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Role of a copper (I)-dependent enzyme in the anti-platelet action of S-nitrosoglutathione

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1 S-nitrosoglutathione (GSNO) is a potent and selective anti-platelet agent, despite the fact that its spontaneous rate of release of nitric oxide (NO) is very slow. Our aim was to investigate the mechanism of the anti-aggregatory action of GSNO.

2 The biological action of GSNO could be mediated by NO released from S-nitrosocystylglycine, following enzymatic cleavage of GSNO by γ-glutamyl transpeptidase. The anti-aggregatory potency of GSNO was not, however, altered by treatment of target platelets with the γ-glutamyl transpeptidase inhibitor acivicin (1 mM). γ-Glutamyl transpeptidase is not, therefore, involved in mediating the action of GSNO.

3 The rate of breakdown of S-nitrosoalbumin was increased from 0.19 ± 0.086 nmol min^{-1} to 1.52 ± 0.24 nmol min^{-1} (mean ± s.e.mean) in the presence of cysteine ($P<0.05$, $n=4$). Inhibition of platelet aggregation by S-nitrosoalbumin was also significantly increased by cysteine ($P<0.05$, $n=4$), suggesting that the biological activity of S-nitrosoalbumin is mediated by exchange of NO from the protein carrier to form the unstable compound cysNO. Breakdown of GSNO showed a non-significant acceleration in the presence of cysteine, from 0.56 ± 0.22 to 1.77 ± 0.27 nmol min^{-1} (mean ± s.e.mean) ($P=0.064$, $n=4$), and its ability to inhibit platelet aggregation was not enhanced by cysteine. This indicates that the anti-platelet action of GSNO is not dependent upon transnitrosation to form cysNO.

4 Platelets pretreated with the copper (I)-specific chelator bathocuproine disulphonic acid (BCS), then resuspended in BCS-free buffer, showed resistance to the inhibitory effect of GSNO. These findings suggest that BCS impedes the action of GSNO by binding to structures on the platelet, rather than by chelating free copper in solution.

5 Release of NO from GSNO was catalysed enzymatically by ultrasonicated platelet suspensions. This enzyme had an apparent K_m for GSNO of 12.4 ± 2.64 μM and a V_{max} of 0.21 ± 0.03 nmol min^{-1} per 10^8 platelets (mean ± s.e.mean, $n=5$). It was inhibited by BCS, but not by the iron chelator bathophenathroline disulphonic acid, nor by acivicin.

6 We conclude that the stable S-nitrosothiol compound GSNO may exert its anti-platelet action via enzymatic, rather than spontaneous release of NO. This is mediated by a copper-dependent mechanism. The potency and platelet-selectivity of GSNO may result from targeted NO release at the platelet surface.

Keywords: S-nitrosothiol; S-nitrosoglutathione; nitric oxide; platelet aggregation; γ-glutamyl transpeptidase; transnitrosation; copper; bathocuproine disulphonate

