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Evidence for a cyclic GMP-independent mechanism in the anti-platelet action of S-nitrosoglutathione

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1 We have measured the ability of a range of NO donor compounds to stimulate cyclic GMP accumulation and inhibit collagen-induced aggregation of human washed platelets. In addition, the rate of spontaneous release of NO from each donor has been measured spectrophotometrically by the oxidation of oxyhaemoglobin to methaemoglobin. The NO donors used were five s-nitrosothiol compounds: S-nitrosoglutathione (GSNO), S-nitrosocysteine (cysNO), S-nitroso-N-acetyl-DL-penicillamine (SNAP), S-nitroso-N-acetyl-cysteine (SNAC), S-nitrosohomocysteine (homocysNO), and two non-nitrosothiol compounds: diethylamine NONOate (DEANO) and sodium nitroprusside (SNP).

2 Using 10 μM of each donor compound, mean $\pm$ s.e.mean rate of NO release ranged from 0.04 ± 0.001 nmol min^{-1} (for SNP) to 3.15 ± 0.29 nmol min^{-1} (for cysNO); cyclic GMP accumulation ranged from 0.43 ± 0.05 pmol per 10^8 platelets (for SNP) to 2.67 ± 0.31 pmol per 10^8 platelets (for cysNO), and inhibition of platelet aggregation ranged from $40 \pm 6.4\%$ (for SNP) to $90 \pm 3.8\%$ (for SNAC).

3 There was a significant positive correlation between the rate of NO release and the ability of the different NO donors to stimulate intra-platelet cyclic GMP accumulation ($r = 0.83$; $P = 0.02$). However, no significant correlation was observed between the rate of NO release and the inhibition of platelet aggregation by the different NO donors ($r = -0.17$), nor was there a significant correlation between cyclic GMP accumulation and inhibition of aggregation by the different NO donor compounds ($r = 0.34$).

4 Comparison of the dose-response curves obtained with GSNO, DEANO and 8-bromo cyclic GMP showed DEANO to be the most potent stimulator of intraplatelet cyclic GMP accumulation ($P < 0.001$ *vs* both GSNO and 8-bromo cyclic GMP), but GSNO to be the most potent inhibitor of platelet aggregation ($P < 0.01$ *vs* DEANO, and $P < 0.001$ *vs* 8-bromo cyclic GMP).

5 The rate of NO release from GSNO, and its ability both to stimulate intra-platelet cyclic GMP accumulation and to inhibit platelet aggregation, were all significantly diminished by the copper (I) (Cu^+) chelating agent bathocuproine disulphonic acid (BCS). In contrast, BCS had no effect on either the rate of NO release, or the anti-platelet action of the non-nitrosothiol compound DEANO.

6 Cyclic GMP accumulation in response to GSNO (10^{-9}–10^{-5}M) was undetectable following treatment of platelets with ODQ (100 μM), a selective inhibitor of soluble guanylate cyclase. Despite this abolition of guanylate cyclase stimulation, GSNO retained some ability to inhibit aggregation, indicating the presence of a cyclic GMP-independent component in its anti-platelet action. However, this component was abolished following treatment of platelets with a combination of both ODQ and BCS, suggesting that Cu^+ ions were required for the cyclic GMP-independent pathway to operate.

7 The cyclic GMP-independent action of GSNO, observed in ODQ-treated platelets, could not be explained by an increase in intra-platelet cyclic AMP.

8 The impermeable thiol modifying agent *p*-chloromercuriphenylsulphonic acid (CMPS) produced a concentration-dependent inhibition of aggregation of ODQ-treated platelets, accompanied by a progressive loss of detectable platelet surface thiol groups. Additional treatment with GSNO failed to increase the degree of aggregation inhibition, suggesting that a common pathway of thiol modification might be utilized by both GSNO and CMPS to elicit cyclic GMP-independent inhibition of platelet aggregation.

9 We conclude that NO donor compounds mediate inhibition of platelet aggregation by both cyclic GMP-dependent and -independent pathways. Cyclic GMP generation is related to the rate of spontaneous release of NO from the donor compound, but transfer of the NO signal to the cyclic GMP-independent pathway may depend upon a cellular system which involves both copper (I) (Cu^+) ions and surface membrane thiol groups. The potent anti-platelet action of GSNO results from its ability to exploit this cyclic GMP-independent mechanism.

Keywords: Nitric oxide; S-nitrosoglutathione; platelet aggregation; guanylate cyclase; cyclic GMP; cyclic GMP-independent; copper; thiols

Introduction

Nitric oxide (NO) is a potent modulator of cellular function and NO donors have proved a useful tool in both experimental and clinical settings. Activation of soluble guanylate cyclase by NO indicates that cellular responses are mediated via the second messenger guanosine 3′:5′-cyclic monophosphate (cyclic GMP), but recent evidence suggests that there may also be cyclic GMP-independent mechanisms (Garg & Hassid, 1991; Clancy *et al*., 1992; Bolotina *et al*., 1994; Lander *et al*., 1997).

