Nitrosylated Bovine Serum Albumin Derivatives as Pharmacologically Active Nitric Oxide Congeners

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ABSTRACT
Although nitrosothiols have been suggested to act as regulators of cell (patho)physiology, little is known about the pharmacology of nitrosylated proteins as nitric oxide (NO) congeners. We describe the molecular consequences of nitrosylating bovine serum albumin (BSA) at multiple specific sites and demonstrate that the product S-nitrosoproteins exert NO-like activity. The content of nucleophilic nitrosylation sites (i.e., free sulfhydryl groups) in native BSA was increased by either reduction with dithiothreitol or thiolation with N-acetylimidazole. Fourteen moles of nitrogen monoxide (NO)/mol BSA equivalent were then selectively positioned on either the endogenous sulfhydryl groups of reduced BSA or the homocysteine moieties of thiolated BSA, respectively. Each resulting S-nitrosoprotein adduct was an oligomeric mixture across the 2000 kDa to 66 kDa molecular mass range. The BSA-derived S-nitrosoproteins were immunoreactive with antibodies against native BSA but evidenced compromised long-chain fatty acid binding. Both types of BSA-derived S-nitrosoproteins suppressed human coronary artery smooth muscle cell proliferation to a similar degree (IC₅₀ ~70 μM NO′ equivalents) and were significantly more effective antiproliferative agents than a standard NO′ donor, DETA NONOate. Antiproliferative bioactivity reflected the NO functionalities carried by each protein, but was independent of molecular mass of the nitrosylated BSA adducts. These data exemplify the rational design and characterization of protein-based S-nitrosothiols as NO′ congeners and suggest that such agents could have therapeutic potential as NO delivery systems.

Extensive research supports diverse roles for NO′ as a cellular mediator, in some instances through second-messenger (i.e., cGMP) formation (Furchgott and Zawadski, 1980; Gross and Wolin, 1995). Nitrogen oxides related to NO′ include NO′-like species, may elicit the nitrosative addition of a NO moiety to nucleophilic centers of biomolecules (Pyror and Squadrito, 1995). Nitrosylation, the covalent attachment of an NO functionality to a thiol nucleophile to yield a nitrosothiol, has been considered a mode of post-translational protein modification (Aronstam et al., 1995; Ewing et al., 1997; Lander et al., 1995). The molecular and pharmacological effects of nitrosylation on protein structure and function are, as yet, not well understood (Simon et al., 1996).

Protein nitrosothiols have been proposed to be bioactive tissue constituents which can modulate cell (patho)physiology (Ignarro et al., 1981; Stamler et al., 1992), perhaps through NO-related intermediates other than NO′ itself (Mathews and Kerr, 1993). One such nitrosothiol, S-nitrosoalbumin, may circulate in mammalian serum as a storage pool of vasoactive NO′ equivalents (Minamiyama et al., 1996; Stamler et al., 1992). A solution of serum albumin bearing multiple S-nitroso groups (<6 mol NO/mol protein) has been reported recently to inhibit neointimal hyperplasia and thrombosis after in vivo mechanical disruption of rabbit femoral artery endothelium (Marks et al., 1995). This observation assumes particular pharmacological significance from the fact that pathogenesis of accelerated atherosclerosis after coronary angioplasty involves, among other factors, vascular cell proliferation, inflammatory cell activation and perhaps cell migration (Mintz et al., 1996).

These limited data suggest that albumin-derived S-nitrosoproteins might have therapeutic utility as a surrogate or congener form of NO′ but do not address the biochemical and molecular properties of such adducts relative to their efficacy and therapeutic potential. Because native albumin across species contains few reduced thiols which tend to be shielded from solvent (Carter and Ho, 1994), its efficient nitrosylation would appear to require chemical derivatization to increase its content of nucleophilic nitrosylation centers (Marks et al., 1995). Assessment of the potential of any such S-NO′-albumin...
adducts to act as NO surrogates necessitates an understanding of their molecular nature and their pharmacological activity in comparison with agents that liberate NO. These considerations led us to synthesize and characterize polynitrosylated BSA and assess its potential bioactivity. We report herein the synthesis of two distinct, stable S-nitroso-BSA preparations reproducibly bearing ~14 mol NO groups per mol BSA equivalent, a more than 2-fold improvement in NO capacity over the previous synthetic effort (Marks et al., 1995). We describe the physical, immunological and biological properties of these derivatives and examine their bioactivity in a model of human vascular cell proliferation. As the first such comprehensive analysis of discrete protein-based NO derivatives, the data exemplify the rational design and analysis of nitrosothiol protein adducts with potential pharmacological utility as NO congeners.

