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

Yu Ishima, Tomohiro Sawa, Ulrich Kragh-Hansen, Yoichi Miyamoto, Sadaharu Matsushita, Takaaki Akaike, and Masaki Otagiri

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences (Y.I., S.M., M.O.) and Department of Microbiology, Graduate School of Medical Sciences (Y.I., T.S., T.A.), Kumamoto University, Kumamoto, Japan; Department of Medical Biochemistry, University of Aarhus, Aarhus, Denmark (U.K.-H.); and Department of Biochemistry, School of Dentistry, Showa University, Tokyo, Japan (Y.M.)

Received October 3, 2006; accepted November 27, 2006

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+) 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-nitrosoalbumin 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 postischemic 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 R410C, 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-nitrosoalbumin 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 postischemic 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 R410C, 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.
SNO-α1-PI were more powerful than those of NO alone and of low-molecular-weight S-nitrosothiols such as S-nitrosoglutathione (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 hydrophobic binding pocket of subdomain ΙΙΑ (Galliano et al., 1998). The amino acid substitution of the variant introduces an additional cysteine residue (Cys-410), which is far from disulfide bonds and from the normal Cys-34. Therefore, the presence of the new cysteine residue does not alter 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 antipototic 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-α1-PI, GSNO, and non-S-nitrosothiol types of NO donors. We also examined the cytotoxic and therapeutic activities of SNO-R410C in an ischemia-reperfusion liver 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 gene stably integrated into the chromosomal DNA was used to produce HSA (Matsushita et al., 2004). We made the R410C mutant by using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the following mutagenic primers (sense and antisense): 5′-GAATGGCTATATTGTT-TGCTACACCAAGAACGTACC-3′ and 3′-GGGTACTTCTTTGTT-GTACGAAACTAATGCGCATTC-5′.

The constructed plasmids (pPIC9-wHSA and pPIC9-HSA R410C) were transferred to XL10-Gold Escherichia coli cells, which were grown in Luria-Bertani medium. After plasmid purification from E. coli, the plasmid sequences were confirmed by use of the dideoxy chain termination method with a PerkinElmer (Boston, MA) ABI Prism 310 Genetic Analyzer. P. pastoris GS115 hiesl 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% biotin, and 1% glycerol) in 1-liter baffled flasks until an A₆₀₀ 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% biotin, and 1% methanol) to an approximate A₆₀₀ 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 M 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 (Akkaike 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-n1-Pi 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 redissolving 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 redissolving.

**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 nitrosothiol binding 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 20 mM sodium phosphate buffer (pH 7.4)/H9258/H9262/H11005 at a dose of 0.1 mg/kg. At appropriate times after injection, blood was obtained from 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.

**Cytotoxic Effect of S-Nitrosothiols.** 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 abdominal 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 (Hnastovich 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 (CLtissue) according to integration plot analysis. By dividing the amount in a tissue at time t (Xt) and the area under the plasma concentration-time curve (AUC) from time 0 to t (AUC0–t) by the plasma concentration at time t (Ct), CLtissue was obtained from the slope of the plot of Xt/Ct versus AUC0–t/Ct. 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 endogenous 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 dif-

- nitrosylation as quantified by the Griess assay with an HPLC flow reactor system. Data are expressed as mean ± S.E.M. (n = 4–6).

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 IC50 in the low micromolar range. In agreement with previous reports, we found that low-molecular-

![Fig. 1. Efficiency of S-nitrosylation of HSA by isoamyl nitrite as a function of R410C content. HSA containing various proportions of R410C was reacted with isoamyl nitrite. The value of the S-nitroso moiety was determined by the Griess assay with an HPLC flow reactor system. Data are expressed as mean ± S.E.M. (n = 4–6).](image-url)
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-$\alpha_1$-PI, used as a positive control, suppressed bacterial growth with an IC$_{50}$ value of approximately 6 mM. SNO-HSA was as potent as SNO-$\alpha_1$-PI, with an IC$_{50}$ value of approximately 8 mM, in suppressing bacterial growth. The corresponding value for SNO-R410C was submicromolar (0.6 mM), which indicated that the antibacterial activity of SNO-R410C was 10 and 13 times stronger than that of SNO-$\alpha_1$-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

---

**Fig. 2.** Analyses of site and rate of S-nitrosylation in R410C produced by GSNO. A, SDS-PAGE of R410C cleaved with CNBr, followed by CBB staining or the biotin switch assay with streptavidin-conjugated horse-radish peroxidase. B, time profile of S-nitrosylation of R410C as determined by the biotin switch assay. Band intensities of fragment 1 to 288 and fragment 299 to 585 were plotted against time of incubation with GSNO. Data are expressed as mean ± S.E.M. (n = 3).

**Fig. 3.** Structural integrity of HSA and R410C with and without S-nitrosylation. A, nonreducing SDS-PAGE, for examination of oligomerization. The gel was stained by CBB. Molecular mass markers are indicated at the left of the gel. B–E, far-UV (B and D) and near-UV (C and E) CD spectra for HSA (B and C) and R410C (D and E) with and without S-nitrosylation.

**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-$\alpha_1$-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-\(\alpha_1\)-PI showed that a quantity of 0.1 \(\mu\)mol/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 \(\mu\)mol/rat had a cytoprotective effect (data not shown). To evaluate liver injury, we measured the extra-cellular release of the liver enzymes AST and ALT via plasma enzyme values. As reported previously for SNO-\(\alpha_1\)-PI and \(\alpha_1\)-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/carrier) 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.
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 $N_2O_3$, a strong $S$-nitrosylating agent.

### Table 1

<p>| Uptake Clearances and Plasma Concentration of Protein and $S$-Nitroso Moieties at 120 min after Injection of $S$-Nitrosylated and Native Proteins |
|---------------------------------|-----------------|-----------------|-----------------|
|                                  | Uptake Clearance |                  | Plasma Concentration after 120 Min |</p>
<table>
<thead>
<tr>
<th>Liver</th>
<th>Kidney</th>
<th>Carrier</th>
<th>S-NO</th>
<th>S-NO/Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA NO (−)</td>
<td>222 ± 20</td>
<td>48 ± 18</td>
<td>62.6 ± 4.4</td>
<td>34.6 ± 8.0</td>
</tr>
<tr>
<td>NO (+)</td>
<td>168 ± 14</td>
<td>126 ± 14</td>
<td>63.4 ± 7.2</td>
<td>42.2 ± 4.7</td>
</tr>
<tr>
<td>R410C NO (−)</td>
<td>324 ± 12</td>
<td>408 ± 17</td>
<td>36.0 ± 4.0</td>
<td>29.3 ± 5.5</td>
</tr>
<tr>
<td>NO (+)</td>
<td>150 ± 13</td>
<td>444 ± 25</td>
<td>42.2 ± 4.7</td>
<td>29.3 ± 5.5</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-R410C to be clinically applicable as a biocompatible pharmacological 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 NO-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-nitrosylated 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.

Acknowledgments

We thank Judith B. Gandy for her excellent editing of our manuscript. We also thank Dr. Yuka Unno at the Center for Drug Discovery, Graduate School of Pharmaceutical Sciences, University of Shizuoka, and Dr. Jun Yoshitake at the Yamagata Promotion Organization for Industrial Technology, for helpful discussions. We thank Dr. Teruo Akuta, at the Effector Cell Institute, Tokyo, and members of the Gene Technology Center in Kumamoto University for their important contributions to the experiments.

References


S-Nitrosylation of Human Serum Albumin R410C


Address correspondence to: Masaki Otagiri, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: ogagiri@gpo.kumamoto-u.ac.jp


