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Inhibition of Human Platelet Aggregation by S-Nitrosothiols

Heme-Dependent Activation of Soluble Guanylate Cyclase and Stimulation of Cyclic GMP Accumulation

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SUMMARY

Recent studies have suggested that cyclic GMP accumulation in platelets mediates the antiaggregatory effects of certain nitrogen oxide-containing agents such as sodium nitroprusside, nitric oxide, nitrosoguanidines, and related agents. The vasodilator effect of these agents may involve the formation of *S*-nitrosothiol intermediates which relax vascular smooth muscle, elevate tissue levels of cyclic GMP, and activate guanylate cyclase. The purpose of this study was to investigate the effects of various synthetic *S*-nitrosothiols on human platelet aggregation. The *S*-nitroso derivatives of *N*-acetylpenicillamine, cysteine, and β-D-thioglucose inhibited human platelet aggregation in a concentration-dependent fashion when ADP, collagen, U46619, or sodium arachidonate was employed as the aggregating agent. The antiaggregatory effects of the *S*-nitrosothiols were associated with a rapid and marked increase in intracellular platelet cyclic GMP levels, whereas cyclic AMP levels remained unchanged. Additionally, *S*-nitrosothiols disaggregated platelets which had been aggregated while concomitantly elevating platelet cyclic GMP levels. Moreover, guanylate cyclase, partially purified from the soluble fraction of human platelets, was markedly activated by *S*-nitrosothiols in a heme-dependent manner. Methemoglobin, a hemoprotein with a high affinity for nitric oxide, partially reversed the antiaggregatory effects, attenuated the accumulation of cyclic GMP, and inhibited the activation of guanylate cyclase by *S*-nitrosothiols. These data are consistent with the hypothesis that *S*-nitrosothiols could serve as active intermediates in the inhibitory action of sodium nitroprusside, nitric oxide, and related nitrogen oxides on platelet aggregation.

INTRODUCTION

The role of cyclic GMP in platelet function is currently a topic of considerable interest. It had previously been observed in several systems that the biological responses associated with an increase in intracellular concentrations of cyclic GMP were opposite in direction to those responses associated with an increase in the formation of cyclic AMP in the same system (1, 2). In view of these findings, and the knowledge that cyclic AMP and agents which stimulate its formation in platelets inhibit platelet function (3, 4), several investigators sought to associate cyclic GMP formation with platelet aggregation and granule secretion. Several agents which induce platelet aggregation and the release reaction were reported to cause small, but measurable, increases in intracellular concentrations of cyclic GMP (5–7). However, additional studies indicated that cyclic GMP formation could be dissociated from platelet aggregation (8, 9) and granule secretion (8–11).

Recent reports from this laboratory (12) and other studies (13–17) have suggested that activation of guanylate cyclase and accumulation of platelet cyclic GMP are closely related to the inhibitory effects of sodium nitroprusside and other nitroso compounds on platelet function. Nitric oxide, cigarette smoke, and a carcinogenic nitrosamine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, all inhibited human platelet aggregation, elevated intracellular concentrations of cyclic GMP, and activated platelet soluble guanylate cyclase (12). Moreover, 8-bromo cyclic GMP, a lipophilic analogue or cyclic GMP, was shown to inhibit human platelet aggregation (12, 14). These data were consistent with the view that these agents inhibit platelet aggregation through a mechanism involving cyclic GMP, and that the effects of nitroso compounds are mediated by a common intermediate such as nitric

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oxide (12). Similarly, the same agents were reported to relax vascular smooth muscle, activate guanylate cyclase, and stimulate cyclic GMP accumulation, thus supporting the hypothesis that cyclic GMP formation is associated with vascular smooth muscle relaxation (18–23).

Extensive investigation of the mechanisms by which sodium nitroprusside, nitric oxide, and related compounds activate guanylate cyclase and relax vascular smooth muscle led to the discovery that such nitrogen oxide-containing agents react with certain thiols to form *S*-nitrosothiols, which are potent activators of soluble guanylate cyclase from a variety of tissues (24–28). *S*-Nitrosothiols have been suggested to function as active intermediates in the vasodilator action of sodium nitroprusside, nitric oxide, and other oxides of nitrogen (29). Since all of the nitrogen oxide-containing compounds which relax vascular smooth muscle also inhibit platelet aggregation (12), it was reasonable to suppose that *S*-nitrosothiols and cyclic GMP could be involved in mediating the inhibitory effect of the former drugs on platelet aggregation. Therefore, the objective of the present study was to investigate the effects of *S*-nitrosothiols on human platelet aggregation, cyclic GMP formation, and on purified preparations of soluble guanylate cyclase from platelets.

MATERIALS AND METHODS

Chemicals and solutions. L-Cysteine, D,L-*N*-acetylpenicillamine, β-D-thioglucose, tetraacetate, adenosine diphosphate (sodium salt), Dowex 50-H^+, and methemoglobin (bovine blood, twice crystallized) were obtained from Sigma Chemical Company (St. Louis, Mo.). Collagen (bovine achilles tendon collagen reagent) was purchased from General Diagnostics, Division of Warner Lambert (Morris Plains, N. J.) and sodium arachidonate (>99% purity) was obtained from NuChek Prep (Elysian Fields, Minn.). The prostaglandin endoperoxide analogue U46619[1] was the generous gift of Dr. John E. Pike, of the Upjohn Company (Kalamazoo, Mich.). O_2-free-N_2 and nitric oxide gas (99.9%) were obtained from Matheson Gas. [^{3}H]Cyclic GMP (2–3 Ci/mmole, ammonium salt) and [α-^{32}P]GTP (10–16 Ci/mmole, triethylammonium salt) were purchased from the New England Nuclear Corporation (Boston, Mass.). Neutral alumina (Woelm) was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio). DE52 cellulose and Matrex gel blue A for column chromatography were purchased from Whatman (Kent, United Kingdom) and the Amicon Corporation (Lexington, Mass.), respectively.

All stock solutions were prepared in cold water (twice glass-distilled). Serial dilutions of stock solutions for aggregation studies and cyclic nucleotide determinations were prepared in 0.154 M saline. Dilutions of all chemicals for use in the guanylate cyclase assay were made in twice-distilled water.

Synthesis of S-nitrosothiols. The *S*-nitroso derivatives of cysteine and β-D-thioglucose were synthesized by a modification of previously described procedures (30, 31) and have been described in detail elsewhere (24, 26, 29). Briefly stated, O_2-free solutions of thiols were saturated with nitric oxide for 10 min at 0–4° and then evacuated and flushed with O_2-free N_2 to remove unreacted nitric oxide. Quantitative conversion of thiols to their respective *S*-nitrosothiols was verified by employing the Ellman test (32) for free sulfhydryls. *S*-Nitrosothiols were identified by the appearance of a sharp peak in the infrared region at 1440–1490 cm^{-1}, characteristic of the *S*-nitroso moiety. Visible absorption spectroscopy indicated that the absorption maxima of the thiols after reaction with nitric oxide were as follows: cysteine (555 nm) and β-D-thioglucose (560 nm). These two *S*-nitrosothiols were highly unstable in the crystalline state but remain stable for many hours at 0–4° under N_2 in neutral aqueous solution not exceeding 50 mM (29). Therefore, the *S*-nitroso derivatives of cysteine and β-D-thioglucose were synthesized and diluted immediately before use. Concentrations of *S*-nitrosothiol solutions were verified by visible absorption spectroscopy (29). Unlike the former *S*-nitrosothiols, *S*-nitroso-*N*-acetylpenicillamine may be prepared as stable crystals with a shelf life of at least 6 months at 0–4°. *S*-Nitroso-*N*-acetylpenicillamine was synthesized according to the method of Field *et al.* (33) by allowing D,L-*N*-acetylpenicillamine to react with $NaNO_2$ at acidic pH. Product formation was quantified and verified by infrared and visible absorption spectroscopy, microscopic crystal identification, and melting point determination (33). The *S*-nitrosothiols tested are stable at 37° in buffered (pH 7.4) aqueous solution for at least 10 min, after which time NO is released (29). *S*-Nitroso-*N*-acetylpenicillamine and *S*-nitrosocysteine are the most and least stable, respectively.

