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Chemical Mechanisms Underlying the Vasodilator and Platelet Anti-Aggregating Properties of *S*-Nitroso-*N*-acetyl-DL-penicillamine and *S*-Nitrosoglutathione

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Abstract—The chemistries of *S*-nitroso-DL-penicillamine (SNAP) and *S*-nitrosoglutathione (GSNO) in relation to their ability to relax vascular smooth muscle and prevent platelet aggregation have been investigated. Metal ion catalysis greatly accelerates the decomposition of SNAP, but has little effect on GSNO. Instead, NO release from GSNO is effected either by NO transfer to a free thiol (e.g. cysteine), or by enzymatic cleavage of the glutamyl–cystyl peptide bond. In both cases the resulting nitrosothiol (i.e. *S*-nitrosocysteine and *S*-nitrosocystylglycine, respectively) is susceptible to metal ion catalysed NO release. We conclude that transnitrosation or enzymatic cleavage are obligatory steps in the mechanism of NO release from GSNO, whereas SNAP needs only the presence of metal ions to effect this process. The different modes of NO production may go some way towards explaining the different physiological effectiveness of these *S*-nitrosothiols as vasodilators and inhibitors of platelet aggregation.

Introduction

The production of nitric oxide (NO) from L-arginine is now firmly established as a bioregulatory pathway of immense physiological and pathophysiological importance.[1] NO release from endothelial cells (cells which line blood vessels) relaxes vascular smooth muscle and is a major determinant of systemic blood pressure.[2] It also inhibits platelet aggregation and their adhesion to the vessel wall.[3] The so-called 'nitro-vasodilators', which have been used clinically for over 100 years, appear to act by releasing NO *in vivo*, either spontaneously or after metabolic biotransformation.[4] *S*-Nitrosothiols (RS-NO) are attractive NO-donor drugs as they decompose to give only NO and a disulfide[5] and it is generally accepted that biological activity depends upon the structure of R.[6,7] If RSH is cysteine, the disulfide

$$2RS\text{–}NO \longrightarrow RS\text{–}SR + 2NO \qquad (1)$$

is a naturally occurring material and so *S*-nitrosocysteine (SNC), acting as an NO donor drug, should have few deleterious side effects. However, SNC is unstable and cannot be isolated as a pure solid. It can be prepared in solution[8] but, even then, it decomposes rapidly at ambient temperatures and so the results of any physiological experiments using SNC must necessarily contain an element of uncertainty.

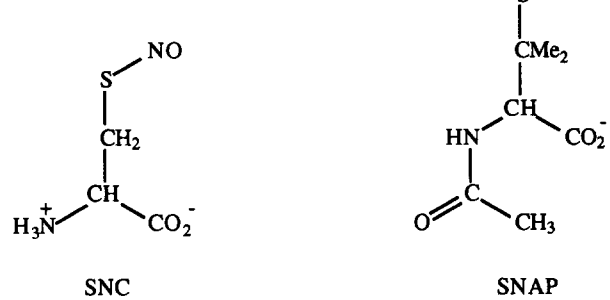
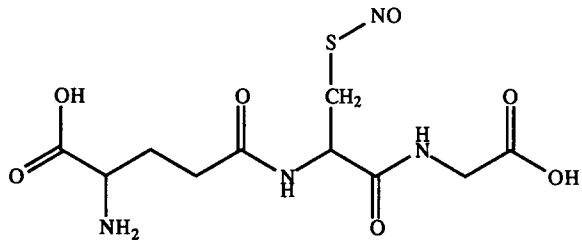


Figure 1.

A simple nitrosothiol which can be obtained as a pure solid is *S*-nitroso-*N*-acetylpenicillamine (SNAP).[9] However, in solution, this decomposes spontaneously yielding the disulfide and NO. Studies by several groups showed that the rate of decomposition *in vitro* is highly variable and half-lives of a few seconds to several hours were observed. We[10] recently showed that this erratic behaviour can be attributed to the presence of variable amounts of metal ion in the buffer solution used for the experiments. Copper(II) and iron(II) are particularly effective catalysts and some samples of ordinary distilled water contain sufficient metal to bring about rapid decomposition of SNAP. It is impractical to remove all metals in *ex vivo* experiments and so an investigation of the vasodilator properties of SNAP[11] is subject to some measure of uncertainty: the observed effects could be due to SNAP itself or to decomposition

products (disulfide and NO) produced by a metal ion catalysed decomposition reaction.

Another nitrosothiol which can be obtained as a pure solid is *S*-nitrosoglutathione (GSNO).[12–14] In order to find out more about the physiological role of nitrosothiols we have compared the chemistry and physiological action of the simple nitrosothiol SNAP with the more complex nitrosothiol GSNO. Some interesting and significant differences have emerged.



GSNO

Figure 2.

We have investigated two possible routes by which the release of NO from GSNO can occur. First, there could be transfer of NO from GSNO to another thiol which is more susceptible than GSNO to decomposition by metal ion catalysed means. Second, enzymatic cleavage of a peptide bond in GSNO occurs to give glutamic acid and a dipeptide with an SNO group more susceptible to metal ion catalysed NO release. We have attempted to distinguish between these possibilities by a series of *in vitro* and *ex vivo* experiments.

