S-Transnitrosation Reactions Are Involved in the Metabolic Fate and Biological Actions of Nitric Oxide

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ABSTRACT

S-Nitrosothiols are a group of potent, bioactive compounds that form through the reaction of nitric oxide (NO) with thiols in the presence of oxygen. These compounds are naturally occurring in vivo, stabilize NO and potentiate its biological effects. S-Nitrosoglutathione is the most abundant intracellular S-nitrosothiol, and the kinetics for its formation favors de novo synthesis. In this analysis, we studied the formation of S-nitrosothiols by S-transnitrosation, or exchange of -NO for -H between sulfur atoms; we synthesized S-nitroso-glutathionyl-Sepharose 4B beads (SNO-4B) as a reagent with which to measure S-transnitrosation reactions. We detected a maximum of 1.57 ± 0.24 pmol NO/bead (n = 5) after S-nitrosation of the beads with acidified nitrite. The stability of the S-NO bond was dependent on temperature, but not pH over the 5 to 9 range (except at pH 5.0). Under the same conditions, the initial rate of -NO transfer to cysteine was 0.72, 3.71 and 4.69 μM/min at pH 5.0, 7.4 and 9.0, respectively (P < .05). There was no appreciable S-transnitrosation between SNO-4B and bovine serum albumin. We further demonstrated that SNO-4B evokes significant vasoconstrictor and platelet inhibitory responses in plasma-free systems and activates platelet soluble guanylyl cyclase. These data suggest a mechanism by which to explain the metabolic fate and distribution of NO among thiol pools in the vasculature, and implicate S-transnitrosation at the cell surface in NO signal transduction.

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ABBREVIATIONS: RSNO, S-nitrosothiol; NO, nitric oxide; GSNO, S-nitrosoglutathione; SNO-4B, S-nitroso-glutathionyl-Sepharose 4B beads; SGC, soluble guanylyl cyclase; GSH, glutathione; SNP, sodium nitroprusside; GTP, guanosine-5'-triphosphate; cGMP, cyclic guanosine-3'-5'-monophosphatase; tris(hydroxymethyl)aminomethane (Tris); IBMX, 3-isobutyl-1-methylxanthine; NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); BSA, bovine serum albumin; DPTA, diethyleneetriaminepentaacetic acid; CySNO, S-nitrosocysteine; SNO-BSA, S-nitrosoalbumin; SCC, soluble guanylyl cyclase; ACD, acid-citrate-dextrose; TCA, trichloroacetic acid; DTT, dithiothreitol.
nium ion (NO\textsuperscript{+}), on a reactive nucleophilic sulfur atom in a suitable molecular environment (Stamler et al., 1992d). A second, somewhat controversial mechanism posits that S-nitrosation by NO is a process mediated by peroxynitrite (ONOO\textsuperscript{−}) or NO\textsubscript{2} (Pryor et al., 1982; Mohr et al., 1994; Wu et al., 1994). Recently, a third mechanism has been proposed in which S-nitrosation of thiols (albumin) and low-molecular-weight thiols is mediated by dinitrosyl-iron complexes (Boese et al., 1995).

The fourth mechanism for the in situ production of RSNOs involves S-transnitrosation reactions, or RSNO-thiol exchange reactions, in which a -NO moiety is transferred from an RSNO to a thiol in exchange for an -H moiety (Feelisch, 1991; Field et al., 1978). This reaction occurs rapidly in vitro and its kinetics are favorable for the formation of GSNO under physiological conditions (Feelisch, 1991; Meyer et al., 1994; Singh et al., 1996).

S-Transnitrosation is considered to be the predominant mechanism for the biological effects of GSNO (27), and this process is believed to lead to the S-nitrosation of thiol-containing proteins (Mohr et al., 1994). We have also clearly demonstrated S-transnitrosation in vivo between S-nitrosated protein thiols (albumin) and low-molecular-weight thiols (L-cysteine and N-acetyl-L-cysteine) (Scharfstein et al., 1994). In the same study, we provided direct evidence that S-transnitrosation reactions are involved in the biological functions of NO in vivo.

Although it is assumed that the release of NO largely contributes to the pharmacological effects of RSNOs (Williams, 1983), several studies show that the rate of NO release from RSNOs cannot account for their biological actions (Mathews and Kerr, 1993; Kowaluk and Fung, 1990; Park et al., 1993). These observations support the concept that S-transnitrosation reactions provide a mechanism for the nitrosation of thiol-containing molecules (especially intracellular and cell-surface thiols) and for the metabolic distribution of NO in vivo, and may represent an important mechanism for the biological actions of NO. However, owing to the instability and low level of parent RSNOs in vivo (in the low μM range or less) (Stamler et al., 1992a; Gaston et al., 1994), it has been difficult to characterize S-transnitrosation and investigate the underlying reaction mechanism(s).

