Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 444–448, January 1992
Pharmacology

S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds

JONATHAN S. STAMLER*†‡, DANIEL I. SIMON*†‡, JOHN A. OSBORNE*†‡, MARK E. MULLINS§, OMAR JARAKI†‡, THOMAS MICHEL*‡, DAVID J. SINGEL§, AND JOSEPH LOSCALZO*†‡¶

*Brigham and Women's Hospital, †Brockton/West Roxbury Veterans Administration Medical Center, Departments of ‡Medicine and §Chemistry, Harvard University, Boston, MA 02115

Communicated by Pedro Cuatrecasas, October 9, 1991 (received for review August 13, 1991)

ABSTRACT **Endothelium-derived relaxing factor (EDRF) activity has been attributed to the highly labile nitric oxide radical (NO). In view of the fact that the plasma and cellular milieux contain reactive species that can rapidly inactivate NO, it has been postulated that NO is stabilized by a carrier molecule that preserves its biological activity. Reduced thiol species are candidates for this role, reacting readily in the presence of NO to yield biologically active *S*-nitrosothiols that are more stable than NO itself. Because sulfhydryl groups in proteins represent an abundant source of reduced thiol in biologic systems, we examined the reaction of several sulfhydryl-containing proteins of diverse nature and function upon exposure to authentic NO and EDRF. We demonstrate that *S*-nitroso proteins form readily under physiologic conditions and possess EDRF-like effects of vasodilation and platelet inhibition. These observations suggest that *S*-nitrosothiol groups in proteins may serve as intermediates in the cellular metabolism of NO and raise the possibility of an additional type of cellular regulatory mechanism.**

The endothelium-dependent relaxation of vascular smooth muscle first observed by Furchgott and Zawadski (1) has been largely attributed to nitric oxide (NO) derived from L-arginine through the action of NO synthase (2–4). This free radical ultimately stimulates guanylate cyclase by the formation of a nitrosyl–heme complex at the activator site of the enzyme (5, 6); however, the molecular mechanism(s) by which NO is transferred from synthase to cyclase remains poorly understood. The rapidity of the reaction of NO with molecular oxygen (7), superoxide anion (8), and heme (2) as well as nonheme iron (9) and the ready availability of these inactivating reactants in the plasma and cellular milieux militate against simple diffusion-limited transport of free NO in this medium. That endothelium-derived relaxing factor (EDRF) has the relatively long half-life of the order of seconds in contrast to the 0.1-sec half-life of authentic NO *in vivo* (10) suggests that NO is stabilized by reaction with a carrier molecule(s) *in vivo* that prolongs its half-life and preserves its biologic activity.

An important class of biologic molecules that may subserve this role is that containing sulfhydryl functional groups. Ample evidence exists in support of the view that low molecular weight thiols react readily with oxides of nitrogen to form *S*-nitrosothiols (11), which are significantly more stable than NO itself (12), and potent vasodilators and platelet inhibitors (12, 13). These adducts have been proposed as biologically active intermediates in the metabolism of organic nitrates (12–14), although one group suggests evidence to the contrary (15). Recent evidence also supports their role in the mechanism of action of EDRF (16–18).

The richest source of reduced thiol in plasma (and a particularly prevalent source in cellular cytosol) is protein sulfhydryl groups (19). The reaction of NO with protein thiols has not been previously studied, and the potential biological significance of this reaction has been neglected because of the exclusion of proteins from (bio)assays of the functional activity and half-life of EDRF and from analyses of its chemical characteristics. We therefore investigated the reaction of protein thiols exposed to NO, and we present data showing that a variety of proteins of biological significance and relative abundance can be S-nitrosylated. S-Nitrosylation of proteins endows these molecules with potent and long-lasting EDRF-like effects of vasodilation and platelet inhibition that are mediated by guanylate cyclase activation. These observations raise the possibility that *S*-nitrosothiol groups in proteins may serve as intermediates in the cellular metabolism or bioactivity of NO and that their formation may represent an important cellular regulatory mechanism.

MATERIALS AND METHODS

Sodium nitrite was purchased from Fisher. NO gas was obtained from Matheson. Tissue-type plasminogen activator was kindly provided by Genentech. Sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride were obtained from Aldrich. Radioimmunoassay kits for the determination of cGMP, $Na^{15}NO_2$, and [^{3}H]arginine were purchased from New England Nuclear. All other chemicals and materials were obtained from Sigma.

Purification of NO Synthetase. NO synthetase was purified from bovine cerebellum by using a 2′,5′-ADP affinity column eluted with NADPH according to the method of Bredt and Snyder (20). NO synthetase activity was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline as described (20).