Platelet aggregation studies. Platelet aggregation was monitored as described previously (12). Briefly stated, whole venous blood was collected from healthy donors, who had not ingested any platelet-affecting drugs for at least 2 weeks, by free-flow venipuncture into 0.1 volume of 3.8% (w/v) trisodium citrate. PRP was prepared by centrifugation of whole blood at 200 × *g* for 20 min at room temperature. PPP, which was used as a reference blank, was prepared by centrifugation of PRP at 2000 × *g* for 25 min. Platelet counts were conducted as described (12) and contamination by leukocytes was less than 5% in all experiments. PRP was exposed only to platic or siliconized glassware and was incubated for 3 min at 37° with stirring (1100 rpm) prior to the addition of any test agents. Platelet aggregation was monitored turbidometrically in Chronolog single- and dual-channel aggregometers (Chrono-log Corporation, Haverston, Pa.). The final volume of all PRP incubation mixtures was 0.5 ml. The following agents and concentrations were used to induce platelet aggregation: ADP (2 μM), collagen (0.24 μg), sodium arachidonate (0.41 mM), and U46619 (2 μM). As the objective of this study was to examine inhibition of platelet aggregation, concentrations of aggregating agents employed were the smallest which elicited sustained, maximal aggregation. Inhibition of platelet aggregation by *S*-nitrosothiols is expressed as the percent inhibition of light transmission at the point of maximal transmittance, as compared with values obtained for control PRP incubations with the same aggregating agent (12). Experiments involving the addition of methemoglobin required a PPP reference blank with an equivalent amount of added methemoglobin. Methemoglobin failed to alter the effects of any of the aggregating agents on platelets.

Purification of platelet soluble guanylate cyclase. Soluble guanylate cyclase was partially purified from human platelets by a modification of procedures employed previously to purify soluble guanylate cyclase from rat liver (34). Washed platelets were prepared from 23 units of fresh packed platelets (no more than 14 days old) obtained from the Southeast Louisiana Blood Center (New Orleans, La.), as previously described (12), except that the buffer employed for successive washings and resuspensions was 50 mM Tris-HCl (pH 7.4) containing 0.5 mM EDTA and 5 mM dithiothreitol (dithiothreitol buffer). Platelets resuspended in dithiothreitol buffer were homogenized using a two-step procedure which involved, first, cellular disruption with the aid of a Brinkman Polytron (No. 3 setting; three 10-sec bursts at 60-sec intervals) and, second, homogenization in a Potter-Elvehjem Teflon-glass tissue grinder (eight passes). The resulting homogenate was centrifuged at 100,000 × *g* for 60 min at 0–4°. All subsequent steps were performed at 0–4°. The 100,000 × *g* supernatant (soluble fraction) was transferred to a glass beaker in an ice bath and subjected to isoelectric precipitation. The guanylate cyclase-rich protein fraction was precipitated from the soluble fraction by slowly adding sodium acetate (200 mM, pH 5) with stirring until the pH of the solution reached 5.2, at which time the addition of sodium acetate was discontinued. Stirring was maintained for an additional 30 min. The resulting sediment was collected by centrifugation at 20,000 × *g* for 10 min and the supernatant was discarded. The sediment was resuspended in 15 ml of dithiothreitol buffer, and solution was achieved by adjusting the pH to 7.4 with 0.1 N NaOH. The solution was applied to a DE52 cellulose column (1.5 × 25

[1] The abbreviations used are: U46619, [(15*S*)-hydroxy-11α,9α-(epoxymethano)-prosta(5*Z*,13*E*)-dienoic acid]; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

cm) previously equilibrated with dithiothreitol buffer and the column was eluted with a linear NaCl gradient (0–0.5 M, 250 ml) in dithiothreitol buffer (20 ml/hr; 4-ml fractions). Fractions containing at least 50% of maximal guanylate cyclase activity were pooled and concentrated by ultrafiltration (Amicon; PM-30 membrane) to 8 ml. This concentrate was applied to a column (1.5 × 20 cm) of Matrex gel blue A previously equilibrated with dithiothreitol buffer. After the entire sample had been incorporated into the resin, column flow was stopped for 60 min to ensure maximal binding of guanylate cyclase to the hydrophobic matrix (34). The column was initially washed with dithiothreitol buffer (150 ml; 40 ml/hr) followed by 0.5 M NaCl in dithiothreitol buffer (250 ml; 40 ml/hr). These two washes eluted most of the protein which did not include guanylate cyclase. Guanylate cyclase was eluted with a linear NaCl gradient (0.5–2.0 M, 400 ml) dithiothreitol buffer (40 ml/hr; 10-ml fractions). Fractions containing greater than 40% of maximal guanylate cyclase activity eluted between 1.1 and 1.3 M NaCl and were divided into two pools as follows. Fractions corresponding to the leading edge of the guanylate cyclase peak were pooled and concentrated to 6.0 ml (heme-deficient fraction). Fractions corresponding to the trailing edge of the guanylate cyclase peak were pooled and concentrated to 4.0 ml (heme-enriched fraction). Both fractions were divided into 250-μl aliquots and stored in 30% glycerol (v/v) containing 5 mM dithiothreitol under an O_2-free N_2 atmosphere at −60°. Protein concentrations of samples throughout enzyme purification were determined by the Bio-Rad Coomassie brilliant blue G-250 method, as described by Bio-Rad Laboratories (Richmond, Calif.).

Determination of guanylate cyclase activity. Guanylate cyclase activity was determined by measuring the formation of [^{32}P]cyclic GMP from [α-^{32}P]GTP in the presence of excess Mg^{2+} by a modification of previously described methods (20, 34, 35). Briefly stated, reaction mixtures (0.2 ml) consisted of 40 mM triethanolamine HCl (pH 7.4), 0.3 mM GTP, [α-^{32}P]GTP (8×10^5 cpm), [^{3}H]cyclic GMP ($3–4 \times 10^4$ cpm), and 3.0 mM Mg^{2+}. Incubation mixtures also contained 2.0 mM dithiothreitol and 5 μg of bovine serum ablumin to stabilize guanylate cyclase activity. Protein concentrations in assays utilizing heme-deficient or heme-enriched enzyme fractions were 0.36 μg or 5 μg, respectively, per assay tube. Reactions were initiated by the addition of enzyme followed immediately by the test agent (*S*-nitrosothiol) and incubated for 10 min at 37°. Reactions were terminated as previously described (32). Cyclic GMP was isolated from reaction mixtures by sequential column chromatography on Dowex 50-H^+ and neutral alumina as described previously (20). Eluates were incorporated into Multisol cocktail, and radioactivity was measured by liquid scintillation spectrometry (Beckman LS-7500).

Determination of platelet cyclic nucleotide levels. Platelet cyclic GMP and cyclic AMP levels were determined by radioimmunoassay (Collaborative Research, Inc., Waltham, Mass.) in homogenized, acetylated samples of PRP exactly as previously described (12).

RESULTS

Platelet aggregation studies. The aggregation of human platelets in PRP induced by ADP, collagen, U46619, and sodium arachidonate was inhibited in a concentration-dependent fashion by *S*-nitroso-*N*-acetylpenicillamine (Fig. 1), *S*-nitrosocysteine (Fig. 2), and *S*-nitroso-

