Role of Thiols in the Targeting of *S*-Nitroso Thiols to Red Blood Cells†

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ABSTRACT: We compared the nitric oxide (•NO)-releasing characteristics of two NO donors, the *S*-nitroso adduct of bovine serum albumin (BSANO) and the *S*-nitroso adduct of L-glutathione (GSNO). In oxygenated phosphate buffer (pH 7.4) and in hemoglobin solution, both NO donors released •NO only in the presence of a low molecular weight thiol (the most active was L-cysteine). The requirement of thiol to release •NO strongly suggests that a transnitrosation reaction occurs between the *S*-nitroso adduct of the NO donor and the sulfhydryl group of the NO acceptor. The reaction produced a labile *S*-nitroso-L-cysteine intermediate that released •NO. As shown by spin-trapping experiments, the transnitrosation reaction involved the formation of •NO (trapped by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) and •S radicals (trapped by 5,5′-dimethyl-1-pyrroline *N*-oxide) of both the NO donors and the NO acceptor (L-cysteine). The reaction leading to •S radical formation was distinct from the transnitrosation reaction, since it was oxygen-dependent. We suggest that •S radicals are formed from oxidizing species produced after a reaction between •NO and molecular oxygen ($•NO_2$ is a likely candidate). As for pure •NO gas, the major oxidation product of NO donors, in phosphate buffer (pH 7.4), was NO_2^-, with no formation of NO_3^-. In the presence of oxyhemoglobin, both NO donors produced only NO_3^-. BSANO and GSNO showed distinct patterns of •NO release both in phosphate buffer and in the presence of hemoglobin. In contrast to BSANO, GSNO oxidized HbO_2 in intact cells at a much slower kinetic rate than with cell lysate or purified hemoglobin. The fast kinetics of BSANO with intact cells suggests binding to the cell surface, where L-cysteine can allow the transport of •NO to the cytoplasm. On account of their ability to oxidize •NO to NO_3^-, red blood cells probably represent the last step in •NO biotransformation or inactivation. The methemoglobin formed in this process was reduced by the NADH-dependent methemoglobin reductase pathway. Our data suggest that sulfhydryl groups, and especially L-cysteine, play a regulatory role in •NO targeting to the red blood cells in plasma, thus buffering the concentration of •NO. Moreover, the *S*-nitroso thiol group of serum albumin may intermediate between cells in the metabolism or bioactivity of •NO.

Nitric oxide (•NO)[1] is a recently discovered messenger molecule involved in at least three important biological processes: vessel smooth muscle relaxation, neurotransmission modulation, and phagocyte cytotoxicity (Moncada, 1992). The •NO is produced from L-arginine and molecular oxygen by an •NO synthase widely distributed in mammalian tissues. •NO is a radical gas molecule that can spread from cell to cell to produce its effects.

Despite the many physiological effects of •NO, excessive amounts (or inappropriate distribution) are detrimental to the organism. The rapidity of its reaction with molecular oxygen or superoxide anion to form toxic oxidizing agents (nitrogen dioxide, $•NO_2$, or peroxynitrite, $ONOO^-$, respectively) and its reaction with heme and nonheme iron militate against simple diffusion-limited transport of free •NO in all tissues.

In appropriate conditions, •NO can react with thiol groups and disulfides to form *S*-nitroso compounds. The predominant redox forms of •NO in (human) plasma are *S*-nitroso thiols, mostly present as *S*-nitroso proteins. These S-nitrosylated proteins, the most abundant of which is serum albumin, are present in micromolar concentrations in normal subjects and show the biological activity of •NO. The generation of NO-containing compounds may be viewed as a means to "package" •NO in forms that are better suited to its intermediary roles. Specifically, the appropriate packaging of •NO might serve to facilitate its transport, prolong its life in the blood and tissues, target its delivery to specific effectors, and mitigate its adverse cytotoxic potential (Hibbs et al., 1987; Hibbs et al., 1988).

Increasing knowledge about the various effects of different NO-donating compounds (NO donors) and their obvious involvement in many bioregulatory systems has renewed both scientific and clinical interest in these compounds. Generally, organic nitrates are metabolized by a combination of enzymatic and nonenzymatic processes. In this regard, *S*-nitroso thiols have been thought of as an exception, since they are able to donate •NO spontaneously to cells and tissues (Stamler et al., 1992a). However, there have been only a few reports in the literature of studies in which the metabolism of NO donors was measured directly (Chung et al., 1992; Feelisch, 1993).

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[1] Abbreviations: BSANO, *S*-nitroso adduct of bovine serum albumin; GSNO, *S*-nitroso adduct of glutathione; CysNO, *S*-nitroso adduct of L-cysteine; •NO, nitric oxide; EPR, electron paramagnetic resonance; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; carboxy-PTI, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; HbO_2, oxyhemoglobin; metHb, methemoglobin; $ONOO^-$, peroxynitrite anion; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid.

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The inactivation of •NO from blood seems to involve oxyhemoglobin (HbO_2), with formation of nitrosylhemoglobin, methemoglobin (metHb), and NO_3^- (Wennmalm et al., 1992; Feelisch & Noack, 1987). The fraction of •NO inactivated as nitrosylhemoglobin or as NO_3^- seems to be determined by the HbO_2/Hb ratio. In arterial blood, •NO was converted to NO_3^- and metHb quantitatively whereas in venous blood about one-third of Hb was transformed into nitrosylhemoglobin (Wennmalm et al., 1992).

The aim of our study was to compare the biochemical characteristics of two NO donors of potential physiologic importance, the *S*-nitroso adduct of bovine serum albumin (BSANO) and the *S*-nitroso adduct of L-glutathione (GSNO). BSANO is an NO donor circulating extracellularly and present in the plasma of normal volunteers (Stamler et al., 1992b). Glutathione is present intracellularly at millimolar concentrations and, as GSNO, is the bioactive intermediate in the NO-dependent activation of the hexose monophosphate shunt of human neutrophils (Clancy et al., 1994). In this work, we found that both NO donors were stable in physiologic buffers and did not spontaneously release •NO in the presence of either red blood cells or purified hemoglobin. The release of •NO and its inactivation to NO_2^- (in physiologic buffers) or to NO_3^- (with hemoglobin or red blood cells) become possible in the presence of L-cysteine. However, BSANO and GSNO showed different biochemical characteristics and reactivity in phosphate buffer, as well as with hemoglobin and red blood cells, thus suggesting different stabilities in biological milieux.

