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Comparative pharmacology of analogues of S-nitroso-*N*-acetyl-DL-penicillamine on human platelets

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1 The effects of two new analogues of S-nitroso-*N*-acetyl-DL-penicillamine (SNAP), S-nitroso-*N*-formyl-DL-penicillamine (SNFP) and S-nitroso-DL-penicillamine (SNPL), on platelet function were examined *in vitro*.

2 SNAP and its analogues were potent inhibitors of platelet aggregation and inducers of disaggregation.

3 All compounds inhibited fibrinogen binding to platelets.

4 They also decreased the release of P-selectin from platelets.

5 Both inhibition of fibrinogen binding and release of P-selectin correlated with an increase in intraplatelet cyclic GMP concentrations.

6 At concentrations sufficient to inhibit platelet function and induce cyclic GMP formation (0.01–3 μM), the release of NO could be detected from SNPL but not from SNAP and SNFP.

7 Release of NO from all compounds was detected at concentrations $\geqslant 10\,\mu M$.

8 Thus, the spontaneous release of NO from SNPL explains the actions of this compound on platelet function; however, platelet-mediated mechanisms may be involved in the release of NO from SNAP and SNFP.

Keywords: Human platelets; S-nitroso-*N*-acetyl-DL-penicillamine analogues; aggregation; fibrinogen binding; P-selectin; cyclic GMP; nitric oxide

Introduction

In vitro, S-nitrosylation of molecules containing functional thiol groups leads to the formation of S-nitrosothiols. Although the biological significance of S-nitrosylation *in vivo* is still uncertain, this reaction may result in stabilization of the chemical and pharmacological properties of NO and generation of compounds with NO-donating properties (Stamler *et al.*, 1992; Venturini *et al.*, 1993). S-nitroso-*N*-acetyl-DL-penicillamine (SNAP) is a stable inhibitor of platelet aggregation (Radomski *et al.*, 1992). We have synthesized two novel analogues of SNAP, S-nitroso-*N*-formyl-DL-penicillamine (SNFP) and S-nitroso-DL-penicillamine (SNPL), and have examined their pharmacological behaviour on human platelets *in vitro*.

Methods

Platelet-rich plasma (PRP) and washed platelet suspensions (WP)

Human blood was collected and PRP and prostacyclin (PGI_2)-washed platelet suspensions were prepared as described by Radomski & Moncada (1983).

Platelet aggregation and release of ATP

These were measured in a platelet-ionized calcium lumi-aggregometer. S-nitrosothiols were incubated with WP (2.5×10^8 platelets ml^{-1}) for 1 min prior to the addition of collagen (1 $\mu g\,ml^{-1}$) and their effects on platelet aggregation studied for 3 min. In some experiments haemoglobin (5 μM) was added 1 min before S-nitrosothiols. In another set of experiments S-nitrosothiols (all at 1 μM) were incubated for 60 min at 37°C.

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Platelet disaggregation

Platelet disaggregation was measured in PRP following initiation of aggregation by ADP (3–5 μM). S-nitrosothiols were added 1 min after ADP and their effects studied for 5 min.

Intraplatelet cyclic GMP and cyclic AMP concentrations

These were measured in WP by enzyme immunoassay as described previously (Radomski *et al.*, 1992). Briefly, WP were incubated in the aggregometer for 10 min at 37°C in the presence or absence of S-nitrosothiols. Following incubation, EGTA (5 mM) was added and platelets lysed by two cycles of freezing in liquid nitrogen and thawing at 37°C. The lysate was centrifuged (10,000 *g* for 5 min) and the supernatant assayed for cyclic nucleotides by the dual range acetylation enzyme immunoassay (Amersham).