Methods

Materials. Molecular weight markers, Sephadex G-25 (coarse), glucose, BSA (≥97% by agarose electrophoresis, essentially fatty acid free), sulfanilamide, N-(1-naphthyl)ethylene diamine dihydrochloride, N-acetylhomocysteine thiolactone, thiourea, TWEEN 20, silver nitrate, rabbit anti-BSA polyclonal antibody, goat anti-rabbit IgG alkaline phosphatase conjugate, SigmaFast alkaline phosphatase sub-strate and mercuric chloride were obtained from Sigma Chemical Co. (St. Louis, MO). DETA NONOate was from Alexis (San Diego, CA). Dialysis tubing (12–14 kDa cut-off) was from Spectrum Medical (St. Louis, MO). DETA NONOate was from Alexis (San Diego, CA). BioGel-5 M gel-filtration media, Dowex AG50W-X4 resin and nitrocellulose (porosity, 0.2 μm) were obtained from BioRad (Richmond, CA). Dialysis tubing (12–14 kDa cut-off) was from Spectrum Medical Industries, Inc. (Los Angeles, CA). [9,10-3H]Palmitic acid (specific activity, 43.0 Ci/mmol) from New England Nuclear (Boston, MA) was purified according to Burczynski et al. (1993) just before use. Other reagents were obtained commercially from previously identified sources (Ewing and Janero, 1995; Ewing et al., 1997; Janero and Hreniuk, 1996). Aqueous solutions were made with 18 megaohm/cm ultrapure water from a Milli-Q system (Millipore, Inc., Bedford, MA).

Protein assays. BSA was routinely quantified by the method of Lowry et al. (1951). Quantification of protein in fractions from gel-filtration chromatography was with a deoxycholate-trichloroacetic acid modified Lowry assay according to the manufacturer (Sigma). BSA derivatives were quantified by spectrophotometric scanning (225–350 nm) of the respective aqueous protein solutions and comparison of the peak heights with those obtained by scanning native BSA solutions over a range of BSA concentrations within which peak height was linearly related to protein concentration.

Synthesis of poly(S-nitroso-N-acetylhomocysteine)-BSA. Thiolation, nitrosylation BSA (p-S-NO-BSA) was prepared with BSA derivatized with N-acetylhomocysteine. Thiolation of up to 25 g BSA was achieved starting with a 50 mg/ml aqueous BSA solution containing 0.65% (w/v) Tween 20 and a 50-fold molar excess of N-acetylhomocysteine thiolactone (pH 8.5) (Benesch and Benesch, 1958). Aqueous silver nitrate was added gradually to this solution during 15 min to a 50-fold molar excess with respect to BSA, and the solution pH was maintained between 7.2 and 7.6 by concurrent addition of 1 M NaOH. Thiolation was terminated by making the reaction 70 mM in thiourea and acidifying it to pH 2.5 with 1 M HCl. Silver was removed through successive treatments with Dowex AG50W-X4 anion-exchange resin (25°C, 4 h) followed by dialysis against 40-liter volumes of 5 mM HCl (24 h, 4°C) until the silver content was <0.01 mg/ml as determined by atomic absorption spectroscopy. The thiolated BSA adduct (p-S-BSA) was then either de-salted on Sephadex G-25 before gel-filtration chromatography (below) or directly nitrosylated within 48 h of synthesis.

Nitrosylation of p-S-BSA was conducted in aqueous 500 mM HCl containing ~15 molar equivalents of sodium nitrite (30 min, 25°C). After nitrosylation, the solution was lyophilized in a foil-wrapped container. Solid p-S-NO-BSA was stable for several months in the dark at ≤4°C. To assess potential positional specificity of S-nitrosylation, BSA was treated as detailed above, except that silver nitrate was omitted from the reaction.

