S-Nitrosylation of Human Variant Albumin Liprizzi (R410C) Confers Potent Antibacterial and Cytoprotective Properties

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ABSTRACT
The S-nitrosylated forms of certain proteins such as albumin have been thought to be circulating endogenous reservoirs of nitric oxide (NO) and may have potential as NO donors in therapeutic applications. In this study, we investigated the characteristics of R410C, a genetic variant of human serum albumin with two free thiols at positions 34 (Cys-34) and 410 (Cys-410), as a NO carrier via S-nitroso formation. A biotin switch assay revealed that Cys-410 was more rapidly and efficiently nitrosylated than was Cys-34. Nitrosylation of Cys-410 introduced only small conformational changes in the protein, which were detected by far-UV circular dichroism but not by near-UV circular dichroism. In addition, both native R410C and S-nitrosylated R410C did not induce molecular heterogeneity through oligomerization. S-Nitrosylated R410C exhibited strong antibacterial activity against Salmonella typhimurium in vitro and suppressed apoptosis of U937 human promonocytic cells induced by Fas ligand. In a rat ischemia-reperfusion liver injury model, S-nitrosylated R410C treatment significantly reduced liver damage, as indicated by markedly decreased release of liver enzymes (aspartate aminotransferase and alanine aminotransferase). Pharmacokinetic analyses indicated retention of the S-nitroso moiety of S-nitrosylated R410C in circulation after i.v. injection, with an approximate half-life of 20.4 min in the mouse. These data suggest that R410C can be a useful NO carrier and can be regarded as a new class of S-nitrosylated proteins possessing antibacterial and cytoprotective properties with a circulation time sufficient for in vivo biological activity.

S-Nitrosothiols are nitric oxide (NO) adducts formed endogenously via reaction of thiols with NO or its reactive metabolites such as N₂O₃ and nitrosonium (NO⁺)-like species (Akaike, 2000). S-Nitrosothiols may function as NO reservoirs and preserve antioxidant properties of NO (Hogg, 2000; Foster et al., 2003). For example, S-nitroso human serum albumin (SNO-HSA) has been suggested to serve in vivo as a reservoir for NO produced by endothelial cells (Stamler et al., 1992). In addition, administration of S-nitroso albumins to animals with ischemia-reperfusion injury minimized the extent of tissue damage associated with reperfusion. This injury is characterized by initial tissue damage during the ischemic period followed by progressive injury during the reperfusion period. Reperfusion is a trigger for the generation of reactive oxygen species, release of cytokines, induction of adhesion molecules on vascular endothelial cells, and the adhesion and extravasation of leukocytes into posts ischemic tissue (Hallstrom et al., 2002; Dworschak et al., 2004). Potent cytoprotection against ischemia-reperfusion liver injury in rats (Ikebe et al., 2000) and antibacterial activity in vitro against several types of bacteria (Miyamoto et al., 2000a,b) resulted after human α₁-protease inhibitor (α₁-PI), an acute phase reactive protein in serum, was S-nitrosylated (SNO-α₁-PI). These cytoprotective and antibacterial activities of
SNO-α$_{1}$-PI were more powerful than those of NO alone and of low-molecular-weight S-nitrosothiols such as S-nitroso glutathione (GSNO) (Miyamoto et al., 2000a,b). In addition to serving as a reservoir for NO, S-nitrosothiols themselves have been suggested to show various biological activities, possibly through transnitrosylation, S-thiolation, and direct action of other biological molecules (Hogg, 2000; Miyamoto et al., 2000a; Foster et al., 2003). These observations suggest many potential clinical uses for S-nitrosothiols, not only as NO-releasing agents but also as members of a new class of therapeutic agents (Richardson and Benjamin, 2002).

Researchers in several laboratories have investigated genetic variants of HSA to define the functional defects and correlate them with molecular and biochemical properties and stability of the molecules. So far, approximately 60 amino acid substitutions have been characterized (Kragh-Hansen et al., 2004). Among them, albumin Liprizzi (R410C) represents the only instance of a mutation located in the disulfide pattern or cause gross conformational changes in the protein (Galliano et al., 1998). These observations led us to hypothesize that Cys-410 might be a target of S-nitrosylation and, if so, multiple S-nitroso sites might be introduced in a single HSA molecule to prepare a new SNO-HSA formulation.