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S-nitrosothiols are adducts of nitric oxide (NO) and free sulphydryl (-SH) groups, for example cysteine residues on proteins and peptides. S-nitrosothiols are detectable in both blood and tissues (Stamler *et al*., 1992; Gaston *et al*., 1993; Naseem *et al*., 1996; Do *et al*., 1996), and it is speculated that they may be a means to preserve NO from inactivation by superoxide and/or haemoglobin within the biological environment (Stamler, 1996). In addition, S-nitrosothiol formation may mediate the bioactivity of nitrovasodilator drugs (Ignarro *et al*., 1981). S-nitrosothiols act as NO donors to inhibit platelet aggregation (Mellion *et al*., 1983) and to relax both vascular (Mathews & kerr, 1993) and non-vascular smooth muscle (Gibson *et al*., 1992; Gaston *et al*., 1994), actions that are accompanied by an increase in cellular cyclic GMP (Lieberman *et al*., 1991). However, there are discrepancies between the rank order of biological effectiveness of different S-nitrosothiol compounds, and (a) their respective rates of spontaneous NO release, and (b) their ability to stimulate guanylate cyclase and elevate intracellular cyclic GMP (Mathews & Kerr, 1993). Recent data have shown that S-nitrosothiol compounds can regulate cellular function in a cyclic GMP-independent fashion, by nitrosative or oxidative modification of protein thiols (Arnelle & Stamler, 1995).

We have previously shown that S-nitrosoglutathione (GSNO), a physiological S-nitrosothiol compound, possesses potent anti-platelet aggregatory activity despite a slow rate of NO release and a modest capacity to stimulate intra-platelet cyclic GMP accumulation. This potency depended on the availability of platelet-associated copper (I) (Cu^+), since it was inhibited by copper (I) selective chelators (Gordge *et al*., 1995; 1996). Our results suggested that the anti-platelet action of GSNO might not be fully explained by cyclic GMP generation.

We have now extended these investigations by examining a range of NO donor compounds and analysing the relationship between their rates of spontaneous NO release, their ability to stimulate cyclic GMP generation, and their inhibition of platelet aggregation. In addition, we have compared in detail the anti-platelet actions of GSNO with those of diethylamine NONOate (DEANO), a non-nitrosothiol compound characterized by a rapid rate of spontaneous NO release (Morley & Keefer, 1993). We have explored the effect of (1) copper chelation, and (2) inhibition of soluble guanylate cyclase activity, on the anti-platelet action of these two compounds. Our results confirm the existence of a cyclic GMP-independent pathway in the anti-platelet action of GSNO, and we have therefore performed experiments to investigate the possible role in this pathway of (a) cyclic AMP and (b) modification of platelet surface thiol groups.

Methods

Preparation of S-nitrosothiols

S-nitrosothiols were prepared as previously described with acidified nitrite as nitrosating agent (Gordge *et al*., 1996). Stock S-nitrosothiol solutions were prepared fresh each day, and stored on ice in 20 mM HCl and protected from light until used. The concentration of each S-nitrosothiol was estimated from the absorbance at 334 nm, with 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 and S-nitrosoglutathione (GSNO) 0.85.

Platelet preparation

Citrated blood was obtained from healthy volunteers who, during the preceding 10 days, had not taken drugs known to affect platelet function. Washed platelets were prepared as previously described (Gordge *et al*., 1995) and suspended at a count of $200-300\times10^9\ l^{-1}$ 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 of platelet aggregation by NO donor compounds was measured essentially as described previously (Gordge *et al*., 1995). Briefly, platelets equilibrated at 37°C were treated with NO donor for 60s before induction of aggregation with collagen ($2\ \mu g\ ml^{-1}$). Aggregation was then monitored turbidometrically for two minutes by use of a PAP4 aggregometer (Biodata Corp., Horsham, PA, U.S.A.), at 37°C with a stirring speed of 1000 r.p.m. The inhibitory action of each NO donor was assessed by comparison with control responses performed in the presence of vehicle alone. In various sets of experiments, the anti-aggregatory actions of the NO donor compounds were assessed in the presence of (a) the copper (I)-specific chelator bathocuproine disulphonic acid (BCS; 100 μM) (Blair & Diehl, 1961), to investigate the influence of copper on platelet inhibition, (b) the selective guanylate cyclase inhibitor ODQ (100 μM) (Garthwaite *et al*., 1995), to investigate the influence of cyclic GMP generation on platelet inhibition, or (c) a combination of BCS and ODQ. Platelets were incubated with BCS and ODQ for 15 min at room temperature before testing. Neither BCS nor ODQ had any significant direct effect on platelet aggregation responses; mean $\pm$ s.e.mean responses to $2\ \mu g\ ml^{-1}$ collagen were 58 ± 2.04, 61 ± 1.58, 58 ± 1.53 and 55 ± 2.11% in the presence of vehicle alone, BCS, ODQ and a combination of BCS and ODQ, respectively ($n=13-16$). In further experiments the role of platelet surface thiol groups on the cyclic GMP-independent action of GSNO (0.1 μM – 1 mM) was assessed by measuring its anti-aggregatory effect on ODQ-treated platelets, in the presence and absence of the membrane impermeable thiol modifying agent CMPS (0.1 μM – 1 mM). The effect of CMPS alone was also measured, and the dose-response curves obtained were compared to determine whether there was any additive inhibitory effect on aggregation. Reagents were incubated with platelets for one minute before the induction of platelet aggregation with collagen ($2\ \mu g\ ml^{-1}$).

