Generation of thiyl radical by nitric oxide: a spin trapping study

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The free radical intermediates formed during the nitrosation of thiols including glutathione (GSH), L-cysteine (CystSH), captopril (CapSH) and *N*-acetylpenicillamine (NAPenSH) by NO$^{\bullet}$ at physiological pH have been investigated using EPR spectroscopy combined with the spin trap DMPO. We have found that NO$^{\bullet}$ in the presence of oxygen reacted with GSH, CystSH and CapSH to generate the corresponding thiyl radicals, which are spin trapped by DMPO to give the spin trapped adducts DMPO-SG, DMPO-SCyst and DMPO-SCap respectively. In the case of NAPenSH no spin trapped adduct is detected. Desferrioxamine, an inhibitor of peroxynitrite ($ONOO^-$), has no effect on the formation of either DMPO-SG or DMPO-SCyst, suggesting that $ONOO^-$ is not involved in the formation of GS$^{\bullet}$ and Cyst$^{\bullet}$. In contrast, desferrioxamine inhibits the detection of DMPO-SCap, pointing to a role for $ONOO^-$ in the formation of CapS$^{\bullet}$. Our results demonstrate that $^{\bullet}NO_2$ is the most likely reactive species in the nitrosation of GSH and CystSH by oxygenated NO$^{\bullet}$, thereby generating the corresponding thiyl radicals. Furthermore, our data indicate that the scavenging of NO$^{\bullet}$ by thiols may lead to the production of superoxide ($O_2^{\bullet -}$) *via* the formation of thiyl radicals.

Introduction

Nitric oxide (NO$^{\bullet}$)[1] is generated through the catalytic action of the monooxygenase, nitric oxide synthase on L-arginine.[1] The resulting free radical has been found to regulate a diversity of physiological functions and to act as a cytotoxic agent.[1e,2] In biological systems, NO$^{\bullet}$ is produced in the presence[3] of O_2 and, subsequently, reacts with this oxidant,[4] $O_2^{\bullet -}$[4e,5] and heme and non-heme iron.[6] In light of these findings, it has been postulated that molecules containing sulfhydryl (thiol) groups may act as carriers of NO$^{\bullet}$ to store, transport and deliver this free radical to sites of action.[7] This hypothesis is based on the long biological half life of *S*-nitrosothiols relative to NO$^{\bullet}$. Consistent with this theory, it has been found that *S*-nitrosohemoglobin is allosterically involved in the control of blood pressure by delivering NO$^{\bullet}$ to its vascular target.[8] In other settings, GSH, by reacting with NO$^{\bullet}$ aerobically to form GSNO, may represent a detoxification pathway for reactive nitrogen oxide species.[9] In support of this hypothesis, it has been demonstrated that *de novo* synthesis of GSH and the recycling of GSSG by glutathione reductase to GSH plays an important role in protecting Ovary (CHO-AA8) cells and human lymphoblastoid (TK6) cells against the cytotoxic effect of NO$^{\bullet}$, by regulating the intracellular concentration of GSH.[10]

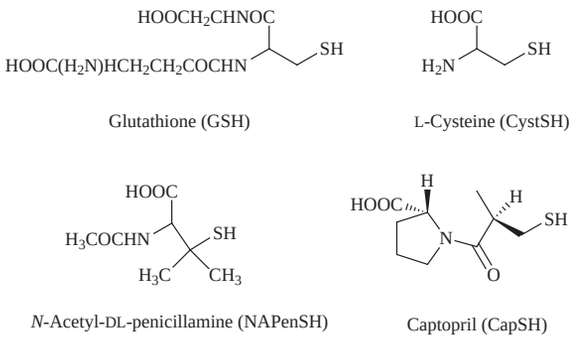
As NO$^{\bullet}$ is produced in an aerobic environment in the presence of high concentrations of GSH, it is not clear whether NO$^{\bullet}$ or GSNO is the actual species responsible for reactions attributed to this free radical. Before this question can adequately be addressed, however, aerobic reactions of NO$^{\bullet}$, especially in the presence of thiols, must initially be defined. For instance, it has been postulated[11] that aerobic nitrosation of thiols, including GSH and CystSH, in aerobic solutions of NO$^{\bullet}$ takes place through N_2O_3. Although this may suggest a free radical reaction, DMPO, which is known to react efficiently with thiyl radicals, did not have an effect on the yield of GSNO.[12] This suggested that GS$^{\bullet}$ does not form during the aerobic reaction of GSH with NO$^{\bullet}$. In support of this theory, Gow *et al.*[13] have proposed that NO$^{\bullet}$ can react anaerobically with cystSH to form an *S*-nitrosothiol in the absence of a thiyl radical. In contrast, Goldstein and Czapski[14] have recently presented a thoughtful discussion on the aerobic reactions of NO$^{\bullet}$ in which $^{\bullet}NO_2$ and N_2O_3 were suggested as possible reactants with thiols, yielding RSNO. One of the possible pathways involves the formation of thiyl radicals. In this manuscript, we demonstrate that thiyl radicals, generated by the aerobic reaction of NO$^{\bullet}$ with GSH, CystSH and CapSH, but not NAPenSH, were spin trapped during the formation of RSNO. Finally, we discuss the biological importance of the aerobic reaction of GSH with NO$^{\bullet}$.