[^{125}I]-fibrinogen binding to platelets

Binding to platelets of [^{125}I]-fibrinogen was measured as described by Marguerie *et al.* (1979) with some modifications. Washed platelets (10^8 ml^{-1}) were incubated in the presence or absence of S-nitrosothiols for 15 min. Binding was initiated by adding [^{125}I]-fibrinogen (50 $\mu g\,ml^{-1}$) and ADP (50 μM) in a final volume of 500 μl, conditions in which B_{max} is approx. 4000 c.p.m./10^6 platelets and $K_D = 13.3 \pm 2.0\,\mu g\,ml^{-1}$ fibrinogen ($n = 3$). After 15 min, samples (50 μl) of the reaction mixture were layered on 200 μl of 15% sucrose solution and centrifuged for 1 min at 10,000 *g*. The samples were immediately frozen in dry ice and the radioactivity associated with the platelet pellet was counted in a γ counter.

P-selectin (CD62, GMP-140, PADGEM)

P-selectin release was measured by enzyme immunoassay in the supernatants of WP (2.5×10^8 platelets ml^{-1}) stimulated with collagen (0.3–10 $\mu g\,ml^{-1}$) in the presence or absence of

S-nitrosothiols or PGI_2. The concentrations of S-nitrosothiols or PGI_2 (3 or 10 μM and 3 or 10 nM, respectively) were selected to achieve a similar inhibitory effect (approx. 95%) on the aggregation induced by 3 μg ml^{-1} of collagen. The inhibitors were added 1 min before collagen and their effects studied for 5 min. At the end of incubation EDTA (5 mM) was added, then the samples were centrifuged at 10,000 g for 1 min and the supernatant assayed for the presence of P-selectin (British Biotechnology).

The release of NO from S-nitrosothiols

NO release from S-nitrosothiols was measured by the method described by Feelisch & Noack (1987). S-nitrosothiols were incubated for 5 min at 37°C with or without intact platelets, platelet lysate, platelet cytosol (100,000 g for 30 min) all prepared from 2.5×10^8 platelets, reduced glutathione (30 μM), ascorbic acid (30 μM) or superoxide dismutase (20 u ml^{-1}) and the rate of NO release was measured in a dual wave spectrophotometer (Shimadzu).

S-nitrosothiols

SNAP was synthesized by the method of Field *et al.* (1978). SNPL and SNFP were synthesized according to the method of Hart (1985). For structures, see Figure 1.

Reagents

Human haemoglobin was prepared by the method of Paterson *et al.* (1976). Adenosine 5'-diphosphate (ADP), adenosine 3':5'-triphosphate (ATP), ethylenediaminetetraacetic acid (EDTA), luciferin-luciferase reagent, sucrose, superoxide dismutase, glutathione, ethyleneglycol-*bis*(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA), Arg-Gly-Asp-Ser (Sigma), prostacyclin sodium salt (Wellcome), L-ascorbic acid (BDH), collagen (Hormon-Chemie), [^{125}I]-fibrinogen (Amersham), guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) enzyme immunoassay (Amersham), Tyrode salt solution (Gibco, BRL) and P-selectin enzyme immunoassay (British Biotechnology) were obtained from the sources indicated.

Statistics

Results are mean ± s.e.mean of at least three separate experiments. They were compared by analysis of variance. The comparisons between all pairs of groups were made using the Bonferroni P value (Computer programme GraphPad 1990), and $P<0.05$ was considered as statistically significant.

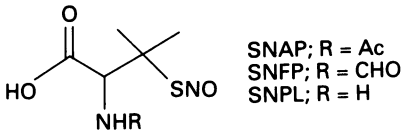

Figure 1 The chemical structures of S-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and its two novel analogues: S-nitroso-DL-penicillamine (SNPL) and S-nitroso-*N*-formyl-DL-penicillamine (SNFP).

