Direct Observation of Trapping and Release of Nitric Oxide by Glutathione and Cysteine with Electron Paramagnetic Resonance Spectroscopy

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ABSTRACT While the biosynthesis of nitric oxide (NO) is well established, one of the key issues that remains to be solved is whether NO participates in the biological responses right after generation through biosynthesis or there is a "secret passage" via which NO itself is trapped, transported, and released to exert its functions. It has been shown that NO reacts with thiol-containing biomolecules (RSH), like cysteine (Cys), glutathione (GSH), etc., to form *S*-nitrosothiols (RSNOs), which then release nitrogen compounds, including NO. The direct observation of trapping of NO and its release by RSNO has not been well documented, as most of the detection techniques measure the content of NO as well as nitrite and nitrate. Here we use spin-trapping electron paramagnetic resonance (EPR) technique to measure NO content directly in the reaction time course of samples of GSH and Cys ($\sim$ mM) mixed with NO ($\sim$ μM) in the presence of metal ion chelator, which pertains to physiological conditions. We demonstrate that NO is readily trapped by these thiols in less than 10 min and $\sim$70–90% is released afterward. These data imply that $\sim$10–30% of the reaction product of NO does not exist in the free radical form. The NO release versus time curves are slightly pH dependent in the presence of metal ion chelator. Because GSH and Cys exist in high molar concentrations in blood and in mammalian cells, the trapping and release passage of NO by these thiols may provide a mechanism for temporal and spatial sequestration of NO to overcome its concentration gradient-dependent diffusion, so as to exert its multiple biological effects by reacting with various targets through regeneration.

INTRODUCTION

The endogenously formed free radical NO has been established as a novel messenger in numerous cellular processes of both physiological and pathological responses (Furchgott and Zawadzki, 1980; Ignarro, 1989; Moncada et al., 1991; Bredt and Snyder, 1994; Nathan and Xie, 1994; Stamler, 1994). Some of these responses have long been postulated to result from a redox-mediated reaction of NO with the thiol-containing biomolecules through thiol/nitrosothiol exchange, leading to disulfide formation and regeneration of NO at the site of action (Girard and Potier, 1993). In an aerobic environment, NO reacts first with oxygen to form higher oxides like N_2O_3, which would then react with the thiol groups of low-molecular-weight peptides and proteins to form *S*-nitrosothiols (RSNOs) (Kharitonov et al., 1995). There is evidence that RSNOs are potent vasodilators and inhibitors of platelet aggregation (Ignarro and Gruetter, 1980; Ignarro et al., 1981; Kelm and Schrader, 1990; Stamler et al., 1992b; Butler and Williams, 1993). On the other hand, it is well established that NO is an endothelium-derived relaxing factor (EDRF) producing vasorelaxation (Palmer et al., 1987; Ignarro et al., 1987). Obviously, the activity of RSNOs is very likely dependent on their ability to regenerate NO, and this suggests the possibility that nitrosated peptides and proteins act as NO transporters and congeners (Minamiyama et al., 1996; Scorza et al., 1997; Ewing et al., 1997). Although the detailed mechanisms of NO release are complicated by the catalytic effect of both trace transition metal ions and the reducing agents thiols and ascorbate (Scorza et al., 1997; McAninly et al., 1993; R. J. Singh et al., 1996), the overall reaction seems to be as follows:

$$2\mathrm{RSNO} \rightarrow \mathrm{RSSR} + 2\mathrm{NO}$$

NO released in this reaction can then react with either other thiol-containing proteins or the heme group of guanylate cyclase to exert its biological activity. In our recent study of the redox reaction between NO and thiol-containing biomolecules, using a chemically modified electrode (CME) for sensitive, selective, and quantitative detection of the oxidation currents of both NO and thiols of biomolecules (Miao et al., 1999), we observed that CME detects Cys at +450 mV, while the oxidation of NO does not occur at this potential but can be optimally detected at +700 mV. The result thus provides a selective control to examine the oxidation of Cys by NO at +450 mV without interference from NO itself. With this CME as a detector, we found that at the completion of the oxidation reaction of Cys by NO as indicated by the decrease in current at +450 mV, the oxidation current at +700 mV remains almost the same as in the initial stage before the reaction. These results are

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consistent with the possibility that NO might be regenerated as a reaction product after its reaction with thiols to form RSNO intermediates, which then decompose to disulfides (RSSRs) and NO.

To further monitor the passage of NO during its reaction with thiol molecules, the technique of electron paramagnetic resonance (EPR) spectroscopy seems to us to be an appropriate tool for analytical studies of the biochemical reaction of NO, in view of the odd electron count (11 valence electrons) of the NO molecule. The application predominantly involves the use of EPR to monitor the content of NO through the observation of spin adducts of NO. In this study we characterize and compare the NO EPR signals during the time course of its reaction with thiol molecules, Cys, and glutathione (GSH). We report that the EPR spectrum of NO is reduced to near baseline following a reaction time course within 10 min at various pH levels and then begins to be regenerated, after a 12-min reaction at a content equal to 70–90% of the original levels before the initiation of reaction.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA, low metal), *S*-nitrosoglutathione (GSNO), Cys, and GSH were obtained from Calbiochem-Novabiochem Co. (San Diego, CA). NO gas (99.9%) was purchased from Matheson Gas (Joliet, IL). Diethyldithiocarbamic acid sodium salt trihydrate (DETC), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), sodium hydrosulfite ($Na_2S_2O_4$), EDTA, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of reagents

Saturated NO solution (2 mM at 25°C and $P_{no} = 1$ atm) (Lantoine et al., 1995) was used for calibrating NO. To produce a saturated NO solution, 50 mM potassium phosphate buffer (pH 7.5) was bubbled with high-purity nitrogen gas for 30 min to remove O_2. The O_2-free buffer was then bubbled with pure NO gas for 30 min in a glass sampling vial. Fresh standards were made for each set of experiments and kept in vials under a NO atmosphere that could be aspirated through a sealed rubber septum with a gas-tight syringe. Dilutions of the saturated solution were made using buffer prepared with deoxygenated phosphate buffer.

DETC (in 10% BSA), $FeSO_4 \cdot 7H_2O$, and $Na_2S_2O_4$ were dissolved in degassed Milli-Q water at 0.01 M, 0.01 M, and 2 M, respectively, before use and served as the spin trap reagents. To initiate the reaction of NO with Cys or GSH, Cys and GSH solutions were prepared in 50 mM phosphate buffer solution (pH 6.5, 7.5, and 8.5 respectively), which was then mixed with NO solution. At reaction time points specified in Figs. 3–5, aliquots of the reaction mixture were taken to mix with the spin trap reagents for 3 min of incubation, and we measured the EPR signal as described below. GSNO was dissolved in 50 mM phosphate buffer (pH 7.5) solution at 10 mM before use. All of the above reagents were freshly prepared. EDTA (100 μM) was added to every phosphate buffer to chelate the trace amounts of transition metals that might be present in the sample solutions.