In the present study, we investigated the kinetics of S-nitrosylation of Cys-34 and Cys-410 in R410C by means of a biotin switch assay. Effects of S-nitrosylation on structural stability of the proteins were examined by using UV spectroscopy and nonreducing SDS-polyacrylamide gel electrophoresis (PAGE). Antibacterial and antiapoptotic activities of the synthesized SNO-R410C were studied in vitro and compared with those of high- and low-molecular-weight S-nitrosothiols, including SNO-HSA, SNO-α$_{1}$-PI, GSNO, and non-S-nitrosothiol types of NO donors. We also examined the cytoprotective capability of SNO-R410C in an ischemia-reperfusion injury model in rats. Finally, we determined the pharmacokinetic parameters of the S-nitrosylated proteins in mice.

**Materials and Methods**

**Materials.** Sephadex G-25 (φ 1.6 × 2.5 cm), Blue Sepharose CL-6B (φ 2.5 × 20 cm), and RESOURCE PHE (φ 0.64 × 3 cm) were from GE Healthcare (Tokyo, Japan). Other chemicals were of the best grades commercially available.

**Nonrecombinant Proteins.** The HSA preparation consisting of different ratios of native HSA and R410C (Galliano et al., 1998) were provided by Drs. M. Galliano and L. Minchiotti (University of Pavia, Italy). The samples were defatted by treatment with a hydroxyalkoxypropyl dextran at pH 3.0 (Kragh-Hansen, 1993). nHSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted by treatment with charcoal as described by Chen (1967).

**Generation of Pichia pastoris Producing HSA and the R410C Mutant.** The expression vector pPIC9-whSA containing the native HSA expression cassette stably integrated into the chromosomal DNA was used to produce HSA (Matsushita et al., 2004). We made the R410C mutant by using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the following mutagenic primers (sense and antisense): 5’-GAATGCGCTATTAGTTCATACAGAAGGAGAAGGAGATTCC-3’ and 3’-GGGTACTTTCTCTTTGGGTGTAGCATAAACATAGCGCATTCC-5’.

The constructed plasmids (pPIC9-whSA and pPIC9-HSA R410C) were transferred to XL10-Gold Escherichia coli organisms, which were grown in Luria-Bertani medium. After plasmid purification from E. coli, the plasmid sequences were confirmed by use of the dyeoxyce chain termination method with a PerkinElmer (Boston, MA) ABI Prism 130 Genetic Analyzer. *P. pastoris* GS115 hst was transformed with Sull-digested pPIC9-whSA or pPIC9-HSA R410C by electroporation according to the manual (EasySelect Pichia Expression Kit Version A; Invitrogen). Histidine-independent transformants were selected and subsequently screened for slow methanol utilization phenotypes. Positive clones were induced with methanol and screened for production of HSA or R410C mutants by 12.5% SDS-PAGE of culture medium.

**Production and Purification of Recombinant Albumins.** The protocol used to express the HSA was a modification of a previously published protocol (Matsushita et al., 2004). Single colonies of *P. pastoris* were grown (30°C, 210 rpm, 48 h) in 300 ml of BMGY growth medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10$^{-2}$% bovine, and 1% glycerol) in 1-liter baffled flasks until an A$_{600}$ value of 2 to 6 was obtained. Cells were then harvested by centrifugation at 3000g, and cell pellets were washed extensively and resuspended in 300 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4 × 10$^{-2}$% bovine, and 1% methanol) to an approximate A$_{600}$ value of 15 to 20. For further culture of this *P. pastoris* suspension, the baffled flasks were shaken (30°C, 190 rpm, 96 h) with daily addition (every 12 h) of methanol at a final concentration of 1% to maintain the induction conditions of the alcohol oxidase 1 promoter.

The recombinant proteins were purified after 96 h of induction, according to the literature (Matsushita et al., 2004). The protein preparation was first subjected to chromatography with the Blue Sepharose CL-6B column (φ 2.5 × 20 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) after dialysis with the same buffer. Proteins were further purified by using the RESOURCE PHE column (φ 0.64 × 3.0 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 1.5 mM ammonium sulfate. The column was washed with the phosphate buffer and then eluted with a 20-ml gradient of ammonium sulfate, 1.5 to 0 M, in the same buffer. The eluted HSA were deionized and defatted via charcoal treatment, freeze-dried, and then stored at −20°C until used. Sample purity was estimated by density analysis of Coomassie Brilliant Blue (CBB)-stained protein bands on 12.5% SDS-PAGE. The recombinant protein samples of HSA and R410C were judged as more than 97% pure.

