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Hepatocyte-specific Distribution of Catalase and Its Inhibitory Effect on Hepatic Ischemia/Reperfusion Injury in Mice

YOSHIYUKI YABE, YASUO KOYAMA, MAKIYA NISHIKAWA, YOSHINOBU TAKAKURA and MITSURU HASHIDA*

Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

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To explore the possibility of using catalase for the treatment of reactive oxygen species (ROS)-mediated injuries, the pharmacokinetics of bovine liver catalase (CAT) labeled with ^{111}In was investigated in mice. At a dose of 0.1 mg/kg, more than 70% of ^{111}In-CAT was recovered in the liver within 10 min after intravenous injection. In addition, ^{111}In-CAT was predominantly recovered from the parenchymal cells (PC) in the liver. Increasing the dose retarded the hepatic uptake of ^{111}In-CAT, suggesting saturation of the uptake process. This cell-specific uptake could not be inhibited by coadministration of various compounds which are known to be taken up by liver PC, indicating that the uptake mechanism of CAT by PC is very specific to this compound. The preventive effect of CAT on a hepatic ischemia/reperfusion injury was examined in mice by measuring the GOT and GPT levels in plasma. A bolus injection of CAT at 5 min prior to the reperfusion attenuated the increase in the levels of these indicators in a dose-dependent manner. These results suggest that catalase can be used for various hepatic injuries caused by ROS.

Keywords: Bovine liver catalase, pharmacokinetics, hepatic uptake, liver, reactive oxygen species, ischemia/reperfusion

INTRODUCTION

Reactive oxygen species (ROS) produced in excess can induce oxidative damage to cells and their production is amplified by pathological events such as neutrophil activation, hyperoxia, metabolism of redox-active drugs, radiation exposure and ischemia–reperfusion treatment. Antioxidant enzymes, therefore, have been considered as therapeutic agents for ROS-mediated injuries and diseases. However, Cu^{2+}-, Zn^{2+}-superoxide dismutase (SOD) and catalase, representative antioxidant enzymes catabolizing superoxide anion and hydrogen peroxide, respectively, have been reported to be rapidly eliminated from the circulation after intravenous bolus injection.[1–3] To increase the plasma half-life of these enzymes, macromolecules such as polyethylene glycol derivatives have been attached to them.[1,4,5] Kawamoto *et al.*[6] also developed a SOD

*Corresponding author. Tel.: +81-75-753-4525. Fax: +81-75-753-4575. E-mail: hashidam@pharm.kyoto-u.ac.jp.

derivative, poly(styrene comaleic acid butyl ester)-linked SOD, which has a high affinity for serum albumin. These chemical modifications improved the plasma retention and biological activities of these enzymes.

Targeted delivery of antioxidant enzymes to sites where ROS are generated should be a promising way of treating ROS-mediated injuries. Since SOD has a molecular weight of about 32 kDa and shows little interaction with any tissues and organs,[7] targeting of this enzyme can be achieved by the introduction of ligands for receptors or by the alteration of its physicochemical properties. In a series of investigations, we synthesized various SOD derivatives and evaluated their *in vivo* disposition and efficacy. Galactosylated and mannosylated SODs showed inhibitory effects superior to unmodified SOD against a hepatic ischemia/reperfusion injury.[8] In contrast, cationized SOD exhibited a significant therapeutic effect which corresponded to a ten-fold higher dose of native SOD on an ischemia/reperfusion injury in rat kidneys.[9] These therapeutic results were considered to correlate with the altered biodistribution characteristics of SOD, since galactosylated and mannosylated SODs were selectively taken up by liver parenchymal cells (PC) and nonparenchymal cells (NPC), respectively,[7] and cationized SOD was taken up by kidneys from their capillary sides.[10]

