Role of ergothioneine on S-nitrosoglutathione catabolism

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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 thiols 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 neuro-transmission 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. Cu2+ and Hg2+) 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), S,5-dimethyl-1-pyrroline N-oxide (DMPO), bathocuproine sodium disulphinate, H3PO4 and 3H2O 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)-(2,2,3,3-2H4)-1-propionate.

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0.75 % (w/w) sodium 3-(trimethylsilyl)-2,2,3,3-d_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 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 the ammonia concentration in the sample. 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.

**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), 1H-NMR spectroscopy (Gemini 300; Varian, Palo Alto, CA, U.S.A.) and UV spectrophotometry (Cary 3E; Varian, Palo Alto, CA, U.S.A.). 1H-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 D_2_0 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.

**Hydroxylamine assay**

The hydroxylamine concentration was determined by the indo-oxine assay [30]. In brief, 300 μl aliquots of incubate sample were added to 300 μl of 50 mM Na_2_ HPO_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_2_ CO_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_434 was measured. The hydroxylamine concentration was determined from a standard curve, calculated in the presence of the initial ESH concentration.

**Nitrile 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](image)

The initial concentration of GSNO was 0.5 mM in 0.4 M phosphate buffer, pH 7.4, plus 0.1 mM DTPA. Symbols: O, 1 mM ESH; A, 1.68 mM ESH; , 2.92 mM ESH; , 4 mM ESH.
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 ($G_i$) as a function of $k''$ and the initial concentrations of GSNO and ESH ($G_0$ and $E_0$ respectively):

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

The equation was derived from the kinetic equation $v = -d[\text{GSNO}]/dt = k''[\text{GSNO}][\text{ESH}]$, by assuming a 1:1 stoichiometry between GSNO and ESH, and thus considering $G_i = G_0 - E_0$. 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 reported in Figure 3. From the fits, a value of $0.178 \pm 0.032 \text{ M}^{-1} \text{s}^{-1}$ (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 $0.023 \pm 0.004 \text{ M}^{-1} \text{s}^{-1}$ ($n = 2$) 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 ($k'' = 10^6 \text{ M}^{-1} \text{s}^{-1}$) with $\text{ES}^\cdot$ free radicals. Under aerobic conditions, a high concentration of DMPO (0.1 M) caused a considerable decrease in the ESH-induced GSNO decomposition rate ($k'' = 0.057 \pm 0.009 \text{ M}^{-1} \text{s}^{-1}$) ($n = 2$) (Table 1). Under anaerobic conditions, the decomposition rate in the presence of DMPO ($k'' = 0.040 \pm 0.007 \text{ M}^{-1} \text{s}^{-1}$; $n = 2$) was not significantly different from that obtained in the absence of DMPO. These results suggest that ESH-induced aerobic GSNO decomposition occurs through an $\text{ES}^\cdot$ 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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**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) $y = 0.9743x - 9.9869$, $r = 0.9931$.

**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.
Anaerobic conditions

NMR spectra. ESH was also analysed by FAB-MS \((\text{A})\). The mixed disulphide (Figure 4) \((\text{FAB-MS, N})\) gave the following "\(\text{A} \)"

\[ \text{GSSG and ESH were identified by direct comparison with authentic specimens and by the analysis of reduced ESH. GSSG and ESH were isolated by preparative TLC, together with the excess of unreacted (determined from the simulation) after 24 h from the reaction of GSNO (160 \(\mu\)M) and ESH (160 \(\mu\)M, 800 \(\mu\)M and 1.6 mM) were incubated at 37 \(^\circ\)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\(\text{GSH}\) concentration, the reaction between GSNO and\(\text{GSH}\) 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 \(0.178 \pm 0.032 \text{ M}^{-1} \cdot \text{s}^{-1}\), which is more than 20-fold the reported rate of \(0.0083 \text{ M}^{-1} \cdot \text{s}^{-1}\) induced by \(\text{GSH}\) under aerobic conditions [17]. The possible nitrosation reaction needs further investigation because we were unable to detect \(\text{S-nitrosoglutathione}\).

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 (\(pK_a\) [32], it has

![Figure 4 Structure of GSSE](image)

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<table>
<thead>
<tr>
<th>Conditions</th>
<th>Second-order kinetic constant (M(^{-1}) \cdot s(^{-1}))</th>
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<tbody>
<tr>
<td>Aerobic</td>
<td>0.178 \pm 0.032 ((n = 7))</td>
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<tr>
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<tr>
<td>Aerobic + DMPO</td>
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<tr>
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<td>0.040 \pm 0.007 ((n = 2))</td>
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![Table 2 Percentages of nitrogen-containing compounds and GSNO unreacted (determined from the simulation) after 24 h from the reaction of GSNO (160 \(\mu\)M) with ESH under aerobic and anaerobic conditions](image)

| Amount remaining (% of initial GSNO) | | | | |
|--------------------------------------|---------------------------------|----------------|----------------|
| **Aerobic**                          | **Anaerobic**                   | **Aerobic**   | **Anaerobic**  |
| ESH (\(\mu\)M)                       | ESH-to-GSNO ratio               | Nitrogen-containing ... | Nitrite | GSNO | Nitrogen-containing ... | Nitrite | GSNO |
| 160                                  | 1:1                             | NH\(_3\)       | 59 \(\pm\) 5 (\(n = 4\)) | 0.3 | 29 | 43 \(\pm\) 11 (\(n = 2\)) | n.d. | 71 |
| 800                                  | 5:1                             | NH\(_3\)       | 77 \(\pm\) 6 (\(n = 4\)) | 0.3 | 0 | 67 \(\pm\) 2 (\(n = 2\)) | n.d. | 24 |
| 1600                                 | 10:1                            | NH\(_3\)       | 87 \(\pm\) 14 (\(n = 3\)) | 0.6 | 0 | 76 \(\pm\) 2 (\(n = 2\)) | 0.8 | 6 |

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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 nitroxylnitrite (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 increased 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' 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