Results and discussion

Our primary objective is to investigate the nature of the free radicals generated during the reaction of NO$^{\bullet}$ with thiols. Kinetic studies suggested that nitrosation of thiols by aerobic solutions of NO$^{\bullet}$ is dependent on the structure of the thiols.[14] Therefore, four different thiols, GSH, CysSH, CapSH and NAPenSH were chosen. As thiyl radicals are one of the likely outcomes from the reaction of NO$^{\bullet}$ with thiols, spin trapping/EPR spectroscopy allows for the identification of these free radicals in a variety of biological environments, not the least of which is *in vivo*.[15] While the spin trapping of thiyl radicals in cells is difficult to achieve,[16] homogenous solutions of DMPO have been successfully employed to detect thiyl radicals,[17] as DMPO[18] does not react with NO$^{\bullet}$. As a source of NO$^{\bullet}$ we used SPER/NO,[19] an NO-releasing compound with an NO$^{\bullet}$ $t_{\frac{1}{2}}$ of 39 min at 37 °C and pH 7.4.

When DMPO (100 mM) was added to a solution containing GSH (1 mM) and SPER/NO (at 0.1 mM or 1 mM), a four line EPR spectrum with $A_N = 15.40$ G and $A_H = 16.20$ G (Table 1)

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GS$^{\bullet}$, glutathionyl radical; CystSH, L-cysteine; CapSH, captopril [(2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline]; NAPenSH, *N*-acetylpenicillamine; $O_2^{\bullet -}$, superoxide; HO$^{\bullet}$, hydroxyl radical; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; NO$^{\bullet}$, nitric oxide; N_2O_3, nitrous anhydride; $ONOO^-$, peroxynitrite; GSNO, *S*-nitrosoglutathione; SPER/NO, 2,2′-bis(hydroxynitrosohydrazono)-ethanamine; DMSO, dimethyl sulfoxide; DMPO, 5,5-dimethylpyrroline *N*-oxide; DMPO-OH, 2,2-dimethyl-5-hydroxypyrrolidine-1-oxyl; DMPO-SG, 2,2-dimethyl-5-glutathionylpyrrolidine-1-oxyl; DMPO-CH_3, 2,2,5-trimethylpyrrolidine-1-oxyl; DMPO-$CH(CH_3)OH$, 2,2-dimethyl-5-(1-hydroxyethylpyrrolidine-1-oxyl; DMPO-SCyst, 2,2-dimethyl-5-cysteinylpyrrolidine-1-oxyl; DMPO-SCap, 2,2-dimethyl-5-captoprilpyrrolidine-1-oxyl; glutathione conjugate *N*-hydroxysulfenamide, GS-N(OH)-SG; glutathione peroxysulfenyl radical, GSOO$^{\bullet}$; glutathione sulfenyl hydroperoxide, GSOOH; glutathione anion, GS^-; glutathione radical anion, $GSSG^{\bullet -}$.



was recorded (Fig. 1A).† The release of NO$^{\bullet}$ by SPER/NO under our conditions was assessed by the oxyhemoglobin method.[20] In the absence of either GSH or SPER/NO, no EPR signal was recorded (Fig. 1B), suggesting that both GSH and SPER/NO are required for the generation of the observed spin trapped adduct. To further confirm that NO$^{\bullet}$ was indeed responsible for the formation of the spin trapped adduct, SPER/NO (1 mM) was first allowed to decompose in buffer for several days until NO$^{\bullet}$ was no longer detected, followed by the addition of GSH (1 mM) and DMPO (1 mM). Under these conditions no EPR signal was detected (data not shown). These results clearly demonstrate that the formation of the spin trapped adduct is due to the aerobic reaction of NO$^{\bullet}$ or a product derived from NO$^{\bullet}$ with GSH. Given that the spin trapped adduct DMPO-OH has similar hyperfine coupling constants,[21] the EPR spectrum shown in Fig. 1A may be attributed to either DMPO-SG or DMPO-OH. Although it is unlikely that HO$^{\bullet}$ is generated under these conditions, it is, nevertheless, essential to unambiguously characterize the spin trapped adduct, whose EPR spectrum is depicted in Fig. 1A. If HO$^{\bullet}$ were generated from either $O_2^{\bullet -}$ or H_2O_2, then SOD or catalase would inhibit DMPO-OH formation. Addition of either SOD (30 U ml^{-1}) or catalase (300 U ml^{-1}) to the reaction mixture containing DMPO (100 mM), GSH (1 mM) and SPER/NO (1 mM) did not alter the peak height of the spin trapped adduct (Fig. 1C). An alternative approach to demonstrate the involvement of HO$^{\bullet}$ is to include high concentrations of scavengers of this free radical, such as DMSO or ethanol, relative to the spin trap in the reaction mixture containing DMPO. Hydroxyl radical is known to react with DMSO and ethanol at diffusion controlled rates to give methyl radical and α-hydroxyethyl radical, respectively.[22] If HO$^{\bullet}$ were generated under these conditions, then either DMPO-CH_3 or DMPO-$CH(CH_3)OH$ would be detected at the expense of DMPO-OH. Inclusion of DMSO (140 mM) or ethanol (1.7 M) in the reaction mixture containing DMPO (100 mM), GSH (1 mM) and SPER/NO (1 mM) did not change either the amplitude or the EPR spectrum of the observed spin trapped adduct (Fig. 1C).‡ Based on these data, we conclude that EPR spectrum shown in Fig. 1A resulted from the spin trapping of GS$^{\bullet}$ by DMPO.