Results and Discussion

In spite of its instability in solution, studies of the effect of SNAP on muscle tone in a length of isolated rat tail artery gave consistent results and a log dose response curve is shown in Figure 3. Similar results were obtained with GSNO (also Fig. 3). The vasodilator actions of SNAP and GSNO *ex vivo* are clearly similar in form but differ in magnitude. The ED_{50} (dose required to produce half-maximal vasodilation) for the former is 3.2 μM and for the latter is 59 μM. SNAP is, therefore > 18 × more effective than GSNO. In contrast, the *in vitro* behaviour of GSNO differs substantially from that of SNAP. The effect of copper(II) ions on the release of NO from SNAP *in vitro* (as shown by the decrease in absorbance at 339 nm) is displayed in Figure 4. Recent work by Barnett and Williams (unpublished results) using an NO electrode to measure the production of NO from SNAP in the presence of trace amounts of copper(II) ions substantiates this claim. Clearly the effect of very low concentrations of metal (5 μM) is quite dramatic. This was not so with GSNO (Fig. 5), where even 50 μM copper(II) causes only a slight increase in the rate of decomposition.

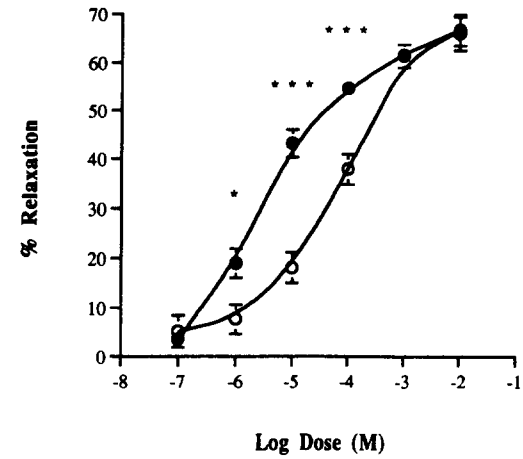


Figure 3. Log[dose]/response curves comparing the vasodilator potencies for 10 μL bolus injections of SNAP (●, n=20) and GSNO (○, n=16). (% Relaxation is the fraction of the pressure drop due to vasodilation by the drug over the precontracted pressure of the artery obtained using phenylephrine mean ± S.E. = 2.7+/- 0.3 μM.) ED_{50} SNAP = 3.2 μM; ED_{50} GSNO = 59 μM (>18 fold difference in ED_{50} values).

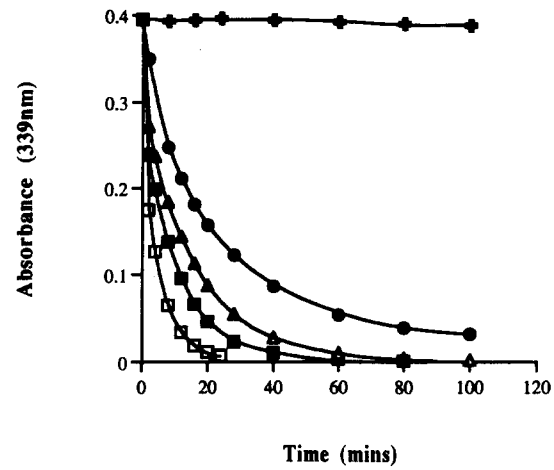


Figure 4. The effect of Cu^{2+} ions on the decomposition rate of SNAP (0.4 mM) [Cu^{2+}] (Δ) 5 μM, (■) 10 μM, (□) 50 μM, (●) no added Cu^{2+}, (✚) [EDTA] 50 μM, 30 °C, KH_2PO_4 buffer pH 7.4.

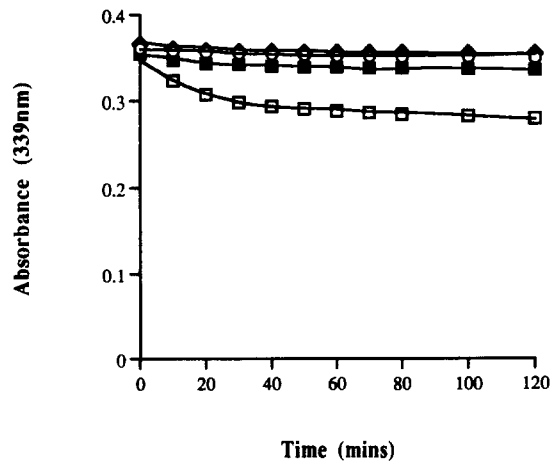


Figure 5. The effect of Cu^{2+} ions on the decomposition rate of GSNO (0.4 mM) [Cu^{2+}] (◊) 1 μM, (■) 10 μM, (□) 50 μM, (○) no added Cu^{2+}, 30 °C, KH_2PO_4 buffer pH 7.4.

We suggest that this is because, to act as a catalyst, the metal ion complexes not only to the sulfur or nitrogen of the SNO group but also to another binding site of the same amino acid. This is possible with SNAP but less likely with a tripeptide derivative like GSNO, which has the cystyl fragment in the middle. GSNO has a greater number of other binding sites compared with SNAP in which metal ions can bind and reduce the amount of metal ion available for binding at the 'catalytic' site. As GSNO has a vasodilator action similar to that of SNAP it must be that there are routes other than a metal catalysed process for the release of NO. Two possibilities have already been mentioned: transnitrosation and enzymic peptide bond cleavage. We investigated transnitrosation first.

