Br. J. Pharmacol. (1992), **107**, 745–749

S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*

Marek W. Radomski, Daryl D. Rees, *Alberto Dutra & [1]Salvador Moncada

Wellcome Research Laboratories, Beckenham, Kent BR3 3BS and *Department of Histopathology University College and Middlesex School of Medicine, London WC1E 6JJ

1 The effect of S-nitroso-glutathione (GSNO), a stable nitrosothiol, on platelet activation was examined *in vitro* and *in vivo*.

2 The adhesion of human platelets to fibrillar collagen and human endothelial cell monolayers was inhibited by GSNO.

3 GSNO caused a concentration-dependent inhibition of collagen-induced platelet aggregation *in vitro* and decreased ADP-induced aggregation in the conscious rat.

4 Inhibition of platelet aggregation *in vitro* correlated with the increase in intraplatelet cyclic GMP levels.

5 The release of NO from GSNO was enhanced by platelet lysate, native glutathione and ascorbate.

6 The results show that GSNO is a carrier of NO and therefore has pharmacological activity as an inhibitor of platelet activation.

Keywords: S-nitroso-glutathione; platelet function *in vitro* and *in vivo*

Introduction

There is now increasing evidence that the physiological and pharmacological release of nitric oxide (NO) regulates haemostatis and inhibits platelet activation (Radomski & Moncada, 1991; Stamler & Loscalzo, 1991). S-nitrosothiols such as S-nitroso-cysteine are formed by S-nitrosylation of thiols in the presence of NO or NO_2^- (Saville, 1958; Ignarro & Gruetter, 1980). With few exceptions, these compounds are usually unstable, even in the crystalline state (Mellion *et al.*, 1983; Park, 1988); however, they are potent inhibitors of platelet aggregation *in vitro* (Mellion *et al.*, 1983; Lieberman *et al.*, 1991). The biological significance of S-nitrosylation has not yet been defined. It has been suggested that highly reactive and unstable NO is stabilized by a reaction with a carrier molecule i.e. R-SH that prolongs its half-life *in vivo* and preserves its biological activity (Stamler *et al.*, 1992). S-nitroso-glutathione (GSNO, Figure 1), a stable S-nitroso-thiol, can be chemically synthesized from one of the most abundant intracellular thiols, glutathione (Meister & Anderson, 1983). This study investigates the pharmacological effects of GSNO on platelet activation both *in vitro* and *in vivo*.

Methods

In vitro

Platelet adhesion Human blood was collected and platelet-rich plasma (PRP) and prostacyclin-washed platelet suspensions (WP) were prepared (Radomski & Moncada, 1983). The adhesion of unlabelled platelets to fibrillar collagen and ^{111}In-labelled platelets to monolayers of human umbilical vein endothelial cells (SGHEC-7) was measured as described previously (Radomski *et al.*, 1987a).

Platelet aggregation and the release of ATP These were measured in a platelet-ionized calcium lumi-aggregometer (Chronolog). The $[Ca^{2+}]_i$ levels were measured in aequorin-loaded platelets prepared by the method of Yamaguchi *et al.* (1986) with a modification of substituting prostaglandin E_1 with prostacyclin (1 μM). All inhibitors of aggregation were incubated with platelets for 1 min prior to the addition of collagen ($1-2\ \mu g\ ml^{-1}$). In some experiments GSNO was incubated for 30 min at 37°C before its effect on collagen-induced platelet aggregation was measured.

Cyclic nucleotides Washed platelets were incubated in the aggregometer for 10 min at 37°C in the presence or absence of GSNO. Following incubation, EGTA (5 mM) was added and platelets lysed by 2 cycles of freezing in liquid N_2 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 system (Amersham).

The release of NO from GSNO This was measured following incubation of GSNO with intact platelets, platelet lysate and platelet cytosol (100,000 *g* for 30 min prepared from 3×10^8 platelets) and assayed in a dual wave spectrophotometer (Shimadzu) by the haemoglobin shift method (Feelisch & Noack, 1987).