Measurement of intra-platelet cyclic GMP and cyclic AMP

NO donor-stimulated accumulation of intra-platelet cyclic GMP and cyclic AMP were measured as previously described (Gordge *et al*., 1995). Briefly, NO donor was incubated with washed platelet suspensions for two minutes at 37°C after which the reaction was stopped by the addition of an equal volume of 10% w/v trichloroacetic acid. Samples were centrifuged and supernatants stored at -20°C until being measured by radioimmunoassay.

In experiments to measure the effects of 8-bromo cyclic GMP, platelets were washed three times (by rapid centrifugation followed by resuspension in Tyrode buffer), in order to remove exogenous cyclic GMP, before the addition of trichloroacetic acid. In experiments performed to measure the

effects of BCS and ODQ on NO donor-induced cyclic GMP and cyclic AMP accumulation, platelets were pretreated for 20 min at room temperature with the non-selective phosphodiesterase inhibitor isobutyl methyl xanthine (IBMX; 0.5 mM), in order to maximize sensitivity of detection of cyclic nucleotides. IBMX was found to have a direct inhibitory effect on platelet aggregation, and in experiments designed to assess the correlation between aggregation inhibition and cyclic GMP accumulation IBMX was not used, so that both aggregation and cyclic GMP, accumulation could be measured under identical conditions.

Measurement of rate of NO release from NO donor compounds

Rate of NO release was measured by incubating 10 μM of each NO donor in 1 ml of Tyrode buffer and monitoring the oxidation of oxyhaemoglobin to methaemoglobin by the difference spectrophotometric method described by Kelm *et al*. (1988), with a Cary 1E spectrophotometer. The rate of NO release in nmol min^{-1} was calculated from the absorbance changes by use of an extinction coefficient ($\sum_{410-419}$) of 38 mM^{-1} cm^{-1}.

Measurement of platelet surface thiol groups

Thiol groups associated with the platelet extracellular membrane were measured by an adaptation of a previously described method (Edwards *et al*., 1995), detecting surface thiols with 3-(N-maleimido-proprionyl) biocytin, a membrane impermeant thiol-specific biotinylating reagent (Bayer *et al*., 1985). Platelet suspensions were incubated for 10 min at 37°C with MBP (50 μM), then washed to remove unbound MPB before being resuspended and incubated for 30 min at 4°C with fluorescein-conjugated avidin (20 $\mu g\ ml^{-1}$) to detect biotinylated membrane thiol groups. After further washing to remove unbound avidin, platelets were solubilized in 1 ml Tyrode buffer containing 0.5% Triton x-100, and fluorescence read in a Perkin Elmer LS 5 luminescence spectrometer, using an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

Surface thiols were measured on platelets following incubation for two minutes at 37°C with (1) CMPS and (2) GSNO (0.1 μM – 1 mM). Results were corrected for non-specific avidin binding, which was measured by omitting the MPB incubation from the experimental procedure.

Reagents

Sources Cysteine, homocysteine, glutathione, N-acetyl cysteine, N-acetyl-DL-penicillamine, bathocuproinedisulphonic acid (BCS), sodium nitroprusside, bovine haemoglobin, 8-bromo cyclic GMP, isobutyl methyl xanthine (IBMX), *p*-chloromercuriphenylsulphonic acid (CMPS) and fluorescein labelled avidin were all purchased from Sigma (Poole, U.K.). 3- (N-maleimido-proprionyl) biocytin (MPB) was from Molecular Probes (Cambridge U.K.), diethylamine NONOate (DEANO) from Cayman Chemicals (Ann Arbor, MI, U.S.A.), collagen from Nycomed (Munich, Germany), 1H-[1,2,4]oxadiazolo [4,3a]quinoxalin-1-one (ODQ) from Tocris Cookson (Bristol, U.K.) and both cyclic GMP and cyclic AMP radioimmunoassay kits from Amersham plc, (High Wycombe, U.K.). Prostacyclin was a kind gift from Prof S. Moncada (Cruciform Project, UCL). All other reagents were of Analar grade and purchased from Merck (Lutterworth UK).

Preparation Stock solutions of reagents were prepared as follows: N-acetyl-DL-penicillamine, 20 mM in 50% (v/v) ethanol; IBMX, 0.5 M in methanol/1 M NaOH (1/1); fluorescein-labelled avidin, 0.5 mM in phosphate-buffered saline (PBS) containing 2% (w/v) bovine serum albumin; MPB, 4 mM in 20% (v/v) dimethylformamide in PBS; DEANO, 10 mM in 10 mM NaOH; ODQ, 10 mM in DMSO; prostacyclin, 10 $\mu g\ ml^{-1}$ in 1 M Tris/HCl pH 9.6. 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 10×1 cm column of Sephadex G25, eluted with 20 mM phosphate buffer and stored at 4°C for not more than seven days before use. All other reagents were dissolved in de-ionized water (milliQ).

Statistics

Pearson coefficients of correlation were calculated to assess the relationships between rate of NO release from different NO donors, donor-mediated inhibition of platelet aggregation and cyclic GMP accumulation. Concentration-response curves were compared by two way ANOVA. Changes from baseline of platelet surface thiols following treatment with GSNO and CMPS, and comparison of cyclic AMP levels in platelets treated with GSNO in the presence and absence of BCS and ODQ, were performed by one way ANOVA followed by Student-Newman-Keuls test.

