Metabolism of S-nitrosoglutathione in intact mitochondria

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S-nitrosation of protein thiol groups by nitric oxide (NO•) is a widely recognized protein modification. Only few intracellular S-nitrosated proteins have been identified and it has been reported that S-nitrosation/denitrosation can serve as a regulatory process in signal-transduction pathways. Given the potential physiological importance of S-nitrosothiols, and considering that mitochondria are endowed with high levels of thiols and the biochemical requisites for synthesizing NO•, we examined the occurrence of S-nitrosoglutathione (GSNO) in intact, coupled rat liver mitochondria. These organelles contained 0.34 nmol of GSNO/mg of protein, detected by HPLC with UV–visible and electrochemical detections. This concentration was dynamically modulated by the availability of NO•; its decay was affected mainly by GSH and superoxide dismutase in a reaction that entailed the generation of GSSG. On the basis of the relatively long half-life of GSNO and the negligible recovery of NO• during

its decay, roles for GSNO as a storage and transport molecule for NO• are discussed. Moreover, the formation of GSNO and its reaction with GSH can be considered to be partly responsible for the catabolism of NO• via a complex mechanism that might result in the formation of hydroxylamine, nitrite or nitrous oxide depending upon the availability of oxygen, superoxide dismutase and glutathione. Finally, the high concentrations of GSH in the cytosol and mitochondria might favour the formation of GSNO by reacting with NO• ‘in excess’, thereby avoiding damaging side reactions (such as peroxynitrite formation), and facilitate the inactivation of NO• by generating other nitrogen-related species without the chemical properties characteristic of NO•.

Key words: nitric oxide, nitric oxide synthase, nitrosothiols, oxygen radicals, transnitrosation.

INTRODUCTION

Nitric oxide (NO•) is a gaseous free radical that has been used since the 1940s as a biochemical probe for haemoprotein binding [1]. However, it was only after the discovery of the L-arginine–nitric oxide pathway that studies on the role of NO• in different biological systems acquired definite biological significance [2,3]. NO• is involved in several, relevant pathophysiological processes such as vasodilation, immune regulation and neurotransmission [2,3]. At the subcellular level it has now been established that short-term exposure to physiological concentrations of NO• rapidly inhibits cytochrome *c* oxidase (Complex IV) in a reversible manner that is competitive with oxygen [4,5]. The relevance of this process has been demonstrated by considering NO• as a physiological regulator of cell respiration and ATP production in isolated mitochondria [6–8] and intact cells [9]. This modulation of respiration has been attributed to the production of NO• by a mitochondrial nitric oxide synthase (NOS) [6–8,10] immunolocalized in these organelles [11]. This enzyme is expressed constitutively, bound to the inner membrane, activated by cofactors required for a NOS activity [6,7] and activated by Ca²⁺ and calmodulin [7,10].

The multiple effects mediated by NO• are essentially based on the chemical properties of this molecule: NO• can form metal–NO• adduct complexes, and can react with other radicals, for example in the production of peroxynitrite (ONOO[−]) from the combination of NO• with superoxide (O₂^{•−}) anion [12,13]. The interaction of NO• with thiol-containing biomolecules has gained considerable importance. A growing body of evidence indicates the relevance of S-nitrosation of peptides and proteins in a variety of biological systems that either produce or were exposed

to NO•. Recent studies demonstrated the inhibition or modulation of important pathways such as apoptosis through the S-nitrosation of proteins [14]. The function of ion channels, G-proteins, respiratory proteins, transcription factors and multiple enzymes can be altered by S-nitrosation ([14] and references therein). The finding that protein modification by nitrosation can be dynamically regulated and, in certain cases, coupled to cell-surface signals has potential implications for other signalling pathways and cellular control mechanisms.

In this regard, the actions of the endothelium-derived relaxing factor were revisited, concluding that they more closely resemble a low-molecular-mass nitrosothiol than NO• itself [15]. S-Nitrosothiols or the delivery of NO• to certain targets might have advantages over a direct production of NO•; some of them might have a relatively longer half-life than that of NO•, facilitating the transport to other cellular sites different from that in which NO• was originally produced, and transient storage of NO• when this species is produced in excess, minimizing adverse effects. However, considering that low-molecular-mass S-nitrosothiols [e.g. S-nitrosocysteine, S-nitrosoglutathione (GSNO)] have a short half-life in systems *in vitro*, it still remains to be proved whether they can be produced under conditions of endogenous NO• production and whether the S-nitrosothiols formed are stable enough to elicit an effect in mitochondria or at other cellular sites.

