Title:

One-step preparation of *S*-nitrosated human serum albumin with high biological activities

Foot Note Title:

One-step preparation of SNO-HSA

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List of Abbreviations:

HSA, human serum albumin; SNO-HSA, *S*-nitrosated HSA; GS-NO, *S*-nitrosoglutathione; N-AcTrp, N-acetyl-L-tryptophanate; CA, caprylic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HO-1, heme oxygenase-1.

ABSTRACT

S-Nitrosated human serum albumin (SNO-HSA) is a large molecular weight nitric oxide carrier in human plasma, and because of its many beneficial effects in different tests, it is currently under investigation as a cytoprotective agent. However, making SNO-HSA preparations is a complicated and time-consuming process. We found that binding of caprylic acid (CA) and N-acetyl-L-tryptophan (N-AcTrp) to defatted mercaptalbumin increased *S*-nitrosation by *S*-nitrosoglutathione (GS-NO) by making Cys-34 of HSA more accessible and by protecting it against oxidation, respectively. Fortunately, HSA solutions for clinical use contain high concentrations of CA and N-AcTrp as stabilizers. By making use of that fact it was possible to work-out a fast and simple procedure for producing SNO-HSA: Incubation of a commercial HSA formulation with GS-NO for only one minute results in *S*-nitrosation of HSA. The biological usefulness of such a preparation was tested in a rat ischemia-reperfusion liver injury model. Although our procedure for making SNO-HSA is fast and straightforward, the cytoprotective effect of the preparation was similar to, or better than, that of a preparation made in a more traditional way. The clinical development of SNO-HSA as a strong cytoprotective agent is under way using this method in collaboration with clinicians and industrial developers.

Keywords:
human serum albumin; nitric oxide; N-acetyl-L-tryptophan; caprylic acid; *S*-nitrosation; ischemia-reperfusion.

INTRODUCTION

Post-translational modifications are essential in their functional regulation. Among these, changes of the redox state of cysteine residues are of great importance. The sulfhydryl moiety can interact with nitric oxide (NO) and thereby form *S*-nitrosothiols [1-3]. *S*-nitrosothiols may function as NO reservoirs and preserve the antioxidant and other activities of NO [4,5]. For example, it has been reported that *S*-nitrosated human serum albumin (SNO-HSA) may serve *in vivo* as a circulating reservoir for NO produced by the endothelial cells [6]. The reservoir function was also reported to be operative when application of SNO-HSA to animals suffering from ischemia-reperfusion injury minimized the extent of tissue damage associated with reperfusion [7-9]. Therefore, SNO-HSA is under investigation as a therapeutic agent in humans. However, efficient SNO-HSA preparations are difficult to produce.

HSA is a single, non-glycosylated polypeptide that organizes to form a heart-shaped protein with approximately 67% α-helix but no β-sheet [10]. Except for Cys-34, all of the 35 cysteine residues are involved in the formation of stabilizing disulfide bonds. Cys-34 represents the largest fraction of free thiols in the circulation (mercaptalbumin). The residue is located in "a crevice" on the surface in the N-terminal part of the protein, and such a location can impede the access of *S*-nitrosating agents to the sulfhydryl group. In addition, normally about half of the Cys-34 residues are oxidized or involved in ligand binding (non-mercaptalbumin), and that kind of hindrance could also make an efficient *S*-nitrosation difficult.

In previous investigations we found that binding of N-acetyl-L-tryptophanate (N-AcTrp) protects the sulfhydryl group of HSA against oxidation [11], and that binding of oleic acid increases *S*-nitrosation of HSA [12]. In the present work we found that simultaneous binding of N-AcTrp and caprylic acid (CA) greatly increased *S*-nitrosation of HSA by *S*-nitrosoglutathione (GS-NO) by protecting the sulfhydryl group against oxidation and by making it more accessible to GS-NO, respectively. HSA solutions for clinical use contain high concentrations of just N-AcTrp and CA, and we took advantage of that fact to work-out a simple, one-step method to make a biological active and stable SNO-HSA preparation which most probable also can be of clinical use, see the illustrative overview in Fig. 1.