Determinations of Cu and Fe concentrations in phosphate buffer and amino acid samples with an inductively coupled plasma-mass spectrometer

The concentrations of Cu and Fe were determined by an inductively coupled plasma-mass spectrometer (ICP-MS) (model ELAN 6000; Perkin-Elmer-Sciex, Norwalk, CT, U.S.A.). The radio frequency power of the instrument was 1000 W. The argon gas flow rates of plasma, auxiliary, and nebulizer were 15, 1, and 0.6 L/min, respectively. The sample solution was introduced into a cross-flow nebulizer with a Scott-type spray chamber. The uptake of sample solution was kept constant by the use of a peristaltic pump, which was operated at a sample uptake rate of 1.5 ml/min.

Commercially available standard solutions (High Purity Standard, Riedel-deHaën, Germany) were used for calibration in the ICP-MS. Different calibration concentrations of the standard solutions of Cu and Fe were prepared in 2% HNO_3 (Riedel-deHaën). The concentration ranges for Cu and Fe were 1–10 ppb and 1–200 ppb, respectively. Linear calibration curves of Cu (mass 63) and Fe (mass 57) were established. The concentrations of Cu and Fe in the samples were calculated from the linear equations of the calibration curves.

Measurement of NO by EPR spectroscopy

NO was trapped by ferrous complex of diethyldithiocarbamate, $Fe^{2+}(DETC)_2$, to form a mononitrosyl adduct, $Fe^{2+}(DETC)_2NO$, which exhibited a characteristic triplet EPR spectrum at $g_\perp = 2.035$ and $g_\parallel = 2.020$ with triplet hyperfine structure (HFS) at $g_\perp$. To overcome the poor solubility of $Fe^{2+}(DETC)_2$ and rapid oxidation of $Fe^{2+}(DETC)_2$ to $Fe^{3+}(DETC)_2$, a modified method (Tsuchiya et al., 1996) was used. Briefly, BSA and $Na_2S_2O_4$ were added to prevent the precipitation of $Fe^{2+}(DETC)_2$ and oxidation of $Fe^{2+}(DETC)_2NO$, respectively. DETC (final concentration, 3.3 mM), BSA (final concentration, 33 mg/ml), $FeSO_4 \cdot 7H_2O$ (final concentration, 3.3 mM), $Na_2S_2O_4$ (final concentration, 1 M), and the NO sample were mixed. Three minutes later, the mixture was introduced into a quartz EPR tube (Wilmad Glass, Buena, NJ) 3 cm in height and put into the finger-tip liquid nitrogen dewar flask for EPR measurement.

EPR measurements were performed with a Bruker ESP 300E spectrometer (X-band) under the following conditions: microwave frequency 9.33 GHz; microwave power 10 mW; modulation frequency 100 kHz; modulation amplitude 0.52 mT; magnetic field 330 $\pm$ 20 mT; time constant 10.24 ms; sweep rate 0.48 mT/s; receiver gain 2×10^4; temperature 77 K. The height of the first peak ($g = 2.047$) to the baseline of the EPR signal was used to represent the size of $Fe^{2+}(DETC)_2NO$. The NO concentration of the sample was then calculated by comparing the height of the first peak ($g = 2.047$) of the EPR signal of the sample with those derived from the standard NO solutions.

RESULTS

Determination of trace metals Fe, Cu in the sample solutions

Using EPR spectroscopy, we inevitably have to add ferrous iron as part of the trapping agent for stabilizing NO. Even in milli-Q water, there could be some tiny trace of metal Cu, Fe as contamination. However, under in vivo situation, transition metals form complexes with biomolecules to avoid metal-catalyzed oxidation. Therefore we first measured the amount of Cu and Fe in the sample solutions being used for our reaction mixtures by employing a mass spec-

trometer (ICP-MS). The molar concentrations found for Cu and Fe (which includes Fe^{2+} and Fe^{3+}) are listed in Table 1 for the nine sample solutions involved. As we read from Table 1, the concentrations of Cu and Fe in our samples are less than 0.05 μM and 2 μM, respectively. We thus add 100 μM EDTA to every phosphate buffer (as stated under Preparation of Reagents). The justification is based on the formation constants for EDTA complexes with Fe^{2+} ($2.1 \times 10^{14}\ M^{-1}$) and Cu^{2+} ($6.3 \times 10^{18}\ M^{-1}$). Assuming there are initially 2 μM Fe^{2+} and 100 μM EDTA in our reaction mixture containing thiols and NO, the final concentration of free Fe^{2+} in the reaction mixture can be calculated to be $\sim 9.7 \times 10^{-17}$ M, which should not significantly affect the reaction of NO with thiols. The free Cu^{2+} in the reaction buffer is calculated in the same way to be $\sim 7.9 \times 10^{-23}$ M.

Calibration of NO concentrations by EPR spectrum of $Fe^{2+}(DETC)_2NO$ in aqueous solution

To quantify NO concentration through a spin-trapping agent $Fe^{2+}(DETC)_2$ complex, we used NO saturated solution as a standard stock and measured the EPR spectrum of diluted NO in the spin-trapping reaction mixture. The EPR spectrum of $Fe^{2+}(DETC)_2NO$ complex is shown in Fig. 1 *A*. It exhibits a characteristic EPR spectrum at $g_\perp = 2.035$ and $g_\parallel = 2.020$ with triplet hyperfine structure (HFS) at $g_\perp$. We used the height of the first peak ($g = 2.047$) to the baseline of the EPR signal to represent the intensity of the $Fe^{2+}(DETC)_2NO$ complex. A linear calibration curve is shown in Fig. 1 *B,* which indicates that the intensity of the EPR signal of $Fe^{2+}(DETC)_2NO$ increased linearly with the various concentrations (0.2–20 μM) of NO added to the reaction mixture (at least within 20 μM). The linear correlation coefficient was 0.998, and the detection limit was 0.2 μM at a signal-to-noise ratio of 3.