Results

Effects of S-nitrosothiols on platelet aggregation, ATP release and disaggregation

Incubation of S-nitrosothiols (0.01–10 μM) with WP resulted in a concentration-dependent inhibition of collagen-induced aggregation and of ATP release from platelets (Figure 2a and Table 1), SNAP and SNFP being significantly more potent ($P<0.05$, $n=3-7$) than SNPL. The inhibitory activity of these S-nitrosothiols was reversed by haemoglobin (5 μM) (Figure 2b). SNAP and SNFP were stable as inhibitors of

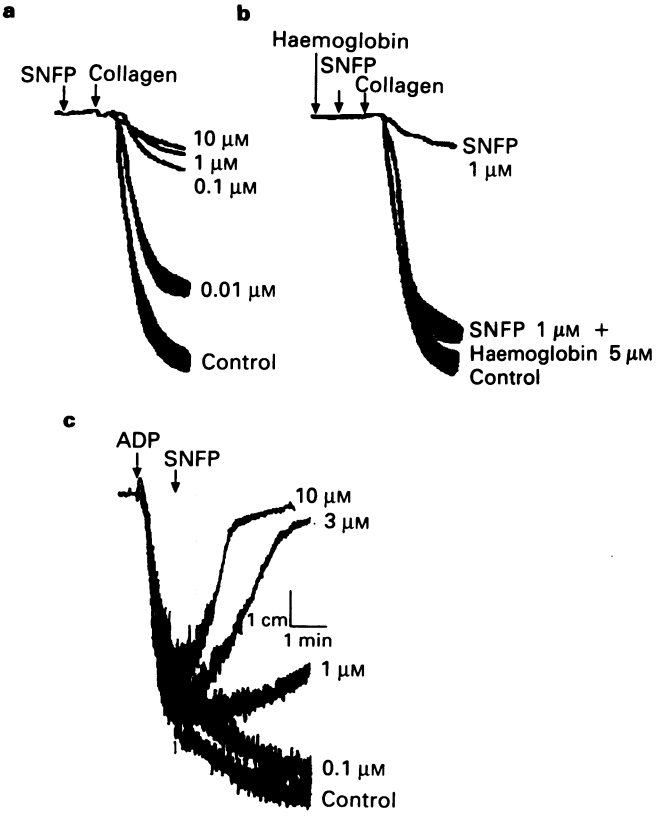

Figure 2 Effect of SNFP on platelet aggregation in washed platelets (WP) (a, b) and platelet-rich plasma (PRP) (c). (a) Inhibition by SNFP (0.01–10 μM) of collagen (1 μg ml^{-1})-induced aggregation. (b) Reversal by haemoglobin (5 μM) of the effect of SNFP (1 μM) on collagen (1 μg ml^{-1})-induced aggregation. (c) Stimulation by SNFP (0.1–10 μM) of disaggregation of platelets aggregated with ADP (5 μM). Representative tracings of five separate experiments.

Table 1 Inhibition of aggregation and ATP release (washed platelets, WP) and stimulation of disaggregation (platelet-rich plasma, PRP) by S-nitrosothiols (R-SNO)

R-SNO ($n=3-7$)	*Inhibition of collagen-induced aggregation* (IC_{50}, μM)	*Inhibition of collagen-induced release of ATP* (IC_{50}, μM)	*Stimulation of disaggregation of platelets aggregated by ADP* (EC_{50}, μM)
SNFP	0.05 ± 0.01	0.15 ± 0.07	0.63 ± 0.21
SNPL	0.38 ± 0.05	0.59 ± 0.13	0.25 ± 0.01
SNAP	0.12 ± 0.02	0.18 ± 0.11	1.09 ± 0.32

SNFP was the most potent inhibitor of collagen-induced aggregation whereas SNPL was the most potent stimulator of disaggregation ($P<0.05$, $n=3-7$). The aggregation was induced in WP by collagen (1 μg ml^{-1}) and in PRP by ADP (3–5 μM). The concentration of aggregating agent was selected in each experiment to produce 75–90% of maximal aggregation.

platelet aggregation. Indeed, the inhibitory activities of these S-nitrosothiols at 1 μM were 94.0 ± 1.2 and 86.8 ± 1.7% before and 92.7 ± 2.3 and 87.9 ± 0.7%, respectively (n = 3–4, $P > 0.05$), after 60 min incubation at 37°C. In contrast, the inhibitory activity of SNPL (1 μM) decayed when stored at 37°C for 60 min from 86.9 ± 6.1 to 56.9 ± 17.5% ($n = 4$, $P < 0.05$).