Microcarrier Endothelial Cell Culture. Endothelial cells were isolated from bovine aorta by established techniques (21) and cultured on a microcarrier system of negatively charged spherical plastic beads (Biosilon) according to the method of Davies and colleagues (22).

Synthesis of *S*-Nitroso Proteins. To investigate the generality of the reaction between NO and protein thiols, we examined several thiol-containing proteins of diverse nature and function: bovine serum albumin (BSA), abundant in plasma and possessing a well studied single free thiol (23); tissue-type plasminogen activator, a representative endothelium-derived enzyme also possessing a single free cysteine residue (24); and cathepsin B, a lysosomal cysteine protease active at pH 5 (25).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EDRF, endothelium-derived relaxing factor; BSA, bovine serum albumin.

¶To whom reprint requests should be addressed at: 75 Francis Street, Boston, MA 02115.

We developed and validated several methods to S-nitrosylate these proteins. All of these methods are established means for generating NO or EDRF bioactivity. In the first method, proteins were exposed to equimolar $NaNO_2$ in 0.5 M HCl (acidified $NaNO_2$) for 30 min at room temperature. Solutions were then titrated to pH 7.4 with 1 M NaOH and 10 mM Tris/0.15 M NaCl (TBS), and dilutions were made as necessary in TBS (pH 7.4). In the second method, protein S-nitrosylation was achieved in helium-deoxygenated solutions of 0.1 M sodium phosphate (pH 7.4) by exposing the protein solution in dialysis tubing to authentic NO gas bubbled into the dialyzate for 15 min. The proteins were then dialyzed against a large excess of 0.01 M phosphate buffer at pH 7.4 to remove excess oxides of nitrogen. In the third method of preparation, proteins were incubated with bovine aortic endothelial cells stimulated by exposure to high shear forces to secrete EDRF as described previously (16, 17). As a complementary biologic method of synthesis, proteins were also incubated directly with NO synthetase in the presence of the substrate L-arginine (5–500 μM) and cofactors required for enzyme activity [Ca^{2+} (250 μM), calmodulin (10 μM), and NADPH (1 mM)].

Spectroscopy. Ultraviolet and visible absorbance spectra of *S*-nitrosoproteins were measured with a Gilford response spectrometer (CIBA–Corning, Oberlin, OH). ^{15}N NMR measurements of *S*-nitrosothiols were made according to the method of Bonnett *et al.* (26), and spectra were recorded with a Bruker 500-MHz spectrometer (Billerica, MA). A deuterium lock was effected with ^2H_2O and the spectra were referenced to a ^{15}N natural abundance spectrum of a saturated solution of $NaNO_2$ at 587 ppm. Spectra were recorded at 50.68 MHz and the nine transients of 16,000 data points were collected with a 30° pulse width and a 10-sec relaxation delay. Data were multiplied by a 2-Hz line broadening factor before Fourier transformation.

Protein Thiol Derivatization. Carboxyamidation of protein sulfhydryls was performed with a 10-fold molar excess of iodoacetamide at neutral pH for 60 min in the dark at 37°C. Proteins were then dialyzed extensively to remove excess iodoacetamide.

Vessel Bioassay. The details of this bioassay system have been published (27). In brief, New Zealand White female rabbits weighing 3–4 kg were anesthetized with sodium pentobarbital (30 mg/kg). Descending thoracic aorta were isolated, the vessels were cleaned of adherent tissue, and the endothelium was removed by gentle rubbing with a cotton-tipped applicator inserted into the lumen. The vessels were cut into 5-mm rings and mounted on stirrups connected to transducers (model TO3C, Grass Instruments, Quincy, MA) by which changes in isometric tension were recorded. Vessel rings were suspended in 7 ml of oxygenated Kreb's buffer (pH 7.5) at 37°C and sustained contractions were induced with 1 μM norepinephrine. In selected experiments, vessel rings were preincubated with 10^{-5} methylene blue.

Platelets. Venous blood, anticoagulated with 3.4 mM sodium citrate, was obtained from volunteers who had not consumed acetylsalicylic acid or any other platelet-active agent for at least 10 days. Platelet-rich plasma was prepared by centrifugation at 150 × *g* for 10 min at 25°C. Platelet counts were determined with a Coulter Counter (model ZM).

Platelet Gel Filtration. Platelets were gel filtered on a column of Sepharose 2B (4 × 10 cm) in Tyrode's Hepes buffer as described (28). Platelets were typically suspended at a concentration of 1.5×10^8 per ml and were used within 30 min of preparation.

