Prevention of Neutrophil-Mediated Hepatic Ischemia/Reperfusion Injury by Superoxide Dismutase and Catalase Derivatives

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ABSTRACT

Our previous study demonstrated that the combination of mannosylated superoxide dismutase (Man-SOD) and succinylated catalase (Suc-CAT), both of which are designed to be targeted to liver nonparenchymal cells, is a promising approach to prevent the initial phase of hepatic ischemia/reperfusion injury induced by occlusion of the portal vein for 30 min followed by a 1-h reperfusion in mice. In this study, the preventive effects of these agents were examined on late-phase injury mediated by infiltrating neutrophils, a more severe condition than the initial one. Administration of Suc-CAT alone or with Man-SOD to mice undergoing hepatic ischemia/reperfusion significantly suppressed the expression of intercellular adhesion molecule-1 along the hepatic sinusoid and prevented neutrophil infiltration in the liver. Man-SOD and Suc-CAT also prevented the increase in plasma glutamic pyruvic transaminase and glutamic oxaloacetic transaminase activities after reperfusion lasting 3 and 6 h. Histological evaluation of liver tissues confirmed the efficacy of this treatment, suggesting that these SOD and catalase derivatives have the ability to suppress neutrophil-induced hepatic injury. These results demonstrate that targeted delivery of antioxidant enzymes to liver nonparenchymal cells is a promising approach to reducing the reactive oxygen species produced by Kupffer cells and neutrophils infiltrating into the tissue. Since Suc-CAT is partially taken up by hepatocytes via a catalase-specific uptake mechanism, such a fraction could also be involved in its preventive effect against the injury.

Hepatic ischemia followed by reperfusion results in severe injuries that contribute to the morbidity and mortality associated with shock, transplantation, and liver surgery. It has been generally accepted that reactive oxygen species (ROS) contribute to hepatic ischemia/reperfusion injury (McCord, 1985), and such injury has been demonstrated to occur in a biphasic pattern involving initial- and subsequent-phase responses (Jaeschke et al., 1991). Activated Kupffer cells generating an increasing amount of ROS mainly mediate the initial phase of injury. These cells are activated during ischemia (Rymsa et al., 1991) and are further stimulated by complement activation during reperfusion (Jaeschke et al., 1993). The initial responses of the ischemia/reperfusion injury trigger the infiltration of neutrophils into postischemic liver (Jaeschke et al., 1991). The recruitment of neutrophils results from a complex series of ischemia-induced cellular responses in the liver and changes in the vasculature that serve to alter the adhesive characteristics of the neutrophils (Jaeschke et al., 1996). These include the increased expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) (Farhood et al., 1995). ICAM-1 is expressed on endothelial cells and plays a key role in the potent adhesion of neutrophils and their transendothelial migration (Lusczinskas et al., 1991). The accumulation of neutrophils in the liver is reported to take place mainly between 30- and 60-min postreperfusion, but they do not spontaneously release ROS in the vasculature (Jaeschke et al., 1991). The respiratory burst of neutrophils adhering to a biological surface, such as endothelial cells or extracellular matrix proteins, is characterized by a lag-phase of 30 to 90 min between the adherence of activated neutrophils and the subsequent long-lasting ROS formation (Jaeschke, 1991). Thus, activated neutrophils play a central role in the later phase of hepatic injury by releasing ROS (Jaeschke, 1991).

In previous studies, we developed various derivatives of superoxide dismutase (SOD) and catalase by carrying out chemical modifications and demonstrated that targeted de-
livery of SOD and catalase to liver nonparenchymal cells is a promising approach to prevent hepatic ischemia/reperfusion injury (Fujita et al., 1992a,b; Yabe et al., 1999b). Among the various combinations, it was that the administration of succinylated catalase (Suc-CAT) and mannosylated SOD (Man-SOD) was very effective in preventing initial hepatic injury at 1 h after reperfusion (Yabe et al., 1999b). However, the preventive effects of such treatments on the later phase of the injury have not been investigated. The subsequent phase of the injury, which is mainly caused by infiltrating neutrophils, is more severe than the initial one and leads to irreversible tissue damage (Jaeschke et al., 1991). Therefore, in the present study, we examined the effect of catalase and SOD derivatives on the late phase of ischemia/reperfusion injury by examining the plasma glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels at 3 and 6 h after reperfusion and ICAM-1 expression and neutrophil accumulation in the liver.

Materials and Methods

Animals. Male ddY mice (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. This study was carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the U.S. National Institutes of Health.