**S-Nitrosylation of the Proteins.** S-Nitrosylated proteins were prepared according to previous reports (Akaike et al., 1997; Ikebe et al., 2000). In brief, protein (300 µM) was incubated with DTT (molar ratio, protein/DTT = 1:10) for 5 min at 37°C. DTT was then quickly 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 DTT-treated proteins (0.1 mM) were incubated with...
isoamyl nitrite (molar ratio, protein/isoamyl nitrite = 1:1.0) in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA for 60 min at 37°C. S-Nitrosylated products were purified by Sephadex G-25 gel filtration, eluted with pure water, and concentrated by ultrafiltration (cutoff size of 7500 Da). These samples were stored at −80°C until use. The protein content of all the protein preparations used in this study was determined by the Bradford assay.

**Determination of S-Nitrosylation Efficiency.** The amounts of the S-nitroso moiety of SNO-HSA, SNO-R410C, and SNO-n2-P1 were quantified by high-performance liquid chromatography (HPLC) coupled with a flow reactor system, as previously reported (Akaike et al., 1997). The stability of the S-nitroso moiety on storage in neutral solution was examined as follows. The modified proteins were dissolved in 100 mM potassium phosphate buffer containing 0.5 mM DTPA (pH 7.4) and left in the dark at room temperature for a maximum of 21 days. At appropriate times after the start of incubation, aliquots of the S-nitrosylated protein solutions were taken and injected into the HPLC flow reactor system to detect S-nitrosylated compounds. The effect of lyophilization was studied by resuspending lyophilized samples in 100 mM potassium phosphate buffer containing 0.5 mM DTPA (pH 7.4). S-Nitroso moieties of the S-nitrosylated proteins were quantified before and after the resuspending.

**Physicochemical Characterization of the S-Nitrosylated Albumins.** Gross conformational changes induced by S-nitrosylation of recombinant HSA were assessed by means of nonreducing SDS-PAGE at 4°C in the dark, with use of a standard curve prepared with a set of marker proteins (Full Range Rainbow Marker; GE Healthcare, Tokyo, Japan). Circular dichroism (CD) spectra of HSA, R410C, and their S-nitrosylated products were measured at 25°C using a J-720-type spectropolarimeter (JASCO, Tokyo, Japan). For calculation of mean residue ellipticity [θ], the molecular weight of the albumins was taken as 66,500. Far-UV and near-UV CD spectra were recorded at protein concentrations of 5 and 15 μM, respectively, in 20 mM sodium phosphate buffer (pH 7.4).

To determine the sites and kinetics of S-nitrosylation, the biotin switch assay was performed, as reported earlier (Jaffrey et al., 2001). Specifically, HSA or R410C (0.3 mM) was incubated with GSNO (1.5 mM) in 100 mM potassium phosphate buffer (pH 7.8) for 5 or 60 min at 37°C. After the incubation, the reaction mixture was immediately applied to a G-25 gel filtration column eluted with HENS buffer (250 mM HEPES, pH 7.7, 0.1 mM EDTA, 0.1 mM neocuproine, and 1% SDS) to terminate the reaction. Fractions containing protein were collected and combined. Protein concentrations were adjusted to 0.5 mg/ml, followed by treatment with MMTS to block free SH. To block free SH groups on the protein without affecting the disulfide bonds, 4 volumes of blocking buffer (225 mM HEPES, pH 7.7, 0.9 mM EDTA, 0.09 mM neocuproine, 2.5% SDS, and 20 mM MMTS) were added. The resulting solutions were agitated for 20 min at 50°C. Proteins were then recovered by precipitation with acetone (final concentration, 50%), and the precipitates were resuspended in 0.1 ml of HENS buffer (protein concentration, 10 mg/ml). To this protein solution was added 0.1 ml of biotin-HPDP (4 mM) in N,N-dimethylformamide and 0.1 ml of aqueous ascorbate (0 or 1 mM), and the samples were incubated for 1 h at room temperature. Proteins were then recovered via acetone precipitation. The biotinylated proteins were dissolved in 0.1 ml of 70% formic acid containing 0.64 mg of CNBr (CNBr/methionine residues = 200:1), and samples were incubated under N2 for 24 h in the dark at room temperature. An aliquot of 1 ml of Milli-Q water was added at the end of the CNBr cleavage to stop the reaction, and the resulting mixture was lyophilized. Lyophilized fragments were resuspended in 0.05 ml of Milli-Q water. The mixture of fragments was separated on 10 to 20% gradient SDS-PAGE gels. The fragment size was determined by use of a calibration curve obtained from size markers, and each fragment was assigned according to its molecular size. To analyze biotinylated fragments, samples prepared as above were separated on 10 to 20% gradient SDS-PAGE gels, transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corporation, Bedford, MA), blocked with 3% bovine serum albumin, and incubated with streptavidin-horseradish peroxidase diluted 1/2000 for 40 min.