Although attempts have already been made to improve the plasma retention of catalase after intravenous injection[3,4] or to increase its affinity for target cells,[11–15] the biodistribution and clearance mechanisms of unmodified catalase are poorly understood. A knowledge of the *in vivo* fate is essential for applying catalase to ROS-mediated injuries as well as for manipulating its biodistribution by chemical modification. In this study, therefore, we investigated the *in vivo* disposition characteristics of bovine liver catalase (CAT) after intravenous injection in mice. The results obtained were analyzed pharmacokinetically to examine its *in vivo* disposition properties, especially hepatic uptake. Furthermore, to clarify the mechanism of its marked accumulation in the liver, the characteristics of its hepatic uptake were examined by coadministration with compounds known to show extensive uptake by the liver. The inhibitory effect of CAT on a hepatic ischemia/reperfusion injury was also examined in mice to elucidate the relationship between its *in vivo* disposition and therapeutic effect.

MATERIALS AND METHODS

Animals

Male ddY mice (5 weeks-old, 25–28 g; 10 weeks-old, 40–50 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. Animals were maintained under conventional housing conditions. Ten weeks-old mice were used in the hepatic ischemia/reperfusion experiment.

Chemicals

CAT, bovine serum albumin (BSA), human transferrin, human milk lactoferrin, horse spleen ferritin, bovine hemoglobin, horse skeletal muscle myoglobin, porcine liver uricase, and collagenase (type IA) were purchased from Sigma Chemical, St. Louis, MO, USA. The molecular weight of CAT was measured by gel filtration chromatography using a G4000SW$_{XL}$ column (Tosoh, Tokyo, Japan) and only the fractions containing a tetramer of the subunits with molecular weight of about 240 kDa were used in the experiments. Galactosylated BSA (Gal-BSA) was synthesized by reacting BSA with 2-imino-2-methoxyethyl 1-thiogalactoside as previously described.[16] Diethylenetriaminepentaacetic acid (DTPA) anhydride was purchased from Dojindo Laboratory, Kumamoto, Japan. ^{111}Indium chloride ([^{111}In]InCl$_3$) was supplied by Nihon Medi-Physics Co., Takarazuka, Japan. All other chemicals were of the finest grade available.

Radiolabeling of CAT

CAT was radiolabeled with ^{111}In using the bifunctional chelating agent, DTPA anhydride, according to the method of Hnatowich *et al.*[17] In brief, CAT (2 mg) was dissolved in 1 ml of 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid (HEPES) buffer (0.1 M, pH 7.0) and a two-fold molar excess of DTPA anhydride in 10 µl of dimethylsulfoxide was added. After stirring for 30 min at room temperature, the mixture was purified by gel-filtration chromatography using a Sephadex G-25 column (1 × 40 cm) and eluted with acetate buffer (0.1 M, pH 6.0) to separate the unreacted DTPA. Fractions containing DTPA-coupled CAT were selected by spectrophotometry and concentrated by ultrafiltration. To 30 µl of sodium acetate buffer, an equivalent volume of $^{111}InCl_3$ solution was added, then 60 µl of DTPA-coupled CAT solution (about 1 mg/ml) was added to the mixture. After 30 min, the mixture was purified by gel filtration chromatography using PD-10 column (Pharmacia, Uppsala, Sweden) and eluted with acetate buffer (0.1 M, pH 6.0). The appropriate fractions were collected based on their radioactivity and concentrated by ultrafiltration. This radiolabeling procedure did not significantly decrease the enzymatic activity of CAT and more than 90% enzymatic activity was retained. ^{111}In-CAT was used only in disposition studies, not in the ischemia/reperfusion experiments.

In Vivo Disposition Experiment

^{111}In-CAT was dissolved in saline and the protein concentration was adjusted by addition of non-radiolabeled CAT to the solution. The solution of ^{111}In-CAT was injected into the tail vein of mice at a dose of 0.1, 1, or 10 mg/kg. At 1, 3, 5, 10, 30, and 60 min after injection, blood was collected from the vena cava under ether anesthesia and the mice were sacrificed. Plasma was obtained by centrifugation of the blood collected. The kidney, liver, spleen, heart, lung and muscle were removed, rinsed with saline and weighed. Urine in the bladder and excreted urine were also collected. The radioactivity in each sample was counted in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo).

