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Copper chelation-induced reduction of the biological activity of S-nitrosothiols

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1 The effect of copper on the activity of the S-nitrosothiol compounds S-nitrosocysteine (cysNO) and S-nitrosoglutathione (GSNO) was investigated, using the specific copper chelator bathocuproine sulphonate (BCS), and human washed platelets as target cells.

2 Chelation of trace copper with BCS (10 μM) in washed platelet suspensions reduced the inhibition of thrombin-induced platelet aggregation by GSNO; however, BCS had no significant effect on the anti-aggregatory action of cysNO. BCS inhibited cyclic GMP generation in response to both cysNO and GSNO.

3 The effect of BCS was rapid (within 30 s), and could be abolished by increasing the platelet concentration to $500 \times 10^9\ l^{-1}$.

4 In BCS-treated platelet suspensions, the addition of Cu^{2+} ions (0.37–2.37 μM) led to a restoration of both guanylate cyclase activation and platelet aggregation inhibition by GSNO.

5 The anti-aggregatory activity of GSNO was reduced in a concentration-dependent manner by the copper (I)-specific chelators BCS and neocuproine, and to a smaller extent by desferal.No effect was observed with the copper (II) specific chelator, cuprizone, the iron-specific chelator, bathophenanthroline sulphonate, or the broader-specificity copper chelator, D-penicillamine.

6 In both BCS-treated and -untreated platelet suspensions, cys NO was more potent than GSNO as a stimulator of guanylate cyclase. In BCS-treated platelet suspensions there was no significant difference between the anti-aggregatory potency of cysNO and GSNO; however, in untreated suspensions, GSNO was significantly more potent than cysNO. Thus, when copper was available, GSNO produced a greater inhibition of aggregation than cysNO, despite being a less potent activator of guanylate cyclase.

7 The breakdown of cysNO and GSNO was measured spectrophotometrically by decrease in absorbance at 334 nm. In Tyrode buffer, cysNO (10 μM) broke down at a rate of 3.3 μM min^{-1}. BCS (10 μM) reduced this to 0.5 μM min^{-1}. GSNO, however, was stable, showing no fall in absorbance over a period of 7 min even in the absence of BCS.

8 We conclude that copper is required for the activity of both cysNO and GSNO, although its influence on anti-aggregatory activity is only evident with GSNO. The stimulatory effect of copper is unlikely to be explained solely by catalysis of S-nitrosothiol breakdown. The enhancement by copper of th anti-aggregatory activity of GSNO, relative to cysNO, suggests that copper may be required for biological activity of GSNO which is independent of guanylate cyclase stimulation.

Keywords: S-nitrosothiol; S-nitrosoglutathione; S-nitrosocysteine; nitric oxide; copper; bathocuproine sulphonate; platelet aggregation; cyclic GMP

Introduction

Nitric oxide (NO) is highly reactive and will combine with a wide array of biological molecules (Stamler *et al.*, 1992b). The reaction of NO with biological thiols to form S-nitrosothiol intermediates is thought to be an important mechanism in the action of nitrovasodilator drugs (Ignarro *et al.*, 1981). Claims that endothelium-derived relaxing factor (EDRF), formed under pharmacological bioassay conditions, is S-nitrosocysteine (cysNO) (Ignarro, 1990), have not been substantiated (Feelisch *et al.*, 1994). Nevertheless, under physiological conditions the stabilization of NO by thiol is likely, and in the blood (Stamler *et al.*, 1992a) and tissues such as the lung (Gaston *et al.*, 1993) NO is found predominantly as S-nitrosothiol adducts of proteins and peptides. The formation of S-nitrosothiols may serve both to stabilize NO during delivery to its target, and to mitigate the toxicity of NO at its site of production.

S-nitrosothiols are effective anti-platelet compounds, inhibiting fibrinogen binding, aggregation and secretion by platelets via an increase in cellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Mellion *et al.*, 1983; Mendelsohn *et al.*, 1990; Lieberman *et al.*, 1991). The most abundant and physiologically relevant S-nitroso adducts are likely to be S-nitrosoproteins, for example, S-nitrosoalbumin and low molecular weight forms such as cysNO and S-nitrosoglutathione (GSNO). There is evidence that the anti-platelet effects of S-nitrosoproteins may be mediated via low molecular weight forms following thiol-nitrosothiol exchange (Simon *et al.*, 1993).

