Therapeutic Potential of Pegylated Hemin for Reactive Oxygen Species-Related Diseases via Induction of Heme Oxygenase-1: Results from a Rat Hepatic Ischemia/Reperfusion Injury Model

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ABSTRACT

Many diseases and pathological conditions, including ischemia/reperfusion (I/R) injury, are the consequence of the actions of reactive oxygen species (ROS). Controlling ROS generation or its level may thus hold promise as a standard therapeutic modality for ROS-related diseases. Here, we assessed heme oxygenase-1 (HO-1), which is a crucial antioxidant, antiapoptotic molecule against intracellular stresses, for its therapeutic potential via its inducer, hemin. To improve the solubility and in vivo pharmacokinetics of hemin for clinical applications, we developed a micellar hemin by conjugating it with poly(ethylene glycol) (PEG) (PEG-hemin). PEG-hemin showed higher solubility in water and significantly prolonged plasma half-life than free hemin, which resulted from its micellar nature with molecular mass of 126 kDa in aqueous media. In a rat I/R model, administration of PEG-hemin significantly elevated HO-1 expression and enzymatic activity. This induction of HO-1 led to significantly improved liver function, reduced apoptosis and thiobarbituric acid reactive substances of the liver, and decreased inflammatory cytokine production. PEG-hemin administration also markedly improved hepatic blood flow. These results suggest that PEG-hemin exerted a significant cytoprotective effect against I/R injury in rat liver by inducing HO-1 and thus seems to be a potential therapeutic for ROS-related diseases, including I/R injury.

Introduction

All aerobic organisms generate reactive oxygen species (ROS), which seem to be indispensable for signal transduction pathways that regulate cell growth and redox status (Davies, 1995). However, overproduction of these highly reactive metabolites can initiate lethal chain reactions and damage cell integrity and survival (Oda et al., 1989; Davies, 1995), which result in reversible and irreversible tissue injury. ROS are known to be involved in many diseases, for example, microbial infections, inflammation, ischemia/reperfusion (I/R) injury, neurological disorders, Parkinson’s disease, hypertension, and cancer (Maeda and Akaike, 1991; McCord, 2000). Developing therapeutics for these ROS-related diseases by suppressing ROS generation or its levels in the body therefore seems to be a reasonable approach. Indeed, many research groups have used this rationale and...
investigated various antioxidative agents and enzymes, such as superoxide dismutase and catalase (Oda et al., 1989; Muzykantov et al., 1996; Fang et al., 2009b). Inhibitors of the ROS-generating enzyme xanthine oxidase (XO) were also the targets along this line (Miyamoto et al., 1996; Fang et al., 2009a, 2010).

In addition to these enzymes, heme oxygenase-1 (HO-1), the antioxidative, antiapoptotic molecule, has attracted great attention. HO is the key enzyme in heme degradation, which generates biliverdin, carbon monoxide (CO), and free iron (Fe\(^{3+}\)) (Maines, 1988; Fang et al., 2004). Biliverdin is subsequently reduced by cytosolic biliverdin reductase to form bilirubin, a potent antioxidant (Baranano et al., 2002). In addition, reports have shown that CO contributes in regulating vascular tone and exhibits antioxidative, anti-inflammatory, and antiapoptotic properties (Abraham and Kappas, 2008). HO-1 is the inducible form of HO, which is a member of the heat shock protein family (Hsp32), and its expression is believed to be associated with fundamental adaptive and defensive responses to oxidative stress and cell stress (Doi et al., 1999; Fang et al., 2004; Abraham and Kappas, 2008). Therefore, induction of HO-1 may become an effective therapeutic strategy for ROS-related diseases. Among HO-1 inducers, hemin is one of the most potent and has few adverse effects to the host. In fact, hemin is used to treat acute hepatic porphyria in Europe and the United States.

However, the very poor water solubility of hemin makes it difficult to achieve a clinically effective dose and develop an optimal therapeutic protocol. To overcome this drawback, we prepared a water-soluble micellar form of hemin by using the biocompatible polymer poly(ethylene glycol) (PEG) (PEG-hemin). This polymer conjugation resulted in an increased in vivo half-life (t\(_{1/2}\)) and reduced antigenicity, as reported previously (Sawa et al., 2000; Fang et al., 2003), and improves pharmacological efficacy significantly.