Dose-dependent release of NO by GSNO

Because previous electrochemical results (Miao et al., 1999) showed that NO might be regenerated after its reaction with Cys and a novel NO target protein in the brain, neurogranin (Ng) (Sheu et al., 1996; Mahoney et al., 1996; Li et al., 1999), we reasoned that RSNO intermediates might be formed in the course of the reaction to account for the NO subsequently released. Here we attempted to determine directly whether an exemplary nitrosothiol, GSNO, could release NO in aqueous solution by quantitatively measuring the ERP spectra of $Fe^{2+}(DETC)_2NO$. Fig. 2 *A* shows a linear relation of initial NO release versus GSNO concentration when solid GSNO was first dissolved in phosphate buffer (50 mM, pH 7.5) and aliquots of various concentrations of GSNO were then taken to mix with the spin-trap reagents. The concentration of NO, released from different amounts of GSNO in liquid phase, was calculated from the standard calibration curve of NO shown in Fig. 1 *B*. In Fig. 2 *A*, the initial rate of NO released from GSNO was shown to be linear as a function of GSNO concentration within 800 μM. Because at higher concentrations of GSNO the EPR signal of $Fe^{2+}(DETC)_2NO$ was out of the linear range of the standard calibration curve, it is not possiblt to accurately estimate the amount of NO released. It should be noted that it is difficult to quantify the exact amount of decomposition of commercial available GSNO in solution because there was a time lag between dissolving solid GSNO in solution and measuring the EPR spectra. During this time lag, various amounts of GSNO might have already been decomposed. However, we further measured a time course EPR signal of $Fe^{2+}(DETC)_2NO$ after the decomposition of GSNO. Fig. 2 *B* demonstrates that for a fixed concentration of GSNO, the NO released shows fluctuation within the first 100 min and becomes stable at $\sim$4 h. Note that as the error bars for high concentrations of GSNO (400, 800 μM) are relatively large, the lines of best fit might not represent the general trends. We observe that there are fluctuations before 100 min. These results indicate that GSNO releases NO in aqueous phase, and certain amounts of NO, once released without further reactions, are stable in solution and can be detected for several hours. We would like to mention that NO can also escape as gas through the liquid-gas interface and what we have measured with EPR is the part of NO that has dissolved in the buffer solution. Our main point in this study is to demonstrate that GSNO can spontaneously release NO directly (Fig. 2). The concentration of GSNO used in our experiment is 100–800 μM. The dissolved NO has been trapped by the trapper DETC and Fe^{2+}. Referring to Fig. 2 *B*, we have discovered that there are fluctuations of NO concentration in the first 100 min or so, implying that what has been lost in the headspace and what has been dissolved or partially autooxidized have not yet reached a steady state. After $\sim$100 min, the rates of loss and release are close to equal, and a steadier steady state is reached. Fig. 2 obviously demonstrates the spontaneous release of NO by GSNO in the solution.

Moreover, some impurity in the commercial reagent GSNO could exist as nitrite. To determine whether our spin-trap reagent can acidify the nitrite to produce NO, we

TABLE 1 Measurement of Cu and Fe concentrations in sample solution by ICP-MS

Concentration (μM) Sample	Cu	Fe
MilliQ H_2O	0.002	0.455
50 mM KPO_4	0.010	0.588
100 mM KPO_4	0.024	1.048
1 mM Cys, 50 mM KPO_4	0.026	0.978
2 mM Cys, 50 mM KPO_4	0.028	1.033
1 mM Cys, 100 mM KPO_4	0.044	1.575
1 mM GSH, 50 mM KPO_4	0.026	1.070
2 mM GSH, 50 mM KPO_4	0.026	1.121
1 mM GSH, 100 mM KPO_4	0.044	1.714