Most S-nitrosothiols are unstable, but measurements of their half-lives depend on the experimental conditions employed. Ignarro and co-workers (1981), reported the half-life of cysNO to be 15 min in oxygenated solutions, compared with more recent estimates of between 4–83 s (Ignarro, 1990; Feelisch *et al.*, 1994; Mathews & Kerr, 1993). The use of Tris-HCl, a metal binding agent (Dawson, 1986), might account for the stabilization of cysNO in the earlier study. Recently, the chemical breakdown of S-nitroso-N-acetyl D,L penicillamine (SNAP) has been shown to be catalysed by Cu^{2+} and Fe^{2+} ions (McAninly *et al.*, 1993), and the stabilization of S-nitrosothiols by EDTA, EGTA and L-cysteine (McAninly *et al.*, 1993; Feelisch *et al.*, 1994) is explained by the ability of these compounds to bind metal ions.

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GSNO is both stable and highly potent as an anti-platelet agent (Radomski *et al.*, 1992) and a smooth muscle relaxant (Kowaluk & Fung, 1990; Jansen *et al.*, 1992). These characteristics suggest that GSNO might not only be an important physiological molecule, but also a useful pharmacological agent. We have therefore chosen to study the low molecular weight S-nitrosothiols GSNO and cysNO. We have used the specific copper (I) chelator bathocuproine sulphonate (BCS) (2,9-dimethyl-4,7-diphenyll,10-phenanthroline) (Blair & Diehl, 1961) to investigate the influence of copper on both the stability and the biological activity of GSNO and cysNO.

Methods

Reagents

Modified Tyrode buffer with the following composition (in mM) was used for suspending washed platelets: NaCl 137.0, glucose 5.55, $CaCl_2$ 1.0, $NaHCO_3$ 11.9, $MgCl_2.6H_2O$ 1.05, $NaH_2PO_4.2H_2O$ 0.36, KCl 2.68 and HEPES 10.0. Using manufacturer's data, the approximate concentrations of copper and iron in the buffer were estimated to be less than 0.37 μM and 0.43 μM respectively. All components of the buffer, and also trisodium citrate and trichloroacetic acid were obtained from Merck (Lutterworth, UK). Bovine thrombin was obtained from Ortho Diagnostics (High Wycombe, UK), cyclic GMP radioimmunoassay kits from Amersham plc (High Wycombe, UK), and prostacyclin was a kind gift from Dr M. Radomski (Wellcome Laboratories, Beckenham, UK). All other reagents were obtained from Sigma (Poole, UK). MilliQ grade water (resistivity 18 MΩ.cm) was used for all solutions.

Preparation of S-nitrosothiols

Fresh S-nitroso-L-cysteine (cysNO) for use in platelet experiments was prepared daily by a modification of the method of Hart (1985), by mixing one volume of 40 mM L-cysteine/40 mM HCl/5 μM BCS with one volume of 44 mM $NaNO_2$ (stock) at 0–4°C for 10 min, followed by dilution with two volumes of 50 mM NaCl, 40 mM sodium phosphate, 5 μM BCS and 5 μM deferoxamine mesylate (desferal), at a pH of 7.4. For experiments to be performed in the absence of BCS, the nitrosating mixture was not neutralised with buffer until the time of dilution and assay.

S-nitrosoglutathione (GSNO) was prepared by mixing one volume of 40 mM glutathione/40 mM HCl with one volume of 44 mM $NaNO_2$ for 120 min at 0°C. GSNO was then precipitated with ice-cold acetone, filtered, and washed three times with further ice-cold acetone. The precipitate was then dried under vacuum and stored at −70°C.

Concentration of each S-nitrosothiol was estimated from the absorbance at 334 nm (extinction coefficients (334 nm): 0.96 and 0.87 mM cm^{-1} for GSNO and cysNO respectively (D.J. Meyer, unpublished data)). Yields were 90–99%.

Platelet preparation

Blood was obtained from healthy volunteers and anticoagulated with trisodium citrate (10.6 mM final concentration). Platelet rich plasma (PRP) was prepared by centrifugation of blood for 15 min at 20°C and 150 *g*. Residual leucocytes and erythrocytes were removed from PRP by centrifugation at 20°C and 110 *g* for 10 min, after which prostacyclin (300 ng ml^{-1}) was added and platelets were pelleted by centrifugation at 20°C and 600 *g* for 10 min. Following resuspension in Tyrode buffer containing 300 ng ml^{-1} prostacyclin, platelets were again pelleted, and finally resuspended at a count of 200 × 10^9 l^{-1} in Tyrode buffer. Washed platelets were stored at room temperature in sealed syringes (to prevent change in pH) for at least 2 h before use to allow recovery from the effects of prostacyclin treatment.

Measurement of cyclic GMP formation

Platelets (180 μl) were equilibrated at 37°C for 5 min before addition of cysNO or GSNO, in a volume of 20 μl. Stimulation of guanylate cyclase was allowed to proceed for 2 min, after which the reaction was stopped by the addition of 200 μl of ice-cold 10% v/v trichloroacetic acid. Samples were mixed, chilled on ice for 5 min then centrifuged at 13 000 *g* for 2 min. Supernatant was removed and stored at −20°C. Prior to assay samples were further extracted using tri-n-octylamine in 1,1,2-trichlorotrifluoroethane, and cyclic GMP measured by radioimmunoassay as previously described (Gordge & Neild, 1992).