**Antibacterial Activity of S-Nitrosothiols.** In vitro antibacterial activities of various S-nitrosothiols and a non-S-nitrosothiol-type NO donor (p-NONOate) were examined according to previously reported methods, with slight modification (Miyamoto et al., 2000a). We used M9 medium (pH 7.4) during incubation of bacteria with S-nitrosothiols and the NO donor. In brief, Salmonella typhimurium LT2 organisms were cultured overnight in M9 medium and were then washed three times with M9 medium. The bacteria were resuspended in Krebs-Ringer phosphate buffer containing 1 mg/ml NH4Cl and 5 mg/ml thiamine hydrochloride. The bacterial suspension (1 × 106 CFU/ml, 0.02 ml in M9 medium) was mixed with 0.18 ml of M9 medium containing various concentrations of S-nitrosothiols or p-NONOate in a 96-well plate. After 1 h of incubation at 37°C, the bacteria were collected by centrifugation (1500g, 5 min), resuspended in 0.18 ml of M9 medium, transferred to another 96-well plate, and incubated for 9 h at 37°C. The numbers of bacteria exposed to various S-nitrosothiols were determined by measuring the turbidity of the culture suspension. A655 value was monitored by using a microplate reader (model 450; Bio-Rad Laboratories, Hercules, CA).

**Effect of S-Nitrosothiol on Apoptosis.** U937 human promonocyctic cells were grown in Dulbecco’s modified Eagle’s medium that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained in a humidified incubator (95% air, 5% CO2) at 37°C. Cells from this culture (5 × 105 cells/ml) were treated with S-nitrosothiols for 6 h in the dark and were then washed with phosphate-buffered saline three times to remove the S-nitrosothiols. After this washing, cells were reacted with 200 ng/ml anti-Fas antibody (Medical and Biological Laboratories, Nagoya, Japan). After 9 h of incubation, the number of apoptotic cells was determined with an annexin V-fluorescein isothiocyanate (FITC) binding assay kit from BD Biosciences (Tokyo, Japan). The fluorescence of annexin V-FITC and propidium iodide was measured via a FACSCalibur flow cytometer (BD Biosciences), using the FL1 and FL2 channels, respectively.

**Cytoprotective Effect of SNO-HSA in Vivo.** A rat ischemia-reperfusion liver injury model served for investigation of the cytotoxic effect of S-nitrosothiol, according to a previous report (Ikebe et al., 2000). 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 various compounds including HSA, R410C, SNO-HSA, and SNO-R410C (usually 0.1 μmol/rat) were given via the portal vein immediately after reperfusion was initiated. The abdomen then 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, 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 the animal experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Graduate School of Medical Sciences, Kumamoto University.

**Pharmacokinetic Experiments.** Proteins were labeled with 111In by using DTPA anhydride as a bifunctional chelating agent (Hastowich et al., 1982; Yamasaki et al., 2002). 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, kidneys, spleen, heart, and muscle 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).

In radioactivity concentrations in plasma were normalized as a percentage of the dose per milliliter and were analyzed by means of the nonlinear least-squares program MULTI (Yamaoka et al., 1981). Tissue distribution profiles were evaluated by using tissue uptake clearance (Cl_{tissue}) according to integration plot analysis. By dividing the amount in a tissue at time t (X_t) and the area under the plasma concentration-time curve (AUC) from time 0 to t (AUC_{0–t}), Cl_{tissue} was obtained from the slope of the plot of X_t/C_t versus AUC_{0–t}/C_t. In addition, we measured S-nitroso moieties in plasma by means of the Saville assay (Akaike et al., 1997). Similar experiments were performed with nonlabeled GSNO. For that purpose, plasma samples were collected into tubes containing DTPA (final concentration, 2 mM) and centrifuged for 5 min at 1500g in the dark. Plasma samples then were analyzed for NO_3^- by means of the Griess reaction.