Synthesis of reduced nitrosylated BSA. r-NO-BSA was prepared by dissolving 1.5 g BSA in 40 ml 20 mM potassium phosphate buffer, pH 7.4, containing 45 mM NaCl and 1.1 μM EDTA. The resulting BSA solution was deoxygenated with nitrogen in an air-tight vessel, care taken to avoid foaming. To this mixture, 4 ml of freshly prepared, deoxygenated, aqueous 0.55 M DTT was added, followed by gentle stirring for 30 min (25°C). The r-BSA solution was dia lyzed (10–14 kDa cut-off) against a total of 6 liters of 100 mM HCl containing 0.1 mM EDTA and 50 mM NaCl at 4°C with three buffer changes during 16 h. The dialysate was recovered for determination of protein (above) and free sulhydryl content with Ellman’s reagent (Sedlak and Lindsay, 1968). The r-BSA solution was diluted to 30 mg protein/ml with dialysis solution and nitrosylated in a sealed amber vial in the presence of a 10% molar excess with respect to Ellman-positive r-BSA sulhydryl content of sodium nitrite in 500 mM HCl for 45 min at 25°C. The r-NO-BSA product was stable for several months in the dark at ≤4°C.

Nitrosothiol determination. Nitrosothiol was routinely quantified as mercury-displaceable nitrite with the Griess reaction by the method of Saville (1958). A chemiluminescence analyzer (Thermied, Inc., Chelmsford, MA) was used in select instances.

Gel-filtration chromatography. Up to 50 mg S-nitroso-BSA was fractionated in a cold room (4°C) under subdued lighting on a calibrated 2.5 × 50 cm BioGel-5 M size-exclusion column. Sample was prepared in column buffer (50 mM Tris, pH 7.5, 100 mM NaCl) containing 5% (v/v) glycerol, neutralized with 1 N NaOH and clarified by centrifugation (500 × g, 5 min) before chromatography. The ~1.4-ml fractions collected under gravity flow were analyzed for their nitrosothiol (Saville, 1958) and protein (Lowry et al., 1951) contents. Column fractions were concentrated in microconcentrators (30 kDa cut-off) (Amicon, Beverly, MA) and stored in the dark at −80°C. Relative peak areas were estimated by the cut-and-weigh technique.

SDS-PAGE and immunoblotting. Protein samples were fractionated on 8% SDS-polyacrylamide gels (Laemmli, 1970). Protein bands were routinely visualized with Coomassie brilliant blue. For Western blotting, bands were transferred to nitrocellulose membranes. Blots were blocked with PBS containing 5% (w/v) nonfat dry milk, 0.2% (v/v) Tween 20 and 0.02% (w/v) sodium azide (16 h, 4°C). After rinsing with PBS, each blot was incubated with rabbit anti-BSA antibody or mouse anti-BSA antibody diluted 1:500 (v/v) in PBS containing 0.2% (v/v) Tween 20 and 0.02% (w/v) sodium azide (1 h, 25°C), with gentle agitation. Excess primary antibody was removed by PBS rinsing, and the blot was incubated as before with the appropriate secondary antibody (goat anti-rabbit immunoglobulin or goat anti-mouse IgG alkaline phosphatase conjugate, respectively) diluted 1:10,000 (v/v) in PBS containing 0.2% (v/v) Tween 20 and 0.02% (w/v) sodium azide (30 min, 25°C). Excess secondary antibody was removed by washing with PBS, and the blot was equilibrated in 25 mM Tris containing 139 mM NaCl and 2.7 mM KCl (pH 8.0) before development. Blots were developed with use of alkaline phosphatase-conjugated goat anti-rabbit antibody with a somewhat extended development to ensure visualization of antigen-antibody complexes over a broad molecular mass range.

Cell culture and proliferation assay. hCASMC from Clonetics Corp. (San Diego, CA) were cultured in complete SmGM-2 medium [containing 5% (v/v) fetal bovine serum, 0.5 mg/ml human recombinant epidermal growth factor, 2 ng/ml human recombinant fibroblast growth factor, 5 μg/ml bovine insulin, 50 μg/ml gentamicin and 50 ng/ml amphotericin B] under humidified 95% air-5% CO2 (37°C).

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Cells were passaged twice weekly (3.5 × 10^6 cells/cm^2 inoculum) and harvested before confluence with 0.025% (w/v) trypsin containing 0.01% (w/v) EDTA. Trypsin activity was blocked with Clonetics Trypsin Neutralizing Solution.

