S-Nitrosogluthathione as a substrate for γ -glutamyl transpeptidase

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S-Nitrosogluthathione (GSNO) has been used as a nitric oxide ($\cdot\text{NO}$) donor compound and has also been postulated to be involved in the transport of $\cdot\text{NO}$ *in vivo*. In this study we have examined the possibility that GSNO is a substrate for γ -glutamyl transpeptidase (γ -GT), an enzyme that hydrolyses the γ -glutamyl moiety of glutathione to give glutamate and cysteinylglycine. γ -GT accelerated the decomposition of GSNO, forming S-nitrosocysteinylglycine (CG-SNO) by a mechanism inhibitable by the γ -GT inhibitors acivicin and S-methylglutathione. The K_m of γ -GT for GSNO was found to be 28 μM . In the presence of contaminating transition metal ions, γ -GT

accelerated the release of $\cdot\text{NO}$ from GSNO, as CG-SNO is more susceptible to transition metal ion-dependent decomposition than GSNO. However, in the presence of the transition metal ion chelator diethylenetriaminepentaacetic acid, neither GSNO nor CG-SNO decomposed to generate $\cdot\text{NO}$. Neither S-methylglutathione nor acivicin affected the vasodilatory response to GSNO in an isolated perfused rat heart. However, rat kidney homogenate stimulated the decomposition of GSNO by an acivicin-inhibitable mechanism. It is likely therefore that γ -GT is involved in the decomposition of GSNO in the kidney but not in the heart.

INTRODUCTION

S-Nitrosogluthathione (GSNO) has been used as a nitric oxide ($\cdot\text{NO}$) donor compound in several studies (see, e.g., [1–4]). The mechanism of GSNO decomposition in chemical or biological systems is not completely understood. In general, S-nitrosothiols (RSNO), such as GSNO, are stable at 37 °C and pH 7.4 [5,6]. However, in the presence of contaminating transition metal ions (especially iron and copper) these compounds become unstable. Transition metal ions decompose RSNO catalytically, forming $\cdot\text{NO}$ and the corresponding disulphide [5–7]. It has been proposed that the thiyl radical ($\text{RS}\cdot$) is an intermediate in the biological decomposition of RSNO [8]. However, we have recently demonstrated that $\text{RS}\cdot$ is not formed as an intermediate during metal-ion-catalysed decomposition of RSNO [6]. In contrast, photolytic decomposition of RSNO gives rise to both $\cdot\text{NO}$ and $\text{RS}\cdot$ by homolytic cleavage of the sulphur–nitrogen bond [9].

GSNO elicits many physiological effects. It exhibits a potent anti-aggregatory effect on platelets while having minimal effects on vascular tone [4] and also improves the functional recovery of reperfused ischaemic rat heart [1]. Although GSNO is able to release $\cdot\text{NO}$ under some conditions [10], it has been postulated that the major mechanism of action involves the transfer of nitrosonium cation (NO^+) to other thiols, a process referred to as transnitrosation [3]. In the presence of thiols, GSNO is slowly reduced to nitroxyl anion (NO^-) [11]. In most studies where RSNO are used as a $\cdot\text{NO}$ source, neither the mechanism of S-nitrosothiol breakdown nor the product distribution have been established. Gordge et al. [12] have implicated the involvement of a membrane-bound copper protein in the GSNO-dependent inhibition of platelet aggregation. Recently it has been speculated that γ -glutamyl transpeptidase (γ -GT), an enzyme involved in glutathione metabolism [13], may be responsible for the decomposition of GSNO. It has been suggested that this enzyme may play an important role both in the effect of GSNO on platelet aggregation [14] and in the cytostatic activity of GSNO to *Salmonella typhimurium* [15].

In this study we have investigated the possibility that GSNO is a substrate for γ -GT. This enzyme hydrolyses the γ -glutamyl moiety of glutathione (GSH) to leave cysteinylglycine (CG-SH) [13]. The analogous reaction, using GSNO as the substrate, would leave S-nitrosocysteinylglycine (CG-SNO), as shown in Figure 1. We show that GSNO is a good substrate for γ -GT, and that γ -GT-dependent GSNO metabolism may be important in tissues, such as the kidney, where γ -GT levels are high.

EXPERIMENTAL

Materials

γ -GT (Type II, from bovine kidney), GSH, CG-SH, acivicin, desferrioxamine (DFO) and L- γ -glutamyl p-nitroanilidine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), sodium nitrite and diethylenetriaminepentaacetic acid (DTPA) were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). RSNO were synthesized using acidified nitrite as previously reported [16]. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl, a nitronyl nitroxide (NNO) was synthesized as previously described [17].