**Statistical Analysis.** Analysis of variance followed by the Newman-Keuls method for more than 2 means were used to evaluate the statistical significance of collected data. Data were expressed as mean ± S.E.M. Differences between groups were evaluated by Student’s t test, and p<0.05 was regarded as statistically significant.

**Results**

**Production of Recombinant R410C.** Because the genetic variant cannot be separated from its normal endogeous counterpart, recombinant R410C was generated, via the *P. pastoris* recombinant system, to be used as a pure R410C preparation. After purification by means of column chromatography, 360 mg of R410C with a purity of >97% was obtained from a 3.6-liter culture. SDS-PAGE under nonreducing conditions revealed only a single band for recombinant R410C with a molecular mass of approximately 67 kDa, which indicated successful generation of a homogeneous monomer protein.

**Physicochemical Properties of S-Nitrosylated R410C (SNO-R410C).** We first examined the efficiency of S-nitrosylation of HSA by using isoamyl nitrite as the nitrosating agent. The S-nitroso moiety of native HSA was determined to be approximately 0.3 mol/mol HSA under the current conditions. When we treated serum-derived HSA containing different proportions of R410C, the S-nitroso content increased linearly according to the increasing content of R410C (Fig. 1). The S-nitroso content reached 1.3 mol/mol protein for recombinant R410C (R410C,total HSA = 1.0). The difference in S-nitroso content between native HSA and R410C was almost 1 mol, which suggests that almost all the Cys-410 residues in R410C may be S-nitrosylated.

To study the relative efficiency of S-nitrosylation at Cys-34 versus that at Cys-410, S-nitrosylation sites were determined by means of the biotin switch assay. Figure 2A shows that CBB staining revealed almost equal amounts of fragments after CNBr treatment of HSA, which confirmed that CNBr protein fragmentation was not affected by S-nitrosylation. The biotin switch assay involves three major steps: blockage of free thiols, specific reduction of S-nitrosothiols to thiols by ascorbate, and labeling the nascent thiols with biotin groups (Jaffrey et al., 2001). The biotin switch assay (Fig. 2A) clearly found fragment 299 to 585, which contains Cys-410, after just 5 min of incubation with GSNO. After 60 min of incubation, fragment 1 to 298, which contains Cys-34, was also detected. To confirm the specificity of the assay, we omitted ascorbate treatment of the protein. As shown in Fig. 2A, no bands were detected for proteins without ascorbate treatment. The time profile of S-nitrosylation as quantified by the biotin switch assay indicated that Cys-410 was S-nitrosylated more rapidly and efficiently than was Cys-34 (Fig. 2B). Similar results were obtained for S-nitrosylation of R410C with use of isoamyl nitrite instead of GSNO (data not shown).

The effect of S-nitrosylation on gross conformation was examined by means of nonreducing SDS-PAGE analysis (Fig. 3A). S-Nitrosylation caused no dimerization via disulfide bond formation, fragmentation, or other gross conformational changes. Potential conformational alterations were investigated in more detail by means of CD spectrometric analyses (Fig. 3, B–E). According to the far-UV spectra, S-nitrosylation of Cys-34 had no detectable effect on the secondary structure of native HSA (Fig. 3B). S-Nitrosylation of both Cys-34 and Cys-410 resulted in a small decrease in the α-helical content of the protein (Fig. 3D). In addition, nitrosylation of the cysteine residues had only a small influence, if any, on the tertiary structure of the protein, as evidenced by UV spectra analyses (Fig. 3, C and E). Thus, our data suggest that R410C was structurally stable in aqueous media and that S-nitrosylation induced no significant conformational changes.

In addition, we examined the stability of S-nitroso moieties in neutral buffer and during lyophilization (data not shown). The half-life of the S-nitroso moiety of SNO-R410C was 23.5 days in phosphate buffer, pH 7.4, in the dark. This half-life provided evidence of a fairly stable compound compared with SNO-α1-PI, which is a stable S-nitrosylated protein (18.9 days). Only a slight decrease (−10%) in S-nitroso content was observed after lyophilization of SNO-R410C.

