Role of ergothioneine on *S*-nitrosoglutathione catabolism

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Ergothioneine (ESH) is a low-molecular-mass thiol present in millimolar concentrations in a limited number of tissues, including erythrocytes, kidney, seminal fluid and liver; however, its biological function is still unclear. In the present study we investigated the role of ESH in the catabolism of *S*-nitrosoglutathione (GSNO). The results show that: (1) GSNO decomposition is strongly influenced by ESH ($k'' = 0.178 \pm 0.032\ M^{-1} \cdot s^{-1}$); (2) ammonia is the main nitrogen-containing compound generated by the reaction; and (3) nitrite is practically absent under both aerobic and anaerobic conditions. These findings are markedly different from those reported for the GSH-induced decomposition of GSNO, in which the nitrogen-containing end products are nitrite, ammonia and nitrous oxide (N_2O) under aerobic conditions but nitrite, ammonia, nitric oxide (NO) and small quantities of hydroxylamine under anaerobic conditions. Considering the high concentration of ESH in specific cells, the reaction with GSNO should be considered as an important molecular event occurring in the cell.

Key words: ammonia, ergothioneine-glutathione disulphide, nitric oxide, thiols.

INTRODUCTION

The chemistry of *S*-nitrosothiols has recently been a topic of intense interest because of these compounds' multiple biological effects and the finding that intracellular thiol nitrosation can result from the exposure of thiol-containing peptide and proteins to nitric oxide (NO) [1]. In fact, it has been shown that *S*-nitrosothiols, in addition to being potent vasodilators [2] and inhibitors of platelet aggregation [3], have a fundamental role in the regulation of many biological processes, including neurotransmission and the cytotoxic actions of immune cells [4]. These activities are probably related to the ability of *S*-nitrosothiols to release NO or related nitrogen species. It is also known that the pharmacological activities of nitroglycerine and other nitrovasodilators, all of which are able to release NO, are associated with the formation of *S*-nitrosothiols [5,6]. Moreover, because *S*-nitrosothiols have a longer half-life than NO, it has been suggested that their formation and decomposition represent mechanisms for the storage and transport respectively of NO *in vivo* [7,8].

Transnitrosation between thiols and *S*-nitrosothiols, which probably occurs through a transfer of NO^+, has also been reported to be a regulatory mechanism of protein function [9–11]. In this regard, because GSH is the most represented cytoplasmic thiol, it has been suggested that the transfer of NO^+ could be essentially unidirectional, from *S*-nitrosothiols to GSH. Thus transnitrosation might represent the basis for a mechanism of 'repair' exerted on *S*-nitrosated proteins [12] by GSH, which can be considered an intracellular thermodynamic sink for these beneficial transnitrosation processes. It is also known that the decomposition of *S*-nitrosothiols is induced by UV radiation, metal ions (e.g. Cu^+ and Hg^{2+} ions) and thiols, through a complicated mechanism that nevertheless remains to be thoroughly investigated [13–19].

Ergothioneine (ESH) (2-mercaptohistidine trimethylbetaine) is an important low-molecular-mass thiol, which is principally present in millimolar concentrations in specific tissues such as erythrocytes, kidney, seminal fluid and liver [20]. Although ESH is present in various plant species, the existence of a biosynthetic pathway in animals has not been demonstrated; the presence of ESH in certain tissues is due mainly to intake in the diet. In fact, it has been shown recently that the uptake of ESH from the surrounding medium occurs in erythrocytes [21]. Despite the fact that a considerable amount of research has been conducted on ESH, its major biological functions are still unclear. It has been reported that it could be involved in the metabolism of iron, copper and zinc [22,23] and in the inhibition of metalloenzymes [24]. It has also been shown that ESH is a scavenger of singlet oxygen [25], hydroxyl radicals [26] and peroxyl radicals [27].

In the present study, we investigated the reaction between *S*-nitrosoglutathione (GSNO) and ESH under aerobic and anaerobic conditions as an additional aspect of the biochemistry of GSNO. The results show that ESH induces GSNO decomposition significantly faster than GSH, suggesting that it might be one of the molecules responsible for the high rate of GSNO decomposition observed in whole blood and, in particular, in the liver and kidney [28,29]. The present study offers an in-depth analysis of the interaction between GSNO and ESH, and it establishes that the ammonia produced by this reaction is the major nitrogen-containing end product.

MATERIALS AND METHODS

Chemicals and reagents

GSNO, ESH, GSH, diethylenetriaminepenta-acetic acid (DTPA), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), bathocuproine sodium disulphonate, H_3PO_4 and ^2H_2O containing

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepenta-acetic acid; ESH, ergothioneine; FAB, fast atom bombardment; GSNO, *S*-nitrosoglutathione; TSP, sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-1-propionate.