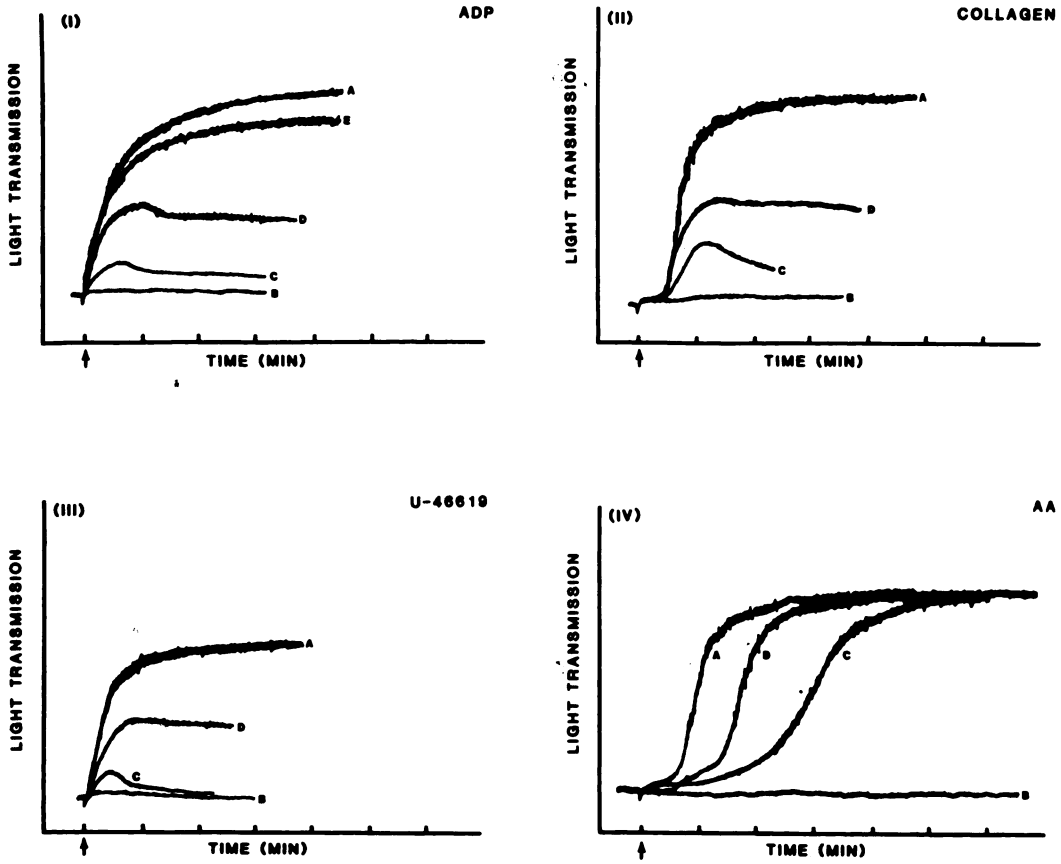

FIG. 1. *Effects of S-nitroso-N-acetylpenicillamine (SNAP) on platelet aggregation induced by various agonists*

SNAP was incubated for 60 sec prior to the addition of aggregating agents. Panel I. Effects of SNAP on ADP-induced aggregation: *A*, ADP alone, 2.0 μM; *B*, 10 μM SNAP; *C*, 5 μM SNAP; *D*, 1.0 μM SNAP; *E*, 0.5 μM SNAP. Panel II. Effects of SNAP on collagen-induced aggregation: *A*, collagen alone, 0.24 μg; *B*, 10 μM SNAP; *C*, 1.0 μM SNAP; *D*, 0.5 μM SNAP. Panel III. Effects of SNAP on U46619-induced aggregation: *A*, U46619 alone, 2 μM; *B*, 10 μM SNAP; *C*, 2.5 μM SNAP; *D*, 1.0 μM SNAP. Panel IV. Effects of SNAP on arachidonate-induced aggregation: *A*, arachidonate alone, 0.41 mM; *B*, 1.0 μM SNAP; *C*, 0.5 μM SNAP; *D*, 0.1 μM SNAP.

β-D-thioglucose (Fig. 3). The data illustrated in Figs. 1–3 are from representative experiments, and at least four separate experiments identical with those represented in these figures were conducted. *S*-Nitrosothiols reduced the maximal extent of platelet aggregation induced by ADP, collagen, and U46619, as measured by a decrease in maximal light transmittance from control. The inhibitory effects of these agents on platelet aggregation induced by sodium arachidonate, however, exhibited an entirely different profile. *S*-Nitro-*N*-acetylpenicillamine, *S*-nitrosocysteine, and *S*-nitroso-β-D-thioglucose, at concentrations of 0.1 or 0.5–10 μM, delayed the onset of platelet aggregation induced by sodium arachidonate (Figs. 1–3, Panel *IV*). The non-nitrosylated derivatives of D,L-*N*-acetylpenicillamine, cysteine, and β-D-thioglucose at concentrations up to 5 mM elicited no observable effects on human platelet aggregation induced by all agents tested (data not shown).

Methemoglobin, a hemoprotein which binds nitric oxide (36) and partially reverses the antiaggregatory effects of sodium nitroprusside, nitric oxide, and other nitroso compounds (12, 17), partially attenuated the inhibitory effects of *S*-nitrosothiols on platelet aggregation induced by ADP, collagen, U46619, and sodium arachidonate, without affecting platelet aggregation per se. This inhibitory effect of methemoglobin was concentration-dependent, and a maximal concentration of 50 μM was employed. Larger concentrations were intensely colored and could not be tested because of interference with measurements of platelet aggregation. The attenuation by 50 μM methemoglobin of the inhibition of ADP-induced platelet aggregation elicited by increasing concentrations of *S*-nitrosocysteine is illustrated in Fig. 4 and summarized in Table 1. Larger concentrations of *S*-nitrosocysteine overcame the inhibitory effect of methemoglobin. Similarly, methemoglobin attenuated the inhibitory effect of *S*-nitroso-*N*-acetylpenicillamine on platelet aggregation induced by sodium arachidonate and collagen (Fig. 5; Table 1). In some experiments, as shown in Fig. 5, larger concentrations of *S*-nitroso-*N*-acetylpenicillamine overcame the inhibitory action of methemoglobin. In other experiments (data now shown) only partial reversal was evident. Methemoglobin also attenuated the antiaggregatory effect of *S*-nitrosocysteine where collagen was employed to induce platelet aggregation (Table 1). In all experiments, the attenuating effect of methemoglobin was at least partially overcome by larger concentrations (10–100 μM) of *S*-nitrosothiols. Although some of the data

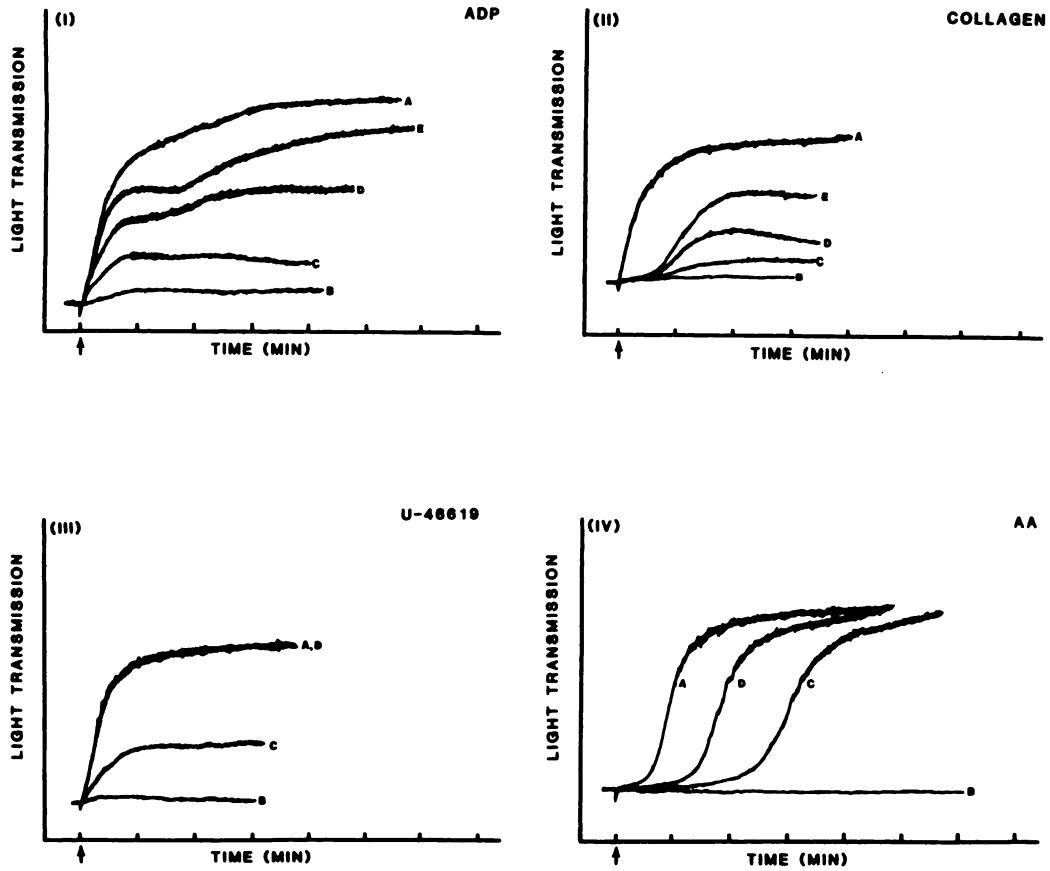

FIG. 2. *Effects of S-nitrosocysteine (NO-Cys) on platelet aggregation induced by various agonists*