Introduction

S-nitrosothiols, with the general formula RSNO, are adducts of NO with sulphydryl (-SH) groups on carrier molecules such as cysteine residues of proteins and peptides. S-nitrosothiol formation can occur under physiological conditions following reaction of biological thiols with both exogenous NO (Kaufmann *et al.*, 1995) and endogenous endothelium-derived relaxing factor (EDRF) (Stamler *et al.*, 1992b). Under aerobic conditions S-nitrosothiols are the primary reaction product of NO/O_2 with cellular constituents (Wink *et al.*, 1994), the principal nitrosylating agent being N_2O_3 (Kharitonov *et al.*, 1995), and S-nitrosothiols are present in both human plasma (Stamler *et al.*, 1992a) and tissue fluid (Gaston *et al.*, 1993). Suggestions that EDRF might be a S-nitrosothiol remain controversial (Feelisch *et al.*, 1994), and the physiological role of endogenous S-nitrosothiol compounds is uncertain. S-nitrosoglutathione (GSNO) is a stable S-nitrosothiol compound which releases NO slowly, but nevertheless possesses potent and selective anti-platelet activity (Radomski *et al.*, 1992; de Belder *et al.*, 1994). This selectivity of action is unexplained. The action of GSNO has been attributed to generation of unstable derivatives, susceptible to metal ion-catalysed NO release, arising either by transnitrosation reactions, perhaps catalysed by glutathione peroxidase (Freedman *et al.*, 1995), or by enzymatic cleavage of GSNO by γ-glutamyl transpeptidase (γGT) to yield S-nitrosocystylglycine (Askew *et al.*, 1995). In earlier studies, we showed that the inhibition of platelet aggregation by GSNO was diminished by the copper(I)-specific chelator bathocuproine disulphonic acid (BCS) (Gordge *et al.*, 1995). This effect of BCS was not, however, explained by inhibition of copper-catalysed NO release. We have now extended our investigations into the anti-platelet action of GSNO, exploring the possible role of both transnitrosation and γGT in its bioactivity, and investigating further the inhibition by BCS of the biological activity of GSNO.

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Methods

Preparation of S-nitrosothiols

Non-protein S-nitrosothiols were prepared by incubating 0.5 ml of 40 mM thiol with 0.5 ml of 40 mM $NaNO_2$ in 40 mM HCl for at least 60 min at 4°C. Stock S-nitrosothiol solutions were prepared fresh each day, and kept at 4°C in the dark until use. Immediately before use, 30 μl aliquots of S-nitrosothiol

were neutralised with 30 μl of 100 mM sodium phosphate pH 7.4 and diluted to the required concentration in water. S-nitrosoalbumin was prepared with n-butyl nitrite as described by Meyer *et al.* (1994), except that bovine, rather than human, serum albumin was used. Concentration of each S-nitrosothiol stock solution was calculated from its absorbance at 334 nm against a water blank, using the following extinction coefficients (mM^{-1} cm^{-1}): S-nitrosocysteine (cysNO) 0.74, S-nitrosohomocysteine (homocysNO) 0.73, S-nitroso-N-acetyl-DL-penicillamine (SNAP) 1.00, S-nitroso-N-acetyl-cysteine (SNAC) 0.87, S-nitrosoglutathione (GSNO) 0.85, and S-nitrosoalbumin (albSNO) 0.87.

Platelet preparation

Citrated blood was obtained from healthy volunteers (of either sex), none of whom had ingested drugs known to affect platelet function in the previous 10 days. Washed platelet suspensions were prepared as previously described (Gordge *et al.*, 1995). Platelets were suspended at a count of $200-300 \times 10^9$ l^{-1} in modified Tyrode buffer with the following composition (in mM): NaCl 137, glucose 5.55, $CaCl_2$ 1.0, $NaHCO_3$ 11.9, $MgCl_2$ 1.05, NaH_2PO_4 0.36, KCl 2.68 and HEPES 10.

Measurement of inhibition of platelet aggregation

Inhibition by S-nitrosothiols of thrombin-induced platelet aggregation was measured as previously described (Gordge *et al.*, 1995). Briefly, platelets equilibrated at 37°C were treated with S-nitrosothiol for 15 s before the induction of aggregation with thrombin ($0.01-0.02$ units ml^{-1}). Aggregation was then monitored turbidometrically for 3 min by a Payton aggregometer, at 37°C and a stirring rate of 1000 r.p.m. The anti-aggregatory action of the S-nitrosothiol was assessed by comparison with control responses obtained in the presence of vehicle alone.

Measurement of rate of S-nitrosothiol breakdown

Rate of breakdown of both GSNO and S-nitrosoalbumin (albSNO) was measured by incubating 100 nmol of S-nitrosothiol in 1 ml of Tyrode buffer and monitoring the decrease in absorbance at 334 nm at 37°C for a period of 20 min. Absorbance was measured with a Cary 1E spectrophotometer. The rate of breakdown of S-nitrosothiol in nmol min^{-1} was calculated from the decrease in absorbance by use of the extinction coefficients quoted above.