In the present study we therefore sought to demonstrate first the occurrence of GSNO in mitochondria given that mitochondria are a source of NO• [6–8,10,11], secondly, that NO• produced by intact, coupled mitochondria contributes to the S-nitrosation of this peptide, and third, the mechanism underlying its catabolism. GSNO is probably the most relevant biological

Abbreviations used: GSNO, S-nitrosoglutathione; L-NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; SOD, superoxide dismutase.

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molecule to perform nitrosation reactions under physiological conditions [16–18]; the study of its metabolism is therefore relevant to a full understanding of its effect on mitochondrial and cellular metabolism.

MATERIALS AND METHODS

Chemicals and biochemicals

EDTA, EGTA, sodium succinate, sodium malate, sodium glutamate, mannitol, sucrose, Hepes, BSA (fatty-acid-free), L-arginine, *N*^G-monomethyl-L-arginine (L-NMMA), GSH, GSSG, cysteine and cystine were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Oxymyoglobin was obtained from horse heart metmyoglobin as described previously [6]. Other reagents were of analytical grade.

Biological materials

Isolation of mitochondria

Liver mitochondria were isolated from adult Wistar rats by differential centrifugation and purified through Percoll centrifugation [6]. This procedure yielded intact mitochondria minimally contaminated with other subcellular compartments [6]. Essentially, male rats (180–220 g body weight) were anaesthetized in a CO₂ chamber. The livers were quickly removed, washed with 0.22 M mannitol/70 mM sucrose/0.5 mM EGTA/2 mM Hepes/0.1 % defatted BSA (pH 7.4) (buffer A) and homogenized in a buffer-to-liver ratio of 10:1 (v/w). Large cell debris and nuclei were pelleted by centrifuging at 600 *g* for 5 min in a Sorvall SS34 rotor. This supernatant was filtered through two layers of cheesecloth to remove fat. Mitochondria were pelleted by centrifuging the supernatant for 10 min at 10 300 *g* in the same rotor. After suspension of the pellet in 5 ml of the previous buffer, the mitochondrial fraction was loaded on 20 ml of 30 % (v/v) Percoll/0.225 M mannitol/1 mM EGTA/25 mM Hepes/0.1 % defatted BSA (pH 7.4) and centrifuged for 30 min at 95 000 *g* in a Beckman 60Ti rotor. Mitochondria were collected from the lowest band and washed twice with buffer A by centrifugation for 10 min at 6300 *g*, then finally washed with 0.15 M KCl. Mitochondrial pellets were gently suspended in a small volume of ice-cold buffer A. The respiratory control and P-to-O ratios of these purified mitochondria were 6.5 ± 0.2 and 2.8 ± 0.1 (means $\pm$ S.E.M.) respectively, assessed in 0.225 M sucrose/5 mM MgCl₂/20 mM KCl/10 mM potassium phosphate/20 mM Hepes/KOH (pH 7.4) (reaction medium) supplemented with 10 mM succinate and 0.25 mM ADP.

Incubation conditions

Coupled mitochondria (30–50 mg/ml protein) were incubated in 2.5 ml of reaction medium plus 10 mM malate/glutamate and, where indicated, 0.1 mM of L-arginine or L-NMMA, at room temperature with constant stirring. At different time points aliquots were withdrawn for analysis of the levels of GSH and GSSG and of GSNO.

Biochemical analyses

Oxygen consumption

The oxygen uptake of mitochondria was measured with a Clark-type O₂ electrode from Hansatech (King's Lynn, Norfolk, U.K.) at 30 °C [6,7]. Intact, purified mitochondria (0.5–2 mg/ml protein) were maintained in 1 ml of reaction medium in the presence of 10 mM malate/glutamate as substrate; where indicated, different amounts of L-Arg or L-NMMA were added.

Evaluation of NOS activity: oxymyoglobin spectrophotometric assay

The production of NO[•] by mitochondria (1–2 mg/ml) was evaluated as the change in *A*_{581–592 nm} at 22 °C with a dual-wavelength, double-beam SLM-Aminco DW-2C UV–visible spectrophotometer. The sample cuvette contained 2 ml of reaction medium, 0.1–0.3 mM oxymyoglobin, 10 mM malate/glutamate and 0.1 mM L-arginine, under constant stirring. When L-NMMA was used in the incubation, the protein concentration was 3–5 mg/ml. Catalase (10 μM) and superoxide dismutase (SOD) were also present in the reaction medium to avoid non-specific side reactions (e.g. NO[•] with O₂^{•−}, oxymyoglobin with ONOO[−] or H₂O₂). The rates of NO[•] production were taken during the first 4–5 min when the ratios of myoglobin to catalase (in haem) to NO[•] were 28:6:1, thereby avoiding a significant inhibition of catalase by NO[•].