Calculation of AUC and Clearances

The ^{111}In-radioactivity concentration in plasma was normalized to percentage of dose/ml and analyzed using the non-linear least-square program MULTI,[18] and the area under the plasma concentration–time curve (AUC) and total body clearance (CL_{total}) were calculated. The tissue distribution pattern of ^{111}In-CAT was evaluated by the organ uptake clearance (CL_{org}) according to the method previously reported,[19] to examine the pharmacokinetic characteristics of the tissue distribution of ^{111}In-CAT. The change in the amount of ^{111}In-radioactivity in an organ with time can be described as

$$\frac{dX(t)}{dt} = CL_{org}C(t) - K_{out}X(t), \qquad (1)$$

where $X(t)$ (percentage of dose) represents the amount of radioactivity in an organ, $C(t)$ (percentage of dose/ml) is the plasma concentration of radioactivity, CL_{org} (ml/h) denotes the organ uptake clearance from the plasma to the organ, and K_{out} (1/h) is the rate constant for an efflux from the tissue. In this paper the efflux process can be considered to be negligible during the initial phase of the experiment, since ^{111}In-radioactivity remains within tissues for a relatively long period.[26,27] Therefore, the Equation (1) can be rewritten as

$$\frac{dX(t)}{dt} = CL_{org}C(t). \qquad (2)$$

Integration of the Equation (2) gives

$$CL_{org} = \frac{X(t_1)}{\int_0^{t_1} C(t)dt} = \frac{X(t_1)}{AUC_{0-t_1}}, \qquad (3)$$

where t_1 (h) is a sampling time after injection, and AUC_{0-t_1} represents AUC from time 0 to t_1. According to the Equation (3), the organ uptake

clearance is calculated using the amount of radioactivity in the organ at an appropriate interval of time and the area under the plasma concentration–time curve (AUC) up to the same time point. When the uptake process follows non-linear kinetics, CL_{org} value is not constant with time due to the change in the plasma concentration. Then a calculated CL_{org} represents an average value for the overall experimental period.

Hepatic Cellular Localization of ^{111}In-CAT in Mice

Groups of three mice each were injected with ^{111}In-CAT at a dose of 0.1 mg/kg and anesthetized by peritoneal administration of pentobarbital sodium. At 10 min after injection, the liver was perfused from the portal vein first with preperfusion buffer (Ca^{2+}-, Mg^{2+}-free HEPES buffer, pH 7.2) for 10 min and then with HEPES buffer (pH 7.5) containing 5 mM $CaCl_2$ and 0.05% (w/v) collagenase for 20 min. Then, the liver was excised and the cells were dispersed by gentle stirring in ice-cold Hank's-HEPES buffer containing 0.1% BSA. The dispersed cells were filtered through cotton mesh sieves and centrifuged for 1 min at 50*g* to sediment the liver PC. The supernatant was removed and kept as a source of NPC. The pellet of PC was resuspended in buffer and centrifuged again to remove other cells. The NPC suspension was centrifuged twice at 50*g* for 1 min to remove the PC, then subjected to centrifugation at 200*g* for 2 min to obtain the pellet of NPC. Both PC and NPC fractions were resuspended in buffer and the number of cells was determined by the trypan blue exclusion method. The radioactivity of both samples was also determined as in the *in vivo* disposition experiment.

Coadministration of Various Compounds with ^{111}In-CAT to Mice

^{111}In-CAT at a dose of 0.1 mg/kg was injected into mice simultaneously with Gal-BSA, iron-carrying proteins (hemoglobin, myoglobin, transferrin, lactoferrin and ferritin), or uricase at a dose of 20 mg/kg. At 5 min after injection, plasma and liver were sampled and assayed for ^{111}In-radioactivity.