It has been shown[7b,11,14] that nitrosation of thiols by NO$^{\bullet}$ to form nitrosothiols required the presence of O_2. When spin trapping experiments were conducted in a N_2 atmosphere, DMPO-SG was not observed (Fig. 1D). While these data demonstrate that GS$^{\bullet}$ is generated during the aerobic reaction of NO$^{\bullet}$ with GSH, they do not rule out the possibility that GS$^{\bullet}$ is formed from the homolytic cleavage of GSNO and subsequently spin trapped by DMPO. In fact several investigators

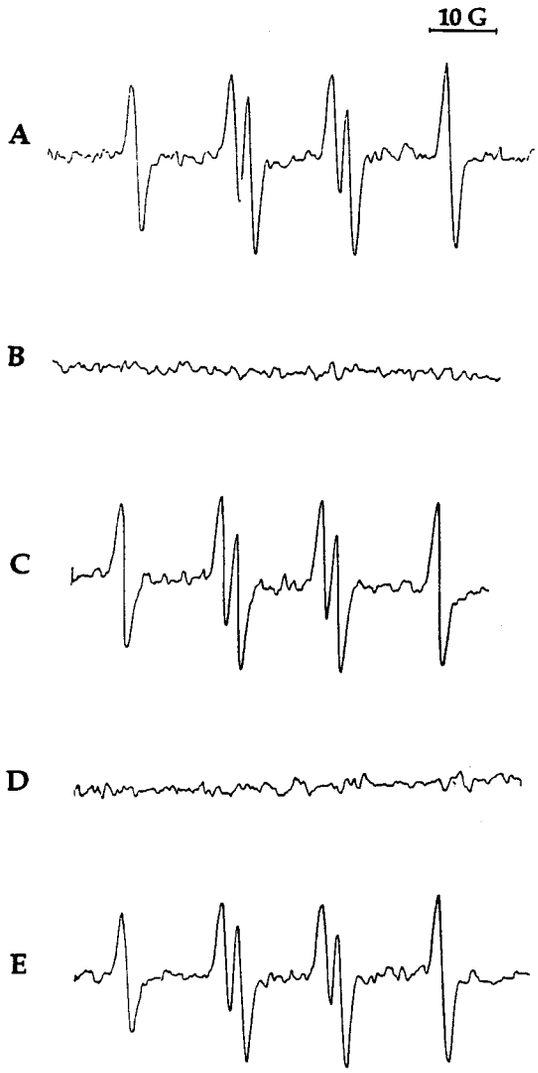


Fig. 1 EPR spectra obtained from the reaction of NO$^{\bullet}$ with GSH in the presence of DMPO. The reaction mixture leading to scan A consisted of SPER/NO (1 mM), GSH (1 mM) and DMPO (100 mM). Scan B was recorded under conditions identical to scan A except that SPER/NO was first allowed to decompose in buffer solution for 3 days. Similar EPR spectra to scan B were obtained when either SPER/NO or GSH were omitted from the solution. Scan C was recorded under conditions identical to scan A except for the addition of SOD (30 U ml^{-1}) to the solution. Similar EPR spectra to scan C were obtained when either catalase (300 U ml^{-1}), DMSO (140 mM) or ethanol (1.7 M) was added to the solution. Scan D was recorded under identical conditions to scan A except the experiment was performed under anaerobic conditions by bubbling the solutions with nitrogen. Scan E was recorded under identical conditions to scan A except for the addition of desferrioxamine (10 mM) to the solution. The receiver gain for the spectrometer was 6.3×10^4.

reported that GSNO can decompose under certain conditions to generate GS$^{\bullet}$ and NO$^{\bullet}$. For instance, Gorren *et al.*,[23] using stopped-flow/rapid-scan spectroscopy to study the decomposition of GSNO, suggested that Cu^+ can efficiently induce the homolytic cleavage of the sulfur nitrogen bond to give NO$^{\bullet}$ and GS$^{\bullet}$ respectively. Singh *et al.*,[17c] using spin trapping as an analytical tool, suggested that *S*-nitrosothiols including GSNO are stable in the dark at 37 °C and pH 7.4 in the presence of transition metal ion chelators. It was shown[17c] that only visible photolysis of *S*-nitrosothiols, including GSNO, led to homolytic cleavage of the nitrogen sulfur bond with subsequent generation of NO$^{\bullet}$ and GS$^{\bullet}$. Because we performed our experiments in room light and in buffer, which might contain trace amounts of redox active transition metal ions, despite having included DTPA (0.1 mM) in the reaction mixture, it is possible that the spin trapped adduct DMPO-SG shown in

† The hyperfine splitting constants for DMPO-SG and DMPO-SCap were determined from a computer simulation. The hyperfine splitting constants for DMPO-SCap have been reported to be $A_N = 15.4$ G and $A_H = 16.0$ G.[26]

‡ Control experiments using well known methods of HO$^{\bullet}$ generation in the presence of excess DMSO and ethanol demonstrated the formation of DMPO-CH_3 and DMPO-$CH(CH_3)OH$ at the expense of DMPO-OH.