Measurements of γ -GT activity

γ -GT activity was determined by following the hydrolysis of L- γ -glutamyl p-nitroanilidine, as previously described [13]. Briefly, purified enzyme or tissue homogenate was incubated with L- γ -glutamyl p-nitroanilidine (1 mM) and glycylglycine (20 mM) in Tris/HCl (0.1 M, pH 8.0) at 37 °C and the increase in absorbance at 410 nm was monitored. Enzyme activity was determined using a molar absorption coefficient of 8800 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for formation of p-nitroanilidine.

HPLC determinations

The reaction of γ -GT and GSNO was quenched at various time points by the addition of trichloroacetic acid (10% v/v). After

Abbreviations used: GSNO, S-nitrosogluthathione; γ -GT, γ -glutamyl transpeptidase; CG-SNO, S-nitrosocysteinylglycine; RSNO, S-nitrosothiols; CG-SH, cysteinylglycine; GSH, glutathione; DFO, desferrioxamine; DTPA, diethylenetriaminepentaacetic acid; NNO, nitronyl nitroxide; KHB, Krebs–Henseleit buffer; INO, imino nitroxide.

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Figure 1 Proposed mechanism of γ -GT-dependent GSNO decomposition

centrifugation, samples were analysed by HPLC, performed on a Hewlett Packard HP1040 system (Palo Alto, CA, U.S.A.) using a Whatman C18 (5 μ m) column (25 cm $\times$ 5 mm) and a mobile phase of 0.05% (v/v) trifluoroacetic acid/methanol (94:6, v/v). Column effluent was monitored by absorbance at 336 nm. GSNO and CG-SNO were quantified by reference to authentic standards.

ESR studies

$\cdot$ NO production from RSNO was monitored by ESR, using NNO, on a Varian E-109 spectrometer employing 100 kHz field modulation [18].

Isolated heart perfusion

Male Sprague–Dawley rats (200–225 g body weight), maintained on a standard diet, were used for this study. Rats were anaesthetized with pentobarbital (60 mg/kg i.p.) and heparinized via the left femoral vein (250 units/kg). The heart was rapidly excised and placed in perfusion medium. Within 30 s, the aorta was attached to a stainless steel cannula, the pulmonary artery was incised to permit adequate drainage, and the heart was perfused normothermically by the method of Langendorff at a perfusion pressure equivalent to 12 kPa (90 mm Hg). The perfusion medium used was Krebs–Henseleit buffer (KHB) having the following composition in mM: NaCl, 118.5; NaHCO₃, 25.0; KCl, 4.8; MgSO₄·7H₂O, 1.2; KH₂PO₄, 1.2; glucose, 11.1; CaCl₂·2H₂O, 1.8 (pH 7.4 when gassed with 95% O₂ and 5% CO₂). During preparation of KHB, the solution was gassed with 5% CO₂ to prevent the precipitation of calcium [19].

Coronary vasodilation protocol

Isolated hearts were perfused with oxygenated KHB for a 20 min equilibration period. During this time, baseline measurements of coronary flow rate were monitored. GSNO (80 nM) was infused via a side arm directly above the site of cannulation of the heart, and the coronary vasodilation response was measured for 5 min.

DFO (50 μ M) and the γ -GT inhibitor S-methylglutathione (50 μ M) were delivered to the perfusion line 5 min before perfusion with GSNO. Acivicin, a slow, irreversible inhibitor of γ -GT, was injected (10 mg/kg i.p.) 2.5 h before excising the heart, as previously described [19].

Preparation of rat tissue homogenates

Kidneys were removed from control and acivicin-treated rats, diced, and immediately frozen in liquid nitrogen. After thawing, tissues were processed as described by Meister et al. [13]. Briefly, tissue was homogenized, by 10 passes of a Potter homogenizer, in 15 vol. of Tris/HCl (2 mM, pH 7.4) at 4 °C. Calcium chloride (10 mM) was added to the homogenate, which was stirred on ice for 10 min. The homogenate was centrifuged at 2500 g for 10 min and the supernatant was retained. Heart tissue homogenate was prepared by a similar protocol.

Statistical analysis

Significance was determined using Student's *t*-test incorporating the Bonferroni correction for multiple comparisons.