MATERIALS AND METHODS

Materials. BSANO was prepared according to Stamler et al. (1992a) and quantitated by measuring its absorption at 335 nm and by the mercury chloride (200 mM) release of nitrites. Bovine serum albumin, used in this study, has a sulfhydryl-to-protein ratio of 0.6 (Isless & Jocelyn, 1963), and the NO-to-sulfhydryl ratio was 0.80 ± 0.05. *S*-Nitroso-L-cysteine (CysNO) was synthesized by combining an equimolar concentration of L-cysteine with sodium nitrite in 0.5 N HCl (Scharfstein et al., 1994). GSNO and the spin trap 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) were obtained from Alexis Corporation (Laufelfingen, Switzerland). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) and *N-tert*-butyl-α-phenylnitrone were obtained from Aldrich Chemical Co. (Milwaukee, WI). DMPO was purified on activated charcoal before use (Buettner & Oberley, 1978). Bovine serum albumin (fraction V), Cu-Zn superoxide dismutase, catalase, NADPH, NADH, riboflavin, L-cysteine, glutathione, and *N*-acetylcysteine were obtained from Sigma Chemical Co. (St. Louis, MO).

Spectrophotometric Determination of NO Donors. The concentrations of BSANO and GSNO were followed by the absorbances at 335 (A_{335} = 3869 M^{-1} cm^{-1}) and 333.5 nm ($A_{333.5}$ = 774 M^{-1} cm^{-1}), respectively.

Determination of NO_2^- and NO_3^-. The concentration of NO_2^- was determined by the Griess reagent (Green et al., 1982). Briefly, 200 μL of solution was incubated with 800 μL of Griess reagent (0.5% sulfanilamide, 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% H_3PO_4). The absorbance was read at 541 nm using a Lambda 17 UV/vis spectrophotometer (Perkin-Elmer, Norwalk, CT). The concentration of NO_3^- was determined indirectly by treating samples with cadmium pillows (Aldrich) to convert NO_3^- to NO_2^- (Gutman & Hollywood, 1992). The concentrations of NO_2^- and NO_3^- were determined from a calibration curve obtained using standard NO_2^- and NO_3^- solutions.

Red Blood Cell Lysate and Hemoglobin Purification. Heparinized human blood was obtained from normal donors following informed consent. After centrifugation, plasma and buffy coat were removed, and the erythrocytes were washed three times with isotonic phosphate-buffered saline (pH 7.4). Erythrocytes were then lysed in 10 vol of ice-cold hypotonic phosphate buffer (5 mM sodium phosphate buffer, pH 8). The hemolysate was obtained by centrifugation for 15 min at 40000*g*, followed by filtration through a 0.2 μm disposable filter holder (Scleicher & Schuell, Dassel, Germany). HbO_2 was purified as described by Winterbourn (1990), except that the ion exchange column was a TSK-GEL, IEX-540 DEAE (Toyo Soda Co., Japan). HbO_2 solutions, bovine serum albumin, and phosphate buffers were treated with Chelex 100 to remove iron contamination (Buettner, 1988). Measurement of HbO_2 oxidation to metHb was determined by the equations described by Winterbourn (1990).

Electron Paramagnetic Resonance (EPR) Spectroscopy. Spectra were measured on a Bruker ECS 106 spectrometer equipped with an ESP 1600 data system. The sample was drawn in a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeuss Industrial Products, Inc., Raritan, NJ). The Teflon tube was folded four times and inserted into a quartz tube open at both ends and fixed to the EPR cavity (4108 TMH). Unless otherwise indicated, the sample was exposed to air and the temperature was 37 °C.

RESULTS

Formation of NO_2^- from BSANO and GSNO in Phosphate Buffer. The principal oxidation product of •NO gas in aerated isotonic phosphate buffer (pH 7.4) is NO_2^- (Ignarro et al., 1993; eqs 1−3). In the presence of oxygen, this reaction is very rapid (*t*/2 of a second; Wink et al., 1993) and allows the complete oxidation of •NO to NO_2^- with little ot no formation of NO_3^-:

$$2^{\bullet}NO + O_2 \rightarrow 2^{\bullet}NO_2 \quad (1)$$

$$2^{\bullet}NO_2 + 2^{\bullet}NO \rightarrow 2N_2O_3 \quad (2)$$

$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (3)$$

Less known is the time required to form NO_2^- from NO donors. These reactions depend on several factors, including pH, oxygen, thiols, transition metals, and hemoproteins [this topic has recently been reviewed by Feelisch (1991)].

Figure 1 shows the time course of NO_2^- release by BSANO incubated in aerated phosphate-buffered saline (pH 7.4). It is worth noting that, after 1 h, BSANO had neither produced NO_2^- (Figure 1) nor released •NO (as measured by the decrease in the absorption at 335 nm), but required the addition of low molecular weight thiols (active thiols tested were L-cysteine > glutathione > *N*-acetylcysteine). This strongly suggests that a transnitrosation reaction occurs between the cysteine residue of the protein and the low

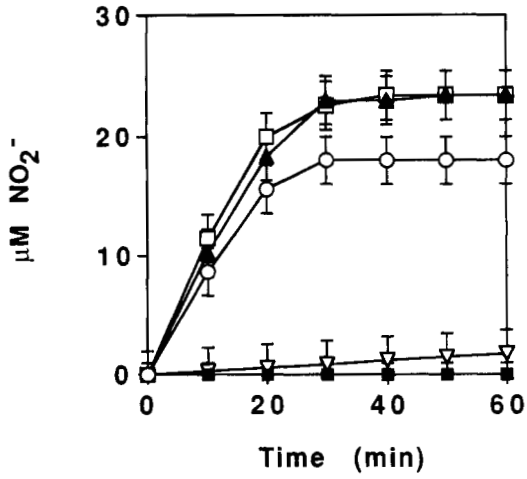

FIGURE 1: Effect of L-cysteine on the time course of NO_2^- formation from BSANO. BSANO (30 μM) was incubated at 37 °C in phosphate buffer (pH 7.4) with 0 (■), 5 (▽), 30 (○), 100 (▲), or 300 (□) μM L-cysteine. At the end of the incubation time, NO_2^- formation was measured by the Griess reagent. Points represent mean value ± SD (n = 4).

molecular weight thiol (NO acceptor), with the formation of a low molecular weight *S*-nitroso thiol intermediate. These intermediates are less stable than BSANO (Feelisch et al., 1994) and decompose to give •NO. Under aerobic conditions and pH > 4, the final fate of the released •NO is the nitrite anion NO_2^-. As shown in Figure 1, when the concentration of L-cysteine was increased, the formation of NO_2^- was strongly induced. When L-cysteine was present in an equimolar amount with BSANO, the half-time required to release 50% of •NO was about 15 min, and the maximum amount of NO_2^- produced was about 60% of the •NO bound to the protein. The NO_2^- produced did not reach 100%, even when the BSANO-to-L-cysteine molar ratio was 1:10 (Figure 1). Since no evidence of NO_3^- formation was found at any L-cysteine concentration, the remaining •NO may be presumed to still be protein-bound.