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0.75% (w/w) sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]-1-propionate (TSP) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Hydroxylamine hydrochloride was obtained from Carlo Erba (Milan, Italy). All solutions were prepared in 0.4 M phosphate buffer adjusted to pH 7.4 with a NaOH solution. Because nitrosothiols can be sensitive to the presence of metal ions, ultra-pure water was used for washing all glassware and for the preparation of solutions. Moreover, because metal ions can be present as a contaminant in the reagents used, 0.1 mM DTPA was added to all solutions as chelating agent. Fresh solutions of GSNO, ESH, and GSH were prepared and used instantly for each experiment.

Kinetic measurements

Kinetic measurements of the reaction between GSNO and ESH were taken with a Cary 3E spectrophotometer (Varian, Palo Alto, CA, U.S.A.) by following the disappearance of the *S*-nitrosothiol absorbance at 370 nm (ϵ_{370} 374 $M^{-1} \cdot cm^{-1}$). Samples were prepared in 1 ml matched quartz cuvettes by mixing appropriate volumes of 7 mM GSNO, 20 mM ESH and 0.4 M phosphate buffer (plus 0.1 mM DTPA) to a final volume of 1 ml and maintained in the dark. Spectra were recorded between 250 and 650 nm at 37 °C. Experiments were performed with a concentration of GSNO ranging from 0.5 to 4 mM and a concentration of ESH ranging from 1 to 4 mM. A fresh solution of GSNO was prepared immediately before each experiment; the concentration was evaluated by measuring A_{335} (ϵ_{335} 677 $M^{-1} \cdot cm^{-1}$). Decomposition rates were studied both in the presence and in the absence of DMPO (0.1 M). In the experiments with DMPO, a solution of DMPO in 0.4 M phosphate buffer was added at the beginning of the reaction. Reactions in the absence of oxygen were performed in cuvettes equipped with rubber septa. Solutions of GSNO and ESH were prepared separately in deoxygenated buffers, bubbled extensively with high-purity argon and then introduced into the cuvette with a gas-tight syringe.

Isolation and structural analysis of reaction end products

GSNO (3.7 mg, 15.6 mM) and ESH (5.4 mg, 32 mM) were combined in 0.7 ml of 0.4 M phosphate buffer (pH 7.4) plus DTPA (0.1 mM) at 37 °C for 24 h in the dark. The reaction mixture was purified by preparative TLC on LiChroprep RP-18 with methanol/water (2:8, v/v) as eluent. The collected fractions were freeze-dried and analysed by fast atom bombardment (FAB) MS (VG 7070EQ), ^{1}H-NMR spectroscopy (Gemini 300; Varian, Palo Alto, CA, U.S.A.) and UV spectrophotometry (Cary 3E; Varian, Palo Alto, CA, U.S.A.). ^{1}H-NMR spectra were recorded at 25 °C and 300 MHz under the following experimental conditions: 2 s of acquisition time, 57° pulse, 1 s of repetition time and 15 p.p.m. spectral width. Samples were dissolved in ^2H_2O containing 7.5 g/kg TSP, which was used as the chemical shift reference (0.0 p.p.m.). Chemical shifts were measured in p.p.m.; coupling constants were measured in Hz.

Ammonia assay

The ammonia concentration was determined spectrophotometrically with a diagnostic kit produced by Sigma. The assay is based on the reductive amination of 2-oxoglutarate plus ammonia in glutamate by glutamate dehydrogenase and NADPH. The decrease in A_{340} due to the oxidation of NADPH is proportional to the ammonia concentration in the sample.

Hydroxylamine assay

The hydroxylamine concentration was determined by the indooxine assay [30]. In brief, 300 μl aliquots of incubate sample were added to 300 μl of 50 mM Na_2HPO_4, pH 6.8; 60 μl of trichloroacetic acid (12%, v/v) and 300 μl of quinoline [50% (v/v) in ethanol] were added to the mixture. After 2–3 min, 300 μl of Na_2CO_3 solution was added. The mixture was heated for approx. 5 min at 95 °C and then cooled at room temperature for approx. 1 h; A_{705} was measured. The hydroxylamine concentration was determined from a standard curve, calculated in the presence of the initial ESH concentration.

Nitrite assay

To determine the nitrite concentration, 200 μl of solution was incubated with 800 μl of Griess reagent [31]; A_{541} was read.