NO-Cys was incubated for 60 sec prior to the addition of aggregating agents. Panel I. Effects of NO-Cys on ADP-induced aggregation: *A*, ADP alone, 2 μM; *B*, 10 μM NO-Cys; *C*, 1.0 μM NO-Cys; *D*, 0.5 μM NO-Cys; *E*, 0.1 μM NO-Cys. Panel II. Effects of NO-Cys on collagen-induced aggregation: *A*, collagen alone, 0.24 μg; *B*, 5 μM NO-Cys; *C*, 1.0 μM NO-Cys; *D*, 0.5 μM NO-Cys; *E*, 0.25 μM NO-Cys. Panel III. Effects of NO-Cys on U46619-induced aggregation: *A*, U46610 alone, 2.0 μM; *B*, 10 μM NO-Cys; *C*, 1.0 μM NO-Cys; *D*, 0.5 μM NO-Cys. Panel IV. Effects of NO-Cys on arachidonate-induced aggregation: *A*, arachidonate alone, 0.41 mM; *B*, 10 μM NO-Cys; *C*, 1.0 μM NO-Cys; *D*, 0.5 μM NO-Cys.

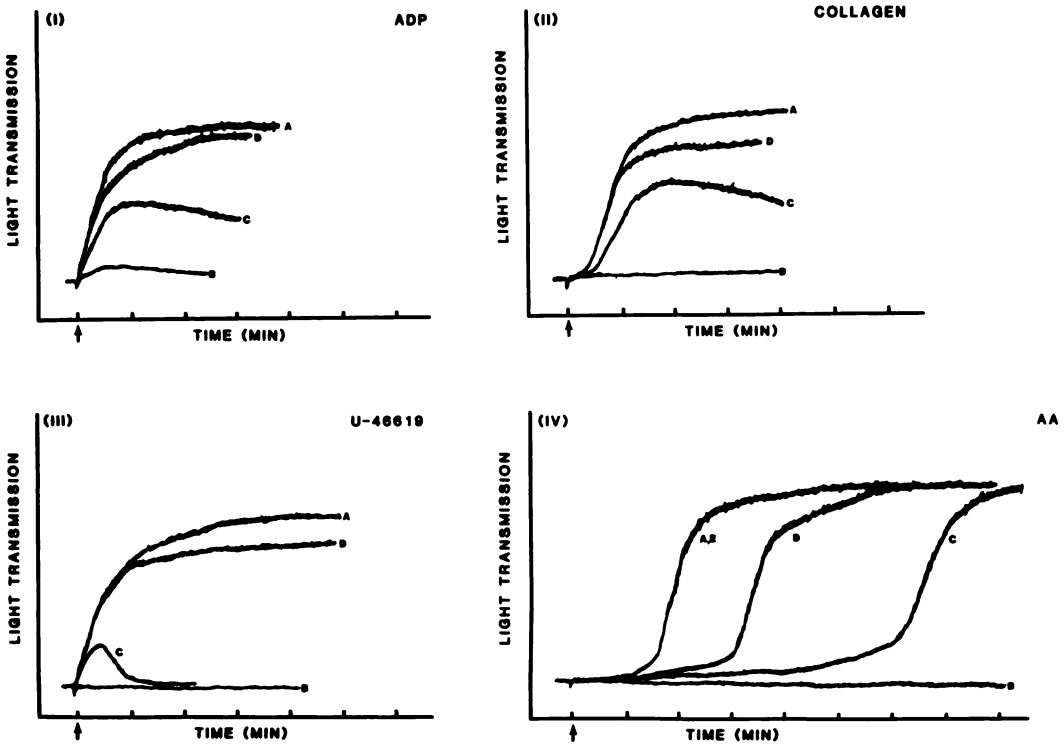

FIG. 3. *Effects of S-nitroso-β-D-thioglucose (NO-TG) on platelet aggregation induced by various agonists*

NO-TG was incubated for 60 sec prior to the addition of aggregating agents. Panel I. Effects of NO-TG on ADP-induced aggregation: *A*, ADP alone, 2 μM; *B*, 10 μM NO-TG; *C*, 1 μM NO-TG; *D*, 0.1 μM NO-TG. Panel II. Effects of NO-TG on collagen-induced aggregation: *A*, collagen alone, 0.24 μg; *B*, 10 μM NO-TG; *C*, 1 μM NO-TG; *D*, 0.1 μM NO-TG. Panel III. Effects of NO-TG on U46619-induced aggregation: *A*, U46619 alone, 2 μM; *B*, 10 μM NO-TG; *C*, 5 μM NO-TG; *D*, 1 μM NO-TG. Panel IV. Effects of NO-TG on arachidonate-induced aggregation: *A*, arachidonate alone, 0.41 mM; *B*, 10 μM NO-TG; *C*, 1 μM NO-TG; *D*, 0.5 μM NO-TG; *E*, 0.1 μM NO-TG.

are not shown, methemoglobin attenuated the inhibitory effects of all three *S*-nitrosothiols on platelet aggregation induced by ADP, collagen, U46619, or sodium arachidonate.

Platelet guanylate cyclase activity. Current studies in this laboratory and another on the purification of soluble guanylate cyclase from bovine lung and rat liver have indicated that guanylate cyclase can be purified in forms

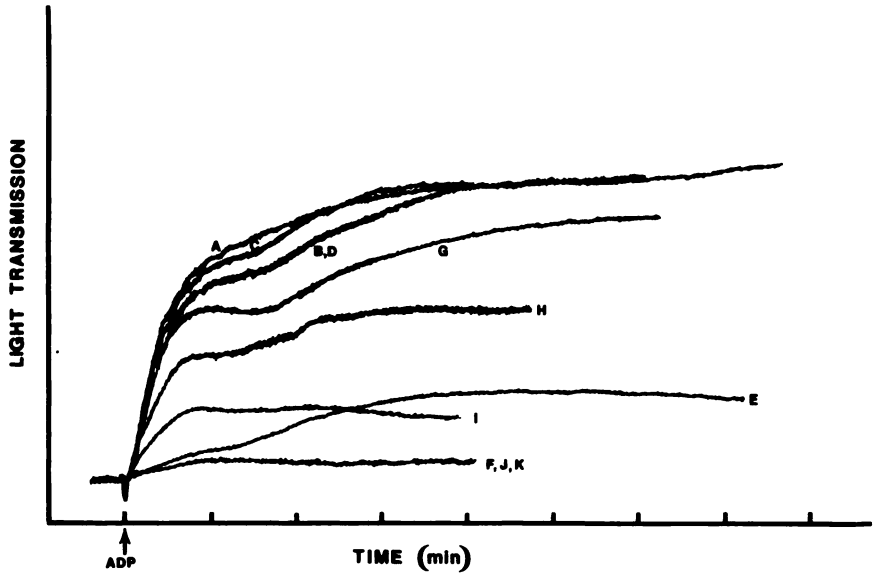

FIG. 4. *Effects of S-nitrosocysteine (NO-Cys) and methemoglobin (MetHb) on ADP-induced human platelet aggregation*

PRP was preincubated at 37° for 3 min prior to the addition of any test agents. MetHb, NO-Cys, and ADP were then added at 60-sec intervals. *A*, ADP alone, 2 μM; *B*, 0.5 μM NO-Cys + 50 μM MetHb; *C*, 0.1 μM NO-Cys + 50 μM MetHb; *D*, 1.0 μM NO-Cys + 50 μM MetHb; *E*, 10 μM NO-Cys + 50 μM MetHb; *F*, 100 μM NO-Cys + 50 μM MetHb; *G*, 0.1 μM NO-Cys; *H*, 0.5 μM NO-Cys; *I*, 1.0 μM NO-Cys; *J*, 10 μM NO-Cys; *K*, 100 μM NO-Cys.