Results

Correlation between rates of NO release, cyclic GMP accumulation and inhibition of platelet aggregation

Values obtained for rate of NO release, cyclic GMP accumulation and % inhibition of collagen-stimulated platelet aggregation, by each NO donor compound, at a concentration of 10 μM, are shown in Table 1. Analysis of results showed a significant positive correlation between rate of NO release (log transformed) and accumulation of cyclic GMP, but no significant correlation between rate of NO release and inhibition of platelet aggregation, nor between cyclic GMP accumulation and inhibition of aggregation (Table 2).

Table 1 NO release, cyclic GMP accumulation and % inhibition of collagen-induced platelet aggregation by NO donor compounds

NO donor (10 μM)	*NO release* (nmol min^{-1})	*Cyclic GMP* (pmol per 10^8 platelets)[a]	*% inhibition of platelet aggregation*[b]
CysNO	3.15 ± 0.29	2.67 ± 0.31	67 ± 7.5
GSNO	0.14 ± 0.03	1.35 ± 0.21	89 ± 2.0
SNAP	0.35 ± 0.06	1.42 ± 0.21	76 ± 7.2
SNAC	0.10 ± 0.005	1.27 ± 0.15	90 ± 3.8
HomocysNO	0.42 ± 0.05	1.86 ± 0.29	89 ± 4.2
SNP	0.04 ± 0.001	0.43 ± 0.05	40 ± 6.4
DEANO	2.34 ± 0.06	2.02 ± 0.25	71 ± 6.2

Values are mean ± s.e.mean from 5–10 observations.
[a]Mean basal cyclic GMP (0.14 pmol per 10^8 platelets) was subtracted so that values represent the NO donor-stimulated elevation of cyclic GMP.
[b]Platelet aggregation was stimulated with 2 $\mu g\ ml^{-1}$ collagen and monitored for two minutes.

Comparison of the biological activities of GSNO, DEANO and 8-bromo cyclic GMP

Statistical comparisons of the dose-response curves obtained with GSNO, DEANO and 8-bromo cyclic GMP for (1) stimulation of intra-platelet cyclic GMP accumulation and (2) inhibition of platelet aggregation, showed that DEANO stimulated the greatest cyclic GMP response (Figure 1a), but that GSNO inhibited platelet aggregation most efficiently (Figure 1b). Inspection of the dose-response curves shows that inhibition of platelet aggregation tended to parallel cyclic GMP accumulation following treatment of platelets with either DEANO or 8-bromo cyclic GMP. In contrast, inhibition of aggregation was evident even at low concentrations of GSNO (<100 nM) which produced no detectable elevation of cyclic GMP.

Table 2 Correlations between (1) rate of spontaneous NO release, (2) stimulation of intra-platelet accumulation of cyclic GMP, and (3) percentage inhibition of platelet aggregation, obtained with different NO donor compounds

Correlation	r *value*	P *value*
NO release* *vs* cyclic GMP accumulation	0.83	0.02
No release* *vs* inhibition of platelet aggregation	−0.17	0.71
Cyclic GMP accumulation *vs* inhibition of platelet aggregation	0.34	0.46

*Log transformed data.

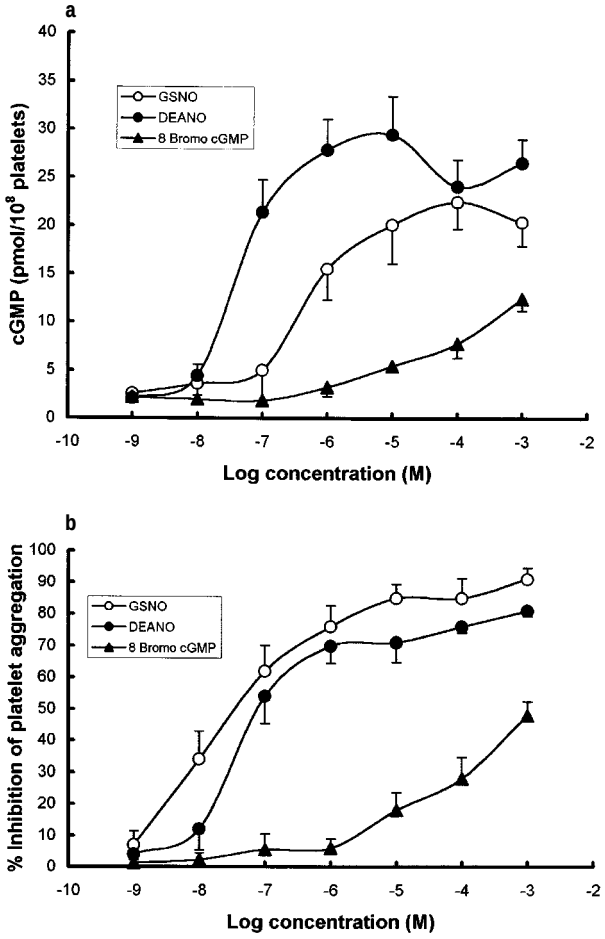

Figure 1 Intra-platelet cyclic GMP (a) and inhibition of platelet aggregation (b) in response to GSNO, DEANO and 8-bromo cyclic GMP (10^{-9}–10^{-3} M). Results are mean and s.e.mean (vertical lines) from five experiments. Comparison of curves by two way ANOVA showed that DEANO stimulated a greater accumulation of cyclic GMP than either GSNO ($P<0.001$) or 8-bromo cyclic GMP ($P<0.001$). In contrast, GSNO inhibited aggregation more strongly than either DEANO ($P<0.01$) or 8-bromo cyclic GMP ($P<0.001$). Note that cyclic GMP measurements were made with IBMX-treated platelets.