The addition of S-nitrosothiols (0.01–10 μM) 1 min after induction of platelet aggregation with ADP (3–5 μM) in PRP resulted in a concentration-dependent induction of platelet disaggregation (Figure 2c and Table 1). SNPL was more potent ($P < 0.05$, $n = 3$–7) than SNAP and SNFP as an inducer of disaggregation. With the exception of SNPL, higher concentrations of these compounds were needed for induction of disaggregation than for inhibition of aggregation (Table 1).

Cyclic GMP and cyclic AMP

The basal concentrations of cyclic GMP and cyclic AMP in platelets were 131 ± 33 and 84 ± 27 fmol/10^8 platelets, respectively ($n = 4$). Incubation of platelets with SNAP, SNFP and SNPL (0.01–10 μM) caused a concentration-dependent increase in intraplatelet cyclic GMP (Figure 3 and Table 2). These compounds were equipotent as stimulators of cyclic GMP in platelets. The concentrations of cyclic AMP were not significantly affected by 10 μM of SNAP, SNFP and SNPL (102 ± 38, 54 ± 28, 69 ± 37 fmol/10^8 platelets respectively, $P > 0.05$, $n = 4$).

[^{125}I]-fibrinogen binding assay

SNAP, SNFP and SNPL caused a concentration-dependent inhibition of [^{125}I]-fibrinogen binding to the platelets (Figure 4 and Table 2). These S-nitrosothiols were equipotent in inhibiting fibrinogen binding to platelets. There was a significant correlation between the concentrations of S-nitrosothiols needed for half-maximal stimulation of intraplatelet cyclic GMP and those required for inhibition of fibrinogen binding (Table 2).

Release of P-selectin

Collagen (0.3–10 μg ml^{-1}) resulted in aggregation and the release of P-selectin from platelets (Figure 5). Both the aggregation and release induced by collagen were inhibited by SNPL, SNFP and SNAP (3–10 μM) (Figure 5). No significant differences were found in the inhibitory activities of these S-nitrosothiols ($n = 3$, $P > 0.05$).

To compare the effects of PGI_2 and SNPL as inhibitors of P-selectin release, the concentrations of PGI_2 (3 or 10 nM) and SNPL (3 or 10 μM) were selected to achieve maximal (approx. 95%) inhibition of collagen (3 μg ml^{-1})-induced aggregation. These concentrations were used to examine their effects on collagen (0.3–10 μg ml^{-1})-induced release of P-selectin. PGI_2 also inhibited both aggregation and P-selectin release induced by collagen. There was no significant difference between the two compounds when tested against 0.3–3 μg ml^{-1} of collagen. However, against the highest concentration of collagen (10 μg ml^{-1}), SNPL (52 ± 3% of maximal release) proved to be significantly ($P < 0.05$, $n = 3$) more effective than PGI_2 (70 ± 4% of maximal release) in inhibiting P-selectin release from platelets.

Release of NO

SNPL when dissolved in Tyrode solution released NO in a concentration-dependent manner (Table 3). The release of

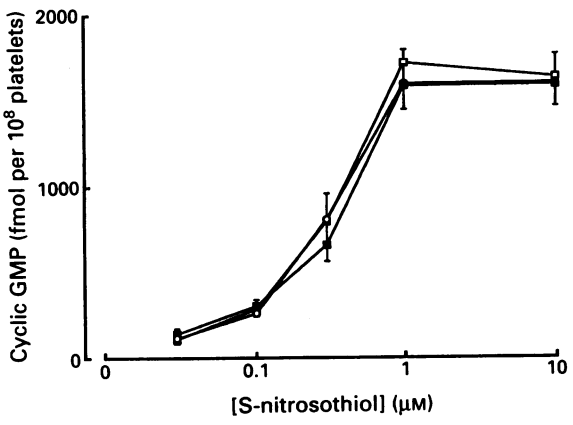

Figure 3 Increase in intraplatelet cyclic GMP concentrations in washed platelets (WP) by S-nitrosothiols. SNAP (□), SNPL (○) and SNFP (■) at concentrations ranging from 0.03 to 3 μM were equipotent as stimulators of the soluble guanylate cyclase. Data are mean ± s.e.mean, n = 3–4.