Effect of acivicin on the anti-platelet action of GSNO

Platelet suspensions were pretreated for 20 min at room temperature with acivicin (1 mM), or vehicle. This treatment abolished γGT activity as measured by a chromogenic assay measuring γ-glutamyl p-nitroanilide breakdown (data not shown), consistent with published accounts of inhibition of platelet γGT (Sexton & Mutus, 1995). The anti-aggregatory action of GSNO ($10^{-9}-10^{-5}$ M) was then assessed as described above, and compared between acivicin-treated and vehicle-treated platelets.

Effect of transnitrosation reactions on S-nitrosothiol stability and bioactivity

The rate of breakdown of the relatively stable S-nitrosothiols albSNO and GSNO was measured in the presence and absence of cysteine (100 μM), in order to assess the effect of transnitrosation on the stability of these compounds. To confirm that changes in absorbance at 334 nm following addition of cysteine were indeed due to release of NO, direct measurements of NO were performed. This was carried out by quantifying the oxidation of oxyhaemoglobin to methaemoglobin by a modification of the difference-spectrophotometic method described by Kelm *et al.* (1988). Briefly, 50 mM sodium phosphate buffer pH 8.2 containing oxyhaemoglobin (5 μM), was equilibrated for 2 min at 37°C, in the presence and absence of cysteine. Release of NO after the additon of S-nitrosothiol (100 μM) was then measured over a period of 5 min by the difference in absorbance at 401 and 419 nm. Absorbance was measured using double beam against a blank containing no S-nitrosothiol, and NO was quantitated by use of an extinction coefficient ($\sum_{401-419}$) of 76 mM^{-1} cm^{-1}. In a similar way, the effect of transnitrosation to cysteine upon the bioactivity of albSNO and GSNO was assessed by measuring the anti-aggregatory activity of the two S-nitrosothiols in the presence and absence of cysteine.

Effect of platelet pretreatment with BCS on the anti-aggregatory activity of GSNO

Washed platelet suspensions were treated with either BCS (100 μM) or vehicle for 5 min, pelleted by centrifugation at 600 *g* for 10 min in the presence of prostacyclin (300 ng ml^{-1}), and re-suspended in BCS-free Tyrode buffer. BCS pretreated and untreated platelets were then left for 2 h at room temperature to allow recovery from the effects of prostacyclin, before being used to assess the anti-aggregatory action of GSNO, as described above.

GSNO breakdown by ultrasonicated platelet suspension

Washed platelets were prepared as described above, and re-suspended in 0.25 M sucrose/20 mM HEPES buffer pH 7.4 at a platelet count of 2000×10^9 l^{-1}. Platelets were then sonicated for 2×30 s at 4°C with a power output of 170 watts and a wavelength of 90 μm, using an Ultrasonics A180 G instrument (Ultrasonics, Shipley U.K.), in the presence of the following protease inhibitors: PMSF (1 mM), leupeptin (1 μM), pepstatin (1 μM) and aprotinin (10 u ml^{-1}). Release of NO from 100 nmol S-nitrosothiol (100 μM in a volume of 1 ml) was quantified in the presence and absence of platelet suspension by its oxidation of oxyhaemoglobin to methaemoglobin, as described above. Platelet-mediated GSNO breakdown was measured as the increase in NO release obtained in the presence of platelet suspension, above 'spontaneous' release in the absence of platelets. Breakdown was measured with both low molecular weight S-nitrosothiols (GSNO, SNAP and SNAC), and a protein S-nitrosothiol (albSNO), as substrate. Values for cysNO and homocysNO are not presented, as their spontaneous rates of NO release were too high to allow accurate measurement of enzymatic breakdown.