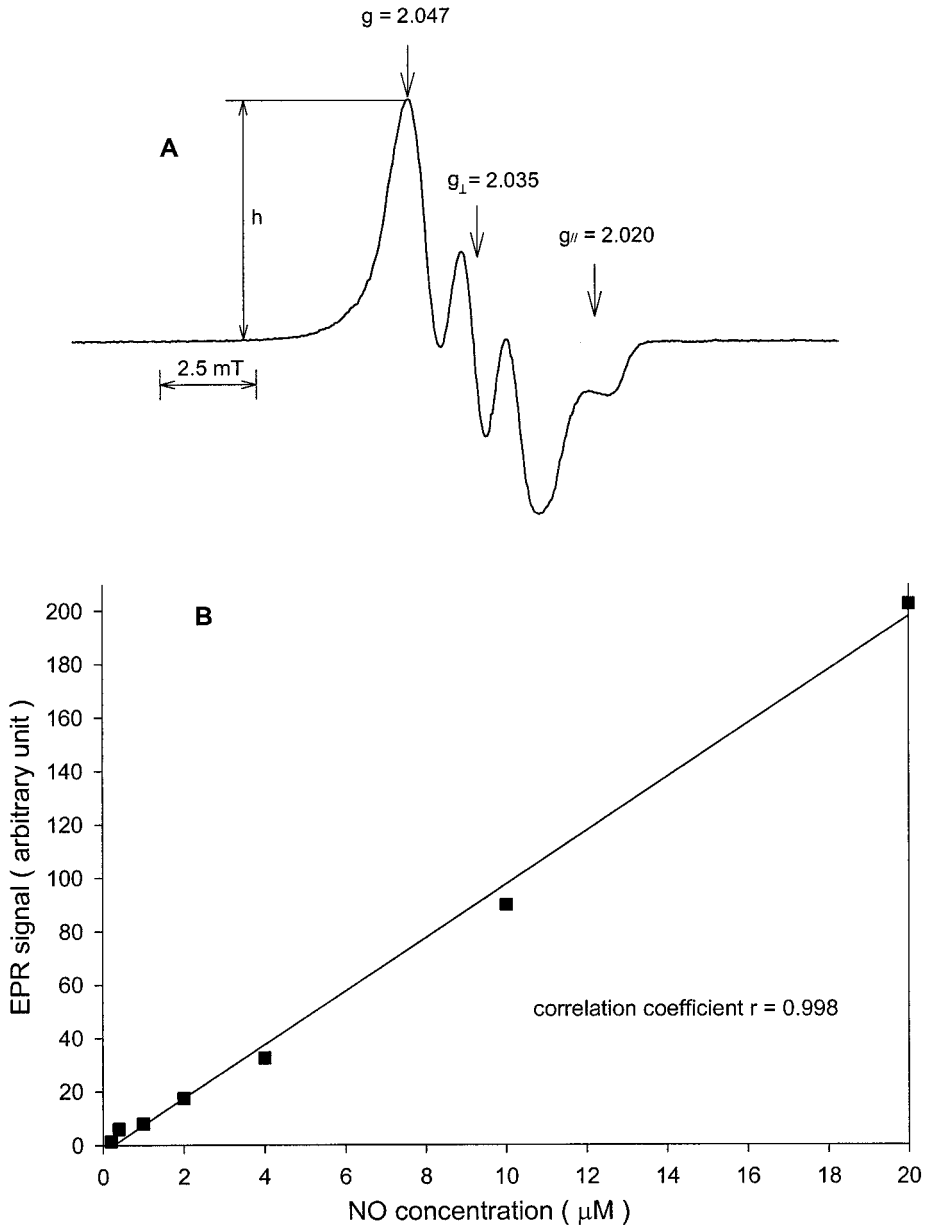


FIGURE 1 Characterization and calibration of EPR spectra of the $Fe^{2+}(DETC)_2NO$ complex. (*A*) A representative EPR signal of the $Fe^{2+}(DETC)_2NO$ complex was detected after a 3-min incubation of 2 μM NO with the spin trapping reagents at 25°C. Concentrations of the spin trapping reagents: $[Fe^{2+}]$ = [DETC] = 3.3 mM, [BSA] = 33 mg/ml, $[Na_2S_2O_4]$ = 1 M. Recording was made at 77 K, microwave frequency = 9.33 GHz, power = 10 mW, modulation frequency = 100 kHz, modulation amplitude = 0.52 mT. The magnetic field was operated to cover the range of 330 ± 20 mT, and the time constant was 10.24 ms with a sweep rate of 0.48 mT/s, and the receiver gain was 2×10^4. The $g_\perp$ and $g_{//}$ values are labeled. The height (*h*) of the first peak (g = 2.047) was taken to measure the NO concentration. (*B*) The strength of the EPR signal against the concentration (μM) of the NO standard solution demonstrates a linear correlation coefficient of 0.998 within 20 μM of NO and a detection limit of 0.2 μM at a signal-to-noise ratio of 3.

put nitrite (2–50 μM) into the spin-trap reagent and carried out EPR measurement. We found that the spin-trap reagent could protonate nitrite to produce NO; however, only ~3% of nitrite was converted to NO by EPR measurement. The GSNO we used was purified by high-performance liquid chromatography to analytical grade in solid form, and the impurity of the nitrite was less than 5%. For a 100 μM GSNO solution sample, if all impurities were nitrite, there could be at most 36 μM nitrite there. Even if 36 μM nitrite was acidified in our spin-trap reagent, only $36 \times 3\% = 1.08$ μM NO might be produced, which contributed to at most ~25% of 4.16 μM NO signals measured in the 100 μM of GSNO solution at the initial release phase (Fig. 2 *B*). So we have confidence that the NO signal detected is mainly contributed by NO released from GSNO rather than nitrite as an impurity.

Trapping and release of NO during reactions of NO with Cys and GSH

It has been shown that NO reacts with thiol-containing biomolecules, Cys, GSH, human serum albumin (HSA), and BSA to form RSNOs through the nitrosating intermediate, N_2O_3, under the aerobic conditions (Kharitonov et al., 1995; Pryor et al., 1982; Wink et al., 1994; Hogg et al., 1996; Vanin et al., 1997). The *S*-nitrosothiols can be further decomposed to NO and disulfide either catalytically by trace

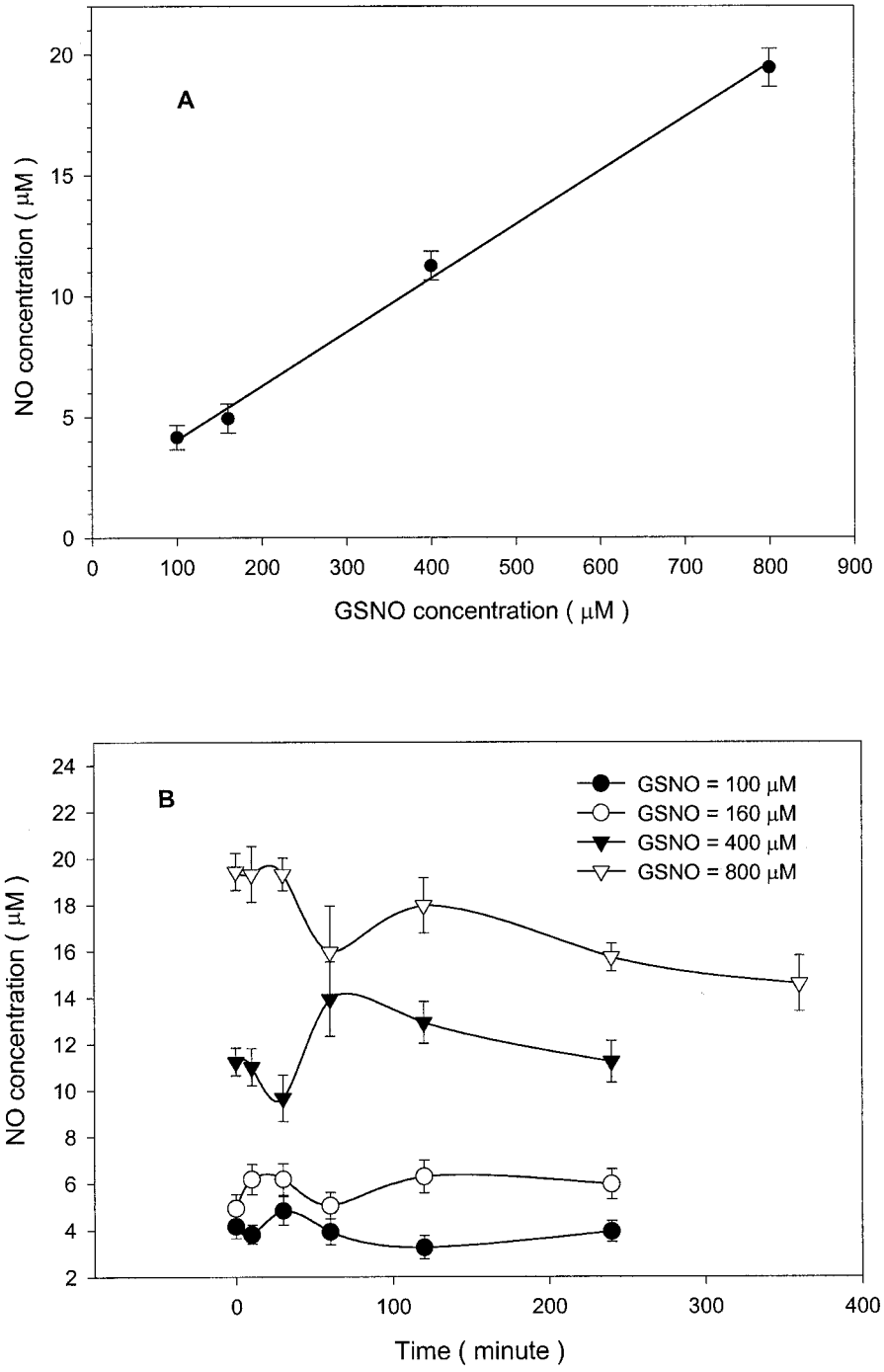


FIGURE 2 Dose- and time-dependent effect of NO release from GSNO. (*A*) Linear relation between the initial NO release and GSNO concentration. Solid GSNO was first dissolved in phosphate buffer (50 mM, pH 7.5), and aliquots of various concentrations of GSNO were mixed with the spin trapping reagents for EPR measurements as described in Fig. 1*A*. (*B*) The concentration of NO released from GSNO is shown as a function of time for four fixed GSNO concentrations, as marked. Data are shown as the mean ± SE of three measurements.

