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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 50, pp. 34966–34975, December 12, 2008

S-Nitrosylated Human Serum Albumin-mediated Cytoprotective Activity Is Enhanced by Fatty Acid Binding*

Received for publication, September 10, 2008, and in revised form, October 20, 2008 Published, JBC Papers in Press, October 20, 2008, DOI 10.1074/jbc.M807009200

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Binding of oleate to *S*-nitrosylated human serum albumin (SNO-HSA) enhances its cytoprotective effect on liver cells in a rat ischemia/reperfusion model. It enhances the antiapoptotic effect of SNO-HSA on HepG2 cells exposed to anti-Fas antibody. To identify some of the reasons for the increased cytoprotective effects, additional experiments were performed with glutathione and HepG2 cells. As indicated by 5,5′-dithiobis-2-nitrobenzoic acid binding, the addition of oleate increased the accessibility of the single thiol group of albumin. Binding of increasing amounts of oleate resulted in increasing and more rapid *S*-transnitrosation of glutathione. Likewise, binding of oleate, or of a mixture of endogenous fatty acids, improved *S*-denitrosation of SNO-HSA by HepG2 cells. Oleate also enhanced *S*-transnitrosation by HepG2 cells, as detected by intracellular fluorescence of diaminofluorescein-FM. All of the *S*-transnitrosation caused by oleate binding was blocked by filipin III. Oleate also increased, in a dose-dependent manner, the binding of SNO-HSA labeled with fluorescein isothiocyanate to the surface of the hepatocytes. A model in two parts was worked out for *S*-transnitrosation, which does not involve low molecular weight thiols. Fatty acid binding facilitates *S*-denitrosation of SNO-HSA, increases its binding to HepG2 cells and greatly increases *S*-transnitrosation by hepatocytes in a way that is sensitive to filipin III. A small nitric oxide transfer takes place in a slow system, which is unaffected by fatty acid binding to SNO-HSA and not influenced by filipin III. Thus, fatty acids could be a novel type of mediator for *S*-transnitrosation.

S-Nitrosothiols may serve as a reservoir of NO in biological systems, and they represent a class of NO donor with many potential biological and clinical uses. In this respect, Cys-34 of human serum albumin (HSA)[2] is important, because it represents the largest fraction of free thiols in circulation (1). In accordance with this proposal, *S*-nitrosylated HSA (SNO-HSA) has been reported to improve systolic and diastolic function, as well as myocardial perfusion and oxygen metabolism, in pigs during reperfusion after severe myocardial ischemia (2, 3) and to reduce ischemia/reperfusion injury in rabbit skeletal muscle (4) and rat liver (5). Among its other beneficial effects, SNO-HSA inhibits the activation of circulating platelets (6), suppresses apoptosis of human promonocytic cells (5), and exhibits antibacterial activity *in vitro* (5).

HSA is a multifunctional protein synthesized and secreted by liver cells. It is a single, non-glycosylated polypeptide that organizes to form a heart-shaped protein with ~67% α-helix but no β-sheet (1). All but one (Cys-34) of the 35 cysteine residues are involved in the formation of stabilizing disulfide bonds. In circulation, approximately half of HSA contains Cys-34 as a free sulfhydryl, whereas the remainder is oxidized or ligand-bound. In addition to forming *S*-nitrosothiols, HSA can interact reversibly with a large number of endogenous and exogenous ligands (1, 7, 8). Thus, one of the important *in vivo* functions of albumin is to transport fatty acids, and usually the protein carries different fatty acid anions, up to a total amount of 1–2 molar equivalents. However, this value can rise to about 4 after maximal exercise or other adrenergic stimulation (1).

In the present work, the effect of oleate (OA) on *S*-transnitrosation from SNO-HSA was studied. OA was used as a representative for the endogenous fatty acids, because quantitatively it is the most important fatty acid in human depot fat, and because it is a major contributor to the albumin-bound fatty acids. First, it was observed that co-binding of OA improved SNO-HSA-mediated cytoprotection against ischemia/reperfusion liver injury in rats and improved its antiapoptotic effect on human hepatocellular carcinoma (HepG2) cells exposed to anti-Fas antibody. In an attempt to explain these effects, *S*-transnitrosation was then investigated in simpler systems, namely from SNO-HSA to glutathione (GSH) and to HepG2 cells. The effect of a mixture of endogenous fatty acids on the latter *S*-transnitrosation was also studied. The influence of OA binding on NO transfer to the HepG2 cells and on the interaction

* This work was supported in part by grants-in-aid from the Japan Society for the Promotion of Science (JSPS), a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology (18390051), Japan, and by Fonden af 1870. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: HSA, human serum albumin; SNO-HSA, *S*-nitrosylated HSA; GS-NO, *S*-nitrosylated GSH; OA, oleic acid/oleate; ALT, alanine aminotransferase; AST, asparatate aminotransferase; DAF-FM DA, diaminofluorescein-FM diacetate; FITC, fluorescein isothiocyanate; DTT, 1,4-dithiothreitol; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DTPA, diethylenetriaminepentaacetic acid; IAN, isoamyl nitrite; DMEM, Dulbecco's modified Eagle's medium.

between SNO-HSA and the hepatocytes was examined. Finally, a model for the OA-induced improvement of NO transfer to HepG2 cells is proposed.

EXPERIMENTAL PROCEDURES

Materials—Non-defatted HSA (96% pure) was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan), and it was defatted by treatment with charcoal as described by Chen (9). Sephadex G-25 (ϕ 1.6 × 2.5 cm) and Blue Sepharose CL-6B (ϕ 2.5 × 20 cm) were from GE Healthcare (Tokyo, Japan). OA, caprylate, stearate, GSH, 1,4-dithiothreitol (DTT), and filipin III were purchased from Sigma-Aldrich. Isoamyl nitrite (IAN) was purchased from Wako Chemicals (Osaka, Japan). Sulfanilamide, naphthylethylenediamine-hydrochloride, $HgCl_2$, and $NaNO_2$ were obtained from Nakalai Tesque (Kyoto, Japan). Diethylenetriaminepentaacetic acid (DTPA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and fluorescein isothiocyanate (FITC) were bought from Dojindo Laboratories (Kumamoto, Japan). Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (Rockville, MD), and diaminofluorescein-FM diacetate (DAF-FM DA) was from Daiichi Pure Chemicals (Tokyo, Japan). $^{111}InCl_3$ (74 MBq/ml in 0.02 N HCl) was a gift from Nihon Medi-Physics Co., Ltd. (Hyogo, Japan). The other chemicals were of the best grades commercially available, and all solutions were made in deionized and distilled water.