The cell proliferation assay involved seeding ~3 × 10^4 hCASMC in 2 ml SmGM-2 medium per well on 24-well plates. After cell attachment and spreading, each test compound (or an appropriate volume of buffer used to solubilize the test compound) was added to triplicate wells in a volume of ≥0.2 ml as a single bolus. On the second or third day after compound addition, the cells were examined microscopically and counted in a hemocytometer after trypsinization. At these times, the cell count was 6.8 ± 0.4 × 10^4 and 14.4 ± 2 × 10^4 hCASMCs/well, respectively (means ± S.D.; n = 3 cultures). Trypan blue dye exclusion was used to discriminate between viable and dead cells. Lactate dehydrogenase release was assessed spectrophotometrically with a commercial kit (Sigma).

**Long-chain fatty acid binding.** Relative palmitic acid binding to native and modified BSA preparations was determined as a heptane/water partition ratio (Burcynski et al., 1993). Each incubation contained 25 ml of a 10 μM protein solution overlaid with 2.0 ml 0.4 μM [3H]palmitic acid in heptane. All protein solutions were in the aqueous buffer described (Burcynski et al., 1993), except for r-BSA and r-NO-BSA, which were in water. After a 24-h incubation with gentle shaking at 37°C, the aqueous and organic phases were sampled for determination of the [3H]-label in each. Appropriate protein-free “blanks” were processed in parallel to quantify any background partitioning of fatty acid into the aqueous phase. The data were calculated as the ratio of total [3H]-label in the heptane phase to net [3H]-label in the aqueous phase.

**Data evaluation.** All data are mean values ± S.D. of three or more independent determinations. Statistical comparison of mean values was performed by the Student’s t-test. A difference at P < .05 was considered statistically significant.

**Results**

Two protein nitrosothiols, designated p-S-NO-BSA and r-NO-BSA, were synthesized by acid nitrosylation of BSA thiolated with N-acetylhomocysteine or BSA reduced with DTT, respectively. In the latter case, at least 40% of the ~34 cysteines in native BSA disulfides became Ellman-positive nucleophilelc thiols theoretically amenable to nitrosylation. Reaction of either BSA-derived matrix with acidic sodium nitrite yielded gram quantities of S-nitrosoprotein reproducibly bearing an average of 12 ± 3 (n = 10) and 15 ± 3 (n = 12) mol NO/mol BSA equivalent for p-S-NO-BSA and r-NO-BSA, respectively. The photolytically displaceable NO of both S-nitrosoproteins was quantitatively removed by mercury pre-treatment, which indicated that BSA nucleophilec centers other than thiols had not become nitrosated (data not shown). This identical degree of nitrosylation demonstrates that introduction of exogenous thiol moieties into BSA did not enhance NO loading relative to BSA having its endogeneous sulfhydryls exposed as reduced thiols. Studies in which the p-S-NO-BSA synthesis protocol was performed with and without silver nitrate before acid nitrosylation followed by comparative analysis of the NO content of these derivatives by the Greiss-Saville reaction revealed a nearly absolute positional specificity (~80%) of nitrosylation to the homocysteine moieties.

p-S-NO-BSA and r-NO-BSA were analyzed to probe select molecular, immunological and biological characteristics of these proteins relevant to their potential pharmacological action as NO congener. By nonreducing SDS-PAGE (fig. 1), p-S-NO-BSA (panel a, lane 3) and r-NO-BSA (panel b, lane 3) were molecularly heterogeneous. In each case, some nitrosylated protein migrated with a molecular mass (~66 kDa) approximating native monomeric BSA. But significant S-nitrosoprotein was retarded in the gels, consistent with the presence of oligomers over a broad molecular mass range. In almost all cases, some very high molecular mass, BSA-derived S-nitrosoprotein did not enter the stacking gel. The high molecular mass species in p-S-BSA (fig. 1, panel a, lane 2) and r-BSA (fig. 1, panel b, lane 2) demonstrate that oligomer formation began before the nitrosylation reaction, i.e., during thioliation or reduction, respectively.

As demonstrated further by the data in figure 1, p-S-BSA (panel a, lane 5), r-BSA (panel b, lane 5), p-S-NO-BSA (panel a, lane 6) and r-NO-BSA (panel b, lane 6), when reduced with DTT before SDS-PAGE, exhibited a banding pattern markedly different than that observed with nonreduced samples. Under reducing conditions essentially all protein present in the samples migrated as monomeric BSA (fig. 1, panels a and b, lane 4), which suggested a major role for intermolecular disulfide bridges in the generation of oligomeric species of p-S-BSA, r-BSA and their nitrosylated counterparts. The slight upward mobility shift on extensive reduction (fig. 1, panels a and b, lanes 4–6) may reflect a conformational change of BSA monomer associated with compromise of intramolecular disulfide bridges and consequently altered detergent binding (Mahoney et al., 1996).