Platelet Aggregation. Aggregation of platelet-rich plasma and gel-filtered platelets was monitored by a standard nephelometric technique, in which 0.3-ml aliquots of platelets were incubated at 37°C and stirred at 1000 rpm in a PAP-4 aggregometer (Biodata, Hatsboro, PA). Whole blood aggregations were performed by impedance aggregometry in a whole blood aggregometer (Chrono-Log, Havertown, PA) (29). *S*-Nitrosoproteins were preincubated with platelets or blood for 10 min and aggregations were induced with 5 μM ADP. Aggregations were quantified by measuring the maximal rate and extent of change of light transmittance and are expressed as a normalized value relative to control aggregations.

Cyclic Nucleotide Assay. Measurements of cGMP were performed as described (30) in a cultured RFL-6 lung fibroblast cell line containing a soluble guanylate cyclase exquisitely sensitive to NO. Reactions were terminated by the addition of ice-cold 0.1 M HCl and, after 20 min, solutions were neutralized for subsequent analysis. Measurements of cGMP were also performed in platelets as described (16). cGMP levels were determined by radioimmunoassay, and acetylation of samples was used to increase the sensitivity of the assay.

Statistics. Paired samples were compared by the Student's *t* test. Dose–effect data were analyzed by two-way analysis of variance. Values of $P < 0.05$ were considered significant.

RESULTS

***S*-Nitrosoproteins: Chemical Analysis.** The formation and stability of the *S*-nitrosoproteins were confirmed by several analytical methods. NO displaced from *S*-nitrosothiol groups with Hg^{2+} was assayed by diazotization of sulfanilamide and subsequent coupling with the chromophore *N*-(1-naphthyl)-ethylenediamine (31). The stoichiometries of *S*-nitrosothiol protein molecules determined by this chemical method are given in Table 1. Confirmatory evidence for *S*-nitrosothiol bond formation in proteins was obtained by spectrophotometry: *S*-nitrosothiols possess dual absorption maxima at 320–360 nm and at ≈550 nm (11–14). As exemplified for *S*-nitroso BSA, absorption maxima were detected at 335 and 545 nm with absorptivities of 3869 $M^{-1}\cdot cm^{-1}$ and 47 $M^{-1}\cdot cm^{-1}$, respectively. As one additional specific measure of protein S-nitrosylation, ^{15}N NMR spectroscopy was used. BSA was S-nitrosylated with ^{15}NO and the ^{15}N NMR spectrum of the resulting species was recorded (Fig. 1). The chemical shift of the observed peak (750 ppm relative to nitrite) was entirely consistent with that of an *S*-nitrosothiol bond (26). In physiologic phosphate buffer (pH 7.4) at 25°C, the half-lives of these compounds are ≈24 hr. The inherent stability of protein *S*-nitrosothiols is quite remarkable and contrasts strikingly with the low molecular weight *S*-nitro-

Table 1. *S*-Nitrosoprotein synthesis

	S-NO/ protein, mol/mol
BSA	0.85 ± 0.04
t-PA	0.88 ± 0.06
Cathepsin B	0.90 ± 0.02
Human plasma	0.87 ± 0.02

BSA (fatty acid free) and cathepsin B were incubated for 30 min with an equimolar concentration of acidified (0.5 M HCl) $NaNO_2$. Tissue-type plasminogen activator (t-PA) was first dialyzed against a large excess of 10 mM HCl for 24 hr to remove excess L-arginine (used to solubilize the protein) and was then S-nitrosylated as described. Freshly obtained human plasma anticoagulated with 3.4 mM trisodium citrate was similarly incubated with 700 μM NO generated from acidified $NaNO_2$, a concentration chosen to approximate the estimated free protein thiol content of plasma (19). *S*-Nitrosothiol formation was also demonstrated with proteins exposed to solutions of authentic NO gas or bovine aortic endothelial cells stimulated to secrete EDRF. *S*-Nitrosothiol content was quantified by the method of Saville (31). The stoichiometries for the individual S-NO/protein molar ratios are given and represent the means ± SEM of three to six determinations. In the case of human plasma, the molar ratio for S-NO/estimated total protein thiol is given.