S-Nitrosylation of HSA—*S*-Nitrosylated protein was prepared with protection against light and according to previously reported methods (10, 11). First, HSA (300 μM) was incubated with DTT (molar ratio, protein:DTT = 1:10) for 5 min at 37 °C. After incubation, DTT was immediately removed by Sephadex G-25 gel filtration and eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA. Samples of 0.1 mM DTT-treated protein (0.8 mol sulfhydryl groups/mol protein) were then incubated with IAN (molar ratio, protein:IAN = 1:10) in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA for 60 min at 37 °C. The amount of the *S*-nitroso moiety of SNO-HSA was quantified by HPLC coupled with a flow-reactor system, as previously reported (10). The HPLC column was a gel filtration column for *S*-nitrosylated 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 *S*-nitrosylated compounds to yield NO_2^- (via NO^+). The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system. Controls performed in the absence of $HgCl_2$ gave no protein-derived absorbency at 540 nm after reaction with the Griess reagent. Therefore, non-covalent association of the nitrite anion with albumin can be excluded. The *S*-nitrosylated product (0.35 ± 0.04 mol SNO-groups/mol protein; mean ± S.E., $n = 53$) was purified by Sephadex G-25 gel filtration, eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA, and concentrated by ultrafiltration (cutoff size of 7500 Da). These samples were stored at −80 °C until use. The protein content of all protein preparations used in this study was determined using the Bradford assay.

Binding of Fatty Acid to HSA—A stock solution of 20 mM OA was made in methanol-H_2O (1:1, v/v). Aliquots of the OA stock solution were added to HSA or SNO-HSA and dissolved in 0.1 M potassium phosphate buffer (pH 7.4) to give OA:HSA molar ratios of 1, 3, or 5; the maximal methanol concentration in the final solutions was 1.25%. Before use, the solutions were held at 37 °C for 30 min. The amount of bound OA was checked using the following approach. The solutions containing albumin and OA were applied to a Sephadex G-25 gel filtration column, the protein-containing fractions were pooled, and both the protein and OA concentrations in the pooled material were determined. The OA concentrations were determined by a colorimetric method using a commercial kit (WAKO NEFA kit) according to the manufacturer's instructions. Three standards (0.5, 1.0, and 1.97 mEq/liter) supplied by the vendor were used to establish a standard curve for determination of the concentration of OA.

Cytoprotective Effect of SNO-HSAs in Vivo—A rat ischemia/reperfusion liver injury model was used to investigate the cytoprotective effect of SNO-HSA, as previously reported (5, 11). 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, or HSA or SNO-HSA (0.1 μmol/rat) with or without bound OA was given via the portal vein immediately after reperfusion was initiated. Because the blood volume of a 200-g rat was estimated to be about 10 ml, we expected blood levels of SNO-HSA to reach ~3 μM after administration of 0.1 μmol/rat of SNO-HSA. This concentration of SNO-HSA is a physiologically relevant one, because levels of *S*-nitrosothiols in normal plasma are at the most 7 μM (12). The abdomen was then 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 function, the animals were euthanized by taking whole circulating blood via the 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 the animal experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Graduate School of Medical Sciences, Kumamoto University.

Antiapoptotic Effect of SNO-HSA in Vitro—HepG2 cells (2×10^5 cells/well) were cultured in 96-well plates (16-mm diameter; Falcon, Lincoln Park, NJ) with DMEM supplemented with 10% fetal bovine serum (Invitrogen). The cells were maintained in a humidified incubator (95% air, 5% CO_2) for 12 h at pH 7.4 and 37 °C. Afterwards, they were treated with different concentrations of HSA or SNO-HSA, with and without bound OA, for 6 h in the dark. The cells were then washed three times with 10 mM phosphate-buffered saline (pH 7.4) to remove the remaining SNO-HSA. After washing, the cells were reacted with 400 ng/ml anti-Fas antibody (Medical and Biological Laboratories,

Nagoya, Japan). After 15 h of incubation, the cultures were treated with 0.05% trypsin, and the cells were transferred to Eppendorf tubes (1.5 ml). The number of apoptotic cells was determined by an annexin V-FITC binding assay kit from BD Biosciences (Tokyo, Japan). The fluorescence of annexin V-FITC and propidium iodide were measured by a FACSCalibur flow cytometer.

Pharmacokinetic Experiments—SNO-HSA, with and without OA, was labeled with ^{111}In, using DTPA anhydride as a bifunctional chelating agent (13, 14). Labeled proteins were injected via the tail vein into male ddY mice (weighing 25–27 g) at a dose of 0.1 mg/kg. At appropriate times after injection, blood was collected from the vena cava with the mouse under ether anesthesia. Heparin sulfate was used as an anticoagulant, and plasma was obtained from the blood by centrifugation. Liver, kidney, and spleen samples were obtained, rinsed with saline, and weighed. The radioactivity in each sample was counted using a well-type NaI scintillation counter ARC-2000 (Aloka, Tokyo, Japan). ^{111}In radioactivity concentrations in plasma were normalized as a percentage of the dose per milliliter and analyzed using the nonlinear least-squares program MULTI (15). Organ distribution profiles were evaluated by relating the radioactivity per gram of tissue to the total amount of injected radioactivity.

Accessibility of Cys-34—We determined the accessibility of Cys-34 in reduced HSA with Ellman's reagent, DTNB (16, 17). HSA (300 μM), with and without OA, and DTNB (5 mM) were mixed in 0.1 M potassium phosphate buffer (pH 7.0) at 20 °C, and the absorbance at 450 nm was registered as a function of time.

S-Denitrosylation of SNO-HSA by GSH and by HepG2 Cells—Solutions with both 100 μM SNO-HSA and 100 μM GSH were made in 10 mM phosphate-buffered saline, pH 7.4. Then 0-, 7.5-, 15-, and 30-min samples were taken, mixed with 1/10 volume of 5 mM DTPA (pH 7.4) and placed at −80 °C. The concentrations of the remaining SNO-HSA and the *S*-nitrosylated GSH (GS-NO) formed were then determined separately by the HPLC flow reactor system.

HepG2 cells (5×10^5 cells/well) were cultured and incubated with DMEM and fetal bovine serum, as described above. After incubation, the culture medium was removed, and the hepatocytes were washed three times with 10 mM phosphate-buffered saline, pH 7.4. Cells were further incubated at 37 °C in the CO_2 incubator with 200 μl of 10 mM phosphate-buffered saline, pH 7.4 and 100 μM SNO-HSA with different molar ratios of bound OA. Samples were taken after incubation for 0, 15, 30, or 60 min, mixed with 1/10 volume of 5 mM DTPA, pH 7.4 and centrifuged at 10,000 × *g* for 10 min at 4 °C. These supernatants were stored at −80 °C until applied to the HPLC flow reactor system.

HSA Obtained from Hemodialysis Patients—It is known that fatty acids bound to HSA rise by dialysis. The content of fatty acids bound to HSA isolated from hemodialysis patients before and after dialysis was analyzed, and the influence on *S*-denitrosation of fatty acid binding was examined. HSA samples were obtained from hemodialysis patients before (HSA-hd (−)) and after dialysis (HSA-hd (+)) according to a previously reported protocol (18). In brief, albumin concentrations in blood plasma were measured using a diagnostic kit (Biotech Reagent) based on the bromcresol green method. Non-esterified fatty acids were measured using a diagnostic kit from Wako Chemicals (Osaka, Japan). To isolate HSA from patient sera, polyethylene glycol fractionation of blood plasma was followed by chromatography on a Blue Sepharose CL-6B column. After isolation, the samples were dialyzed against deionized water for 48 h at 4 °C, followed by lyophilization. The purity of the HSA-hd samples was at least 95%, and the percentage of dimers did not exceed 7%, as evidenced by SDS-PAGE and native PAGE, respectively. The protocol used in this study was approved by the Institutional Review Board, and informed consent was obtained from all subjects.