In this study, we propose that RSNOs are generated through S-transnitrosation reactions or exchange of -NO for -H between sulfur atoms. The specific objectives of the present study are to: 1) synthesize the stable and easily separable SNO-4B as a source of RSNO in transnitrosation reactions; 2) characterize the SNO-4B both chemically and biologically; 3) characterize transnitrosation reactions between SNO-4B and biologically relevant thiols, such as GSH and cysteine, and the possible mechanism(s) involved in the reactions; and 4) investigate the possible relationship of S-transnitrosation reactions to NO metabolism and bioactivities.

**Methods**

**Chemicals and Solutions**

SNP, GTP, cGMP, Tris, IBMX, piperazone-N, N\textsuperscript{-}bis(2-ethanesulfonyl acid), L-cysteine, phosphocreatine, creatine phosphokinase, neutral alumina (WN 3), phenylmethylsulfonyl fluoride, NEM, DTNB, DTPA, BSA, reduced GSH and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). Mercuric chloride was purchased from Fisher Co. (Fair Lawn, NJ). Sulfanilamide, ammonium sulfamate and N-1-naphthyl ethylenediamine dihydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Activated thiol Sepharose 4B was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). \textsuperscript{32P}GTP (3000 Ci/mmol) and \textsuperscript{3H}GMP (11 Ci/mmol) were purchased from Du Pont NEN (Boston, MA). EcoLite liquid scintillation cocktail was supplied by ICN (Costa Mesa, CA).

All other chemicals were reagent grade or better and obtained from Fisher or Aldrich Chemical Co. Krebs's solution consisted of (mM): NaCl (120), KCl (5.6), MgSO\textsubscript{4} (1.2), NaH\textsubscript{2}PO\textsubscript{4} (1.2), CaCl\textsubscript{2} (2.5), NaHCO\textsubscript{3} (25), dextrose (10) and EDTA (0.03) and was equilibrated with 95%O\textsubscript{2}/5%CO\textsubscript{2}. PBS (10 mM NaH\textsubscript{2}PO\textsubscript{4}, 150 NaCl, pH 7.4) and Tris-buffered saline (10 mM Trizma base, 150 mM NaCl, pH 8.0) were prepared as stock solutions. Calcium-free Tyrode's solution consisted of (mM): NaCl (140), KCl (2.5), NaHCO\textsubscript{3} (10), MgCl\textsubscript{2} (1), dextrose (5.5), piperazone-N, N\textsuperscript{-}bis(2-ethanesulfonic acid) (5, pH 6.5), EGTA (0.26) and BSA (0.35 mg/ml).

**Preparation of S-nitroso activated thiol Sepharose 4B.** Freeze-dried, activated thiol Sepharose 4B (4B) (fig. 1) was swollen in a 13-ml test tube with PBS (10 ml/g 4B) for 15 min and washed twice with PBS. The excess liquid in the tube was gently removed and free thiols on the Sepharose 4B were generated by suspending the 4B in Tris-buffered saline (pH 8.0; 5 ml/g 4B) containing 1% dithiothreitol, 0.3 M sodium bicarbonate and 1 mM EDTA for 40 min at room temperature with occasional gentle mixing. The 4B was then transferred into a 30-ml column and washed with 0.1 M acetic acid solution containing 0.5 M NaCl and 1 mM EDTA using a total volume of 400 ml of washing solution per gram of 4B, followed by PBS with a total volume equivalent to two column beds. The 4B was then suspended in PBS (5 ml/g 4B) in a test tube and the free thiol level determined using a modified DTNB assay (Albano et al., 1983). The S-nitrosation of the free thiol on the 4B was achieved with NaN\textsubscript{O} in the presence of 0.5 N HCl. The S-nitrosated, activated thiol Sepharose 4B (SNO-4B) (fig. 1) was then washed thoroughly with PBS and suspended in PBS at concentrations as desired for specific experimental purposes. S-Nitroso content of the SNO-4B was quantitated by Saville assay (Saville, 1958). The control 4B was prepared as follows: after recovering the free thiols, the 4B was incubated with 10 mM NEM for 10 min to alkylate the sulphydryl group; the NEM-treated 4B (NEM-4B) was then thoroughly washed with PBS and exposed to conditions of S-nitrosation as described for SNO-4B.