RESULTS

γ -GT-catalysed decomposition of GSNO

GSNO has a broad absorbance band that is centred at 336 nm (molar absorption coefficient = 770 M⁻¹·cm⁻¹) [2]. Figure 2 shows the effect of γ -GT on the stability of GSNO in Dulbecco's phosphate-buffered saline (PBS) at 37 °C. Incubation of GSNO in PBS resulted in a slight loss of absorbance, at 336 nm, over several hours. This absorbance change was inhibited by DTPA

Figure 2 Decomposition of GSNO by γ -GT

(A) GSNO (700 μ M) was incubated in PBS at 37 °C and the absorbance at 336 nm was monitored continuously. γ -GT (a, 1 unit/ml; b, 2 units/ml; c, 3 units/ml) was added where indicated by the arrow. (B) CG-SNO (1 mM) was incubated in PBS at 37 °C in the presence (a) and absence (b) of DTPA (100 μ M) and the absorbance at 336 nm was monitored continuously.

Figure 3 Kinetics of γ -GT-catalysed decomposition of GSNO

(A) GSNO (25 μM) was incubated at 37 $^{\circ}\text{C}$ with γ -GT (0.5 unit/ml) in the presence of DTPA (100 μM). Aliquots were taken at the indicated time points and separated by HPLC. GSNO and CG-SNO were quantified by reference to authentic standards. (B) Double-reciprocal plot of the initial rate of GSNO decomposition as a function of GSNO concentration. Linear regression analysis gave a K_m of 28 μM .

(results not shown), indicating that this decomposition of GSNO was due to contaminating transition metal ions [5]. Addition of γ -GT dramatically enhanced the decomposition of GSNO, as shown in Figure 2(A). The loss of absorbance cannot be attributed solely to hydrolysis of the γ -glutamyl residue of GSNO as the product, CG-SNO, has a very similar absorbance band centred at 330 nm (results not shown). However, as shown in Figure 2(B), CG-SNO is more susceptible to transition metal ion-dependent decomposition than GSNO (cf. Figure 2A). The presence of DTPA (Figure 2B) totally inhibited CG-SNO decomposition. Hence, the γ -GT-catalysed decomposition of GSNO represents both hydrolysis of GSNO to give CG-SNO and transition metal ion-dependent decomposition of CG-SNO. As it is unclear which process is rate limiting, direct spectrophotometric methods were deemed unsuitable for monitoring enzyme kinetics.

K_m of γ -GT for GSNO

In the presence of the transition metal ion chelator DTPA, γ -GT did not stimulate $\cdot\text{NO}$ formation from GSNO and had little effect on the change in absorbance at 336 nm (results not shown). However, under these conditions, the hydrolysis of the γ -glutamate residue of GSNO can be monitored by HPLC. Addition of γ -GT to GSNO resulted in the time-dependent

Figure 4 Inhibition of γ -GT-catalysed decomposition of GSNO by inhibitors of γ -GT

GSNO was incubated with γ -GT (1 unit/ml) in PBS at 37 $^{\circ}\text{C}$ in the presence of DTPA (100 μM) either alone (diamond) or in the presence of S -methylglutathione ($\blacktriangle$, 1 mM), γ -glutamyl-(α -carboxy)phenylhydrazine ($\circ$, 1 mM) or acivicin ($\bullet$, 1 mM). Where acivicin was used, the enzyme was pre-incubated with the inhibitor for 1 h before use. GSNO concentration was determined by HPLC.

decomposition of GSNO and the concomitant formation of CG-SNO. Quantification of the HPLC data revealed a stoichiometric conversion of GSNO to CG-SNO (Figure 3A). The kinetics of the γ -GT-catalysed hydrolysis of GSNO are shown in Figure 3. A reciprocal plot of the initial rate of GSNO decomposition versus GSNO concentration is shown in Figure 3(B). The linearity of this curve indicates that the enzyme exhibits Michaelis–Menten kinetics with a K_m of 28 μM . The K_m of γ -GT for glutathione is 5–10 μM [9].

γ -GT-dependent decomposition of GSNO was inhibited by known inhibitors of γ -GT, as shown in Figure 4. Both S -methylglutathione and γ -glutamyl-(α -carboxy)phenylhydrazine, reversible inhibitors of γ -GT [13], slowed the decomposition of GSNO. Acivicin, a slow-acting non-competitive inhibitor of γ -GT [19], inhibited GSNO decomposition after preincubation with the enzyme. These data imply that the active site for GSNO decomposition is the same as that for GSH.

γ -GT-catalysed $\cdot\text{NO}$ release

ESR was used to detect γ -GT-induced $\cdot\text{NO}$ production from GSNO. NNO reacts with nitric oxide to form the corresponding imino nitroxide (INO). NNO and INO have distinctly different ESR spectra and the conversion of NNO to INO can be used to monitor $\cdot\text{NO}$ formation (Figure 5) [11]. Addition of GSNO to NNO did not result in any significant change in the ESR spectrum of NNO. However, immediately after the addition of γ -GT, the formation of INO was observed. After 120 min complete conversion of NNO to INO occurred (Figure 5). INO formation was not observed in the presence of DTPA (results not shown).