RESULTS

ESH-induced aerobic decomposition of GSNO

All kinetic measurements were performed at 370 nm because during the reaction between GSNO and ESH we observed an increase in A_{335} whose extent depended on the ESH concentration; this increase was due to the formation of oxidized ESH. The time course of the reaction between GSNO and ESH was followed in the presence of 0.1 mM DTPA, 0.5 mM DTPA, and 0.5 mM DTPA plus 50 μM bathocuproine sodium disulphonate. The results of the experiments showed that 0.1 mM DTPA was able to remove all of the metal ions present as contaminants in both the phosphate buffer and the GSNO and ESH preparations; it was thus possible to exclude the contribution of these ions to the observed decomposition of GSNO. Moreover, under these experimental conditions we observed that the GSNO solution [0.5 mM GSNO in 0.4 M phosphate buffer (pH 7.4)] without ESH was stable at 37 °C for 2 h of incubation, as shown in previous studies [14], whereas under the same experimental conditions but in the presence of ESH, GSNO decomposition occurred at a rate depending on the ESH concentration. Figure 1 shows the time course of the decomposition of 0.5 mM GSNO at five different ESH concentrations (range 1.0–4.0 mM). Figure 2(A) shows the logarithmic plot of the initial decomposition rates

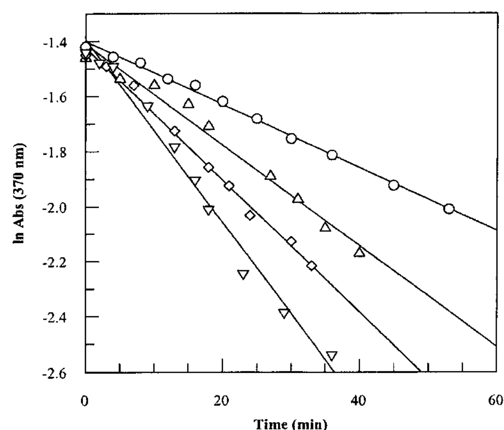

Figure 1 Time course of aerobic GSNO decomposition at four different ESH concentrations

The initial concentration of GSNO was 0.5 mM in 0.4 M phosphate buffer, pH 7.4, plus 0.1 mM DTPA. Symbols: ○, 1 mM ESH; △, 1.68 mM ESH; ◇, 2.92 mM ESH; ▽, 4 mM ESH.

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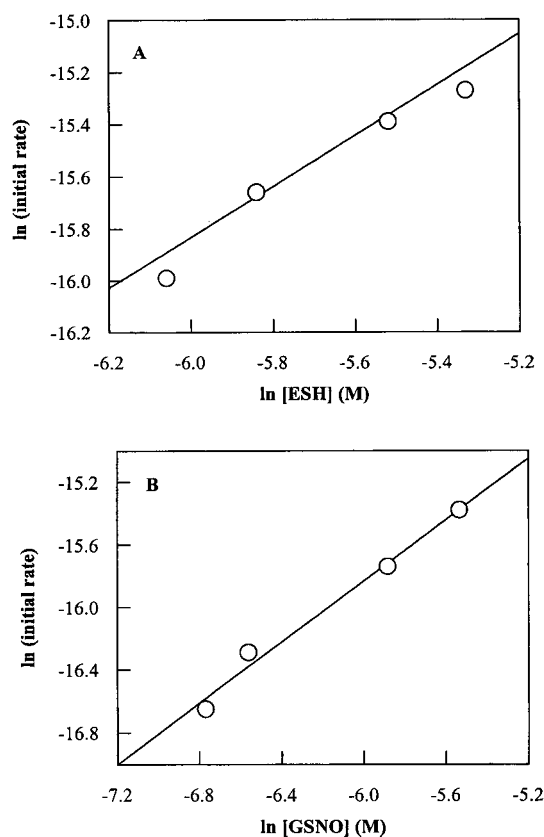

Figure 2 First-order dependence of reaction rate on ESH (A) and GSNO (B) concentrations

The initial ESH and GSNO concentrations (after mixing) were 0.3 mM and 0.6 mM respectively for each run; all runs were done in 0.4 M phosphate buffer, pH 7.4, plus 0.1 mM DTPA. Regressions: (**A**) $y = 0.9676x - 10.0743$, $r = 0.9851$; (**B**) $y = 0.9743x - 9.9869$, $r = 0.9931$.