TABLE 1

Effects of S-nitroso-N-acetylpenicillamine, S-nitrosocysteine, and methemoglobin on human platelet aggregation

Inhibition of platelet aggregation in human PRP was measured as described under Materials and Methods.

Aggregating agent	SNAP[a]	NO-Cys[b]	% Inhibition of aggregation[c]	
			No MetHb[d]	+50 μM MetHb
	μM	μM		
ADP (2 μM)	10		86.5 ± 1.6	33.7 ± 8.7
	1		57.5 ± 5.1	16.3 ± 4.8
	0.1		5.7 ± 4.2	2.3 ± 2
		10	91.3 ± 2.1	70.8 ± 8.9
		1	77.3 ± 2.0	8.2 ± 3.7
		0.1	9.5 ± 1.5	0
Collagen (0.24 μg)	10		97.8 ± 0.8	42.8 ± 6.7
	1		83.0 ± 7.3	2.7 ± 2.7
	0.1		0	0
		10	98.0 ± 1.2	95.5 ± 1.9
		1	98.3 ± 1.0	25.0 ± 10.4
		0.1	20.0 ± 10.7	15.0 ± 9.0

[a] *S*-Nitroso-*N*-acetylpenicillamine.

[b] *S*-Nitrosocysteine.

[c] Data are expressed as the percentage inhibition of platelet aggregation (mean ± standard error of the mean) in three to six separate experiments.

[d] Methemoglobin.

which contain varying amounts of bound heme (34, 35, 37, 38). Guanylate cyclase containing maximal (stoichiometric) amounts of bound heme is markedly activated by nitric oxide and nitroso compounds (35, 37), whereas enzyme containing little or no bound heme is only marginally activated (34, 38). The latter enzyme preparation, however, is markedly activated by nitroso compounds after the addition of heme to enzyme reaction mixtures. In the present study, heme-deficient fraction refers to enzyme fraction that is only partially (7- to 9-fold) activated by *S*-nitrosothiols, and where activation is markedly enhanced to almost 50-fold by the addition of hematin (which exists as heme in the presence of excess dithiothreitol) to reaction mixtures. Heme-enriched fraction refers to enzyme fraction that is markedly (up to 26-fold) activated by *S*-nitrosothiols, and where activation is enhanced only an additional 25–50% by the addition of hematin to reaction mixtures. Since these enzyme fractions are only partially purified, it is unknown whether the apparent varying amount of heme is a reflection of enzyme-bound heme or merely due to different amounts of unbound heme in the enzyme fractions. In view of the knowledge that guanylate cyclase binds heme avidly (28, 34, 35, 38) and stoichiometrically (35, 37, 38), however, it is more likely that any heme present in the fractions is enzyme-bound. Previous experiments with purified bovine lung guanylate cyclase indicated that a close correlation exists between the amount of enzyme-bound heme and the percent maximal enzyme activation attainable by *S*-nitrosothiols, nitric oxide, and other nitroso compounds (38).

Specific activities (nanomoles of cyclic GMP per minute per milligram of protein) of heme-deficient and heme-enriched fractions, respectively, were 0.74 ± 0.08 (excess Mg^{2+}) or 6.2 ± 0.5 (excess Mn^{2+}) and 0.16 ± 0.04 (excess Mg^{2+}) or 1.0 ± 0.02 (excess Mn^{2+}). Initial experiments indicated that in the presence of 2 mM dithiothreitol and 5 μg of bovine serum albumin, cyclic GMP formation was linearly related to both incubation time and protein concentration of partially purified enzyme fractions in the absence or presence of *S*-nitrosothiols and hematin. Large concentrations of *S*-nitroso-*N*-acetylpenicillamine

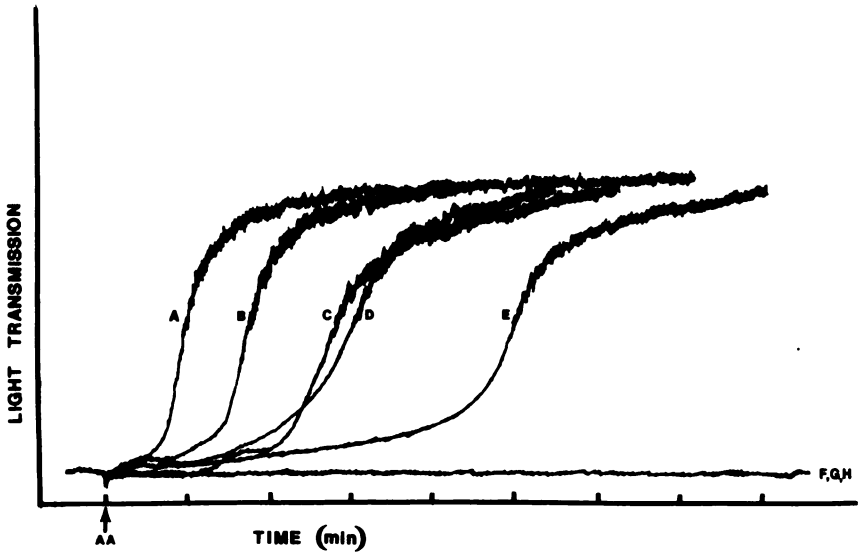

FIG. 5. *Effects of S-nitroso-N-acetylpenicillamine (SNAP) and methemoglobin (MetHb) on arachidonate-induced human platelet aggregation*

PRP was preincubated at 37° for 3 min prior to the addition of any test agents. MetHb, SNAP, and arachidonate were then added at 60-sec intervals. *A*, arachidonate alone, 0.41 mM; *B*, 0.1 μM SNAP; *C*, 1.0 μM SNAP + 50 μM MetHb; *D*, 0.5 μM SNAP + 50 μM MetHb; *E*, 0.5 μM SNAP; *F*, 1.0 μM SNAP; *G*, 10 μM SNAP; *H*, 10 μM SNAP + 50 μM MetHb.

activated the heme-deficient fraction approximately 7-fold in the absence of added hematin, and activation was markedly increased to almost 50-fold in the presence of optimal concentrations (0.5 μM) of hematin (Table 2). On the other hand, *S*-nitroso-*N*-acetylpenicillamine activated the heme-enriched fraction 26-fold in the absence, and 44-fold in the presence, of 0.5 μM hematin (Table 2). The optimal concentration of hematin was found to be 0.5 μM for both heme-deficient and heme-enriched enzyme fractions (data not shown). Similar relationships between the two enzyme fractions were observed with *S*-nitrosocysteine and *S*-nitroso-β-D-thioglucose (Table 2). In addition, activation of guanylate cyclase was dependent on the concentration of each *S*-nitrosothiol. Concentration of hematin in excess of 0.5 μM produced less enhancement of guanylate cyclase activation by *S*-nitroso-*N*-acetylpenicillamine or other *S*-nitrosothiols, and also inhibited basal enzymatic activity (data not shown).

Methemoglobin inhibits platelet guanylate cyclase activation by sodium nitroprusside, nitric oxide, and related nitroso compounds (12, 17). Similarly, methemoglobin has been reported to inhibit guanylate cyclase activation by *S*-nitrosothiols in a variety of tissues (24–26). In the present study, 25 μM methemoglobin inhibited the activation of heme-deficient enzyme fractions by *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosocysteine, and *S*-nitroso-β-D-thioglucose without affecting basal enzymatic activity (Table 3).

TABLE 2

Activation of platelet soluble guanylate cyclase: effects of S-nitrosothiols and hematin on heme-enriched and heme-deficient enzyme fractions

Guanylate cyclase activity was assayed as described under Materials and Methods. Reactions were initiated by the addition of fraction (5–8 μg and 0.36 μg of protein, respectively, for heme-enriched and heme-deficient fractions) followed immediately by the test agents, and incubated for 10 min at 37°. Basal (unstimulated) and stimulated activities were measured in the presence of 3 mM Mg^{2+}.