**Stability of the SNO-4B.** The stability of the S-NO bond on the SNO-4B was assessed by monitoring the S-nitroso concentration over time using the Saville assay under different conditions. The SNO-4B was placed in PBS at a pH of 5.0, 7.4, or 9.0 at 0°C, at room temperature, or 37°C. Fixed aliquots of the SNO-4B from each group (total of nine groups) were removed to quantitate the S-nitroso concentration as a function of time. In some experiments, stability of the RSNOs on the SNO-4B was assessed in the presence of 0.2 or 2.0 mM DTPA, a transition metal ion chelator, to evaluate the role of copper ion in the decomposition of the RSNOs on SNO-4B, as proposed for other RSNOs such as GSNO (Singh et al., 1996).

**S-transnitrosation between the SNO-4B and GSH, cysteine or BSA.** Time-dependent cumulative formation of GSNO, CySNO or SNO-BSA was measured after incubating the SNO-4B with GSH, cysteine or BSA to monitor the S-transnitrosation reactions between the SNO-4B and these thiol-bearing molecules. A fixed amount of SNO-4B (usually 150,000 Sepharose 4B beads) was incubated with 1.0 mM thiols at room temperature at pH 5.0, 7.4 or 9.0 for a fixed period of time. The yields of GSNO, CySNO and SNO-BSA were determined by the Saville assay at different time points (5, 10, 30, 60 and 120 min). The SNO-4B was conveniently separated from the reaction mixture by gravity; and the S-nitroso level remaining on the SNO-4B after the incubation also measured by the Saville assay for each thiol-containing substance under different conditions. The concentration-dependence of the S-transnitrosation reactions is presented for each thiol-containing substance under different conditions.

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*Note: The above text is a segment of a larger scientific article and is presented for context and understanding.*
reaction between SNO-4B and GSH was investigated by incubating SNO-4B with a variety of GSH concentrations ranging from 10 nM to 1 mM for 30 min (maximal yield at this time point). The GSNO production after the incubation was quantitated by both the Saville assay and photolysis-chemiluminescence analysis (Alpert et al., 1997). The production of RSNOs from SNO-4B during the S-trans-nitrosation reactions between SNO-4B and the thiol-containing substances was also confirmed by UV-visible absorption spectroscopy in the wavelength range of 260 to 500 nm at room temperature on a Cary 4E UV-visible spectrophotometer (Varian, Inc., Australia Pty., Ltd., Mulgrove Vic, Australia).

SNO-4B-induced vasorelaxation. Rabbit aortic rings with intact endothelium were prepared from New Zealand White male rabbits and mounted between a fixed support and a force transducer (model FT-03, Grass Instrument Co., Quincy, MA) in an organ chamber (Radnoti Glass Co., Inc., Monrovia, CA) containing 10 ml of Krebs's solution (37°C, pH 7.4) aerated with 95% O2 - 5% CO2 gas mixture. The aortic rings were prestretched with 2 g tension and equilibrated for 1 hr with changes of the Krebs's solution in the chamber every 20 min. The rings were then maximally contracted with phenylephrine (10 μM) and washed thoroughly with Krebs's solution until the tension returned to baseline. After 20 min equilibration, the rings were submaximally (60-80% of maximum) contracted with 0.1 μM phenylephrine, and a cumulative dose-response curve of vasorelaxation induced by SNO-4B then conducted. In a parallel experiment, a dose-response curve for SNO-4B-induced vasorelaxation was obtained in the presence of 0.1 mM GSH in the media to evaluate the possible role of S-transnitrosation in SNO-4B-induced vasorelaxation. NEM-4B was used as a nonnitrosated control in all cases.