Measurement of L-citrulline

L-Citrulline was measured with a colorimetric assay essentially as described by Prescott and Jones [19].

Detection of GSH and GSSG

Intact, purified rat liver mitochondria (approx. 100 μl; 30–50 mg/ml protein) were treated with 0.5 ml of 1 mM γ-Glu-Glu (as internal standard)/1 mM bathophenanthroline disulphonate/10 % (v/v) HClO₄. The samples were centrifuged for 15 min at 3620 *g*. A 0.5 ml aliquot of the supernatant was treated with 50 μl of 10 mM iodoacetic acid in 0.2 mM *m*-Cresol Purple plus 0.5 ml of 2 M KOH/2.4 M KHCO₃. After 10 min at room temperature in the dark, 1 ml of 1 % (w/v) fluorodinitrobenzene in ethanol was added to the samples. The samples were maintained at 4 °C overnight to allow the reaction to proceed, then centrifuged at 28 384 *g* and passed through 0.22 μm pore size filters. The thiol derivatives were separated by HPLC with a 3-aminopropyl column [column specifications: YMC-Pack PA from Waters (Milford, MA, U.S.A.), 25 cm × 4.6 mm internal diam.; particle size 5 μm; pore size 120 Å] equipped with a guard column from Supelco[®] (Bellefonte, PA, U.S.A.) (1 cm × 4.6 mm internal diam., filled with Waters[®] Spherisorb 3-aminopropyl), eluted with gradient of mobile phases A [80 % (v/v) methanol] and B [0.5 M sodium acetate in 64 % (v/v) methanol filtered through 0.4 μm filters] essentially as described by Fariss and Reed [20]. The chromatography equipment consisted of a Class-VP Shimadzu HPLC system equipped with two high-pressure LC10ADvp pumps, an automatic injector SIL-10ADvp and an SPD-M10Avp diode array detector (215 and 365 nm). The chromatograms were analysed with the software provided with the system (version 5.03).

Detection of GSNO

Aliquots of the reactions were withdrawn at different time points. The aliquots were treated to avoid thiol oxidation or thiol–disulphide exchange, to terminate any metabolic process and to prevent any artifactual GSNO production. To satisfy these criteria, the samples were kept on ice protected from light for the duration of the procedure, promptly precipitated with either HClO₄ (methods I and II) or cold acetone (method III) in the presence of a metal chelator, desferrioxamine. Method I consisted of the treatment of 0.8 ml of sample with 20 μl of 10 mM desferrioxamine and 36 μl of 70 % (v/v) HClO₄. The samples were kept on ice for 10 min, then centrifuged at 28 384 *g* for 10 min at 4 °C. The supernatants were kept at −80 °C until HPLC analysis was performed. Method II consisted of the same precipitation protocol as that for method I except that the

supernatants were treated with 1 vol. of 0.15 M K_2HPO_4 /10 mM sodium citrate/5 mM EDTA [phosphate/citrate/EDTA (PCE) buffer]. The samples were kept on ice for 30 min and then centrifuged at 28 384 g for 15 min at 4 °C. The supernatants were kept at -80 °C until HPLC analysis. Method III consisted of a precipitation of the samples with cold acetone (9 vol. of acetone at -20 °C to 1 vol. of sample), followed by incubation on ice with occasional stirring for 30 min. The samples were centrifuged at 28 384 g at 4 °C for 10 min; the supernatants were freeze-dried with a SpeedVac (Savant). Dried samples were kept at -80 °C and were reconstituted with 100 μ l of mobile phase for HPLC analysis.

The samples obtained with any of these methods were processed immediately or stored at -80 °C for no more than 24 h because some degradation was noticeable after that time. The samples were passed through 0.2 μ m-pore-size filters before injection and analysed by HPLC with a method modified from Stamler and Feelisch [21]. The separation and detection of GSNO were performed with a C_{18} Aqua HPLC column (column specifications: 250 mm $\times$ 4.6 mm internal diam.; particle size 5 μ m; Phenomenex[®], Torrance, CA, U.S.A.); connected with a SecurityGuard[™] column from the same manufacturer (4 mm $\times$ 3 mm C_{18} cartridge). The various components were eluted with an isocratic gradient of 99% (v/v) 0.1 M monochloroacetic acid/0.125 mM EDTA/1.5 mM sodium octyl sulphate (pH 2.8)/1% (v/v) acetonitrile. The mobile phase was purged with helium; the flow rate was set at 0.8 ml/min. The peaks were monitored by a photodiode array detector (215 and 336 nm) and an electrochemical detector (L-ECD-6A from Shimadzu set at -300 mV) connected in series. The identification of the derivatives was performed by comparison with synthetic GSNO. GSNO was prepared by the method of Hart [22] by the acid-catalysed nitrosation of GSH (elemental analysis: found: C, 34.5; H, 4.9; N, 15.2; S, 9.0; H_2O , 0.94; $C_{10}H_{16}N_4O_7S.H_2O$ requires C, 34; H, 5.1; N, 15.8; S, 9.1; H_2O , 5.1%). Fresh solutions of GSNO were prepared immediately before each experiment; the concentrations were confirmed spectrophotometrically at 334 nm.