EXPERIMENTAL PROCEDURES

Materials

20% HSA solutions were donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). As stabilizers the formulation contained N-AcTrp (6.2 mol/mol HSA) and CA (5.3 mol/mol HSA). Part of the albumin was defatted by treatment with charcoal as described by Chen [13], dialyzed against deionized water, freeze-dried, and then stored at -20°C until used. According to density analysis of Coomassie Brilliant Blue (CBB)-stained protein bands on 12.5% SDS-PAGE, the purity of albumin in the original solutions and of the defatted samples was more than 97%. N-AcTrp, CA and 1,4-dithiothreitol (DTT) were purchased from Sigma–Aldrich (St. Louis, MO). Sulfanilamide, naphthylethylenediamine-hydrochloride, $HgCl_2$, $NaNO_2$ and $NaNO_3$ were obtained from Nakalai Tesque (Kyoto, Japan). GS-NO, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and diethylenetriaminepentaacetic acid (DTPA) were obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of the best grades commercially available, and all solutions were made in deionized water. Sephadex G-25 desalting column (ϕ 1.6 × 2.5 cm) was from Amersham Pharmacia Biotech (Tokyo, Japan).

Preparation of HSA-ligand solutions

First, Cys-34 of defatted HSA was reduced by treatment with DTT as follows. HSA (300 μM) was incubated with DTT (molar ratio, protein:DTT = 1:10) for 5 min at 37°C. After incubation, DTT was quickly removed by Sephadex G-25 gel filtration using 10 mM phosphate-buffered saline (pH 7.4) (PBS; Ca^{2+}, Mg^{2+} free). We checked that the final SH/HSA ratio was less than 1 by using the DTNB assay described below. Stock solutions of 20 mM CA and 20 mM N-AcTrp were made in methanol-H_2O (1:1, v/v) and H_2O, respectively. Later, these stock-solutions were diluted with PBS. In all cases, the resulting solutions were mixed with PBS containing HSA. The final methanol concentrations in the CA-containing solutions were 0.75% or less. The protein content of all protein preparations used in this study was determined by the Bicinchoninic acid protein assay.

Accessibility of Cys-34 in the presence and absence of ligands

DTT-treated HSA (100 μM) was incubated with 0.5 mM Ellman's reagent, DTNB, for 60 min at room temperature. During incubation, the absorbance of the samples was monitored at 405 nm. The accessibility was evaluated as A_5/A_{60}, where A_5 and A_{60} is the sample absorbance after 5 min and 60 min (maximal absorbance), respectively, of incubation with DTNB [14].

SH content of Cys-34 in the presence and absence of ligands

The amount of free SH-groups was also estimated with Ellman's reagent, DTNB. Briefly, HSA (100 μM) was incubated with 0.5 mM DTNB for 60 min at room temperature, and afterwards the absorbance at 405 nm was measured. The thiol concentration was calculated from a standard curve prepared with cysteine.

Oxidation of Cys-34 and protein polymerization by heat treatment in the presence and absence of ligands

DTT-treated HSA (100 μM) without or with ligand (5 mol/mol HSA) was incubated in PBS (pH 7.4) for 15 or 60 min at 60°C. The mercaptalbumin fraction was determined from a standard curve prepared with cysteine using the DTNB assay. Any changes in molecular weight and aggregation of heated HSA samples were visualized by non-reduced 10% SDS-PAGE with 3 μg of protein in each lane.

S-nitrosation of HSA in the presence and absence of ligands

SNO-HSA was prepared with protection against light and according to previous reports [15,16]. Briefly, DTT-treated HSA (100 μM) without or with ligand (5 mol/mol HSA) or the original solution from Chemo-Sera-Therapeutic Research Institute was incubated with GS-NO as NO donor (molar ratio, protein:NO donor = 1:5) in PBS for 1, 15 or 60 min at 37°C. To remove the NO donor, the *S*-nitrosated products were applied to a Sephadex G-25 column, eluted with PBS containing 0.5 mM DTPA, and concentrated by ultrafiltration. These samples were stored at -80°C until analyzed. For the *in vivo* ischemia-reperfusion studies, the SNO-HSA samples with and without ligand were adjusted to the same *S*-nitrosation efficiency by means of different incubation times with GS-NO. Actually, the HSA samples with or without ligand were incubated with GS-NO for 1 or 60 min, respectively.