I/R injury, a typical ROS-related pathological process, is a major cause of organ damage in many fatal diseases such as cardiac infarction, cerebral ischemia, and thrombosis, as well as in surgical procedures. Many studies reported that ROS, especially superoxide anion radical (O\(_2^−\)), is produced excessively in many tissues, mostly by XO, during I/R injury (McCord, 1985). Under normal conditions most XO is present as xanthine dehydrogenase type D, which has very low O\(_2^−\)-generating activity; however, during ischemia, XO activity rapidly increases by conversion from XO type D to type O, which leads to rapid production of O\(_2^−\) (Roy and McCord, 1983). O\(_2^−\) with highly cytotoxic activity, is converted to H\(_2\)O\(_2\) by superoxide dismutase, and then to hydroxyl radicals in the presence of transition metals (e.g., Fe\(^{3+}\)), if no catalase is available. All of these ROS can readily cross cell membranes and cause oxidative damage to DNA, proteins, and lipids (Halliwell and Gutteridge, 1984; Beckman and Ames, 1997; Berlett and Stadtman, 1997). In addition, O\(_2^−\) can react rapidly with NO and form the more toxic species peroxynitrite (ONOO\(^−\)), which further exacerbates tissue injury or leads to complications. Furthermore, removal of NO by reactions with O\(_2^−\) on the vascular endothelial surface results in vasoconstriction (hypertension) and triggers neutrophil adherence and accumulation, which will exacerbate reperfusion injury (Beckman and Koppenol, 1996; Akaike and Maeda, 2000). All of these data together indicate that ROS are the major cause of I/R-induced tissue injury and subsequent pathological manifestations.

The present study describes the synthesis of PEG-hemin and the physicochemical and biological characterization of the conjugate. In view of the therapeutic potential of this agent for ROS-related diseases, this study also evaluated the cytoprotective effect of PEG-hemin in vivo in a rat liver I/R model.

Materials and Methods

Materials. Hemin was purchased from Sigma-Aldrich (St. Louis, MO). The succinimidyl glutarate derivative of PEG (MEG-50HS), with a mean molecular weight of 5250, was from NOP Co. (Tokyo, Japan). PEG used in this experiment had a molecular weight dispersity index of 1.025. Other chemicals of reagent grade were from Wako Pure Chemicals (Osaka, Japan) and used without additional purification.

Cell Culture. Human hepatocyte Hc cells (DS Pharma Biomedical Co. Ltd, Osaka, Japan) were cultured in CSC Serum-Free Medium (DS Pharma Biomedical Co. Ltd.) at 37°C in a 5% CO\(_2/\)95% air atmosphere.

Animals. Male Wistar rats, 6 to 7 weeks old and weighing between 200 and 230 g, and 6-week-old male ddY mice, weighing between 20 and 25 g, were obtained from Kyudo Inc. (Kumamoto, Japan). All animals were maintained under standard conditions and fed water and murine chow ad libitum. All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Faculty of Pharmaceutical Sciences, Sojo University.

Synthesis of PEG-Hemin. PEG-hemin was synthesized according to the protocol for PEG-zinc protoporphyrin (PEG-ZnP) synthesis described in our previous work (Sahoo et al., 2002), with some modifications. In brief, an aminated derivative of hemin was synthesized by using ethylenediamine, and then succinimidyl PEG was cross-linked to hemin via the amide bond (Scheme 1). The resultant PEG-hemin was characterized by means of a UV spectrophotometer (model UV/Vis-550; Jasco, Tokyo, Japan) and infrared spectrometer (FT/IR-4200; Jasco), as well as dynamic light scattering (DLS) and Sephadex column chromatography, as described below.

Quantification of the Free Amino Group. To determine whether PEG reacted with the amino group introduced into hemin, loss of the primary amino group after the reaction was quantified by the use of fluorescamine, an amino group-reactive fluorescent agent (Stocks et al., 1986). In brief, 2 μM PEG-hemin (hemin equivalent) and diethylaminoheemin (aminated derivative of hemin) were dissolved in deionized water and then reacted with fluorescamine. Fluorescence was detected at 475 nm with excitation at 390 nm. The concentration of free amino groups in PEG-hemin was estimated by using glycine as the standard.