Liquid chromatography with MS detection

The characterization of GSNO was done with a Finnigan-MAT (San Jose, CA, U.S.A.) model TSQ-700 triple-quadrupole mass spectrometer equipped with a Finnigan-MAT electrospray source and interfaced to a Beckman Instruments (Fullerton, CA, U.S.A.) System Gold liquid chromatograph which had been modified for low eluant-flow operation. A Phenomenex Aqua column (C_{18} , 1 mm $\times$ 75 mm, particle size 3 μ m) was used for all analyte separations. The eluant used for the characterization of GSNO was A [0.5% (v/v) methanol/0.1% (v/v) formic acid] and B [50% (v/v) methanol/0.1% (v/v) formic acid], flowing at 40 μ l/min from 1% (v/v) B (held for 5 min) to 70% (v/v) B (in 10 min). A sheath liquid [80% (v/v) methanol/0.1% (v/v) formic acid, 20 μ l/min] was used along with a sheath gas (N_2 at 35 ml/min) to enhance ion formation. The electrospray needle was held at 3.5 kV and the capillary lens was at 135 °C. The TSQ-700 was used in the Q3 scanning mode (60–560 Da/s). The sample injection volume was 10 μ l.

Evaluation of H_2O_2 production

The rate of H_2O_2 production was evaluated with horseradish peroxidase and with *p*-hydroxyphenylacetic acid as electron donor. The fluorescence of the dimer was measured in a Shimadzu RF-10 spectrofluorometer. The reaction mixture contained 1 mM GSNO in 2 ml of PBS. Aliquots were taken at timed intervals

and H_2O_2 was measured by adding 1 ml of the reaction buffer (5 units/ml horseradish peroxidase and 1 mM *p*-hydroxyphenylacetic acid in 0.1 M sodium phosphate, pH 7.4). Where indicated, 20 mM GSH and/or GSH and 10 μ M SOD were added to the samples and incubated for 30–60 min. Calibration curves of H_2O_2 were run with a standardized solution (ϵ_{240} 42.8 $M^{-1} \cdot cm^{-1}$) in the same buffer.

Protein determination

Protein concentration was determined by biuret assay [23] with the modifications introduced by Yonetani [24], with bovine serum albumin as standard.

Statistics

Data are expressed as mean $\pm$ S.E.M. for at least three independent experiments. Statistical analysis was performed by one-way analysis of variance.

RESULTS

Evidence for a nitrosoglutathione pool in mitochondria

Recent reports from our laboratory [6–8,25] and others [10,11] indicated the presence of a NOS in mitochondria. We hypothesized that part of the $NO^{\bullet}$ produced by this enzyme could be stored/scavenged by GSH as GSNO, given the high concentration of this thiol in mitochondria [26]. The occurrence of GSNO and other low-molecular-mass nitrosothiols was therefore analysed in intact mitochondria by HPLC with UV and electrochemical detections (see the Materials and methods section). Three different methods were used for the preparation of mitochondrial samples for HPLC analysis. These methods were assessed to identify the best in terms of avoidance of artifactual GSNO production, and recovery and reproducibility. Method I consisted of the $HClO_4$ precipitation of mitochondrial proteins, followed by the direct injection of an aliquot of the supernatant into the HPLC. Method II followed essentially method I with the addition of a neutralization step with PCE buffer. Method III consisted of the precipitation of proteins with cold (-20 °C) acetone.

Given that GSH in the presence of nitrite at acidic pH results in the formation of GSNO [22], and that both of these reactants are present in mitochondria, it was important to evaluate the contribution of this reaction to the putative pool of mitochondrial GSNO. The production of GSNO was therefore evaluated by the three methods described above, in the presence and absence of permeabilized mitochondria, supplemented with [GSH]/[$NaNO_2$] (18.5:1) (Table 1). This ratio was chosen because it matches the concentration of nitrite produced by L-arginine-supplemented mitochondria at 30 min of incubation (the rate of nitrite production by $NO^{\bullet}$ -producing mitochondria was 0.018 ± 0.003 nmol/min per mg of protein, evaluated by Griess's assay [27]), which was usually the last time point taken during our experiments. In the absence of mitochondria, the percentage of GSNO formed from GSH obtained with methods I and II was significantly higher (520-fold and 275-fold higher respectively) than that with method III (Table 1). These results can be explained considering the acidic pH that methods I and II use to precipitate proteins, favouring the nitrosation of GSH by $NaNO_2$ [22]. In the presence of mitochondria, the percentage of GSNO obtained from GSH decreased 27-fold when using methods I and II, whereas it remained constant with method III. The decrease in GSNO production under these conditions can be understood considering the reactivity of nitrite towards other substrates