**Antibacterial Activity of SNO-R410C.** NO and related species including S-nitrosothiols reportedly inhibit the growth of a wide variety of microorganisms, including viruses, bacteria, parasites, and fungi (Fang, 2004). Miyamoto et al. (2000b) showed that SNO-α1-PI has a strong bacteriostatic effect against both Gram-negative and Gram-positive bacteria, with the IC_{50} in the low micromolar range. In agreement with previous reports, we found that low-molecular-
weight NO donors such as GSNO and p-NONOate had a very weak antibacterial effect, with an IC_{50} value of approximately 3 mM (Fig. 4). SNO-α_{1}-PI, used as a positive control, suppressed bacterial growth with an IC_{50} value of approximately 6 μM. SNO-HSA was as potent as SNO-α_{1}-PI, with an IC_{50} value of approximately 8 μM, in suppressing bacterial growth. The corresponding value for SNO-R410C was submicromolar (0.6 μM), which indicated that the antibacterial activity of SNO-R410C was 10 and 13 times stronger than that of SNO-α_{1}-PI and SNO-HSA, respectively. Parental R410C had no antibacterial activity (data not shown).

**Effect of SNO-R410C on Apoptosis of Cells Induced by Fas Ligand.** NO and related species reportedly induce both antiapoptotic and proapoptotic responses in cells, the type of response depending on the concentrations of the NO donors, cell types, and apoptosis-inducing reagents (Kim et al., 1999). In the present study, we examined the effect of SNO-R410C on apoptosis of U937 cells induced by Fas ligand (FasL). Under the present experimental conditions, 65% of the total number of cells underwent apoptotic cell death after incubation with or without various compounds. Data are expressed as mean ± S.E.M. (n = 3).

**Fig. 4.** Concentration-dependent antibacterial effects of S-nitrosothiols and p-NONOate. Bacterial growth, assessed by means of turbidity and expressed as a percentage of control, was determined at 12 h after incubation with or without various compounds. Data are expressed as mean ± S.E.M. (n = 3–6). *, p < 0.05 and **, p < 0.01 compared with SNO-α_{1}-PI.
treatment with FasL (Fig. 5). This apoptotic change was significantly suppressed by SNO-R410C; however, the other S-nitrosylated compounds—SNO-HSA and GSNO—showed only a marginal inhibitory effect.

**Cytoprotective Effect of SNO-R410C against Ischemia-Reperfusion Liver Injury in Rats.** We used an ischemia-reperfusion liver injury model in rats (Ikebe et al., 2000) to examine the cytoprotective effect of SNO-R410C. Previous studies with SNO-α1-PI showed that a quantity of 0.1 μmol/rat was most suitable for this kind of experiment (Ikebe et al., 2000). Therefore, we used the same quantity of SNO-R410C. However, the effect was dose-dependent, but even a dose of 0.01 μmol/rat had a cytoprotective effect (data not shown). To evaluate liver injury, we measured the extracellular release of the liver enzymes AST and ALT via plasma enzyme values. As reported previously for SNO-α1-PI and α1-PI (Ikebe et al., 2000), these values increased to a maximum at 2 h after reperfusion and then decreased gradually during 24 h (data not shown). Native HSA and R410C did not modify liver damage in this model (Fig. 6). However, a significant reduction in release of AST and ALT was observed in rats treated with SNO-R410C, with this reduction also significantly greater than that noted for SNO-HSA. Even at 12 h after reperfusion, the levels of AST and ALT remained significantly lower in SNO-R410C-treated animals than in control animals, although the difference was not so great compared with that observed at 2 h after reperfusion (data not shown). At 24 h after reperfusion, no significant difference in the levels of AST and ALT was observed between SNO-R410C-treated animals and control animals because the liver damage in both groups was almost recovered to the basal level (data not shown).

**Pharmacological Properties of SNO-R410C.** We determined pharmacokinetic characteristics of HSA, R410C, and their S-nitrosylated counterparts in mice. Figure 7A indicates that the plasma concentration of R410C decreased more rapidly than that of HSA. S-Nitrosylation of R410C slightly prolonged the plasma half-life of R410C (Fig. 7A). Kidney uptake of R410C (Fig. 7C) was not affected by S-nitrosylation, whereas liver uptake of R410C (Fig. 7D) was significantly suppressed after S-nitrosylation. As shown in Table 1, R410C had higher clearance rates than did HSA in both liver and kidney, which may explain the more rapid clearance of R410C from circulation compared with clearance of HSA. Despite this relatively higher clearance rate, the decrease in the S-nitroso moiety was only slightly faster for SNO-R410C than for SNO-HSA. Consequently, the S-nitroso content per carrier (S-NO/carryer) remained appreciably higher for SNO-R410C compared with SNO-HSA, as shown in Table 1. These data suggest that the stability of SNO-R410C is higher than that of SNO-HSA in the circulation. No tissue-specific accumulation of R410C and HSA was observed (data not shown). The plasma S-nitroso level became undetectable at 10 min after i.v. injection of GSNO (Fig. 7B). However, even at 120 min after administration, appreciable levels of the S-nitroso moieties were detected in plasma of mice receiving SNO-R410C and SNO-HSA (Fig. 7B). The apparent half-lives of the S-nitroso moieties were 4.2, 20.4, and 46.3 min for GSNO, SNO-R410C, and SNO-HSA, respectively.