To verify whether the •NO not released as NO_2^- was still bound to BSA, the samples in Figure 1 were incubated for 40 min (a time sufficient to obtain a plateau level of NO_2^-) and further tested for the presence of residual BSANO. The presence of BSANO was quantitated (i) by measuring the mercury chloride release of nitrite in the Griess reaction and (ii) by the formation of metHb in the presence of 1 mM L-cysteine (Feelisch & Noack, 1987). As shown in Figure 2, the BASNO-to-L-cysteine molar ratio regulates the amount of •NO released from BSANO. When L-cysteine was 2−10 times more concentrated than BSANO, the formation of NO_2^- was about 80% of the total •NO, and 20% of the initial BSANO was detected. Interestingly, in the presence of a 100-fold amount of L-cysteine, the release of NO_2^- was not increased further but was only 60% of the total •NO. This result was similar to that observed by Feelisch et al. (1994) and was due to the chemical stabilization of *S*-nitroso-L-cysteine in the presence of a large amount of L-cysteine.

Figure 3 shows the time course of NO_2^- formation from GSNO in aerated phosphate buffer (pH 7.4). Similar to BSANO, GSNO did not release NO_2^- without L-cysteine. Although we used the same thiol (L-cysteine) to remove •NO from GSNO, the pattern of NO_2^- formation was distinct from that of BSANO, suggesting that the transnitrosation reaction is dependent on the NO donor. The rate of NO_2^-

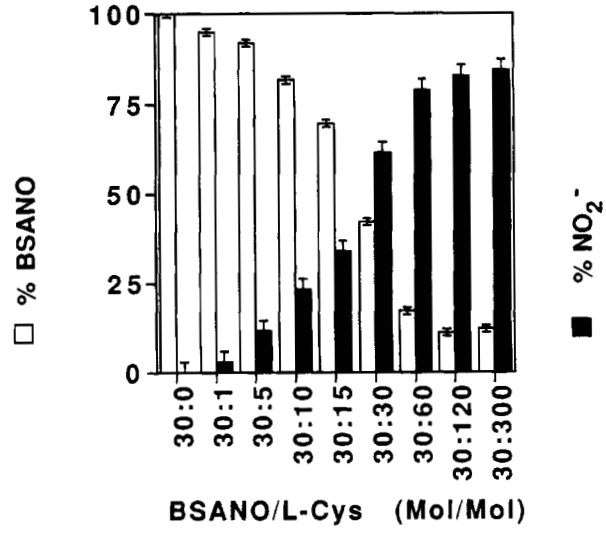

FIGURE 2: Effect of the BSANO-to-L-cysteine molar ratio on the production of NO_2^- in phosphate buffer (pH 7.4). BSANO (30 μM) was incubated at 37 °C with the indicated concentrations of thiol, and NO_2^- production and residual BSANO were measured after 40 min. NO_2^- formation was measured by the Griess reagent. Points represent mean value ± SD (n = 4).

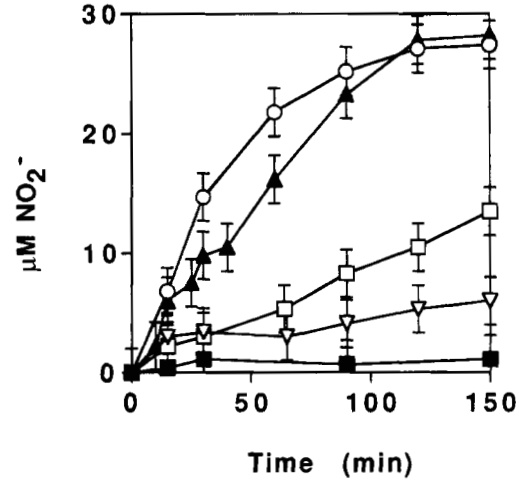

FIGURE 3: Effect of L-cysteine on the time course of NO_2^- formation from GSNO. GSNO (30 μM) was incubated at 37 °C in phosphate buffer (pH 7.4) with 0 (■), 5 (▽), 30 (○), 120 (▲), or 300 (□), μM L-cysteine. At the end of the incubation time, NO_2^- formation was measured by the Griess reagent. Points represent mean value ± SD (n = 4).

formation from GSNO was significantly slower than that observed with BSANO. With an equimolar GNSO-to-L-cysteine ratio, a plateau level was reached only after 120 min (Figure 3). Moreover, the rate of NO_2^- formation was lower when a 10-fold amount of L-cysteine was added (Figure 3). As illustrated in Figure 4, the maximum release of NO_2^- from GSNO was obtained when L-cysteine was present in equimolar amounts or slightly in excess (1:2 or 1:4). When a large excess of L-cysteine was present, NO_2^- formation decreased significantly (Figure 4).

Spin Trapping of Free Radicals Produced by the NO Donors. It is known that •NO is a labile free radical that reacts with nitronyl nitroxides such as carboxy-PTIO (a group of organic compounds with nitronyl and nitroxide functional groups) to produce imino nitroxides (Akaike et al., 1993). The EPR spectra of nitronyl nitroxide and imino nitroxides are characteristic and differ from one another.

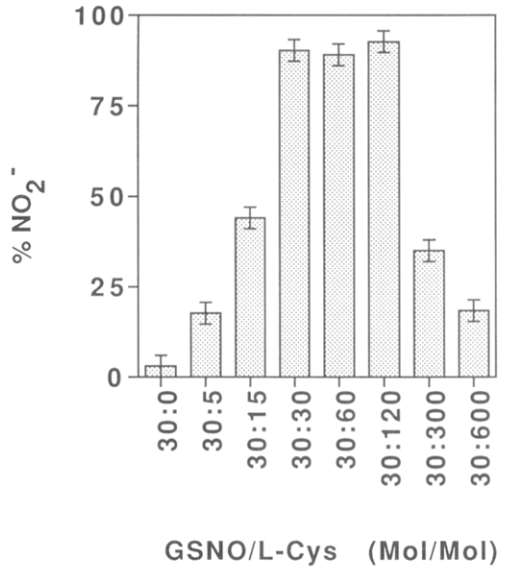

FIGURE 4: Effect of the GSNO-to-L-cysteine molar ratio on the production of NO_2^- in phosphate buffer (pH 7.4). GSNO (30 μM) was incubated at 37 °C with the indicated concentrations of thiol, and NO_2^- production was measured by the Griess reagent after 150 min. Points represent mean value ± SD (n = 4).