In vivo

Platelet aggregation *in vivo* was measured by use of the disappearance of single platelets as an index of aggregation (Radomski *et al.*, 1990). Male Wistar rats (250–300 g) were briefly anaesthetized with isoflurane (2%). A double-lumen catheter was implanted in the femoral vein for drug administration and a single-lumen catheter implanted in the femoral artery for blood pressure and heart rate measurements. The catheters were fed subcutaneously to exit at the lower back and were connected to the cage housing via a flexible spring. Experiments were started at least 2 h later, when the rats were conscious and unrestrained in their cages. Adenosine 5′-diphosphate (ADP, $0.1-3\ \mu mol\ kg^{-1}$, i.v.) was administered in the presence or absence of a continuous infusion of GSNO ($0.03-1\ \mu mol\ kg^{-1}\ min^{-1}$, i.v.). Blood samples (50 μl) were collected over a period of 60 min, diluted with an equal volume of Tyrode solution containing EGTA (5 mM) and immediately (1 min following each collection) counted in a

[1] Author for correspondence.

whole blood platelet counter, Coulter T-540 (Coulter Electronics Ltd.).

Blood gases and pH levels remained within the normal range for the duration of the experiments.

Reagents

S-nitroso-glutathione (GSNO) was synthesized and human haemoglobin prepared by the methods of Hart (1985) and Paterson *et al.* (1976) respectively. Solutions of NO gas were prepared as described by Palmer *et al.* (1987). Glutathione, ascorbic acid (BDH), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), luciferin-luciferase reagent, human thrombin, Arg-Gly-Asp-Ser, Arg-Gly-Glu-Ser (Sigma), aequorin (Dr Blinks, Mayo Foundation), prostacyclin sodium salt (Wellcome), S-nitroso-acetylpenicillamine (SNAP, Wellcome), SIN-1 (N-ethoxycarbonyl-3-morpholino-sydnonimine; Therabel) and collagen (Hormon-Chemie) were obtained from the sources indicated.

Statistics

Results are mean ± s.e.mean of at least 3 separate experiments. They were compared by analysis of variance and $P<0.05$ was considered as statistically significant.

Results

In vitro

S-nitroso-glutathione (0.1–3 μM) inhibited, in a concentration-dependent manner, platelet adhesion to fibrillar collagen (Figure 1a) and thrombin-stimulated adhesion to SGHEC-7 cells (Figure 1b). The inhibition by the maximally effective concentration of GSNO (3 μM) was completely reversed by haemoglobin (3 μM, Figure 1a,b).

Incubation of GSNO (0.01–10 μM) with PRP or WP for 1 min resulted in a concentration-dependent inhibition of the collagen-induced increase in platelet aggregation and ATP release from platelets (Figure 2a and Table 1). Incubation of GSNO in WP for 30 min did not change significantly the antiaggregating activity of this compound ($IC_{50} = 0.11 \pm 0.04$ μM). S-nitroso-glutathione was approx. 2–3 times more potent in WP than in PRP. This inhibitory activity of GSNO was reversed by haemoglobin (Figure 2b). The aggregation of aequorin-loaded platelets by collagen was also inhibited by GSNO (0.03–0.3 μM, Figure 3). A subthreshold concentration of prostacyclin (0.1 nM) acted synergistically with a threshold concentration of GSNO (0.03 μM) to inhibit aggregation (Figure 4). However, no synergistic interactions were observed between GSNO and two tetrapeptide antagonists of the fibrinogen receptor (Gartner *et al.*, 1985; Pierschbacher & Ruoslahti, 1984) Arg-Gly-Asp-Ser or Arg-Gly-Glu-Ser (3–300 μM) as inhibitors of platelet aggregation ($n = 3$, data not shown). Nitric oxide, GSNO, SNAP and SIN-1 (0.01–30 μM) all inhibited collagen-induced platelet aggregation in a concentration-dependent manner. Comparison of the antiaggregatory activity of these NO donors is shown in Table 1. The order of inhibitory potency was: GSNO > NO > SNAP > SIN-1.

The basal levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) were 34 ± 6 and 16 ± 5 fmol/10^8 platelets respectively. Incubation of platelets with GSNO (0.01–100 μM) caused a concentration-dependent increase in intraplatelet cyclic GMP to a maximum of 2078 ± 140 fmol/10^8 platelets (Figure 5). There was a significant correlation ($r = 0.9561$, $P = 0.0439$) between inhibition of platelet aggregation and increases in cyclic GMP induced by GSNO. The levels of cyclic AMP were slightly but significantly increased ($P = 0.0412$) at concentrations of GSNO > 0.3 μM, however, there was no significant correlation ($r = 0.9102$, $P = 0.0898$) between inhibition of platelet aggregation and increase in cyclic AMP caused by GSNO.