transition metal ions (McAninly et al., 1993; R. J. Singh et al., 1996; Vanin et al., 1997) that are present in all buffers or photolytically by irradiation with visible light through homolytic cleavage of the S-N bond to generate NO and a thiyl radical, $RS^{\cdot}$ (R. J. Singh et al., 1996). In previous studies either extensive NO bubbling (Kharitonov et al., 1995; Pryor et al., 1982; Vanin et al., 1997) or NO donors (Hogg et al., 1996) were used as an NO source added to the thiol-containing reaction mixture. The exact concentration of NO in the reaction mixture was therefore obscured and might well deviate from the physiological conditions of NO generation, which was estimated to be in the submicro to micromolar range (Shibuki and Okada, 1991; Malinski et al., 1993). Moreover, the overall reaction that starts from thiols mixed with NO and proceeds to the final step of NO regeneration has not been reported.

We determined the reaction of 2 μM NO with 2 mM Cys or 2 mM GSH by using diluted NO derived from the freshly

prepared saturate standard solution. NO concentrations were calibrated according to the linear range of NO EPR signals shown in Fig. 1 *B*. Fig. 3 shows representative time-course EPR spectra of NO obtained from the reaction of 2 μM NO with 2 mM GSH at pH 7.5 under aerobic condition (i.e., the reaction mixture was mixed in a sample tube exposed to air). In these experiments, a solution containing GSH was mixed with a diluted solution of NO to initiate the reaction. At every time point, an aliquot of reaction mixture was taken to mix with the NO trapping reagents for EPR measurement. The results demonstrate that NO signals readily decline to 50% at 2 min and further decline to baseline at 6 min of reaction. Interestingly, the NO signals appear again at 8 min, rise to nearly 70% of the original level at 15 min, and remain stable afterward up to 60 min of reaction. Figs. 4 *B* and 5 *B* are time-course results of the NO concentration normalized to the original NO concentration before the reaction of NO with Cys and GSH, respectively, at pH 7.5, with the reaction initiated under aerobic condition. The EPR signals of NO are readily reduced within 2–5 min, then start to regenerate and reach a plateau value around 10–12 min thereafter.

In addition to neutral pH, we also assayed the reaction in both basic and acidic conditions. Figs. 4 *A* and 5 *A* are the time-course EPR signals of NO determined at pH 8.5 for Cys and GSH, respectively, and Figs. 4 *C* and 5 *C* are time-course reaction curves determined at pH 6.5. Under acidic conditions, the amplitudes of NO signals in the EPR

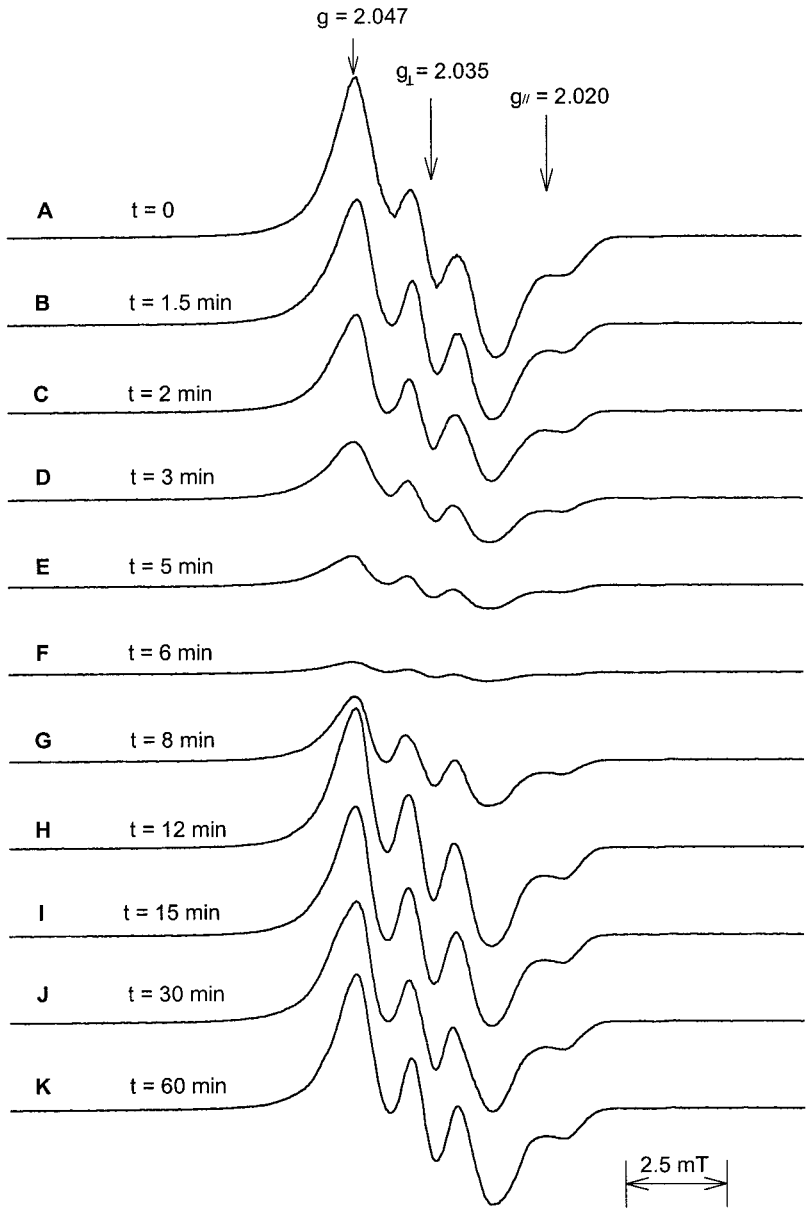
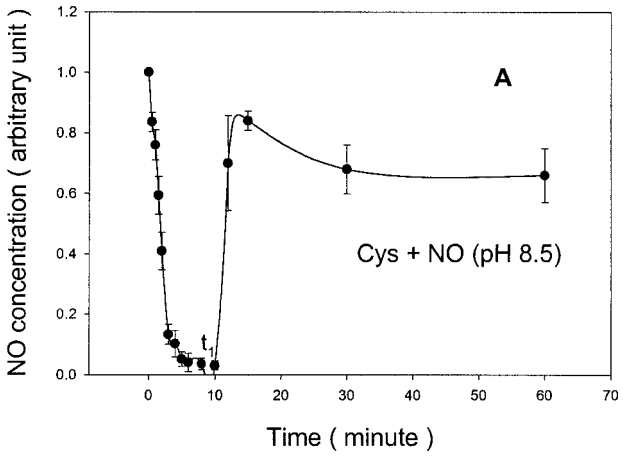
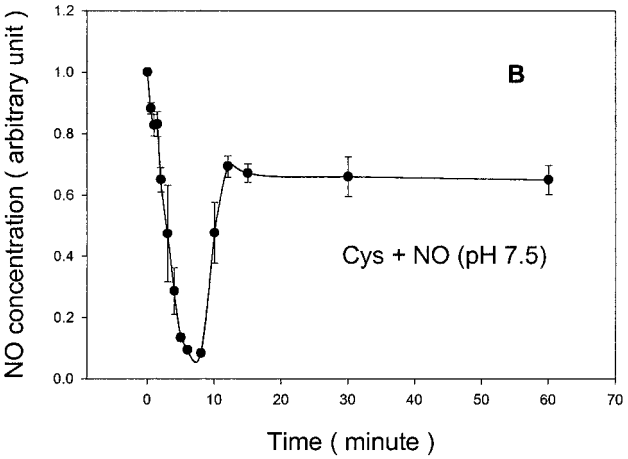