Measurement of platelet aggregation inhibition

Aggregation of washed platelets was induced with a submaximal concentration of bovine thrombin (0.012–0.025 u ml^{-1}); 240 μl of platelet suspension was equilibrated for 2 min at 37°C, then treated with either nitrosothiol or vehicle control in a volume of 30 μl for 15 s, after which 30 μl of thrombin was added. Platelet aggregation was measured at 37°C at a stirring rate of 1000 r.p.m., with a Payton turbidometric aggregometer, as the maximum change in light transmission occurring within 3 min, expressed as a percentage of the difference in light transmission between Tyrode buffer and starting platelet suspension. Inhibition of this reaction by cysNO and GSNO was assessed by comparison with the aggregation response obtained in the presence of vehicle alone.

Effect of copper chelation with BCS on the action of GSNO and CysNO

Platelet guanylate cyclase stimulation by GSNO and CysNO (10^{-9}–10^{-5} M) was measured, as described above, in the presence of BCS (10 μM final concentration). Platelets were suspended in Tyrode buffer containing BCS, and all reagents used in the experiments were diluted in water containing BCS. Parallel assessments of the action of GSNO and cysNO were made in the absence of BCS, and their potencies compared in the presence and absence of the copper chelator. The effect of BCS on the anti-aggregatory action of the two S-nitrosothiol compounds was also assessed in a similar way.

Time-course of the action of BCS to reduce the anti-aggregatory activity of GSNO

In order to assess the time required for BCS to reduce the activity of GSNO, platelets were pre-incubated at room temperature with BCS (10 μM) or vehicle for periods of 30 s to 60 min, after which the ability of GSNO (1 μM) to inhibit thrombin-induced platelet aggregation was assessed as described above.

Effect of increasing platelet count on the action of BCS to reduce the anti-aggregatory action of GSNO

Platelet suspensions with counts varying from 100–500 × 10^9 l^{-1} were prepared in Tyrode buffer. BCS (10 μM) was then pe-incubated for 3 min with aliquots of these suspensions, after which the ability of GSNO (1 μM) to inhibit thrombin-induced aggregation was assessed as described above.

Effect of different chelating agents on the action of GSNO

The effects on the anti-aggregatory action of GSNO of the following chelating agents were investigated: the copper (I) specific agents BCS (Blair & Diehl, 1961) and neocuproine (2,9-dimethyl 1,10-phenanthroline) (Diehl & Smith, 1958);

the copper (II) specific agent cuprizone (*bis*cyclohexanone-oxalyldihydrazone) (Peterson & Bollier, 1955), the iron specific agent bathophenanthroline sulphonate (BPS) (4,7-diphenyl, 1,10 phenanthroline) (Blair & Diehl, 1961), and the less specific agents D-penicillamine and desferal. Stock solutions of chelating agents (1 mM) were prepared in water, except for cuprizone, which was dissolved in 50% (v / v) ethanol. Platelets were pre-incubated for 3 min with chelator (100 μM final concentration) or vehicle, prior to the addition of either GSNO (1 μM final concentration) or vehicle. After 15 s platelet aggregation was induced with thrombin and the inhibitory action of GSNO was assessed as described above. Control experiments were also performed in which GSNO was excluded, in order to assess the direct effects of each chelator on platelet aggregation.

Dose-response experiments were performed with BCS, neocuproine and desferal, in which the final concentration of each chelator was increased from 10^{-7} to 10^{-4} M. The effect of this on the action of GSNO (1 μM) both to inhibit platelet aggregation and to elevate platelet cyclic GMP was assessed as described above.

Effect of addition of copper to BCS-treated platelets on the action of GSNO

In further experiments, the effect on the activity of GSNO of restoring Cu^{2+} ions to BCS-chelated platelet suspensions was assessed. Platelets were pelleted from PRP as described above, after which they were washed twice in Tyrode buffer, the first wash in the presence of 10 μM BCS and the second in the presence of 1 μM BCS. Platelets were finally suspended in BCS-free Tyrode in which the concentration of copper from contamination of the buffer salts was estimated to be <0.37 μM. Aliquots of the platelet suspension were then supplemented with 10 μM BCS (to remove all free copper), or $CuSO_4$ to give final concentrations of Cu^{2+} of approximately 0.37–2.37 μM. After 10 min equilibration at 37°C the effect of GSNO (1 μM) on both stimulation of soluble guanylate cyclase and inhibition of platelet aggregation were measured, as described above. In these experiments, platelets used for measurement of guanylate cyclase stimulation were treated wtih isobutyl methyl xanthine (IBMX) (1 mM) for 15 min prior to use in order to inhibit phosphodiesterase activity.