Effects of BCS and ODQ on the biological actions of GSNO

In a cell free system, BCS (100 μM) inhibited NO release from GSNO (10 μM) from a mean ($\pm$s.e.mean) value of 0.14 ± 0.03 nmol min^{-1} to an undetectable level. NO release was not significantly altered by ODQ (100 μM); mean ($\pm$s.e.mean) value was 0.15 ± 0.09 nmol min^{-1} ($n=6$ in all experiments).

Platelet accumulation of cyclic GMP was significantly inhibited by BCS (100 μM) ($P<0.001$ by two way ANOVA), and was undetectable in platelets treated with either ODQ alone (100 μM), or with a combination of BCS and ODQ (Figure 2a). The ability of GSNO to inhibit platelet aggregation was significantly diminished, but not abolished, by either BCS alone or ODQ alone ($P<0.001$ by two way ANOVA). Anti-aggregatory activity was diminished to an

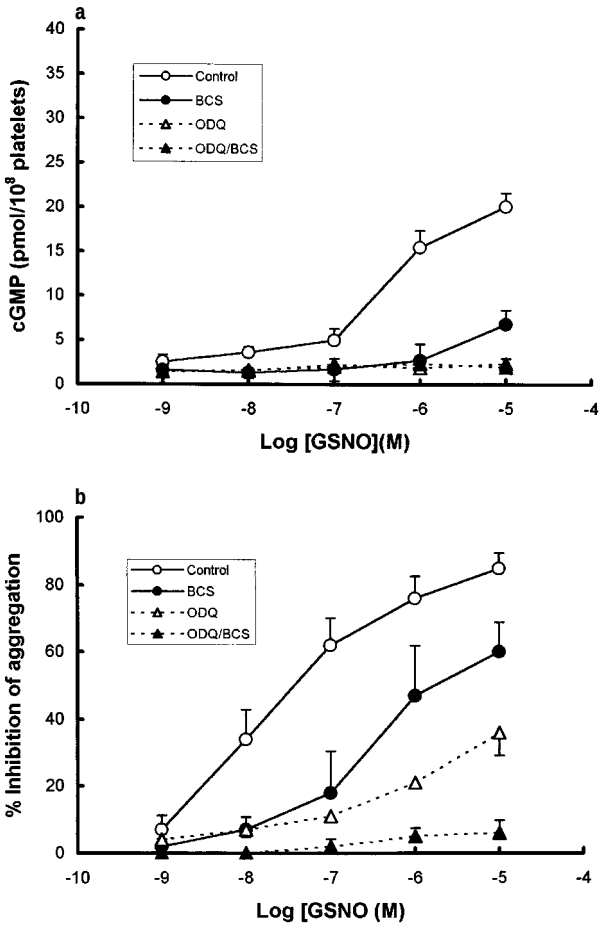

Figure 2 Intra-platelet cyclic GMP (a) and inhibition of platelet aggregation (b) in response to GSNO (10^{-9}–10^{-5} M) in platelets treated with vehicle (control), BCS (100 μM), ODQ (100 μM), or a combination of BCS and ODQ. All treatments were significantly different from control ($P<0.001$ by 2 way ANOVA). Results are mean and s.e.mean (vertical lines) from 5 experiments. Note that cyclic GMP measurements were made with IBMX-treated platelets.

undetectable level by a combination of ODQ and BCS (Figure 2b).

Effects of BCS and ODQ on the biological actions of DEANO

Mean (±s.e.mean) rate of NO release from DEANO was 2.34 ± 0.06 nmol min^{-1}. This was not significantly altered by either BCS (100 μM) (2.38 ± 0.06 nmol min^{-1}), or ODQ (100 μM) (2.02 ± 0.21 nmol min^{-1}) ($n=5$ in all experiments). Cyclic GMP accumulation was not affected by BCS alone, but was significantly inhibited by ODQ when added either alone or in combination with BCS ($P<0.001$ by two way ANOVA) (Figure 3a).

The ability of DEANO to inhibit platelet aggregation was unaffected by BCS, but was significantly diminished by ODQ, both alone and in combination with BCS ($P<0.001$ by two way ANOVA) (Figure 3b).

Stimulation of intra-platelet cyclic AMP accumulation by GSNO

There was no significant alteration of intra-platelet cyclic AMP concentrations following treatment with GSNO (10 μM), either in the presence or absence of BCS and ODQ (Table 3).