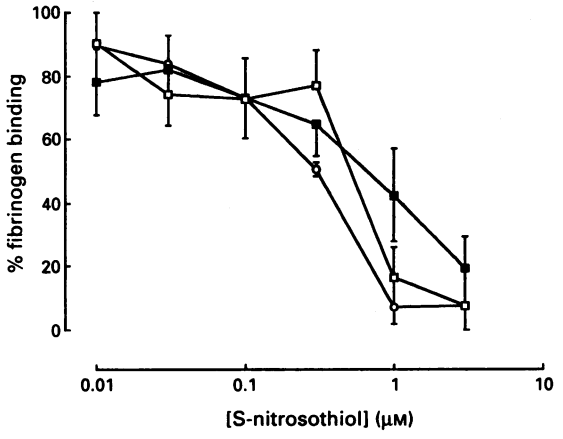

Figure 4 Inhibition by S-nitrosothiols of ADP (50 μM)-induced [^{125}I]-fibrinogen binding to washed platelets, for which B_{max} is approx. 4000 c.p.m./10^6 platelets and K_D = 13.3 ± 2.0 μg ml^{-1} fibrinogen (n = 3). SNAP (□), SNPL (○) and SNFP (■) were equipotent in inhibiting fibrinogen binding to platelets. Data are mean ± s.e.mean, n = 3.

Table 2 Correlation between intraplatelet cyclic GMP (cGMP) concentrations and inhibition of [^{125}I]-fibrinogen binding to washed platelets (WP)

R-SNO (n = 3–7)	*Half maximal stimulation of intraplatelet cGMP* (EC_{50}, μM)	*Inhibition of [^{125}I]-fibrinogen binding* (IC_{50}, μM)	*Correlation between cGMP and fibrinogen binding* (r)
SNFP	0.35	0.64 ± 0.13	0.88
SNPL	0.29	0.34 ± 0.07	0.95
SNAP	0.40	0.34 ± 0.07	0.97

There was a significant correlation between inhibition of fibrinogen binding and increases in cyclic GMP concentrations induced by each of the S-nitrosothiols (R-SNO).

NO from this compound was detected at concentrations as low as 0.1 μM. However, the release of NO from SNAP and SNFP was not detected at concentrations lower than 10 μM.