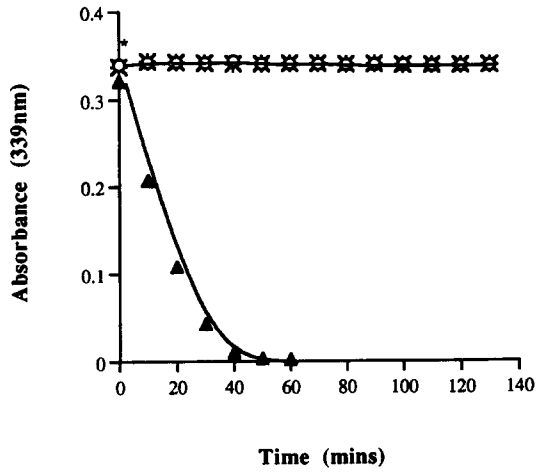


Figure 6. The effect of cysteine (1 mM) and S-methylcysteine (1 mM) on the decomposition of GSNO. GSNO (O, n=6), GSNO + cysteine (▲, n=4), GSNO + S-methylcysteine (⊠, n=2). GSNO + cysteine data is significantly different from GSNO and GSNO+ S-methylcysteine data at P=0.001 confidence level. Conditions: 30 °C, KH_2PO_4 buffer pH 7.4.

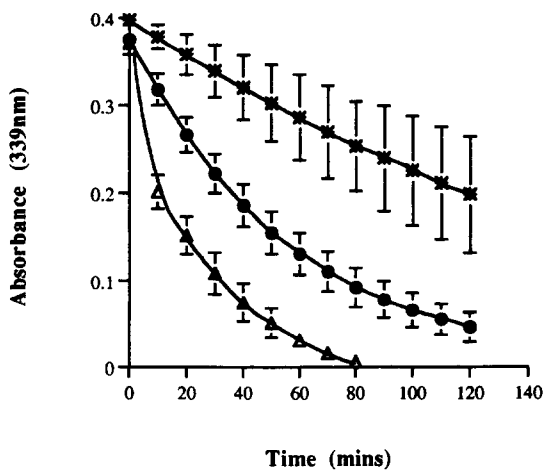
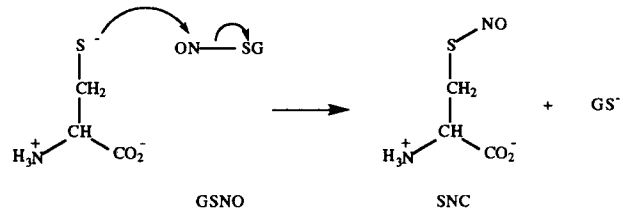


Figure 7. The effect of cysteine (1 mM) and S-methylcysteine (1 mM) on the decomposition of SNAP. SNAP (●, n=9), SNAP + cysteine (Δ, n=7), SNAP + S-methylcysteine (✱, n=4). SNAP + cysteine data is significantly different from SNAP and SNAP + S-methylcysteine data at P=0.01 (**) and at P=0.001 (***) confidence level respectively, and SNAP data is significantly different from SNAP + S-methylcysteine data at P=0.01 (**) confidence level. Conditions: 30 °C, KH_2PO_4 buffer pH 7.4.

Addition of 20 mM cysteine to a solution of 20 mM GSNO produced a red solution with an absorption spectrum identical to that of *S*-nitrosocysteine (made by the action of acidified sodium nitrite upon cysteine[8]). This effect could not be observed visually at 0.4 mM concentrations of GSNO but the enhanced rate of decomposition (monitored spectrophotometrically) of GSNO in the presence of cysteine is evidence that it does occur (Fig. 6). There is no difference in decomposition rate for GSNO and GSNO in the presence of 1 mM *S*-methylcysteine (Fig. 6), which implies that NO transfer can occur only if there is a free thiolate group. A similar effect is seen on the decomposition rate of SNAP when cysteine is added (Fig. 7). In contrast, addition of *S*-methylcysteine to SNAP causes a small decrease in the rate of NO release. This would be consistent with the removal of adventitious metal ions in the buffer solution owing to binding to *S*-methylcysteine and so less is available for catalysis of SNAP decomposition (Figs 4 and 5).

Transnitrosation reactions are known[15] to be very rapid. The rate-determining step in the cysteine catalysed decomposition of GSNO must be loss of NO from *S*-nitroso- cysteine. Formally transnitrosation may be seen as transfer of NO^+ from GSNO to cysteine (Scheme 1) and here we acknowledge the suggestion by Lipton *et al.* [16] that NO^+, as well as NO, has a role in animal physiology.



Scheme 1.

However, the free nitrosonium ion (NO^+) can never exist in an aqueous physiological environment. The *ex vivo* vasodilator action of bolus injections of GSNO was enhanced when cysteine was allowed to perfuse through the artery via the internal Krebs buffer supply (Fig. 8).

The ED_{50} value for GSNO is 120 μM compared with 19 μM in the presence of 1 mM cysteine. As GSNO has only 3–5 s to react with cysteine before reaching the artery, it is reasonable to suppose that NO^+ transfer to form a less stable thiol occurs rapidly.