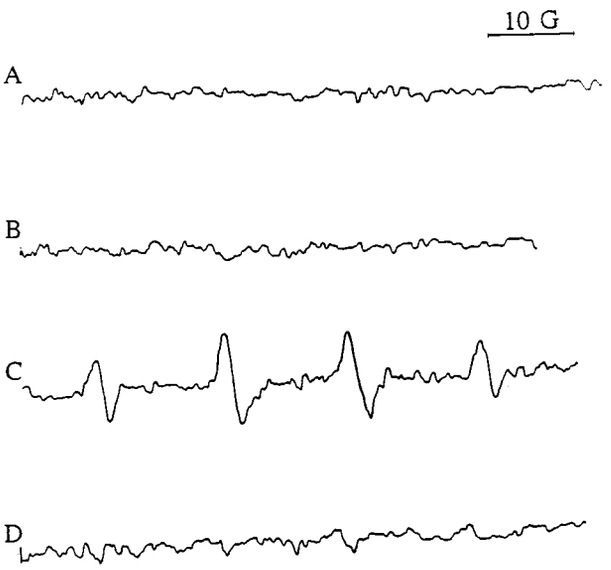


Fig. 2 EPR spectra obtained from the reaction of GSNO. The reaction mixture leading to scan A consisted of GSNO (1 mM) and DMPO (100 mM). Scan B was recorded under conditions identical to scan A except for the addition of SPER/NO (1 mM). Scan C was recorded under conditions identical to scan A except for the addition of GSH (1 mM). Scan D was recorded under conditions identical to scan C except the concentration of GSH was 100 mM. No spin trapped adduct was detected in the presence of GSH (1 mM) and GSNO (100 mM). Receiver gain for the spectrometer was 6.3×10^4.

Table 1 Hyperfine splitting constants (G) of thiol-containing spin trapped adducts of DMPO

	$GS^{\bullet}$		$CystS^{\bullet}$		$CapS^{\bullet}$	
	A_N	A_H	A_N	A_H	A_N	A_H
DMPO	15.40	16.20	14.80	17.40	15.40	16.00

Fig. 1A is derived from homolytic cleavage of GSNO. Another potential source of $GS^{\bullet}$ is the homolytic cleavage of the S–N bond of the glutathione conjugate *N*-hydroxysulfenamide, GS-N(OH)-SG, formed from the reaction of GSH with GSNO as proposed by Singh *et al.*[17c] To rule out these possibilities several experiments were undertaken. First, when GSNO (1 mM) was incubated with DMPO (100 mM) under our experimental conditions, we did not observe an EPR spectrum (Fig. 2A). Similarly, no spin trapped adduct was detected in the presence of GSNO (1 mM), SPER/NO (1 mM) and DMPO (100 mM) (Fig. 2B). In contrast, when GSH (1 mM) was added to GSNO (1 mM) in the presence of DMPO (100 mM), a small EPR signal corresponding to DMPO-SG was observed (Fig. 2C). The amplitude of this spectrum was estimated to be about 10–20% of the EPR spectral intensity shown in Fig. 1A. When either the concentration of GSH or the concentration of GSNO was decreased to 100 μM, no EPR signal was detected (Fig. 2D). Given the weak EPR signal shown in Fig. 2C and the fact that concentrations of both GSH and GSNO in our reaction solutions were less than 1 mM, we believe that DMPO-SG, depicted in Fig. 2A, did not result from homolytic cleavage of GSNO, but rather it arrived from the spin trapping of $GS^{\bullet}$, generated by the aerobic reaction of GSH with $NO^{\bullet}$. It is possible that DMPO-SG, shown in Fig. 2C, is derived from the trapping of $GS^{\bullet}$ which is generated from the homolytic cleavage of the S–N bond of GS-N(OH)-SG.[24] Finally, it is interesting to note that under our conditions, the rate of DMPO-SG formation reached a plateau after 10 min and lasted for at least 60 min (data not shown).