Preparation of S-Nitrosylated HSA-hd—HSA-hd (−) or HSA-hd (+) was *S*-nitrosylated using IAN, as described above. The content of SNO groups in SNO-HSA-hd was determined to be (0.35 ± 0.04 for HSA-hd (−) or 0.39 ± 0.06 for HSA-hd (+) mol SNO-groups/mol protein; mean ± S.E., $n = 7$).

S-Denitrosylation of Hemodialysis Patient SNO-HSA by HepG2 Cells: Effects of HSA-bound Fatty Acids—One-hundred micromolar of SNO-HSA-hd (−) and SNO-HSA-hd (+) dissolved in phosphate-buffered saline was incubated with HepG2 cells (5×10^5 cells/well). After 15-, 30-, and 60-min incubation, culture supernatant was collected, and the remaining SNO content was measured and compared with control SNO-HSA samples before incubation.

NO Uptake by HepG2 Cells—HepG2 cells (5×10^5 cells/well) were cultured and incubated with DMEM and fetal bovine serum, as described above. After incubation, the culture medium was removed, and the hepatocytes were washed three times with 10 mM phosphate-buffered saline, pH 7.4. After adding phosphate-buffered saline containing 5 μM DAF-FM DA, the cells were incubated for 1 h in the dark at 37 °C. The cells were again washed three times with phosphate-buffered saline, and further incubated at 37 °C with 100 μM HSA or SNO-HSA with different molar ratios of bound OA. Upon cellular uptake, ester groups in DAF-FM DA are cleaved by esterases, resulting in the formation of DAF-FM that can react with NO to give fluorescence. The fluorescence was determined with excitation at 385 nm and monitored at 535 nm using a monochromator (TECAN SPECTRA FLUOR). In some experiments, 50 μM filipin III (caveolae formation inhibitor) dissolved in phosphate-buffered saline was added after 30 min of DAF-FM DA exposure (19).

Binding of FITC-HSA to HepG2 Cells—The binding of HSA to HepG2 cells, with or without *S*-nitrosylation and its modulation by fatty acid binding, was analyzed by means of fluorescent microscopy with HSA labeled with FITC. FITC-HSA was prepared according to a previous report (20). In short, HSA (60 μM) was incubated with FITC (2 mM) for 3 h at 25 °C in 0.1 M potassium phosphate buffer (pH 7.4). After incubation, FITC-HSA was isolated from unreacted FITC using a Sephadex G-25 gel with the phosphate buffer, and stored at −80 °C until use. To prepare FITC-SNO-HSA, HSA was first *S*-nitrosylated as described above, followed by FITC labeling. After purification of FITC-SNO-HSA, the SNO content was determined to be (0.33 ± 0.03 mol SNO-groups/mol protein; mean ± S.E., $n = 3$),

suggesting that FITC labeling did not affect the SNO content to a significant extent.

For the binding assay, HepG2 cells (5×10^5 cells/well) were preincubated with serum-free DMEM for 2 h at 37 °C. In some experiments, cells were further treated with 50 μM filipin III for 30 min, which inhibits caveolae formation. Cells were then maintained at 4 °C to block the uptake of macromolecules through energy-dependent mechanisms, including endocytosis. FITC-SNO-HSA with varying OA content (0, 3, 5 OA/HSA) was dissolved in 10 mM phosphate-buffered saline, pH 7.4 and added to the culture wells to give a final concentration of 50 μg/ml. After 10 min, the cells were washed twice with phosphate-buffered saline to remove unbound FITC-SNO-HSA and fixed with 4% paraformaldehyde in phosphate-buffered saline at 25 °C for 30 min. After washing twice in MilliQ water, these cells were analyzed using a fluorescence microscope (Biozero-8000, Keyence, Osaka, Japan) with the combination of excitation at 385 nm and emission at 535 nm. Fluorescent intensity was quantified using an NIH Image. Similar experiments were conducted using FITC-HSA without *S*-nitrosylation.

Statistical Analysis—The statistical significance of the collected data were evaluated using analysis of variance, followed by the Newman-Keuls method for more than 2 means. Data are expressed as means ± S.E. Differences between groups were evaluated using a Student's *t* test. $p < 0.05$ was regarded as statistically significant.

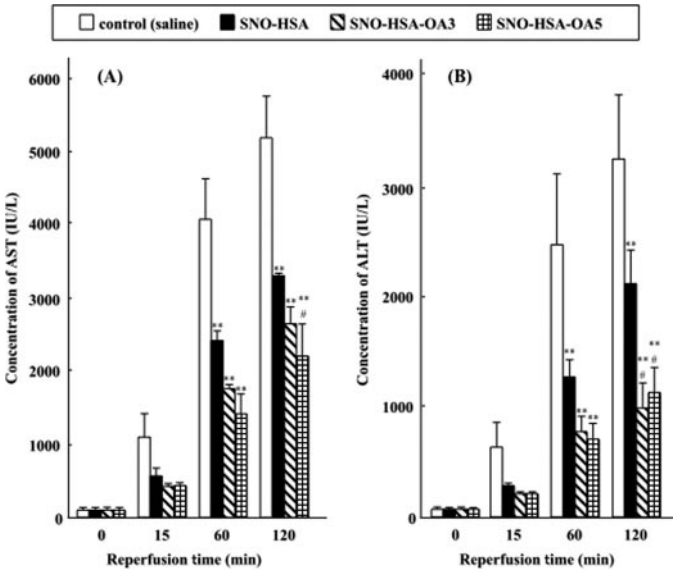

FIGURE 1. **Time profile of changes in serum levels of AST (*A*) and ALT (*B*) after hepatic ischemia/reperfusion in rats.** Ischemia was induced by occluding both the portal vein and hepatic artery for 30 min. After that period of time reperfusion was established. Control (saline), 0.1 μmol of SNO-HSA per rat, or 0.1 μmol of SNO-HSA-OA (OA/HSA = 3 or 5) per rat was administered via the portal vein immediately after initiation of reperfusion. Blood was collected from the portal vein at various time points after reperfusion. ALT and AST activities were measured by using a sequential multiple AutoAnalyzer system. Data are expressed as means ± S.E. ($n = 4$ at each time point). *, $p < 0.05$ and **, $p < 0.01$, compared with control. #, $p < 0.05$, compared with the SNO-HSA-treated group. (OA/HSA = 3 or 5: HSA with 3 or 5 bound OA molecules per protein molecule.)