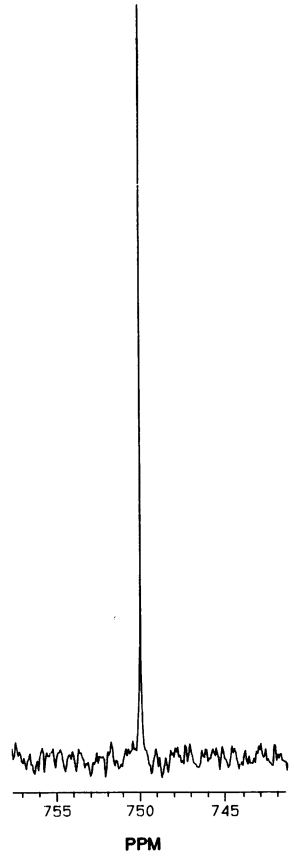

FIG. 1. ^{15}N NMR spectrum of ^{15}N-labeled *S*-Nitroso BSA. *S*-Nitroso BSA was synthesized by incubating BSA (200 mg/ml) with an equivalent amount of $Na^{15}NO_2$ in acidified aqueous solution at 25°C. The chemical shift for *S*-nitroso BSA (referenced to sodium nitrite) was 750 ppm, which falls into the same range as other *S*-nitrosothiols (e.g., *S*-nitroso L-cysteine) prepared under similar conditions. No additional chemical shifts were noted.

sothiols, which are exceedingly unstable under physiologic conditions (12, 32).

S-Nitrosoproteins: Effects on Blood Vessels. The vasodilatory actions of *S*-nitrosoproteins were examined in a bioassay with endothelium-denuded rabbit aortic strips in Kreb's buffer (pH 7.5) at 37°C. Dose-dependent relaxations (expressed as percentage of tone induced by 1.0 μM epinephrine) were observed over the range of 15 nM to 15 μM *S*-nitrosoproteins, and representative mean data for *S*-nitroso BSA are provided in Fig. 2. *S*-Nitrosoproteins synthesized with acidified $NaNO_2$, with NO gas, or by exposure to bovine aortic endothelial cells stimulated to secrete EDRF were essentially equipotent; this is again exemplified for *S*-nitroso BSA in Fig. 2. With the notable exception of *S*-nitroso cathepsin, the relaxation responses to *S*-nitrosoproteins differed from those generally ascribed to EDRF, authentic NO, and the relatively labile low molecular weight biological *S*-nitrosothiols, all of which are characterized by rapid, transient relaxations. In marked contrast, *S*-nitrosoproteins induced a less rapid, but much more persistent, relaxation response (Fig. 3*a*). Furthermore, BSA incubated with NO synthetase in the presence of cofactors required for enzyme activity (calmodulin, NADPH, Ca^{2+}) showed an L-arginine-dependent ability to induce persistent vasorelaxation characteristic of *S*-nitrosoproteins.

The half-life of *S*-nitroso BSA as determined by its addition to the bioassay at various time points after synthesis corresponded with chemical measurements (31) of half-life in phosphate buffer (pH 7.4; 25°C) and is ≈24 hr. In plasma, the half-life is ≈40 min. This half-life is significantly longer than

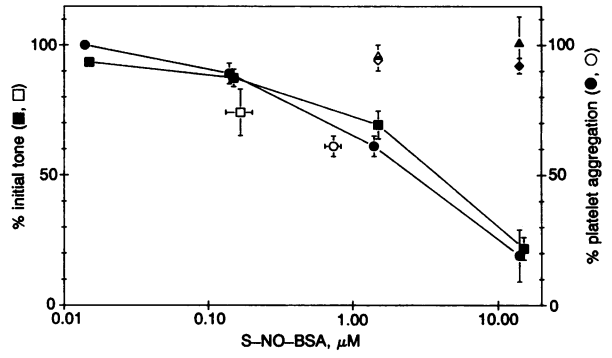

FIG. 2. Dose-dependent relaxation of vascular smooth muscle and inhibition of platelet aggregation with *S*-nitroso BSA (S-NO-BSA). Dose–effect curves for vessel relaxation (■) and platelet inhibition (●), the latter reported as extent of aggregation relative to control, were generated with S-NO-BSA synthesized with equimolar $NaNO_2$ in acidified aqueous solution as described in the text and then neutralized to pH 7.4. Data are presented as means ± SEM (n = 6–18). Open symbols represent experiments in the vessel (□) and platelet (○) bioassays in which S-NO-BSA was synthesized by exposure of BSA to bovine aortic endothelial cells stimulated to secrete EDRF (14, 16). These data are presented as means ± SEM (n = 3–8), with the x axis error bars indicating the variance in the concentration of S-NO-BSA generated from EDRF and the y axis error bars indicating the variance in the bioassay response. In these latter experiments, effluent from cells stimulated to secrete EDRF in the absence of BSA had no significant effect in either the vessel or platelet bioassay. In other control experiments in vessels (△) and platelets (▲) (n = 4–13), $NaNO_2$ at concentrations up to 15 μM had no significant effect on either vessel tone or platelet aggregation. All nonnitrosylated proteins (as shown for BSA) had a small nonspecific effect on vessel tone (◇) and platelet aggregation (◆) at the highest concentration alone. Dose–response curves to S-NO-BSA were significantly different from dose–response curves to BSA (data not shown) and $NaNO_2$ (data not shown) ($P < 0.001$) by two-way analysis of variance.