obtained at a constant initial GSNO concentration (0.6 mM) and different ESH concentrations (range 2.33–4.85 mM). Figure 2(B) shows the analogous plot obtained at a constant initial ESH concentration (0.3 mM) and different GSNO concentrations (range 1.15–3.95 mM). In Figure 2(A) the slope value of the least-squares line was 0.9676; in Figure 2(B) it was 0.9743, demonstrating a first-order dependence for both reagents. Although it is possible to obtain an initial estimate of the second-order rate constant k'' from the y-intercept of the two least-square lines, a more accurate estimate was obtained by performing a parametric fit of the various reaction time courses observed at different concentrations of GSNO and ESH. The following equation, which was used to fit experimental data, describes the time-dependent concentration of GSNO (G_t) as a function of k'' and the initial concentrations of GSNO and ESH (G_0 and E_0 respectively):

$$G_t = \frac{G_0}{E_0}(E_0 - G_0)\frac{e^{-(E_0 - G_0)k''t}}{1 - e^{-(G_0/E_0)k''t}} \quad (1)$$

The equation was derived from the kinetic equation $v = -d[GSNO]/dt = k''[GSNO][ESH]$, by assuming a 1:1 stoichiometry between GSNO and ESH, and thus considering $G_t = G_0 - E_0 + E_t$.

As an example, the time course of the reaction between 0.5 mM GSNO and 2.5 mM ESH with the result of the fit is

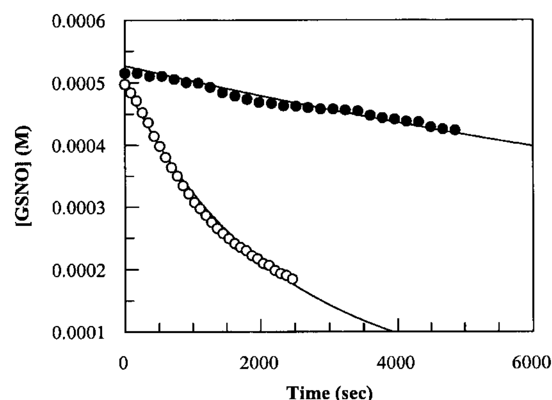

Figure 3 Time course of GSNO concentration in the presence of 2.5 mM ESH under aerobic (○) and anaerobic (●) conditions

Measurements were performed in 0.4 M phosphate buffer, pH 7.4, plus 0.1 mM DTPA.

reported in Figure 3. From the fits, a value of $0.178 \pm 0.032\ M^{-1} \cdot s^{-1}$ (means ± S.D.; $n = 7$) was calculated for k''.

ESH-induced anaerobic decomposition of GSNO

The role of oxygen in the reaction between GSNO and ESH was investigated by following the decomposition of GSNO at pH 7.4 in 0.4 M phosphate buffer in the presence of DTPA (0.1 mM) under anaerobic conditions. Figure 3 shows the time course of the reaction between GSNO (0.5 mM) and ESH (2.5 mM) in the presence (○) and in the absence (●) of oxygen, clearly showing that oxygen has a crucial role in the reaction rate. The time course of GSNO decomposition under anaerobic conditions was fitted with eqn (1); a value of $0.023 \pm 0.004\ M^{-1} \cdot s^{-1}$ ($n = 2$) was obtained for k'', which is approx. 8-fold lower than that measured in the same experimental conditions in the presence of oxygen. The influence of oxygen on the reaction rate between GSNO and GSH was also investigated: as found in other studies [18,19], it was affected by oxygen only slightly (results not shown).

Effect of DMPO on ESH-induced aerobic and anaerobic decomposition of GSNO

To investigate further the mechanism of ESH-induced GSNO decomposition, the effect of DMPO on the reaction was studied. DMPO is a spin-trapping agent that reacts rapidly ($k'' = 10^7\ M^{-1} \cdot s^{-1}$) with $RS^{\bullet}$ free radicals. Under aerobic conditions, a high concentration of DMPO (0.1 M) caused a considerable decrease in the ESH-induced GSNO decomposition rate ($k'' = 0.057 \pm 0.009\ M^{-1} \cdot s^{-1}$) ($n = 2$) (Table 1). Under anaerobic conditions, the decomposition rate in the presence of DMPO ($k'' = 0.040 \pm 0.007\ M^{-1} \cdot s^{-1}$; $n = 2$) was not significantly different from that obtained in the absence of DMPO. These results suggest that ESH-induced aerobic GSNO decomposition occurs through an $ES^{\bullet}$ intermediate, which could be easily formed owing to its stability deriving from aromatic electronic conjugation, in the presence of singlet oxygen [25].

NMR and MS spectroscopy characterization of end products

The reaction between GSNO and ESH produced the following three end products: GSSG, the mixed disulphide ESH-glutathione disulphide (GSSE) and oxidized ESH. The reaction mixture was prepared in 0.4 M phosphate buffer, pH 7.4, in

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Table 1 Second-order kinetic constants determined by fitting with eqn (1) the time course of decomposition of GSNO under four different experimental conditions

Results shown are means ± S.D.