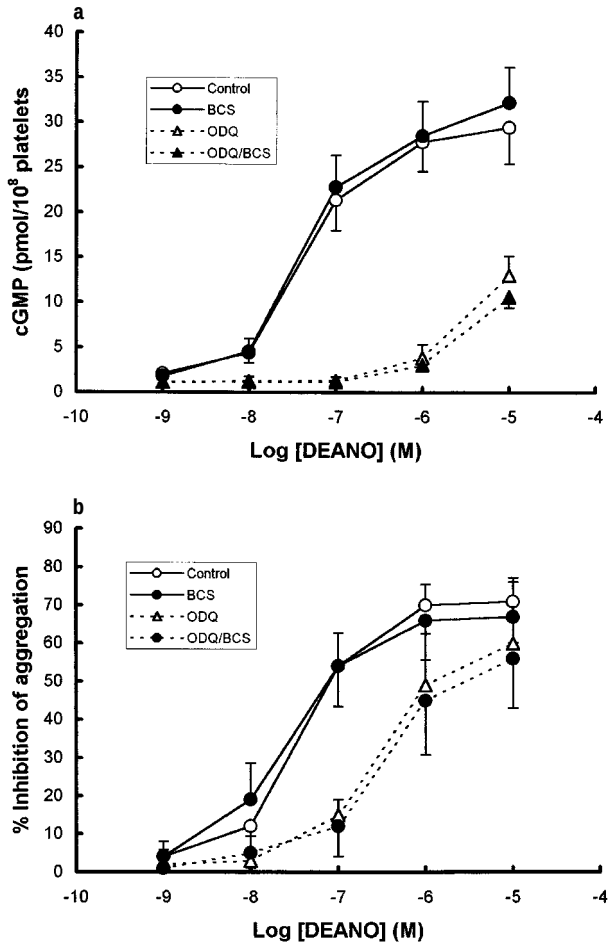

Figure 3 Intra-platelet cyclic GMP (a) and inhibition of platelet aggregation (b) in response to DEANO ($10^{-9}-10^{-5}$ M) in platelets treated with vehicle (contol), BCS (100 μM), ODQ (100 μM), or a combination of BCS and ODQ. Results obtained in the presence of ODQ, and a combination of BCS and ODQ, were significantly different from control ($P<0.001$, by 2 way ANOVA). Results are mean and s.e.mean (vertical lines) from 5 experiments. Note that cyclic GMP measurements were made with IBMX-treated platelets.

Effect of GSNO and CMPS on (1) platelet surface thiols, and (2) cyclic GMP-independent inhibition of platelet aggregation

To investigate whether modification of membrane thiol groups could explain the cyclic GMP-independent action of GSNO, surface thiols were measured on ODQ-treated platelets following treatment with (1) GSNO (0.1 μM – 1 mM) and (2) CMPS (0.1 μM – 1 mM), as a positive control. No significant loss of surface thiols was detectable following treatment with GSNO, but CMPS caused a progressive loss of detectable thiol groups on the platelet surface, which was significant at concentrations of 0.1 and 1 mM (Figure 4a). In preliminary experiments, detection of surface thiols was unaffected by pretreatment of platelets with ODQ.

Both GSNO and CMPS produced a concentration-dependent inhibition of aggregation in ODQ-treated platelets. However, treatment of platelets with a combination of GSNO and CMPS did not significantly increase the degree of inhibition of platelet aggregation (Figure 4b).

Discussion

Our study provides direct evidence that GSNO is able to exploit a cyclic GMP-independent mechanism to increase its inhibition of platelet function. Our data suggest that targeting of GSNO to this cyclic GMP-independent pathway may depend upon the availability of copper, and of platelet surface thiol groups.

There was a divergence in the rank order of potency of different NO donors (a) to increase intra-platelet cyclic GMP, and (b) to inhibit platelet aggregation. The ability of a donor to raise cyclic GMP was significantly correlated with its spontaneous rate of NO release, consistent with a model of guanylate cyclase stimulation in which enzyme activation is brought about by binding of free NO to ferrous haem (Ignarro, 1992). However, there was no similar correlation between rate of NO release and the ability of the NO donor compounds to inhibit platelet aggregation, nor between cyclic GMP elevation and aggregation inhibition. These results are consistent with those previously obtained by Mathews and Kerr (1993). Since all measurements were made under identical conditions of buffer composition and temperature, the divergence between NO release and aggregation inhibition could not be explained by discrepancies in rates of copper-catalyzed or thermal breakdown (Singh *et al*., 1996; Dicks *et al*., 1996). We did not find any of the carrier molecules for the NO donor compounds used (cysteine, glutathione, etc) to possess any significant anti-aggregatory activity (data not

Table 3 Intra-platelet cyclic AMP following treatment of platelets with GSNO, in the presence and absence of BCS and ODQ

	Intra-platelet cyclic AMP (pmol/10^8 platelets)
Baseline	4.76 ± 1.06
GSNO	5.13 ± 0.24
GSNO + BCS	4.76 ± 0.16
GSNO + ODQ	4.70 ± 0.16
GSNO + BCS + ODQ	4.70 ± 0.20

Values are mean and s.e.mean from 10 experiments with IBMX-treated platelets. Experiments were performed with 10 μM GSNO and 100 μM of both BCS and ODQ. None of the results obtained was significantly different from baseline.