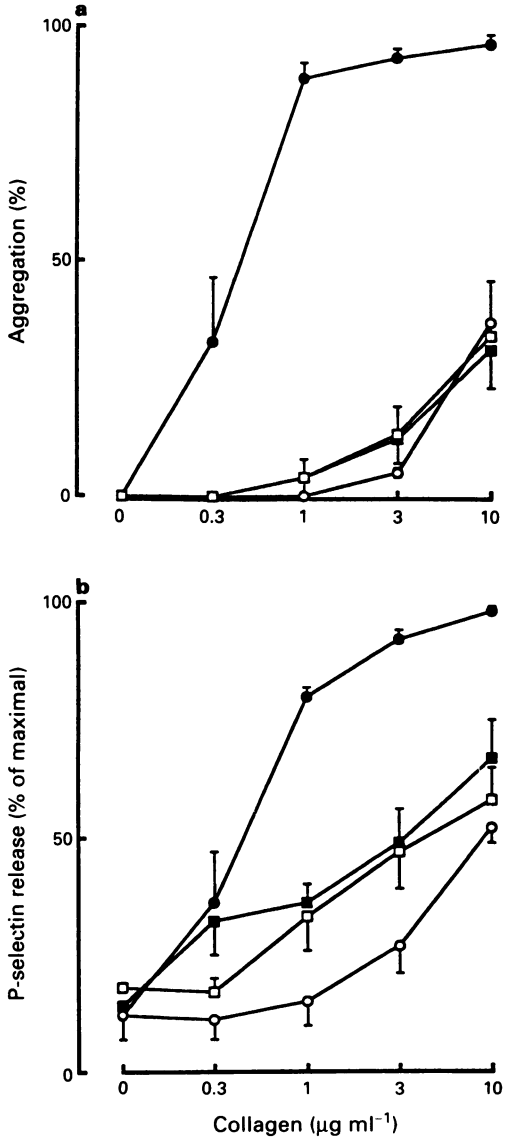

Figure 5 Inhibition by SNAP and its analogues of collagen (0.3–10 μg ml^{-1})-induced platelet aggregation (a) and P-selectin release (b) from washed platelets. The concentration (3 or 10 μM) of SNAP (□), SNPL (○) and SNFP (■) was selected in each experiment in order to achieve maximal (90–95%) inhibitory effect on the aggregation induced by 3 μg ml^{-1} of collagen (●). Data are mean ± s.e.mean, $n = 3$–4.

Table 3 Formation of NO from S-nitrosothiols (R-SNO)

R-SNO	*NO release* (pmol min^{-1})		
(μM, $n = 4$)	*SNPL*	*SNFP*	*SNAP*
0.01	ND	ND	ND
0.1	1.29 ± 0.02	ND	ND
1.0	52.34 ± 1.23	ND	ND
3.0	253.70 ± 2.73	ND	ND
10.0	743.00 ± 5.60	2.15 ± 0.02	5.15 ± 0.64

ND: not detectable (<0.1 pmol min^{-1}).
SNPL (0.1–10 μM) resulted in the release of NO.
The release of NO from SNAP and SNFP was detected only at concentrations higher than 3 μM. Nitric oxide was detected spectrophotometrically by monitoring the rate of NO-induced conversion of oxyhaemoglobin to methaemoglobin.

The addition of platelet lysate, intact platelets, platelet cytosol, reduced glutathione, ascorbic acid or superoxide dismutase did not increase the rate of NO release from these compounds at concentrations up to 10 μM ($n = 3$, data not shown).

Discussion

We have shown that the analogues of SNAP, SNFP and SNPL, are inhibitors of platelet aggregation and the release reaction induced by collagen. Thus, modification of the chemical structure of SNAP resulted in S-nitrosothiols capable of inhibiting platelet function *in vitro*. The potency of these S-nitrosothiols was comparable to that of NO, since their anti-aggregating and disaggregating effects were detected at submicromolar concentrations. However, in contrast to NO (Radomski *et al.*, 1987), their anti-aggregating activity was stable for at least 60 min when incubated at 37°C. The order of stability was SNAP = SNFP > SNPL, indicating that the presence of formyl or acetyl groups results in stabilization of the pharmacological properties of these molecules. Platelet aggregation induced by ADP in plasma depends on the exposure of the fibrinogen receptor (GP IIb/IIIa) and formation of fibrinogen bridges between platelets (Bennet & Vilaire, 1979; Marguerie *et al.*, 1979; Nachman *et al.*, 1984). Since S-nitrosothiols proved to be effective inducers of disaggregation of ADP-aggregated platelets, we examined the effect of these compounds on fibrinogen binding to platelets. SNAP, SNFP and SNPL inhibited fibrinogen binding to platelets at concentrations that also inhibited platelet aggregation and caused disaggregation. Moreover, these actions correlated well with the formation of cyclic GMP elicited by these compounds. Similar observations have been published for S-nitrosoacetylcysteine by Mendelsohn and colleagues (1990). Thus, it is likely that the platelet-inhibitory activity of S-nitrosothiols depends on cyclic GMP-mediated inhibition of fibrinogen binding to platelets. Since S-nitrosothiols (≤ 10 μM) did not increase intraplatelet cyclic AMP concentrations, it is unlikely that the pharmacological effects of SNAP and its analogues were mediated via inhibition by cyclic GMP of low K_M cyclic AMP phosphodiesterase and subsequent increase in cyclic AMP concentrations (Maurice & Haslam, 1990). The mechanism by which cyclic GMP inhibits fibrinogen binding is unclear. It has been suggested that increases in cyclic GMP lead to the phosphorylation of a number of platelet proteins including a 46-kDa vasodilator-stimulated phosphoprotein (VASP). This may result in inhibition of phosphatidylinositol turnover, protein kinase C activity and the exposure of the fibrinogen receptor (Takai *et al.*, 1981; Mellion *et al.*, 1983; Halbrugge *et al.*, 1990).