Analytical gel-filtration chromatography of p-S-NO-BSA revealed three broad classes of molecules between >2000 kDa and <1 kDa molecular mass (fig. 2, panel a). The first class, protein of ≥1000 kDa, typically represented some 35% of total eluted protein area. Most of the remaining protein

**Fig. 1.** SDS-PAGE analysis of r-NO-BSA and p-S-NO-BSA. Protein samples were fractionated by SDS-PAGE. (a) lanes 1 and 4, BSA; lanes 2 and 5, p-S-BSA; lanes 3 and 6, p-S-NO-BSA. (b) lanes 1 and 4, BSA; lanes 2 and 5, r-BSA; lanes 3 and 6, r-NO-BSA. Lanes 1 to 3 and 4 to 6 in each panel are samples run under nonreducing and reducing conditions, respectively. Each lane = 5 μg protein. Molecular mass markers (kDa) are indicated to the left of the gel series. The gels shown are representative of four or more independent experiments.
eluted in the <1000 kDa to 29 kDa mass range. Mercury-displaceable NO content tracked the protein elution profile, demonstrating that all species were indeed BSA-derived S-nitrosoproteins, regardless of their apparent molecular mass or the degree of oligomerization relative to native BSA. A third class of molecules contained <5% of the recovered NO equivalents as low molecular mass (<1 kDa), protein-deficient material. Whereas this material was positive in the Griess reaction for nitrite, it was essentially devoid of mercury-displaceable NO (i.e., nitrosothiol). These data suggest that the nonprotein material is largely free nitrite, a stable solution end-product of NO (Gross and Wolin, 1995). A similar distribution of nitrosylated protein species was observed in r-NO-BSA (data not shown).

On nonreducing SDS-PAGE, fractions of either p-S-NO-BSA (fig. 2, panel b, lanes 1–3) or r-NO-BSA (data not shown) generally migrated with an apparent molecular mass consistent with their elution position during size-exclusion column chromatography. Reduction altered the migration of the nitrosoproteins to a position approximating that of monomeric BSA (fig. 2, panel b, lanes 4–6). The equivalent amount of protein applied to each gel lane (fig. 2, panel b) further demonstrates that overall protein staining intensity need not reflect total nitrosylated protein load, which suggests that nitrosylation alters the dye-binding characteristics of BSA. For this reason, size-exclusion chromatography, and not SDS-PAGE, appears to be a more reliable index of the molecular mass distribution of p-S-NO-BSA species (cf. fig. 2, panel a). Similar results were obtained for r-NO-BSA (data not shown).

Polyclonal antiserum raised against native BSA which, by definition, contains a mixture of antibodies that likely recog-
ize a variety of antigenic epitopes of the protein was used in Western blot analyses to probe whether nitrosoprotein formation affected the immunoreactivity of S-nitroso-BSAs such that a derangement in higher order structure of the molecules might be suggested. As shown in figure 3 (panels a and d), neither thiolation, reduction nor nitrosylation altered the immunoreactivity pattern of the BSA-derived S-nitrosoproteins with polyclonal antibody. Immunoreactive material was observed in both p-S-NO-BSA (panel b, lane 1) and r-NO-BSA (panel d, lane 2) over the entire molecular mass range of the Coomassie-stained protein profile (panel a, lane 2; panel c, lane 2, respectively). Analysis of p-S-NO-BSA and r-NO-BSA immunoreactivity to monoclonal antibody against native BSA yielded similar results (data not shown), although the monoclonal preparation would be predicted to recognize only a single epitope in the BSA molecule.

Transport of fatty acid substrate to peripheral tissues is a major physiological function of serum albumin (Carter and Ho, 1994). Consequently, we used [3H]palmitic acid to assess the potential effect of BSA nitrosylation on the molecule’s ability to carry long-chain fatty acid. A dramatic, near total loss of fatty acid binding capacity to BSA followed either BSA thiolation (p-S-BSA) or reduction (r-BSA) (table 1). Fatty acid binding capability remained diminished relative to native BSA yielded similar results (data not shown), although the monoclonal preparation would be predicted to recognize only a single epitope in the BSA molecule.