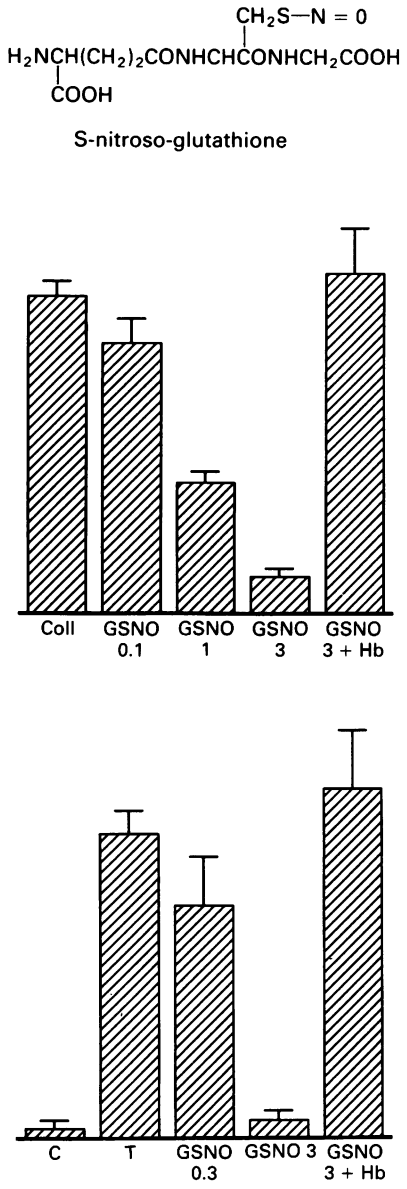

Figure 1 Inhibition of human platelet adhesion to fibrillar collagen (a) and to human umbilical endothelial cell monolayer (b) by S-nitroso-glutathione (GSNO). (a) Collagen (Coll)-induced platelet adhesion was inhibited in a concentration-dependent manner by GSNO (0.1–3 μM). The inhibitory activity of a maximally effective concentration of GSNO (3 μM) was reversed by haemoglobin (3 μM, GSNO 3 + Hb). (b) Platelet adhesion to endothelium (C) was enhanced by thrombin (0.005 u ml^{-1}, T). GSNO (0.3 and 3 μM) caused a concentration-dependent inhibition of adhesion. This action of GSNO was reversed by haemoglobin (GSNO 3 + Hb). The results are mean (± s.e.mean, vertical bars) of 4 experiments.

The release of NO from GSNO (10 μM) dissolved in Tyrode solution was low (0.01 ± 0.01 nmol min^{-1}). The addition of platelet lysate, but not intact platelets or cytosol, caused a concentration-dependent increase in the rate of NO release from GSNO (Figure 6a). Nitric oxide was also released by glutathione and ascorbic acid (Figure 6b).

In vivo

Preliminary experiments demonstrated that ADP (0.3–10 μmol kg^{-1}, i.v.) caused a dose-dependent decrease in the free platelet number and GSNO (0.03–3 μmol kg^{-1} min^{-1}, i.v.)