the half-lives of low molecular weight *S*-nitrosothiols and suggests that the temporal profile of the relaxation response for *S*-nitrosothiols correlates with the lability of the S-NO bond. Blockade of protein thiols by carboxyamidation with iodoacetamide prevented *S*-nitrosothiol formation as determined chemically and rendered the proteins exposed to NO or EDRF biologically inactive (Fig. 3*a*). Consonant with the mechanism of other nitro(so) vasodilators (26), relaxations were abolished by methylene blue, an inhibitor of guanylate cyclase (Fig. 3*a*).

S-Nitrosoproteins: Effects on Platelets. The effects of *S*-nitrosoproteins were first studied in gel-filtered platelets. Dose-dependent inhibition of ADP-induced platelet aggregation was observed over the range of 150 nM to 15 μM *S*-nitrosoproteins; a nitrosylated protein plasma fraction was even more potent, manifesting inhibition at estimated S-NO concentrations of 150 pM. *S*-Nitrosoproteins synthesized with acidified $NaNO_2$, with NO gas, or by exposure to bovine aortic endothelial cells stimulated to secrete EDRF were essentially equipotent, as shown for *S*-nitroso BSA in Fig. 2. Furthermore, the platelet inhibitory effects of *S*-nitrosoproteins (1.4 μM) were confirmed both in platelet-rich plasma and in whole blood. Representative mean data and illustrative aggregation tracings for *S*-nitroso BSA are provided in Figs. 2 and 3*b*, respectively. Carboxyamidation of protein thiols with iodoacetamide or pretreatment of platelets with the guanylate cyclase inhibitor methylene blue abolished the antiplatelet effects of *S*-nitrosoproteins (Fig. 3*b*). In addition, the half-life of the antiplatelet effects correlated with the observations for vascular smooth muscle relaxation. Specifically, the platelet inhibitory potency of *S*-nitrosoproteins 24 hr ($t_{1/2}$) after synthesis in physiologic buffer at pH 7.4 corre-

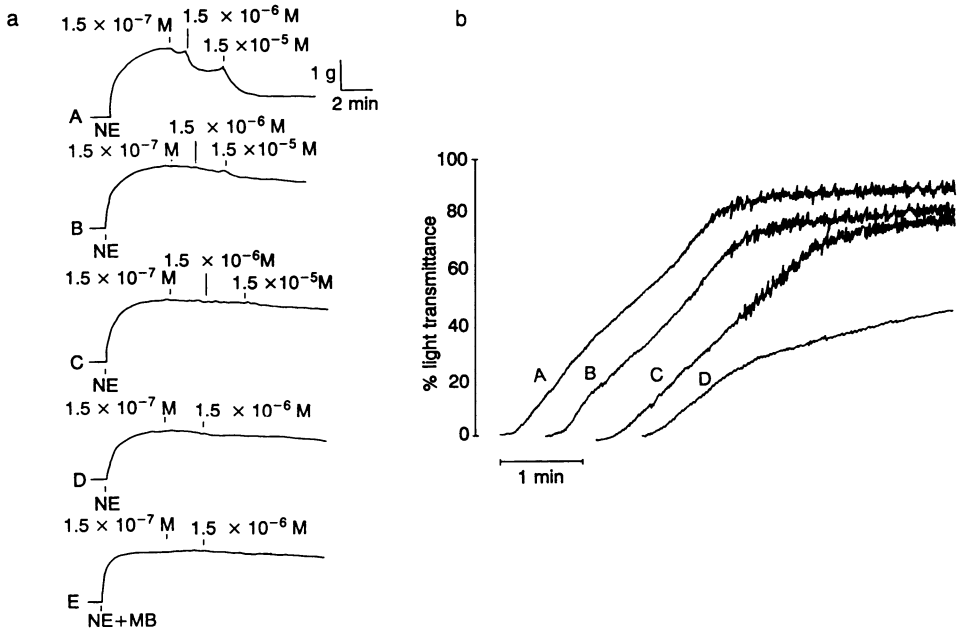

FIG. 3. Representative tracings of vessel relaxation and platelet inhibition to *S*-nitroso BSA (S-NO-BSA). S-NO-BSA was synthesized with acidified $NaNO_2$ as described in the text. Samples were added to vessel and platelet bioassays as described in Fig. 2. (*a*) Illustrative tracings comparing the effects of S-NO-BSA (trace A), $NaNO_2$ (trace B), BSA (trace C), iodoacetamide-treated BSA exposed to acidified $NaNO_2$ (trace D), and S-NO-BSA after vessel rings were pretreated with 10 μM methylene blue (trace E). NE, norepinephrine; MB, methylene blue. (*b*) Illustrative tracings in gel-filtered platelets using the agonist ADP (5 μM), comparing the effects of BSA (1.4 μM) (trace A), iodoacetamide-treated BSA treated with acidified $NaNO_2$ (trace B), S-NO-BSA (1.4 μM) after platelets were pretreated with 1 μM methylene blue for 10 min (trace C), and S-NO-BSA (1.4 μM) (trace D). In additional control experiments, $NaNO_2$ at concentrations up to 15 μM had no significant effect on platelet aggregation (data not shown).