Chemicals. Bovine liver catalase (40,000 U/mg) was purchased from Sigma (St. Louis, MO). Recombinant human SOD (111-Ser) was supplied by Asahi Kasei (Tokyo, Japan). Rat anti-mouse ICAM-1 antibody (KAT-1) was purchased from Daizinippon Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals were of the highest grade available.

Synthesis and Characterization of Catalase and SOD Derivatives. The synthesis of Suc-CAT and Man-SOD was performed as reported previously (Fujita et al., 1992b; Yabe et al., 1999b). In brief, 100 mg of catalase was dissolved in 0.2 M Tris buffer (pH 8.65), and 46 mg of succinic anhydride was added. The mixture was then stirred for 18 h at room temperature. The reaction mixture was subjected to column chromatography (Toyopearl HW-55S, Tosoh Co.) and fractions with larger molecular weights were collected. Man-SOD was synthesized by reacting SOD with 2-imino-2-methoxyethyl 1-thiomannoside. Each derivative was washed, concentrated by ultrafiltration (molecular weight cut-off, 200,000 for Suc-CAT and 20,000 for Man-SOD, respectively) against distilled water, and lyophilized.

The number of amino groups was determined using trinitrobenzenesulfonic acid with glycine as a standard (Habeeb, 1966), and about 70% of the total protein amino groups were calculated to be about 70% of the total protein amino groups were calculated to be used for chemical modification for both Suc-CAT and Man-SOD. The apparent molecular weight was estimated by high-performance liquid chromatography using a G4000SWXL column (Tosoh Co., Tokyo, Japan), and fractions with larger molecular weights were collected. Man-SOD was synthesized by reacting SOD with 2-imino-2-methoxyethyl 1-thiomannoside. Each derivative was washed, concentrated by ultrafiltration (molecular weight cut-off, 200,000 for Suc-CAT and 20,000 for Man-SOD, respectively) against distilled water, and lyophilized.

Hepatic Ischemia/Reperfusion Experiment. Male ddY mice were anesthetized with a peritoneal injection of pentobarbital sodium (50 mg/kg). An incision was made in the abdomen, and the portal vein and hepatic artery were occluded with a vascular clamp for 30 min to induce hepatic ischemia. Then, blood was allowed to flow through the liver again (reperfusion). Saline (control), catalase derivatives (10,000 U/kg), Man-SOD (10,000 U/kg), or a combination of Suc-CAT and Man-SOD (10,000 U/kg for each) was administered twice through the tail vein 5 min before and 60 min after re-establishing blood flow. After an appropriate period of reperfusion (30, 45, 60, 120, 180, 360, 720 min), blood was collected from the vena cava, and plasma was obtained by centrifugation. GPT and GOT activities, as indicators of hepatocyte injury during reperfusion, were assayed using commercial test reagents.

Histological Examination. Liver tissues after 30 or 180 min of reperfusion were excised and subjected to histological examination. Tissue samples were fixed with 10% neutral buffered formalin, embedded in paraffin blocks, and 5-μm-thick sections were cut. For immunohistochemical staining of ICAM-1, the sections from the liver were stained after 30 min of reperfusion with a rat anti-mouse ICAM-1 antibody, according to the method reported previously (Hsu et al., 1981). Furthermore, the sections from the liver were stained after 180 min of reperfusion with hematoxylin and eosin to observe hepatic injury.

Evaluation of Neutrophil Infiltration. Neutrophil infiltration into liver tissue was evaluated by a method reported previously (Parhood et al., 1995). Briefly, liver tissues were excised after 30 or 45 min of reperfusion to make the sections, and neutrophils in the liver section were stained using the AS-D chloroacetate esterase technique (Moloney, 1960). Neutrophils were identified by positive staining and morphology and were counted in 25 high-power fields (HPF) (400×) using a Nikon Labophot microscope (Tokyo, Japan). Only those neutrophils were counted that were present within sinusoids or extravasated into the tissue, those in large hepatic vessels such as venules were not.

Statistical Analysis. Differences were statistically evaluated by one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test at a significance level of p < 0.05.