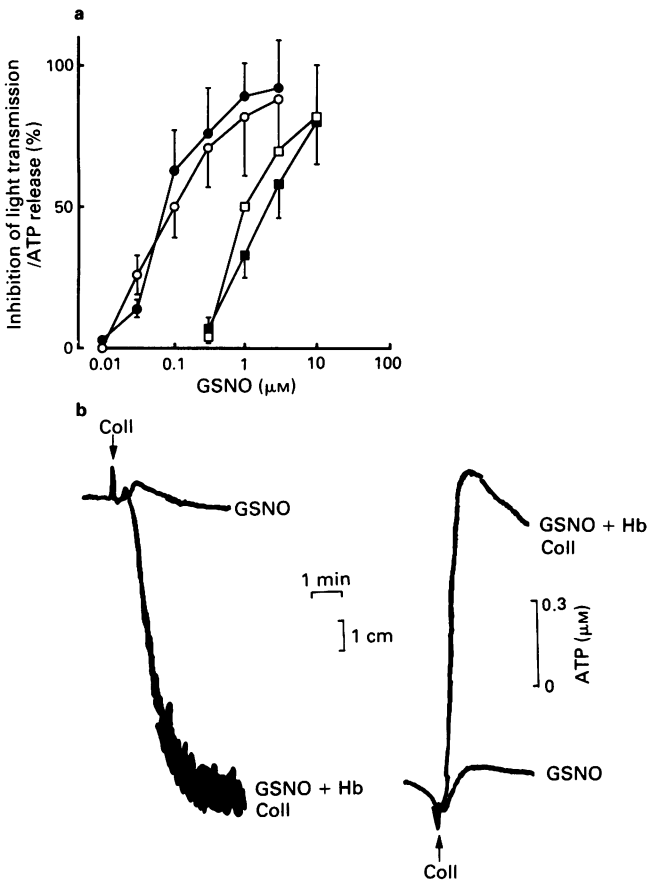

Figure 2 Inhibition of platelet aggregation and the release reaction in washed platelets (WP) and platelet-rich plasma (PRP) by S-nitroso-glutathione (GSNO) (a) and its reversal by haemoglobin (b). (a) Collagen (1 μg ml^{-1})-induced increase in light transmission in WP (○) and PRP (□) and ATP release in WP (●) and PRP (■) was inhibited by GSNO. Results are mean (± s.e.mean, vertical bars) of 3–5 experiments. (b) Haemoglobin (3 μM, GSNO + Hb) reversed the inhibitory effect of GSNO (1 μM) on collagen (1 μg ml^{-1}, Coll)-induced increases in light transmission (left hand tracings) and ATP release (right hand tracings). Tracings representative of 3 similar experiments.

Table 1 Inhibition of platelet activation by GSNO and other NO donors

Compounds	*Aggregation* IC_{50} (μM)	*ATP release*
Nitric oxide	0.36 ± 0.10	0.30 ± 0.09
S-nitroso-glutathione	0.12 ± 0.04	0.09 ± 0.04
S-nitroso-acetylpenicillamine	1.05 ± 0.27	0.79 ± 0.25
SIN-1	2.16 ± 0.41	3.41 ± 0.49

caused a dose-dependent fall in mean arterial blood pressure (data not shown).

ADP (1 μmol kg^{-1}, i.v.) caused a sub-maximal decrease in the free platelet number (27–34%) which recovered to control levels within 60 min. S-nitroso-glutathione (0.3 μmol kg^{-1} min^{-1}, i.v.) produced a small decrease in blood pressure (8 ± 3 mmHg) and inhibited significantly ($P = 0.0094$, $n = 3$) the ADP-induced fall in platelet number and its duration of action (Figure 7). These inhibitory effects were transient, since 1 h after the termination of the GSNO infusion, ADP induced a fall in platelet number similar to the control level (Figure 7).