Hepatic Ischemia/Reperfusion Experiment

Mice were anesthetized by peritoneal administration of pentobarbital sodium. An incision was made in the abdomen and the hepatic artery and the portal vein were occluded with a clamp for 30 min. At 5 min before reperfusion, saline (control), BSA (5 mg/kg) or CAT (40,000 units/mg; 10,000–200,000 units/kg) was administered through the tail vein. After 60 min of reperfusion, blood was collected from the vena cava and plasma was obtained in the same manner as above. GPT and GOT activities in plasma were assayed using commercial test reagents (GPT-UV test Wako, GOT-UV test Wako) purchased from Wako pure chemicals, Japan.

RESULTS

In Vivo Disposition of ^{111}In-CAT

Figure 1 shows the plasma concentration- and liver accumulation-time courses of radioactivity following intravenous injection of ^{111}In-CAT into mice at various doses. ^{111}In-CAT was rapidly taken up by the liver after injection at doses of 0.1 and 1 mg/kg. In accordance with this rapid uptake, ^{111}In-CAT was instantly eliminated from plasma. Increasing the dose to 10 mg/kg, however, reduced the uptake rate of ^{111}In-CAT by the liver as well as its elimination rate from plasma. Although the rate of hepatic uptake was slow at this dose, more than 60% of the radioactivity was finally recovered in the liver within 1 h after administration.

Figure 2 shows the tissue distribution and urinary excretion of radioactivity after intravenous injection of ^{111}In-CAT into mice at a dose of 0.1 mg/kg. Except for the liver, all the other

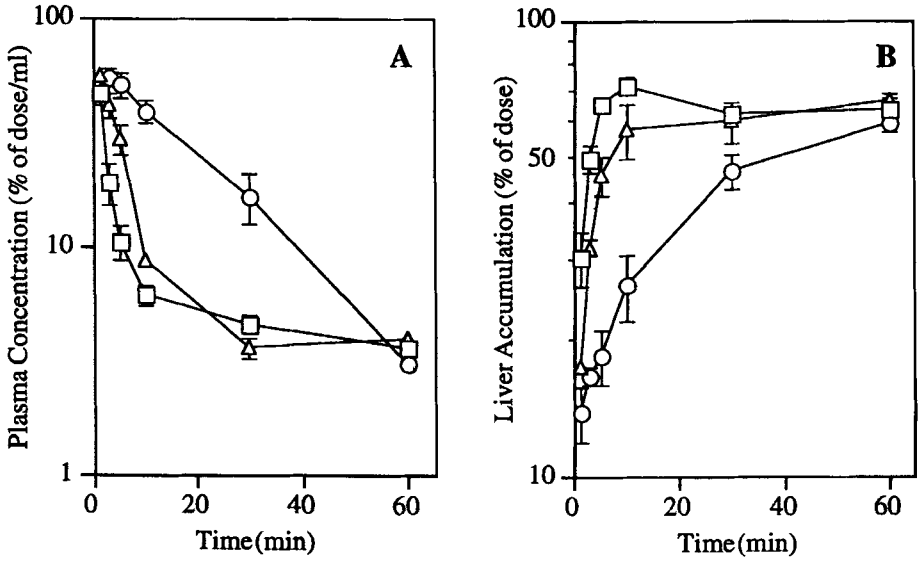

FIGURE 1 (A) Plasma concentration and (B) liver accumulation of ^{111}In-CAT after intravenous injection into mice at doses of 0.1 mg/kg (□), 1 mg/kg (△), or 10 mg/kg (○). Results are expressed as the mean ± SD of three mice.