Ferrohemoglobin is a scavenger for NO.[17,18] Experiments performed using ferrohemoglobin (Hb) in the internal perfusate show (Figs 9 and 10) that the vasodilator action of GSNO is substantially less inhibited than that of SNAP. Ferrohemoglobin reduces the ED_{50} value for GSNO by a factor of only 11 whereas that for SNAP is reduced by a factor of 73. These results are consistent with the finding that SNAP is the more unstable

compound in solution. The vasodilator actions of both SNAP and GSNO are only partially inhibited by 15 μM Hb. This suggests that a significant percentage of each drug reaches the vascular smooth muscle cells before releasing NO, probably at the membrane surface.[19]

When isolated rat tail artery is perfused with Krebs buffer containing superoxide dismutase (SOD, 150 units mL^{-1}) and given a bolus injection of GSNO there is no potentiation of the vasodilator response (Fig. 11). The ED_{50} value for GSNO of 17 μM remains unchanged in the presence of SOD. This observation is consistent with the view that GSNO does not release NO spontaneously in the lumen of the artery but requires tissue and/or tissue fluid to effect its release. On the other hand, the vasodilator action of SNAP is slightly enhanced by SOD (Fig. 12), suggesting that SNAP does

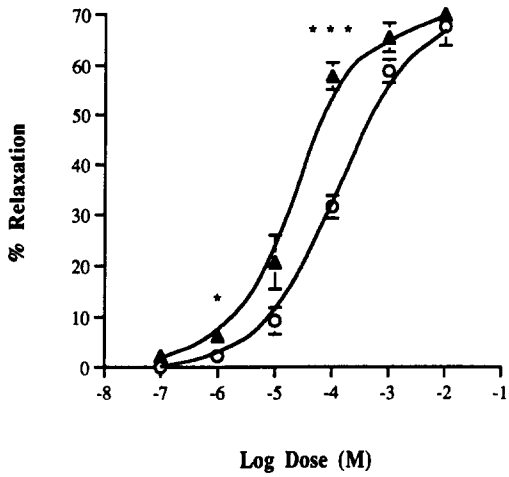


Figure 8. The effect of adding cysteine (1 mM) to the internal perfusate on the vasodilator responses to bolus injections of GSNO. GSNO (O, n=9), GSNO/cysteine (▲, n=9), ED_{50} GSNO = 120 μM, ED_{50} GSNO/cysteine = 19 μM.

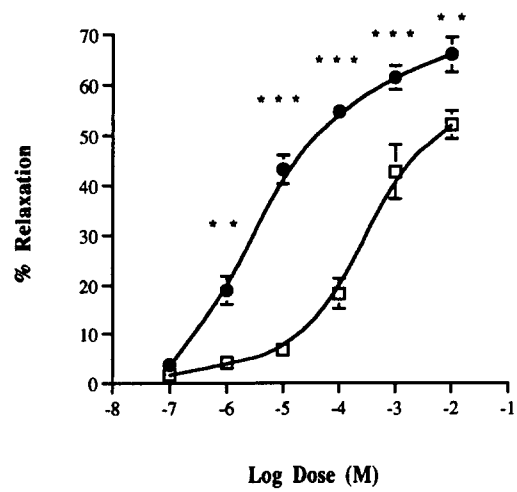


Figure 9. The effect of adding ferrohemoglobin (15 μM) to the internal perfusate on the vasodilator responses to bolus injections of SNAP. SNAP (●, n=20), SNAP/Hb (□, n=10), ED_{50} SNAP = 3.2 μM, ED_{50} SNAP/Hb = 230 μM.

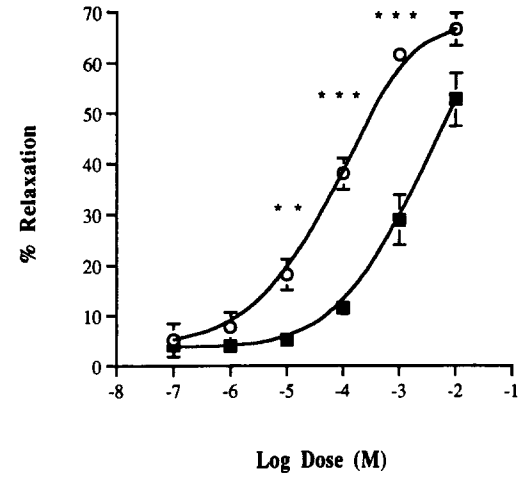


Figure 10. The effect of adding ferrohemoglobin (15 μM) to the internal perfusate on the vasodilator reponses to bolus injections of GSNO. GSNO (O, n=16), GSNO/Hb (■, n=8). ED_{50} GSNO = 59 μM, ED_{50} GSNO/Hb = 660 μM.

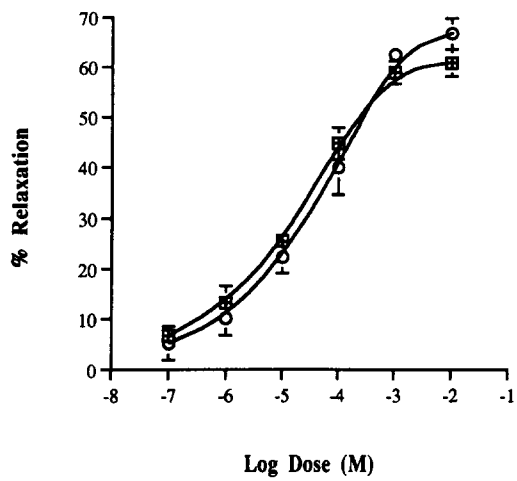


Figure 11. The effect of adding SOD (150 units mL^{-1} to the internal perfusate on the vasodilator response to bolus injections of GSNO. GSNO (O, n=5), GSNO/SOD (⊕, n=5), ED_{50} GSNO and GSNO/SOD = 17 μM.