The spectrum of carboxy-PTIO consists of a five-line pattern with an intensity ratio of 1:2:3:2:1 (Figure 5a). This spectrum is attributed to an electron interacting with two equivalent nitrogens (a_N = 8.14 G). When carboxy-PTIO was mixed with BSANO or GSNO and L-cysteine, the distinctly different EPR spectra of both carboxy-PTIO and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl) (carboxy-PTI) were observed (Figure 5b,e). The hyperfine coupling constants of the two inequivalent nitrogens of carboxy-PTI (a_N^1 = 4.4 G, a_N^2 = 9.8 G) confirmed the •NO trapping. As expected, the transnitrosation reaction did not occur without L-cysteine, and carboxy-PTI was not observed (Figure 5d). It is interesting that the release of •NO from the NO donors was oxygen-independent. As shown in Figure 5c, anoxic conditions did not affect the spectra of carboxy-PTIO or carboxy-PTI obtained from BSANO and L-cysteine. The reaction of carboxy-PTIO with the NO donors increased in a time-dependent manner: it was slower with GSNO, and, when the NO donors were present in excess, it produced a complete transformation of carboxy-PTIO to carboxy-PTI (spectrum not shown).

We used the spin-trapping agent DMPO to study the intermediates formed during the reaction of NO donors with L-cysteine. Figure 6a shows the radical formed 2−5 min after the addition of an equimolar amount of L-cysteine to an aerobic solution of BSANO. The hyperfine coupling constants of the DMPO adduct (a_N = 15.2 G and a_H = 17.6 G; Harman et al., 1984) suggest the formation of the •S radical of L-cysteine trapped by DMPO. The •S adducts of DMPO−glutathione and DMPO−*N*-acetylcysteine were also observed when these thiols were used in 10-fold greater amounts than BSANO (spectra not shown). However, with these thiols the intensity of DMPO adducts was lower than those of L-cysteine (about one-half for glutathione and one-tenth for *N*-acetylcysteine).

After about 10 min, the DMPO−L-cysteine adduct decayed, and we observed a highly anisotropic immobilized signal (Figure 6b). This radical adduct consists of a triplet of doublets (a_H = 16 G), with considerable broadening of the high-field lines. This is consistent with the trapping by DMPO of a very large radical resulting in the formation of a slowly tumbling BSA−nitroxide radical ($2A_{zz}$ = 52 G) similar to that previously observed by Gatti et al. (1994). This signal was more persistent than that of DMPO−L-cysteine and was not eliminated by extensive dialysis. These data suggest the formation of a BSA−cysteinyl adduct of DMPO produced by the trapping of the only free cysteine of BSA (Cys-34, which is also the only cysteine that is reactive for •NO; Stamler et al., 1992a). The use of NO-free BSA or the omission of L-cysteine resulted in the loss of any DMPO adduct. We used another spin trap, the α-phenyl-*N*-*tert*-butylnitrone, to demonstrate further that the radical of Figure 6b was a BSA •S radical adduct. In the reaction between BSANO and L-cysteine, this nitrone trapped a free radical characteristic of a strongly immobilized nitroxide spin label with $2A_{zz}$ = 62 G assigned to the α-phenyl-*N*-*tert*-butylnitrone−BSA adduct (spectrum not shown). Notably, the identical adduct was first obtained by Graceffa using α-phenyl-*N*-*tert*-butylnitrone as the trapping agent in the reduction−oxidation reaction between Ce(IV) and BSA (Graceffa, 1983). Ce(IV) is reported to oxidize thiols via the •S radical intermediate (Hill & McAuley, 1968; Kertsz et al., 1974).

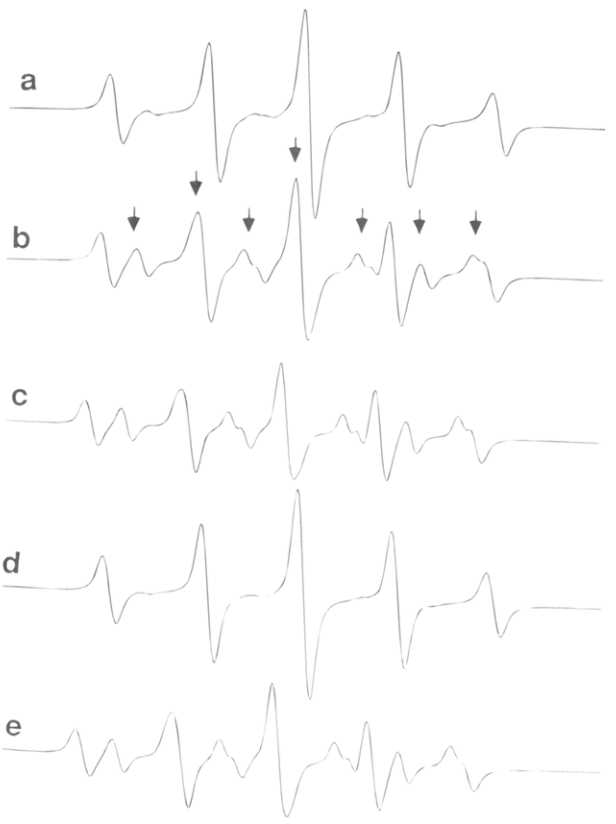

FIGURE 5: EPR spectra of 100 μM carboxy-PTIO in the presence or absence of NO donors: (a) no added NO donors: (b) spectrum obtained 5 min after the addition of 100 μM BSANO and 100 μM L-cysteine in aerobic phosphate buffer; (c) sample from (b), but in N_2; (d) sample from (b), but without L-cysteine; (e) spectrum obtained 30 min after the addition of 100 μM GSNO and 100 μM L-cysteine in aerobic phosphate buffer. The arrow denotes the line position of carboxy-PTI. Spectrometer conditions: frequency, 9.4 GHz; field modulation, 100 kHz; power, 20 mW; scan range, 50 G; time constant, 328 ms; gain, 6.3 × 10^3 (a, d) or 1.25 × 10^4 (b, c, e); modulation amplitude, 0.5 G; scan time, 84 s; number of scans, 1.