**Discussion**

NO is a powerful biological agent (Moncada and Higgs, 1993; Liu and Stamler, 1999), but its short in vivo half-life (~0.1 s) sometimes limits its potential biological usefulness.

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**Fig. 5.** Antiapoptotic effects of S-nitrosothiols. U937 cells were treated with anti-Fas antibody to induce apoptotic cell death. Numbers of dead cells were determined by means of flow cytometry with annexin V-FITC and propidium iodide. SNO-HSA, SNO-R410C, and GSNO were added to cell cultures at the indicated concentrations. Data are expressed as mean ± S.E.M. (n = 5–9). *, p < 0.05 and **, p < 0.01 compared with SNO-HSA.

**Fig. 6.** 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 portal vein and hepatic artery for 30 min, followed by reperfusion. Vehicle (saline), HSA, R410C, SNO-HSA, and SNO-R410C were administered via the portal vein immediately after initiation of reperfusion. Data are expressed as mean ± S.E.M. (n = 4–6 at each time point). *, p < 0.05 and **, p < 0.01 compared with the vehicle-treated group. #, p < 0.05 compared with the SNO-HSA-treated group.
However, the half-life can be greatly prolonged by forming S-nitrosothiols with cysteine residues of proteins, so that, for example, plasma may contain a long-lasting, circulating reservoir. In the present study, we investigated S-nitrosylation of R410C, a genetic variant of HSA possessing an additional free cysteine at position 410, and examined the physicochemical, biological, and pharmacokinetic properties of the resultant SNO-R410C. The biotin switch assay revealed that Cys-410 was more rapidly and efficiently S-nitrosylated than was Cys-34 in R410C (Fig. 2B), with the S-nitroso content increased from 0.3 mol/mol normal HSA to 1.3 mol/mol R410C.

Earlier structural analysis (Carter and Ho, 1994; Sugio et al., 1999) reported that Cys-34 is located in a crevice on the surface of the albumin molecule but seems to be surrounded and protected by several other residues. In contrast, Cys-410 lies in a tunnel region in subdomain IIIA and seems to be freely accessible to the extracellular milieu (Bhattacharya et al., 2000). This molecular environment of the Cys-410 residue in R410C is indeed thought to be favorable for S-nitrosylation.

As illustrated in Fig. 8, Cys-410 is surrounded by acidic (Glu) and basic (Lys) residues, which may constitute the acid-base motif that may facilitate deprotonation of thiol to form a nucleophilic thyl anion (−S⁻), as suggested recently by Hess et al. (2005). In addition, the presence of hydrophobic moieties (Tyr) near Cys-410 may stabilize NO and thereby promote formation of N₂O₃, a strong S-nitrosylating agent.

Table 1

<table>
<thead>
<tr>
<th>Uptake Clearances</th>
<th>Plasma Concentration after 120 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>ml/h</td>
</tr>
<tr>
<td>HSA NO (−)</td>
<td>222 ± 20 a</td>
</tr>
<tr>
<td>HSA NO (+)</td>
<td>168 ± 14</td>
</tr>
<tr>
<td>R410C NO (−)</td>
<td>324 ± 12</td>
</tr>
<tr>
<td>R410C NO (+)</td>
<td>150 ± 13</td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± S.E.M. (n = 3).*
via reaction with molecular oxygen. Together, these factors should thus contribute to effective S-nitrosylation of R410C at Cys-410.