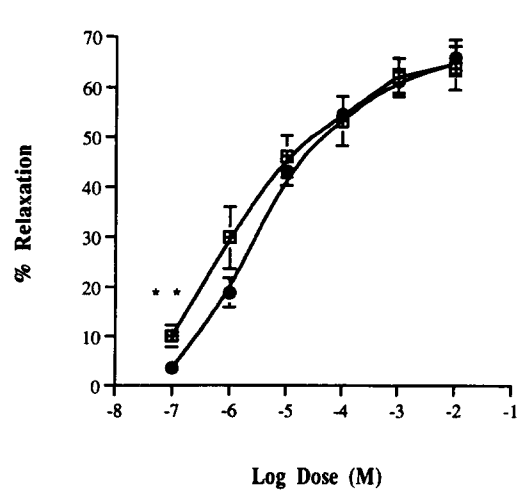


Figure 12. The effect of adding SOD (150 units mL^{-1} to the internal perfusate on the vasodilator response to bolus injections of SNAP. SNAP (●, n=5), SNAP/SOD (⊕, n=5), ED_{50} SNAP = 3.2 μM, ED_{50} SNAP/SOD = 1.5 μM.

release NO in the lumen, where it can then be destroyed by superoxide. Release of NO from SNAP in the lumen could be due to the presence of metal ions. A further possibility that cannot be ruled out is that GSNO and SNAP enter vascular smooth muscle cells and activate guanylate cyclase directly. However, after treatment of isolated rat tail artery with ethacrynic acid, a known alkylator of thiol groups, there is complete inhibition of the vasodilator action of GSNO and so direct activation appears unlikely. Some attenuation of responses to 'authentic' NO also occurred, but this was not unexpected as the activation of guanylate cyclase is critically dependent upon SH groups.[20]

The other possibility mentioned earlier is that NO release from GSNO is brought about by the action of γ-glutamyltranspeptidase or a related enzyme. This enzyme cleaves one of the peptide bonds in glutathione with release of glycylcysteine and transfer of the glutamyl moiety to another amine (generally glutamine). We found that a parallel reaction occurs with GSNO as a substrate. GSNO was incubated with γ-glutamyltranspeptidase for 30–60 min and the resulting solution examined by capillary zone electrophoresis (CZE) (Fig. 13). Peaks were observed at the elution times corresponding to glycylcysteine and the disulfide of glycylcysteine. However, no peaks were observed corresponding to GSNO, glutathione, and the disulfide of glutathione. The absence of the first shows that reaction has occurred but this cannot be due to the direct release of NO from GSNO since this would have led to formation of the disulfide of glutathione. However, if the enzyme effects cleavage of the cysteine–glutamic acid peptide bond to give *S*-nitrosoglycylcysteine, which then decomposes with release of NO, formation of the disulfide of glycylcysteine is to be expected. We then examined the kinetics of breakdown of GSNO (monitored by the disappearance of the absorption peak at 335 nm) as a function of enzyme concentration. The results are displayed in Figures 14 and 15. Clearly the rate of decomposition is enzyme dependent (Fig. 14) and decomposition occurs in both the presence and absence of the acceptor amino acid (glutamine) for the glutamyl fragment (Fig. 15). The results obtained are consistent with the reaction shown in Scheme 2. One would expect the rate of release of NO from *S*-nitrosocystylglycine to be greater