Table 1 GSNO formed from GSH and nitrite detected by different preparation methods

Reaction mixtures containing 10 mM GSH and 0.6 mM NaNO₂ in reaction medium without (no mitochondria) or with 50 mg/ml protein from permeabilized rat liver mitochondria (with mitochondria) were processed by the three methods explained in the text. The amount of GSNO was quantified by HPLC as described in the Materials and methods section; 10 mM GSH is the concentration of thiol present in mitochondria. The concentration of nitrite was matched to that obtained after the incubation of L-arginine-supplemented mitochondria for 30 min (see the text).

Additions	Method ...	[GSNO]/[GSH] (%)		
		I	II	III
No mitochondria		10.4	5.5	0.02
With mitochondria		0.39	0.2	0.01

present in mitochondria, not necessarily GSH. These experiments indicated that method III was less prone to the production of artificial GSNO, whereas methods I and II exhibited some formation of GSNO, being 2-fold lower with the additional neutralization step.

The recovery of GSNO by each method was calculated by spiking mitochondria with a known amount of GSNO and following the same procedure as for the samples. The results obtained with the three methods are summarized in Table 2. The recovery of GSNO was best with method II, followed by methods I and III. Although method III did not show any artifactual

Table 2 Characteristics of the preparation methods for GSNO detection in intact mitochondria

Concentrations of GSNO shown were corrected for artifactual GSNO (from Table 1, with mitochondria) and for the corresponding recoveries.

Method	[GSNO] (nmol/mg of protein)	Recovery (%)
I	0.25 ± 0.05	51
II	0.34 ± 0.05	85
III	0.21 ± 0.08	23

Table 3 Levels of GSH, GSSG and GSNO in rat liver mitochondria

Species	Concentration (nmol/mg of protein)	Ratio (glutathione equivalents)
GSH	7.7 ± 0.5	1
GSSG	0.8 ± 0.2	5
GSNO	0.34 ± 0.03	23

GSNO, the recovery of GSNO from mitochondria was very low (especially considering the low pool of GSNO present in mitochondria) and in addition the HPLC chromatograms exhibited more peaks than those obtained with the other two methods; it was sometimes difficult to obtain good resolution. On the basis of our results we therefore concluded that method II represented a good compromise between artifactual GSNO production (0.2%; Table 1) and recovery (85%; Table 2), in addition to resulting in a more reliable method based on reproducibility when using these biological samples with low endogenous GSNO levels.

The analysis of mitochondrial samples with method II indicated the presence of three main peaks at 12, 20 and 22.5 min (Figure 1A, middle trace). The peak that was eluted at 22.5 min was identified as GSNO on the basis of the identical retention time to that of synthetic GSNO under analogous chromatographic conditions (Figure 1A), co-elution of the peak with GSNO co-injected with the sample (results not shown) and an identical absorption spectrum indicating that both compounds contained the same type of chromophore, an S-nitroso moiety (Figure 1B). Definite characterization was obtained from liquid chromatography/MS indicating that the peak observed with the mitochondrial samples had the same molecular ion and fragmentation pattern as synthetic GSNO (results not shown). The other two major peaks observed in the chromatogram were attributed to desferal (12 min), added to the samples to avoid metal-catalysed decay of GSNO, and GSSG (20 min). On the basis of the UV-visible absorption spectra, no other peak in these chromatograms was identified as a nitrosothiol.

The concentration of GSNO in mitochondria was calculated as 340 ± 30 pmol/mg of protein, estimated from a calibration

Figure 1 HPLC characterization of GSNO in mitochondria

(A) HPLC chromatograms of pure GSNO (top trace), or mitochondrial supernatants [obtained with mitochondria supplemented with L-arginine (middle trace) or L-NMMA (bottom trace)] after incubation for 1 h. Incubation of mitochondria with L-arginine and 1 mM oxyhaemoglobin resulted in a chromatogram similar to that obtained with L-NMMA (results not shown). The chromatograms were detected at 336 nm with a photodiode array detector or at -300 mV with an electrochemical detector (results not shown). Other experimental conditions were described in the text. (B) Absorption spectrum of the peak eluted at 22.5 min. The absorption spectrum was obtained by using a photodiode array detector. Data processing was performed with the software provided with the apparatus.