SNO-4B-induced SGC activation. Platelet SGC was prepared from the blood of healthy volunteers. Blood was collected into syringes containing acid-citrate-dextrose (NIH formula A) anticoagulant. Platelet-rich plasma was prepared by centrifugation of the blood at 280 × g for 15 min at 4°C. Platelets were isolated from the plasma by centrifugation at 3400 × g for 15 min at 4°C. The pelleted platelets were resuspended in and washed with calcium-free Tyrode's solution three times in order to remove all traces of contaminating erythrocytes and free hemoglobin. The washed platelets were resuspended in 150 mM Tris.HCl (pH 7.6) at a concentration of 5 mg wet weight of platelets/ml. This suspension was frozen in a dry ice/ethanol bath and then gently shaken in a 30°C water bath until completely thawed. This freeze-thaw cycle was repeated twice and the platelet lysate centrifuged at 100,000 × g in a fixed-angle rotor (Beckman Ti 50) for 60 min at 4°C. Platelet cytosol was isolated and used immediately for the SGC assay. A 100 μl aliquot of the cytosol was added to the assay mixture containing (mM): Tris.HCl (40, pH 7.6), MnCl2 (6), IBMX (2), cGMP (2), L-cysteine (1), phosphocreatine (5), 5 U creatine kinase, 1 mg/ml BSA and [32P]GTP (0.2; 500,000 dpm) in a total volume of 250 μl. A variable amount of SNO-4B was introduced into the reaction mixture. NEM-4B served as control in all cases. In another group, the assay mixture included SNP as a positive control. The assay was initiated by transfer of the tubes containing the reaction mixture to a 30°C water bath. The reaction was allowed to proceed for 20 min, after which it was terminated by the addition of 250 μl of ice-cold 20% (w/v) TCA and transfer of the tubes to an ice bath. After the addition of 250 μl of [3H]cGMP to each sample and the removal of the precipitated proteins from the acidified sample by centrifugation (12,000 × g), the supernatant was applied to a column containing 0.5 g neutral alumina that was equilibrated with 5% (w/v) TCA. Each column was washed with 1 ml 5% (w/v) TCA, 2 ml water and 0.75 ml 0.2 M sodium acetate (pH 6.0). [32P]cGMP and [3H]cGMP were eluted with an additional 1 ml 0.2 M sodium acetate (pH 6.0) and counted by liquid scintillation spectrometry using 10.0 ml of EcoLite. After correction for crossover of the [32P] into the [3H] channel and the recovery of [3H]cGMP from each column (60-70%), the amount of cGMP formed during the incubation was calculated from the specific radioactivity of the [32P]GTP.

Platelet aggregation in the presence of SNO-4B. Platelets were isolated from platelet-rich plasma of healthy volunteers centrifugation of whole blood at room temperature at 280 × g for 15 min.

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Fig. 1. The chemical structures of Sepharose 4B (4B), deprotected activated Sepharose (HS-4B) and S-nitrosated Sepharose 4B (SNO-4B).
The platelets were then gel-filtered with Sepharose 2B and suspended in balanced Hepes buffer. The gel-filtered platelets were then incubated with various concentrations of SNO-4B for 5 min at 37°C. Aggregation was induced by the addition of thrombin (1 U/ml), which caused approximately 80% aggregation of control platelets and quantitated by the change in light transmittance through the stirred platelet suspension. Gly-Pro-Arg-Pro, an inhibitor of fibrin polymerization, was added (1.1 mM) before the addition of thrombin. The aggregation experiment was repeated in the presence of 100 μM GSH to investigate a possible role for S-transnitrosation in SNO-4B-induced inhibition of platelet aggregation; NEM-4B was used as control in all cases.

**Protein concentration determination.** Protein concentration of the platelet cytosol was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard.

**Data analysis.** The data are expressed as the mean ± S.D. for individual experiments. Statistical analysis involved the unpaired Student’s t test for two-group data, one-way analysis of variance followed by the Newman-Keuls test for more than two groups of data and two-way analysis of variance for data with two variables. The results of any analysis were considered to be significant when P < 0.05.

**Results**

**Characteristics of SNO-4B**

**Free thiol level and S-nitroso content of the 4B.** The free thiol level detected on the 4B after DTT deprotection and activation was estimated by DTNB assay to be 1.86 ± 0.31 pmol/ bead (n = 5). The S-nitroso content of the SNO-4B after nitrosation was measured by Saville assay and found to be 1.57 ± 0.24 pmol/ bead (n = 5). Thus, approximately 85% of the total thiols on the 4B is nitrosated under these experimental conditions. There was no detectable free thiol on the SNO-4B during formation of -SNO on the SNO-4B and GSH after incubation with SNO-4B was both pH- and time-dependent, as shown in figure 2 (P < .05). The initial rate of GSNO formation in the first 5 min was calculated to be 0.53, 3.03 and 5.14 μM/min at pH 5.0, 7.4 and 9.0, respectively (P < .05); however, there is no statistical difference in the initial rate of formation at pH 7.4 and pH 9.0 (P > .05). The maximal production of GSNO was achieved within 30 min at pH 7.4 and pH 9.0 and within 60 min at pH 5.0 (P < .05). No difference in the maximal production of GSNO was observed at all three pHs. The GSNO concentration decreased slowly over time after maximal concentrations were achieved at all three pHs. As shown in figure 2A, the S-nitroso content remaining on the SNO-4B declined in parallel with the increase in GSNO, consistent with the transfer of -NO from the SNO-4B to GSH. The efficiency of NO transfer was estimated to be 15.5, 24.5 and 27.1% at pH 5.0, 7.4 and 9.0, respectively.