Conditions	Second-order kinetic constant ($M^{-1} \cdot s^{-1}$)
Aerobic	0.178 ± 0.032 ($n = 7$)
Anaerobic	0.023 ± 0.004 ($n = 2$)
Aerobic + DMPO	0.057 ± 0.009 ($n = 2$)
Anaerobic + DMPO	0.040 ± 0.007 ($n = 2$)

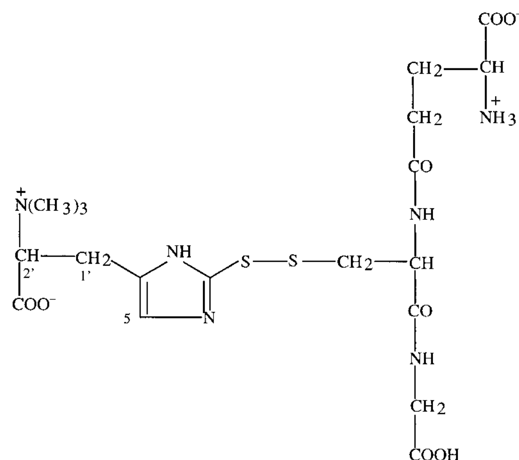

Figure 4 Structure of GSSE

the presence of 0.1 mM DTPA and incubated in the dark at 37 °C for 24 h. At the end of the reaction the products were isolated by preparative TLC, together with the excess of reduced ESH. GSSG and ESH were identified by direct comparison with authentic specimens and by the analysis of ^{1}H-NMR spectra. ESH was also analysed by FAB-MS ($[M+H]^+$ at m/z 230). Oxidized ESH was identified by FAB-MS ($[M+H]^+$ at m/z 457) and ^{1}H-NMR analysis. The observed ^{1}H-NMR signals were as follows: 3.22 (2H, m, H-B); 3.27 [9H, s, $N^+(CH_3)_3$]; 3.88 (1H, dd, J 11.2 and 4.7, H-C); 6.80 (1H, s, H-A). The mixed disulphide (Figure 4) (FAB-MS, $[M+H]^+$ at m/z 613) gave the following ^{1}H-NMR signals: 2.11 (2H, m, H-β-Glu); 2.47 (2H, t, J 7.4, H-γ-Glu); 3.11 (1H, dd, J 14.5 and 10.0, H_a-β-Cys); 3.25 (2H, m, H-1′); 3.34 [9H, s, $N^+(CH_3)_3$]; 3.42 (1H, dd, J 14.5 and 5.2, H_b-β-Cys); 3.74 (1H, m, H-α-Glu); 3.77 (2H, H-α-Gly); 3.89 (1H, dd, J 10.7 and 4.5, H-2′); 4.77 (1H, dd, J 10.0 and 5.2, H-α-Cys); 7.09 (1H, s, H-5).

Nitrogen-containing end products

GSNO (160 μM) and ESH (160 μM, 800 μM and 1.6 mM) were incubated at 37 °C under aerobic and anaerobic conditions in 0.4 M phosphate buffer, pH 7.4, in the presence of 0.1 mM DTPA for 24 h in the dark. Ammonia was found to be the major nitrogen-containing compound generated by the reaction (Table 2); its percentage increased with ESH concentration. The ammonia yield was slightly lower under anaerobic conditions than under aerobic conditions. Nitrite was also detected under both aerobic and anaerobic conditions but it accounted for only a negligible percentage (less than 1 %) of the initial nitrogen concentration. Hydroxylamine was not detected under either aerobic or anaerobic conditions.

DISCUSSION

S-Nitrosothiols have been defined as a novel class of signalling molecules that might act independently of homolytic cleavage to NO or be metabolized to other bioactive nitrogen oxides [1]. It has been demonstrated that the stability of *S*-nitrosothiols is affected by a variety of factors including light, pH of the medium, metal ions, oxygen and low-molecular-mass thiol concentration. In view of the possible physiological significance of the high intracellular GSH concentration, the reaction between GSNO and GSH has been studied extensively [18,19]. Here we have shown that ESH, another physiologically important low-molecular-mass thiol present in millimolar concentrations in a limited number of tissues and organs, influences GSNO stability. The reaction between GSNO and ESH was established to occur by overall second-order kinetics. The mean rate of GSNO decomposition induced by ESH under aerobic conditions was $0.178 \pm 0.032\ M^{-1} \cdot s^{-1}$, which is more than 20-fold the reported rate of $0.0083\ M^{-1} \cdot s^{-1}$ induced by GSH under aerobic conditions [17]. The possible transnitrosation reaction needs further investigation because we were unable to detect *S*-nitrosoergothioneine.