Figure 2 Effect of L-arginine on GSNO production

Intact mitochondria were incubated in reaction mixture without (○) or with L-arginine (●). The production of GSNO was detected in mitochondrial aliquots, processed by using method II and analysed by HPLC with UV detection.

curve with pure GSNO. Comparing levels of GSNO with those of other relevant thiol-containing low-molecular-mass compounds, the concentration of GSNO was 2.4-fold lower than that of GSSG and represented approx. 3–4% of total glutathione equivalents (Table 3).

Changes in GSNO concentration with time were followed in mitochondria incubated with L-arginine by using method II (Figure 2). Mitochondria were incubated with a respiratory substrate (to sustain the NADPH pool) and supplemented with L-arginine (a substrate of NOS) to promote NO[•] production. Enough Ca²⁺ was present in our reaction medium to activate the enzyme; this observation was confirmed by the lack of activation of NO[•] production by subsequent additions of exogenous Ca²⁺ (up to 30 μM) to the medium. In addition, the procedure followed to isolate mitochondria and the buffers used throughout our experiments did not comply with the conditions required for a minimum Ca²⁺ level, such as the use of EGTA/*N*-hydroxyethylethylenediaminetriacetic acid [28].

Although we did not expect to detect GSNO in mitochondria without supplying L-arginine, especially after a relatively lengthy isolation, a measurable amount of GSNO was detected in mitochondrial samples at zero time (Figure 2). This apparent discrepancy can be bridged by considering that GSNO could have been maintained by transnitrosation in which the NO[•] moiety was directly transferred from one thiol to another or that the decay of GSNO was halted or minimized by the conditions of isolation (low temperature, use of metal chelators and protection from light). The occurrence of the latter possibility was supported by the negligible production of GSNO in mitochondria incubated with either L-arginine and 1 mM oxymyoglobin (the latter as a scavenger of NO[•]), D-arginine (the isomer of L-arginine) or L-NMMA (Figure 1A, bottom trace, and Figure 2), suggesting that transnitrosation reactions might have a secondary role in the maintenance of the GSNO pool.

In the presence of L-Arg, the concentration of GSNO increased linearly with time from 0.34 to 0.81 nmol/mg of protein. The initial velocity was calculated as 14.7 pmol of GSNO/min per mg of protein (Figure 2). This velocity represented the rate of GSNO production stimulated by the endogenous production of NO[•]. These results indicated that the production of GSNO was associated with the endogenous formation of NO[•] by mito-

Figure 3 Decay of GSNO in mitochondria

The decay of GSNO in mitochondria incubated with L-NMMA was followed by HPLC with UV detection. The results were plotted on a semilogarithmic scale to show the pseudo-first-order kinetics. Other experimental details were as described in the Materials and methods section.

chondria. The slow production of GSNO observed under these conditions could have been attributed to two factors: first, that the generation of GSNO was not significant in kinetic terms, or second, that the formation of GSNO was limited by the amount of NO[•] available to drive this reaction. To test these possibilities, GSNO formation was evaluated in mitochondria supplemented with pure NO[•] gas, incubated in the absence of a respiratory substrate and KCN. These conditions precluded any type of reaction between NO[•] and the respiratory chain components, leaving enough NO[•] to react with GSH. Most of the NO[•] was recovered as GSNO (80–90%) after 30 min of incubation, indicating that the generation of GSNO in mitochondria was limited by the availability of NO[•].

Decay pathways of GSNO

The decay of GSNO was followed in mitochondria incubated with L-NMMA. The slow decay (3–5 pmol/min per mg of protein) was in contrast with the reported high lability of this compound under conditions *in vitro*. The decay followed first-order kinetics (Figure 3) with a rate constant of $1.2 \times 10^{-4} \text{ s}^{-1}$. This rate constant was double that obtained with a chemical system ($k_{\text{mitochondria}} = 0.012 \text{ M}^{-1} \cdot \text{s}^{-1}$, compared with $6.0 \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$; Table 4) consisting of GSNO and GSH at molar ratios relevant to this system (k in mitochondria was calculated from the experimental k' , with $[\text{GSH}] = 10 \text{ mM}$ ([26], and this study), and 35-fold that obtained with GSNO in PBS without GSH ($k = 3.4 \times 10^{-6} \text{ s}^{-1}$). These results agreed with those published previously in which the presence of thiols accelerated the decay of GSNO in the presence of metal chelators [29–31]. The addition of bathophenanthroline or bathocuproine to mitochondria did not significantly change the decay of GSNO, which excluded a role for a metal-catalysed reaction [32] under our experimental conditions. The lack of metal chelation effect on the decay of GSNO was in line with the limited (virtually absent) availability of free metal in cells owing to the presence of metallochaperones that transfer the corresponding metal by protein–protein interactions [33].