A kinetic analysis was also conducted to characterize the S-transnitrosation reaction between SNO-4B and cysteine. (fig. 2B). The initial rate of CysNO generation was determined to be 0.72, 3.71 and 4.69 μM/min at pH 5.0, 7.4 and 9.0, respectively (P < .05). Similar to S-transnitrosation between SNO-4B and GSH, no statistical difference in the initial rate of CysNO formation was observed at pH 7.4 and pH 9.0. As shown in figure 2B, the S-transnitrosation between the SNO-4B and cysteine occurred rapidly (faster than that between SNO-4B and GSH under the same conditions). The cumulative production of CysNO peaked within 10 min at pH 9.0 and within 30 min at pH 5.0 and pH 7.4. Similar to that of GSNO, the CysNO concentration decreased gradually after peak concentrations were attained under all conditions. Similarly, a corresponding loss of S-nitroso content on the SNO-4B was observed during formation of CysNO, again consistent with the transfer of -NO from the SNO-4B to cysteine. The efficiency of transfer was calculated to be 21.6, 29.5 and 39.7% at pH 5.0, 7.4 and 9.0, respectively. Under the same conditions, no S-transnitrosation was observed between SNO-4B and BSA after incubation for a period of up to 3 hr.

The formation of GSNO and CysNO through S-transnitrosation reactions was further confirmed by demonstrating the characteristic absorbance spectra in the wavelength range of 300 to 400 nm. Both GSH and cysteine reaction solutions exhibited specific absorbance spectra characteristic to GSNO and CysNO, respectively, after incubation with SNO-4B (fig. 3), with absorption maxima of 335 and 336 nm, respectively.

The concentration-dependence of S-nitrosation reactions was demonstrated by incubating a fixed amount of SNO-4B with a range of concentrations of GSH. As illustrated in figure 4, the formation of GSNO increased as the concentration of GSH in the incubation mixture increased, while the S-nitroso content of the SNO-4B decreased correspondingly.

In an effort to understand the chemical mechanism of S-transnitrosation between the SNO-4B and the thiols, the

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**TABLE 1**

Estimated half-life of -SNO on SNO-4B under different conditions of pH and temperature

<table>
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<td>pH 5.0</td>
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thiol level on the 4B was measured after the incubation with GSH or cysteine. The percent recovery of free thiols on the 4B after transnitrosation was estimated to be 28.9, 50.2 and 66.9% after incubation with GSH at pH 5.0, 7.4 and 9.0, respectively and 63.5, 70.2 and 39.8% after incubation with cysteine at pH 5.0, 7.4 and 9.0, respectively.

**SNO-4B-induced vasorelaxation.** To evaluate its biological activity and to investigate the possible role of S-transnitrosation reactions in the bioactivity of NO, the vascular smooth muscle relaxing effects of SNO-4B were investigated using rabbit aortic rings. As shown in figure 5, SNO-4B induced a dose-dependent relaxation of the rings with an estimated IC₅₀ of 1,000 SNO-4B beads per ml (final concentration), which equal approximately 1.5 μM RSNO equivalent (1.57 pmol RSNO/bead). A representative tracing of the SNO-4B-induced vasorelaxation is shown in figure 5A. We observed that the action of the SNO-4B on the vascular rings is rapid and short-lived, which is reflected by the rapid changes in the vascular tension recorded when the SNO-4B is added to the organ chamber. When GSH (0.1 mM) was present in the organ chamber, the dose-response curve for SNO-4B-induced vasorelaxation was significantly shifted to the left with an estimated IC₅₀ of 250 SNO-4B beads per ml (approximately 0.4 μM RSNO equivalent) (P < .05, two-way analysis of variance). However, there was no difference between of pH 7.4 and 9.0 in the transfer of -NO to either GSH or cysteine (P > .05). Each point represents the mean of five experiments. S.D. are omitted for the sake of clarity.