Vascular smooth muscle cell proliferation can be inhibited by gaseous NO’ and NONOate NO’ donors (Mooradian et al., 1995; Sarkar et al., 1996). We assessed the bioactivity of polynitrosylated BSAs by evaluating whether they could inhibit hCASMC proliferation in vitro. As shown in figure 4a, a single bolus of either p-S-NO-BSA or r-NO-BSA suppressed serum-stimulated hCASMC proliferation for 3 days. Neither p-S-BSA, r-BSA nor BSA itself was cytostatic, which demonstrated an absolute requirement for the NO functionality in the hCASMC antiproliferative response. Nitrite and nitrate, the stable solution end-products of NO oxidation, were likewise without antiproliferative effect (data not shown). Inhibition of hCASMC proliferation by p-S-NO-BSA and r-NO-BSA was concentration-dependent (fig. 4, panel b). Both S-nitrosoproteins inhibited cell growth with an IC_{50} \approx 70 \mu M NO equivalents. The standard NO’ donor, DETA NONOate, was 2-fold less potent an antiproliferative agent. In all cases, maximal inhibition of hCASMC proliferation was 80%, and the hCASMC density in the maximally inhibited cultures approximated the original cell inoculum. There was no net lactate dehydrogenase release into the medium of the NO-inhibited cell cultures, and removal of either NO-BSA adduct reinitiated serum-stimulated cell proliferation (data not shown). These data and the viability of the NO-treated cells as judged by trypan blue exclusion indicate that the BSA-derived S-nitrosoproteins did not exert their antiproliferative effect as cytotoxins, i.e., did not induce necrotic cell death.

The extensive molecular heterogeneity observed in p-S-NO-BSA and r-NO-BSA prompted investigation as to whether their cytostatic activity was related to their molecular mass. Direct examination of high (~2000–100 kDa) and low (~100–30 kDa) molecular mass fractions of p-S-NO-BSA (fig. 5) and r-NO-BSA (data not shown) at ~80 \mu M NO equivalents demonstrated that antiproliferative activity was independent of S-nitrosoprotein molecular mass.

### Discussion

Although bioactivity has been attributed to at least some nitrosothiols (Ignarro et al., 1981; Mathews and Kerr, 1993; Stamler et al., 1992), the potential of nitrosylation to yield

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heptane/Water Ratio</th>
<th>Relative Fatty Acid Binding</th>
</tr>
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<tbody>
<tr>
<td>BSA (in buffer)</td>
<td>0.011 ± 0.004</td>
<td>1</td>
</tr>
<tr>
<td>p-S-BSA</td>
<td>33.6 ± 1.4</td>
<td>0.003</td>
</tr>
<tr>
<td>p-S-NO-BSA</td>
<td>1.99 ± 0.1</td>
<td>0.006</td>
</tr>
<tr>
<td>BSA (in water)</td>
<td>0.021 ± 0.002</td>
<td>1</td>
</tr>
<tr>
<td>r-BSA</td>
<td>3.2 ± 0.45</td>
<td>0.007</td>
</tr>
<tr>
<td>r-NO-BSA</td>
<td>1.64 ± 0.32</td>
<td>0.013</td>
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*Binding of palmitic acid to BSA and modified BSA preparations was determined as a heptane/water partition ratio after 10 µM aqueous protein solutions were overlaid with 0.4 µM [3H]palmitic acid in heptane and incubated for 24 h at 37°C. The BSA, p-S-BSA and p-S-NO-BSA group was solubilized in the buffer described (Burczynski et al., 1993), whereas the BSA, r-BSA and r-NO-BSA group was solubilized in water. Fatty acid binding to protein is also expressed relative to the appropriately solubilized, native BSA.

*Data are means ± S.D. (n = 4).*

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**Fig. 3.** Assessment of p-S-NO-BSA and r-NO-BSA immunoreactivity by Western blot analysis. (a and c) SDS-PAGE protein gel stained with Coomassie brilliant blue; (b and d) Western blot probed with goat anti-BSA polyclonal antisera. Lanes 1 to 3 in panels a and b are p-S-BSA, p-S-NO-BSA and BSA, respectively. Lanes 1 to 3 in panels c and d are r-BSA, r-NO-BSA and BSA, respectively. Each lane = 5 µg protein. Molecular mass markers (kDa) are indicated to the left of the gel series. The Western blots shown are representative of four or more independent experiments and have been developed to allow visualization of antigen-antibody complexes across a broad molecular mass range on the original gels.
pharmacologically useful chemical entities acting as NO surrogates remains ill-defined, particularly at the molecular level. These considerations prompted our efforts to synthesize and characterize stable, BSA-derived S-nitrosoproteins as prototype study-objects amenable to detailed biochemical and functional characterization at the molecular level. The present findings constitute the first such characterization of a nitrosoprotein adduct and demonstrate that distinct BSA adducts bearing NO moieties on endogenous thiols or (predominantly) on thiols introduced by chemical modification can be generated as bioactive NO delivery agents.