Published second-order rate constants [or recalculated from these papers (Table 4)] for the reaction of GSNO with GSH were 1.2–1.4-fold our experimental value obtained with identical

Table 4 Second-order rate constants for the decay of GSNO with GSH

Reaction	Rate constant ($M^{-1} \cdot s^{-1}$)	Reference
Decay of GSNO with GSH	$8.3 \times 10^{-3*}$	[29]
	$8.0 \times 10^{-3\dagger}$	[30]
	7.0×10^{-3}	[31]
	$6.0 \times 10^{-3\dagger}$	This study
	$10.0 \times 10^{-3}\S$	This study
Decay of GSNO in mitochondria	$12 \times 10^{-3}\parallel$	This study

* Calculated from Figure 5C in [29].

† Calculated from Figure 1 in [30].

‡ This rate constant was obtained by measuring the A_{334} of aliquots withdrawn from a reaction mixture containing 1 mM GSNO, various GSH concentrations (5–20 mM) and 0.1 mM desferal in PBS, pH 7.4. The reaction mixture was incubated in the dark at 25 °C.§ This rate constant was calculated as described in the previous footnote, except that the reaction mixture contained 10 μ M SOD.|| This rate constant was calculated from $k' = 1.2 \times 10^{-4} s^{-1}$.**Table 5** Changes in reduced glutathione and GSNO concentrations under different experimental conditions

Mitochondria were incubated with the corresponding compound (L-NMMA or L-arginine) and the concentrations of GSH and GSNO were analysed by HPLC every 10 min. The values are differences in concentrations between 0 and 30 min: production is positive; consumption is negative.

Species	L-NMMA	L-Arginine
GSH production (nmol/30 min per mg of protein)	−0.33	−0.53
GSNO production (nmol/30 min per mg of protein)	−0.13	+0.44
[GSH]/[GSNO]	2.5	1.2

reactant concentrations and pH (Table 4). This discrepancy can be explained by considering that our values were obtained by measuring GSNO in aliquots taken from a reaction mixture kept in the dark, whereas others were obtained under continuous irradiation, a condition that might have accelerated the decay of GSNO, given the photosensitivity of nitroso compounds.

Our results showed that the second-order rate constant for the decay of GSNO in mitochondria was double that obtained with a chemical system under similar conditions; however, when the reaction mixture of GSNO with GSH was supplemented with

10 μ M SOD (the concentration of SOD in rat liver mitochondria), the resulting rate constant was comparable to that obtained with intact mitochondria. These results indicated that the main catabolism of GSNO in mitochondria could be attributed to its reaction with GSH, further accelerated by the presence of SOD. Although the decay of GSNO was enhanced 1.7-fold by the presence of SOD and GSH, its decay was relatively slow. In fact, from the experimental decay rate constant, the half-life of GSNO was calculated as 101 min at 25 °C and 48 min at 37 °C. From the Einstein–Smoluchowski equation ($d^2 = 2Dt$, in which d is the root-mean-square distance, D is the diffusion coefficient for GSNO, and t is time), and assuming that the diffusion coefficient for GSNO is similar to those of other amino acids and small molecules (approx. $9 \times 10^{-6} cm^2/s$), then the average distance a GSNO molecule can diffuse in 1 s is approx. 40 μ m, which is comparable to the average size of four cells. Thus the relatively long lifetime (i.e. $1/k$) of GSNO results in a 6–30-fold larger diffusion distance than that of NO $\cdot$ [34], significantly increasing its expected target radius.

Previous studies reported that thiols decreased the release of NO $\cdot$ from GSNO, favouring the production of other products (NH $_3$, N $_2$ O, NH $_2$ OH, NO $_x$ [29–31]). In agreement with those results, the rate of NO $\cdot$ production in mitochondria (in the presence of L-NMMA or in the chemical system consisting of GSNO, GSH and SOD) represented a negligible fraction of the rate of GSNO decay. Singh et al. [30] proposed a complex mechanism for the reaction of GSNO with GSH, entailing homolytic and heterolytic pathways. To elucidate the main pathway that mitochondrial GSNO followed on reaction with GSH, the molar ratios of these species obtained from mitochondria incubated with either L-arginine or L-NMMA were evaluated (Table 5). The changes in GSNO and GSH concentrations were followed over 30 min; the concentrations were analysed by HPLC as described in the Materials and methods section. In the presence of L-NMMA, GSH was consumed in a 2.5:1 ratio to GSNO. Although the mechanism described by Singh et al. [30] was proposed for a simple, well-defined chemical system, our ratio was compatible with the homolytic decomposition of an intermediate [GS–N(GS)OH] formed on the reaction of GSH with GSNO (Scheme 1). Further experiments will be needed to confirm our modified mechanism or the occurrence of this mechanism in intact mitochondria. The original mechanism involved a complex chemistry [30] and suggested the participation of glutathionyl radicals (GS $\cdot$). According to our pathway, the fate of glutathionyl radicals will depend essentially on the

Scheme 1 Postulated reactions of GSNO with GSH in mitochondria

The scheme shows the mechanism postulated to occur in mitochondria (based on [30]).