Size Exclusion Chromatography. Size exclusion chromatography was performed with a Sephadex G-100 column, 40 cm (length) × 1.3 cm (diameter) (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), to determine the apparent molecular mass of PEG-hemin. Various globular proteins of known molecular mass were used as reference standards. The mobile phase was 0.25 M NaHCO\(_3\), pH 8.2, and 2.5-ml fractions were collected per tube.

DLS Measurement of PEG-Hemin Particle Size. DLS measurement was performed with the Phtotal DLS-7000 HLs laser spectrophotometer (Otsuka Electronics, Osaka, Japan), equipped with a 10-mW He/Ne laser, at a wavelength of 632.8 nm. The scattering angle was fixed at 90°, and the temperature was at 25°C ± 0.05°C. Particle size was determined with 0.1 mg/ml samples prepared in deionized water (filtered through a 0.45-μm filter).

In Vivo Pharmacokinetics of PEG-Hemin. ddY mice were used in the determination of PEG-hemin plasma t\(_{1/2}\) area under the concentration versus time curve (AUC), and total body clearance. PEG-hemin (dissolved in physiological saline) or free hemin (dissolved in 0.01 M NaOH with 10% dimethyl sulfoxide) was injected intravenously at 10 mg/kg (hemin equivalent). After scheduled in-
tervals, mice were killed and blood was collected in the presence of heparin, so that plasma could be obtained after centrifugation at 5000g for 20 min at 4°C. Then, 1.8 ml of ethanol with 0.25 M HCl was added to 0.2 ml of plasma to extract PEG-hemin or hemin. After vigorous vortexing and centrifugation at 20,000g for 20 min, supranatant UV spectra between 300 and 600 nm were measured, and peak absorbance at 385 nm was used to calculate the concentration of hemin according to the standard curve of hemin.

In Vitro Cytotoxicity Assay of PEG-Hemin. Human hepatocytes (Hc) were plated at 3000 cells/well in a 96-well plate (NUNC A/S, Roskilde, Denmark). After overnight preincubation, different concentrations of PEG-hemin were added to the cells. After an additional 48-h incubation, cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Dojindo Laboratories, Kumamoto, Japan).

Induction of HO-1 Expression in Human Hepatocytes by PEG-Hemin. To investigate the HO-1-inducing activity of PEG-hemin, HO-1 expression in human Hc was determined with or without PEG-hemin treatment. Hc cells were plated in six-well culture plates (50,000 cells/well), incubated overnight at 37°C, and treated with the indicated concentrations of PEG-hemin for 24 h. Cells were then collected, and total RNA and protein were extracted by using TRizol reagent (Invitrogen, Carlsbad, CA) and CelLytic MT (Sigma-Aldrich) reagent, according to the manufacturers’ instructions. Reverse transcription-PCR was performed for quantification of HO-1 copies in Hc cells. Primers used for PCR were as follows: HO-1 antisense 21-mer, 5'-GATGTTGAG-GTGAGGAGTCCACCC-3', and sense 26-mer, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3', to obtain a 555-bp HO-1 cDNA (nucleotides 79–633); and GAPDH antisense 24-mer, 5'-CATGTTGGCCATGAGGTCCACCAC-TG-3', and sense 26-mer, 5'-CATGTTGGCCATGAGGTCCACCAC-TG-3', to obtain a 983-bp GAPDH cDNA fragment. After an initial denaturing step at 94°C, 25 PCR cycles (30 cycles for GAPDH) were performed as follows: denaturing for 1 min at 94°C, primer annealing for 1 min at 56°C, and DNA synthesis for 1 min at 72°C. PCR products then underwent electrophoresis on ethidium bromide-stained 1% agarose gels.

Experimental Protocol for Hepatic I/R Injury. Rats were fasted overnight before the experiment but were allowed free access to water. Animals were anesthetized with isoflurane during the operation by using an anesthesia system (SF-B01; DS Pharma Biomedical Co. Ltd.). After the abdomen was shaved and disinfected with 70% ethanol, a complete midline incision was performed. The portal vein and hepatic artery were exposed and clamped for 30 min with a noncrushing microvascular clip. Subsequently, reperfusion was initiated by removing the clips, and the abdomen was closed in two layers with 2-0 silk. Rats were kept warmed until they awoke and became active. Two hours before surgery for I/R, saline or PEG-hemin at different doses (2 or 10 mg/kg) was injected into each rat via the tail vein. In some experiments, a polymeric micellar HO-1 inhibitor, ZnPP, encapsulated with styrene maleic acid (SMA) copolymer, namely SMA-ZnPP (Iyer et al., 2007), was administered intravenously (5 mg/kg) just before I/R.