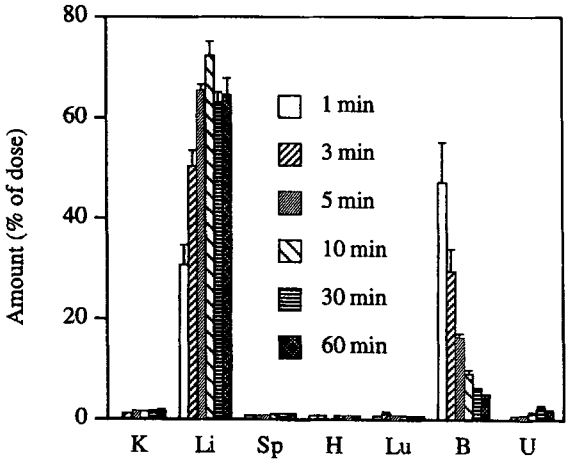

FIGURE 2 Tissue distribution of ^{111}In-CAT after intravenous injection into mice at a dose of 0.1 mg/kg. Results are expressed as the mean ± SD of three mice. Abbreviations: K, kidney; Li, liver; Sp, spleen; Lu, lung; M, muscle; U, urine.

samples contained very little radioactivity at any time point investigated. At different doses (1 and 10 mg/kg), similar profiles of tissue distribution were observed (data not shown).

Pharmacokinetic Analysis of ^{111}In-CAT

Table I summarizes the AUC, CL_{total}, uptake clearances of the liver (CL_{liver}) and kidney (CL_{kidney}), and urinary excretion clearance (CL_{urine}) of ^{111}In-CAT. At any dose, CL_{liver} accounted for a large portion of CL_{total}, and both CL_{kidney} and CL_{urine} were much smaller than CL_{liver}. CL_{liver} decreased with an increase in dose, indicating that the hepatic uptake of ^{111}In-CAT involves a saturable process as observed in the hepatic uptake of glycosylated proteins.[16]

Hepatic Cellular Localization of ^{111}In-CAT

After injection of a dose of 0.1 mg/kg, ^{111}In-CAT was mostly recovered in the PC of the liver (Figure 3). The amount recovered in PC was 4.88 ± 1.07% of the dose/10^7 cells, and that in NPC was 0.115 ± 0.028% of dose/10^7 cells. When calculated using the numbers of liver cells reported (PC, 1.25×10^8 cells/g liver; NPC, 0.65×10^8 cells/g liver),[20] uptake by PC accounted for more than 98% of the hepatic uptake.

TABLE I Pharmacokinetic parameters for ^{111}In-labeled CAT after intravenous injection in mice

Dose (mg/kg)	AUC (% of dose hr/ml)	Clearance (μl/hr)			
		Total	Liver	Kidney	Urine
0.1	2.87	34900	25100	350	270
1	6.37	15700	9500	190	310
10	21.4	4700	2800	70	80

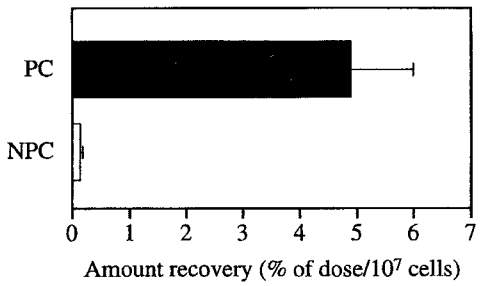

FIGURE 3 Amount of ^{111}In-CAT recovered in liver parenchymal cells (PC) and nonparenchymal cells (NPC) after intravenous injection into mice at a dose of 0.1 mg/kg. Results are expressed as the mean ± SD of three mice.

Coadministration of Gal-BSA, Iron-carrying Proteins or Uricase

Figure 4 shows the amount in the liver of ^{111}In-CAT 5 min after co-injection into mice of an excess of various compounds. As already shown in Figure 1, the increase in the dose of CAT reduced the hepatic uptake of ^{111}In-CAT in a dose-dependent manner. Coadministration of Gal-BSA, a specific ligand for the asialoglycoprotein receptors on hepatocytes,[16] did not affect the hepatic uptake of ^{111}In-CAT. Neither transferrin, lactoferrin nor ferritin produced any significant change in the amount of ^{111}In-CAT in the liver. In addition, the administration of hemoglobin or myoglobin with ^{111}In-CAT had little effect on the distribution of ^{111}In-CAT. Finally, coadministration of uricase, a protein sorted to peroxisomes after protein synthesis, failed to reduce the hepatic uptake of ^{111}In-CAT.