RESULTS

Effect of OA Binding on SNO-HSA-mediated Cytoprotection against Ischemia/Reperfusion Liver Injury in Rats—An ischemia/reperfusion liver injury model (5) was used to examine the *in vivo* effect of OA binding on SNO-HSA-mediated cytoprotection. Because previous studies with SNO-HSA showed that a quantity of 0.1 μmol/rat had the greatest protective effect (5), the same quantity was used in this study. To evaluate liver injury, the extracellular release of the liver enzymes AST and ALT was measured via plasma enzyme values. Injecting HSA, OA, or HSA-OA into the portal vein immediately after reperfusion was initiated did not affect the plasma concentrations of AST and ALT (data not shown). However, administration of SNO-HSA diminished, to a significant extent, the enzyme concentrations measured after 60 and 120 min (Fig. 1). The protection of the liver cells by SNO-HSA was more pronounced if the protein also carried OA; the additive effect was most evident after 120 min of reperfusion. The effect of OA on SNO-HSA-mediated cytoprotection appears to depend on OA content, *e.g.* AST release was reduced significantly more by treatment with SNO-HSA-OA5 than with SNO-HSA-OA3 120 min after reperfusion (Fig. 1*A*). Even at 12 h after reperfusion, the levels of AST and ALT remained significantly lower in SNO-HSA-OA-treated animals than in control animals, although the differences were not so pronounced as those observed at 120 min after reperfusion (data not shown). We also examined the effect of other fatty acid on cytoprotective effect for SNO-HSA. In the ischemia/reperfusion injury model, we found that caprylate (C8:0), a short-chain and saturated fatty acid, potentiated the cytoprotective effect of SNO-HSA. The potentiation effect of caprylate was slightly weaker than that of OA. AST levels at 120 min after reperfusion were 3256 ± 55, 2586 ± 105, 2202 ± 253 for treatment with SNO-HSA, SNO-HSA-caprylate (HSA: caprylate = 1:5), SNO-HSA-OA5, respectively (The values are means ± S.E., $n = 4$).

Effect of OA Binding on SNO-HSA-mediated Cytoprotection of HepG2 Cells Exposed to Anti-Fas Antibody—NO and related species reportedly induce both antiapoptotic and proapoptotic responses in cells, the type of response depending on the concentration of the NO donors and on the type of cell and apoptosis-inducing reagent (5). In the present study, the influence of OA binding on the antiapoptotic effect of SNO-HSA on HepG2 cells treated with anti-Fas antibody was examined. As seen in Fig. 2, the presence of HSA, with or without bound OA, had no effect on the induced apoptosis. OA alone possessed no antiapoptotic activity in our HepG2 cell study (data not shown). By contrast, the addition of SNO-HSA resulted in concentration-dependent protection of the cells. This protection was greatly increased by binding 5 mol of OA per mol of SNO-HSA. Thus, fatty acid binding can also improve the cytoprotective effect of SNO-HSA in an *in vitro* system.

Pharmacokinetic Experiments—The pharmacokinetic characteristics of SNO-HSA with and without bound OA were determined in mice. The results in Fig. 3*A* indicate that OA binding did not affect the elimination of SNO-HSA from the circulation. The plasma half-lives were 269.3 ± 1.7 min, 270.6 ± 1.2 min, and 271.6 ± 5.5 min for SNO-HSA, SNO-HSA-OA3, and SNO-HSA-OA5, respectively. Likewise, the uptake of SNO-HSA by liver, kidney, and spleen was unaffected by OA binding (Fig. 3, *B–D*). These data suggest that binding of

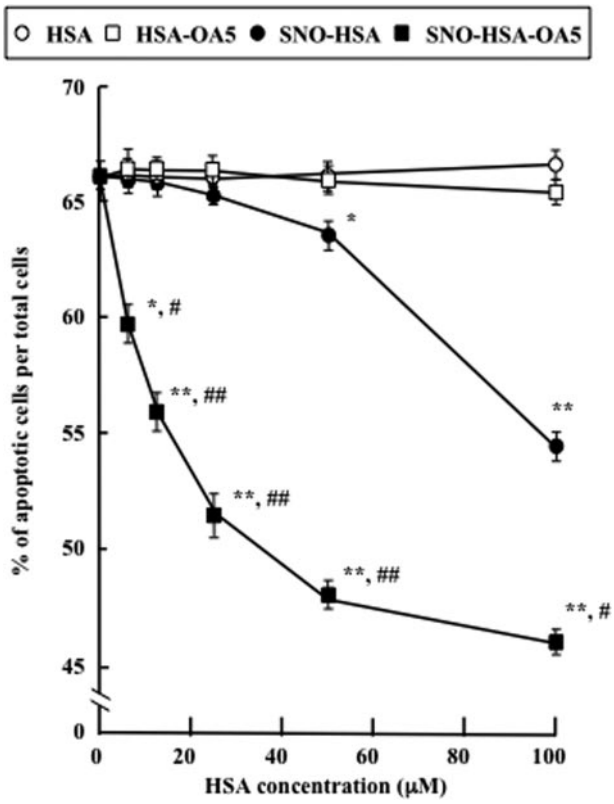

FIGURE 2. **Antiapoptotic effect of albumin without and with 5 mol OA/mol protein.** HepG2 cells (2×10^5 cells/well) were treated with different concentrations (0, 6.25, 12.5, 25, 50, 100 μM) of HSA or SNO-HSA, with and without bound OA, for 6 h at 37 °C in the dark. After incubation, the cells were washed three times with 10 mM phosphate-buffered saline, pH 7.4 to remove the remaining protein. Afterwards, the HepG2 cells were treated with anti-Fas antibody to induce apoptotic cell death. The number of apoptotic cells was determined by means of flow cytometry with annexin V-FITC and propidium iodide. Data are expressed as means ± S.E. ($n = 6$). *, $p < 0.05$ and **, $p < 0.01$, compared with the HSA-treated group. #, $p < 0.05$, ##, $p < 0.01$, compared with the SNO-HSA-treated group.

as much as 5 mol of OA per mol of protein does not modify the pharmacokinetic properties of SNO-HSA.

Accessibility of Cys-34 in HSA-OA—Crystallographic studies have revealed the existence of seven OA binding sites in HSA, and showed that none of these sites involve Cys-34 (21) (Fig. 4*A*). Cys-34 is located in a crevice on the surface of the protein in subdomain IA (1). Therefore, the improvement effect of even the highest OA concentration on the cytoprotection of SNO-HSA must be caused by indirect means. To test this hypothesis, the effect of OA on the readiness of Cys-34 in reduced HSA to bind DTNB was investigated. As seen in Fig. 4*B*, because the amount of DTNB bound after 120 min of incubation was the same without OA and with different concentrations of OA, OA did not mask the thiol in Cys-34. On the contrary, the OA-induced conformational changes enhanced the accessibility of the SH group of Cys-34, because the absorbance at 450 nm increased in a concentration-dependent manner at shorter incubation times. For example, the ratio between the absorbencies after 5 and 120 min of incubation increased from 0.171 ± 0.017 (OA/HSA = 0) to 0.211 ± 0.057 ($p < 0.05$) at OA/HSA = 3 and to 0.821 ± 0.054 ($p < 0.01$) at OA/HSA = 5; the results are given as means ± S.E. ($n = 4$). Non-reducing SDS-PAGE showed that OA binding did not result in dimerization of the protein (data not shown).

S-Denitrosylation of SNO-HSA by GSH and by HepG2 Cells—In Fig. 5*A* it can be seen that OA facilitated *S*-transnitrosation from SNO-HSA to GSH. However, the effect was not dependent on the concentration of OA. The amount of GS-NO formed after 30 min of incubation was higher when OA was bound to SNO-HSA, but the amounts were similar, whether SNO-HSA bound 1, 3, or 5 mol of OA per mol of protein. OA also increased the velocity with which GSH was *S*-nitrosylated. For the sake of comparison, $T_{½}$ values for *S*-transnitrosation were estimated. $T_{½}$ for *S*-nitrosylation of GSH by SNO-HSA was 22 min, whereas $T_{½}$ was only 16 min when *S*-nitrosylation was brought about by SNO-HSA binding 1, 3, or 5 mol OA/mol protein. These findings suggest that only the protein conformational changes caused by binding of the first OA molecule are essential for *S*-transnitrosation to GSH.