Thiolation or reduction of native BSA before nitrosylation led to extensive molecular heterogeneity via oligomerization (figs. 1 and 2). Despite this change in higher-order structure, at least partial immunoreactivity of the nitrosylated proteins with anti-BSA polyclonal and monoclonal antibodies was retained. Unlike the marked BSA fragmentation induced by oxidant stress (Davies et al., 1992), nitrosylation did not appear to alter BSA primary structure, although the nitrosylation reaction used is oxidative in nature. Long-chain fatty acid binding, an important physiological characteristic of albumins (Carter and Ho, 1994), was almost totally abrogated by thiolating or reducing BSA and was not restored upon nitrosylation (table 1). These findings invite reinterpretation of a recent report (Burczynski et al., 1995) attributing diminished palmitic acid binding by BSA to nitrosylation per se of BSA's single free cysteine. In the more general context of designing protein-based NOz congeners/delivery systems, the changes in some intrinsic properties of native BSA consequent to p-S-NO-BSA and r-NO-BSA synthesis raise consideration as to what extent nitrosylation is compatible with preservation of the structure/function of any given natural protein. Consequently, a biologically inert, “synthetic” (genetically engineered or chemically derived) peptide could represent a more advantageous nitrosylation substrate in certain instances.

The unusual abundance of disulfide-linked cysteines in native albumins (Carter and Ho, 1994) makes them attractive nitrosylation matrices upon reduction. The location of the albumin disulfides almost exclusively between helical domains contributes to the marked stability of this protein (Carter and Ho, 1994). Consistent with this fact is the known resistance of albumin disulfides to reduction (Katchalski et al., 1957) such that we indeed reduce only a portion of the BSA disulfides by DTT treatment. Nevertheless, the NO equivalents in r-NO-BSA approximated those following ni-
trosylation of BSA thiolated with N-acetylhomocysteine. This result does not necessarily imply that each BSA derivative bears the identical NO content after nitrosylation on a per-molecule basis. It does, however, demonstrate that under our nitrosylation conditions BSA, which had been thiolated to increase its reduced sulfhydryl content, afforded no apparent advantage as a nitrosylation substrate over BSA whose endogenous disulfides were partly reduced to thiols. Consistent with results of Simon et al. (1996), we observed that exposure of r-BSA to a 20-fold molar excess of nitrosating agent did not potentiate r-BSA nitrosylation above ~4 mol NO/mol BSA equivalents (data not shown). In contrast to that report, though, a 20-fold excess of nitrosating agent altered r-BSA solubility to generate in our hands an intractable material (data not shown). From a design perspective targeted toward a therapeutically useful S-nitrosoprotein, these data demonstrate the need to balance NO loading with other characteristics (physicochemical, biochemical) of the target protein for nitrosylation deemed essential to its intended pharmacological application. In light of this reasoning, the comparatively more facile synthesis, and recent findings that elevated levels of homocysteine and its derivatives have pathological consequences (Jakubowski, 1997; Mayer et al., 1996), r-NO-BSA may be of greater clinical and scientific interest than p-S-NO-BSA.

The similarity in NO loading and antiproliferative activity of r-NO-BSA and p-S-NO-BSA raised a possibility that the chemically introduced sulfhydrys in the latter preparation were not nitrosylated, i.e., that endogenous sulfhydryl groups potentially exposed during the course of derivatization were the NO acceptor sites. Our studies in which the BSA thiolation reaction with N-acetylhomocysteine thiocarbamoyl chloride was performed in the presence and absence of silver nitrate demonstrate that this is not the case. Nitrosylation of p-S-BSA occurred almost exclusively at its N-acetylhomocysteine moieties and not at endogenous thiols. It is thus now established by our work that r-NO-BSA and p-S-NO-BSA are molecularly distinct S-nitrosoproteins. The site-preferential nature of S-nitrosylation we observe in vitro is reminiscent of instances in vivo whereby cells selectively modify only certain cysteine residues in a given protein with a NO functionality (Aronstam et al., 1995; Lander et al., 1995). By analogy, our data provide the first demonstration that target (protein) modification before nitrosylation by in vitro chemical means is a useful strategy to target the intramolecular sites of NO-moiety addition and help obviate the limited molecular specificity of the nitrosation reaction.