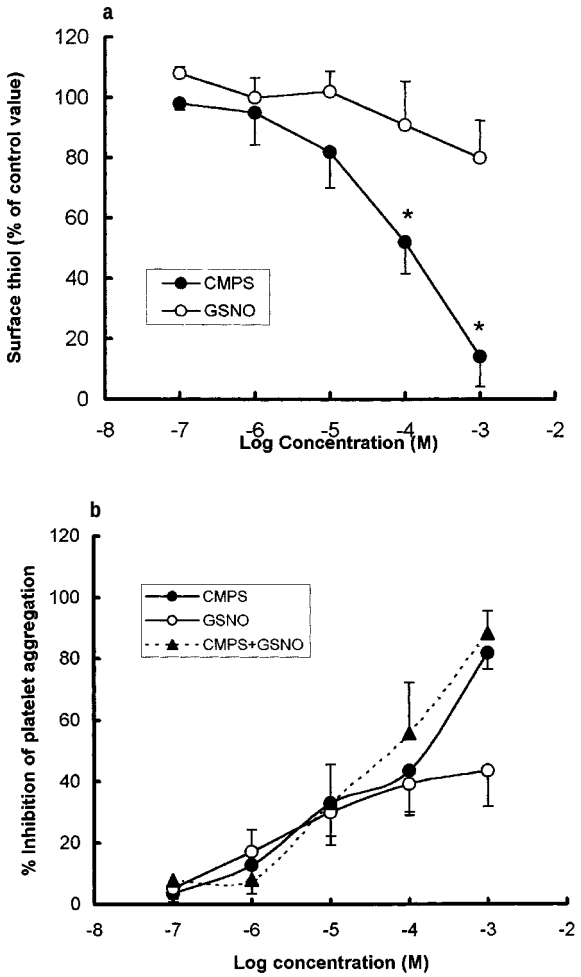

Figure 4 Surface thiols (a), expressed as a percentage of control, and inhibition of platelet aggregation (b) following treatment with CMPS or GSNO ($10^{-7}-10^{-3}$ M). Results are mean and s.e.mean (vertical lines) from 3–4 experiments. Surface thiols were significantly lower than control following treatment with 0.1 and 1 mM CMPS ($P<0.05$ by Student-Newman-Keuls test). Statistical comparison by 2 way ANOVA showed no significant difference between inhibition of platelet aggregation in the presence of GSNO, CMPS or a combination of both. Note that platelets were treated with ODQ (100 μM) to inhibit guanylate cyclase before the experiments were performed.

shown), thus there was no evidence for NO-independent anti-platelet effects. Taken together, our results suggest that platelet inhibition by NO donors is mediated not only by elevation of cyclic GMP, but also by a cyclic GMP-independent pathway(s).

Comparison of GSNO with DEANO showed GSNO to be a more potent inhibitor of aggregation despite the fact that DEANO both released NO at a faster rate and stimulated a greater increase in platelet cyclic GMP. This suggests that GSNO is able to target NO to platelets by some other mechanism than spontaneous release of NO into solution. Our dose-response data showed that GSNO inhibited aggregation even at low concentrations, which produced no detectable accumulation of cyclic GMP, suggesting a cyclic GMP-independent action. In contrast, inhibition of aggregation by both DEANO and 8-bromo cyclic GMP tended to parallel the degree of cyclic GMP accumulation. We have previously shown similar results when comparing GSNO with cysNO, which, like DEANO, breaks down rapidly to release NO (Gordge *et al.*, 1995). These results raise the question how the NO signal is transmitted from stable nitrosothiols, such as GSNO, to the target cell. Cellular uptake of intact GSNO by an active transport system would remove the need for extracellular NO release and explain how GSNO could inhibit platelet function without first releasing NO. Platelets possess a glutathione uptake system, but GSNO does not appear to be a substrate, since it fails to compete significantly with GSH for transport (Sexton & Mutus, 1995). Previous studies of our own (Gordge *et al.*, 1996) and others (Kowaluk & Fung, 1990) suggest rather that NO transfer is accomplished enzymatically. The identity of the enzyme involved is not yet clear. However, we have shown (a) that the enzyme is copper-dependent, and (b) that it is not γ-glutamyl transpeptidase, an extracellular enzyme known to metabolize GSNO (Hogg *et al.*, 1997). Other workers have shown the presence of stereoselective nitrosothiol receptors (Davisson *et al.*, 1996).

GSNO is a naturally occurring S-nitrosothiol compound, which participates in the storage and/or transport of NO within the body (Gaston *et al.*, 1993; Jia *et al.*, 1996). It is used therapeutically as an arterioselective vasodilator (MacAllister *et al.*, 1995), and a platelet-selective anti-thrombotic agent (de Belder *et al.*, 1994; Langford *et al.*, 1994), and we have therefore made a detailed investigation of its action. Using the selective guanylate cyclase inhibitor ODQ (Garthwaite *et al.*, 1995), we have demonstrated that GSNO acts, in part, by a cyclic GMP-independent pathway, since there was a component in its anti-aggregatory action that survived complete abolition of any detectable cyclic GMP generation. This is consistent with earlier work by Brunner *et al.* showing a similar component in the vasorelaxant action of GSNO (Brunner *et al.*, 1996). In addition, Moro and co-workers have shown that high concentrations of another S-nitrosothiol compound, SNAP, may exert platelet inhibitory effects via cyclic GMP-independent mechanisms (Moro *et al.*, 1996). It must be conceded that measurement of cyclic GMP in whole cells may be misleading, since cellular responses can be determined by the rate of turnover of the cyclic nucleotide, or by targeting to a specific subcellular compartment (Nakatsu & Diamond, 1989; Barsony & Marx, 1990). We attempted to account for these possibilities by using high concentrations of IBMX to block phosphodiesterase-mediated breakdown of cyclic GMP, and were still able to show aggregation inhibition by GSNO under conditions in which there was an absence of any detectable rise in cyclic GMP.