**Inhibitory effects of SNO-4B on platelet aggregation.** SNO-4B was also examined for its ability to inhibit platelet aggregation. As shown in figure 6, SNO-4B inhibited collagen-induced platelet aggregation in a dose-dependent manner.
with an estimated IC\textsubscript{50} of 1,500 SNO-4B beads per ml, or 2.4 \( \mu \)M RSNO equivalent. Complete inhibition of platelet aggregation was achieved at a concentration of 6000 SNO-4B beads per ml, or 9.4 \( \mu \)M RSNO equivalent. The presence of 0.1 mM GSH significantly potentiated the inhibitory effects of SNO-4B on platelet aggregation with an estimated IC\textsubscript{50} of 300 SNO-4B beads per ml, or 0.5 \( \mu \)M RSNO equivalent. NEM-4B had no effect on platelet aggregation, as shown in figure 6.

**Solute guanylyl cyclase activation by SNO-4B.** sGC activity in platelets is stimulated by SNO-4B and shown in figure 7 and expressed as cumulative production of cGMP. Incubation of the sGC with SNO-4B produced a concentration-dependent increase in enzymatic activity. The sGC activity began to increase when the concentration of the SNO-4B was 1,500 beads per ml, or 2.4 \( \mu \)M RSNO equivalent and achieved activities greater than 13-fold of basal levels when 12,000 beads per ml, or 19 \( \mu \)M RSNO, equivalent was present in the reaction mixture. Similarly, as a positive control, incubation of the platelet cytosolic fraction with different concentrations of SNP (from 10 to 1000 \( \mu \)M) resulted in a dose-dependent increase in the catalytic activity of the enzyme (data not shown). No stimulation of sGC was observed by NEM-4B as there is no increase in the cGMP formation after incubation with enzyme under these conditions (fig. 7).

**Discussion**

NO is a reactive free radical gas that undergoes either oxidation or reduction reactions depending upon the redox status of its micro-environment (Stamler et al., 1992d; Gross and Wolin, 1995). Among the variety of reactions involving NO is the S-nitrosation of thiol-containing molecules (Stamler et al., 1992b, 1992c, 1992d). The products of this reaction are RSNOs, a group of potent, bioactive compounds involved in a wide range of biological activities (Stamler, 1994). It is generally believed that RSNOs play an important role(s) in NO-mediated bio-activities (Keaney et al., 1993; Simon et al., 1993; Mathews and Kerr, 1993). To understand better the biological actions of RSNOs and their regulation, it is important to investigate the mechanism(s) and characteristics of S-nitrosation of thiols. Although several mechanisms have been proposed for the S-nitrosation of thiols, the S-nitrrosation reaction is considered to be important under physiological conditions and closely related to the biological effects of RSNOs (Stamler et al., 1992c, 1992d; Park et al., 1993). It is well known, however, that the majority of RSNOs are thermodynamically and photolytically unstable, and sensitive to high pH and redox-active transition metal ions (Feelisch, 1991, Oae and Shinhama, 1983). The concentrations of RSNOs have also been found to be low \textit{in vivo} (Stamler et al., 1992a; Gaston et al., 1994). Taken together, these observations rendered the characterization of S-nitrrosation reactions technically difficult. To improve upon these technical limitations, we identified an RSNO as an -NO source that is relatively stable and easily separated from other RSNOs.

In this study, we described and characterized a newly synthesized RSNO, S-nitroso-glutathionyl-Sepharose 4B.
and used this SNO-4B as the source of -NO to study the role(s) of S-transnitrosation reactions in the metabolic distribution of NO and their potential relationship to the biological actions of NO. SNO-4B contained significant amount of RSNOs (average 1.57 pmol/bead) that could serve as an efficient, stable source of -NO for S-transnitrosation reactions. The RSNO level on the SNO-4B represented approximately 85% of total thiols. This result was comparable to that of Clancy and Abramson (1992) who covalently attached a thiol group to agarose and nitrosated it with acidified NaN3O2. In their study, approximately 82% of total thiols were nitrosated.

One of the striking advantages for SNO-4B is its stability in physiological solutions with an estimated t1/2 of more than 2 wk at 0°C and 7 days at 37°C. Therefore, the RSNO on SNO-4B is among the few known RSNOs that have a relatively long half-life, including S-nitrosothioglycerol, GSNO and S-nitroso-N-acetyl-L-cysteine with estimated t1/2 of approximately 12, 7 and 4 days at pH 7.4 and 37°C, respectively (Mathews and Kerr, 1993). However, a major difference between the RSNO on SNO-4B and other RSNOs is that the RSNO on the SNO-4B have comparable stability at pHs from 5.0 to 9.0. Although very little is known about the factors