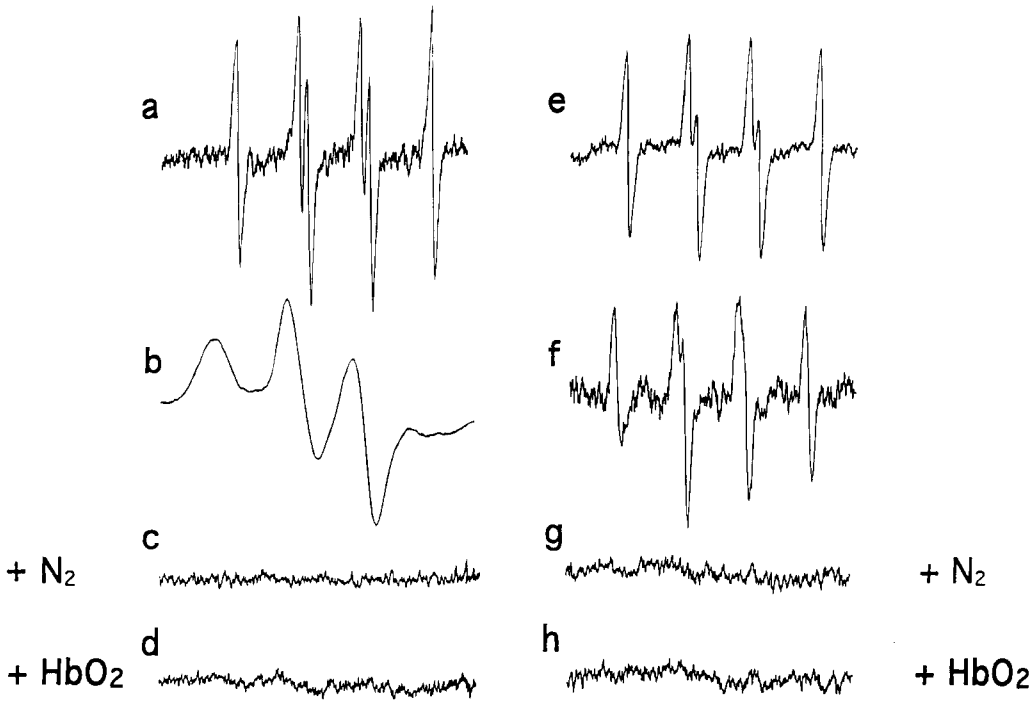

FIGURE 6: EPR spectra of DMPO radical adducts produced by BSANO/L-cysteine or GSNO/L-cysteine: (a) DMPO−L-cysteine adduct from 300 μM BSANO, 300 μM L-cysteine, and 0.1 M DMPO incubated for 3 min at 37 °C in aerobic phosphate buffer (pH 7.4); (b) DMPO−BSA adduct observed from (a) recorded 15 min after mixing; (c) sample from (a), but in N_2; (d) sample from (a), but in the presence of 300 μM HbO_2; (e) DMPO−L-cysteine adduct from 5 mM GSNO and 10 mM L-cysteine incubated for 3 min at 37 °C in aerobic phosphate buffer (pH 7.4); (f) DMPO−L-cysteine and DMPO−glutathione adducts from 1 mM GSNO and 0.3 mM L-cysteine incubated at 37 °C in aerobic phosphate buffer (pH 7.4); (g) sample from (e), but in N_2; (h) sample from (e), but in the presence of 300 μM HbO_2. Spectrometer conditions: frequency, 9.4 GHz; field modulation, 100 kHz; power, 20 mW; scan range, 70 G; time constant, 328 ms; gain, 1.25×10^6 (b, e, f, g, h) or 6.3×10^5 (a, c, d); modulation amplitude, 1.0 (a, c, d−h) or 4.13 G (b); scan time, 84 (a, c, d−h) or 42 s (b); number of scans 1 (a, c, d), 5 (e, g, h), 6 (f), or 40 (b).

It should be noted that BSA is frequently contaminated by transition metals, particularly iron, which is present in phosphate buffers and copper and is "physiologically" bound to serum albumin (Scudder et al., 1978). Fe^{2+} and Cu^{2+} strongly accelerated the formation of •S radicals and the rate of HbO_2 oxidation (our unpublished results). A characteristic Fe^{2+}-NO−BSA paramagnetic complex was produced by non-demetalized BSANO in the presence of L-cysteine (Henry et al., 1991; our unpublished results). To avoid the contamination of transition metals, the BSA used in this study was prepared in transition metal-free buffers and treated extensively with Chelex 100. The copper content of a solution of 700 μM BSANO was reduced from 5.7 μM to 1.2 μM after the Chelex 100 treatment (thus the contamination of copper in our BSANO preparation was about 0.17%). After treatment with Chelex 100, the BSA used in this study did not show detectable concentrations of the Fe^{2+}-NO−BSA complex. Since *S*-nitroso thiol decomposition is known to be metal-dependent (McAninly et al., 1993), we tested the effect of 10 μM DTPA or 10 μM EDTA on the formation of •S radicals produced by BSANO and L-cysteine. DTPA did not abolish the L-cysteine−DMPO and BSA−DMPO adducts, but reduced the signal intensity by 30−40%. This result suggests that transition metals (possibly the residual metal content of BSA) can accelerate the reaction of *S*-nitroso thiol decomposition, with the formation of •S radicals. EDTA did not modify the intensity of •S radicals.

The spectra in Figure 6e−f show the radical species produced by GSNO in the presence of L-cysteine and trapped by DMPO. Figure 6e shows the radical formed after the addition of L-cysteine to an aerobic solution of GSNO. The hyperfine coupling constants (a_N = 15.2 G and a_H = 17.5 G) of the DMPO adduct suggest the formation of the •S adduct of L-cysteine. However, the slight difference between the spectrum of Figure 6e and the spectrum of Figure 6a suggests the possible formation of a low amount of glutathione−DMPO adduct. The hyperfine coupling constants of glutathione−DMPO (a_N = 15.4 G and a_H = 16.2 G; Harman et al., 1986) are similar to those of L-cysteine−DMPO. The formation of the glutathione−DMPO adduct is suggested by the partial overlap of central lines. The likely formation of the glutathione−DMPO adduct is more evident in Figure 6f, when GSNO was added in excess to L-cysteine.

Molecular oxygen was necessary to obtain the spectra in Figure 6a,b,e,f. If the samples were collected in a gas-permeable Teflon tube and inserted in the EPR cavity equilibrated with nitrogen and not in air, none of the DMPO−S adducts were formed. Similarly, the addition of HbO_2 completely eliminated the DMPO radical adducts (Figure 6d,h), presumably due to the removal of •NO from the reaction.