Scheme 1 illustrates the chemical reaction that might occur. The first step of the reaction is assumed to be a nucleophilic attack on either the nitrogen (reaction 1) or the sulphur (reaction 2) of the *S*-nitroso group. Although the anionic form of ESH is present at a high percentage only at pH 10.75 (pK_{a3}) [32], it has

Table 2 Percentages of nitrogen-containing compounds and GSNO unreacted (determined from the simulation) after 24 h from the reaction of GSNO (160 μM) with ESH under aerobic and anaerobic conditions

Amounts of GSNO shown are those still present in the reaction mixture, as calculated from the simulation. Percentages were calculated with respect to initial GSNO concentration. Abbreviation: n.d., not detected.

			Amount remaining (% of initial GSNO)					
			Aerobic conditions			Anaerobic conditions		
ESH (μM)	ESH-to-GSNO ratio	Nitrogen-containing compound …	NH_3	Nitrite	GSNO	NH_3	Nitrite	GSNO
160	1:1		59 ± 5 ($n = 4$)	0.3	29	43 ± 11 ($n = 2$)	n.d.	71
800	5:1		77 ± 6 ($n = 4$)	0.3	0	67 ± 2 ($n = 2$)	n.d.	24
1600	10:1		87 ± 14 ($n = 3$)	0.6	0	76 ± 2 ($n = 2$)	0.8	6

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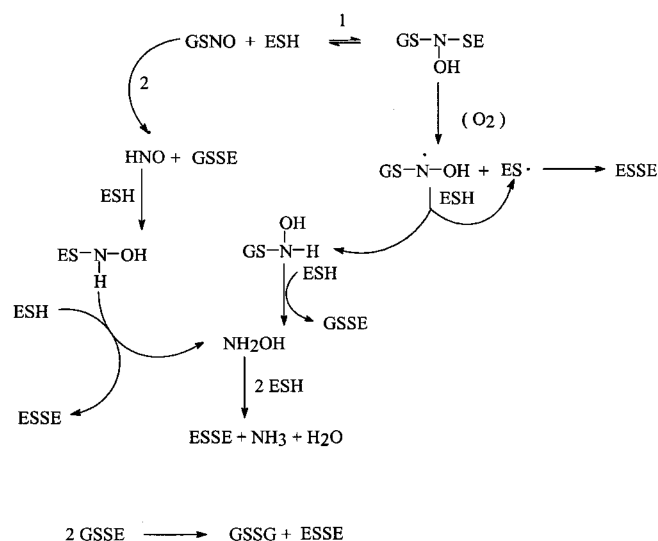

Scheme 1 Reactions of GSNO with ESH under aerobic and anaerobic conditions

been demonstrated that ESH also possesses considerable nucleophilic character in its neutral thiol and thione forms [33]. The attack of ESH on the sulphur atom of GSNO should lead to the formation of the mixed disulphide GSSE and nitroxyl (HNO), as already proposed by Wong et al. for the reaction between GSNO and GSH [19]. The attack on the nitrogen atom should lead to the production of the *N*-hydroxysulphenamide intermediate, proposed by Singh et al. as a possible intermediate in the reaction between GSNO and GSH [18]. This intermediate should decompose through the homolytic cleavage of the sulphur–nitrogen bond, giving rise to the formation of two radicals, one of which corresponds to the ESH radical, which is highly stable because of aromatic electronic conjugation. Consistent with such a mechanism was our finding that oxidized ESH, the mixed disulphide GSSE and GSSG were the main final products of the reaction. As far as the fate of the nitrogen is concerned, we found that after 24 h the major nitrogen-containing end product was ammonia under both aerobic and anaerobic conditions. The results in Table 2 show that the ammonia determined after 24 h increases with ESH concentration under both aerobic and anaerobic conditions. When GSNO and GSH were reacted in a 1:1 ratio, the ammonia concentration (measured at 22, 32 and 46 h) increased from 66 % at 22 h to 84 % at 46 h. These results suggest that the dependence of the ammonia concentration after 24 h on the ESH-to-GSNO ratio is due to a different extent of GSNO decomposition because of different reaction rates. In fact, when simulating GSNO decomposition considering the initial GSNO and ESH concentrations and the value obtained for k'', after 24 h under aerobic conditions and with a 1:1 ratio, 29 % of GSNO was still present in the reaction mixture. The reaction between GSNO and ESH also produced nitrite, but only in a negligible amount (less than 1 %). Under our experimental conditions, i.e. 24 h of incubation of 0.5 μM nitrite and 2.8 mM ESH in 0.4 M phosphate buffer, pH 7.4, plus 0.1 mM DTPA, nitrite was not reduced to ammonia by ESH; neither could ESH-induced reduction of nitrite to NO justify the very low percentage of nitrite also found under aerobic conditions. In fact, as reported by Arduini et al. [34], although ESH inhibits the nitrite-induced oxidation of oxyhaemoglobin, this is not the result of a direct reaction between ESH and nitrite.