Results

ICAM-1 Expression in the Liver Detected by Immunohistochemical Staining. In a previous study, pretreatment with catalase was effective in maintaining low GPT and GOT levels only at high doses after 60 min of reperfusion (Yabe et al., 1999a). Suc-CAT, which is taken up by liver nonparenchymal cells, was very effective in inhibiting the production of hepatic injury at lower doses, and its effect was increased by coadministering Man-SOD (Yabe et al., 1999b). First we evaluated the effects of Suc-CAT and/or Man-SOD on ICAM-1 expression in the liver section from the mice subjected to 30 min of ischemia followed by 30 min of reperfusion. Staining of control liver sections with a monoclonal anti-ICAM-1 antibody showed weak expression of ICAM-1 on sinusoidal endothelial cells and no expression on hepatocytes (Fig. 1A). Ischemia/reperfusion followed by saline injection resulted in an increased expression of ICAM-1 on both endothelial cells and hepatocytes (Fig. 1B). The increase in ICAM-1 expression was prevented to some extent by catalase administration (Fig. 1C). The pattern of ICAM-1 expression in the specimens of mouse liver receiving Suc-CAT and Man-SOD was indistinguishable from that of the control group (Fig. 1D).

Neutrophil Accumulation in the Liver. The number of neutrophils accumulating in the liver after reperfusion was counted under a microscope. After 30 or 45 min of reperfusion, 2.3 or 3.5 times as many neutrophils, respectively, accumulated in the liver compared with control mice (p < 0.05, p < 0.001) (Fig. 2). After 180 min of reperfusion, the number
of neutrophils increased to 373 ± 68 (per 25 HPF), which was 7.1 times as many as that of control mice. At 45 min of reperfusion (Fig. 2), treatment with Man-SOD, catalase, and Suc-CAT reduced the number of neutrophils in the liver by 44, 57, and 62%, respectively. Significant differences were observed between each treated group and saline-treated group (*p < 0.05; **p < 0.001, significantly different from the control group).

**Time Course of Plasma GOT and GPT Levels after Reperfusion following a 30-Min Period of Ischemia.**

During the 30-min period of ischemia, plasma GOT and GPT levels did not significantly increase. After re-establishing hepatic blood flow, however, both activities were continuously elevated. Their activities became significantly higher after 30 min of reperfusion (p < 0.01) and reached a maximum after 6 h of reperfusion (Fig. 3). After 6 h of reperfusion, the values of GOT and GPT were 9 and 35 times greater, respectively, than those before ischemia and were significantly higher than those after 60 or 360 min of reperfusion (GOT, p < 0.05; GPT, p < 0.01), indicating more severe injury occurring at the later phase of the reperfusion.

**Prevention of Later Phase Hepatic Ischemia/Reperfusion Injury by Catalase and SOD Derivatives.**

The effect of catalase and SOD derivatives on plasma GOT and GPT activities after a longer reperfusion of 180 or 360 min was examined. Measured at 180 min after reperfusion, the plasma GOT and GPT levels remained significantly lower than those in saline-treated mice following injection of Suc-CAT or coinjection of Suc-CAT and Man-SOD (p < 0.01) (Fig. 4, A and B, panel a). These preventive effects were maintained until 360 min after reperfusion (Fig. 4, A and B, panel b).

**Liver Tissue Damage.** Figure 5 shows the liver specimens from mice suffering from ischemia (30 min)/reperfusion (180 min) followed by treatment with saline (A), catalase (B), Suc-CAT (C), or Man-SOD and Suc-CAT (D). The sections from the saline- and CAT-treated mice had lots of necrotic and/or damaged cells. In contrast, hepatocellular damage was prevented, and the integrity of sinusoids was almost completely maintained in the livers of mice treated with Suc-CAT alone or Suc-CAT and Man-SOD.