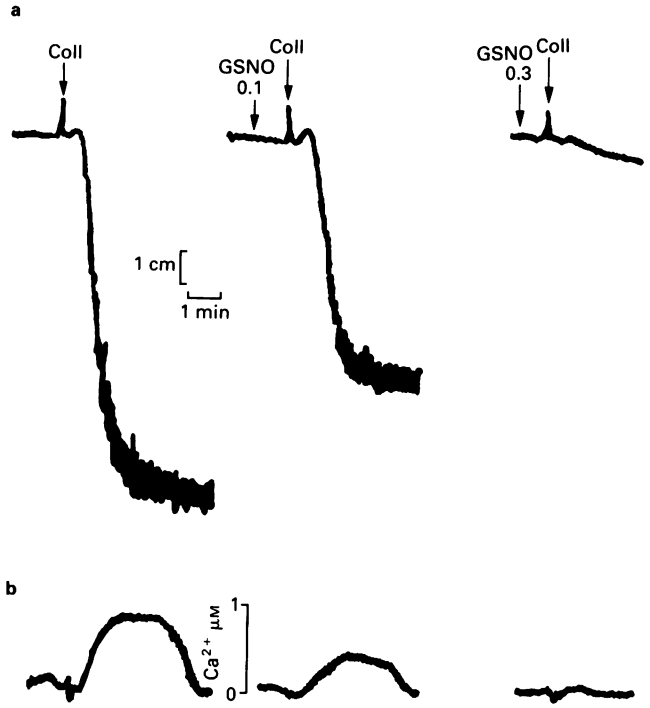

Figure 3 Inhibition of S-nitroso-glutathione (GSNO) of collagen-induced increase in $[Ca^{2+}]_i$ in aequorin-loaded platelets. Collagen (4 μg ml^{-1}, Coll)-induced increases in the light transmission (a) and $[Ca^{2+}]_i$ (b) were inhibited by GSNO (0.1 and 0.3 μM). Tracings representative of 3 similar experiments.

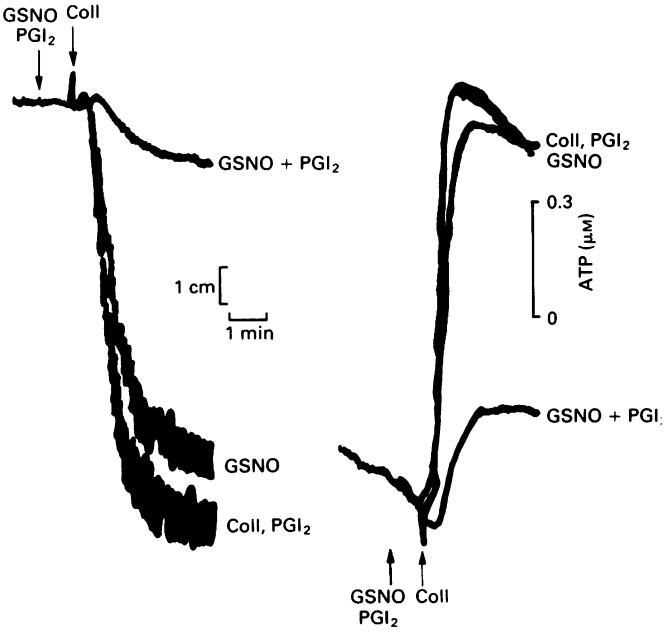

Figure 4 Interactions between S-nitroso-glutathione (GSNO) and prostacyclin as inhibitors of platelet aggregation. A subthreshold concentration of prostacyclin (PGI_2, 0.1 nM) and threshold concentration of GSNO (0.03 μM) acted synergistically (GSNO + PGI_2) to inhibit collagen (Coll, 1 μg ml^{-1})-induced increase in light transmission (left hand tracings) and ATP release (right hand tracings). Tracings representative of 3 similar experiments.

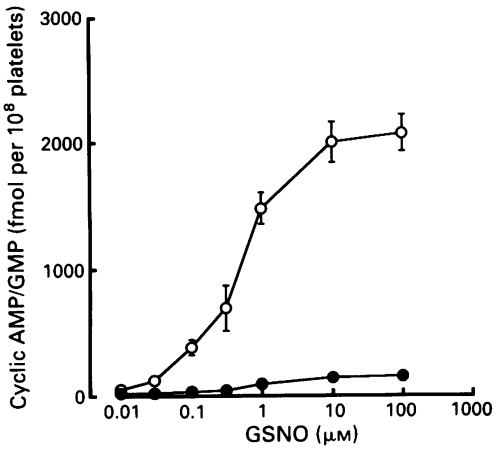

Figure 5 The increase in intraplatelet cyclic nucleotide levels by S-nitroso-glutathione (GSNO). Incubation of platelets with GSNO caused a concentration-dependent increase in cyclic GMP levels (○). A small but significant elevation of cyclic AMP (●) was observed at concentrations of GSNO > 0.3 μM. Each point is mean (± s.e.mean, vertical bars) of 3–4 experiments.