sponded with that predicted from the chemical measurements as determined from the dose–response curve in Fig. 2.

***S*-Nitrosoproteins: Effects on Guanylate Cyclase Activity.** The vasodilatory and antiplatelet actions of low molecular weight *S*-nitrosothiols are mediated by cGMP. This mechanism was therefore studied by using *S*-nitrosoproteins. The activation of guanylate cyclase by *S*-nitroso BSA is shown in Fig. 4. RFL-6 lung fibroblasts incubated with 16 μM *S*-nitroso BSA for 5 min induced 4.6-fold increases in cGMP over basal levels relative to BSA alone. This stimulation by *S*-nitroso BSA was attenuated by the guanylate cyclase inhibitor methylene blue (10^{-4} M). Intracellular platelet increases in cGMP of 70% above basal levels ($n = 4$; $P < 0.025$) were also documented 2 min after the addition of 15 μM *S*-nitroso BSA.

DISCUSSION

Our data demonstrate that (*i*) NO can react either directly or by way of a closely related oxidized derivative thereof (such as NO^+ or N_2O_3) with thiol groups in proteins to form *S*-nitrosothiols; (*ii*) this reaction occurs under physiologic conditions; (*iii*) these compounds are biologically active, exhibiting vasodilatory and antiplatelet properties that are independent of their method of synthesis; (*iv*) the long chemical half-lives of *S*-nitrosoproteins, vis-à-vis the half-life of NO, are reflected in their different relaxation kinetics: *S*-nitrosoproteins induce slower but much more persistent relaxations than does pure NO; and (*v*) the biochemical mechanism of action of *S*-nitrosoproteins, through activation of guanylate cyclase, is fully consistent with that of other nitroso compounds (5, 6, 32).

In view of the rapid reactivity of NO, the means by which it is delivered to target sites in physiologic media remain unknown. In this regard, the reaction of reduced thiol species with NO_x, is of particular interest as it preserves the bioactivity of NO and increases its half-life (12, 17, 33). This reaction has been extensively investigated in the case of low molecular weight *S*-nitrosothiols, which are more stable than NO itself and exhibit antiplatelet and vasodilatory properties mediated by cGMP (12–14, 32). In favor of the biological relevance of these findings, we have shown that low molecular weight thiols potentiate the antiplatelet and vasorelaxant effects of EDRF in a cGMP-dependent manner and also significantly prolong its half-life (16, 17). Moreover, one group has also suggested that EDRF more closely resembles *S*-nitroso L-cysteine than NO (18). A key element lacking in these studies of low molecular weight thiols is the demonstration of *S*-nitrosothiol formation from endogenous NO.

Our study documents *S*-nitrosothiol formation from endogenously derived NO. Thus, our demonstration of S-nitrosylation of biological sulfhydryl groups with EDRF represents critically needed evidence in favor of the role of *S*-nitrosothiols in the biochemical mechanism of endogenously derived oxides of nitrogen. The potential biological importance of this reaction is well exemplified by plasma

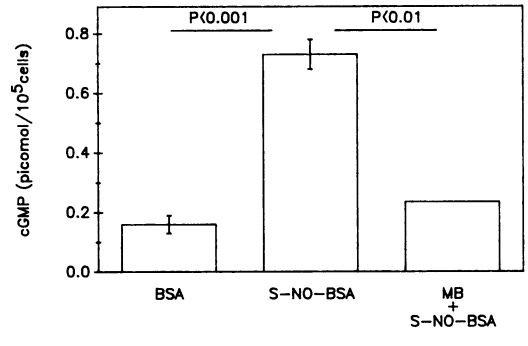

FIG. 4. Stimulation of cGMP production in RFL-6 lung fibroblasts. Exposure of cells to 16 μM *S*-nitroso BSA (S-NO-BSA) (n = 7) for 5 min significantly increased cGMP production above resting levels in the presence of 16 μM BSA alone (n = 6). Stimulation of cGMP production is attenuated by preincubation with methylene blue (MB) (n = 2). Results are expressed as means ± SEM. cGMP assays were performed in duplicate.

experiments in which the protein thiol content, largely determined by albumin, far exceeds in concentration other reduced thiol species (19). Thus, the remarkable stability of *S*-nitroso BSA under physiologic conditions may provide a mechanism for accumulating a pool of molecules active for vasorelaxation and platelet inhibition in plasma.