To demonstrate the dependence of NO-releasing activity on platelet concentration, the volume of platelet suspension used in the assay was varied from 50–200 μl (equivalent to $1-4 \times 10^8$ platelets). In a similar way, the dependence on pH was investigated by adjusting the pH of the assay buffer to give a range of values from 6.0 to 9.5, and measuring the enzymatic activity of platelet sonicates over this pH range. Phosphate buffer was used for pH values between 6.0 and 8.0, and borate buffer for values above 8.0. The difference in buffer system did not affect enzymatic activity, which gave the same value at pH 8.0 when measured in either phosphate or borate buffer (data not shown). The effect of heat inactivation was assessed by assaying platelet suspensions following incubation at 90°C for 5 min. To investigate whether platelet-related NO-releasing activity was due to a dialysable factor, ultrasonicates (1 ml) were dialysed in 6.3 mm diameter visking tubing (Medicell International, London N1, U.K.) with a 12,000 molecular weight cut-off, against two changes of 1000 ml of 50 mM phosphate buffer pH 7.4 for 60 min each at 4°C. Platelet-related NO-releasing activity was also measured at a range of GSNO concentrations and kinetic analysis performed by means of a Hanes plot ([S]/V versus [S]), which gives a more reliable estimate of the Michaelis-Menton kinetic constants than the Lineweaver-Burk method of plotting 1/V against 1/[S] (Dowd & Riggs, 1965). The role of copper availability on enzymatic activity was assessed by measuring enzymatic activity

in the presence and absence of the copper (I)-specific chelator BCS ($10^{-7}-10^{-3}$ M) (Blair & Diehl, 1961). In a similar way, the role of iron was assessed by using bathophenanthroline disulphonic acid (BPS), an iron-specific chelator of similar structure to BCS (Blair & Diehl, 1961). The contribution of γGT to the enzymatic activity of platelet ultrasonicates was assessed by comparing activity before and after incubation for 20 min at room temperature with acivicin (1 mM).

Reagents

Cysteine, glutathione, homocysteine, N-acetyl cysteine, N-acetyl-DL-penicillamine, bovine serum albumin, bovine haemoglobin, BCS, acivicin, phenylmethylsulphonyl fluoride (PMSF), leupeptin, pepstatin and bovine thrombin were all purchased from Sigma (Poole, U.K.). Aprotinin was from Bayer (Newbury, U.K.). Prostacyclin was a kind gift from Dr S. Moncada (Wellcome Laboratories). All other reagents were of Analar grade and purchased from Merck (Lutterworth, U.K.). Oxyhaemoglobin was prepared by adding 3 mg of sodium dithionite to 16 mg of haemoglobin in 0.5 ml of 20 mM sodium phosphate buffer pH 7.4. The reduced haemoglobin was then applied to a 10 x 1 cm column of Sephadex G25 and eluted with 20 mM phosphate buffer. It was then stored at 4°C and used within 5 days.

Statistics

Comparisons of single responses were performed by paired Student's *t* test, or Wilcoxon test if the data were not normally distributed. Concentration-response curves were compared by two way ANOVA. *P* values of <0.05 were taken to indicate statistical significance.

Results

Effect of acivicin on the anti-aggregatory action of GSNO

Incubation of platelets with the γGT inhibitor, acivicin (1 mM for 20 min), had no effect on the anti-aggregatory action of GSNO. There was no significant difference between the concentration-response curves obtained in the presence and absence of acivicin, when compared by two way ANOVA ($P=0.60$, $n=4$) (data not shown).

Effect of transnitrosation on S-nitrosothiol stability and bioactivity

Spontaneous breakdown of albSNO (100 μM), measured by decrease in absorbance at 334 nm, was 0.19 ± 0.086 nmol·min^{-1} (mean±s.e.mean). This increased significantly to 1.52 ± 0.24 nmol min^{-1} in the presence of cysteine (100 μM) ($P<0.05$, $n=4$). By use of the oxyhaemoglobin assay, spontaneous NO release was estimated to be 0.28 ± 0.02 nmol·min^{-1}, increasing to 1.10 ± 0.20 nmol min^{-1} in the presence of cysteine. Breakdown of GSNO, measured by fall in absorbance at 334 nm, was increased by cysteine in a similar manner, from 0.56 ± 0.22 nmol min^{-1} (mean±s.e.mean) to 1.77 ± 0.23 nmol min^{-1}, although in this case the difference was not significant ($P=0.064$, $n=4$). NO release was confirmed by the oxyhaemoglobin assay, showing an increase from 0.24 ± 0.10 nmol min^{-1} to 5.56 ± 0.40 nmol min^{-1} in the presence of cysteine.