Fig. 5. Cumulative dose-dependent vasorelaxation of rabbit aortic rings by SNO-4B. The rings with intact endothelium were submaximally contracted with phenylephrine and exposed to various amounts of SNO-4B or NEM-4B in the presence or absence of .1 mM GSH. SNO-4B induced a dose-dependent decrease in tension, and GSH significantly potentiated SNO-4B-induced vasorelaxation. A shows representative tracings of SNO-4B-induced vasorelaxation in the presence and absence of 0.1 mM GSH. The numbers 1, 2, 3, 4, 5 present the cumulative concentration of 100, 300, 700, 1,700, 3,700 SNO-4B beads per ml in the organ chamber, respectively. B shows the analyzed dose-response for vasorelaxation. Data are presented as mean ± S.D. (n = 3).

Fig. 6. Effect of SNO-4B on platelet soluble guanylyl cyclase activity. The platelet cytosolic fraction containing sGC was incubated with various amounts of SNO-4B for 20 min at 30°C. Reactions were terminated by addition of 250 μl ice cold 20% (v/v) trichloroacetic acid. The cumulative production of cGMP formed during the incubation was used as an indicator of sGC activity. In a parallel experiment, the cytosolic fraction was incubated with various concentrations of SNP as a positive control. SNP stimulated sGC in a dose-dependent manner (data not shown). Values are expressed as mean ± S.D. (n = 3).

Fig. 7. Inhibitory effects of SNO-4B on platelet aggregation. Gel-filtered platelets were pre-incubated with various amounts of SNO-4B for 5 min in the presence or absence of 0.1 mM GSH and aggregated with thrombin. The rate of aggregation was compared among the groups. SNO-4B significantly inhibited thrombin-induced platelet aggregation in a dose-dependent manner, which was significantly potentiated by GSH. Values are expressed as mean ± S.D. (n = 4).
contributing to the stability of RSNOs, evidence indicates that the overall structure of S-nitrosothiol molecules plays an important role in their biological activity and chemical stability; however, there appears to be no correlation between activity and stability (Mathews and Kerr, 1993). The RSNOs on the SNO-4B are linked to a polysaccharide matrix through the tripeptide, as illustrated in figure 1. This unique structure of the SNO-4B might impart stability to the RSNOs, or the glutathionyl moiety may form intramolecular associations and cyclize when covalently bound to this matrix. More studies are warranted to establish a relationship among the molecular structures, chemical stability and biological activity of RSNOs.

Singh et al. (1996) proposed that RSNOs are susceptible to transition metal ion-catalyzed decomposition. In our study, an attempt was made to evaluate the role of transition metal ions in the stability of the RSNOs on SNO-4B by introducing a transition metal ion chelator, DTPA, into the mixture. No changes in the stability of SNO-4B were observed in the presence of DTPA. These data are interpreted as suggesting transition metal ions play a minimal role, if any, in the decomposition of this particular RSNO. This is consistent with previous studies (Askew et al., 1995). A similar result was obtained for S-nitrosated albumin (Alpert et al., 1997). The reason for the discrepancy between our results and those of Singh and colleagues, again, might be attributable to the formation of relatively stable intramolecular conformations of individual RSNOs.

In our study, RSNOs (GSNO and CysNO) were generated through S-transnitrosation between SNO-4B and corresponding thiols under physiological conditions. The existence of GSNO and CysNO after incubation with SNO-4B was confirmed by both Saville assay and the characteristic absorbance spectra by UV-visible spectrophotometry. The formation of both GSNO and CysNO was pH, time and concentration dependent. These results are consistent with those reported previously with red agarose as a source of -NO (Clancy and Abramson, 1992). The initial rate of S-transnitrosation reactions seemed to correlate with the pKa of the thiols (the pKa of SH was 8.7 and 8.3 in GSH and cysteine, respectively). At high pH (9.0), the nucleophilic thiolate concentration would accordingly be higher than at neutral or acidic pH. These data are consistent with the argument that the formation of RSNOs involves electrophilic attack of the -NO on the SNO-4B by the thiolate anion.

In our study, the nature of the chemical reactions between -NO and thiols is complex. One of the reactions involved the exchange of -NO and -H between the SNO-4B and thiols similar to S-transnitrosation reactions in other studies (Scharfstein et al., 1994; Meyer et al., 1994). This outcome was reflected by the loss of -NO from SNO-4B and the generation of GSNO and CySNO, as well as the recovery of thiols on the 4B. However, the efficiency of the exchange was moderate (less than 30% for GSH and less than 40% for cysteine). The remainder of the -NO on the SNO-4B was presumably metabolized to other nitrogen oxides because there was no Hg-displaceable NO on the 4B after incubation with thiols (Stamler et al., 1992b; Alpert et al., 1997).

