.4 ± 6.6</td>
</tr>
</tbody>
</table>

BE, base excess; HbCO, Carbonyl hemoglobin; Lac, lactate; ND, not determined.

*P < 0.05 vs. baseline.

**P < 0.01 vs. baseline.

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![Fig. 2. Expression of hepatic protein P450 isoforms (A, CYP1A2; B, CYP2C11; C, CYP2E1; D, CYP3A2) in sham and hemorrhagic-shock rats at 1 hour and 24 hours after resuscitation by rHSA, RBCs, or CO-RBCs. Protein expression of each P450 isoform/β-actin in sham rat defined as 100%. In the rHSA group, three of ten rats died during the experiment. Therefore, the number of rats in the rHSA group was four and three at 1 and 24 hours, respectively. The other group of rats survived during experiment (n = 5). The values are means ± S.D. *P < 0.05, **P < 0.01, compared with sham rats group. ***P < 0.01, compares with RBC resuscitation group.](image-url)
midazolam were negatively correlated with the expression levels of the respective P450 isoforms (Figs. 4 and 5). These results suggest that such alternations in pharmacokinetic properties and the pharmacodynamic action of P450 substrates is attributable to metabolic inhibition and not the suppression of renal excretions. P450-metabolizing drugs are frequently used in the treatment of hemorrhagic shock. For example, fentanyl and propofol are frequently used as surgical narcotics. Therefore, maintaining drug metabolism with use of CO-RBCs is of

![Fig. 3.](image)

**Fig. 3.** Plasma concentration profile for the P450 cocktail (A, caffeine [CYP1A2]; B, tolbutamide [CYP2C11]; C, chlorzoxazone [CYP2E1]; D, midazolam [CYP3A2]) in sham (opened circles) and hemorrhagic-shock rats at 1 hour after re-suscitation by rHSA (hatched circles), RBCs (closed circles), or CO-RBCs (gray circles). In the rHSA group, two of five rats died during experiment. Therefore, the number of rats in rHSA group was three. The other group rats survived during experiment (n = 5). Each point represents the mean ± S.D.

### TABLE 2

Pharmacokinetic parameters for the of P450 cocktail after intravenous injection to sham rats and hemorrhagic-shock rats resuscitated by rHSA, RBCs, or CO-RBCs

<table>
<thead>
<tr>
<th>Cocktail</th>
<th>T1/2 (h)</th>
<th>K (10^6 min^{-1})</th>
<th>AUC (h × ng/ml)</th>
<th>CL (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine (CYP1A2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>4.8 ± 2.0</td>
<td>2.7 ± 1.0</td>
<td>279.7 ± 25.3</td>
<td>1.86 ± 0.15</td>
</tr>
<tr>
<td>rHSA</td>
<td>5.1 ± 2.3</td>
<td>2.1 ± 0.9</td>
<td>384.5 ± 71.3</td>
<td>1.54 ± 0.48</td>
</tr>
<tr>
<td>RBC</td>
<td>8.2 ± 3.3*</td>
<td>1.6 ± 0.8*</td>
<td>509.6 ± 82.7**</td>
<td>1.04 ± 0.16**</td>
</tr>
<tr>
<td>CO-RBC</td>
<td>5.4 ± 3.7</td>
<td>1.4 ± 0.2</td>
<td>406.1 ± 4.9</td>
<td>1.28 ± 0.09</td>
</tr>
<tr>
<td>Tolbutamide (CYP2C11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>2.7 ± 0.6</td>
<td>4.5 ± 1.0</td>
<td>224.4 ± 43.1</td>
<td>2.39 ± 0.40</td>
</tr>
<tr>
<td>rHSA</td>
<td>2.8 ± 1.4</td>
<td>4.2 ± 0.7</td>
<td>264.4 ± 40.2</td>
<td>2.15 ± 0.56</td>
</tr>
<tr>
<td>RBC</td>
<td>6.8 ± 0.7**</td>
<td>1.7 ± 0.2**</td>
<td>401.2 ± 39.0**</td>
<td>1.29 ± 0.13**</td>
</tr>
<tr>
<td>CO-RBC</td>
<td>2.9 ± 0.6</td>
<td>4.0 ± 0.7</td>
<td>255.3 ± 36.9</td>
<td>2.07 ± 0.31</td>
</tr>
<tr>
<td>Chlorzoxazone (CYP2E1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>4.8 ± 1.1</td>
<td>2.5 ± 0.6</td>
<td>287.3 ± 61.5</td>
<td>1.87 ± 0.36</td>
</tr>
<tr>
<td>rHSA</td>
<td>3.6 ± 1.3</td>
<td>3.2 ± 1.1</td>
<td>304.8 ± 41.5</td>
<td>1.77 ± 0.44</td>
</tr>
<tr>
<td>RBC</td>
<td>6.7 ± 0.9**</td>
<td>1.7 ± 0.2**</td>
<td>593.0 ± 64.8**</td>
<td>0.95 ± 0.12**</td>
</tr>
<tr>
<td>CO-RBC</td>
<td>3.3 ± 0.8</td>
<td>3.6 ± 0.8</td>
<td>315.9 ± 95.0</td>
<td>1.73 ± 0.37</td>
</tr>
<tr>
<td>Midazolam (CYP3A2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>3.2 ± 0.4</td>
<td>3.7 ± 0.4</td>
<td>211.3 ± 19.2</td>
<td>2.47 ± 0.24</td>
</tr>
<tr>
<td>rHSA</td>
<td>3.6 ± 1.7</td>
<td>3.6 ± 1.4</td>
<td>316.4 ± 73.2</td>
<td>1.62 ± 0.86</td>
</tr>
<tr>
<td>RBC</td>
<td>4.5 ± 1.2*</td>
<td>2.7 ± 0.6*</td>
<td>496.5 ± 116.1**</td>
<td>1.08 ± 0.22**</td>
</tr>
<tr>
<td>CO-RBC</td>
<td>3.2 ± 0.7</td>
<td>3.7 ± 0.7</td>
<td>293.8 ± 14.8</td>
<td>1.78 ± 0.16</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. sham.