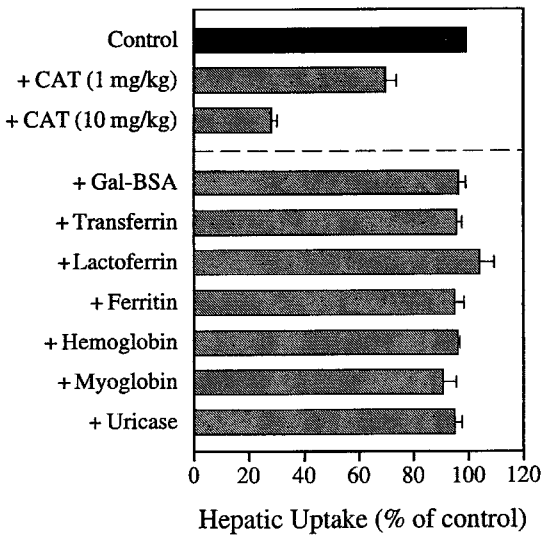

FIGURE 4 Amount of ^{111}In-CAT recovered in the liver at 5 min after intravenous injection of various compounds into mice. ^{111}In-CAT was injected at a dose of 0.1 mg/kg with a different amounts of unlabeled CAT (1 or 10 mg/kg) or a 20 mg/kg dose of these compounds. Results are expressed as the mean ± SD of three mice.

Inhibitory Effect of CAT against Hepatic Ischemia/Reperfusion Injury

Figure 5 shows the GPT and GOT in the plasma of hepatic ischemia/reperfusion-injured mice. Occlusion of the hepatic artery and the portal vein followed by reperfusion resulted in marked increases in both GPT and GOT in the plasma: GPT increased from 8.67 to 107 IU/l, and GOT from 32.3 to 185 IU/l. Administration of CAT prior to reperfusion effectively reduced the GOT and GPT in a dose-dependent manner. The GPT and GOT of mice injected with a large amount of CAT (200,000 units/kg) were two to three times higher than those of normal mice.

DISCUSSION

Various endogenous macromolecular substances have become a new class of therapeutic agents following recent progress in biotechnology. Catalase is an enzyme having the ability to detoxify