Measurement of Liver Enzyme Activity in Serum. Three hours after reperfusion resumed, rats were killed under anesthesia, and whole blood was withdrawn from the inferior vena cava. Activities of alanine aminotransferase (ALT), aspartate aminotransferase, and bilirubin reductase, 33 M hemin, and 333 M NADPH in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, plus 290 mM sucrose, 2 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, and 10 μM leupeptin (Merck, Darmstadt, Germany). Homogenates were centrifuged at 10,000g for 30 min at 4°C, and the resultant supernatant was ultracentrifuged at 105,000g for 1 h at 4°C. The microsomal fraction in the precipitates was suspended in 0.1 M potassium phosphate buffer, pH 7.4, followed by sonication for 2 s at 4°C.

Supernatant of the 10,000g fraction, with 50 μg of protein in each sample, was used for analysis of HO-1 expression by Western blot. In brief, total protein was separated by electrophoresis with 12% SDS-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA). This process was followed by reaction with a monoclonal antibody for HO-1 (GTS-1; Takara Bio Inc., Otsu, Shiga, Japan). Activities were expressed as IU/l.

Measurement of HO-1 Expression and Activity in Rat Liver after I/R. Liver tissues collected from rats receiving the above-described treatment with PEG-hemin or no treatment were homogenized with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) in ice-cold homogenizing buffer (20 mM potassium phosphate buffer, pH 7.4, plus 250 mM sucrose, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin). Homogenates were centrifuged at 10,000g for 30 min at 4°C, and the resultant supernatant was ultracentrifuged at 105,000g for 1 h at 4°C. The microsomal fraction in the precipitates was suspended in 0.1 M potassium phosphate buffer, pH 7.4, followed by sonication for 2 s at 4°C.

The microsomal fraction was used for measurement of HO-1 activity. The HO reaction mixture consisted of the microsomal fraction (1 mg of protein), cysteine fraction (supernatant after ultracentrifugation described above) of rat liver (1 mg of protein) as a source of biliverdin reductase, 33 μM hemin, and 333 μM NADPH in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4. The mixture was incubated for 15 min at 37°C, after which the reaction was terminated by addition of 33 μl of 0.01 M HCl. Bilirubin formed in the reaction was extracted with 1.0 ml of chloroform, and bilirubin concentration was measured by means of the AutoAnalyzer system (Hitachi Ltd., Tokyo, Japan) activities were expressed as IU/l.
determined spectroscopically by measuring the difference in absorbance between 465 nm (absorbance of bilirubin) and 530 nm (background), with a molar extinction coefficient of 40 mM⁻¹ cm⁻¹ at 465 nm.

Moreover, HO-2 expression in this experimental protocol was measured by reverse transcription-PCR. In brief, total RNA of each liver tissue was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Primers used for PCR were as follows: antisense, 5'-AGTAAAGTCAGTGGTGCGCC3', and sense, 5'-CAGCAACAATGGTCCATGAGG-3' to obtain a 230-bp HO-2 cDNA. After an initial denaturing step at 94°C, 35 PCR cycles were performed as follows: one cycle of denaturing for 2 min at 92°C, primer annealing for 1 min at 54°C, and DNA synthesis for 1 min at 72°C. PCR products then underwent electrophoresis on ethidium bromide-stained 1% agarose gels.

Quantification of CO in Blood. After the I/R procedure just described, rats were killed and blood was collected. Each 0.35-ml sample of blood was diluted with 3.65 ml of phosphate-buffered saline and placed in a 10-ml glass test tube on ice. The test tubes were then sealed, and the air in the tubes was replaced by purging nitrogen gas into the tubes, after which the NO donor 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine was added, to a final concentration of 1 mM. Because NO has a much higher affinity than CO for hemoglobin, CO bound to hemoglobin would be liberated. After 2 h of incubation at room temperature, 1 ml of the gas in the test tubes was collected and processed in a gas chromatography CO analyzer (TRIlyzer mBA-3000; TAIYO Instruments, Inc., Osaka, Japan) equipped with a semiconductor gas sensor.