**Discussion**

SOD is an enzyme that converts superoxide anion to hydrogen peroxide, and catalase is an enzyme that can detoxify hydrogen peroxide. Although these enzymes have been used to prevent various ROS-mediated injuries, inadequate delivery to target sites may be the cause of the controversial results obtained so far (Atalla et al., 1985; Chiu and Toledo-Pereyra, 1987; Flye and Yu, 1987) because these enzymes can detoxify ROS only at the sites to which they are delivered. Cell-specific targeting is a promising approach to improve the pharmacological activity of these biologically active agents.
In previous studies, the targeted delivery of SOD and catalase to liver nonparenchymal cells by mannosylation or succinylation has been demonstrated (Fujita et al., 1992b, 1999b; Yabe et al., 1999b). Kupffer and sinusoidal endothelial cells, which account for the majority of liver nonparenchymal cells, possess mannose receptors that recognize and internalize ligands containing mannose, such as Man-SOD and Man-CAT, and scavenger receptors that recognize polyanions, such as succinylated proteins (e.g., Suc-CAT). Therefore, mannosylation or succinylation greatly increases the amount of these enzymes targeted to the liver nonparenchymal cells where ROS are generated in large quantities during the initial phase of the ischemia/reperfusion injury. Compared with other types of enzyme derivatives, these liver nonparenchymal cells targeted SOD, and catalase derivatives exhibited more promising effects in preventing the initial phase of hepatic ischemia/reperfusion injuries (Fujita et al., 1992a, 1999b). Among the various combinations, Man-SOD and Suc-CAT have shown the greatest efficacy in preventing hepatic injury (Yabe et al., 1999b). Previous results reported from our laboratory demonstrated that the amount (on a cell-number basis) of succinylated proteins taken up by liver endothelial cells was 3.3 times as large as Kupffer cells in rats (Furitsu et al., 1997). On the other hand, the uptake of mannosylated proteins took place on both the endothelial cells and Kupffer cells at a similar level (Ogawara et al., 1999). Therefore, after the administration of Suc-CAT and Man-SOD, liver endothelial cells could possess a higher level of catalase activity than that of SOD activity, whereas Kupffer cells could mainly have SOD activity. Therefore, a plausible mechanism of the protection by Suc-CAT and Man-SOD is the dismutation of superoxide anion that Kupffer cells generate by Man-SOD followed by Suc-CAT-mediated elimination of hydrogen peroxide, which is a stable amphiphilic molecule that can diffuse cellular membrane.

Ischemia/reperfusion injury is supposed to consist of an initial and a subsequent phase. In the initial phase of the injury, ROS are mainly released from Kupffer cells (Jaeschke, 1991). Then, these ROS recruit and activate neutrophils (Jaeschke et al., 1991). Once activated and attached to endothelial cells, the neutrophils may exacerbate tissue injury by generating ROS and secreting several proteases, such as myeloperoxidase, elastase, and collagenase (Jaeschke, 1991). Neutrophil sequestration may be explained by two mechanisms: 1) exposure of serum to oxidants results in generation of chemotaxin(s) that contribute to directed migration of neutrophils into inflamed tissue (McCord, 1985), and 2) activation of cell-surface adhesion molecules by oxidants mediates adhesive interactions between neutrophils and endothelial cells (Lewis et al., 1988; Lo et al., 1993). It has been reported that cell adhesion molecules play a central role in leukocyte-endothelial interactions (Farhood et al., 1995). ICAM-1 is constitutively present on endothelial cells and on some other cell types, including lymphocytes, fibroblasts, hepatocytes, and epithelial cells (Jaeschke et al., 1996). ICAM-1 is known to be involved as a counter receptor of various adhesion molecules, such as VCAM-1, E-selectin, and L-selectin.

![Fig. 3](image3.png)

**Fig. 3.** Plasma GOT (A) and GPT (B) activities in mice undergoing a 30 min-period of ischemia followed by reperfusion up to 12 h. Results are expressed as the mean ± S.E.M. of at least three mice. Zero time indicates the start of reperfusion. Those activities before ischemia are GOT = 2.11 ± 0.61 (IU/l) and GPT = 8.01 ± 2.11 (IU/l). After reperfusion, all data were significantly different from the values at the start of reperfusion (p < 0.01).

![Fig. 4](image4.png)

**Fig. 4.** Effect of Man-SOD, catalase, Suc-CAT, and Suc-CAT with Man-SOD on plasma GOT (A) and GPT (B) levels in mice following 180 min (a) or 360 min (b) of reperfusion. Each derivative was administered twice at a dose of 10,000 U/kg. Results are expressed as the mean ± S.E.M. of at least three mice. *p < 0.05; **p < 0.1; ***p < 0.001, significantly different from the saline-treated group.
al., 1995), and hydrogen peroxide increases ICAM-1 mRNA and protein expression (Lo et al., 1993; Farhood et al., 1995).

Based on such evidence, we examined ICAM-1 expression (Fig. 1) and neutrophil accumulation (Fig. 2) in the mice liver after hepatic ischemia/reperfusion. Hepatic ischemia/reperfusion resulted in a marked increase in ICAM-1 expression on the endothelial cells and hepatocytes. Furthermore, many neutrophils were found infiltrating in liver tissues. Neutrophil accumulation began at 30 to 45 min of reperfusion and continued to 3 h of reperfusion. These results were not consistent with a previous study (Jaeschke, 1991), but experimental procedures could account for such differences. Administration of catalase, Suc-CAT, and Man-SOD reduced both the ICAM-1 expression and neutrophil infiltration into the liver. Among them, administration of Suc-CAT alone or with Man-SOD markedly reduced both. Since up-regulation of ICAM-1 is brought by intracellular transcription, these results suggest that these catalase and SOD derivatives could eliminate intracellular ROS in the sinusoidal endothelial cells.