Effect of γ -GT inhibitors and metal ion chelators on GSNO-mediated biological effects

The observation that GSNO is a substrate for γ -GT suggests that this may be one possible pathway by which GSNO is metabolized *in vivo*. We tested for this possibility in two systems: (i)

Figure 5 γ -GT-dependent $\cdot$ NO production from GSNO determined by ESR

GSNO (100 μ M) was incubated with NNO (50 μ M) in the absence (A) and presence (B–E) of γ -GT (1 unit/ml) and the ESR spectrum was taken at the indicated time. ESR conditions were: scan width, 100 G; modulation amplitude, 1 G; microwave power, 1 mW.

decomposition of GSNO by rat kidney homogenate and (ii) GSNO-dependent vasodilation in an isolated heart.

Kidney homogenate contained 2.52 units of γ -GT activity/mg of protein (Table 1), consistent with published observations [13]. Acivicin (10 mg/kg injected i.p. 2.5 h before sacrifice) decreased γ -GT activity by approx. 95% (Table 1 and Figure 6). GSNO decomposition was markedly inhibited in kidney homogenate from acivicin-treated rats, as shown in Table 1. The presence of glycylglycine dramatically accelerated GSNO decomposition in control homogenate but only slightly enhanced GSNO decomposition by acivicin-treated homogenate (Table 1 and Figure 6). The observations that acivicin treatment inhibits GSNO decay and that glycylglycine, a co-substrate in the transpeptidation reaction, accelerates GSNO decay strongly indicate that γ -GT is responsible for GSNO decomposition in kidney homogenate.

The effect of GSNO on coronary flow is shown in Table 2. GSNO (80 nM) elicits a 60% increase in coronary flow during aerobic isolated rat heart perfusion. We have previously shown that this concentration of GSNO approximately corresponds to the EC_{50} and leads to a sub-maximal vasodilatory response in this model [1]. Neither DFO (50 μ M), nor the γ -GT inhibitors acivicin and *S*-methylglutathione, affected the GSNO-induced increase in coronary flow. Moreover, the combination of acivicin and DFO also did not affect the vasodilatory response of GSNO.

Table 1 Decomposition of GSNO by rat kidney homogenate

γ -GT activity was measured in the homogenate of kidneys from acivicin-treated and control animals. Homogenate (5 μ l) was added to GSNO (1 mM) in the presence or absence of glycylglycine (20 mM) in Tris/HCl (100 mM, pH 8.0) and the decomposition of GSNO was monitored spectrophotometrically at 336 nm. Data are means $\pm$ S.E.M. ($n = 3$).

Conditions	γ -GT activity (units/mg of protein)	Initial rate of GSNO decay (μ M/min)	
		Without glycylglycine	With glycylglycine
Control	2.52 $\pm$ 0.13	6.07 $\pm$ 0.40	38 $\pm$ 8.8
+ Acivicin	0.15 $\pm$ 0.04	2.28 $\pm$ 0.12	3.68 $\pm$ 1.26

Figure 6 GSNO decomposition by rat kidney homogenate

GSNO (1 mM) was incubated in Tris/HCl (100 mM, pH 8.0) in the presence of rat kidney homogenate (approx. 20 μ g of protein/ml) from either acivicin-treated (A) or control animals in the absence (B) or presence (C) of glycylglycine. GSNO decay was monitored spectrophotometrically at 336 nm.

γ -GT activity could not be measured in heart homogenate. This indicates that neither γ -GT nor transition metal ions are responsible for the vasodilatory effects of GSNO.

DISCUSSION

γ -GT is a central enzyme of the γ -glutamyl cycle, the pathway for the synthesis and degradation of glutathione. This enzyme is also important in the processing of glutathione conjugates such as in the synthesis of leukotriene D [20]. γ -GT is membrane-bound and appears to act during the translocation of GSH across cell membranes to deliver extracellular cysteine. Many *S*-substituted derivatives of glutathione, including GSSG, are also substrates for γ -GT, and this enzyme acts to degrade and recycle both GSSG and the products of glutathione *S*-transferase activity [13]. We show here that γ -GT catalyses the conversion of GSNO to CG-SNO with a K_m of 28 μ M. This value is similar to those for other *S*-substituted GSH derivatives [21]. The enzymic mechanism does not result in the release of $\cdot$ NO if care is taken to chelate contaminating metal ions in the reaction buffers. However, in the absence of a transition metal ion chelator, γ -GT stimulates the release of $\cdot$ NO, as CG-SNO is more susceptible to transition metal ion-catalysed decomposition than GSNO.