Preparation of Liver Tissue Sections for Histological Examination and Detection of Apoptosis. Rat livers were collected 3 h after I/R and cut into small tissue blocks (approximately 3–5 mm in length, width, and height). After the blocks were fixed with 6% buffered neutral formalin solution, they were embedded in paraffin. Paraffin-embedded sections (6 µm thick) were prepared as usual for histological examination after hematoxylin and eosin staining and for apoptosis staining (TUNEL) as described below.

In Situ Detection of Apoptosis in the Liver. TUNEL staining was used to investigate apoptosis in the paraffin-embedded sections described above, after I/R with or without PEG-hemin treatment, with an in situ apoptosis detection kit (TACS; Trevigen, Gaithersburg, MD), according to the manufacturer’s instructions. TUNEL-positive cells in four different fields per sample were counted, and results were expressed per mm² of tissue section.

Detection of Caspase 3/7 Activities in the Liver. Apoptosis in the livers of different treatment groups were further examined in their caspase 3/7 activities, with a caspase assay kit that contains the substrate peptide DEVD (Caspase-Glo 3/7 Assay; Promega, Madison, WI). In brief, the liver tissue homogenates were prepared by using 1 g of wet tissue added with 4 ml of hypotonic extraction buffer (25 mM HEPES buffer, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin) with the Polytron homogenizer (Kinematica). Homogenates were centrifuged at 10,000g for 30 min at 4°C, and the supernatant was used for caspase activity assay according to the manufacturer’s instructions.

Measurement of Liver Tissue Blood Flow. A laser Doppler flowmeter (ALF21; Advance Co. Ltd., Tokyo, Japan) was used to measure liver tissue blood flow in anesthetized animals during I/R (until 1 h after initiating reperfusion) with or without PEG-hemin treatment (10 mg/kg i.v.). The flowmeter probe was inserted at the same site in the median lobe of the liver in each animal. The real-time change in blood flow was monitored, and the mean blood flow at specific time points was calculated and expressed as a percentage of the preischemic initial blood flow value as the control.

Thiobarbituric Acid-Reactive Substance Assay. Oxidative cell damage in the liver after I/R with or without PEG-hemin treatment was quantified by assay of lipid peroxide formation via the thiobarbituric acid reaction (Ohkawa et al., 1979). In brief, liver tissue homogenates were prepared at a ratio of 1 g of wet tissue to 9 ml of 1.15% KCl in a Polytron homogenizer. Then, 0.1 ml of homog-
Plasma t1/2 of PEG-Hemin. We expected the macromolecular micellar formulation of PEG-hemin to have superior in vivo pharmacokinetics (e.g., prolonged t1/2), and we investigated this possibility by using ddY mice. Compared with free hemin, PEG-hemin had a relatively long circulation time: even 20 h after intravenous injection, approximately 30% remained in circulation; almost no hemin was detected 2 h after administration (Fig. 3). The t1/2 of PEG-hemin was calculated to be 18.2 h, which is approximately 20 times longer than that of hemin (0.96 h); in parallel, the AUC of PEG-hemin increased to 16 times of that of free hemin, and the total body clearance of PEG-hemin was approximately 16 times slower than that of free hemin (Table 1).

Amelioration of Liver I/R Injury by PEG-Hemin. The potential therapeutic or tissue protective effect of PEG-hemin against I/R injury of the liver was evaluated by measuring plasma concentrations of the liver enzymes AST, ALT, and LDH. In our previous study, we reported that these enzymes increased to a maximum level at 3 h after reperfusion (Ikebe et al., 2000); thus, in this study all experiments were performed at 3 h after reperfusion. As anticipated, I/R caused a large increase in the levels of all three liver enzymes, to more than 20 times of normal levels. AST, ALT, and LDH values for I/R versus those for normal rat were as follows: 2647.4 ± 460.4 IU/l versus 65.3 ± 0.7 IU/l, 1356.4 ± 182.1 IU/l versus 54.3 ± 1.2 IU/l, and 6932.6 ± 1131.0 IU/l versus 62.3 ± 3.4 IU/l, respectively (Fig. 6A).