Addition (mM)	Guanylate cyclase activity[a]			
	Heme-enriched		Heme-deficient	
	−Hematin	+Hematin (0.5 μM)	−Hematin	+Hematin (0.5 μM)
	nmoles cyclic GMP/min/mg protein			
None (basal activity)	0.14 ± 0.004	0.12 ± 0.001	0.72 ± 0.06	0.71 ± 0.06
SNAP[b]				
0.1	3.6 ± 0.2	5.3 ± 0.4	5.4 ± 0.2	35 ± 1.2
0.01	2.8 ± 0.2	3.7 ± 0.1	3.7 ± 0.1	18 ± 0.7
0.001	0.25 ± 0.01	0.27 ± 0.01	0.72 ± 0.04	0.88 ± 0.02
NO-Cys[c]				
0.1	2.9 ± 0.08	3.6 ± 0.07	7.4 ± 0.3	27 ± 1.1
0.01	2.4 ± 0.12	2.9 ± 0.08	7.0 ± 0.4	18 ± 0.8
0.001	—	—	0.86 ± 0.05	1.2 ± 0.04
NO-TG[d]				
0.1	2.9 ± 0.07	3.8 ± 0.1	5.9 ± 0.2	30 ± 1.7
0.01	2.4 ± 0.05	2.6 ± 0.07	3.4 ± 0.2	17 ± 1.0
0.001	—	—	0.7 ± 0.03	1.2 ± 0.08

[a] All values represent the mean ± standard error of the mean for four to six determinations in two or three separate experiments.
[b] *S*-Nitroso-*N*-acetylpenicillamine.
[c] *S*-Nitrosocysteine.
[d] *S*-Nitroso-β-D-thioglucose.

TABLE 3

Methemoglobin inhibition of heme-deficient platelet soluble guanylate cyclase activation by S-nitrosothiols

Guanylate cyclase activity was assayed as described under Materials and Methods. Additionally, all incubations contained 0.5 μM hematin. Reactions were initiated by the addition of fraction (0.36 μg of protein) followed immediately by the test agents and incubated for 10 min at 37°. Basal (unstimulated) and stimulated activities were measured in the presence of 3 mM Mg^{2+}.

S-Nitrosothiol	Guanylate cyclase activity[a]	
	No addition	+MetHb[b] (25 μM)
	nmoles cyclic GMP/min/mg protein	
None (basal activity)	0.71 ± 0.06	0.72 ± 0.07
SNAP[c] (10 μM)	18 ± 0.7	2.1 ± 0.2
NO-Cys[d] (10 μM)	18 ± 0.8	3.5 ± 0.1
NO-TG[e] (10 μM)	17 ± 1.0	3.2 ± 0.3

[a] All values represent the mean ± standard error of the mean for four to six determinations in two or three separate experiments.
[b] Methemoglobin.
[c] *S*-Nitroso-*N*-acetylpenicillamine.
[d] *S*-Nitrosocysteine.
[e] *S*-Nitroso-β-*D*-thioglucose.

Platelet cyclic nucleotide accumulation. The objective of these experiments was to ascertain whether the inhibition of platelet aggregation by *S*-nitrosothiols is associated with a concomitant accumulation of platelet cyclic GMP. The effects of *S*-nitrosothiols on platelet cyclic GMP and cyclic AMP levels in PRP were evaluated by radioimmunoassay under conditions identical with those under which these compounds exhibited antiaggregatory activity (see Materials and Methods). Basal cyclic GMP levels in PRP (0.5 ml containing 1.4–3.2 × 10^8 platelets) were 0.46 ± 0.06 pmole/10^9 platelets, after correction for the small amounts of cyclic GMP in PPP (12). *S*-Nitroso-*N*-acetylpenicillamine, *S*-nitrosocysteine, and *S*-nitroso-β-D-thioglucose failed to alter platelet cyclic AMP levels, even at concentrations (up to 10 μM) which completely abolished platelet aggregation induced by all aggregatory agents tested. Under these conditions, platelet cyclic AMP levels (36.1 ± 1.7 pmoles/10^9 platelets) remained unchanged for at least 20 min following the addition of the *S*-nitrosothiols. At 15–45 sec (10 μM *S*-nitrosocysteine; 10 μM *S*-nitroso-β-D-thioglucose) and 60–90 sec (10 μM *S*-nitroso-*N*-acetylpenicillamine) after the addition of *S*-nitrosothiols, cyclic AMP levels (picomoles/10^9 platelets) were 34.7 ± 3.5 (*S*-nitroso-*N*-acetylpenicillamine), 40.1 ± 1.2 (*S*-nitroso-β-D-thioglucose), and 46.0 ± 12 (*S*-nitrosocysteine). Cyclic AMP levels did not exceed these values for up to 20 min after addition of *S*-nitrosothiols.

S-Nitroso-*N*-acetylpenicillamine (10 μM) induced a rapid, unsustained rise in platelet intracellular cyclic GMP levels within 45–90 sec which returned toward basal values over the next 3 min (Fig. 6). Methemoglobin, which reversed (either partially or completely) the inhibitory effects of *S*-nitrosothiols on platelet aggregation and inhibited the activation of guanylate cyclase by these compounds, likewise inhibited the increase in cyclic GMP accumulation elicited by *S*-nitroso-*N*-acetylpenicillamine (Fig. 6). The data shown in Fig. 6 are from one series of experiments conducted with platelets obtained from a

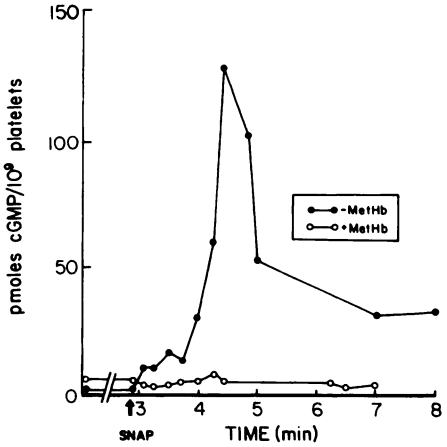

FIG. 6. *Effects of S-nitroso-N-acetylpenicillamine (SNAP) and methemoglobin (MetHb) on platelet cyclic GMP levels*

PRP was preincubated at 37° for 3 min prior to the addition of any agents. Buffer (in place of aggregating agent), MetHb, and 10 μM SNAP were then added at 60-sec intervals. Addition of SNAP was, therefore, at 3 min after preincubation of PRP. The incubations were terminated at the time indicated for determination of cyclic GMP levels in the presence or absence of 50 μM MetHb. Data are from one representative experiment.

single individual. Five additional series of experiments yielded qualitatively similar data, although the inhibition of *S*-nitroso-*N*-acetylpenicillamine-elicited cyclic GMP accumulation by methemoglobin varied considerably from 20% to 95%. Similarly, *S*-nitrocysteine (10 μM) stimulated a marked increase in platelet cyclic GMP concentrations. This increase appeared within 5–30 sec following the addition of *S*-nitrosocysteine to PRP (Fig. 7). Methemoglobin partially inhibited the increase in cyclic GMP accumulation produced by *S*-nitrosocysteine (Fig. 7). In some experiments the inhibition observed with methemoglobin was more marked (up to 75%) than that illustrated in Fig. 7. *S*-Nitroso-ν-D-thioglucose (10 μM)

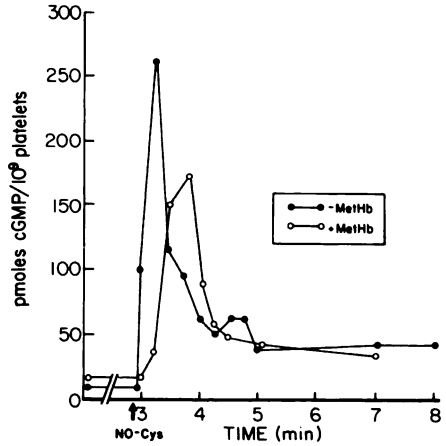

FIG. 7. *Effects of S-nitrosocysteine (NO-Cys) and methemoglobin (MetHb) on platelet cyclic GMP levels*

PRP was preincubated at 37° for 3 min prior to the addition of any agents. Buffer (in place of aggregating agent), MetHb, and 10 μM NO-Cys were then added at 60-sec intervals. Addition of NO-Cys was, therefore, at 3 min after preincubation of PRP. The incubations were terminated at the times indicated for determination of cyclic GMP levels in the presence or absence of 50 μM MetHb. Data are from one representative experiment.