The spectra in Figure 7 show the radical species produced by *S*-nitroso-L-cysteine (CysNO) in the presence of DMPO. The L-cysteine−DMPO adduct was observed when CysNO and L-cysteine were added simultaneously (Figure 7a). Notably, no radical species were detected if only CysNO or only L-cysteine was incubated with DMPO (Figure 7b,c). If the sample was equilibrated in nitrogen (Figure 7d), or if HbO_2 was added in air (Figure 7e), the DMPO radical adduct was not detected. The effect of HbO_2 was likely due to the removal of •NO. We observed the formation of the EPR signal of •NO hemoglobin (Eriksson, 1994) soon after mixing

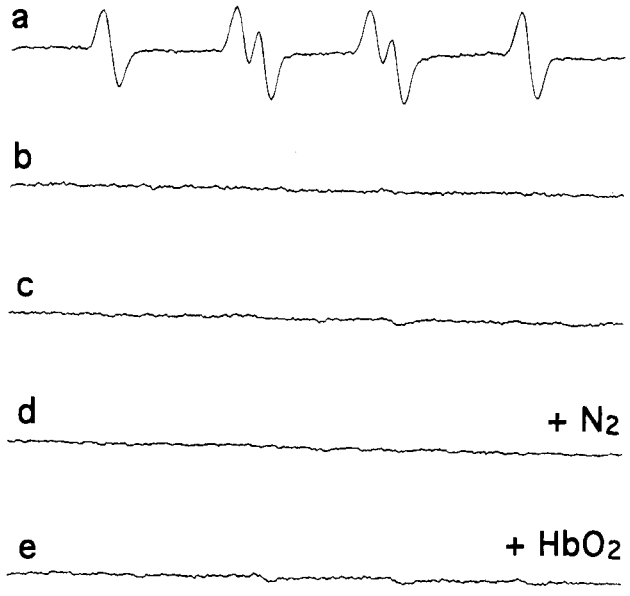

FIGURE 7: EPR spectra of DMPO radical adducts produced by CysNO/L-cysteine: (a) DMPO-L-cysteine adduct from 300 μM CysNO, 300 μM L-cysteine, and 0.1 M DMPO incubated for 2 min at 37 °C in aerobic phosphate buffer (pH 7.4); (b) sample from (a), but without L-cysteine; (c) sample from (a), but without CysNO; (d) sample from (a), but in N_2; (e) sample from (a) but in the presence of 300 μM HbO_2. Spectrometer conditions: frequency, 9.4 GHz; field modulation, 100 kHz; power, 20 mW; scan range, 70 G; time constant, 328 ms; gain, 6.3×10^5; modulation amplitude, 1.0 G; scan time, 84 s; number of scans, 1.

300 μM CysNO or 300 μM BSANO with equimolar amounts of L-cysteine and HbO_2 (spectra not shown).

These results suggest that molecular oxygen, as well as •NO, is involved in the formation of the free radical adducts of DMPO. The addition of 0.1 M dimethyl sulfoxide, 20 μg/mL superoxide dismutase, or 20 μg/mL superoxide dismutase plus 40 μg/mL catalase did not modify the intensity or the type of radical trapped by DMPO from BSANO/L-cysteine or from GSNO/L-cysteine. The formation of DMPO radical adducts did not depend on the laboratory light, since no difference in the signal intensity was observed if the reaction occurred in the dark (spectra not shown).

Reaction of NO Donors with Hemoglobin or Red Blood Cells. NO donors, as well as •NO gas, are known to induce the oxidation of HbO_2 to metHb with complete oxidation of •NO to NO_3^- (Feelisch & Noack, 1987; Ignarro et al., 1993). However, without L-cysteine, BSANO and GSNO did not form metHb (Figure 8) or release NO_3^-, irrespective of whether we used purified HbO_2, red blood cell lysate, or intact cells.

In the presence of L-cysteine, both NO donors produced metHb from HbO_2 (Figure 8) and NO_3^- (the amount of NO_3^- was equimolar to metHb; Ignarro et al., 1993; our unpublished results). BSANO and GSNO showed comparable kinetics of metHb formation with equimolar quantities of L-cysteine. No evidence of other Hb oxidation products, such as hemichromes, choleglobin, or ferrylhemoglobin, was detected [as measured by the spectrophotometric method described by Winterbourn (1990)]. Interestingly, when a 10-fold molar excess of L-cysteine was present, the rate of metHb formation from BSANO was strongly accelerated (50% metHb was produced after 5 min; Figure 8). The reaction with BSANO was quantitative, as 30 μM BSANO

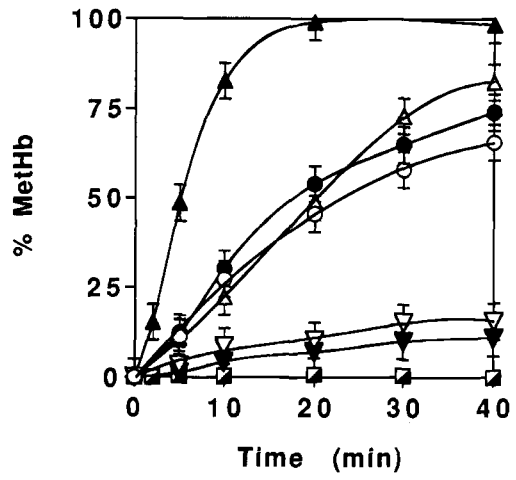

FIGURE 8: Effect of L-cysteine on the time course of metHb absorption spectral changes during oxidation induced by BSANO (closed symbols) or GSNO (open symbols). NO donor (30 μM) and 30 μM purified HbO_2 were incubated at 37 °C in phosphate buffer (pH 7.4) with 0 (□, ■), 5 (▽, ▼), 30 (○, ●), or 300 (△, ▲) μM L-cysteine. The percentage of metHb was determined as described in Winterbourn (1990). Points represent mean value ± SD (n = 4).

consumed, after 15 min, 29.7 ± 1.5 μM HbO_2 (mean ± SD, n = 4). This behavior clearly distinguished BSANO from GSNO, since the latter did not accelerate the rate of metHb formation when L-cysteine was present in larger amounts (Figure 8).