More important, PEG-hemin treatment, at 10 mg/kg (hemin equivalent), significantly lowered the elevated levels of AST, ALT, and LDH, with liver enzyme levels almost recovering to normal (Fig. 6A). Furthermore, this cytoprotective effect of PEG-hemin was almost nullified by administration of SMA-ZnPP, which is a macromolecular water-soluble HO inhibitor that we had prepared previously (Iyer et al., 2007) (Fig. 6A). These results suggest that the cytoprotective effect of PEG-hemin was through an HO-1-mediated pathway, as

![Fig. 1. IR spectra of hemin and PEG-hemin. Amide I (1654 cm⁻¹) and amide II (1542 cm⁻¹) were clearly observed in PEG-hemin, which suggested an amide bond formation between hemin and PEG through bis-(ethylenediamino) hemin.](image-url)
One major reason for liver tissue injury by I/R is reduced blood flow, as clearly seen in Fig. 7, which shows that hepatic blood flow decreased to 80% of normal at 1 h after reperfusion and recovered to only 20% of normal at 20 min after ischemia and recovered to only 30% of normal at 1 h after reperfusion was initiated. The tissue-protective effect of PEG-hemin treatment largely increased hepatic blood flow, which reached 80% of normal at 1 h after the start of reperfusion (Fig. 7).

Apoptosis in the Liver after I/R. The TUNEL assay for apoptosis allowed further elucidation of the pathological events caused by I/R with or without PEG-hemin treatment. I/R clearly induced apoptosis at 3 h after reperfusion, whereas PEG-hemin significantly lowered the number of apoptotic cells in the liver after I/R (Fig. 8A), findings that correlated well with the liver enzyme profiles. This was further supported by caspase 3/7 activity assay, by which the caspase 3/7 activities were found to be remarkably increased in the livers of I/R, whereas it was significantly inhibited by PEG-hemin (Fig. 8B).

Oxidative Injury Induced by I/R in the Liver. To investigate the role of ROS in liver I/R injury, the TBARS assay, which is a standard method of detecting oxidative injury of tissue involving lipid peroxidation, was performed. As Fig. 9 shows, I/R greatly increased the TBARS level in the rat liver. This increase was markedly inhibited by PEG-hemin treatment in a dose-dependent manner (Fig. 9): at 10 mg/kg PEG-hemin, the TBARS value was almost normal, which suggests that liver injury caused by I/R involved the increased generation of ROS. Similar results were obtained when 8-OHdG, which is a common index for oxidative injury of DNA, was detected in the liver of rat after I/R procedure with or without PEG-hemin treatment. These findings indicate the involvement of ROS in I/R injury and the protective effect of PEG-hemin in this process.

Change in Serum Inflammatory Cytokine MCP-1 Level after I/R With or Without PEG-Hemin. Inflammation is a major consequence of ROS-related diseases and thus plays a crucial role in the pathological process of I/R. Among the many inflammatory cytokines known to be involved in I/R injury, MCP-1, a proinflammatory chemokine and key mediator in the inflammatory process, is strongly associated with ROS-induced pathological conditions including I/R injury (Melgarejo et al., 2009). We thus measured the levels of MCP-1 in rat serum after I/R with or without PEG-hemin administration. Figure 10 shows a significantly increased serum MCP-1 level at 3 h after I/R. However, PEG-hemin treatment before I/R markedly inhibited this increase, which was consistent with the liver function results, as well as necrosis and apoptosis in liver tissue.

Discussion

During the past few decades, the clinical importance of ROS has been discussed extensively, and development of drugs to control the generation of ROS or scavenge ROS has become a focus of great interest (Maeda and Akaike, 1991; McCord, 2000). One approach is to block ROS generation. We in fact developed an XO inhibitor, 4-amino-6-hydroxyypyrazolo[3,4-d]pyrimidine, and its polymer conjugate to inhibit O$_2^-$ production by XO (Miyamoto et al., 1996; Fang et al., 2009a, 2010). An alternative approach that has attracted our