**P < 0.01 vs. sham.
great significance from the viewpoint of achieving a safe and effective drug therapy during a massive hemorrhage. Such protective effects of CO-RBCs against hepatic P450 isoforms might be exerted in pathologic situations involving not only systemic hemorrhagic shock but also various local ischemia, such as liver transplantation, in which P450-metabolizing immunosuppressive agents (e.g., tacrolimus and cyclosporine) are frequently used (Tamura et al., 1997; Abu-Amara et al., 2010). Because the results reported here involved the use of a rat model of hemorrhagic shock, it is not entirely clear that the results would be directly applicable to humans. However, similar results were reported for human subjects by Harbrecht et al., 2005, such as changes in the activities of P450 isoforms (CYP2C9, 2C19, and 2E1) and hepatic injury in patients with RBC transfusions. Therefore, this shared aspect between human and animal models supports the hypothesis that the results reported here may well be applicable to humans.

Another finding of note in the present study was the early manifestation of the effect of CO, at 1 hour after resuscitation. Ischemia-reperfusion injury in the liver has been demonstrated to show a biphasic pattern, consisting of both acute- and subacute-phase responses (Izuishi et al., 2000; Eum and Lee, 2004; Aitken and Morgan, 2007). The former is characterized by hepatocellular injury after 3–6 hours of reperfusion, and the latter peaks after 18–24 hours of reperfusion. In ischemic rats, hepatic P450 levels were significantly decreased after both 5 and 24 hours of reperfusion. However, no information is available concerning the effect of hepatic P450 at the more acute phase of ischemia-reperfusion, such as at 1 hour after reperfusion. Present findings clearly show that, at least 1–24 hours after RBC resuscitation, the hepatic P450 levels of all isoforms and their activities were significantly decreased by RBC transfusion-induced ischemia-reperfusion during hemorrhagic shock, and this was effectively attenuated by the CO-RBC treatment. Therefore, it would be expected that CO-RBC treatment would also exert a protective effect against hepatic P450 levels and activity during the acute and subacute phase of ischemia-reperfusion.