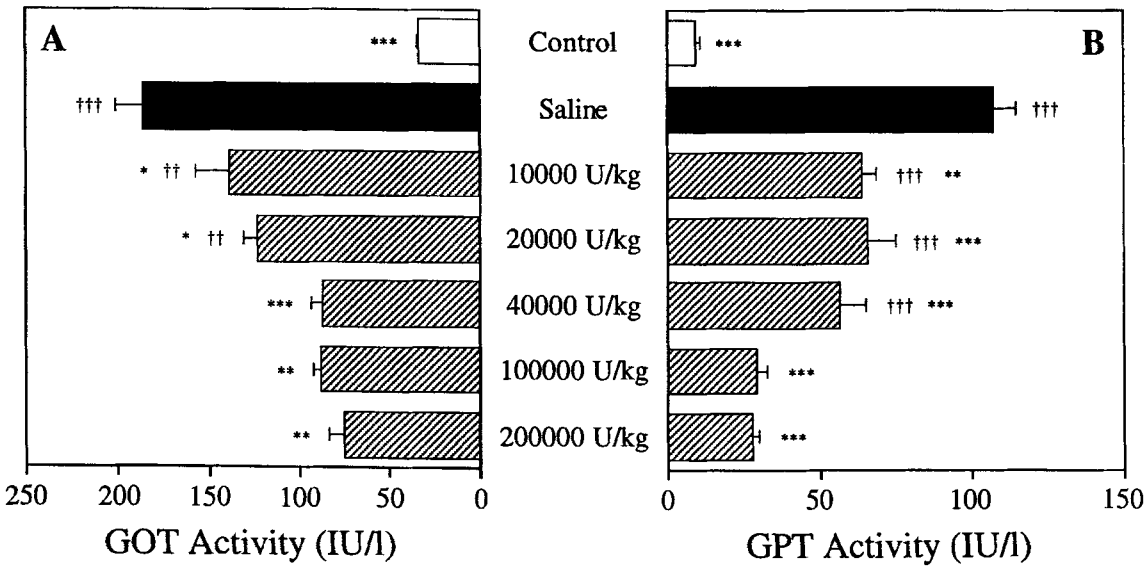

FIGURE 5 Effect of CAT administration on plasma GOT (A) and GPT (B) of mice with an ischemia/reperfusion injury. Results are expressed as the mean ± SEM for at least three mice. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$: significantly different from no treatment (saline administration). $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$: significantly different from that of control mice.

hydrogen peroxide which is involved in various ROS-mediated injuries. Therefore, treatment with catalase alone or its combination with SOD have been applied to ROS-mediated injuries such as ischemia/reperfusion injuries, but its reported pharmacological effects are controversial so far.[21–25] Experimental differences in design including the duration of ischemia and reperfusion, dosing schedules of the enzymes, and animals used could account for such discrepancies. However, although catalase can metabolize hydrogen peroxide at local sites where the enzyme distributes, its *in vivo* distribution as well as its pharmacokinetic behavior have been hardly considered in previous reports. The purpose of this study is, therefore, (1) to clarify the clearance mechanisms and disposition characteristics of CAT, and (2) to examine its effect on a ROS-mediated injury in the liver since the biodistribution data in this paper revealed that CAT is specifically taken up by liver PC.

In the disposition studies, CAT was labeled with ^{111}In using DTPA anhydride as a bifunctional chelating agent. This radiolabeling method is suitable to examine the distribution phase of macromolecules including CAT from plasma to various tissues, since radioactive metabolites, if produced after cellular uptake, are retained within cells where the uptake takes place.[26,27]

In general, proteins with molecular weights less than 40 kDa, such as SOD and soybean trypsin inhibitor, are susceptible to glomerular filtration and rapidly eliminated to urine from plasma. On the other hand, BSA (67 kDa) and bovine γ-immunoglobulins (150 kDa) having large molecular weights and a weak negative charge circulate in plasma for a long time due to low filtration at the glomerulus and little interaction with various tissues. Although CAT, which has a molecular weight of 240 kDa and an isoelectric point of 5.7, possesses similar physicochemical characteristics to these proteins, ^{111}In-CAT rapidly accumulated in the liver after intravenous injection. Although it has been speculated that catalase may be taken up by the liver,[3,28] to our knowledge, this is the first report describing the rapid uptake of CAT by the liver PC. In any mouse, about 70–80% of radioactivity injected was

recovered in the samples shown in Figure 2, i.e., liver, kidney, spleen, heart, lung, whole blood, and urine. Distribution to other tissues such as intestines, muscle, fat, bone, skin and vessels could explain the recovery of less than 100%; the amount recovered in muscle would be around 4–8% of dose since the concentration of radioactivity in muscle was observed to be about 0.4–0.7% of dose/g tissue (data not shown).