In a similar way the influence of Fe^{3+} ions on the activity of GSNO was assessed. Platelet suspensions were washed in the presence of 10 μM and 1 μM desferal, an iron chelator, and then supplemented with either desferal (10 μM) or $FeCl_3$. The concentration of iron in the Tyrode buffer used in the experiments was estimated to be <0.43 μM.

Effect of copper chelation on the anti-aggregatory action of prostacyclin, sodium nitroprusside and dibutyryl cyclic GMP

In control experiments, the inhibitory effect of prostacyclin (10^{-2}–10^{2} ng ml^{-1}), sodium nitroprusside (10^{-7}–10^{-3} M) and dibutyryl cyclic GMP (10^{-5}–10^{-3} M) on thrombin-induced aggregation of washed platelets was assessed in the presence and absence of BCS (10 μM), as described above.

Effect of BCS on the rate of breakdown of cysNO and GSNO in Tyrode buffer

CysNO and GSNO (both 10 μM) were prepared as described above, but without the addition of BCS. Under acid conditions, both were stable for at least 2 h (less than 0.001 units fall in absorbance at 334 nm). Each compound was then diluted to a final concentration of 10 μM in Tyrode buffer, in the presence and absence of BCS (10 μM) and their breakdown monitored over a period of 7 min by the fall in absorbance at 334 nm, with a Cary 1E spectrophotometer.

Statistics

Dose-response curves were compared by two-way analysis of variance. In experiments in which the action of GSNO was assessed after restoration of Cu^{2+} and Fe^{3+} ions to BCS- or desferal-washed platelets, variation in intra-platelet cyclic GMP and aggregation inhibition obtained at different metal ion concentrations was analysed by Kruskal Wallis one-way analysis of variance.

Results

Effect of copper chelation on platelet cyclic GMP concentrations following stimulation with cysNO and GSNO

BCS caused a significant shift to the right in the dose-response curves of cyclic GMP generation in response to both cysNO ($P=0.027$) and GSNO ($P<0.001$), The influence of copper chelation was greater with GSNO than with cysNO (Figure 1). Comparison of dose-response data obtained with the two different S-nitrosothiols showed that, both in the presence and absence of BCS the elevation of cyclic GMP in platelets was significantly greater following stimulation with cysNO than with GSNO ($P<0.001$).

Effect of copper chelation on the inhibition of platelet aggregation by cysNO and GSNO

BCS caused a significant shift to the right in the anti-aggregatory dose-response curve of GSNO ($P<0.001$), indicating a loss of inhibitory activity. The anti-aggregatory activity of cysNO, however, was not significantly altered by BCS ($P=0.455$) (Figure 2). Comparison of the dose-response data obtained with the two different S-nitrosothiols showed that in the presence of BCS there was no significant difference between their anti-aggregatory potency. In the absence of BCS, however, GSNO was significantly more potent than cysNO ($P<0.001$).

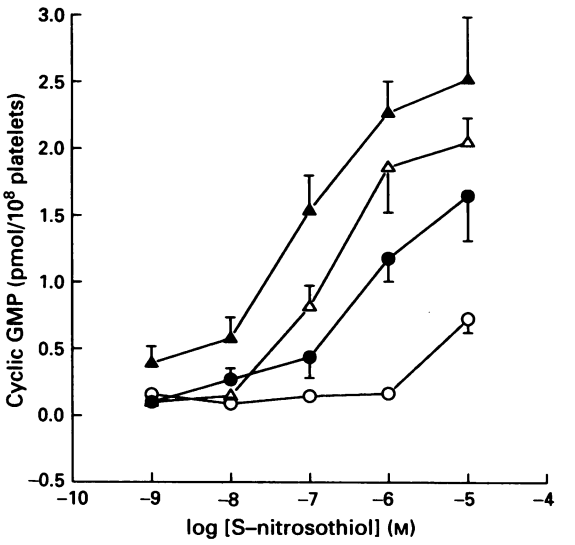

Figure 1 Intraplatelet cyclic GMP concentrations following stimulation for 2 min with cysNO (10^{-9}–10^{-5} M) in the presence (Δ) and absence (▲) of BCS (10 μM), and GSNO (10^{-9}–10^{-5} M) in the presence (○) and absence (●) of BCS (10 μM). Copper concentration in the absence of BCS was estimated to be <0.37 μM. The shift to the right of the dose-response curves in the presence of BCS, analysed by two-way ANOVA, was significant for both cysNO ($P=0.027$) and GSNO ($P<0.001$). Results are mean (±s.e.mean) from four experiments. For abbreviations, see text.