Details of the mechanism by which CO-RBCs ameliorate hepatic P450 content induced by ischemia-reperfusion are currently unclear.
There are two possibilities that explain the decrease in P450 expression after RBC transfusion: (1) degradation of P450 enzymes by ischemia-reperfusion and (2) inhibition of the biosynthesis of P450 enzymes by ischemia-reperfusion. There is now a substantial body of evidence to support the notion that many of the pathophysiological events triggered by ischemia-reperfusion injury are mediated through the production of reactive oxygen species (ROS) and to the subsequent secretion of inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Carcillo et al., 2003). Previous studies clearly showed that these cytokines and ROS cause a decrease in P450 expression resulting from an enhancement in P450 degradation (Thelen and Dressman, 2009; Vee et al., 2009; Weaver, 2009). These findings led us to hypothesize that the reduction in P450 expression after RBC resuscitation may be caused by the degradation of P450 enzymes rather than the inhibition of P450 synthesis and that CO-RBC may inhibit P450 degradation via the suppression of ROS production or the expression of IL-6 and TNF-α, because it has been demonstrated that CO suppresses the production of both ROS and inflammatory cytokines (Motterlini and Otterbein, 2010). In fact, at 1 hour after resuscitation, the mRNA levels of each P450 isoform remained relatively unchanged among the sham, rHSA, RBC, and CO-RBC resuscitation groups (data not shown). We plan to investigate this mechanism in more detail in a future study.

Cabrales et al. (2007) and Sakai et al. (2009) prepared CO-RBCs under the same conditions as were used in the present study and compared their resuscitative effect and safety, with those of RBC transfusion with use of a rodent model of 50% hemorrhagic shock. As a result, they concluded that CO-RBCs could be as effective as RBCs in recovering from hemorrhagic shock, in terms of their influence on blood pressure, laboratory parameters, and microhemodynamics, and the treatments suppressed tissue damage induced by reperfusion, an effect not seen in the case of RBC resuscitation. The present experimental conditions also show that CO-RBCs possess the same resuscitative effect as RBCs, including survival and blood gas parameters (Fig. 1;Table 1). However, from a clinical perspective, the toxicity of CO-RBCs is of concern because of the potential for CO poisoning (Weaver, 2009). In this study, no symptoms of CO poisoning were observed with 40% hemorrhagic-shock rats. Similarly, no adverse or toxic effects of CO were evident in the aforementioned study with 50% hemorrhagic-shock hamsters and rats (Cabrales et al., 2007; Sakai et al., 2009). Such little toxicity of CO treatment during ischemia-reperfusion is probably attributable to the rapid elimination of CO. In fact, hemoglobin CO levels in blood, an indicator of CO poisoning, were found to increase transiently just after CO-RBC administration, but then decreased to baseline levels within 1 hour. Judging from these comprehensive findings, we concluded that the risk of CO poisoning due to CO-RBC resuscitation is low and that CO-RBC has the potential for use as a promising resuscitative fluid.

Although our results demonstrated the usefulness of CO-RBC, some limitations must also be taken into account. First, the bleeding volume was fixed at 40% in the present study. In actual clinical settings involving RBC transfusions, more than 40% of the total blood supply is frequently lost, which can necessitate the transfusion of blood to an extent that the entire circulating blood volume is exceeded. For this reason, it will be necessary to assess the resuscitation and hepatic P450 protective effects of CO-RBCs under conditions involving more than a 40% blood loss. Second, the present study did not examine the changes in P450 levels in other organs. Although the majority of P450...
is located in the liver, it is well known that intestinal P450 also plays an important role in the bioavailability of orally administered drugs (Provenzani et al., 2011). Therefore, it will be necessary to investigate the changes in P450 levels in the gastrointestinal tract and evaluate the suppressive effects of CO-RBCs thereafter.

In conclusion, we provide the first demonstration that RBC resuscitation–induced ischemia-reperfusion causes a decrease in the levels of isoforms of hepatic P450 and that this significantly influences the disposition of P450-metabolizing drugs and their pharmacological action, from at least 1–24 hours after resuscitation. In addition, CO-RBCs are as effective as RBCs as a resuscitation fluid for hemorrhagic shock, but also minimize the unfavorable effects of drug therapy associated with RBC-induced ischemia-reperfusion by protecting against decreases in hepatic P450 isoforms. These findings show that CO-RBCs have the potential for serving as a novel blood transfusion medium that improves the limitations associated with the currently used RBC transfusions.

Authorship Contributions

Participated in research design: Ogaki, Taguchi, Otagiri, Maruyama.
Conducted experiments: Ogaki, Taguchi.
Contributed new reagents or analytic tools: Otagiri, Maruyama.
Performed data analysis: Watanabe.
Wrote or contributed to the writing of the manuscript: Ogaki, Taguchi, Maruyama.

References


Address correspondence to: Toru Maruyama, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: tomaru@gpu.kumamoto-u.ac.jp.