The role of NO from the tunica intima (endothelium) in helping maintain blood vessel tone is well documented (Purchgott and Zawadski, 1980; Gross and Wolin, 1995). Mechanical injury stimulates neointimal proliferation, which may contribute to restenosis of atherosclerotic vessels after balloon angioplasty (Mintz et al., 1996). An adduct bearing <6 mol NO/mol BSA equivalent has been shown by Loscalzo and co-workers to affect vascular tissue, including smooth muscle relaxation (Keaney et al., 1993). Furthermore, local application of a solution of this material reduced intimal proliferation and platelet deposition after balloon injury to the femoral artery in the rabbit (Marks et al., 1995). Because the BSA derivative used in these studies (Keaney et al., 1993; Marks et al., 1995) was not characterized, the extent to which it resembles our polynitrosylated BSA preparations remains unknown. Although our r-NO-BSA and p-S-NO-BSA nitrosoproteins have not been examined for their effects on femoral artery restenosis, their antiproliferative effect on hCASMCs suggests that nitrosylated BSAs deliver bioactive NO. The independence between cytotoxic activity and the molecular mass of the BSA-deriv derived nitrosylated adducts indeed points to NO content as the critical determinant of their antiproliferative activity.

The antiproliferative mechanism of action of r-NO-BSA and p-S-NO-BSA remains to be detailed. Our findings that these BSA-derived S-nitrosoproteins did not alter hCASMC dye exclusion and the cells’ ability to reinitiate serum-stimulated proliferation upon removal of the NO-BSA adduct argue against a cytotoxic mechanism of action. Since redox forms of NO other than NO- may arise from nitrosothiol decomposition (Scorza et al., 1997; Singh et al., 1996), the antiproliferative effect of polynitrosylated BSAs need not involve an NO- induced increase in cGMP (Dierks and Burstyn, 1996; Myers et al., 1990). Nitrosothiols may participate in intermolecular transnitrosylation reactions, transferring an NO moiety to a reduced thiol acceptor (Scorza et al., 1997). The BSA-derived S-nitrosoproteins could thus exert their antiproliferative activity through “NO transfer” to the hCASMCs, in contrast to the direct NO- liberation characteristic of NONOates and other NO- donors (Mooradian et al., 1995). This putative difference may underly the greater antiproliferative efficacy of r-NO-BSA and p-S-NO-BSA in our human cell culture model as compared with DETA NONOate (fig. 4). The mode of bioactive NO release from BSA-derived S-nitrosoproteins notwithstanding, their potency as inhibitors of hCASMC mitogenicity clearly exceeds that of DETA NONOate, the most effective antiproliferative NONOate heretofore known (Mooradian et al., 1995).

Restenosis is a multifactorial disease state likely to extend beyond the well established proliferative component to include, for example, smooth muscle cell migration and differentiation, local vasomotor tone and endothelial dysfunction (Mintz et al., 1996; Shaw et al., 1995). At the cellular level, multiple NO-sensitive targets exist which may potentially be involved in the net suppression of hCASMC proliferation by NO-BSA adducts observed herein (e.g., ribonucleotide reductase, soluble guanylate cyclase) (Dinerman et al., 1993). Furthermore, the bioactivity of the BSA-derived S-nitrosoproteins need not be limited to an antiproliferative effect and may encompass, for instance, vasorelaxation. The present study provides a firm foundation for future investigations addressing these issues.

In conclusion, two novel S-nitrosoproteins prepared from reduced or thiolated BSA have been characterized as molecularly distinct, bioactive NO- congeners. During the course of r-NO-BSA and p-S-NO-BSA generation, significant changes in the intrinsic molecular and physiological properties of BSA were documented, as was the ability of the BSA-derived S-nitrosoproteins to act as antiproliferative NO delivery systems with greater potency than DETA NONOate. Such agents may have important therapeutic advantages over classic NO- donors in the therapy of proliferative vascular disease syndromes. Our study further illustrates important considerations for the rational design and application of NO-based pharmacological agents.
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References


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