γ -GT is the major route of GSNO decomposition in rat kidney homogenate. Acivicin, a specific inhibitor of γ -GT, decreased

Table 2 Coronary vasodilation of isolated rat heart in response to GSNO

Coronary vasodilation was monitored in an isolated perfused rat heart in response to GSNO (80 nM) in the absence and presence of DFO (50 μ M), S-methylglutathione (50 μ M) and acivicin (10 mg/kg body weight injected i.p. 2.5 h before heart excision). Data are means $\pm$ S.E.M. ($n = 4$). All conditions are not significantly different ($P > 0.05$).

Conditions	Increase in coronary flow rate (%)
GSNO	63 $\pm$ 2
GSNO + DFO	63 $\pm$ 7
GSNO + acivicin	72 $\pm$ 4
GSNO + acivicin + DFO	57 $\pm$ 5
GSNO + S-methylglutathione	64 $\pm$ 4

enzyme activity by almost 95 % and inhibited the decomposition of GSNO. γ -GT has been shown to both hydrolyse the γ -glutamyl residue of GSH (Figure 1) and also to transfer this residue to a second substrate, such as glycylglycine, in a transpeptidation reaction [13]. The presence of glycylglycine resulted in a 6-fold increase in the initial rate of GSNO decomposition by control homogenate and only marginally affected the decomposition of GSNO by acivicin-treated homogenate. This indicates that GSNO is more susceptible to decomposition by transpeptidation rather than hydrolysis if a suitable co-substrate is present.

γ -GT does not appear to be responsible for the metabolism of GSNO in cardiac tissue and is not responsible for GSNO decomposition in heart homogenate (N. Hogg, E. Konorev and B. Kalyanaraman, unpublished work). Inhibitors of γ -GT did not affect the vasodilatory response to GSNO in an isolated heart model. It appears unlikely that γ -GT represents a route of GSNO metabolism in this system. We have previously demonstrated that NNO will antagonize GSNO-dependent coronary vasodilation, indicating that $\cdot$ NO release is responsible for the vasodilatory effect [1]. Butler et al. [22] suggested that variable levels of contaminating transition metal ions in Krebs buffer may be responsible for vasodilation in response to RSNO. Although this may be true for S-nitroso-N-acetylpenicillamine and S-nitrosocysteine, which are more sensitive to transition metal ion-catalysed decomposition than GSNO, the transition metal ion chelator DFO did not affect the vasodilatory response to GSNO. The mechanism of GSNO-dependent vasodilation is, at present, unknown and may involve either transnitrosation to a membrane protein thiol or decomposition by a cell-surface metalloprotein.

There is evidence that the cytostatic effect of GSNO on *S. typhimurium* is mediated by a dipeptide transporter. This implicates the formation and uptake of CG-SNO by a γ -GT-dependent mechanism [15]. More recently Gordge et al. [23] have demonstrated that the anti-platelet properties of GSNO are not inhibited by acivicin and they concluded that γ -GT was not responsible for this effect.

GSNO is formed from the reaction between $\cdot$ NO and GSH in the presence of oxygen [24]. GSNO has been detected in the lung lavage of patients undergoing $\cdot$ NO inhalation therapy [25]. However, as yet, GSNO has not been identified *in vivo* or in cell culture. It has been postulated that this molecule may represent a stable pool of nitric oxide within cells [26]. However, S-substituted derivatives of glutathione are generally exported from the cell by carrier proteins and this also may be the case for

GSNO. Thus if GSNO is synthesized intracellularly the major route of catabolism may be excretion followed by γ -GT-dependent hydrolysis to glutamate and CG-SNO. This pathway may be of particular importance in the kidney where the high levels of γ -GT are responsible for the catabolism of other S-substituted glutathione derivatives [13]. Consequently, the kidney may be a particular target for exogenously administered GSNO. Further studies are required to elucidate the role of γ -GT in the metabolism of GSNO.

In conclusion, γ -GT catalyses the decomposition of GSNO to form CG-SNO with a similar K_m to that of glutathione. This process does not generate $\cdot$ NO, as CG-SNO is stable in the absence of transition metal ions, but may represent a mechanism by which the nitrosyl functional group is transferred into the cytosolic compartment. This process may be the major route of GSNO metabolism in the kidney, but does not appear to mediate GSNO-induced coronary vasodilation.

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