By contrast, hydroxylamine was reduced to ammonia under the same conditions, suggesting that ammonia could, in part, also be generated by the reduction of hydroxylamine. Thus the ammonia detected in our samples might have been the result of both direct ammonia production and hydroxylamine reduction. In this respect, the nitrogen-containing compounds produced by the reaction between GSNO and ESH differ from those produced by the reaction with GSH (i.e. nitrite, ammonia and nitrous oxide (N_2O) under aerobic conditions, and ammonia, NO and small quantities of hydroxylamine under anaerobic conditions) [18,19]. The effect of oxygen and the spin-trap DMPO on reaction rate was also studied. As expected, it was found that the decomposition rate decreased under anaerobic conditions and in the presence of spin-trapping. That is, if $RS^{\bullet}$ radicals are generated by the reaction between GSNO and ESH in the presence of oxygen, then they react rapidly with DMPO, with a decrease in the reaction rate. The finding that DMPO did not significantly influence the reaction rate under anaerobic conditions confirms that, in the absence of oxygen, the reaction proceeds preferentially through ionic intermediates instead of radical intermediates, by a slower pathway.

In conclusion, our results show that ESH has a greater effect than GSH on the stability of GSNO, through a reaction that differs from that proposed for GSH in terms of both kinetic behaviour and the nitrogen-containing compounds produced. We found that, in the presence of ESH, ammonia was the main nitrogen-containing end product, even under aerobic conditions. On the basis of the ability of ESH to drive the decomposition of R-SNO to ammonia without any significant presence of nitrite, we postulate that this molecule can act as a protective cellular agent in specific tissues.

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REFERENCES

1 Gaston, B. (1999) Nitric oxide and thiol groups. Biochim. Biophys. Acta **1411**, 323–333

2 Mathews, W. R. and Kerr, S. W. (1993) Biological activity of S-nitrosothiols: the role of nitric oxide. J. Pharmacol. Exp. Ther. **267**, 1529–1537

3 Simon, D. I., Stamler, J. S., Jaraki, O., Keaney, J. F., Osborne, J. A., Francis, S. A., Singel, D. J. and Loscalzo, J. (1993) Antiplatelet properties of protein S-nitrosothiols derived from nitric oxide and endothelium-derived relaxing factor. Arteroscler. Thromb. **13**, 791–799

4 Hobbs, A. J. and Ignarro, L. J. (1996) Nitric oxide–cyclic GMP signal transduction system. Methods Enzymol. **269**, 134–148

5 Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. J., Kadowitz, P. J. and Gruetter, C. A. (1981) Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. J. Pharmacol. Exp. Ther. **218**, 739–749

6 Stamler, J. S. and Loscalzo, J. (1991) The antithrombotic effects of organic nitrates. Trends Cardiovasc. Med. **1**, 346–353

7 Myers, P. R., Minor, Jr., R. L., Guerra, Jr., R., Bates, J. N. and Harrison, G. (1990) Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble *S*-nitrosocysteine than nitric oxide. Nature (London) **345**, 161–163

8 Girard, P. and Potier, P. (1993) NO, thiols and disulfides. FEBS Lett. **320**, 7–8

9 Liu, Z., Rudd, M. A., Freedman, J. E. and Loscalzo, J. (1998) S-transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. J. Pharmacol. Exp. Ther. **284**, 526–534

10 Meyer, D. J., Kramer, H., Ozer, N., Coles, B. and Ketterer, B. (1994) Kinetics and equilibria of S-nitrosothiol-thiol exchange between glutathione, cysteine, penicillamines and serum albumin. FEBS Lett. **345**, 177–180

11 Arnelle, D. R. and Stamler, J. (1995) NO^+, $NO^{\cdot}$, and NO^- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. Arch. Biochem. Biophys. **318**, 279–285

12 Singh, R. J., Hogg, N., Joseph, J. and Kalyanaraman, B. (1996) Mechanism of nitric oxide release from S-nitrosothiols. J. Biol. Chem. **271**, 18596–18603