Time course of the action of BCS, and the effect of platelet count on the action of BCS to reduce the anti-aggregatory activity of GSNO

BCS acted rapidly; inhibition of the anti-aggregatory action of GSNO was maximal after pre-incubation of platelets with BCS for 30 s. There was no further increase in the effect of BCS when pre-incubation periods were prolonged up to 60 min ($n=2$; data not shown).

The effectiveness of BCS was diminished by increasing platelet concentration. At a platelet count of $100 \times 10^9\ l^{-1}$ BCS reduced the platelet inhibitory action of GSNO from 92% to 57%. Increasing the platelet count led to a progressive loss of the ability of BCS to reduce the action of GSNO (Figure 3).

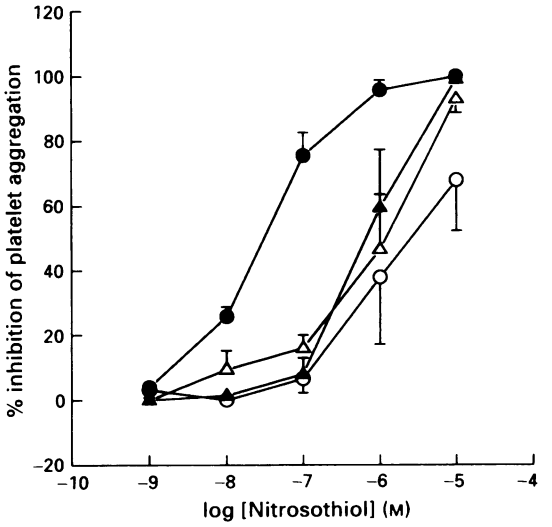

Figure 2 Inhibition of thrombin-induced platelet aggregation by cysNO (10^{-9}–10^{-5} M) in the presence (Δ) and absence (▲) of BCS (10 μM), and by GSNO (10^{-9}–10^{-5} M) in the presence (○) and absence (●) of BCS (10 μM). Copper concentration in the absence of BCS was estimated to be <0.37 μM. BCS caused no significant alteration in the dose-response data for cysNO, but the dose-response curve for GSNO was significantly shifted to the right in the presence of BCS ($P<0.001$) (two-way ANOVA). Results are mean (±s.e.mean) from four experiments. For abbreviations, see text.

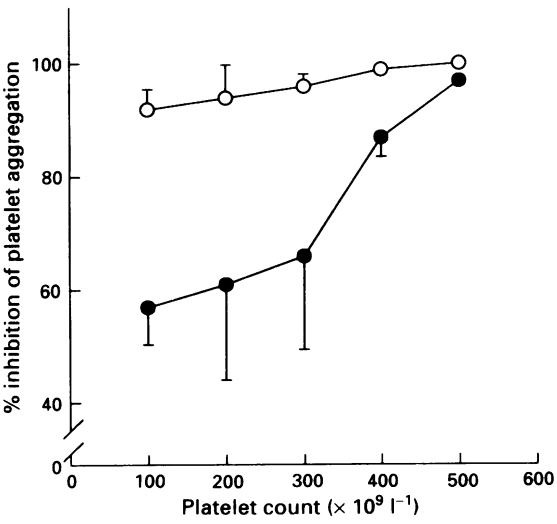

Figure 3 Effect of increasing platelet count on the action of BCS. Platelet suspensions were prepared with counts ranging from $100-500 \times 10^9\ l^{-1}$. The ability of GSNO (1 μM) to inhibit thrombin-induced platelet aggregation was then tested in the presence (●) and absence (○) of BCS (10 μM). Results are mean (±s.e.mean) from four experiments. For abbreviations, see text.

Effect of different chelating agents on the action of GSNO

At concentrations up to 100 μM, none of the chelating agents used in these experiments had any direct effect on platelet aggregation, except for neocuproine, which at concentrations >10 μM, inhibited aggregation by approximately 30%.

In the absence of chelating agents, 1 μM GSNO produced 93–99% inhibition of platelet aggregation. Pre-incubation of platelets with BPS, penicillamine or cuprizone (all at 100 μM) failed to alter this anti-aggregatory activity (mean aggregation inhibition by GSNO 100%, 99% and 96% respectively) ($n=4$). The action of GSNO was, however, reduced by 100 μM concentrations of BCS, neocuproine and, to a smaller extent, desferal (mean aggregation inhibition by GSNO reduced to 43%, 71% and 79% respectively) ($n=4$).

Dose-response experiments showed a progressive reduction in the aggregation inhibitory action of GSNO in the presence of increasing concentrations of BCS, neocuproine and desferal, although in the case of neocuproine this trend was reversed at concentrations above 10 μM (Figure 4). This reversal was probably due to the direct inhibitory effect on platelet aggregation of high concentrations of neocuproine.