Earlier work showed that introduction of multiple S-nitroso residues in albumins led to formation of aggregates through intermolecular disulfide formation (Marks et al., 1995; Ewing et al., 1997). Aggregate formation results in induction of molecular heterogeneity, which limits the therapeutic application of S-nitroso albumins that have multiple S-nitroso residues. Katsumi et al. (2005) reported that conjugation of the water-soluble synthetic polymer polyethylene glycol to albumins with multiple S-nitroso residues prevented molecular aggregate formation (Katsumi et al., 2005). In the present study, we found that S-nitrosylation did not induce molecular aggregation of R410C, as shown by nonreducing SDS-PAGE analysis (Fig. 3A). Furthermore, CD spectroscopy showed that S-nitrosylation had only a small influence on the tertiary structure of the protein (Fig. 3, B–E). These results suggest that multiple S-nitroso sites can be introduced in R410C without causing any major conformational changes. It is also important to note that no apparent clinical disorders were observed in human subjects with R410C alloalbuminemia (Galliano et al., 1998). Therefore, we expect SNO-410C to be clinically applicable as a biocompatible pharmaceutical agent, although further study is required to clarify other issues, including antigenicity of this variant protein.

Our investigation confirmed that S-nitrosylation added new biological functions to R410C that were not observed with parental R410C. For example, the present in vitro study with Salmonella revealed that SNO-R410C possessed the strongest antibacterial activity among various S-nitrosothiols that we have tested (Miyamoto et al., 2000a). Our previous data also showed that transnitrosylation from SNO-α1-PI to bacteria is a critical step for the antibacterial action of SNO-α1-PI (Miyamoto et al., 2000a). In addition, the N0-releasing agent p-NONOate had very weak antibacterial activity (Fig. 4). These results suggest that, for its antibacterial function, SNO-R410C may act as a nitroso donor rather than a pure NO donor. Likewise, SNO-R410C was the most potent S-nitrosothiol in terms of antiapoptotic activity in FasL-induced apoptosis of U937 cells (Fig. 5).

Reperfusion of ischemic tissue leads to inflammatory responses and endothelial cell dysfunction. S-Nitrosothiols have been shown to improve ischemia-reperfusion injury in various organs in animals (Konorev et al., 1996; Ikebe et al., 2000; Hallstrom et al., 2002; Mittermayr et al., 2003; Semroth et al., 2005). We previously used SNO-α1-PI as an S-nitrosothiol derivative to show that the cytoprotective effects of S-nitrosothiol may involve multiple mechanisms, including 1) maintenance of tissue blood flow; 2) induction of heme oxygenase-1, a cytoprotective enzyme; 3) suppression of neutrophil infiltration; and 4) reduction of apoptosis in the liver (Ikebe et al., 2000). The present study clearly showed that SNO-R410C also had a potent cytoprotective effect on ischemia-reperfusion liver injury, with its effect being stronger than that of SNO-HSA in this reperfusion model, despite its shorter in vivo half-life compared with that of SNO-HSA, as discussed below. Ikebe et al. (2000) observed that administration of GSNO had no cytoprotective effect. We found that the blood concentration of the SNO moiety after GSNO administration decreased very rapidly compared with that of SNO-HSA (Fig. 7). These findings suggest that sufficient half-life in the circulation of S-nitrosothiols is important for the cytoprotective effect. It remains unclear, however, whether mechanisms similar to those found for SNO-α1-PI or alternative or additional ones may be involved in cytoprotection mediated by SNO-R410C.

Although S-nitroso levels in blood after administration of GSNO showed a transient increase and rapidly returned to the basal level, appreciable S-nitroso levels could be detected, even after 120 min, in blood of mice receiving SNO-R410C and SNO-HSA (Fig. 7). Experiments with radiolabeled proteins explained this result because carrier proteins that can escape renal excretion because of their large molecular size have a long half-life. However, we observed that R410C was more rapidly cleared from circulation than was HSA. A similar result was reported by Iwao et al. (2006): a single amino acid substitution at position 410 from arginine to alanine (R410A) shortened the half-life of the HSA mutant in circulation. It is of interest that S-nitrosylation improved the in vivo half-life of R410C, which may be because of the reduced liver uptake of SNO-R410C compared with that of R410C (Fig. 7; Table 1). All these findings indicate that using SNO-R410C can achieve prolonged delivery of NO, which is sufficient for in vivo biological activity.

In conclusion, our data suggest that SNO-R410C is not only a useful NO carrier but also a member of a new class of S-nitrosoylated proteins possessing beneficial biological properties—antibacterial, antiapoptotic, and cytoprotective. Advantages of using SNO-R410C as an RNSO include 1) a high degree of S-nitrosylation, 2) physicochemical stability in solution and during lyophilization, 3) physiological degradation products (R410C and NO), and 4) sufficient half-life in circulation. Based on these advantages, we believe that further investigation in clinical settings is warranted.

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