The obvious question raised by these experiments is how presumably impermeable extracellular protein *S*-nitrosothiols effect intracellular activation of guanylate cyclase. The observations of Kowaluk and Fung (33), revealing a lack of correlation between the bioactivity of RS-NO species, the extracellular stability of the S-NO bond, and their lipophilicity are of particular interest in this regard. The further observation by Mordivinstev and coworkers (34) that protein–sulfur–NO complexes form on the platelet surface in correlation with platelet inhibition by iron–nitrosyl complexes raises intriguing mechanistic possibilities. Thus, mechanistic issues for consideration include bioactivity of NO mediated by initiation of signal transduction processes through interaction with surface thiols or iron–sulfur-centered proteins; transfer of NO across cell membranes by way of a *S*-nitrosothiol or by *S*-nitrosothiol–thiol (or disulfide) exchange reactions; interaction with surface protein thiols to directly impair activation of platelets or smooth muscle through cGMP-independent events, such as might occur by S-nitrosylation of the ADP receptor of the platelet (35) or the critical thiol of myosin (36). We also suggest that intracellular protein S-nitrosylation may serve as a signal transduction mechanism analogous to phosphorylation.

The *S*-nitrosoproteins we have synthesized are meant to be exemplary compounds. The existence and identity of *S*-nitrosoproteins *in vivo* as well as their role in cellular metabolism, stabilization, or transport of EDRF remain to be determined. Nevertheless, we believe that evidence presented here in favor of biological relevance for these compounds is compelling. Additional indications of the existence and biologic importance of S-nitrosylated proteins can be inferred from several other studies. Chong and Fung (37) observed that the denitrification of nitroglycerin in plasma is catalyzed by the thiol of albumin, suggesting an analogy between this mechanism and the thiol-dependent enzymatic denitrification of nitroglycerin with glutathione-*S*-transferase in a reaction generating thionitrates (38); these adducts may undergo spontaneous reduction to biologically active thionitrites (*S*-nitrosothiols) (39). Hemoproteins also catalyze denitrification of nitroglycerin (40) and, interestingly, have been shown to react by way of thiol groups with certain nitroso compounds as part of the hypothesized detoxification pathway for arylhydroxylamines (41).

In summary, S-nitrosylation of proteins is a favorable reaction under physiologic conditions that stabilizes NO in a uniquely bioactive form. These results suggest unusual biochemical and regulatory pathways of endogenous NO metabolism and present a potential mechanism for modification of proteins with important pharmacologic, as well as biologic, consequences.

We thank Ms. Stephanie Francis and Ms. Stephanie Tribuna for excellent technical assistance. This work was supported in part by National Institutes of Health Grants R01-H140411, HL43344, and RRO4870; a Grant-in-Aid from the American Heart Association with funds contributed in part by the Massachusetts Affiliate; by a Merit Review Award from the Veterans Administration; and by National Science Foundation Grant CHE88-14019. J.S.S. is the recipient of a National Research Service Award from the National Institutes of Health (HL 10877); D.I.S. is a Samuel A. Levine Fellow of the Massachusetts Affiliate of the American Heart Association; T.M. is the recipient of a Clinician Scientist Award from the American Heart Association; and J.L. is the recipient of a Research Career Development Award from the National Institutes of Health (HL02273).