Table 6 H₂O₂ production and oxygen consumption during the decay of GSNO in the presence of GSH and/or SOD

The rates of GSNO decay were followed at 334 nm in a Cary 1E spectrophotometer. The experimental conditions included (1) 1 mM GSNO, (2) 1 mM GSNO plus 20 mM GSH or (3) 1 mM GSNO plus 20 mM GSH and 10 μ M SOD in PBS. The rates of H₂O₂ were evaluated as described in the Materials and methods section. The oxygen consumption was followed with a Clark-type oxygen electrode.

Addition	Rate of production or consumption (μ M/min)		
	$-d[\text{GSNO}]/dt$	$+d[\text{H}_2\text{O}_2]/dt$	$-d[\text{O}_2]/dt$
GSNO	3.0 ± 0.7	0.18 ± 0.06	0.05 ± 0.01
GSNO + GSH	7.4 ± 0.2	0.67 ± 0.02	0.56 ± 0.05
GSNO + GSH + SOD	11 ± 1	1.21 ± 0.06	2.17 ± 0.06

availability of GSH, SOD and oxygen [30,35]; the involvement of GS $\cdot$ in our system might explain the enhanced rate of H₂O₂ production observed in a chemical system consisting of GSNO, GSH and SOD (Scheme 1 and Table 6).

In the presence of L-arginine, the concentration of GSH consumed was almost equimolar to that of GSNO formed, indicating that the reaction of NO $\cdot$ (or NO $\cdot$ -derived species) with thiols under aerobic conditions results in the formation of GSNO, possibly through oxidized nitrogen species such as NO₂ and N₂O₃ (Table 5); however, this ratio did not preclude a contribution from transnitrosation reactions between GSNO and other thiol-containing molecules. In all the experimental conditions indicated in Table 4, the only significant product was GSSG; no sulphinamide or sulphinic acid was detected by HPLC with UV-visible detection or with liquid chromatography/MS.

DISCUSSION

In this study we have identified for the first time the presence of GSNO in mitochondria. The concentration of this metabolite was similar to that of GSSG and was effectively modulated by endogenous NO $\cdot$. Other nitrosothiols were found to be endogenous products in different mammalian systems: extracellularly in plasma [36] and airway lining fluid [37], and intracellularly in neutrophils [38]. However, no studies reported the presence of GSNO in mitochondria or of the mechanism underlying its metabolism. Nitrosothiols are endowed with a variety of biological activities. These compounds apparently release NO $\cdot$ in most biological settings; they are therefore capable of eliciting some (if not all) NO $\cdot$ functions. However, this assumption has not been supported by other reports and our results [39,40]. Furthermore, nitrosothiols have been found to be effective nitrosating agents eliciting additional NO $\cdot$ -related responses mediated through interactions with thiol-containing targets. S-nitrosylation has been suggested to constitute a post-translational modification of proteins implicated in the regulation of important pathways.

In agreement with these reports and given our present results (decay rate constant, concentration of GSNO and release of NO $\cdot$), it seems reasonable to exclude a function for GSNO as a donor of 'free' NO $\cdot$, especially considering the high GSH concentration in mitochondria (and in most cellular settings) and SOD, which will favour the production of other final products, not necessarily NO $\cdot$. However, if we take into account its relatively long lifetime, even in the presence of high concentrations of GSH, GSNO could be considered a significant storage

and a transport molecule for NO $\cdot$ to fulfil its role in transnitrosation reactions.

The reactions of GSNO with thiols might have a significant role in NO $\cdot$ -mediated signal transduction pathways: the reaction of GSNO with GSH (or with other thiols with high pK_a) could be envisaged as the method of NO $\cdot$ inactivation, whereas the reaction of GSNO with other thiol-containing molecules, probably with lower pK_a values, might represent a way to activate or deactivate proteins by transnitrosation. The contribution of GSNO in either pathway will depend essentially on the concentration and chemical properties (net charge of neighbouring groups, steric hindrance, pK_a values of the thiol groups involved [41,42]) of reactants (GSH and other thiol-containing proteins) at a particular time and subcellular location.

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