The effect of OA on the decay of SNO-HSA was also tested in a biological system using HepG2 cells. From Fig. 5*B*, it is apparent that the decrease in SNO-HSA caused by this cell type was faster and quantitatively more pronounced in the presence of OA, and that the effects increased with OA concentration. Thus, the decrease after 60 min of incubation was significantly greater between 0 and 1, and between 3 and 5 mol OA/mol protein, whereas the increment was smaller when increasing from 1 to 3 mol OA/mol protein. The $T_{½}$ values decreased from 52 min to 24, 22, and 15 min when the amount of OA increased from 0 to 1, 3, and 5 mol/mol protein, respectively. The OA-mediated promotion of SNO-HSA decay can be explained by increased accessibility to the *S*-nitroso moiety of HSA and/or by an intensified interaction between SNO-HSA and cell surface thiols.

S-Denitrosylation of SNO-HSA using HSA Isolated from Hemodialysis Patients—HSA samples obtained from hemodialysis patients were used to study the effect of endogenous fatty acids on *S*-denitrosylation of SNO-HSA. Serum fatty acid concentrations were significantly higher after dialysis among the patients examined (Table 1). Previous studies have shown that the primary fatty acid component in serum from dialysis patients is OA, and that the increased concentration is caused by activation of lipoprotein lipase by administered heparin (22, 23). As shown in Fig. 6, *A* and *B*, the decrease in SNO-HSA caused by HepG2 cells occurred more rapidly for SNO-HSA-hd (+) than SNO-HSA-hd (−). When the $T_{½}$ values of *S*-denitrosylation were plotted against the fatty acid contents in HSA, a good linear fit was obtained (Fig. 6*C*). Taken together, these findings indicate that in addition to OA, endogenous fatty acids can also facilitate the decay of SNO-HSA by HepG2 cells.

NO Uptake of HepG2 Cells—Figs. 5*B* and 6 show that fatty acid binding accelerates the SNO-HSA decomposition by HepG2 cells. To investigate whether this decomposition is accompanied by NO uptake by the cells, we used intracellular DAF-FM fluorescence. From Fig. 7 it is apparent that the intracellular NO concentration increases with incubation time and with increasing OA/SNO-HSA molar ratios. Control experiments performed by incubating HepG2 cells with HSA alone showed no increase in DAF-FM fluorescence (data not shown). To clarify the *S*-transnitrosation properties of other fatty acids,

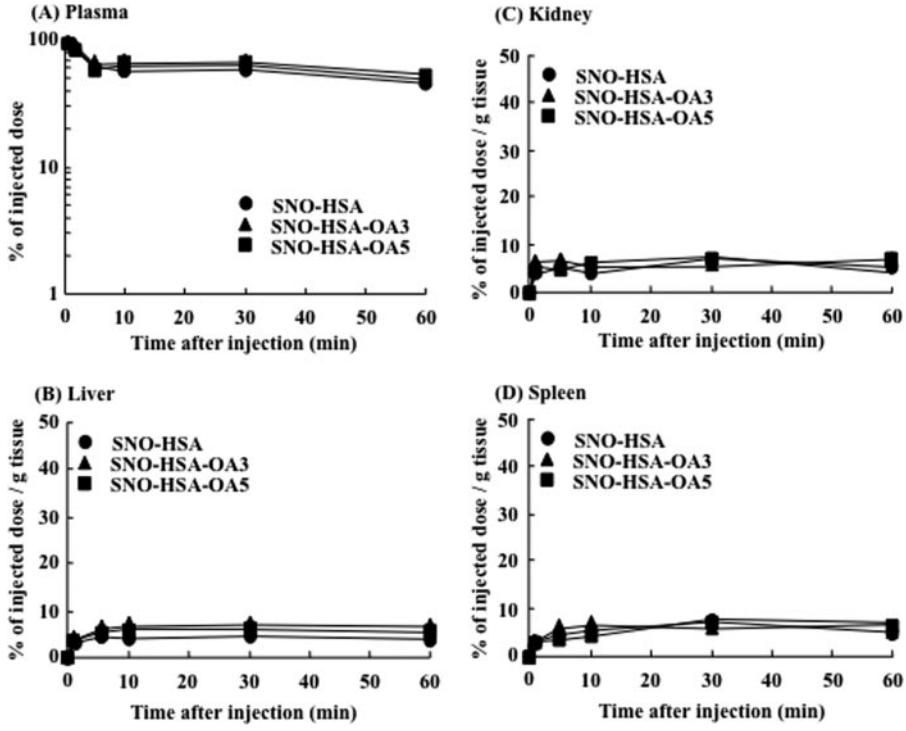

FIGURE 3. **Plasma concentrations of ^{111}In radioactivity (*A*) and tissue accumulation of radioactivity in liver (*B*), kidney (*C*), and spleen (*D*) after i.v. injection of SNO-HSA with different molar ratios of bound OA.** ^{111}In-SNO-HSAs with and without OA were injected via the tail vein into male ddY mice at a dose of 0.1 mg/kg. At different times thereafter, mice were taken for collection of blood from the vena cava with the animal under ether anesthesia; plasma was obtained from the blood by centrifugation. After blood collection, the mice were euthanized, liver, kidney, and spleen samples were obtained, rinsed with saline, and weighed. The radioactivity in each sample was counted using a well-type NaI scintillation counter ARC-2000. Data are expressed as means ± S.E. ($n = 3$); the *bars* showing S.E. were smaller than the size of the symbols.