The platelet inhibitory action of albSNO was increased in the presence of cysteine (100 μM), as shown by a significant ($P<0.05$, $n=4$) shift to the left in the concentration-response curve for inhibition of platelet aggregation. In contrast, the anti-aggregatory action of GSNO was significantly ($P<0.05$, $n=4$) reduced in the presence of cysteine (Figure 1).

Effect of platelet pretreatment with BCS on the anti-aggregatory activity of GSNO

Platelets which had been pretreated with BCS and then re-suspended in BCS-free buffer showed resistance to the action of GSNO, demonstrated by a significant ($P<0.05$) shift to the right in their concentration-response curve to GSNO, when compared with untreated platelets (Figure 2).

S-nitrosothiol breakdown by enzymatic activity in platelet ultrasonicates

Enzymatic breakdown of S-nitrosothiols by platelet ultrasonicates, above the rate of spontaneous breakdown, was de-

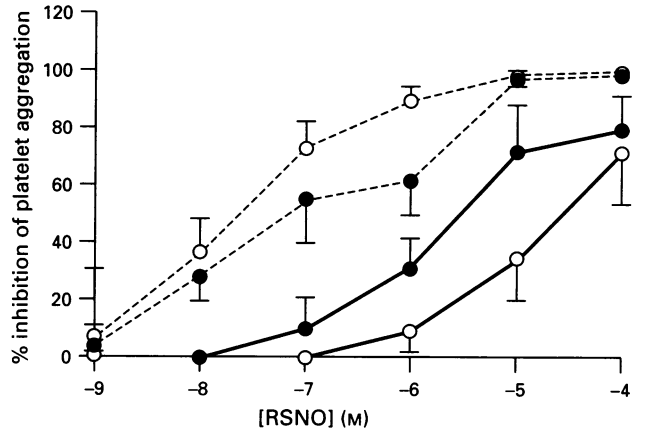

Figure 1 Inhibition of thrombin-induced platelet aggregation by S-nitrosoalbumin (albSNO; $10^{-9}-10^{-4}$ M) in the absence (○–○) and presence (●–●) of cysteine (100 μM), and by S-nitrosoglutathione (GSNO; $10^{-9}-10^{-4}$ M) in the absence (○- -○) and presence (●- -●) of cysteine (100 μM). Cysteine (or vehicle) was added to the platelet suspension 15 s before the addition of albSNO or GSNO. After a further 15 s platelet aggregation was induced with thrombin (0.01–0.02 units ml^{-1}). The concentration-response curve for albSNO was significantly shifted to the left in the presence of cysteine ($P<0.05$ by 2 way ANOVA), whereas the curve for GSNO was significantly shifted to the right in the presence of cysteine ($P<0.05$ by 2 way ANOVA). Values are mean from 4 experiments; vertical lines show s.e.mean.