When L-cysteine was present in larger amounts, the oxidation of metHb by BSANO was faster than the formation of NO_2^- (compare Figures 1 and 8). This could suggest that the oxidation of HbO_2 to metHb by the NO donors was not due to previously formed NO_2^-. This interpretation was strengthened by the observation that, when 30 μM NO_2^- and 300 μM L-cysteine were added to intact red blood cells, cell lysate, and purified HbO_2, no appreciable amounts of metHb were formed after 1 h at 37 °C.

Since BSANO is a macromolecular NO donor, it may differ from GSNO with regard to transport inside the cells. It was, therefore, interesting to compare the rate of metHb formation with equimolar amounts of NO donors and L-cysteine in red blood cell lysate and in intact cells (Figure 9). BSANO produced superimposable rates of metHb formation in red blood cell lysate, in intact cells, and in purified HbO_2 (purified HbO_2 not reported in Figure 9). By contrast, the rate of metHb formation for GSNO in intact cells was about 7 times slower than that for BSANO (0.84 and 0.11 nmol mL^{-1} min^{-1} for BSANO and GSNO, respectively), while the rate of metHb formation for red blood cell lysate was comparable for both GSNO and BSANO (Figure 9).

Methemoglobin Reduction from Red Blood Cells Treated with BSANO. Normal red cells reduce metHb by means of two enzymatic pathways. The NADH-methemoglobin reductase is the predominant pathway, and the second pathway is NADPH-dependent (Jandl, 1987). The NADPH-methemoglobin reductase pathway accounts for less than 5% of metHb reduction and seems to be involved when redox agents and/or various quinone derivatives are present.

Red cells containing 90% of metHb produced by BSANO when incubated in a phosphate buffer (pH 7.4) very slowly reduced metHb to HbO_2 (Figure 10). The addition of

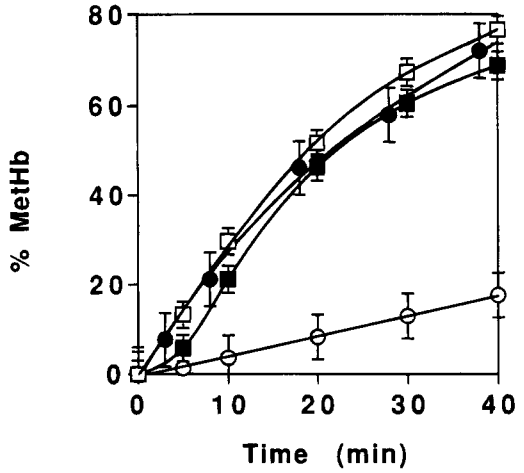

FIGURE 9: Time course of metHb absorption spectral changes during oxidation induced by BSANO (closed symbols) or GSNO (open symbols) in red blood cell lysate or intact cells. The different preparations contained 30 μM NO donors, 30 μM HbO_2, and 30 μM L-cysteine. Red blood cell lysate (□, ■) or intact red cells (○, ●) were incubated at 37 °C in phosphate buffer (pH 7.4). The percentage of metHb was determined as described in Winterbourn (1990). Points represent mean value ± SD (n = 4).

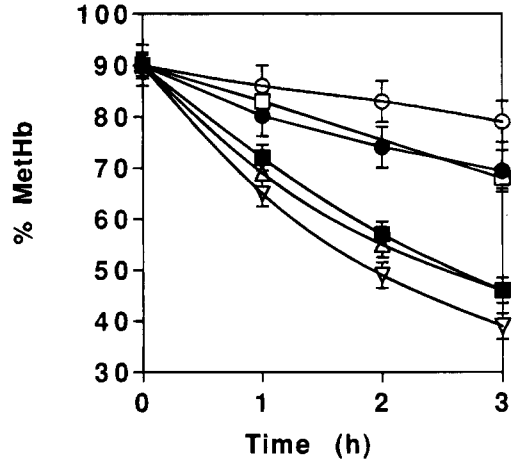

FIGURE 10: Time course of metHb absorption spectral changes during reduction induced by different agents. MetHb was produced by 30 μM BSANO and 1 mM L-cysteine at 37 °C for 10 min. Then, red blood cells (open symbols) were washed and resuspended in autologous plasma (▽), phosphate buffer (pH 7.4) (○), phosphate buffer containing 1 mM riboflavin (□), or phosphate buffer containing 1 mM glucose (△). Red blood cell lysate (closed symbols), obtained from BSANO-treated intact cells, was incubated with 10 mM NADPH (●) or 1 mM NADH (■). The percentage of metHb was determined as described in Winterbourn (1990). Points represent mean value ± SD (n = 4).

autologous fresh plasma to intact cells increased the rate of metHb reduction, and, after 3 h, about 50% of the metHb was reduced to HbO_2 (Figure 10). The addition of glucose (0.1−10 mM) to intact red blood cells incubated in phosphate buffer was sufficient to recover the energy necessary for the reduction of metHb (Figure 10). The involvement of the major NADH-dependent metHb reductase pathway of red cells was shown by the ability of NADH to reduce metHb. NADH (0.1−10 mM) added to the cell lysate reduced metHb at a rate comparable to those of glucose and plasma (Figure 10). NADPH (10 mM) added to the cell lysate and riboflavin (1 mM) added to intact cells failed to catalyze metHb reduction (Figure 10), thus ruling out the participation of the NADPH-metHb reductase pathway.

DISCUSSION

Generally, organic nitrates have to undergo metabolic activation to generate •NO. One class of organic nitrates that, under some circumstances, can generate •NO spontaneously or with the participation of thiols is that of S-nitroso thiols. It has been suggested that one of these compounds, S-nitrosocysteine, whose biological stability is very similar to that of •NO, may account for the vasorelaxant properties of endothelium-derived relaxing factor (Myers et al., 1990). However, evidence against this hypothesis has been published recently (Feelisch et al., 1994).

In this work, we used two S-nitroso thiols that require L-cysteine to release •NO in phosphate buffer, in HbO_2 solution, or with intact red blood cells. In other words, both thiols required a transnitrosation reaction to be biologically active:

$$R'S{-}NO + RSH \rightleftarrows R'SH + RS{-}NO \qquad (4)$$

It was significant that the •NO release (as measured by carboxy-PTIO) occurred in both the presence and absence of molecular oxygen, thus preceding further oxidation reactions of •NO in aerated solutions.