1. Furchgott, R. F. & Zawadski, J. V. (1980) *Nature (London)* **288,** 373–376.
2. Palmer, R. M., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327,** 524–526.
3. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. & Chaudhuri, G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9265–9269.
4. Marletta, M. A. (1989) *Trends Biochem. Sci.* **14,** 488–492.
5. Craven, P. A. & DeRubertis, F. R. (1978) *J. Biol. Chem.* **253,** 8433–8443.
6. Katsuki, S., Arnold, W., Mittal, C. & Murad, F. (1977) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **3,** 23–35.
7. Bunton, C. A., Llewellyn, D. R. & Stedman, G. (1957) in *Recent Aspects of the Inorganic Chemistry of Nitrogen* (Chemical Society, London), Spec. Publ. No. 10, pp. 113–120.
8. Blough, N. V. & Zafiriou, O. C. (1985) *Inorg. Chem.* **24,** 3504–3505.
9. Lancaster, J. R., Jr., & Hibbs, J. B., Jr. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1223–1227.
10. Kelm, M. & Schrader, J. (1990) *Circ. Res.* **66,** 1561–1575.
11. Oae, S. & Shinhama, K. (1983) *Org. Prep. Proced. Int.* **15,** 165–198.
12. Ignarro, L. J., Lipton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J. & Gruetter, C. A. (1981) *J. Pharmacol. Exp. Ther.* **218,** 739–749.
13. Mellion, B. T., Ignarro, L. J., Myers, C. B., Ohlstein, E. H., Ballot, B. A., Hyman, A. L. & Kadowitz, P. J. (1983) *Mol. Pharmacol.* **23,** 653–664.
14. Loscalzo, J. (1985) *J. Clin. Invest.* **76,** 703–708.
15. Feelisch, M. & Noack, E. (1987) *Eur. J. Pharmacol.* **139,** 19–30.
16. Stamler, J., Mendelsohn, M. E., Amarante, P., Smick, D., Andon, N., Davies, P. F., Cooke, J. P. & Loscalzo, J. (1989) *Circ. Res.* **65,** 789–795.
17. Cooke, J. P., Stamler, J., Andon, N., Davies, P. F. & Loscalzo, J. (1990) *Am. J. Physiol.* **28,** H804–H812.
18. Myers, P. R., Minor, R. L., Jr., Guerra, R., Jr., Bates, J. N. & Harrison, D. G. (1990) *Nature (London)* **345,** 161–163.
19. Jocelyn, P. C. (1972) in *Biochemistry of the SH Group* (Academic, London), pp. 240–260.
20. Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 682–685.
21. Schwartz, S. M. (1978) *In Vitro* **14,** 966–980.
22. Davies, P. F., Truskey, G. A., Warren, H. B., O'Connor, S. E. & Eisenhaure, B. H. (1985) *J. Cell Biol.* **101,** 871–879.
23. Simpson, R. B. & Saroff, H. A. (1958) *J. Am. Chem. Soc.* **80,** 2129–2131.
24. Loscalzo, J. (1988) *J. Clin. Invest.* **82,** 1391–1397.
25. Hu, L. Y. & Abeles, R. H. (1990) *Arch. Biochem. Biophys.* **281,** 271–274.
26. Bonnett, R., Hollyhead, R., Johnson, B. L. & Randall, E. W. (1975) *J. Chem. Soc. Perkins Trans.* **1,** 2261–2264.
27. Osborne, J. A., Lento, P. H., Siegrfield, M. R., Stahl, H. G., Fusman, B. & Lefer, A. M. (1989) *J. Clin. Invest.* **83,** 465–473.
28. Hawiger, J., Parkinson, S. & Timmons, S. (1980) *Nature (London)* **283,** 195–198.
29. Feinman, R. D., Lubowsky, J., Charo, I. & Zabinski, M. P. (1977) *J. Lab. Clin. Med.* **90,** 125–129.
30. Forstermann, U., Gorsky, L. D., Pollock, J. S., Ishii, K., Schmidt, H., Heller, M. & Murad, F. (1990) *Mol. Pharmacol.* **38,** 7–13.
31. Saville, B. (1958) *Analyst (London)* **83,** 670–672.
32. Ignarro, L. J. (1989) *Circ. Res.* **65,** 1–21.
33. Kowaluk, E. A. & Fung, H.-L. (1990) *J. Pharmacol. Exp. Ther.* **256,** 1256–1264.
34. Mordivinstev, P. I., Rubnevar, G., Vanin, A. F., Shimkevitch, L. L. & Kodorov, B. I. (1986) *Biokhimiia* **51,** 1851–1857.
35. McFarlane, D. E. & Mills, D. C. B. (1981) *J. Cyclic Nucleotide Res.* **7,** 1–11.
36. Kubberod, G., Cassens, R. C. & Greaser, J. (1974) *Food Sci.* **39,** 1228–1231.
37. Chong, S. & Fung, H.-L. (1990) *Drug Metab. Dispos.* **18,** 61–67.
38. Keen, J. H., Habig, W. H. & Jakoby, W. B. (1976) *J. Biol. Chem.* **251,** 6183–6188.
39. Yeates, R. A., Lauffen, H. & Leitold, M. (1985) *Mol. Pharmacol.* **28,** 555–559.
40. Bennett, B. M., Kobus, S. M., Brien, J. F., Nakatsu, K. & Marks, G. S. (1986) *J. Pharmacol. Exp. Ther.* **237,** 629–635.
41. Umemoto, A., Monden, Y., Tsuda, M. T., Grivas, S. & Sugimura, T. (1988) *Biochem. Biophys. Res. Commun.* **151,** 1326–1331.