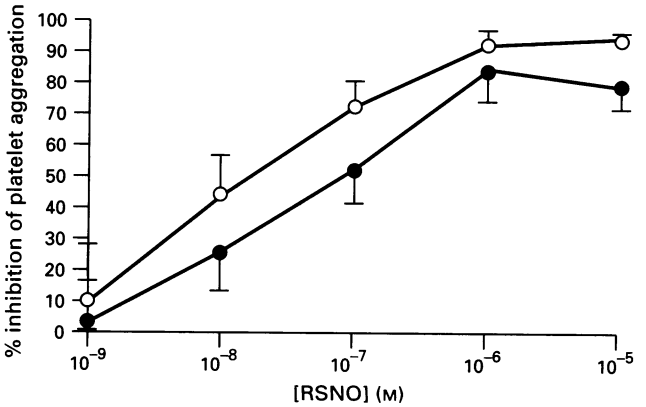

Figure 2 Effect of pretreatment of platelets with bathocuproine disulphonic acid (BCS) on the anti-aggregatory action of S-nitrosoglutathione (GSNO). Platelets were incubated with BCS (100 μM) for 5 min, washed and re-suspended in BCS-free buffer before measurement of the anti-aggregatory action of GSNO ($10^{-9}-10^{-5}$ M). Concentration-response curves obtained for pretreated (●) and untreated platelets (○) were significantly different ($P<0.05$ by 2 way ANOVA). Values are mean from 6 experiments; vertical lines show s.e.mean.

monstrable for each of the low molecular weight S-nitrosothiols tested, but albSNO was a poor substrate (Table 1). Incubation of GSNO with increasing amounts of platelet suspension (equivalent to 1, 2 and 4×10^8 platelets) was associated with a concentration-dependent increase in NO release of 0.19 ± 0.04 ($n=8$), 0.36 ± 0.105 ($n=7$) and 0.61 ± 0.084 ($n=4$) nmol min^{-1}, respectively (mean ± s.e.mean). This activity showed a pH optimum of between 8.0 and 8.5 (Figure 3), was sensitive to heat inactivation (Figure 4), but was not removed by dialysis, or by the γGT inhibitor acivicin (Figure 4). Activity was inhibited in a concentration-dependent manner by the copper chelator BCS ($10^{-7}-10^{-3}$ M), but not by the iron chelator BPS (Figure 5). Kinetic analysis of GSNO breakdown by use of a Hanes plot gave an apparent K_m of 12.4 ± 2.64 μM (mean ± s.e.mean, $n=5$) and a V_{max} of 0.21 ± 0.03 nmol min^{-1} per 10^8 platelets.

Table 1 Rate of enzyme-mediated NO release from four S-nitrosothiol compounds (100 nmol) in the presence of platelet ultrasonicates

S-nitrosothiol	*Number of observations*	*Initial rate of NO release* (nmol min^{-1} per 10^8 platelets)
GSNO	7	0.14 ± 0.016
SNAC	5	0.10 ± 0.011
SNAP	5	0.10 ± 0.012
AlbSNO	6	0.03 ± 0.014

Values are mean ± s.e.mean. Abbreviations: GSNO, S-nitrosoglutathione; SNAC, S-nitroso-N-acetyl-cysteine; SNAP, S-nitroso-N-acetyl-DL-pencillamine; albSNO, S-nitrosoalbumin.

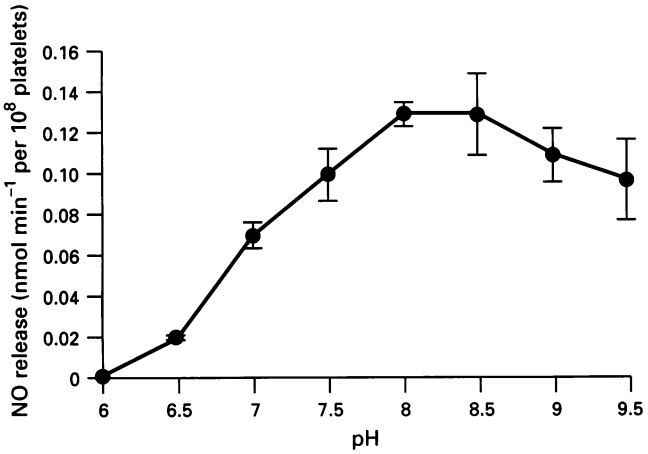

Figure 3 Effect of pH on enzymatic breakdown of S-nitrosoglutathione (GSNO) by platelet ultrasonicate. Activity of ultrasonicates was measured by use of assay buffer with pH adjusted to give a range of 6.0 to 9.5. Values are mean from 4 experiments; vertical lines show s.e.mean.