Determination of S-nitrosation efficiency

The amounts of the *S*-nitroso moiety of SNO-HSA were quantified by HPLC coupled with a flow-reactor system, as previously reported [15,17]. The HPLC column was a gel filtration column for *S*-nitrosated proteins (ϕ 8 × 300 mm), Diol-120, YMC, Kyoto, Japan. Briefly, the eluate from the HPLC column was mixed with a $HgCl_2$ solution to decompose SNO compounds to yield NO_2^- (via NO^+). The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system.

CD spectroscopy

CD spectra were measured at 25°C using a J-720-type spectropolarimeter (JASCO, Tokyo, Japan). Far-UV and near-UV spectra were recorded at protein concentrations of 5 μM and 15 μM, respectively, in 20 mM sodium phosphate buffer (pH 7.4). For calculation of mean residue ellipticity, [θ], the molecular weight of the albumins was taken as 66,500.

Cytoprotective effect of SNO-HSA in vivo

A rat ischemia-reperfusion liver injury model served for investigation of the cytoprotective effect of SNO-HSA, according to a previous report [16]. Male Wistar rats weighing between 200 and 230 g (Kyudo, Inc., Kumamoto, Japan) were used. The animals were fasted for 9 h before surgery but were allowed access to water. The rats were anesthetized with ether during the operation. After the abdomen was shaved and disinfected with 70% ethanol, a complete midline incision was made. The portal vein and hepatic artery were exposed and cross-clamped for 30 min with a noncrushing microvascular clip. Saline, as the vehicle control, GS-NO or DTT-treated HSA without or with *S*-nitrosation or the original formulation without or with *S*-nitrosation (0.1 μmol protein (0.03 μmol SNO)/rat) were given via the portal vein immediately after reperfusion was initiated. Then the abdomen was closed in two layers with 2-0 silk. The rats were kept under warming lamps until they awakened and became active. Because blood loss caused by frequent blood sampling could affect liver functions, the animals were sacrificed by taking whole circulating blood via abdominal aorta under anesthesia at various time points after reperfusion was initiated. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were

measured by using a sequential multiple AutoAnalyzer system from Wako Chemicals, with activities expressed in international units per liter. All animal experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Graduate School of Medical Sciences, Kumamoto University.

Purification of intracellular proteins and Western blot analysis

Frozen liver samples, originally taken 6 h after hepatic ischemia-reperfusion, were homogenized in a lysis buffer (50 mM HEPES, 5 mM EDTA, and 50 mM NaCl; pH 7.5) containing protease inhibitors (10 mg/mL aprotinin, 1 mM PMSF, and 10 mg/mL leupeptin). Supernatant was taken after centrifugation at 15,000 × g. Equal amounts of protein from the supernatant were loaded onto 12.5% SDS-PAGE gels and blotted onto polyvinylidine difluoride membranes (Millipore). Polyclonal antibody against rat heme oxygenase-1 (HO-1; StressGen) and horseradish conjugated secondary antibody (Jackson ImmunoResearch) were used at 1:2,000 and 1:5,000 dilutions, respectively. The ECL Western blotting system (Amersham Life Sciences) was used for detection. Bands were visualized and quantified using a lumino-analyzer (LAS-1000, Fuji photo Film). Density of the bands for HO-1 and β-actin was quantitatively analyzed using the NIH Image J Software.

Statistical Analysis

The statistical significance of collected data was evaluated using the ANOVA analysis followed by Newman–Keuls method for more than 2 means. Differences between groups were evaluated by the Student's t test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of ligand binding on the SH-accessibility of Cys-34

The effect of CA and/or N-AcTrp binding on the SH-accessibility of defatted, DTT-treated HSA was evaluated by the DTNB assay. Binding of 1-5 mol CA/mol HSA increased significantly and in a dose-dependent manner the accessibility of the SH-group (Fig. 2A). By contrast, addition of 5 mol of N-AcTrp per mol of HSA had no significant effect on SH accessibility (Fig. 2B). Furthermore, addition of N-AcTrp had no influence on the improving effect of CA (Fig. 2B).