FIGURE 3 The trapping and release of NO in the time course of NO reaction with GSH. The EPR spectra of a reaction mixture of GSH (2 mM) plus NO (2 μM) at pH 7.5 under aerobic condition were recorded at the indicated reaction time points during a time course up to 60 min. The operation conditions were the same as those specified in Fig. 1. The EPR signal was found to reduce to almost baseline level at $t = 6$ min and to reappear at $t = 8$ min.





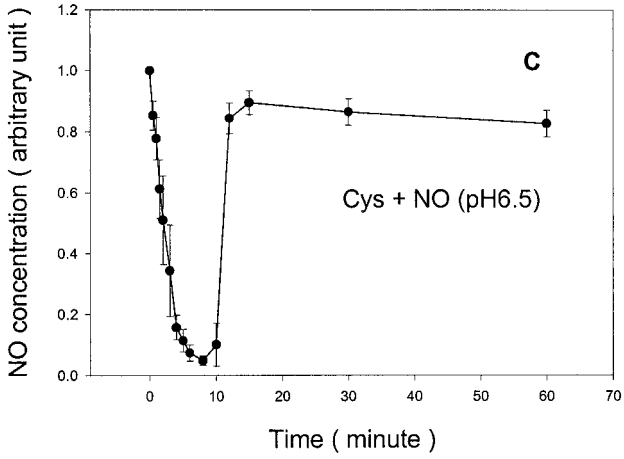
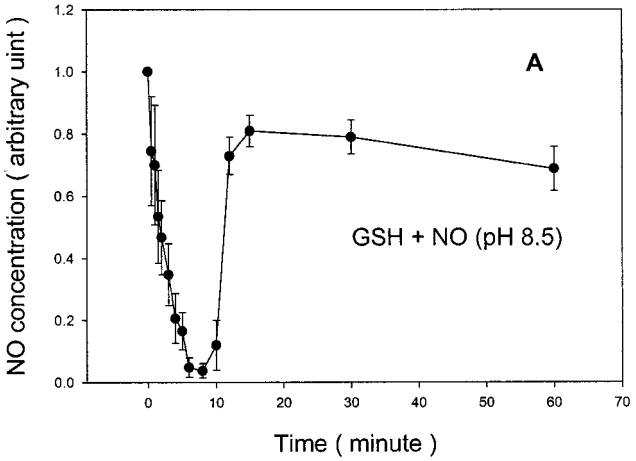
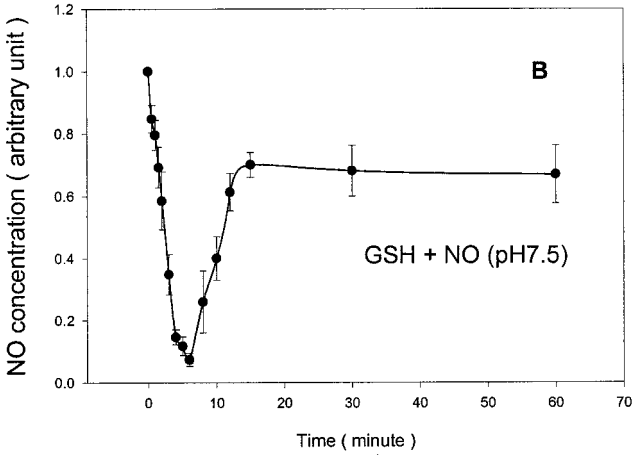


FIGURE 4 The effect of pH on the trapping and release of NO from its reaction with Cys. A time course reaction of NO (2 μM) with Cys (2 mM) was conducted at (*A*) pH 8.5, (*B*) pH 7.5, and (*C*) pH 6.5 under aerobic conditions. At each time point, the NO concentration of an aliquot of the reaction mixture was measured by EPR. Each data point of NO concentrations was normalized to the original NO concentration before the reaction and represented as the mean ± SE of three independent experiments.





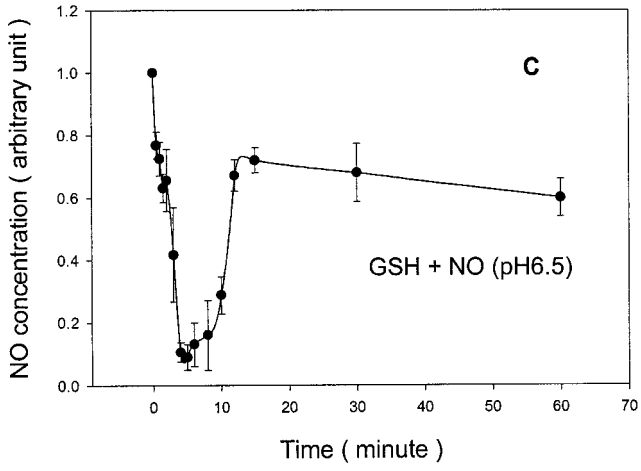


FIGURE 5 The effect of pH on the trapping and release of NO from its reaction with GSH. A time course reaction of NO (2 μM) with GSH (2 mM) was conducted at (*A*) pH 8.5, (*B*) pH 7.5, and (*C*) pH 6.5 under aerobic conditions. At each time point, the NO concentration of an aliquot of the reaction mixture was measured by EPR. Each data point of NO concentrations was normalized to the original NO concentration before the reaction and represented as the mean ± SE of three independent experiments.

spectra are gradually reduced during the 5 min of reaction, stay near baseline levels up to 10 min, and rapidly start to increase thereafter. Similar observations were obtained for reactions carried out under basic conditions at pH 8.5. It should be noted that under basic conditions the average NO signals at earlier time points (reaction time < 10 min) fluctuate more than those obtained under neutral and acidic conditions. This suggests that RSNOs formed under basic conditions might be less stable than those formed under neutral or acidic conditions. In all of the various conditions tested, the amounts of NO regenerated are around 70–90% of its original levels before the initiation of the redox reactions with thiols. The oxygen molecules might be involved in the chain of electron transport and serve as electron carriers and acceptors in the redox reactions.