Although we used the same thiol as NO acceptor (i.e., L-cysteine), we observed different kinetics of NO_2^- formation in phosphate buffer for the two NO donors. GSNO produced NO_2^- more slowly than BSANO. At high L-cysteine concentrations, the inhibition of NO_2^- release observed with both NO donors may involve the chemical stabilization of S-nitroso-L-cysteine. As reported by Feelisch et al. (1994), the half-life of 30 μM S-nitrosocysteine in phosphate buffer (pH 7.4) at 37 °C was 0.31 ± 0.05 min. The addition of L-cysteine increased the stability of S-nitrosocysteine in a concentration-dependent manner, so that at a molar ratio of S-nitrosocysteine-to-L-cysteine of 1:333 the half-life was 36.9 ± 3.7 min. However, with BSANO an inhibition of NO_2^- production was observed when a very large excess (100-fold) of L-cysteine was added. On the contrary, with GSNO the inhibition of NO_2^- production was observed above a 10-fold excess of L-cysteine. This result may be due to a different accessibility of L-cysteine to the S-nitroso group of serum albumin. X-ray crystallographic analysis of serum albumin (He & Carter, 1992) showed that Cys-34 is located near the surface of the molecule and is partially protected from the solvent.

The reaction between NO donors and L-cysteine produced the formation of •S radicals, which was related to the transnitrosation reaction and required molecular oxygen and •NO. Although Josephy et al. (1984) and Stamler et al. (1992b) suggested that a homolytic mechanism was involved in S-nitroso thiol decomposition (see eq 5), our results suggest that this mechanism has no role in the formation of •S radicals.

$$RSNO \rightarrow RS^{\bullet} + {}^{\bullet}NO \qquad (5)$$

Whereas Josephy et al. (1984) observed that the homolytic decomposition of thionitrites produced •S radicals after oxygen removal, we observed the opposite, i.e., •S radicals were trapped only in the presence of oxygen. Moreover, eq 5 should be accelerated by HbO_2. The removal of •NO should accelerate the homolytic scission of S-nitroso com-

pounds, whereas our results showed that HbO_2 completely inhibited the formation of •S radicals.

We suggest that •NO can be released from *S*-nitroso-L-cysteine according to eq 6 and that •S radicals are formed from strongly oxidizing species produced after a reaction of •NO with molecular oxygen. Possible candidates are $•NO_2$ (eq 1) or $ONOO^-$ (formed by the interaction of •NO with superoxide anion; Blough & Zafiriou, 1985). Thiols are readily oxidized to •S radicals by either $•NO_2$ or $ONOO^-$ (Pryor et al., 1982; Radi et al., 1991; Gatti et al., 1994). Although the reaction of $ONOO^-$ with superoxide is very fast ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$; Huie & Padmaia, 1993), and some superoxide is not scavenged by superoxide dismutase, the complete lack of any inhibition by superoxide dismutase militates against the involvement of $ONOO^-$ as the oxidizing agent. $•NO_2$ reacts more rapidly than $ONOO^-$ with sulfhydryls ($k = 10^8$ and $1500-5000\ M^{-1}\ s^{-1}$, respectively; Prutz et al., 1985; Radi et al., 1991), and thus we support the hypothesis that $•NO_2$ is the oxidizing species responsible for •S radical formation according to the scheme proposed by Pryor et al. (1982) (eq 7).

$$2RSNO \rightarrow RSSR + 2•NO \quad (6)$$

The $RSN(OH)_2$ is unstable and decomposes to *S*-nitroso

$$RSH + •NO_2 \rightarrow [RS-\overset{\bullet}{\underset{|}{\overset{O}{N}}}-OH] \xrightarrow{RSH} RS-\underset{|}{\overset{OH}{N}}-OH + RS• \quad (7)$$

thiols and water (eq 8). The subsequent fate of the •S radicals is the production of disulfides (eq 9).

$$RSN(OH)_2 \rightarrow RSNO + H_2O \quad (8)$$

$$2RS• \rightarrow RSSR \quad (9)$$

•S radicals were not inhibited by dimethyl sulfoxide (hydroxyl radical scavenger) or by catalase. Thus, the involvement of other reactive oxygen species seems to be unlikely. However, dimethyl sulfoxide reacts with $•NO_2$ (Pace & Kalyanaraman, 1994); thus, to explain our finding, we suggest that the reaction rate with dimethyl sulfoxide may be slower than the reaction with •S. Furthermore, no radical formation was observed with only L-cysteine and DMPO (Figure 7c), thus excluding the idea that •S radicals were due to L-cysteine air autoxidation.

Characteristics of BSANO and GSNO as NO Donors. The first characteristic that distinguishes BSANO from GSNO is that the release of •NO from the former was strongly accelerated by a molar excess of L-cysteine. BSANO is present in the plasma of normal subjects at micromolar concentrations (about 6 μM; Stamler et al., 1992b), and the level of L-cysteine is about 30 μM (Saran & Bors, 1994). Our data suggest that sulfhydryl groups, and especially L-cysteine, play a regulatory role in plasma in the targeting of •NO to red blood cells, thus buffering the concentration of •NO. Recently, Scharfstein et al. (1994) obtained direct evidence for the biochemical transnitrosation of •NO from a plasma albumin pool to a low molecular weight thiol pool in vivo.

The oxidation of •NO to NO_3^- by the red blood cells probably represents the last step in the biotransformation or inactivation of •NO. The metHb formed in this process can be reduced by the NADH-dependent metHb reductase pathway of red cells. The energy required to reduce metHb is obtained by the release of free energy from phosphorylated glucose (Embden–Meyerhof pathway).

Significantly, other cells, such as platelets and rabbit aortic strips, do not require L-cysteine in order to release •NO from BSANO or GSNO (Radomski et al., 1992; Stamler et al., 1992a). Thus, it is likely that platelets and rabbit aortic strips possess, on their surfaces, a reactive thiol(s) that can allow the exchange from the NO donor and the intracellular compartment.

A second difference between BSANO and GSNO is the different rate of formation of metHb with intact red blood cells. In contrast to BSANO, GSNO oxidized HbO_2 in intact cells with kinetics significantly slower than that with cell lysate or purified hemoglobin. The finding that BSANO oxidizes HbO_2 in intact cells as well as in cell lysate suggests that albumin can bind to the red blood cell surface, where L-cysteine allows •NO to be transported to the cytoplasm. It is possible that reactive membrane thiols are also involved in the signal transduction pathway of •NO, as shown for glyceraldehyde-3-phosphate dehydrogenase from human erythrocytes (Kots et al., 1992). GSNO is not efficiently bound on the cell surface; thus, the •NO transport to the red blood cell cytoplasm is not as efficient as for BSANO. These observations raise the possibility that *S*-nitroso thiol groups in proteins may serve as intermediates in the cellular metabolism or bioactivity of •NO. The formation of *S*-nitroso proteins may represent an important cellular regulatory mechanism.

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