### Table 1

<table>
<thead>
<tr>
<th>Agent</th>
<th>$t_{1/2}$</th>
<th>AUC</th>
<th>Total Body Clearance</th>
</tr>
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<tbody>
<tr>
<td>Hemin</td>
<td>0.96</td>
<td>203.3</td>
<td>49.2</td>
</tr>
<tr>
<td>PEG-hemin</td>
<td>18.25</td>
<td>3289.3</td>
<td>3.0</td>
</tr>
</tbody>
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Pharmacokinetic parameters of free hemin and PEG-hemin. Plasma $t_{1/2}$ indicates time required to reach to half-concentration at time zero by interpolation.
attention is to take advantage of the products of HO-1, which is a major antiapoptotic antioxidative molecule (Maines, 1988; Fang et al., 2004; Abraham and Kappas, 2008). Induction of HO-1 either by its inducers such as cobalt protoporphyrin and hemin or by HO-1 gene transfer showed beneficial effects in many ROS-related diseases (Fang et al., 2004; Abraham and Kappas, 2008). We followed this approach by using a modified hemin and developed a macromolecular micellar hemin formulation to improve its water solubility and in vivo pharmacokinetics.

We used PEG, which is a widely used biocompatible polymer, to prepare hemin micelles. Consistent with our previous reports of PEG-ZnPP (Sahoo et al., 2002; Fang et al., 2003), which is chemically similar to PEG-hemin, PEG modification of hemin led to markedly increased solubility (more than 15 mM; hemin equivalent) in physiological solutions, in which free hemin can be hardly solubilized. More important, PEG-hemin exhibited a very long circulation time: its $t_{1/2}$ in ddY mice was approximately 18 h, which was approximately 20 times longer than that of free hemin (Fig. 3; Table 1). Furthermore, PEG-hemin had no or very little toxicity; PEG-hemin treatment, up to 250 µg of PEG-hemin (µM) produced no cell death (Fig. 4A). Its safety was also verified in vivo: injection of 100 mg/kg i.v. PEG-hemin into ddY mice did not cause the death of the mice or any apparent side effects such as loss of body weight and change in blood biochemistry (data not shown). These findings warrant further clinical applications of PEG-hemin.

The improved water solubility and plasma $t_{1/2}$ are caused by the micellar formulation of PEG-hemin, which had an apparent molecular mass of 126 kDa in aqueous solution as judged by Sephadex G-100 column chromatography (Fig. 2A). This PEG-hemin presents as an aggregated micellar form, as observed by DLS, which showed a mean particle size of 121.5 nm in physiological solution (Fig. 2B). Macromolecular drugs
The macromolecular PEG-hemin micelles developed on the basis of the EPR effect and are now not only at the laboratory stage of development but also in the clinical stage of development (Maeda, 1986; Maeda et al., 2000, 2001; Duncan, 2003; Fang et al., 2011). Many macromolecular drugs have been enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Matsumura and Maeda, 2004; Abraham and Kappas, 2008). Heme and free iron are pro-oxidants that would induce or enhance ROS generation (Akaike et al., 1992; Jeney et al., 2002), whereas bilirubin, which is a major product of heme degradation, is one of the most abundant endogenous antioxidants in mammals and accounts for the major antioxidative activity in human serum (Baranano et al., 2002).

Mechanisms involved in the tissue-protective and therapeutic effects of PEG-hemin were thought to rely mostly on the antioxidative role of HO-1, which would take advantage of the enzyme reactions in heme catabolism (Fang et al., 2004; Abraham and Kappas, 2008). Heme and free iron are pro-oxidants that would induce or enhance ROS generation (Akaike et al., 1992; Jeney et al., 2002), whereas bilirubin, which is a major product of heme degradation, is one of the most abundant endogenous antioxidants in mammals and accounts for the major antioxidative activity in human serum (Baranano et al., 2002).

More recently, CO generated during heme degradation is also believed to account for the major, if not all, biological properties of HO-1. HO-catalyzed heme catabolism is the major source of CO (i.e., more than 80%) in the mammalian system, and the roles of CO include, similarly to NO, regulating vascular tone (vasorelaxant), participating in antiapoptosis, serving an antioxidative, anti-inflammatory function, and inhibiting the activation of monocyte/macrophages, inducing angiogenesis, and others (Abraham and Kappas, 2008). In our present experiment, we found the CO concentration in circulation increased after PEG-hemin treatment (Fig. 5C), which was paralleled with the improved hepatic
blood flow (Fig. 7) and decreased apoptosis in the liver in the I/R process (Fig. 8), even though we did not show direct evidence of its role on the cytoprotective effect against I/R injury. However, a recent study by Wei et al. (2010) demonstrated a direct effect of CO on I/R injury by using a CO-releasing molecule, although the effect was less significant, probably because of the short in vivo $t_{1/2}$ of CO-releasing agent.