Interestingly, under the same conditions, S-transnitrosation between SNO-4B and albumin did not readily occur. This lack of reactivity is possibly a consequence of the lack of accessibility of the thiol group in albumin to -NO on the SNO-4B. In an earlier study, it was reported that there is no measurable S-nitroso-BSA formation when BSA is incubated with GSNO, while a significant amount of S-nitroso-BSA was generated when the BSA was incubated with CysNO (Boese et al., 1995).

As a group, RSNOs are potent, bioactive compounds with NO-like effects (Mathews and Kerr, 1993). In our study, we clearly demonstrate that SNO-4B is a rapidly acting and potent vasodilator, platelet aggregation inhibitor and sGC stimulator. SNO-4B, therefore, functioned fully as a typical RSNO in biological systems and appeared to do so through a cGMP-dependent mechanism. However, its profound biological effects could not be explained by the spontaneous release of NO because it was very stable under physiological conditions, or could its effects be explained by entering directly into the target cells to activate the intracellular signaling systems owing to its extremely large physical size and particular nature. It was claimed that even low molecular weight RSNOs such as CysNO are unlikely to permeate cell membranes readily because they exhibit negligible distribution from aqueous solution to nonpolar phases at pH 7.4, with partition coefficients of 0.02-0.05 (Kowaluk and Fung, 1990) consistent with their highly polar nature under physiological conditions. Thus, one possible explanation for the biological activity of SNO-4B is that SNO-4B interacts with the components of the cell membrane surface through the following possible mechanisms: 1) NO is released at the membrane surface, diffuses into cells and then initiates related biological events such as stimulation of sGC. In support of this mechanism, membrane fractions of vascular smooth muscle cells have been demonstrated to be capable of catalyzing the release of NO from S-nitrosothiols (Kowaluk and Fung, 1990). This action could be achieved through a one-electron reduction of RSNOs to release NO and regenerate the parent thiol by cell-surface electron transport chains (Gross and Wolin, 1995; Crane et al., 1985). 2) NO is transferred to other thiol-containing species on the cell surface through S-transnitrosation reactions and the nitrosated cell-surface thiols initiate the intracellular signal transduction process in the target cells. S-Transnitrosation reactions occur naturally in vivo and participate in physiological and pharmacological actions of S-nitrosothiols (Park et al., 1993; Scharfstein et al., 1994). It was demonstrated previously that S-nitrosation of thiol-containing membrane ion channels partially contributed to NO-induced vasorelaxation (Bolotina et al., 1994). This mechanism also likely accounts for other NO-related bioactions (Stamler, 1994). Although sGC, rich in cysteine residues, is considered to be the primary target site, it is unlikely to participate directly in the cell surface events. However, sGC might play a role in intracellular S-transnitrosation processes, which might constitute a critical aspect of the NO:cGMP pathway. The specificity and extent of the S-transnitrosation reaction-related biological activities could be determined by the redox status of the local micro-environment and the amount and types of thiol-containing molecules on the target cell surface. In the present study, we observed that GSH (0.1 mM) significantly potentiated SNO-4B-induced vasorelaxation. GSH might facilitate the S-transnitrosation reactions between the SNO-4B and cell surface thiols and/or help preserve the thiols on the cell surface. However, further studies are needed to investigate this mechanism in detail. 3) RSNOs on the SNO-4B may interact with other cell
membrane components, such as non-heme-iron moieties and specific receptor systems (Hu and el-Fakahany, 1993), to modulate cellular function.

In conclusion, a stable and easily separable S-nitrosothiol, SNO-4B, was synthesized and served as a source of NO. S-Transnitrosation reactions occurred rapidly between SNO-4B and GSH and cysteine under physiological conditions in a pH-dependent manner. SNO-4B exhibited NO-like properties including vasorelaxation, platelet aggregation inhibition and sGC stimulation. The biological effects of SNO-4B may, therefore, be partly related to a cGMP-dependent mechanism. SNO-4B-induced vasorelaxation and platelet inhibition were enhanced significantly by GSH. These results are consistent with the hypothesis that S-transnitrosation reactions are important in the generation of RNSOs, the metabolic fate and distribution of NO among thiol pools in the vasculature and the biological actions of NO. These results also implicate S-transnitrosation reactions at the cell surface in NO signal transduction pathways.

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