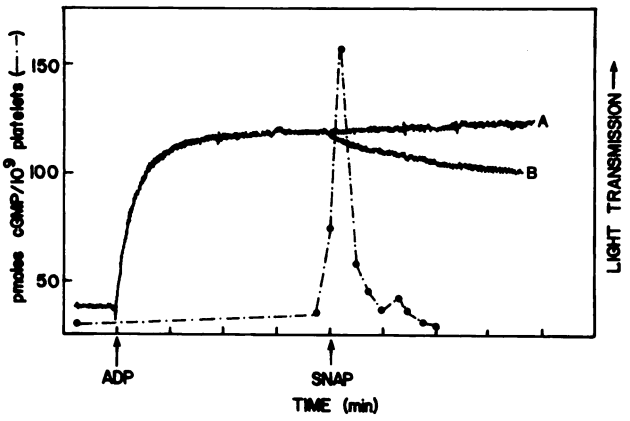

FIG. 8. *Effects of S-nitroso-N-acetylpenicillamine (SNAP) on cyclic GMP levels in preaggregated platelets*

PRP was preincubated at 37° for 3 min prior to the addition of ADP (2 μM). SNAP (10 μM) was added 4 min after ADP, and cyclic GMP concentrations were measured at the time points indicated. Aggregometer tracings represent (*A*) ADP alone (2 μM); (*B*) ADP + SNAP (10 μM). Data are from one representative experiment. Three additional experiments yielded qualitatively and quantitatively similar results.

also stimulated the accumulation of platelet cyclic GMP in a time course similar to that observed with *S*-nitrosocysteine, which was partially inhibited (40–90%) by 50 μM methemoglobin (data not shown). The experiments shown in Figs. 6 and 7 were conducted in the absence of platelet-aggregating agents. Previous studies indicated that the presence of ADP failed to alter the profiles (time course and magnitude) of cyclic GMP accumulation characteristic of nitric oxide and nitroso compounds (12). Similarly, in the present study the profiles of cyclic GMP accumulation generated by the three *S*-nitrosothiols were very similar in the absence or presence of ADP, collagen, U46619, or sodium arachidonate.

S-Nitrosothiols, when added to ADP-preaggregated platelets, resulted in the partial disaggregation of these cells, as measured by a decrease in light transmission. Figure 8 shows the results of such an experiment in which 10 μM *S*-nitroso-*N*-acetylpenicillamine partially disaggregated the platelets and caused a concomitant increase in the platelet concentration of cyclic GMP. It should be noted that the profile of cyclic GMP accumulation in Fig. 8 is sharper than that in Fig. 6. This difference may be due to the difference in experimental designs. For example, *S*-nitroso-*N*-acetylpenicillamine was added to preaggregated platelets in the experiment shown in Fig. 8 but to unaggregated platelets in the experiment shown in Fig. 6. These differences have been consistently observed. Both the disaggregation response and the increased cyclic GMP accumulation were partially inhibited by 50 μM methemoglobin (data not shown). Qualitatively similar results were obtained with *S*-nitrosocysteine and *S*-nitroso-β-D-thioglucose, whether platelet aggregation had been elicited by ADP or sodium arachidonate.

DISCUSSION

Results of the present study show that *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosocysteine, and *S*-nitroso-β-D-thioglucose, in a concentration-dependent manner, inhibited platelet aggregation in PRP induced by a variety

of agents, each capable of inducing aggregation by different mechanisms. Platelet aggregation induced by ADP and collagen is independent of the arachidonate metabolites prostaglandin G_2, prostaglandin H_2, and thromboxane A_2, but granule secretion induced by these agents appears to be dependent on prostaglandin endoperoxide, and subsequent thromboxane A_2 synthesis initiated as a result of aggregation (39, 40). Platelet aggregation and secretion induced by sodium arachidonate, on the other hand, is absolutely dependent on prostaglandin endoperoxide and or thromboxane A_2 formation (39, 40). U46619, however, appears to aggregate platelets by a mechanism which differs from that of other aggregatory agents (41, 42), perhaps by mimicking thromboxane A_2 at the receptor level (41, 43, 44). Since *S*-nitrosothiols inhibited aggregation induced by all agents, it is reasonable to suspect that the process(es) affected are common to all aggregating agents.

S-Nitrosothiols attenuated the maximal extent of platelet aggregation induced by ADP, collagen, and U46619. Aggregation induced by sodium arachidonate, however, was affected in a dissimilar fashion. When sodium arachidonate was added to PRP which had been preincubated with *S*-nitrosothiols, a delay in the onset of aggregation was observed without a decline in the maximal extent of aggregation. This effect is not likely attributed to interference with the oxidation of arachidonic acid, since *S*-nitrosothiols inhibited aggregation induced by U46619, which is thought to act by stimulating thromboxane A_2 receptors. Interestingly, Gorman and co-workers (45, 46) observed a similar inhibitory pattern on arachidonic acid-induced platelet aggregation by thromboxane synthetase inhibitors which suggested that the delay in the onset of aggregation was associated with the inhibition of thromboxane A_2 formation. Information on the effects of *S*-nitrosothiols and cyclic GMP on thromboxane formation could prove useful in elucidating the mechanism(s) by which *S*-nitrosothiols inhibit sodium arachidonate-induced platelet aggregation.

Methemoglobin, a hemoprotein with high binding affinity for nitric oxide (36), has been used in studies on the mechanisms by which nitrogen oxides affect platelet function (12, 17). Nitric oxide has been implicated as a common reactive intermediate in the antiaggregatory effects of sodium nitroprusside, nitrosoguanidine, cigarette smoke (12), and the antianginal drug molsidomine and its metabolites (17). Methemoglobin attenuated the inhibitory effects of all of these antiaggregatory agents. In the present study, methemoglobin attenuated the inhibitory effects of *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosocysteine, and *S*-nitroso-β-D-thioglucose on platelet aggregation induced by a variety of aggregatory agents. *S*-Nitrosothiols are somewhat unstable in neutral aqueous medium and break down to the corresponding disulfide and nitric oxide (29). Inhibition of the antiaggregatory effects of *S*-nitrosothiols by methemoglobin is likely due to the binding and sequestration of nitric oxide by the hemoprotein. Indeed, *S*-nitrosothiols react rapidly with methemoglobin to form bright red solutions characteristic of nitrosyl-hemoglobin.[2] In addition to their inhibiting effects on platelet aggregation, *S*-nitrosothiols were observed to stimulate a large, short-lived elevation in platelet cyclic GMP levels, which was inhibited by methemoglobin. This inhibition was variable. These results suggest that an accumulation of cyclic GMP in platelets may be associated with the antiaggregatory effect of these compounds. *S*-Nitrosothiols, in this study, and other nitroso compounds (12) elicited transient accumulations of platelet cyclic GMP whereas their inhibitory effects on aggregation were long-lasting. Similar observations were made on relaxation of vascular smooth muscle by nitroso compounds (23, 29). The reason for these observations is unknown. One possible explanation, however, is that the transient elevation of cyclic GMP levels triggers a subsequent intracellular event(s) which is longer lasting and leads to inhibition of platelet aggregation. Other explanations are not ruled out.

Considerable variability in the effects of methemoglobin was often observed when comparing platelets from one individual with those from another. For example, 50 μM methemoglobin variably inhibited platelet cyclic GMP accumulation by 95% (Fig. 6) to only 20%. Similar variability was observed with the inhibitory effect of methemoglobin on *S*-nitrosothiol-elicited antiaggregation. Therefore, it is difficult to compare the effects of methemoglobin in one experimental protocol with those in another.

S-Nitrosothiols elevated platelet cyclic GMP levels to similar values whether or not aggregating agents were present. Similar observations were made with nitric oxide, sodium nitroprusside, and nitrosoguanidine (12). Aggregating agents alone inconsistently caused only little (less than 3-fold) or no cyclic GMP accumulation. Therefore, one might expect platelet cyclic GMP accumulation caused by *S*-nitrosothiols to be similar in the absence or presence of aggregating agents. In support of this view are the observations that cyclic GMP accumulation in aggregated platelets was similar to that in unaggregated platelets after addition of *S*-nitroso-*N*-acetylpenicillamine (Fig. 8) or the other *S*-nitrosothiols. *S*-Nitrosothiols also reversed platelet aggregation, as did nitric oxide and related nitroso compounds (12), and this effect occurred concomitantly with platelet cyclic GMP accumulation.