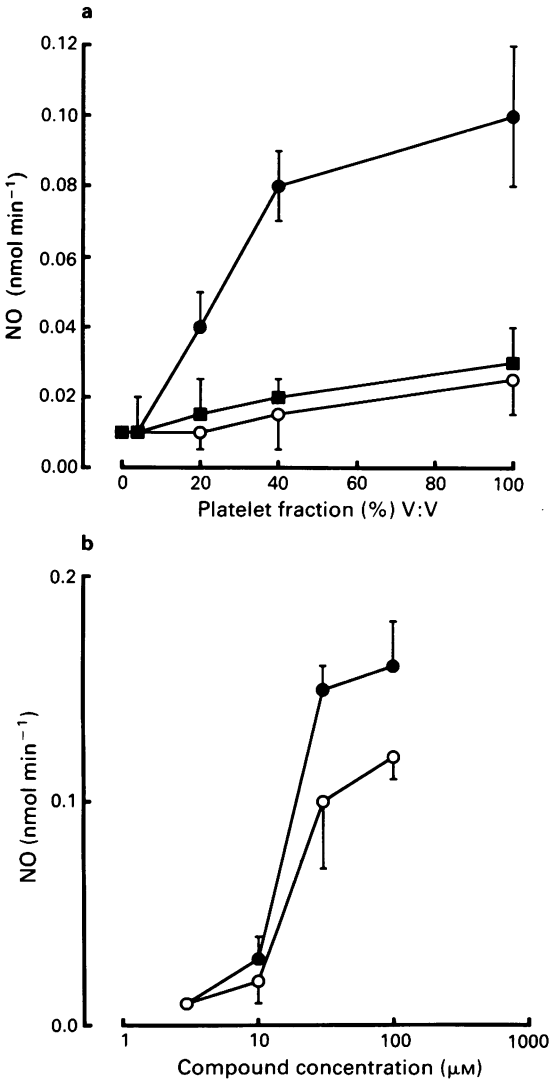

Figure 6 Release of NO from S-nitroso-glutathione (GSNO) by platelet fractions (a) or by reduced glutathione and ascorbic acid (b). (a) Spontaneous release of NO from GSNO (10 μM) was low (0.01 ± 0.01 nmol min^{-1}). The addition of platelet lysate (●) but not cytosol (○) or intact platelets (■) caused a significant increase in NO release from GSNO. (b) Reduced glutathione (●) or ascorbic acid (○) caused a concentration-dependent release of NO from GSNO (10 μM). Each point is mean (± s.e.mean, vertical bars) of 3 experiments.

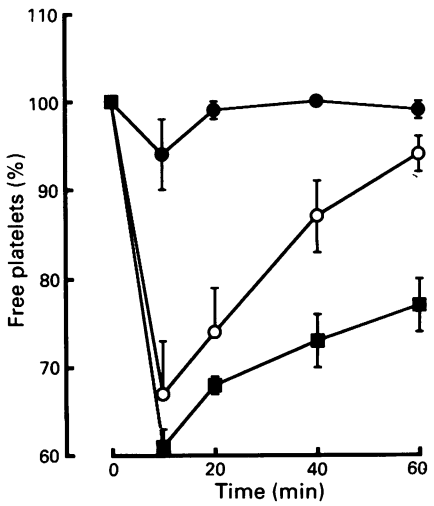

Figure 7 Inhibition of ADP-induced platelet aggregation in the conscious rat by S-nitroso-glutathione (GSNO). ADP (1 μmol kg^{-1}, i.a) caused a time-dependent decrease in free platelet numbers which recovered over the period of 60 min (○). Infusion of GSNO (0.3 μmol kg^{-1} min^{-1}, i.a) attenuated both the extent and duration of this effect (●). Following GSNO washout (1 h) the aggregating activity of ADP was restored (■). Each point is mean (± s.e.mean, vertical bars) of 3 experiments.