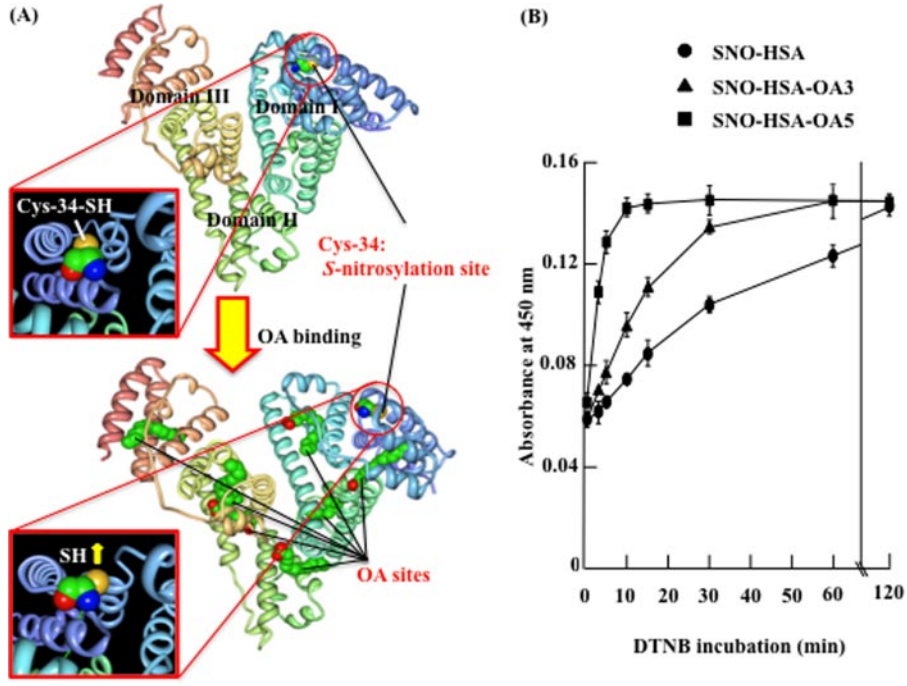

FIGURE 4. **Crystal structure of HSA showing locations of OA binding sites and the *S*-nitrosylated site (Cys-34) (*A*), and the effect of OA binding on the accessibility of Cys-34 (*B*).** *A*, as seen, OA binding results in a more open protein structure. The subdivision of HSA into domains (I-III) is indicated. The structures were simulated on the basis of x-ray crystallographic data for HSA and HSA-OA (PDB ID codes 1bmo and 1gni, respectively) and modified with the use of Rasmol. *B*, SNO-HSA (300 μM), without and with different amounts of bound OA, and DTNB (5 mM) were mixed in 0.1 M potassium phosphate buffer, pH 7.0 at 20 °C, and the absorbance at 450 nm was registered as a function of time. Data are expressed as means ± S.E. ($n = 4$).

we tested the effect of the saturated fatty acid stearate on NO uptake by HepG2 cells. The results showed that stearate has an effect on the *S*-transnitrosation of SNO-HSA, which is very similar to that of OA (data not shown). From Fig. 7 it can also be seen that the OA-induced increment in the transfer of NO from SNO-HSA into the hepatocytes can be completely blocked by the addition of filipin III.

Binding of SNO-HSA to HepG2 Cells—FITC-fluorescence was used to monitor albumin binding to the cell membrane or membrane components of HepG2 cells. Fig. 8*A* shows a pronounced binding of FITC-SNO-HSA to the hepatocytes. However, 5 equivalents of OA more than doubled albumin binding, to an extent that was significantly higher than that observed upon albumin binding of 3 OA equivalents, which again was significantly higher than that observed in the absence of OA (Fig. 8*E*). Similar results were obtained with albumin that was not *S*-nitrosylated (data not shown). Therefore, albumin binding to HepG2 cells is not affected by *S*-nitrosylation. Finally, the influence of filipin III was investigated. Even though a small decrease in albumin binding was observed (Fig. 8*E*), the decrease was not significant. The present data lead to the proposal that OA binding enhances, in a dose-dependent manner, the interaction between SNO-HSA and HepG2 cells.

DISCUSSION

The *S*-nitrosothiol fraction in plasma is largely composed of SNO-HSA (24). The biological importance of this form of albumin is illustrated by an increasing number of examples of its beneficial effects (2–6). In the present work, its cytoprotective effect on liver cells was used as an example. In addition to forming SNO-HSA, albumin can reversibly bind a large number of endogenous and exogenous ligands (1, 7, 8). Therefore, we sought to investigate whether ligand binding can influence biological activities of

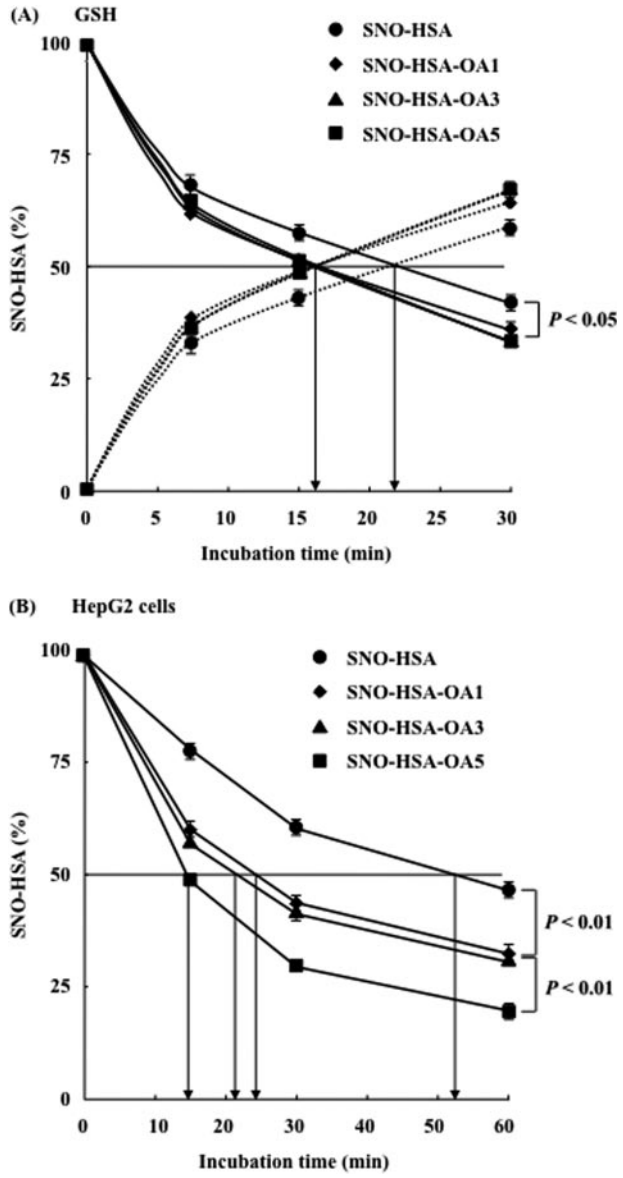

FIGURE 5. ***S*-Denitrosylation of SNO-HSA by GSH (*A*) and HepG2 cells (*B*).** *A*, SNO-HSA (100 μM), without and with different amounts of bound OA, was incubated with GSH (100 μM) in 10 mM phosphate-buffered saline, pH 7.4 at 37 °C. After 0, 7.5, 15, and 30 min of incubation, samples were taken, and the concentrations of the remaining SNO-HSA (*full curves*) and the GS-NO formed (*dotted curves*) were determined separately by a HPLC flow reactor system. The $T_{1/2}$ values for the decline of SNO-HSAs are indicated by the *arrows*. *B*, SNO-HSA (100 μM), without and with different amounts of bound OA, was incubated with HepG2 cells (5×10^5 cells/well) in 10 mM phosphate-buffered saline, pH 7.4 at 37 °C. After 0, 15, 30, or 60 min of incubation, the concentrations of the remaining SNO-HSA were determined by the HPLC flow reactor system. The $T_{1/2}$ values for the decline of SNO-HSAs are indicated. Data are expressed as means ± S.E. ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$, compared with SNO-HSA without OA binding.

SNO-HSA with particular reference to its cytoprotective effects. As seen in Fig. 1, binding of the important endogenous fatty acid OA increased the cytoprotective effect on liver cells in a dose-dependent manner. The effect may involve multiple mechanisms, including maintenance of tissue blood flow, induction of heme oxygenase-1 (a cytoprotective enzyme), suppression of neutrophil infiltration and reduction of apoptosis (11).