Substitution of CystSH (1 mM) for GSH in the reaction mixture gave a characteristic six line EPR spectrum with $A_N = 14.8$ G and $A_H = 17.4$ G (Table 1) corresponding to DMPO-SCyst (Fig. 3A).[17a–c] As in the case of GSH, the presence of both CystSH, SPER/NO and O_2 are required for the generation of DMPO-SCyst (Fig. 3B and D). Finally, similar to the results obtained with GSH, addition of SOD (30 U ml^{-1}), catalase (300 U ml^{-1}), DMSO (140 mM) or ethanol (1.7 M) in the reaction mixture containing DMPO (100 mM), CystSH (1 mM) and SPER/NO (1 mM) did not inhibit the formation of DMPO-SCyst (Fig. 3C). These data demonstrate that $NO^{\bullet}$ reacts with GSH and CystSH in the presence of O_2 to give the corresponding thiyl radicals.

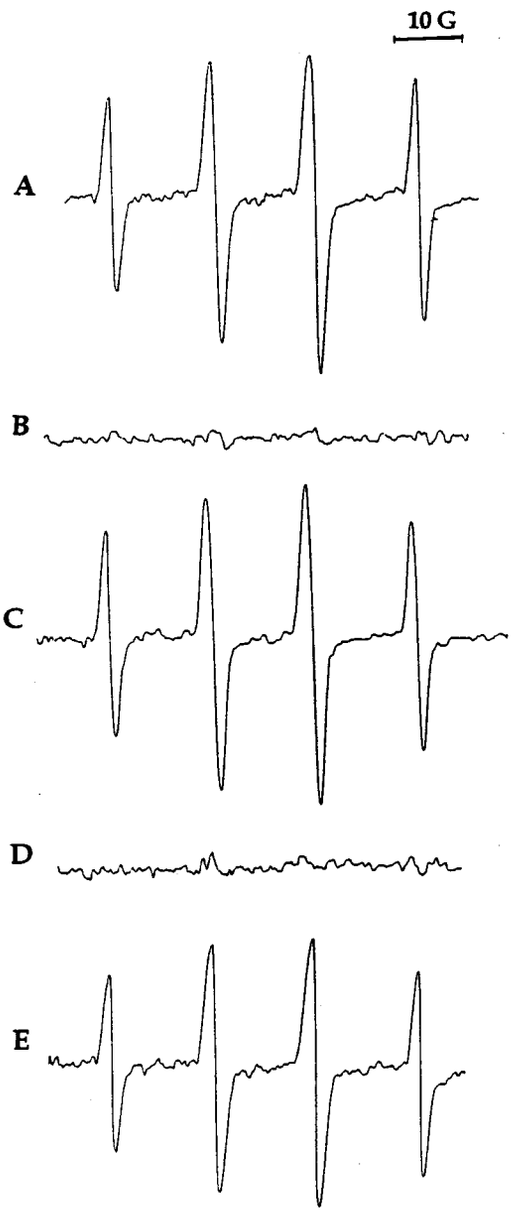


Fig. 3 EPR spectra obtained from the reaction of $NO^{\bullet}$ with CystSH in the presence of DMPO. The reaction mixture resulting in scan A consisted of SPER/NO (1 mM), CystSH (1 mM) and DMPO (100 mM). Scan B was recorded under conditions identical to scan A except that SPER/NO was first allowed to decompose in buffer solution for 3 days. Similar EPR spectra to scan B were obtained when either SPER/NO or CystSH were omitted from the solution. Scan C was recorded under conditions identical to scan A except for the addition of SOD (30 U ml^{-1}) to the solution. Similar EPR spectra to scan C were obtained when either catalase (300 U ml^{-1}), DMSO (140 mM) or ethanol (1.7 M) was added to the solution. Scan D was recorded under identical conditions to scan A except the experiment was performed under anaerobic conditions by bubbling the solutions with nitrogen. Scan E was recorded under identical conditions to scan A except for the addition of desferrioxamine (10 mM) to the solution. Receiver gain for the spectrometer was 8×10^4.

Based on the known rate of autooxidation of NO$^{\bullet}$, several mechanisms to account for the formation of nitrosothiols by oxygenated NO$^{\bullet}$ solutions have been proposed.[14] One mechanism involves ONOONO, an intermediate formed in the reaction of O_2 with NO$^{\bullet}$, as the nitrosating agent according to the following sequence of reactions.

$$ONOONO + RS^- \longrightarrow RSNO + ONOO^- \quad (1)$$

$$ONOO^- + RSH \longrightarrow RS^{\bullet} + {}^{\bullet}NO_2 + OH^- \quad (2)$$