Discussion

We have shown that GSNO is a potent inhibitor of platelet adhesion to fibrillar collagen and to human endothelial cells (SGHEC-7). This action is similar to that reported for exogenous NO and endogenous, endothelium-derived NO (Radomski *et al.*, 1987a), confirming that this mediator is an important regulator of platelet adhesion to the vascular wall. In addition, GSNO was a potent inhibitor of collagen-induced aggregation *in vitro*. The inhibition of platelet aggregation *in vitro* has been demonstrated for S-nitrosothiols such as S-nitroso-cysteine, S-nitroso-acetylcysteine, SNAP and S-nitroso-β-D-thioglucose (Mellion *et al.*, 1983; Loscalzo, 1985; Mendelsohn *et al.*, 1990; Lieberman *et al.*, 1991). With the notable exception of SNAP, the remaining S-nitrosothiols are very unstable, which probably accounts for the reported variation in their antiaggregating potency, with IC_{50}s ranging from 6 nM (Loscalzo, 1985) to 20 μM (Lieberman *et al.*, 1991). In accordance with Park (1988), we found that the rate of NO release from GSNO dissolved in an aqueous solution was low but could be enhanced in the presence of thiols such as glutathione or ascorbate (Ignarro & Gruetter, 1980). In addition, the release of NO was significantly increased in the presence of platelet lysate but not by intact platelets or platelet cytosol. This suggests that the release of NO from GSNO may also be catalyzed by an enzyme present in platelet membranes which becomes activated following platelet stimulation. Interestingly, Kowaluk & Fung (1990) have recently suggested that an enzymic rather than spontaneous liberation of NO accounts for the relaxation of rat aortic rings by GSNO.

The biological half-life of GSNO on platelets was several times longer than that of NO (Radomski *et al.*, 1987b), although GSNO was only 2–3 times more potent as an inhibitor of aggregation. Moreover, SNAP and SIN-1, two known NO donors, were several times less potent than GSNO as inhibitors of aggregation. Thus, GSNO was a potent NO donor with a long-lasting platelet-inhibitory effect *in vitro*. Prostacyclin effectively potentiated the antiaggregating activity of GSNO, confirming well-recognized synergistic interactions between the agonists of adenylate and guanylate cyclase systems in platelets (Radomski & Moncada, 1991). Interestingly, we did not observe additive or

synergistic interactions between two tetrapeptide antagonists of the fibrinogen receptor (Pierschbacher & Ruoslahti, 1984; Gartner *et al.*, 1985) and GSNO. Whether the phenomenon is confined to the interactions between these agents *in vitro* or is a part of a general lack of synergy between agonists of guanylate cyclase, which are known to inhibit fibrinogen binding to platelets (Mendelsohn *et al.*, 1990) and inhibitors of the IIb/IIIa receptor remains to be investigated. Incubation of GSNO with WP resulted in a concentration-dependent increase in cyclic GMP and cyclic AMP levels. There was a good correlation between inhibition of platelet aggregation and increases in cyclic GMP caused by GSNO, suggesting that the biological effect of this nitrosothiol on platelets, like that of S-nitroso-cysteine (Lieberman *et al.*, 1991), depended on the stimulation of the soluble guanylate cyclase. Maurice & Haslam (1990) have recently suggested that the increases in cyclic AMP levels caused by cyclic GMP-induced inhibition of low K_m cyclic AMP phosphodiesterase contribute to the platelet-inhibitory activity of nitrovasodilators. Indeed, we have observed that GSNO increases cyclic AMP levels. However, this increase was significant only at concentrations $>0.3\,\mu M$ and failed to correlate with GSNO-induced inhibition of platelet aggregation.

S-nitroso-glutathione was also an effective inhibitor of ADP-induced platelet aggregation *in vivo*. This action was short-lasting and disappeared within 1 h following termination of the GSNO infusion which may be due to the distribution, metabolism and inactivation of GSNO by haemoglobin. It is important to note that a platelet-inhibitory dose of GSNO ($0.3\,\mu mol\,kg^{-1}\,min^{-1}$) had only a small effect on blood pressure. Thus, it may be possible to design platelet-selective NO donors based on the structure of stable S-nitrosothiols such as GSNO.

A low-molecular weight thiol, S-nitroso-cysteine has been suggested as the chemical identity of endothelium-derived relaxing factor (Myers *et al.*, 1990); however, biological generation of this compound has not been demonstrated. S-nitroso-glutathione is yet another candidate for an endogenous NO-carrying molecule. Indeed, glutathione, present in high amounts in the cell (Meister & Anderson, 1983), could be available to form GSNO and prolong the biological activity of NO. Endogenous NO has been shown to S-nitrosylate high-molecular weight thiol-containing proteins (Stamler *et al.*, 1992). Although the S-nitrosylation of low-molecular weight thiols by endogenous NO has not been yet demonstrated, it is also likely to occur. The conditions under which this might happen and the biological relevance of compounds that might act either as biological 'sinks' or carriers for NO remains to be investigated.

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(Received June 17, 1992
Accepted July 10, 1992)