Discussion

We have confirmed and extended our earlier findings on the inhibitory effect of BCS, a Cu(I) specific chelator (Blair & Diehl, 1961), on the action of GSNO (Gordge *et al.*, 1995). Resistance to the anti-aggregatory action of GSNO remained evident in BCS-treated platelets even after re-suspension in BCS-free buffer. This supports our earlier conclusion that the action of BCS is not mediated by chelation of free Cu(I) ions, but by binding of BCS to a structure on the target platelet. It should be noted that the degree of inhibition of the action of GSNO was smaller than that observed in our earlier study (Gordge *et al.*, 1995), in which BCS was present in the surrounding buffer, and this suggests that the binding of BCS to the platelet is relatively weak. The exact nature of the platelet receptor for BCS remains unknown. However, our demonstration of a BCS-inhibitable enzymatic activity in platelet ultrasonicates which releases NO from low molecular weight S-nitrosothiols, suggests that the effect of BCS may be mediated by its ability to block the processing of low molecular weight S-nitrosothiols by a copper-dependent enzyme.

We also found albSNO to be a poor substrate for this enzyme, suggesting that the enzymatic processing mechanism may be inefficient for protein S-nitrosothiols. Anti-platelet activity of protein S-nitrosothiols may therefore depend upon other mechanisms, for example transnitrosation to acceptors such as cysteine. Thiol-nitrosothiol exchange is an established mechanism mediating the biological action of high molecular weight S-nitrosothiol compounds. Both the hypotensive and the platelet inhibitory actions of albSNO are accelerated and augmented by the addition of cysteine, and transfer of NO from the protein to the amino acid carrier has been directly demonstrated (Simon *et al.*, 1993; Scharfstein *et al.*, 1994). Our results are consistent with this mechanism. We found an accelerated decline in absorbance at 334 nm following addition of cysteine to albSNO, and interpret this as evidence of

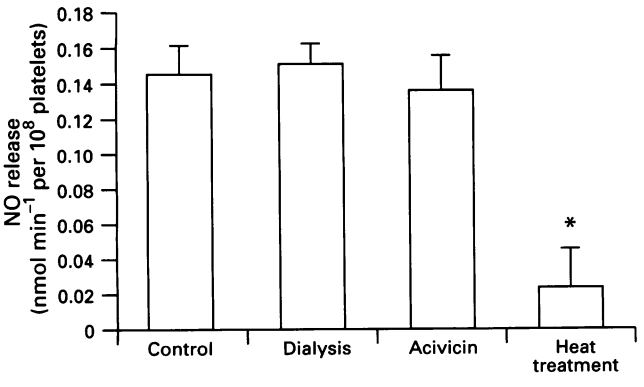

Figure 4 Effect on GSNO breakdown by enzymatic activity in platelet ultrasonicates of dialysis, pre-incubation for 20 min with acivicin (1 mM), and of heat treatment (90°C for 5 min). Values are mean from 4–6 experiments; vertical lines show s.e.mean. $*P<0.05$.

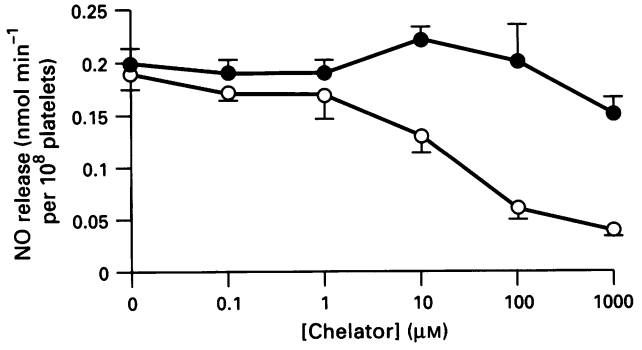

Figure 5 Effect of the copper chelator bathocuproine disulphonic acid (BCS; $10^{-7}-10^{-3}$ M) (○) and the iron chelator bathophenanthroline disulphonic acid (BPS; $10^{-7}-10^{-3}$ M) (●) on S-nitrosoglutathione (GSNO) breakdown by enzymatic activity in platelet ultrasonicates. Values are mean from 4 experiments; vertical lines show s.e.mean.