Only hepatocytes contribute to the hepatic uptake of CAT, and this uptake is saturated by increasing the dose. It is well known that galactosylated compounds such as asialoglycoproteins are rapidly taken up by the hepatocytes via the asialoglycoprotein receptors in a dose-dependent manner.[16] However, the asialoglycoprotein receptor should not be involved in the hepatic uptake of ^{111}In-CAT, since excess Gal-BSA, a ligand for the receptor, had no effect on its distribution to liver. CAT is a homotetramer which contains one heme per subunit. Proteins containing heme such as hemoglobin and myoglobin are known to be transported to liver PC by serum proteins.[29–32] In addition, other iron-carrying proteins, such as transferrin, lactoferrin and ferritin, are also taken up by liver PC.[33–35] Based on these facts, the possibility that CAT is recognized by liver PC as an iron-carrying protein was examined by coadministering them. However, these compounds did not significantly inhibit the hepatic uptake of ^{111}In-CAT. After being synthesized intracellularly, CAT is transported to peroxisome since it has a targeting signal to this organelle.[36,37] To investigate whether any common mechanism for proteins targeted to the organelle is involved in the hepatic uptake of ^{111}In-CAT, coadministration of uricase, which also localizes in peroxisome after biosynthesis, was carried out. However, it also failed to inhibit the uptake of ^{111}In-CAT suggesting that transporting mechanisms of these proteins to peroxisome would not be important for their hepatic uptake. These results strongly suggest that a saturable and CAT-specific mechanism is involved in the recognition of CAT by hepatocytes. In preliminary studies using perfused rat livers, ^{111}In-CAT showed a serum protein-independent and temperature-dependent hepatic uptake suggesting that ^{111}In-CAT is internalized via endocytosis after binding to the surface of liver cells (unpublished data). A better understanding of the uptake mechanism of CAT by liver PC, however, requires further studies.

Since CAT was shown to be delivered to liver PC after intravenous injection, its potential as a therapeutic agent for hepatic injuries caused by ROS was investigated using experimental ischemia/reperfusion injury in mice. It is well known that ROS are involved in ischemia/reperfusion injury of the liver.[38,39] Neutrophils and Kupffer cells are considered to mainly contribute to ROS generation in the liver after reperfusion. In addition, the contribution of neutrophils to ROS production can be excluded in an early phase of reperfusion after ischemia, since the recruitment of neutrophils to the liver[40] and their activation to producing ROS[41,42] requires 30–60 min and 30–90 min, respectively. Although these findings have been mostly obtained in rats and there are few studies dealing with hepatic ischemia/reperfusion injuries in mice,[43] we can assume that Kupffer cells are the major source of ROS in the liver in the present experiments involving 30 min of ischemia followed by a 60 min reperfusion.

The ischemia followed by reperfusion resulted in a striking increase in plasma GPT and GOT, indicating that liver PC were damaged by the treatment. The administration of CAT attenuated the increase in plasma GPT and GOT in a dose-dependent manner. In addition, the administration of BSA failed to suppress the increase in the GPT and GOT (data not shown), suggesting that the protection is actually due to catalase activity. After intravenous injection, CAT can be considered to localize on the surface of hepatocytes and inside them since ^{111}In-CAT was rapidly eliminated from plasma and recovered in liver PC. Therefore, these results suggest that CAT bound to and/or internalized by PC prevents the injury

caused by the ischemia/reperfusion. It has been demonstrated that intracellular oxidative stress in hepatocytes has little pathophysiological relevance since hepatocytes are able to detoxify ROS orders of magnitude more than is formed during perfusion.[38] However, when CAT is internalized by cells, the intracellular distribution will differ from the endogenous catalase since extracellular CAT could be internalized via an endocytotic process. Further investigations are required to identify whether surface bound, internalized or both forms of CAT contribute to prevent the injury. Considering that most ROS are generated from Kupffer cells in the early phase of reperfusion as in this study, CAT associated to hepatocytes protects the cells from the attack by ROS.

In conclusion, CAT can be delivered to liver PC via a CAT-specific mechanism and it prevents ROS-mediated injuries in the liver. This study is the first to clarify the relationship between the *in vivo* distribution of CAT and its pharmacological activity in experimental ischemia/reperfusion injury. The pharmacological activity of CAT could be improved by controlling its *in vivo* distribution characteristics by chemical modification. For example, targeting to liver NPC such as Kupffer cells is a promising approach to achieve injury prevention since ROS is mainly generated from Kupffer cells in the initial phase of the injury. We have already reported an enhanced inhibitory effect of SOD derivatives which can be delivered to liver NPC in a rat model.[8] Further improvements can be achieved by using two antioxidant enzyme derivatives, SOD and catalase, through the optimization of their *in vivo* distribution profiles by chemical modification.

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