Cyclic GMP measurements showed that both BCS and neocuproine, but not desferal, inhibited the elevation of cyclic GMP by GSNO in a dose-dependent fashion (Figure 5). The baseline level of cyclic GMP formation was lower in the experiments with desferal. The reason for this is uncertain.

Effect of restoring Cu^{2+} and Fe^{3+} ions to platelets washed in the presence of either BCS or desferal

The presence of BCS (10 μM) was associated with a significant reduction of both the anti-aggregatory activity and stimulation of intra-platelet cyclic GMP by GSNO (1 μM) ($P<0.05$). Restoration of Cu^{2+} ions to platelets which had been washed in the presence of BCS resulted in a return to full activity of GSNO.

Iron chelation with desferal (10 μM) resulted in only a small, and statistically insignificant reduction in the activity of GSNO (Figure 6a and b).

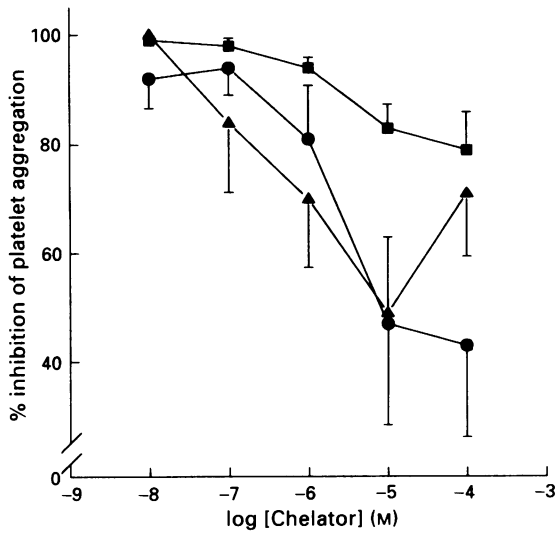

Figure 4 Inhibition of the anti-aggregatory action of GSNO by different chelating agents. The ability of GSNO (1 μM) to inhibit thrombin-induced aggregation was tested in platelet suspensions pre-incubated with increasing concentrations of either BCS (●), neocuproine (▲) or desferal (■). (Note that neocuproine at a concentration $>10^{-5}$ M showed a direct inhibitory effect on platelet aggregation, independent of GSNO). Results are mean (±s.e.mean) of four experiments. For abbreviations, see text.

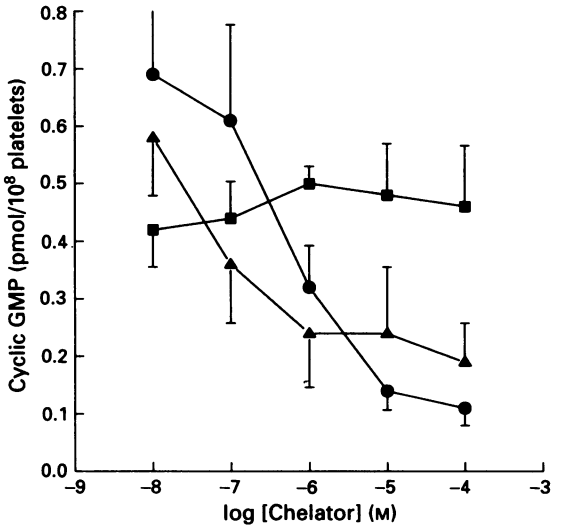

Figure 5 Inhibition of GSNO-stimulated cyclic GMP formation by different chelating agents. The ability of GSNO (1 μM) to stimulate cyclic GMP formation was tested in platelet suspensions pre-incubated with increasing concentrations of either BCS (●), neocuproine (▲) or desferal (■). Results are mean (±s.e.mean) of four experiments. For abbreviations, see text.

Effect of copper chelation on the anti-aggregatory action of prostacyclin, sodium nitroprusside and dibutyryl cyclic GMP

There was no significant alteration in the anti-aggregatory action of either prostacyclin ($n=3$), sodium nitroprusside ($n=3$), or dibutyryl cyclic GMP ($n=2$), following treatment of washed platelet suspensions with 10 μM BCS (data not shown).

Effect of BCS on the breakdown of cysNO and GSNO in Tyrode buffer

CysNo (10 μM) was unstable in Tyrode buffer, breaking down at a rate of 3.3 μM min^{-1}. In the presence of BCS (10 μM), however, this rate of breakdown was reduced to 0.5 μM min^{-1}. In contrast GSNO was stable under these conditions, showing no change in absorbance over a period of 7 min whether or not BCS was present.