The identity of the cyclic GMP-independent pathway activated by GSNO is not yet known. S-nitrosothiol compounds donate NO in different redox forms (NO, NO^-, NO^+), depending on local conditions (Arnelle & Stamler, 1995), and this versatility may be utilized in redox signalling (Stamler, 1994). Thus, in addition to interaction with ferrous haem in guanylate cyclase, biological action may be mediated via transfer of NO to other targets. Nitrosation of both low molecular weight (Clancy *et al.*, 1994) and protein sulphydryls can alter cellular behaviour, independent of cyclic GMP generation. Examples include inhibition of NADPH oxidase (Clancy *et al.*, 1992), glyceraldehyde-3-phosphate dehydrogenase (Mohr *et al.*, 1994) and cytosolic calcium accumulation (Garg & Hassid, 1991), activation of calcium-dependent potassium channels (Bolotina *et al.*, 1994), and stimulation of guanine nucleotide exchange on $p21^{ras}$ (Lander *et al.*, 1997). Tyrosine phosphorylation is an important signal transduction mechanism during platelet aggregation (Law *et al.*, 1996), and its disruption by nitration of tyrosine residues is a further possible pathway of cyclic GMP-independent action.

The fact that the anti-platelet activity of GSNO can be inhibited by oxyhaemoglobin (Radomski *et al.*, 1992), an

impermeable NO scavenger, suggests that NO must become available at an extracellular site, perhaps at the external cell surface, during delivery from GSNO. We speculated that GSNO might transmit a cyclic GMP-independent signal by nitrosating a cell surface thiol group to modulate platelet aggregation. To test this hypothesis we used the impermeable thiol modifying agent CMPS, which caused concentration-dependent inhibition of aggregation of ODQ-treated platelets (in which cyclic GMP generation had been abolished), associated with a progressive loss of platelet surface thiols. Platelet inhibition by thiol modifying agents can result from dysregulation of receptors for fibrinogen (Zucker & Mauss, 1986) and thromboxane (Dorn 1990), and inhibition of calpain (Puri *et al*., 1993). We found that treatment of platelets with a combination of CMPS and GSNO produced no significant increase in the degree of aggregation inhibition, over that obtained with each agent singly. This absence of any additive effect suggests that a common pathway of thiol modification was utilized by both GSNO and CMPS to elicit cyclic GMP-independent inhibition of platelet aggregation. However, we were unable to obtain direct evidence for this since there was no significant loss of platelet surface thiol groups following treatment with GSNO alone, and therefore this interpretation must remain speculative.

An alternative explanation of cyclic GMP-independent platelet inhibition by GSNO is that blockade of guanylate cyclase allowed cyclic AMP to accumulate as a result of diminished activity of the cyclic GMP-stimulated phosphodiesterase (PDE2) (Dickinson *et al*., 1997). However, our results failed to support this hypothesis, since cyclic AMP was not elevated in platelets treated with a combination of ODQ and GSNO, conditions under which cyclic GMP-independent inhibition of aggregation was apparent.

Release of NO from S-nitrosothiol compounds is catalyzed by copper (I) (Cu^+) ions, and we have demonstrated inhibition of GSNO breakdown by the copper (I) chelator BCS. GSNO-mediated inhibition of platelet aggregation was partially suppressed by ODQ (despite complete suppression of any cyclic GMP response), but completely abolished by a combination of ODQ and BCS. This suggests that the cyclic GMP-independent component of GSNO action (evident in platelets treated with ODQ alone) is dependent upon the availability of copper. We have previously provided evidence for a copper-dependent enzyme in platelet homogenates which catalyzes the breakdown of GSNO (Gordge *et al*., 1996). The purpose of this system might therefore be to target the NO signal from GSNO to a cyclic GMP-independent pathway. At present this interpretation can only be tentative, since, in our experiments with platelets treated with BCS alone, low concentrations of GSNO retained the ability to inhibit aggregation, despite the fact that cyclic GMP generation was almost completely suppressed. Thus aggregation inhibition was evident despite both copper chelation and absence of a cyclic GMP response. Further work is therefore required to define the role of copper in cyclic GMP-independent signalling by GSNO.

BCS failed to influence either the breakdown of the non-nitrosothiol compound DEANO, or its anti-platelet action. NO release from DEANO is not, therefore, catalyzed by copper ions, nor is copper required for it to inhibit platelet aggregation. It was not possible to achieve complete suppression of DEANO-mediated cyclic GMP accumulation with ODQ, which is a haem-binding inhibitor of guanylate cyclase (Schrammel *et al*., 1996). The most likely explanation is that the rapid rate of NO release from DEANO allowed NO to compete effectively for the haem group in guanylate cyclase. We could not, therefore, determine directly whether DEANO utilizes a cyclic GMP-independent pathway to inhibit platelets.

In conclusion, targeting and transmission of the NO signal from GSNO to platelets appears to involve both cyclic GMP-dependent and -independent pathways of cellular response. There is preliminary evidence for the involvement of copper ions, cell surface thiol groups and enzymes/receptors. Elucidation of the mechanism of action of GSNO is important, first to our understanding of NO biology, and secondly because of the potential of GSNO in the therapeutic delivery of NO.

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