DISCUSSION

RSNOs were discovered in the early 1980s to be potent vasodilators and inhibitors of platelet aggregation (Ignarro and Gruetter, 1980; Ignarro et al., 1981). On the other hand, it is well established that NO is an EDRF (Palmer et al., 1987; Ignarro et al., 1987). Therefore, it is important to study the nitrosation processes of thiols and their subsequent release of NO to understand the kinetics of smooth muscle relaxation. Active debates and research on the existence of intermediate states of nitrogen oxides during nitrosation have been reported in the past several years (Kharitonov et al., 1995; Vanin et al., 1997; Gow et al., 1997). In particular, it was found (Stamler et al., 1992b) that at pH 7.0, NO does not react directly with thiols to yield nitrosothiols, but nitrosation of thiols occurs in the presence of oxygen, suggesting that N_2O_3 or the like is formed in the intermediate steps (Kharitonov et al., 1995). In contrast, NO was also proposed to react directly with a reduced thiol to produce a radical intermediate, R-S-N˙-O-H, which in turn is converted to RSNO by the reduction of an electron acceptor such as oxygen (Gow et al., 1997). A different nitrosation mechanism of thiol was also proposed (Vanin et al., 1997), in which transition metal ions catalyze the conversion of NO to NO^+ in neutral pH aqueous solution in the absence of oxygen but potentiated with air. In line with our present results showing that the EPR signals of μM NO are readily reduced and trapped by mM Cys and GSH, it is tempting to suggest that NO can react with the abundant Cys or GSH to form RSNO whichever proposed mechanism might prevail.

Although it has been established that serum albumin and hemoglobin are thiol proteins in blood plasma that are responsible for trapping (part) of NO released through the usual biosynthesis process (Stamler et al., 1992a; Gow and Stamler, 1998), there are other thiols, particularly low M_r ones, circulating in the blood and tissue cells. For instance, GSH is abundant intracellularly in mammalian cells, ranging from 0.5 to 10 mM (Meister and Anderson, 1983), as compared to a micromolar concentration in blood plasma. Another thiol of physiological and practical interest is Cys, which is the precursor of GSH and is estimated to be present in cells in a concentration one magnitude less than that of GSH, but still much greater than that of blood plasma. Considering just the vasodilation process, NO has to travel through these four thiols (and others too) when it diffuses from the location of generation to one of the target molecules—guanylate cyclase in other surrounding smooth muscle cells. It is naturally of fundamental interest to study the biochemical reaction rates of the plausible reactions involved under physiological conditions.

Based on earlier kinetic studies (Kharitonov et al., 1995; R. J. Singh et al., 1996; Wink et al., 1993, 1994; S. P. Singh et al., 1996), the following reactions were proposed for the reaction of GSH with NO in aqueous solution under aerobic conditions:

$$2NO + O_2 \rightarrow 2NO_2 \text{ (Reaction 1)}$$

$$NO_2 + NO \rightarrow N_2O_3 \text{ (Reaction 2)}$$

$$N_2O_3 + H_2O \rightarrow 2H^+ + 2NO_2^- \text{ (Reaction 3)}$$

$$N_2O_3 + GSH \rightarrow H^+ + NO_2^- + GSNO \text{ (Reaction 4)}$$

$$2GSNO \rightarrow GSSG + 2NO \text{ (Reaction 5)}$$

Analysis of kinetic data of reactions 1 and 2 indicates that the rate-limiting step was reaction 1, with N_2O_3 as the main nitrosating species. Because the hydrolysis rate of N_2O_3 is on the same order as the rate of its reaction with GSH, the formation of GSNO through N_2O_3 is very likely. It was found that nitrosation of GSH at high concentration ($\geq$ mM, like that under physiological conditions) occurs at a rate about one order of magnitude greater than that of nitrosation of HSA. Reaction 4 for Cys is close to that of GSH and would be important in situations where the concentration of Cys is high.

With respect to the reaction of NO with Cys, Kharitonov et al. (1995) reported that the reaction of NO with Cys in the presence of O_2 was complex and different from that of NO with GSH. The difference is due to the fact that although O_2 is essential for the reaction to take place, the initial rate of NO reaction with Cys is dependent on NO concentration in a first-order manner, while the reaction rate of NO with GSH is dependent on both NO and O_2, with first order for O_2 but second order for NO. Namely, in the reaction of NO with Cys, the micromolar contamination of iron impurity in the phosphate buffer system was indicated to mediate the redox reaction of converting some NO to NO^+, which can react directly with Cys to form CysNO (Kharitonov et al., 1995; Vanin et al., 1997; Vanin and Malenkova, 1996).

Apart from the mechanisms discussed above, moreover, the ferrous iron catalyzes both the synthesis and release of NO from GSNO and CysNO: the catalysis rate is dependent on the ratios between RSNO, thiols, iron, and NO in the

reaction system (Vanin et al., 1997; Vanin and Malenkova, 1996). Mass spectroscopy in the present study shows that the concentrations of transition metals Fe and Cu are smaller than 2 μM and 0.05 μM, respectively. Other transition metals, if present, would not have concentrations greater than this kind of order of magnitude. To chelate these metal ions, 100 μM EDTA was added to every sample solution to eliminate the possibility that the presence of these ions might influence the reaction rates involved. It is interesting to note that the general trends of Figs. 4 and 5 are very similar with or without EDTA. The reported synthesis time of both GSNO and CysNO is around 10 min (Vanin et al., 1997; Vanin and Malenkova, 1996), which is within the same order of magnitude of the trapping time of NO by GSH and Cys as described here. However, the trapping and release times of NO are pH dependent and can vary somewhat (comparing Figs. 4 and 5).