We have also shown that these S-nitrosothiols are effective inhibitors of the release of P-selectin from platelets. P-selectin is a glycoprotein first identified in the platelet α-granules (Stenberg *et al.*, 1985) that mediates the interactions between platelets, leukocytes and endothelium (for review, see Bevilacqua & Nelson, 1993). When platelets are stimulated by aggregating agents such as collagen, P-selectin is rapidly selected to the plasma membrane to promote adhesion of cells to vascular endothelium or to neutrophils and monocytes at the site of tissue injury (Johnston *et al.*, 1989; Larsen *et al.*, 1989; Hamburger & McEver, 1990; Geng *et al.*, 1990; Palabrica *et al.*, 1992). Thus, if endogenous NO is a regulator of P-selectin expression, our results provide an explanation for the finding that inhibitors of NO synthase *in vivo* promote the formation of platelet-leukocyte aggregates (Kurose *et al.*, 1993). As was the case with fibrinogen binding, the inhibition of P-selectin release correlated with the increase in platelet cyclic GMP concentrations. Although SNPL appeared more effective than PGI_2 as an inhibitor of P-selectin release, full concentration-response curves will be required to substantiate this point. This is specially interesting in view of the suggestion that elevations of cyclic GMP may be more

significant than increases in cyclic AMP as a mechanism for the regulation of interaction between cells (Radomski *et al.*, 1987).

The release of NO, with the subsequent NO-mediated increase in cyclic GMP, is considered necessary for the pharmacological activity of S-nitrosothiols (Feelisch & Noack, 1987). This release can be spontaneous, stimulated by some thiols, or may require enzymatic activity, as is the case with S-nitrosoglutathione (Feelisch & Noack, 1987; Kowaluk & Fung, 1990; Radomski *et al.*, 1992). In our experiments there was a good correlation between NO release from SNPL and the pharmacological and biochemical effects of this S-nitrosothiol. However, using the oxyhaemoglobin assay, we failed to detect NO release from SNAP and SNFP at concentrations ($<10\,\mu M$) that produced both an anti-platelet effect and stimulation of the soluble guanylate cyclase. The release could not be detected even in the presence of intact platelets, platelet fractions or reducing agents known to enhance the rate of NO release from other NO donors (Feelisch & Noack, 1987; Radomski *et al.*, 1992). It is important to note that higher concentrations of these S-nitrosothiols ($\geq 10\,\mu M$) did result in the release of detectable amounts of NO.

Thus, SNPL differs from SNAP and SNFP because it: (a) releases NO at lower concentrations, (b) is less stable, (c) is more potent as an inducer of platelet disaggregation, and (d) is less potent as an inhibitor of collagen-induced aggregation and ATP release from platelets. In other respects, i.e. stimulation of the soluble guanylate cyclase, inhibition of fibrinogen binding to platelets and inhibition of P-selectin release, all three S-nitrosothiols are equipotent. These results suggest that the release of NO from SNAP and SNFP occurs inside the platelet while SNPL releases NO spontaneously. Spontaneous release of NO from SNPL may explain its increased potency as an inducer of disaggregation, since a freely diffusible molecule such as NO will probably reach and exert its action on most of the aggregated platelets, whereas SNFP and SNAP would have a more restricted access. This might indeed be the case, since it has been suggested that SNAP requires activation of glyceraldehyde-3-phosphate dehydrogenase for the transport of NO in platelets (McDonald *et al.*, 1993). Alternatively, if SNAP and SNFP stimulate the soluble guanylate cyclase directly, the release of NO would not be a necessary prerequisite for their pharmacological action.

In summary, the analogues of SNAP, SNPL and SNFP, although different from each other in their chemical stability and rate of NO release, are potent stimulators of the soluble guanylate cyclase and inhibitors of platelet function.

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