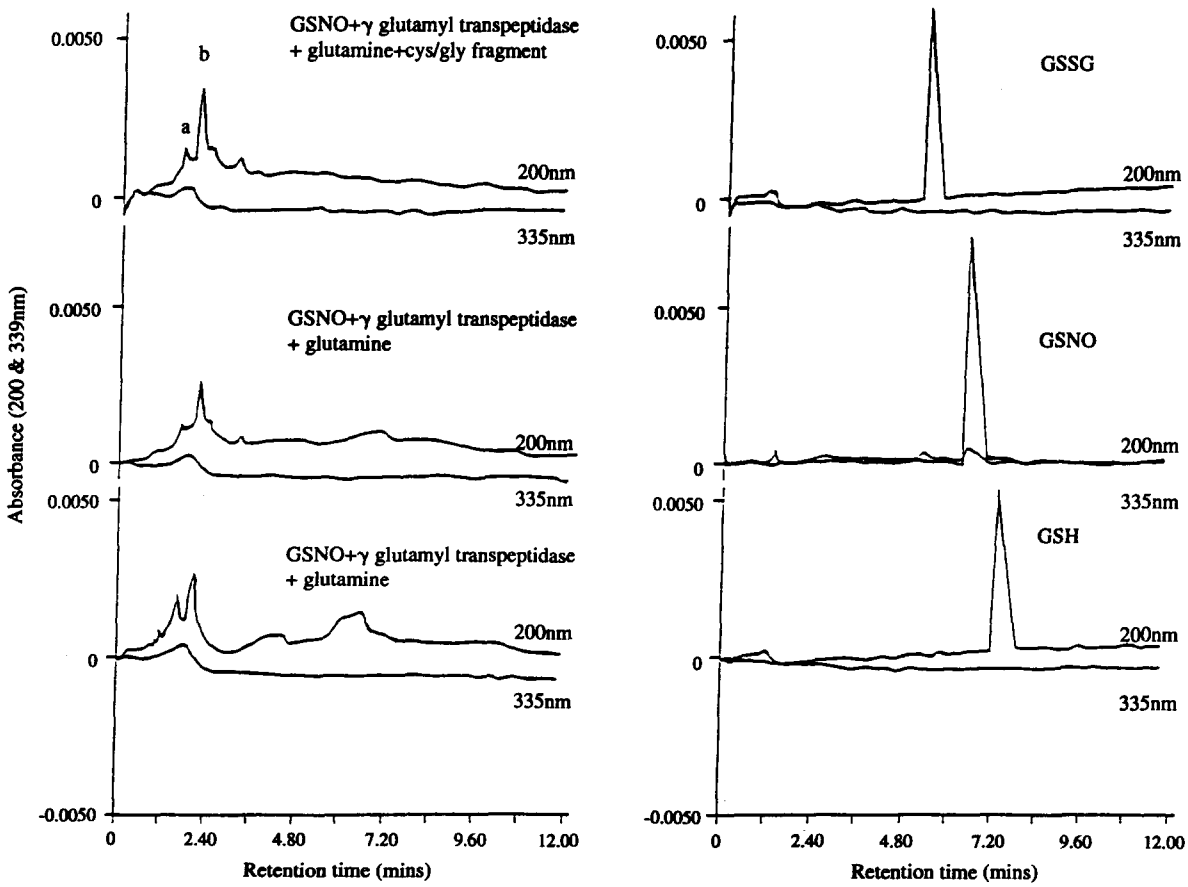


Figure 13. CZE traces for glutathione (GSH), *S*-nitrosoglutathione (GSNO) and oxidised glutathione (GSSG) (all 1 mg mL^{-1}), comparing them to the γ glutamyl transpeptidase enzyme (3.3 units mL^{-1}) digest products of glutathione and *S*-nitrosoglutathione in the presence of the acceptor amino acid glutamine (1 mg mL^{-1}). The cystylglycine (cys/gly) fragment of glutathione was also used to spike the enzyme digest of *S*-nitrosoglutathione and glutamine, giving rise to an increase in peaks a and b. It was shown that a and b were the reduced and oxidised forms of the cys/gly fragment. Note the complete disappearance of the GSNO peak and no formation of the peak corresponding to GSSG. This also indicates that cleavage of the glutamyl/cystyl peptide bond has taken place.

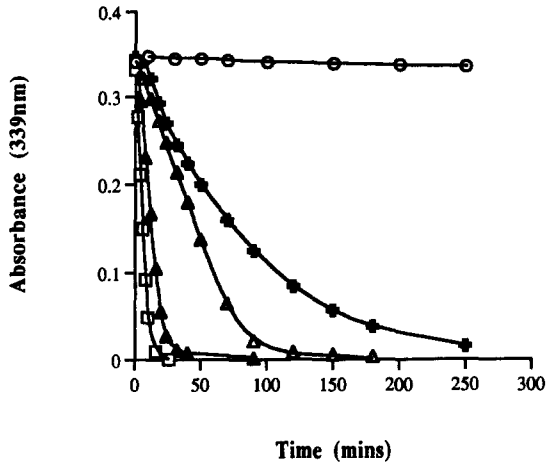


Figure 14. The effect of the enzyme γ glutamyl transpeptidase (γGT) on the decomposition rate of GSNO (0.4 mM) in the presence and absence of EDTA. (O) GSNO, (Δ) GSNO/γGT (0.7 units mL^{-1}, (▲) GSNO/γGT (2.8 units mL^{-1}), (□) GSNO/γGT (5.6 units mL^{-1}), (✚) GSNO/γGT (5.6 units mL^{-1}) + EDTA (50 μM). Conditions: 30 °C, KH_2PO_4 buffer pH 7.4.

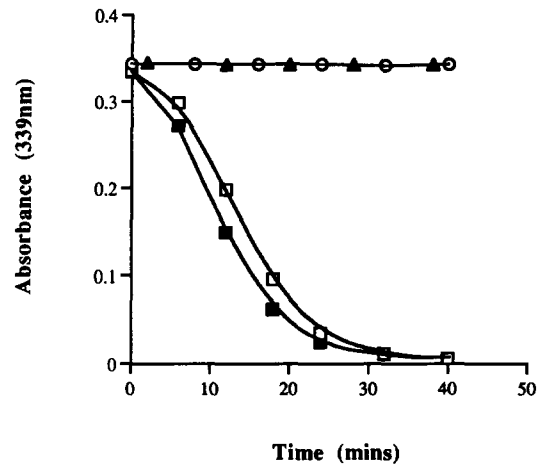
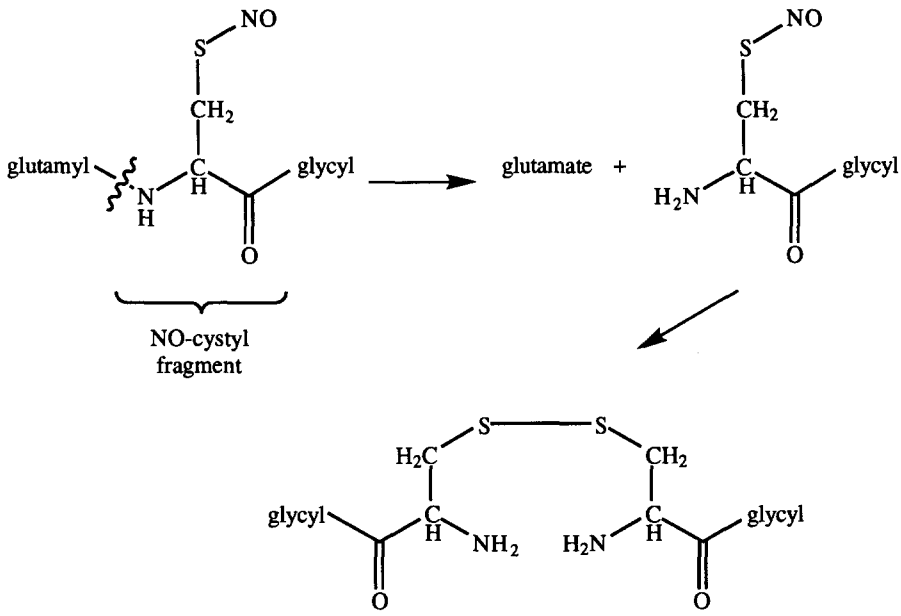