Protective effect of ligands on oxidation and aggregation of mercaptalbumin exposed to heat treatment

Defatted, DTT-treated HSA without and with ligands (N-AcTrp and/or CA) was incubated at 60°C. Heat incubation was used, because in this way we could study both thiol oxidation and protein aggregation [11,18,19]. From Table 1 it is seen that N-AcTrp is an effective protector of the sulfhydryl group against heat-facilitated oxidation; less than 10% of the groups have been oxidized after 60 min of incubation. By contrast, binding of CA increases oxidation; after 60 min of heat treatment ca. 75% of Cys-34 have been oxidized, whereas only ca. 35% of the groups have been modified in the absence of ligands. Finally, simultaneous binding of CA diminishes somewhat the protective effect of N-AcTrp. The increasing effect of CA on oxidation is most probably due to the fact that CA binding increases the accessibility of Cys-34 (see above). Often oxidation of cysteine residues is accompanied by protein aggregation. Whether or not this has taken place in our heat-treated HSA samples was analyzed for by using non-reduced SDS-PAGE (Fig. 3). Lane 1 shows no clear presence of light or heavy aggregated HSA in the non-heated sample. Heating for 15 min at 60°C actually results in formation of both types of aggregations (lane 2), and heating for 60 min results in a major fraction of heavy aggregation (lane 6). Addition of N-AcTrp diminishes aggregation (lanes 3 and 7); especially the formation of heavy aggregation is strongly depressed. By contrast, binding of CA has no inhibitory effect on aggregation (lanes 4 and 8). Interestingly, apparently CA has no influence on the protective effect of N-AcTrp (lanes 5 and 9). Thus, N-AcTrp, but not CA, minimizes HSA aggregation and protects Cys-34 against oxidation.

Improving effects of ligand binding on S-nitrosation of HSA

Now, we investigated whether an increased accessibility to Cys-34 (caused by CA binding) and/or protection of the residue against oxidation and aggregation (caused by N-AcTrp binding) can promote *S*-nitrosation of HSA. Incubation of defatted, DTT-treated HSA alone with GS-NO for 60 min resulted in the formation of 0.31 mol *S*-nitroso moieties per mol of HSA (Table 2). This value, and those determined at 1 and 15 min of incubation, is not changed by addition of N-AcTrp. By contrast, at all incubation times CA significantly increased *S*-nitrosation. Simultaneous addition of both ligands increased this effect further, and after 1 min of incubation the number of *S*-nitroso moieties (0.42 mol SNO/mol HSA) is even higher than that obtained after 60 min of HSA without ligand. Thus, co-binding of CA and N-AcTrp results in efficient and fast *S*-nitrosation of HSA. HSA formulations for clinical applications usually contain high concentrations of CA and N-AcTrp for stabilizing and protecting the protein against oxidation during the heat treatment performed for destroying potential contamination with viral or other biological agents [11]. We used the solutions from Chemo-Sera-Therapeutic Research Institute as an illustrative example, and this formulation has 5-6 mol of each of the two ligands per mol of HSA. Furthermore, it has a high percentage of mercaptalbumin, i.e. 71% ± 8% (n = 6). Therefore, we investigated whether a simple incubation of the formulation with GS-NO would result in significant amounts of SNO-HSA. As seen from Table 2, this one-step procedure also resulted in an efficient and fast *S*-nitrosation of HSA.

Physicochemical properties and stability of S-nitrosated HSA

According to reduced and non-reduced SDS-PAGE, the one-step *S*-nitrosation of formulation HSA did not result in the formation of dimer via disulfide bond formation, fragmentation or other gross conformational changes (Fig. 4A). Any minor conformational changes were examined for by using circular dichroism spectroscopy. The far-UV spectra (Fig. 4B) and the near-UV spectra (Fig. 4C) show that *S*-nitrosation of Cys-34 had no evident effect on the secondary or tertiary structure of HSA. Previous examinations with non-reducing SDS-PAGE revealed that *S*-nitrosation of defatted, DTT-treated HSA only results in, if any, changes in the tertiary structure of the protein [9]. Thus, our data propose that *S*-nitrosation does not induce significant conformational changes in any of our protein preparations. The stability of SNO-HSA with 5 mol of CA and N-AcTrp per mol of protein was tested in two different ways. First, the half-life of the *S*-nitroso moiety of SNO-HSA with the

ligands was 50 days in phosphate buffer, pH 7.4, in the dark. Without the ligands the half-life was only 25 days. Second, lyophilization with CA and N-AcTrp resulted in only a slight decrease (10%) in *S*-nitroso content. Thus, SNO-HSA with CA and N-AcTrp is very stable.