$${}^{\bullet}NO_2 + RS^- \longrightarrow RS^{\bullet} + NO_2^- \quad (3)$$

$$RS^{\bullet} + {}^{\bullet}NO \longrightarrow RSNO \quad (4)$$

In this scheme [reactions (1)–(4)], thiyl radical is initially generated by the oxidation of thiols by $ONOO^-$. With the subsequent formation of ${}^{\bullet}NO_2$, which can then accept an electron from RS^-, another thiyl radical molecule is generated. Peroxynitrite is known to oxidize thiols including CystSH, GSH and NAPenSH, to form the corresponding thiyl radicals.[25] Based on the stoichiometric analysis of the aerobic nitrosation of thiols by NO$^{\bullet}$, Goldstein and Czapski[14] suggested that $ONOO^-$ is not involved with the generation of thiyl radicals during the nitrosation reaction. To confirm this hypothesis, we reasoned that if $ONOO^-$ is not the species responsible for the formation of thiyl radicals, then addition of $ONOO^-$ scavengers would not impact on the generation of DMPO-SG and DMPO-SCyst. Desferrioxamine, an iron chelator, has been shown to react directly with $ONOO^-$ rather than by metal chelation.[19] When desferrioxamine (10 mM) was included in the reaction mixture, no significant change in the intensity of EPR spectra for DMPO-SG and DMPO-SCyst was observed (Fig. 1E and Fig. 3E). For GSH and CystSH, this result confirms the hypothesis of Goldstein and Czapski.[14] Two other intermediates, ${}^{\bullet}NO_2$ and/or N_2O_3, which are both derived from ONOONO, have been suggested as possible nitrosating species.[14] For N_2O_3, nitrosation of thiols can proceed through a nucleophilic attack without the formation of a free radical [reaction (5)].

$$N_2O_3 + RS^- \longrightarrow RSNO + NO_2^- \quad (5)$$

As GS$^{\bullet}$ and CsytS$^{\bullet}$ were spin trapped, our results suggest that among the intermediates for the reactive species in the nitrosation of GSH and CysSH by oxygenated NO$^{\bullet}$ solutions, ${}^{\bullet}NO_2$ is the most likely candidate, which by acting as an oxidant, can generate the corresponding thiyl radical.

For NAPenSH and CapSH, it has been suggested[14] that the nitrosation proceeds directly through N_2O_3 as in these cases the rate of oxidation of these thiols by ${}^{\bullet}NO_2$ is too slow at pH 7.4 to compete effectively with the reaction of ${}^{\bullet}NO_2$ with NO$^{\bullet}$. To test this hypothesis, CapSH (1 mM) was substituted for GSH in the reaction solution containing SPER/NO (1 mM) and DMPO (100 mM). To our surprise a four line EPR spectrum with $A_N = 15.4$ G and $A_H = 16.0$ G (Table 1) was recorded† (Fig. 4A). As in the case with GSH, the presence of both CapSH, SPER/NO and O_2 are required for the generation of DMPO-SCap (Fig. 4B and D). Finally, similar to the results obtained with GSH, addition of SOD (30 U ml^{-1}), catalase (300 U ml^{-1}), DMSO (140 mM) or ethanol (1.7 M) in the mixture containing DMPO (100 mM), CapSH (1 mM) and SPER/NO (1 mM) did not inhibit the formation of the spin trapped adduct (Fig. 4C). These results eliminate the possibility that HO$^{\bullet}$ is generated under the experimental conditions and suggest that CapS$^{\bullet}$ is formed and spin trapped by DMPO, giving DMPO-SCap in the aerobic reaction of CapSH with NO$^{\bullet}$. To explore the mechanism of CapS$^{\bullet}$ generation, experiments were performed in the presence of desferrioxamine. As shown in Fig. 5, desferrioxamine at 5 mM and 10 mM inhibited DMPO-SCap. This finding is in contrast with those obtained for GSH and CystSH where desferrioxamine (10 mM) did not significantly alter the intensity of the corresponding spin trapped adducts. Although the involvement of contaminating reactive transition metals cannot be completely ruled out, our results are consistent with the possibility that generation of CapS$^{\bullet}$ in the aerobic nitrosation of CapSH by NO$^{\bullet}$ takes place predominantly through $ONOO^-$. It is important to emphasize that the detection of CapS$^{\bullet}$ does not disprove the proposed mechanism of the nitrosation of CapSH.[14] It is conceivable that the generation of CapS$^{\bullet}$ represents only a minor pathway in the nitrosation reaction. Finally, substitution of NAPenSH (1 mM) for GSH in the reaction mixture did not give a detectable EPR signal (data not shown). Despite the fact that NAPenS$^{\bullet}$ is more sterically hindered than is GS$^{\bullet}$, CystS$^{\bullet}$ or CapS$^{\bullet}$, NAPenS$^{\bullet}$ has previously been spin trapped by DMPO.[17c] If NAPenS$^{\bullet}$ were formed under our experimental conditions, then DMPO-SNAPen should have been detected. Therefore, NAPenS$^{\bullet}$ appears not to be an intermediate in the aerobic nitrosation of NAPenSH by NO$^{\bullet}$.