Figure 15. The effect of the enzyme γ glutamyl transpeptidase (γGT) on the decomposition rate of GSNO (0.4 mM) in the presence and absence of an acceptor peptide (glutamine), for the transfer of the glutamyl fragment of GSNO. (O) GSNO, (▲) GSNO + glutamine (0.4 mM), (□) GSNO/γGT (2.1 units mL^{-1}), (■) GSNO/γGT (2.1 units mL^{-1}) + glutamine (0.4 mM). Conditions: 30 °C, KH_2PO_4 buffer pH 7.4.



Scheme 2.

than from GSNO as the catalysing metal can complex with two favourable binding sites in the former (–NH_2 and –SNO) while the latter is resistant to metal ion catalysis for reasons mentioned earlier.

That metal ion catalysed NO release occurs is seen from the data displayed in Figure 14. Addition of EDTA to a mixture of GSNO and γ-glutamyltranspeptidase diminishes the rate of decomposition of the nitrosothiol to NO, while having no effect on the action of the enzyme (results not shown).

Conclusion

The chemical and biochemical evidence suggests that both transnitrosation to give SNC and enzymatic peptide cleavage to *S*-nitrosoglycylcysteine could contribute to the release of NO from GSNO. Once NO has been transferred to cysteine, metal ion catalysed NO release can occur. Equally, metal ion catalysed decomposition can occur once the –SNO group has become part of a terminal amino acid.

As reported in this paper, GSNO is a less effective vasodilator than SNAP but in work carried out in collaboration with Salas *et al.*[21,22] it was found that the reverse is the case in respect of their abilities to prevent platelet aggregation. We suggest that this difference is due to the presence in platelets of an enzyme which can cleave the glutamic acid from

GSNO to give a species from which NO release can readily occur.

Experimental

Chemicals

L-Cysteine, glutathione, *N*-acetyl-DL-penicillamine and sodium nitrite were purchased from Aldrich Chemical Co. Copper(II) nitrate and all the buffer components (Aristar where appropriate) were from BDH. Glutamine was obtained from Lancaster Synthesis Ltd and *S*-methycysteine γ-glutamyltranspeptidase, bovine hemoglobin, glycylcysteine, superoxide dismutase, EDTA, ethacrynic acid, and L-phenylephrine hydrochloride from Sigma Chemical Co.

S-*Nitroso*-N-*acetylpenicillamine (1; SNAP).* Prepared by the method of Field *et al*.[9]

S-*Nitrosoglutathione (3; GSNO).* Prepared by the method of Hart *et al.*[12]

HPLC

The purities of the above compounds were examined by reverse phase HPLC at 215 and 340 nm and found to give only one peak for each wavelength at the same retention time. Conditions: a Lichroscorb RP-18 column (0.46 × 25 cm, 10 μm) was used with a solvent system of 0.05% trifluoroacetic acid/water (A) and 0.035% trifluoroacetic acid/acetonitrile (B) using a linear gradient of 90% (A): 10% (B) for 10 min going to 80% (A): 20% (B) over the next 20 min and then to 70% (A): 30% (B) over the following 10 min.

Solution preparation

Solutions of known concentrations were obtained by serial dilution of a standard solution prepared by weighing. The buffered solutions were kept on ice (at this temperature metal ion-catalysed release of NO was negligible over the time of the experiment) until used and protected from light to prevent their photochemical decomposition. Buffered solutions of copper nitrate (1–50 μM), cysteine (1 mM), *S*-methylcysteine (1 mM), γ-glutamyl-transpeptidase (for concentrations see Figs 13–15), and EDTA (50 μM) were also prepared by serial dilution.

Kinetic studies

The kinetics of decomposition of SNAP and GSNO were monitored by following the reduction in absorbance at 339 nm using a Phillips PU8700 UV/Vis scanning spectrophotometer with a Pye Unicam cell temperature control unit. All experiments were carried out at 30 °C in a phosphate buffer at pH 7.4 (0.1 M KH_2PO_4/ 0.1 M NaOH) made up using distilled water which had undetectable copper and iron content on the ppm (parts per million) scale. Therefore, trace metal ions present in the buffer solution must come from the buffer system itself.

Enzyme kinetics

The activity of the enzyme γ-glutamyltranspeptidase was tested by the use of 4-nitrobenzanilide as the standard substrate and showed expected activity. Measured amounts of the enzyme (see Figs 14, 15) were then added to buffered solutions of GSNO, in the absence and presence of glutamine (4×10^{-4} M), and the decomposition monitored spectrophoto-metrically as above.