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13 Singh, R. J., Hogg, N., Joseph, J. and Kalyanaraman, B. (1995) Photosensitized decomposition of S-nitrosothiols and 2-methyl-2-nitrosopropane. Possible use for site-directed nitric oxide production. FEBS Lett. **360**, 47–51
14 Gorren, A. C., Schrammel, A., Schmidt, K. and Mayer, B. (1996) Decomposition of S-nitrosoglutathione in the presence of copper ions and glutathione. Arch. Biochem. Biophys. **330**, 219–228
15 Williams, D. L. H. (1996) The mechanism of nitric oxide formation from S-nitrosothiols (thionitrites). Chem. Commun. 1085–1091
16 Butler, A. R. and Rhodes, P. (1997) Chemistry, analysis, and biological roles of S-nitrosothiols. Anal. Biochem. **249**, 1–9
17 Hogg, N., Singh, R. J. and Kalyanaraman, B. (1996) The role of glutathione in the transport and catabolism of nitric oxide. FEBS Lett. **382**, 223–228
18 Singh, S. P., Wishnok, J. S., Keshive, M., Deen, W. M. and Tannenbaum, S. R. (1996) The chemistry of the S-nitrosoglutathione/glutathione system. Proc. Natl. Acad. Sci. U.S.A. **93**, 14428–14433
19 Wong, P. S., Hyun, J., Fukuto, J. M., Shirota, F. N., DeMaster, E. G., Shoeman, D. W. and Nagasawa, H. T. (1998) Reaction between S-nitrosothiols and thiols: generation of nitroxyl (HNO) and subsequent chemistry. Biochemistry **37**, 5362–5371
20 Kaneko, I., Takeuchi, Y., Yamaoka, Y., Tanaka, Y., Fukuda, T., Fukumori, Y., Mayumi, T. and Hama, T. (1980) Quantitative determination of ergothioneine in plasma and tissues by TLC-densitometry. Chem. Pharm. Bull. **28**, 3093–3097
21 Mitsuyama, H. and May, J. M. (1999) Uptake and antioxidant effects of ergothioneine in human erythrocytes. Clin. Sci. **97**, 407–411
22 Hartman, P. E. (1990) Ergothioneine as antioxidant. Methods Enzymol. **186**, 310–318
23 Motohashi, N., Mori, I. and Sugiura, Y. (1976) Complexing of copper ion by ergothioneine. Chem. Pharm. Bull. **24**, 2364–2368
24 Hanlon, D. P. (1971) Interaction of ergothioneine with metal ions and metalloenzymes. J. Med. Chem. **14**, 1084–1087
25 Dahl, T. A., Midden, W. R. and Hartman, P. E. (1988) Some prevalent biomolecules as defenses against singlet oxygen damage. Photochem. Photobiol. **47**, 357–362
26 Akanmu, D., Cecchini, R., Aruoma, O. I. and Halliwell, B. (1991) The antioxidant action of ergothioneine. Arch. Biochem. Biophys. **288**, 10–16
27 Aruoma, O. I., Whiteman, M., England, T. G. and Halliwell, B. (1997) Antioxidant action of ergothioneine: assessment of its ability to scavenge peroxynitrite. Biochem. Biophys. Res. Commun. **231**, 389–391
28 Jourd'heuil, D., Hallen, K., Feelish, M. and Grisham, M. B. (2000) Dynamic state of S-nitrosothiols in human plasma and whole blood. Free Radical Biol. Med. **28**, 409–417
29 Kashiba-Iwatsuki, M., Kitoh, K., Kasahara, E., Yu, H., Nisikawa, M., Matsuo, M. and Inoue, M. (1997) Ascorbic acid and reducing agents regulate the fates and functions of S-nitrosothiols. J. Biochem. (Tokyo) **122**, 1208–1214
30 Magee, W. E. and Burris, R. H. (1954) Fixation of N_2 and utilization of combined nitrogen by *Nostoc muscorum*. Am. J. Bot. **41**, 777–782
31 Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal. Biochem. **126**, 131–138
32 Stanovnik, B. and Tisler, M. (1964) Dissociation constants and structure of ergothioneine. Anal. Biochem. **9**, 68–74
33 Carlsson, J., Kierstan, M. P. J. and Brocklehurst, K. (1974) Reactions of L-ergothioneine and some other aminothiones with 2,2′- and 4,4′-dipyridyl disulphides and of L-ergothioneine with iodoacetamide. 2-Mercaptoimidazoles, 2- and 4-thiopyridones, thiourea and thioacetamide as highly reactive neutral sulphur nucleophils. Biochem. J. **139**, 221–235
34 Arduini, A., Mancinelli, G., Radatti, G. L., Hochstein, P. and Cadenas, E. (1992) Possible mechanism of inhibition of nitrite-induced oxidation of oxyhemoglobin by ergothioneine and uric acid. Arch. Biochem. Biophys. **294**, 398–402

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