As a first step, we tested whether binding of OA could also increase the cytoprotective effect of SNO-HSA in an *in vitro* system. This was found to be the case, because OA enhanced the antiapoptotic effect of SNO-HSA on HepG2 cells exposed to anti-Fas antibody (Fig. 2). One explanation for the improvement effect of OA binding is an increased accessibility to the *S*-nitrosothiol group of HSA (Fig. 4). From the crystal structure analyses (16, 21, 25), it has been suggested that the reactive SH group of Cys-34 is located in a crevice on the surface of the HSA. Binding of oleate to HSA has been reported to induce conformational changes in the protein, leading to the slight opening of the interface between the two halves of the albumin molecule and a rotation of domain I to open the crevice that contains Cys-34, results in the increase of the accessibility of the Cys-34 SH group (25). The binding of only one mol of OA per mol of SNO-HSA was sufficient to increase *S*-transnitrosation to the relatively small GSH molecule (Fig. 5*A*). By contrast, the decay of SNO-HSA caused by incubation with HepG2 cells increased with the OA concentration up to a SNO-HSA:OA molar ratio of 5 (Fig. 5*B*). Essentially, the same effect was observed with SNO-HSA to which a mixture of endogenous fatty acids was bound (Fig. 6). This finding has biological and clinical implications, because the plasma concentration of non-esterified fatty acids can be increased in a number of situations. In addition to hemodialysis (Table 1), the increase in fatty acids is seen in connection with exercise and other adrenergic stimulation, and in pathological conditions such as the metabolic syndrome and diabetes mellitus.

TABLE 1

Plasma concentrations of HSA and fatty acids in 7 patients before and after hemodialysis

The concentrations of albumin are after dialysis, but they were not significantly affected by dialysis. Results are expressed as means ± S.E. ($n = 3$–4). p values for before and after dialysis.

	Sex	Albumin	Number of dialysis	Fatty acids		
				Before dialysis	After dialysis	p values
		g/dl		*mM*	*mM*	
No.1	F	2.9 ± 0.2	4	0.070 ± 0.003	0.159 ± 0.003	0.000973
No.2	F	3.9 ± 0.4	12	0.114 ± 0.004	0.614 ± 0.006	0.000624
No.3	F	4.0 ± 0.3	10	0.174 ± 0.005	0.261 ± 0.003	0.000323
No.4	F	3.6 ± 0.3	4	0.182 ± 0.006	0.934 ± 0.007	0.000546
No.5	M	4.1 ± 0.2	3	0.322 ± 0.005	0.718 ± 0.005	0.000478
No.6	M	3.9 ± 0.4	8	0.230 ± 0.005	0.380 ± 0.003	0.000556
No.7	F	4.1 ± 0.3	18	0.130 ± 0.005	0.651 ± 0.003	0.000236

Two additional factors in the improvement effect of OA binding are proposed by the present work. First, the decomposition of SNO-HSA seen in the presence of HepG2 cells is accompanied by a pronounced filipin III-sensitive *S*-transnitrosation of intracellular components (Fig. 7). A basal mechanism, not affected by filipin III addition, results in the slow transfer of small amounts of NO or modifications thereof (*e.g.* NO^+, Fig. 7). The filipin III-sensitive system is faster and quantitatively more important; it is activated by OA. Both systems may involve a membrane protein, and they may operate by transferring NO^+ from one thiol to another. The second factor is an OA-mediated increased interaction between SNO-HSA and the hepatocytes (Fig. 8). As established by the seminal study of Stremmel *et al.*, HSA-fatty acid complexes possess a high affinity to the caveolae (27, 28). Therefore, the structure of caveolae is essential for cellular fatty acid uptake.

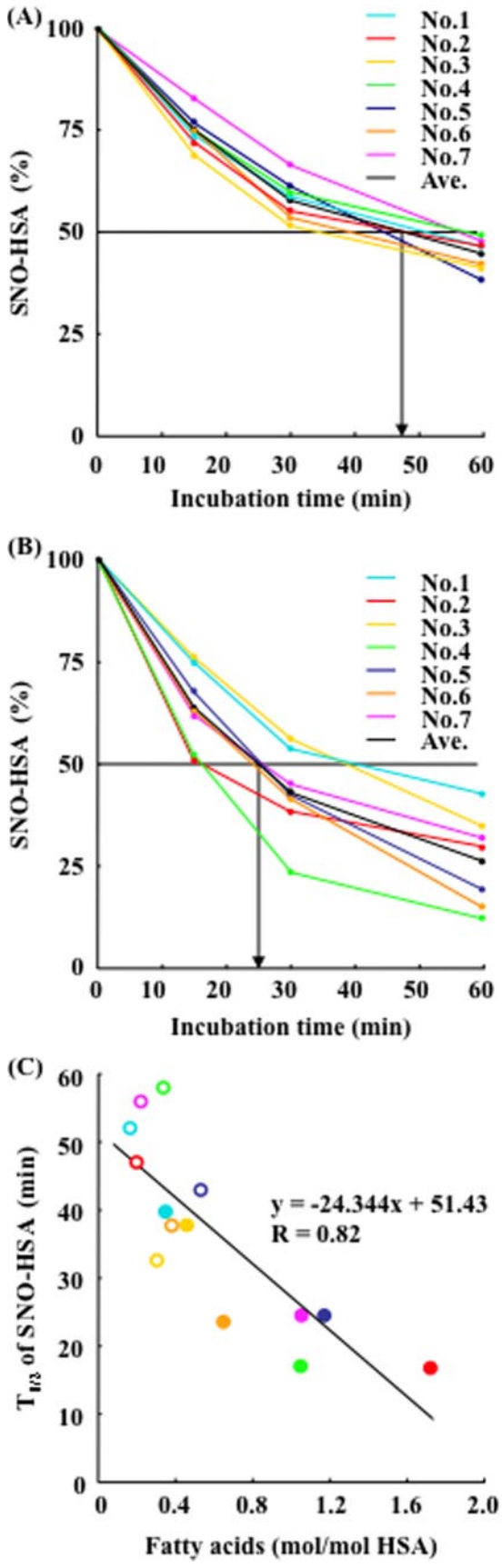

FIGURE 6. ***S*-Denitrosylation of SNO-HSA made from HSA isolated from hemodialytic patients before (*A*) and after (*B*) dialysis by HepG2 cells.** HSA samples were obtained from hemodialytic patients before and after dialysis. The HSA samples were *S*-nitrosylated using IAN, as described above. HepG2 cells (5×10^5 cells/well) and 100 μM SNO-HSA having different amounts of bound endogenous fatty acids were incubated in 10 mM phosphate-buffered saline, pH 7.4 at 37 °C. After 15, 30, and 60 min of incubation, the concentration of the remaining SNO-HSA was measured by the HPLC flow reactor system. The average $T_{1/2}$ for the *S*-denitrosylation is indicated, *i.e.* 48 min in *A* and 25 min in *B*. Data are expressed as means of four experiments. *C*, relationship between the individual $T_{1/2}$ values and the fatty acid contents of the HSA samples used. Samples are represented by *open circles* (before dialysis) or by *closed circles* (after dialysis); the colors correspond to the hemodialytic patients' number and color in *panel A* and *panel B*.