In addition to HO-1 (the inducible enzyme), the constitutive form of HO, namely HO-2 is a major source of HO activity in most tissues. Both are alike in terms of enzymatic mechanisms of heme degradation and cofactor and substrate specificity (Maines, 1988; Abraham and Kappas, 2008). The HO activities in liver tissues measured in this study (Fig. 5B) may thus include HO-2 activity as well. Unlike HO-1, whose expression is relatively low in most normal tissues unless exposed to various stresses, such as I/R injury and hemin treatment as shown in this study (Fig. 5), HO-2 displays, in general, a constitutive expression in many normal tissues but it is not up-regulated upon stress or injury, which was also confirmed in this study (Supplemental Fig. 1). HO-2 is thus believed to contribute to normal housekeeping. And it has been reported that HO-2 behaves as a basal tone of anti-inflammatory signals, and deletion of HO-2 disables execution of the acute inflammatory and reparative response, leading to an exaggerated inflammatory response including increased oxidative stress and angiogenesis (Seta et al., 2006; Bellner et al., 2009). Moreover, it is known that HO-2 is induced by the treatment of corticosteroids (Maines et al., 1996), which is an important protective response during acute illness or stress, and is widely used as a treatment of inflammatory diseases. Thus, HO-2, along with HO-1, may also play important role as a protective response against I/R and inflammatory distress, facilitating the therapeutic response induced by PEG-hemin, which warranted further investigations.

I/R injury is a typical ROS-related inflammatory disorder, in which $O_2^-$, as a highly reactive radical, reacts with and oxidizes many biological molecules including NO (McCord, 1985; Maeda and Akaike, 1991; Ikebe et al., 2000) and thereby can trigger cell death. These pathological roles were clearly demonstrated in the present study using the I/R procedure, as evidenced by increased levels of the inflammatory cytokine MCP-1 (Fig. 10), elevated TBARS and 8-OHdG (Fig.
cause PEG-hemin itself demonstrated no or very little toxicity (Fig. 4A).

In addition, many disorders other than I/R injury are associated with ROS including inflammation, hypertension, and bacterial and viral infections (Maeda and Akaike, 1991; McCord, 2000). Therefore PEG-hemin may be applicable for the treatment of these diseases as well.

In conclusion, findings from the present study demonstrated that the macromolecular, water-soluble, micellar form of HO-1 inducer PEG-hemin exerted a potent cytoprotective effect against I/R injury of the liver in rats, as evidenced by low serum liver enzyme values (Fig. 6A), decreased numbers of apoptotic cells in the liver (Fig. 8), increased hepatic blood flow (Fig. 7), and reduced inflammatory cytokine levels in serum (Fig. 10). The cytoprotective effects of PEG-hemin can be attributed to augmented HO-1 activity (e.g., increased CO production, as Fig. 5C showed) through its antiapoptotic, antioxidative actions, because the HO-1 inhibitor SMA-ZnP almost completely nullified the effect of PEG-hemin (Fig. 6A). This finding was also supported by the increased HO-1 mRNA and protein expression and elevated CO levels in blood and HO-1 enzyme activity (Figs. 4B and 5) and reduced tissue peroxidation in the liver after PEG-hemin treatment (Fig. 9). Therefore, the data presented herein suggest a therapeutic potential of PEG-hemin for I/R injury, and other ROS-related diseases, such as inflammatory disorders and hypertension, so PEG-hemin warrants further investigation as a therapeutic agent.

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Authorship Contributions

Participated in research design: Fang and Maeda.

Conducted experiments: Fang, Qin, Seki, Nakamura, and Tsukigawa.

Wrote or contributed to the writing of the manuscript: Fang, Qin, Shin, and Maeda.

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Therapeutic potential of pegylated hemin for ROS-related diseases via induction of heme oxygenase-1: results from a rat hepatic ischemia/reperfusion injury model

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**Figure 1.** Expression of HO-2 in liver tissues of I/R with or without PEG-hemin treatment. I/R protocol and PEG-hemin treatment are same as that shown in Fig. 6. RT-PCR was performed for HO-2 mRNA expression. See “Materials and Methods” for details.