Capillary zone electrophoresis

The apparatus used was a Biofocus 3000 Capillary Electrophoresis System (Bio-Rad, Richmond, CA) fitted with a polyacrylamide coated capillary (17 cm × 25 μm). Electrophoretic separations were detected on-line by computer. The analyses were performed at 20 °C. To initiate analyses the capillary and electrode reservoirs were filled with electrophoresis buffer (0.01 M Na_3PO_4/0.03 HCl, pH 2.3, as used by Stamler *et al.*[23]). The capillary was first flushed with HPLC grade water (Rathburn Chemicals, Walkerburn, Scotland), secondly with capillary wash (Bio-Rad), and thirdly by the electrophoresis running buffer. This procedure was carried out between each sample run. The voltage field strength was 11 kV and the polarity of the internal power supply was set for migration of cations towards the detector (positive polarity). The samples were diluted 1:10 with HPLC water. The detector wavelength was set at 200 nm. Loss of NO from GSNO was also monitored at 335 nm (the absorption maximum of GSNO).

Physiological testing

Preparation. The vasodilator actions of SNAP and GSNO were determined by experiments performed on segments of tail artery taken from normotensive adult Wistar rat (250–460 g) which were killed by cervical dislocation. A length of artery (0.8–1.5 cm) was dissected free, cannulated (Portex cannula) and transferred to a perspex bath, part of the apparatus shown in Figure 16.

Apparatus. The cannula (C) forms part of a constant flow perfusion driven by a peristaltic pump P(1). (Gilson Minipulse). The length of artery (A) was perfused internally (flow rate 2 mL min^{-1}) with the solution pre-warmed by passage through a heat exchanger. Drugs were introduced into the lumen of the artery by bolus injection [10 μL through a side tube (I)]. The outer surface of the artery was superfused continuously with a solution from a second peristaltic pump P(2). The temperature of the bath was held at 30–33 °C by adjustment of the flow rate of the external circuit (*ca* 8 mL min^{-1}). This temperature range was chosen since phenylepherine-induced perfusion pressures remained more stable than at 37 °C over the

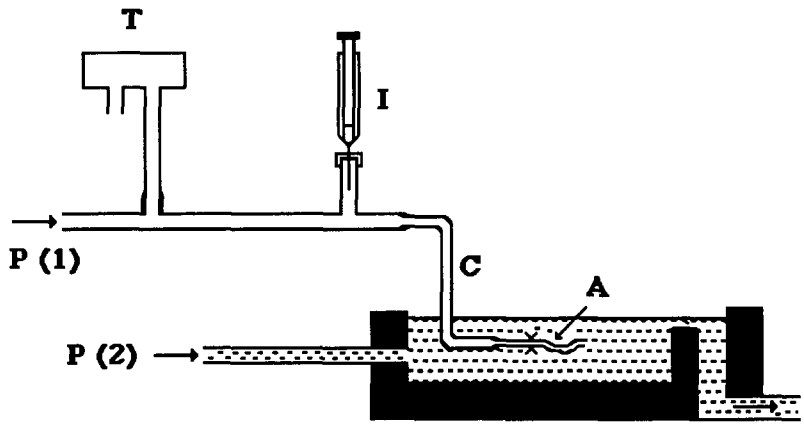


Figure 16.

time periods (up to 8 h) required to complete the experiments. A differential pressure transducer (T; sensym type SCX 150NC Farnell Electronic Components, Leeds, U. K.) detected changes in back pressure due to changes in arterial tone. Responses were displayed on a computer and chart recorder.

Experimental protocol. Arteries were perfused internally with Krebs solution (composition, mM NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with O_2:CO_2 (95%:5%) to maintain pH at 7.4). The initial flow rate was low but was gradually increased over 20 min to reach a final value 2 mL min^{-1}. The preparation was allowed to stabilize for 20–30 min, after which the artery was precontracted by addition of phenylepherine hydrochloride to the Krebs buffer ([P.E]: mean (± S.E.) = 2.7 ± 0.3 μM gave agonist induced perfusion pressure: mean (± S.E.) = 102 ± 2.9 mmHg).

Responses to bolus injections of SNAP and GSNO were recorded and compared with those obtained when the following were added to the internal perfusate: 1 mM cysteine, 15 μM ferrohemoglobin (an NO scavenger), and 150 units mL^{-1} superoxide dismutase (a scavenger of superoxide anions which rapidly inactivate NO).

All experiments were conducted in a darkened laboratory with a red safelight (60 W) as the sole means of illumination.

Drugs: synthetic procedures and use

SNAP and GSNO. Solutions (10^{-2} M) of SNAP and GSNO were made up in Krebs buffer and serially diluted to the concentration required for injection immediately prior to use. Both *S*-nitrosothiols solutions were kept in the dark and on ice at all times to minimize any mode of decomposition.

N.B. The concentrations of the drugs injected (indicated in the figures) are virtually the same as the concentrations which reach the tissue due to the rapid injection time (< 0.5 s) and the drug travelling through the narrow bore tubing as a bolus. However, a submaximal response produced by 7 μM SNAP administered by perfusion was matched by a 3 mM injection of SNAP. This suggests that less than 1/400th of the drug delivered to the artery by injection (as a bolus) is reaching its site of action.

Ferrohemoglobin (M_r 64500). Prepared by reduction of 100 mL of 1 mM bovine hemoglobin using 10 mM sodium dithionite. The dithionite was dialysed out of solution against 3 × 2 L volumes of deaerated distilled water at 0–5 °C. The stock ferrohemoglobin was then split into 3 mL samples and frozen (–10 °C). Samples were used within 2 weeks.

Statistical analysis of data in figures

Statistical analysis on the data was carried out using an unpaired Student's *t*-test: * indicates data is significantly different at the $P = 0.05$ confidence level; ** indicates data is significantly different at the $P = 0.01$ confidence level; *** indicates data is significantly different at the $P = 0.001$ confidence level.

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