transnitrosation between albSNO and cysteine, resulting in the formation of the unstable species cysNO. Direct detection of NO release using the oxyhaemoglobin assay supported this interpretation. Our results showed a small discrepancy in the absolute values obtained with the two techniques, but such differences have been found by others (Arnelle & Stamler, 1995). The anti-aggregatory activity of albSNO was also increased by cysteine, implying that cysNO is a more potent anti-platelet agent than albSNO. This mechanism could be important in the transport, targeting and metabolism of NO from protein S-nitrosothiols, although the slow kinetics of these reactions have raised questions about their relevance *in vivo* (Meyer *et al.*, 1994). Such transnitrosation events have also been suggested to explain the action of GSNO (Askew *et al.*, 1995). The accelerated decline in absorbance at 334 nm we observed following addition of cysteine to GSNO is consistent with transnitrosation from GSNO to cysteine, but the result of this was to reduce, rather than enhance, the inhibition of platelet aggregation. We have previously demonstrated (Gordge *et al.*, 1995), that cysNO is less potent than GSNO as an anti-aggregatory agent, and these findings, together with our present results, indicate that transnitrosation reactions to form cysNO cannot explain the biological potency of GSNO.

The exact mechanisms by which different S-nitrosothiols donate NO to target cells remain to be identified. S-nitrosothiols inhibit platelet aggregation (Mellion *et al.*, 1983) and relax both vascular (Mathews & Kerr, 1993) and non-vascular smooth muscle (Gibson *et al.*, 1992; Gaston *et al.*, 1994) via an increase in cellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Lieberman *et al.*, 1991). Biological activity is also, in part, mediated via cyclic GMP-independent mechanisms involving transnitrosation events (Park, 1988). S-nitrosothiols decompose spontaneously in solution to release NO, a reaction that is accelerated by both light (Sexton *et al.*, 1994) and transition metals (McAninly *et al.*, 1993; Dicks *et al.*, 1996). Earlier assumptions that S-nitrosothiols acted via spontaneous release of NO have not been supported by pharmacological studies (Kowaluk & Fung, 1990; Mathews & Kerr, 1993; Gaston *et al.*, 1994), and there is evidence that the action of S-nitrosothiols involves enzymatic, rather than spontaneous, release of bioactive NO (Kowaluk & Fung, 1990; Radomski *et al.*, 1992; Clancy & Abramson, 1992). Processing of GSNO by membrane-bound γGT has been suggested to explain its potent biological activity, in view of its slow spontaneous release of NO (Askew *et al.*, 1995). Our results do not, however, support such a mechanism. An acivicin-inhibitable form of γGT is present on platelets (Sexton & Mutus, 1995), nevertheless we found that acivicin treatment failed to inhibit either the platelet-mediated breakdown or the anti-aggregatory action of GSNO. The enzyme responsible for GSNO processing is therefore distinct from γGT.

Patterns of response to S-nitrosothiols indicate that the processing and targeting of NO from S-nitrosothiol carriers may be controlled by membrane enzymes or receptors that differ between various target tissues (Mathews & Kerr, 1993). Such mechanisms would be crucial in the biological control of NO signalling via endogenous S-nitrosothiol carriers, and open the possibility of developing tissue-specific NO donors targeted to a particular organ or cell type. The platelet-selective features of GSNO have already been exploited in the clinical arena, both in a severe variant of pre-eclampsia (de Belder *et al.*, 1995) and in patients undergoing cardiopulmonary bypass (Langford *et al.*, 1994). This platelet selectivity might be explained by the copper-dependent NO-releasing mechanism shown in our study.

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