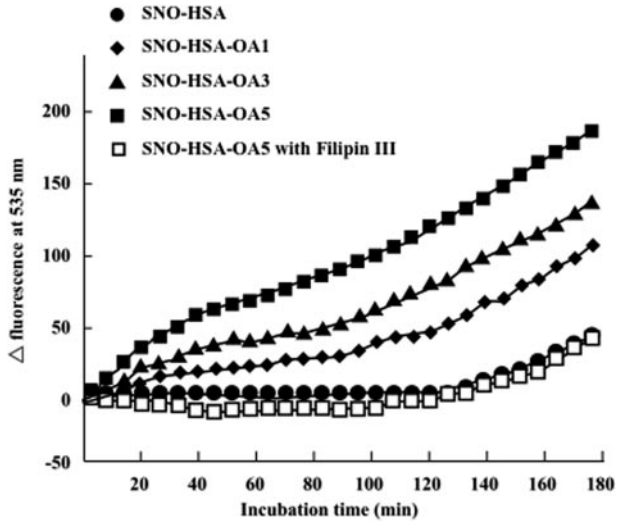

FIGURE 7. **Intracellular NO concentration of HepG2 cells exposed to SNO-HSA with different molar ratios of OA.** The HepG2 cells (5×10^5 cells/well) were first incubated with 5 μM DAF-FM DA for 1 h and then treated with 100 μM SNO-HSA with different amounts of bound OA in 10 mM phosphate-buffered saline, pH 7.4 in the dark at 37 °C. Some of the experiments with the highest OA concentration were also performed in the presence of 50 μM filipin III. Intracellular NO was monitored with DAF-FM fluorescence (ex/em of 385/535 nm). Δ*fluorescence* represents DAF-FM fluorescence in cells incubated with different preparations of SNO-HSA, minus the fluorescence in cells that had been incubated with buffer only. Data are expressed as means ± S.E. ($n = 4$); the *bars* showing S.E. were smaller than the size of the symbols.

Fig. 9 proposes a model for *S*-transnitrosation from SNO-HSA. Binding of OA, or of a mixture of endogenous fatty acids, introduces conformational changes in SNO-HSA that render the *S*-nitroso moiety more accessible. OA binding also facilitates the binding of albumin to a receptor on the hepatocytes, perhaps the albumin binding adaptor protein gp60 (29). When bound to the receptor, SNO-HSA-OA can *S*-transnitrosate to HepG2 cells via two (or more) systems. Because OA-induced transnitrosation could be completely blocked by filipin III, an inhibitor of caveolae (19), it is proposed that caveolae are important for this type of *S*-transnitrosation. These findings strongly suggest that OA and NO of SNO-HSA-OA are transported by caveolae-associated proteins. Further studies are needed to identify and clarify the mechanism of the caveolae-associated proteins.

It is widely assumed that *S*-transnitrosation from SNO-HSA to cells takes place solely or mainly via low molecular weight thiols (6, 26, 30). In the present work, *S*-transnitrosation to HepG2 cells was studied in the absence of GSH and other low molecular weight thiols. In accordance with the assumption mentioned above, only a slight *S*-transnitrosation was detected

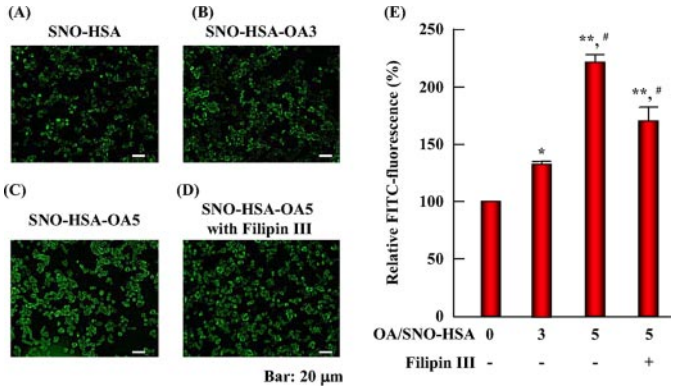

FIGURE 8. **Effect of OA and filipin III on binding of FITC-SNO-HSA to HepG2 cells.** To prepare FITC-SNO-HSA, HSA was first *S*-nitrosylated as described above, followed by FITC labeling. FITC-SNO-HSA (50 μg/ml) with varying OA content (0, 3, 5 OA/HSA) was dissolved in 10 mM phosphate-buffered saline (pH 7.4) and added to HepG2 cells (5×10^5 cells/well) for 10 min at 4 °C. In some experiments, the cells had been pretreated with 50 μM filipin III for 30 min. After incubation, the cells were washed twice with the phosphate-buffered saline to remove unbound FITC-SNO-HSA. After washing, the cells were analyzed using a fluorescence microscope. *E*, four columns quantify the FITC-fluorescence in *panels A*, *B*, *C*, and *D*, respectively, using the fluorescence in *panel A* as a reference value, *i.e.* 100%. The values are means ± S.E. ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$, compared with the fluorescence of FITC-SNO-HSA without OA. #, $p < 0.05$, compared with the fluorescence of FITC-SNO-HSA with 3 mol of OA/HSA.

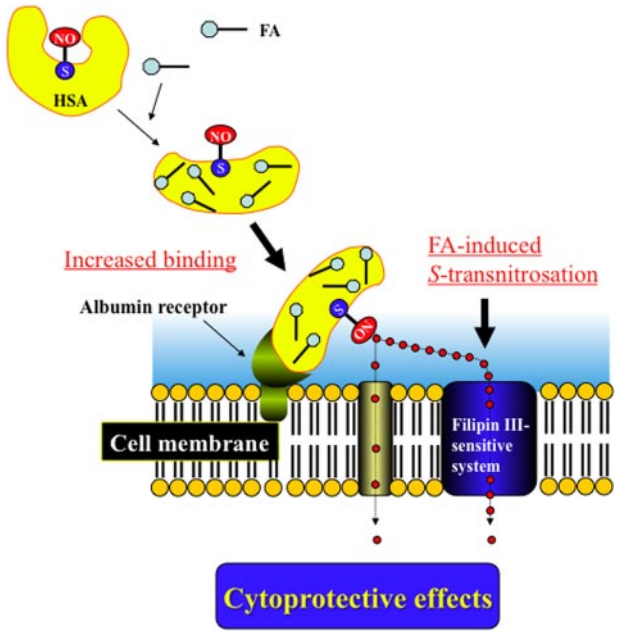

FIGURE 9. **Proposed model for the OA-mediated increase in *S*-transnitrosation of HepG2 cells by SNO-HSA.** The *S*-transnitrosation can lead to cytoprotective effects, see Figs. 1 and 2. *FA*, free fatty acid.

in the absence of OA. However, OA binding to SNO-HSA greatly increased the process.

In conclusion, fatty acid binding can improve the cytoprotective effect of SNO-HSA *in vivo*, and reinforcement of an antiapoptotic effect by fatty acid binding contributed to this result. Fatty acid bound to SNO-HSA can enhance the interaction between SNO-HSA and HepG2 cells, and the *S*-denitrosylation of SNO-HSA. This results in the enhancement of NO transfer from SNO-HSA into the hepatocytes, and to the antiapoptotic effect. Moreover, we found a novel filipin III-sensitive mechanism of the transfer of NO from SNO-HSA into the hepatocytes. Taken together, further study is now warranted to explore the roles of fatty acid binding in the pharmacological benefits of SNO-HSA, for which a clinical application is expected as a potent NO supplementary therapy.

Acknowledgments—We thank the members of the Gene Technology Center at Kumamoto University for important contributions to these experiments.

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