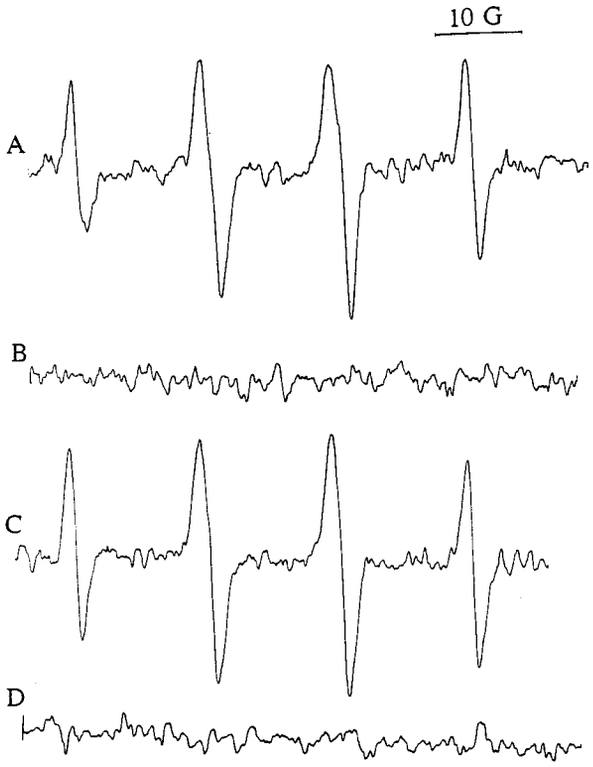


Fig. 4 EPR spectra obtained from the reaction of NO$^{\bullet}$ with CapSH in the presence of DMPO. The reaction mixture resulting in scan A consisted of SPER/NO (1 mM), CapSH (1 mM) and DMPO (100 mM). Scan B was recorded under conditions identical to scan A except that SPER/NO was first allowed to decompose in buffer solution for 3 days. Similar EPR spectra to scan B were obtained when either SPER/NO or CapSH were omitted from the solution. Scan C was recorded under conditions identical to scan A except for the addition of SOD (30 U ml^{-1}) to the solution. Similar EPR spectra to scan C were obtained when either catalase (300 U ml^{-1}), DMSO (140 mM) or ethanol (1.7 M) was added to the solution. Scan D was recorded under identical conditions to scan A except the experiment was performed under anaerobic conditions by bubbling the solutions with nitrogen. Receiver gain for the spectrometer was 10×10^4.

In summary, we have shown that thiyl radicals derived from GSH, CystSH and CapSH have been formed and spin trapped in the aerobic nitrosation reaction. Although the spin trapping of thiyl radicals will not define a complete picture of the complex chemistry of this nitrosation reaction, it establishes the existence of these reactive free radical intermediates, which may have physiological relevance. The chemistry of GS$^{\bullet}$ in the presence of O_2 and excess GSH is well established.[27] Fig. 6 shows multiple pathways which could lead to the formation of GS$^{\bullet}$.

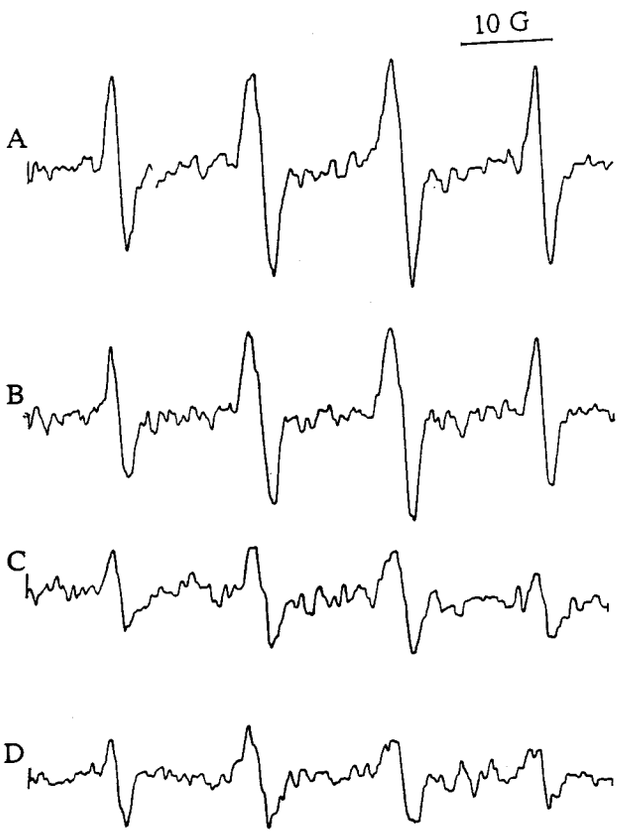


Fig. 5 Effect of desferrioxamine on the spin trapped adduct DMPO-SCap. The reaction mixture resulting in scan A consisted of SPER/NO (1 mM), CapSH (1 mM) and DMPO (100 mM). Scan B was recorded under conditions identical to scan A except for the addition of desferrioxamine (1 mM) to the solution. Scan C was recorded under identical conditions to scan B except the concentration of desferrioxamine was 5 mM. Scan C was recorded under identical conditions to scan B except the concentration of desferrioxamine was 10 mM. Receiver gain for the spectrometer was 10×10^4.