Discussion

We have shown that specific chelation of copper with BCS in human platelet suspensions significantly inhibits the anti-aggregatory activity of GSNO. In addition, BCS significantly reduces the stimulation of platelet guanylate cyclase by both cysNO and GSNO. Copper is therefore required for the full activity of these S-nitrosothiol compounds.

What is the mechanism by which copper augments S-nitrosothiol action? The simplest possibility is via catalysis of S-nitrosothiol breakdown, leading to an increase in the availability of NO. Our data do not, however, support such an explanation. We measured the chemical breakdown of both cysNO and GSNO in Tyrode buffer identical to that used in our platelet experiments. We observed no spontaneous breakdown of GSNO, but the breakdown of cysNO under these conditions was rapid, and could be inhibited by copper chelation with BCS. In contrast, BCS had a profound effect on the anti-aggregatory activity of GSNO but did not significantly alter that of cysNO. There was therefore a discrepancy between the effects of copper chelation on the chemical breakdown and the biological activity of the two compounds. Other authors have demonstrated a discrepancy between the

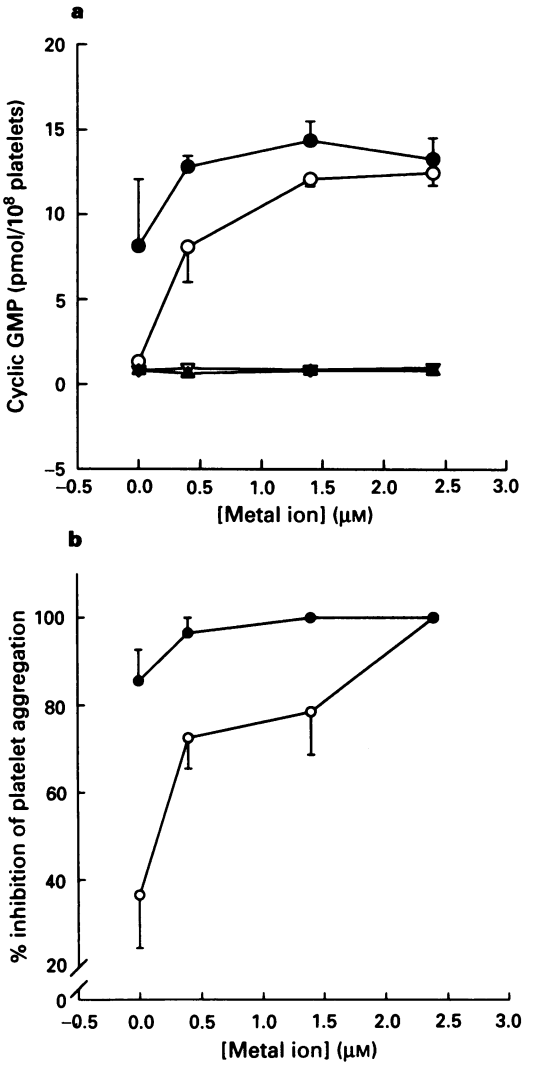

Figure 6 Intraplatelet cyclic GMP concentrations (a), and inhibition of thrombin-induced platelet aggregation (b) induced by GSNO (1 μM) in platelet suspensions containing different concentrations of Cu^{2+} (○) and Fe^{3+} ions (●). Basal levels of cyclic GMP were unaffected by either Cu^{2+} (△) or Fe^{3+} ions (▲).

Metal ions were removed from platelet suspensions by washing in either BCS or desferal, and then restored at the concentrations indicated. For values of zero free Cu^{2+} or Fe^{3+}, experiments were performed in the presence of BCS (10 μM) or desferal (10 μM) respectively. The variation in both cyclic GMP response and percentage inhibition of aggregation associated with alteration of Cu^{2+} concentration was statistically significant ($P<0.05$) (Kruskal Wallis one-way ANOVA). Variation in responses associated with different Fe^{3+} concentrations was not significant. Results are mean (±s.e.mean) from four experiments. Note that, in these experiments, measurements of cyclic GMP were made with platelet suspensions pretreated with IBMX (1 mM). For abbreviations, see text.

biological activity of S-nitrosothiols and their rate of spontaneous liberation of NO (Kowaluk & Fung, 1990; Mathews & Kerr, 1993). Kowaluk & Fung (1990), using bovine smooth muscle cells, found evidence of a membrane-associated enzyme catalysing the generation of NO from SNAP. A similar, membrane-associated activity accelerating the release of NO from GSNO has also been described in human platelets (Radomski *et al.*, 1992). It is not yet known whether copper influences this enzyme activity.

Copper might alter the redox properties of NO released from S-nitrosothiol carriers. It has recently been shown that optimal activation of guanylate cyclase via its haem group requires NO to be in its oxidised (NO^+, nitrosonium) form

(Severina *et al.*, 1992). Such an oxidation could occur during the conversion of Cu(II) to Cu(I).