Cytoprotective effect of SNO-HSA against ischemia-reperfusion injury

We tested the biological usefulness of the one-step SNO-HSA preparation by studying its cytoprotective effect in an ischemia-reperfusion liver injury model in rats [9,16,20]. Previous studies with SNO-HSA showed that a quantity of 0.1 μmol protein/rat was most suitable for this kind of experiment [9]. Therefore, we used the same quantity of SNO-HSA in this study. To evaluate liver injury, we measured the extracellular release of the liver enzymes ALT and AST via plasma enzyme values. Without adding albumin, the ALT-values increased to a maximum at 1 h after reperfusion. After 2 h the value was slightly lower and then decreased further and gradually during 24 h. Principally the same results were obtained for AST (data not shown). Administration of DTT-treated HSA, of the original HSA formulation or of GS-NO at the beginning of reperfusion did not modify liver damage (Fig. 5A). However, a significant reduction in the release of ALT was observed in rats treated with SNO-HSA, which had been made by incubating defatted, DTT-treated HSA (5 mol CA and N-AcTrp/mol HSA) with GS-NO for 60 min. The same, or even better, cytoprotection was obtained after injecting SNO-HSA, which had been made by incubating the original HSA formulation with GS-NO for only 1 min. Again, principally the same results were obtained for AST (data not shown). We have also determined the expression of the intracellular, cytoprotective enzyme HO-1 in the liver cells 6 h after reperfusion. Fig. 5B shows that if DTT-treated HSA, HSA formulation or GS-NO was injected at the beginning of reperfusion, a pronounced induction of HO-1 took place. However, the most pronounced induction took place after injection of SNO-HSA. As an internal control we determined the amount of β-actin (Fig. 5B). The density of the HO-1 and β-actin bands were quantified and related to each other. Also this type of analysis showed that administration of SNO-HSA resulted in a very pronounced induction of HO-1. The HO-1 expression increased as follows: DTT-treated HSA = HSA formulation < GS-NO < SNO-HSA, which had been made by incubating DTT-treated HSA (5 mol CA and N-AcTrp/mol HSA) with GS-NO for 60 min < SNO-HSA, which had been made by incubating the original HSA formulation with GS-NO for only 1 min. Thus, in addition to *S*-nitrosation, binding of CA and

N-AcTrp improved the cytoprotective effect. *S*-nitrosothiols such as SNO-HSA can exert cytoprotective effects in different ways. In addition to inducing HO-1, the effect can be brought about by, for example, maintenance of tissue blood flow, suppression of neutrophil infiltration and reduction of apoptosis in the liver [16].

DISCUSSION

SNO-HSA has been shown to be cytoprotective against free radical mediated damage and microvascular injury associated with ischemia-reperfusion or hemorrhagic shock as well as acute lung injury in a murine model of sickle cell disease. Therefore, SNO-HSA is under investigation as a therapeutic agent in humans. However, existing methods for making SNO-HSA preparations are complicated and time-consuming, see the illustrative overview in Fig. 1. In the first parts of this work, we observed that binding of CA and N-AcTrp resulted in a very pronounced increment in *S*-nitrosation of HSA when incubated with GS-NO. The increment is most probably caused by an easier access of GS-NO to the sulfhydryl group of Cys-34 and protection of the residue against oxidation. In addition, the presence of the ligands resulted in an increased stability of the *S*-nitrosated product. We have previously found that binding of oleic acid resulted in an almost linear increment in the reactivity of DTNB to Cys-34 [12]. Thus, even though oleic acid and CA most probably have both common and private binding sites [21], they exert principally the same effect on the accessibility of Cys-34. Because none of the fatty acids bind to Cys-34, their effects must be due to binding-induced conformational changes of HSA making Cys-34 more accessible to electrophilic molecules [12,14]. These findings show that fatty acid binding can ease the access to the sulfhydryl group of Cys-34 and thereby improve the *S*-nitrosation of HSA. HSA solutions for clinical use contain high concentrations of CA and N-AcTrp as stabilizers. Therefore, we decided to test, whether such formulations could be turned into biological useful SNO-HSA preparations by a simple one-step procedure, namely by incubation with GS-NO (Fig. 1). This was found to be the case, because incubation with GS-NO for only 1 min *S*-nitrosated about one third of the albumin. It is not necessary to remove any unreacted GS-NO from the product by, e.g., gel filtration, because GS-NO is eliminated fast and apparently without secondary effects from the circulation [9]. We tested the biological usefulness of the preparation by studying its cytoprotective effect in an ischemia-reperfusion liver injury model. The results showed that it had a superior biological activity, and that CA and N-AcTrp binding improved the effect. The present findings can probably be of practical use, because instead of giving patients a simple HSA formulation, the formulation can easily and fast be upgraded to a solution with a high concentration of SNO-HSA, which could be of greater help to the patient and have a broader application. The clinical development of SNO-HSA as a strong cytoprotecting agent is under way using our simple method in collaboration with clinicians