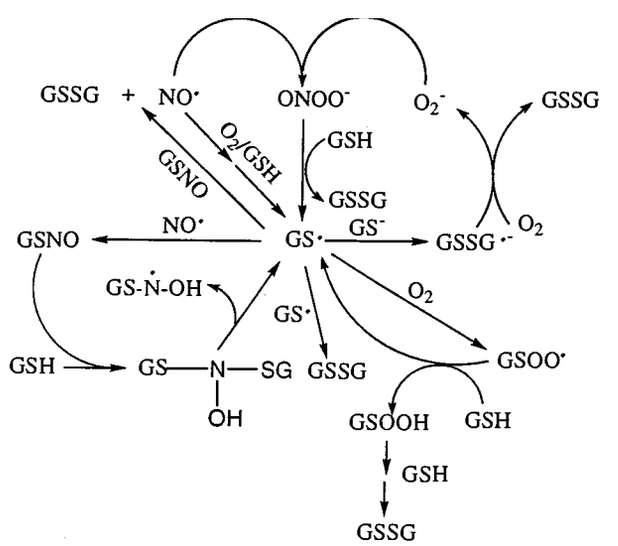


Fig. 6 Hypothetical scheme showing the interaction of NO• with O_2/GSH and the subsequent chemical reactions involving GS• (adapted from Singh *et al.*,[24] Karoui *et al.*[25b] and Ross *et al.*[27a])

In this scheme, there are several potential cyclic reaction sequences, which can result in the formation of GS•. First, GS• can be formed from the homolytic cleavage of the S–N bond of GS-H(OH)-SG, depending upon the availability of GSH and GSNO.[24] Second, GS• can react with O_2 to form the glutathione peroxysulfenyl radical (GSOO•) which then can abstract a hydrogen atom from GSH to give another GS• and glutathionesulfenyl hydroperoxide (GSOOH).[27] Third, GS• can react[27] with glutathione anion (GS^-) to form glutathione radical anion ($GSSG^{\bullet -}$) which can then reduce O_2 to $O_2^{\bullet -}$. Subsequent reaction[25b] of $O_2^{\bullet -}$ with NO• gives $ONOO^-$ which can interact with GSH to form another GS•. Clearly, based on these cyclic reaction sequences and the availability of O_2 and GSH, low fluxes of GS• could potentially lead to extensive thiol oxidation. Our results suggest that in the presence of NO•, the major source of GS• is derived from the aerobic reaction of GSH with NO•. Finally, GS• can either dimerize to form GSSG or react with GSNO to give NO• and GSSG.[24]

The formation of sulfur centered free radicals has several important biological implications. First, thiyl radicals have the potential to participate in a number of one-electron reactions, including the initiation of lipid peroxidation.[28] Alternatively, the formation of $O_2^{\bullet -}$ may represent a more important biological consequence of thiyl radical generation. While the formation of $O_2^{\bullet -}$ in the presence of NO• will ultimately lead to $ONOO^-$, we believe that the major source of $ONOO^-$ in biological systems may results from NOS.[29]

Recently, it has been found[9,10,29c,30] that GSH-depleted cells are susceptible to killing by NO•. Based on data presented herein, one might suggest that GSH, by acting as a scavenger for NO• and products derived from the aerobic oxidation of this free radical, has broadened its well established role as a cytoprotective agent against oxidant stress.[31]

As the formation of *S*-nitrosothiol cannot proceed in the absence of O_2, the concentration of O_2 becomes critical in predicting biological outcomes. At low concentrations of NO• compared to O_2, the half life for the nitrosation step is very slow, suggesting the improbability of this reaction.[14] Despite this, GS• may indeed be generated in NO• producing cells, where the levels of GSH are high.

One rather interesting caveat from these studies is the possibility that $O_2^{\bullet -}$ may be produced as the result of thiyl radical formation. If so, then an additional burden is placed on SOD to afford protection against $O_2^{\bullet -}$ mediated cytotoxicity. We are currently exploring the biological implications of the reaction between GSH and NO•.

Experimental

All chemicals obtained from commercial sources were used as received without further purification. GSH, CystSH, CapSH, NAPenSH, GSNO, oxyhemoglobin (human Ao, ferrous), *N*,*N*,*N*′,*N*″,*N*″-diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD) and catalase were purchased from Sigma (St. Louis, MO). Pure ethanol U.S.P was obtained from Warner-Graham (Cockeysville, MD). 2,2′-Bis(hydroxynitrosohydrazono)ethanamine (SPER/NO), was obtained from Midwest Research Institute (Kansas City, MO). Hanks' balanced salt solution (HBSS) was obtained from Gibco Laboratories (Grand Island, NY). The spin trap DMPO was synthesized according to Bonnett *et al.*[32] and was purified by vacuum distillation. The buffer used in our study was HBSS, pH 7.4, containing DTPA (0.1 mM) and sodium phosphate (30 mM). Stock solution of SPER/NO was prepared in 1 mM NaOH. The pH of the solution after the addition of all reagents was 7.4–7.6.

Spin trapping experiments were performed by mixing all the components described in the figure legends to a final volume of 0.5 ml. The reaction was initiated by the addition of SPER/NO. Anaerobic experiments were performed by bubbling the solutions with N_2 for 15 min. Reaction mixtures were then transferred to a flat EPR quartz cell, fitted into the cavity of the spectrometer (Varian Associates E-109), and spectra were recorded 5 min after the addition of SPER/NO at 25 °C. Spectrometer settings were microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude,

1.0 G; sweep time, 12.5 G min^{-1}; and response time, 1 s. Receiver gain for each experiment is presented in the figure legends.

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