Our findings using a range of chelating agents suggest that copper in its reduced form (Cu(I)) is required for the full activity of GSNO. Concentration-dependent inhibition of both the anti-aggregatory and the guanylate cyclase-stimulating activity of GSNO was shown by the Cu(I) specific agents BCS and neocuproine, but not by the Cu(II) specific agent cuprizone, nor by the iron-specific agent BPS (structurally very similar to BCS) (Diehl & Smith, 1958; Blair & Diehl, 1961). Desferal produced a small, concentration-dependent inhibition of the anti-aggregatory action of GSNO, but this was not matched by changes in cyclic GMP formation. Desferal may display effects unrelated to its metal-binding properties (Halliwell, 1989), and its influence on the anti-aggregatory action of GSNO may therefore be non-specific. D-Penicillamine was ineffective, despite the fact that it can complex Cu(I) ions. This might be explained by its inability to mobilize protein-bound copper (Laurie & Prime, 1979), or by the fact that copper bound to D-penicillamine is rapidly exchangeable and can participate in cellular processes such as uptake on a transport system (McArdle *et al.*, 1989). Thus the Cu(I)-binding properties of D-penicillamine may be inadequate to interfere with the action of GSNO.

It is unlikely that the action of BCS is mediated via chelation of free Cu(I), since such ions are unstable and would be oxidized to copper (II) in solution. In addition, the concentration of BCS required to bind $<0.37\ \mu M$ free copper in Tyrode buffer would be lower than the maximally effective concentration observed ($10-100\ \mu M$). Instead, the target for BCS may be a copper (I)-containing protein or enzyme on the platelet. Consistent with this, the effect of a fixed concentration BCS could be 'diluted out' by increasing the platelet concentration, suggesting that BCS was interacting with a platelet, rather than a buffer, component. Protein-bound copper can be held in the form of reduced Cu(I) (Poillon & Dawson, 1963), and this may explain our finding that incubation of BCS-washed platelets with Cu(II) ions (as $CuSO_4$) could increase the action of GSNO.

BCS failed to influence the action of either sodium nitroprusside or dibutyryl cyclic GMP. The effect of BCS on the activity of S-nitrosothiols cannot therefore be explained by interference with redox regulation of soluble guanylate cyclase (White *et al.*, 1976) or cyclic GMP-dependent protein kinase (Landgraf *et al.*, 1991), nor by binding of copper in the guanylate cyclase enzyme (Gerzer *et al.*, 1981). In addition, the likelihood that BCS is acting by some non-specific mechanism is weakened by its failure to influence platelet inhibition by prostacyclin.

Our study has also shown that the anti-aggregatory potency of GSNO was greater than that of cysNO, despite the fact that cysNO appeared to be a more efficient stimulator of guanylate cyclase. This augmentation of the inhibitory action of GSNO depended upon the availability of copper, since it was abolished by BCS. In a recent comparison of the properties of eight S-nitrosothiol compounds, Mathews & Kerr (1993) found that the biological activities of these agents did not correlate with their different abilities to stimulate soluble guanylate cyclase. They found GSNO to be the second most potent inhibitor of platelet aggregation but the least active stimulator of guanylate cyclase. These findings raise the possibility that the biological activity of GSNO (and other S-nitrosothiols) may be mediated in part via pathways independent of cyclic GMP. Covalent modification of protein sulphydryl groups by rapid transnitrosation may contribute to the activity of GSNO and other S-nitrosothiols (Park, 1988). In human platelets NO-donors inactivate the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GADPH) by S-nitrosylation and subsequent NAD-dependent covalent modification of a specific cysteine residue (cys-149). This modification appears to influence the transport of NO into platelets from some, but not all NO-donors (McDonald *et al.*, 1993). Thus differences between the biological activities of different S-nitrosothiols (and other NO-donors) might arise from variation in the pattern of protein modification. Our observations of the influence of BCS on the relative activities of cysNO and GSNO suggest that copper participates in these cyclic GMP-independent interactions.

Despite the fact that S-nitrosothiols are potent mediators of NO signalling, the mechanism(s) by which they donate NO and alter cellular function are poorly understood. Our results indicate that copper is a cofactor which increases the biological activity of these compounds. It is important to note that nitrosothiols as generally prepared may contain significant levels of copper (from contamination of reagents such as HCl) which may alter their stability compared with nitrosothiols occurring *in vivo*. It is also of interest that rats fed a copper-deficient diet develop a defect in NO-mediated vasodilatation (Schuschke *et al.*, 1992), an observation which supports the physiological relevance of our findings. Although the mechanism of action of copper is still unclear, it appears that both cyclic GMP-dependent and -independent pathways may be involved.

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