Note also that we have added BSA to the trapping reagent to avoid precipitation of $FeSO_4 \cdot 7H_2O$. The reaction rate of BSA with NO is slower than the reaction rate of HAS with NO (Kharitonov et al., 1995), which in turn is less than the reaction rates of GSH and Cys with NO. Therefore, the presence of BSA in our experiments is unlikely to affect the estimates of the amount of NO trapped and released.

The pH dependence of trapping and release of NO by GSH and Cys may result from an equilibrium between $RS^- + H^+$ and thiol-containing biomolecules (RSHs) at various pH levels. In the acidic case, pH 6.5, the equilibrium is favorable for the formation of RSHs, resulting in a lower RSNO formation, as indicated by the observed slightly lower reaction rate of NO with Cys or GSH (Figs. 4 *C* and 5 *C*). In contrast, at alkaline pH (pH 8.5), the lower H^+ in the reaction mixture favors the formation of RS^-, which in turn speeds up the reaction of NO with Cys or GSH for RSNO formation (Figs. 4 *A* and 5 *A*). It is interesting to note that at the more neutral condition of pH 7.5, we detected a higher portion of free NO left in the reaction mixture. The time intervals in which NO was trapped by both Cys and GSH were slightly shorter at neutral pH than those at pH 8.5 and pH 6.5.

Based on the persistent observation that NO is trapped and released in the course of time, and for convenience in the following discussion, we define Phase I to be the time interval between the initial instant ($t = 0$) and the instant when the EPR signal just begins to increase after passing the minimum (practically zero). If we designated this time as t_1 (Fig. 4 *A*), then phase II refers to the time domain $t > t_1$.

In the following, we shall compare the rate of initial NO disappearance with NO autooxidation rates published by others. Kharitonov et al. (1995) and other authors have shown that the rate of reaction of NO with thiol is determined by the rate of the initial reaction of NO with oxygen. The NO autooxidation reaction rate is dependent on O_2 concentration in the first order and dependent on NO concentration in the second order, respectively. $d[NO]/dt$ satisfies a third-order kinetic law (pH independence) for the NO autooxidation reaction rate:

$$d[NO]/dt = -k[O_2][NO]^2 \quad (1)$$

Several rate constants determined from various sources are in fairly good agreement: $k = (6.3 \pm 0.4) \times 10^6\ M^{-2}\ S^{-1}$ at 25°C (Kharitonov et al., 1994); $(6.0 \pm 1.5) \times 10^6\ M^{-2}\ S^{-1}$ at 22°C (Wink et al., 1993); and $8.4 \times 10^6\ M^{-2}\ S^{-1}$ at 23°C (Lews and Deen, 1994). In our experimental environment at 22°C, we substitute $k = 6.0 \times 10^6\ M^{-2}\ S^{-1}$, $[O_2]_{t=0} = 210\ \mu M$ (Schmidt et al., 1998), and $[NO]_{t=0} = 2\ \mu M$ into Eq. 1 and obtain the initial NO disappearance rate $d[NO]/dt|_{t=0} = 5.1 \times 10^{-9}\ MS^{-1}$, which is close to the range of $8–17 \times 10^{-9}\ MS^{-1}$ as calculated from the slopes of the [NO]-time graphs at $t = 0$ in Figs. 4 and 5.

In the work of S. P. Singh et al. (1996) it is proposed that during the reaction of GSNO and GSH, NO would be released from GSNO and conjugate with excess GSH, forming a series of possible intermediate compounds. After 24 h of reaction, the end products are nitrite, nitrous oxide, and ammonia. In contrast to the analysis of Singh et al., our EPR observation of NO signal reduction (i.e., phase I) would mainly result from the reaction of NO with oxygen, forming N_2O_3 and possible other higher oxygen compounds, which in turn would react with thiols to form nitrosothiols. At the end of phase I, NO would disappear, and we believe there could be 1–2 μM S-nitrosothiol compounds and excess free thiols with concentrations near 2 mM. The study of S. P. Singh et al. (1996) focused on the period starting from the end of the stated phase I to the end of phase II: regeneration of NO from the reaction of S-nitrosothiols with thiols. We note, however, there is a tremendous difference in the concentration ratio of the reactants. In their case, the ratio of GSNO/GSH is 1:10, whereas in our case this ratio is 1:1000. Based on their analysis and proposed mechanism, the concentration of NO_2^- formed is dependent on the excess concentration of GSH: the higher the excess of GSH is, the less NO_2^- is formed. Therefore, their proposed mechanism predicts that the amount of NO_2^- formed as the end product would be very limited if the excess concentration of GSH is increased from 10- to 1000-fold. Hence, under our experimental conditions, the final product that exists as nitrite would be negligible (compared to the NO concentration). They also propose that the half-life of GSNO is dependent on the concentration of GSH as the decomposition rate of GSNO increases with the excess concentration of free thiols (S. P. Singh et al., 1996). Because in our reaction mixture, the excess of GSH is 1000-fold greater than the amount of GSNO, we expect the rate of NO release from GSNO will be greatly accelerated, as from the result of Singh et al. We have observed NO regeneration within several minutes of the beginning of phase II. Our contribution in the present study is to provide a direct observation of NO regeneration within a comparatively short time domain during the whole

reaction time course. The end products found 24 h after the beginning of phase II are outside the scope of the present investigation.

It is well known that many proteins are bound to membranes by acyl linkage involving cysteine residues. In fact, neuromodulin (a.k.a. GAP-43, protein F1) is another neuron-specific protein kinase C substrate that has two cysteines in its N-terminal (Cys^3 and Cys^4) end as Ng. However, unlike Ng as a soluble protein, the two cysteines of neuromodulin are involved in fatty acylation to anchor it to the presynaptic membranes (Chapman et al., 1992). Skene's group further reported that exposure of cultured dorsal root ganglion neurons and PC-12 cells to NO donors resulted in inhibition of protein fatty acylation by way of a thioester linkage to cysteine residues of neuromodulin (Hess et al., 1993). Because the results of the present study indicate that nitrosothiol is formed as an unstable intermediate in the reaction of NO with the thiol of cysteine, it is therefore possible that protein S-NO might be replaced by the abundant glutathione, thus affecting fatty acylation of cysteines to the membrane phospholipids.

In summary, this study presents evidence that the physiologically generated levels of NO can be trapped and regenerated directly by abundant free thiol-containing biomolecules such as Cys and GSH and determined through direct measurement of the characteristic EPR spectra of NO under aerobic and various pH conditions. The trapping and release of NO by these free thiols may provide a mechanism for temporal and spatial sequestration of NO to stabilize the NO supply, so that NO can overcome the diffusion gradient to exert its versatile effects by reacting with various targets through regeneration. Such a consequence has the potential to be of medical relevance.

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