After transendothelial migration, neutrophils attached to the hepatocyte aggravate the later phase of reperfusion injury by generating cytotoxic mediators, such as ROS (Komatsu et al., 1992; Jaeschke et al., 1996). Therefore, as the next step, we examined the protective effect of SOD and catalase derivatives targeted to the liver nonparenchymal cells in long-term reperfusion injury. The plasma GOT and GPT activities gradually increased with time after reperfusion and reached a maximum at 6 h of reperfusion, indicating that more severe injury occurs at the late phase of reperfusion (Fig. 3). Although the catalase and SOD derivatives, Suc-CAT and Man-SOD, used in the present study were effective in preventing the leakage of GOT and GPT up to 1 h of reperfusion when given as a single administration of 10,000 U/kg (Yabe et al., 1999b), the same dose of these derivatives administered once at 5 min before reperfusion had no significant preventive effect on the long-term injury, determined at 3 or 6 h (data not shown). These results suggest that the reduction in ROS generation by a single administration of these enzymes or their combination is not sufficient to prevent the subsequent phase of the injury. Ligands internalized by receptor-mediated endocytosis are known to be sorted to endosomes, then to lysosomes where they are degraded by proteolysis (Pontow et al., 1992). This degradation might limit the duration of the efficacy of the enzyme derivatives if ROS are continuously released. We then tried to administer these derivatives on two occasions, at 5 min before and 60 min after reperfusion. This protocol made it possible to maintain the plasma GOT and GPT levels of mice treated with Suc-CAT alone or with Man-SOD at significantly lower levels than those of saline- or CAT-treated mice, following injection at least 6 h after reperfusion (Fig. 4). Histological evaluation of liver tissues also confirmed the efficacy of Man-SOD and Suc-CAT (Fig. 5D). In the Man-SOD and Suc-CAT group, the integrity of sinusoid was almost completely maintained, suggesting that protection of sinusoidal endothelial cells is important in preventing hepatic injury. Prevention of ICAM-1 expression and subsequent neutrophil accumulation in the liver could be one reason why the combined use of Man-SOD and Suc-CAT is effective on both the initial and subsequent phases of ischemia/reperfusion injury in the liver. These findings indicate that the simultaneous cell-specific targeting of catalase and SOD to liver nonparenchymal cells is an effective approach for preventing not only the initial hepatic ischemia/reperfusion injury but also that occurring later by eliminating both extracellular ROS generated by Kupffer cells and intracellular ROS in sinusoidal endothelial cells.

Recent studies have demonstrated that intracellular oxidative stress in hepatocytes is also important in hepatocyte cell death (Jaeschke et al., 1999; Kumamoto et al., 1999). Mitochondria seem to be the major source that produces ROS intracellularly (Dawson et al., 1993). Superoxide induces hepatocyte apoptosis during the early phase of reperfusion after hepatic ischemia (Sasaki et al., 1998), and apoptosis of hepatocytes acts as a signal for sinusoidal sequestration and transendothelial migration of neutrophils (Lawson et al.,
1998). Although the contribution and importance of apoptosis in the hepatic injury are still controversial (Cursio et al., 1999; Gujral et al., 2001), there is no doubt that intracellular ROS contribute to the reperfusion injury after hepatic ischemia. Our previous study suggests that In-Man-CAT and Suc-CAT are recognized by not only the mannose and scavenger receptors on liver nonparenchymal cells but also by the mechanism specific for catalase on hepatocytes (Yabe et al., 1999b). Therefore, a part of Suc-CAT is taken up by hepatocytes after systemic administration, and it may show the enzymatic activity against intracellular ROS following hepatic ischemia/reperfusion. Further studies are needed to evaluate how much these catalase derivatives, which are delivered to hepatocytes, can eliminate intracellular ROS.

In conclusion, we have demonstrated that cell-specific targeted delivery of catalase and SOD to liver nonparenchymal cells is a promising strategy for inhibiting hepatic ischemia/reperfusion injuries. This approach is effective not only in eliminating ROS produced by Kupffer cell but also in decreasing neutrophil accumulation in the liver by inhibiting expression of ICAM-1 along sinusoidal endothelial cells.

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References


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