After having demonstrated the effects of *S*-nitrosothiols on platelet aggregation and cyclic nucleotide levels, it was of interest to examine the effects of these compounds on guanylate cyclase activity. Guanylate cyclase was partially purified from the soluble fraction of human platelets by dye-ligand hydrophobic affinity chromatography. Two fractions of partially purified enzyme were recovered. One fraction was heme-deficient and the other fraction was heme-enriched. Preliminary studies by Gerzer and Garbers (47) and a report from this laboratory (38) indicate that bovine lung soluble guanylate cyclase can be purified into heme-deficient and heme-containing forms of the same enzyme. Partially purified guanylate cyclase, as described in this report, showed the largest specific activities reported thus far for purified preparations of platelet soluble guanylate cyclase (17, 48).

The heme-deficient guanylate cyclase fraction was partially activated by *S*-nitrosothiols, and this activation was markedly enhanced by the addition of hematin to assay reaction mixtures. On the other hand, the heme-

[2] B. T. Mellion, L. J. Ignarro, C. B. Myers, E. H. Ohlstein, B. A. Ballot, A. L. Hyman, and P. J. Kadowitz, unpublished observations.

enriched enzyme fraction was markedly activated by *S*-nitrosothiols, and added hematin only slightly enhanced enzyme activation. Hematin, at concentrations which maximally enhanced enzyme activation by *S*-nitrosothiols, did not affect basal guanylate cyclase activity. Larger hematin concentrations were less effective in enhancing enzyme activation. This observation is consistent with the findings that hemoproteins and large concentrations of hematin inhibit the activation of soluble guanylate cyclase by nitroso compounds (20, 21, 34, 49–51), probably by competing with NO-heme complexes for a common binding site on the enzyme (52). These observations are consistent with the present findings that methemoglobin inhibited the activation of platelet guanylate cyclase by *S*-nitrosothiols.

The present findings suggest that, as appears to be the case for hepatic guanylate cyclase (34, 49), platelet guanylate cyclase probably requires heme for the full expression of enzyme activation by nitroso compounds. These findings are consistent with other reports that preformed NO-heme binds to (28) and markedly activates (34, 49, 52) guanylate cyclase. It is plausible that *S*-nitrosothiols react with the heme associated with guanylate cyclase to form NO-heme, which in turn activates the enzyme.

The two different fractions of guanylate cyclase possessed different (about 6-fold) specific activities for both the unactivated (basal) and activated forms. This difference could be due to a number of factors, including intrinsic differences in the two enzyme forms, differences in stability, differences in purity, and differences in the amount of enzyme-bound heme. Pertaining to the latter point, one might expect that the heme-enriched fraction would possess a lower basal specific activity than that of the heme-deficient fraction because heme is a potent inhibitor of guanylate cyclase (27, 28, 34, 35, 38). However, since the stimulated specific activities of the two enzyme fractions in the presence of excess heme are different, the first three factors listed above must be considered. Experiments with homogeneous soluble platelet guanylate cyclase should provide more definitive explanations of the present observations.

Activation of platelet guanylate cyclase by *S*-nitrosothiols as well as the inhibitory effect of methemoglobin are consistent with the effects of these agents on platelet cyclic GMP accumulation. These data support and extend our hypothesis that elevated platelet cyclic GMP levels are associated with the antiaggregatory effects of nitrogen oxide-containing vasodilators and related agents (12). Moreover, the possibility that *S*-nitrosothiols serve as intermediates of certain nitrogen oxides such as nitric oxide, sodium nitroprusside, nitrosoguanidines, and cigarette smoke in the inhibition of human platelet aggregation is supported by the following observations: (a) normal human serum contains between 400 and 600 μmoles/liter of sulfhydryl, mostly as mercaptoalbumin (53); (b) cigarette smoke contains nitric oxide (54), which reacts with thiols to form *S*-nitrosothiols (31, 33); (c) thiols promote the release of NO from nitrosoguanidines (25), with the subsequent formation of *S*-nitrosothiols (29); (d) *S*-nitrosothiol formation in neutral, aqueous solution from nitrosoguanidines (31) and sodium nitroprusside (29) has been reported. It can, therefore, be hypothesized that the addition of sodium nitroprusside, nitrosoguanidines, nitric oxide, or cigarette smoke to PRP could result in the formation of *S*-nitrosothiols which, in turn, cross the plasma membrane, activate cytosolic guanylate cyclase, elevate intracellular cyclic GMP levels, and inhibit platelet aggregation. Alternatively, sodium nitroprusside, nitrosoguanidines, and nitric oxide may first penetrate the cell membrane, subsequently combining with free sulfhydryls in the intracellular compartment of the platelet to form *S*-nitrosothiols, which then activate guanylate cyclase and increase cyclic GMP levels. The latter explanation is unlikely, however, in view of an earlier study (12) where methemoglobin, which does not penetrate cell membranes, inhibited the antiaggregatory effects and the increased cyclic GMP formation in PRP elicited by nitroso compounds.

Although it is clear that *S*-nitrosothiols markedly elevated platelet cyclic GMP levels and that this effect occurred concomitantly with inhibition or reversal of platelet aggregation (Fig. 8), it is not clear from these studies whether a quantitative relationship exists between the two effects. Concentration-effect relationships of *S*-nitrosothiols on platelet cyclic GMP accumulation were not studied. In adtition, the variable effects of methemoglobin on cyclic GMP accumulation and platelet aggregation in the presence of *S*-nitrosothiols with platelets from one individual to another preclude any assessment of quantitative relationships. The results of the present investigation, however, demonstrate clearly that *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosocysteine, and *S*-nitroso-β-D-thioglucose inhibit aggregation, activate partially purified guanylate cyclase, and stimulate the formation of cyclic GMP in human platelets. These data are consistent with the hypothesis that cyclic GMP is associated with the inhibitory action of certain nitroso-containing compounds (12, 15, 17, 55), rather than as a mediator of platelet activation as previously suggested (6, 7, 56).

We have been unable to observe appreciable elevations of platelet cyclic GMP levels consistently during onset of platelet aggregation caused by the aggregating agents tested. Cyclic GMP accumulations ranged inconsistently from zero to 3-fold. Indeed, Haslam and co-workers (10) have concluded that the small increase in platelet cyclic GMP levels by certain aggregating agents is probably an effect, rather than a cause, of aggregation. Therefore, the implication is that cyclic GMP may serve as a feedback inhibitor of platelet aggregation and other platelet functions (55). Such a role for cyclic GMP in vascular smooth muscle relaxation has been proposed by others (57, 58), who demonstrated that some contractile agents, as well as certain relaxant drugs such as sodium nitroprusside, increase cyclic GMP formation in this tissue. Sodium nitroprusside inhibits platelet aggregation and elevates cyclic GMP levels without significantly altering cyclic AMP levels in human PRP (12). In the present study, several *S*-nitrosothiols also inhibited platelet aggregation and elevated cyclic GMP levels without altering cyclic AMP levels. It has been reported recently that at lower concentrations (0.1–10 μM), the effects of sodium nitroprusside in washed human platelets may be mediated by cyclic AMP (59). In previous studies with PRP (12), as

well as in the present study, cyclic AMP was not elevated significantly by the nitrogen oxide-containing agents. The reason for this apparent difference in results is unknown but may be related to differences in preparations and procedures used in these studies, since Haslam and Davidson (59) employed prelabeling techniques in washed human platelets whereas the present studies were carried out in human PRP in which basal cyclic AMP levels were higher. Takai *et al.* (60, 61) have recently shown that increased platelet cyclic GMP levels is associated with the inhibition of both the production of diacyl glycerol from phosphatidylinositol and the concomitant phosphorylation of a 40,000 M_r protein, which results from platelet stimulation by thrombin. However, a 50,000 M_r protein is phosphorylated in the presence of elevated intracellular cyclic GMP levels. Haslam *et al.* (55) have also reported an increase in the phosphorylation of 40,000 and 50,000 M_r proteins by sodium nitroprusside and 8-bromo-cyclic GMP. Collectively, these studies imply that cyclic GMP may function as a feedback inhibitor in platelets exposed to aggregating agents.

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