of hospital and developers of drug industry.

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Figure Legends

Figure 1. **Mechanism of a novel SNO-HSA preparation strategy using HSA formulation and GS-NO**

The conventional method (A) contains two time-consuming steps (SH reduction and NO addition), which take more than 4-6 h to complete. On the other hand, our new method (B) contains only one step (GS-NO incubation), which takes less than 1 min. In case of our method, CA and N-AcTrp contained in HSA formulations play the roles of not only stabilizers but also as a Cys-34 accessible inducer and a Cys-34 oxidation inhibitor, respectively. The presence of these two ligands makes it possible to *S*-nitrosate HSA within 1 min.

Figure 2. **Effect of ligand binding on the SH-accessibility of Cys-34**

The effect of CA (CA/HSA = 0, 1, 2, 3, 4, 5) on the SH-accessibility of defatted, DTT-treated HSA was studied in the absence (A) and presence of N-AcTrp (N-AcTrp/HSA = 5) (B). DTT-treated HSA (100 μM) was incubated with 0.5 mM Ellman's reagent, DTNB, for 60 min at room temperature. The accessibility was evaluated as the ratio of DTNB absorbance at 405 nm after 5 and 60 min of incubation (A_5/A_{60}). A5 and A60 is the sample absorbance at 405 nm after 5 min (initial rate period) and 60 min (maximal absorbancy), respectively, of incubation with DTNB. Data are expressed as means ± SEM (n = 4). *, $p < 0.05$, **, $p < 0.01$, compared with control (CA = 0).

Figure 3. **Effect of ligands on aggregation of heat-treated mercaptalbumin**

Defatted, DTT-treated HSA without or with ligand (5 mol/mol HSA) was incubated in PBS (pH 7.4) for 15 or 60 min at 60 °C. Afterwards, protein samples (3 μg) were analyzed by non-reduced 10% SDS-PAGE; the gel was stained by CBB. Lane 1 represents non-heated HSA. The arrows indicate heavy aggregation, light aggregation, dimer and monomer from the top.

Figure 4. **Structural integrity of HSA and SNO-HSA**

A, Reduced and non-reduced SDS-PAGE of HSA and SNO-HSA. 1 μg of protein was added to each lane, and the gel was stained by CBB. Molecular mass markers are indicated at the left of the gel. B and C show far-UV and near-UV CD spectra, respectively, of HSA and SNO-HSA. The

proteins were unmodified and *S*-nitrosated HSA formulation.

Figure 5. **Serum levels of ALT and Western blot of HO-1 and β-actin in liver cells after hepatic ischemia-reperfusion in rats**

A, Saline (control), HSA, HSA formulation, SNO-HSA, SNO-HSA formulation or GS-NO (the same amount of SNO administration as with SNO-HSA) was injected at the beginning of reperfusion (0 min), and the activities of ALT were measured at 60 min after reperfusion. B, Western blot of HO-1 and β-actin in liver cells 6 h after hepatic ischemia-reperfusion. I/R− are results from rats, which had not been subject to ischemia-reperfusion. The density of the bands for HO-1 and β-actin was quantitatively analyzed using the NIH Image J Software. Data are expressed as means ± SEM (n = 4). *, $p < 0.05$, **, $p < 0.01$, compared with I/R−. $^{\#}$, $p < 0.05$, compared with SNO-HSA.