S-Nitrosocysteine Inhibition of Human Platelet Secretion Is Correlated With Increases in Platelet cGMP Levels

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Platelet inhibition by exogenous and endogenous nitrovasodilators has been shown to be associated with increases in cGMP, but proof of a role for cGMP in this process is lacking. We therefore studied the effects of cGMP and guanylate cyclase stimulation on human platelet secretion by pharmacologically modulating intraplatelet cGMP levels. The endothelium-derived relaxing factor (EDRF)–like activator of guanylate cyclase, S-nitrosocysteine (SNOC), led to a dose-dependent inhibition of secretion in intact human platelets (IC_{50}=10^{-8} M). The cGMP phosphodiesterase inhibitor M&B 22,948 augmented SNOC-induced inhibition of secretion through elevations in cGMP without affecting cAMP levels (from 50% to 81% inhibition versus control, p=0.02). Methylene blue reversed the inhibitory effects of SNOC on platelet secretion (p=0.03). Dibutryl-cGMP and 8-bromo-cGMP also significantly inhibited secretion in this system. Incubation of platelets with exogenous cGMP to achieve intraplatelet cGMP levels comparable to those after SNOC treatment resulted in similar degrees of inhibition of secretion (32% inhibition versus control, p=0.01) and was also potentiated by M&B 22,948 (from 32% to 68% inhibition, p=0.003). In addition, a highly significant correlation between intraplatelet cGMP levels and the degree of inhibition of secretion was demonstrable in these studies (r=0.94, p=0.016). These data demonstrate that elevation of intraplatelet cGMP levels by the EDRF-like compound SNOC is correlated with inhibition of human platelet secretion. (Circulation Research 1991;68:1722–1728)

The importance of platelet activation in the pathogenesis of acute ischemic events, including unstable angina and myocardial infarction, is well established.1,2 Normal hemostasis and endogenous thrombolysis are dependent on a dynamic equilibrium that exists between the endothelium and platelets, influenced by at least two endothelial vasodilators with known antiplatelet effects, prostacyclin and endothelium-derived relaxing factor (EDRF).3

Although EDRF has been demonstrated to be a potent inhibitor of platelet activation, the mechanism of this inhibition is not well understood. It has been suggested by a number of investigators that cGMP plays an important role in this inhibition, and in both in vitro and in vivo models, an association between platelet inhibition by EDRF and elevations in cGMP levels has been reported.4–11 However, the precise biochemical relation between cGMP and inhibition of platelet function has not been rigorously established and remains controversial.

We therefore sought to define the role of cGMP in the inhibition of platelet function by using S-nitrosothiol (SNOC), which has recently been suggested to be one form of EDRF,12 and pharmacological agents capable of altering intraplatelet cGMP levels. We have recently reported that guanylate cyclase activation by S-nitrosothiol nitrovasodilator compounds inhibits fibrinogen binding to platelets via a cGMP-mediated mechanism.13 We now provide direct evidence that the putative EDRF, SNOC, inhibits platelet secretion by a cGMP-mediated mechanism.

Materials and Methods

Materials

ADP and l-cysteine were obtained from Sigma Chemical Co., St. Louis. Human thrombin was obtained from Enzyme Research Laboratories, South Bend, Ind. Radioimmunoassay supplies for cGMP
and cAMP determinations were purchased from New England Nuclear, Boston. [14C]Serotonin was obtained from Amersham Corp., Arlington Heights, Ill. 2-o-Propoxyphenyl-8-azapurin-6-one (M&B 22,948) was a kind gift from Rhone-Poulenc, Dagenham, England. Methylene blue was obtained from American Regent Laboratories Inc., Shirley, N.Y. Distilled, deionized water was used throughout all experiments. All other materials used were the highest reagent grade available.

Platelets

Venous blood was obtained from healthy human volunteers who had not ingested acetylsalicylic acid for at least 10 days nor caffeine-containing beverages for at least 12 hours. The blood was collected into citrate-phosphate-dextrose at a dilution of 9:1 and used within 1 hour after collection. Platelet-rich plasma (PRP) was prepared by differential centrifugation at 160g for 10 minutes at 22°C. The top two thirds of the PRP was removed for aggregation or secretion experiments. Platelet counts were determined with a Coulter Counter (model ZM, Coulter Electronics, Hialeah, Fla.).

Platelet Aggregation

Platelet aggregation was performed in either a Payton dual-channel aggregometer (Payton Associates, Inc., Buffalo, N.Y.) or a Bio/Data four-channel aggregometer (model PAP-4, Bio/Data, Hatboro, Pa.). Aggregation was performed in 0.5-ml aliquots of PRP that were incubated at 37°C and stirred at 900 rpm, as described.14 Aggregation was initiated by the addition of 10 μM ADP unless otherwise noted. In some experiments platelets were preincubated with SNOC for 2 minutes before the initiation of aggregation.

Platelet Secretion

Platelet secretion was determined using 14C-radio-labeled serotonin as previously described.15 Briefly, PRP was incubated with [14C]serotonin at 37°C for 10 minutes. Imipramine was added just before the initiation of secretion to prevent reuptake of secreted serotonin.15 Secretion was then initiated by the addition of thrombin (0.5 unit/ml) to 0.25-ml aliquots of the [14C]serotonin-loaded PRP. Secretion was allowed to proceed for 2 minutes and was terminated by the addition of 50 μl ice-cold formaldehyde in 0.05 M EDTA; samples were then isolated in a tabletop microcentrifuge (model 235C, Fisher Scientific Co., Pittsburgh, Pa.) at 14,000g for 3 minutes.

Percent secretion was determined as described previously.15 Briefly, secretion was calculated as a percentage of the total radioactivity present in each sample

\[
\text{Secretion (\%) = \frac{cpm \text{ in } S_{\text{test}} - cpm \text{ in } S_{\text{control}}}{cpm \text{ in } T_{\text{control}} - cpm \text{ in } S_{\text{control}}} }
\]

where the supernatants obtained contained the [14C]serotonin (S\text{test}) or the “background” level of 14C from resting platelets (S\text{control}). Additional aliquots for total content of [14C]serotonin, which remained uncentrifuged, were simultaneously prepared (T\text{control}) for each experiment and treated in an identical fashion.

In certain experiments, platelets were preincubated with pharmacological agents capable of affecting cGMP, or cGMP itself, for specific times. In all instances, platelets were first loaded with serotonin as described, and thrombin exposure to induce secretion was for 2 minutes. Pretreatment with M&B 22,948, dibutylryl-cGMP, 8-bromo-cGMP, or exogenous cGMP itself was for 15 minutes at room temperature after the serotonin was loaded. M&B 22,948 was dissolved first in 0.1 M NaOH and then was diluted serially into distilled, deionized water at the time of the experiment.

In every experiment, control points were included of serotonin-labeled platelets to which parallel additions of buffer were added simultaneously to the additions of individual agonists and/or antagonists to treated platelets. The final volumes and incubation times for these controls and the treated platelets were therefore identical. In addition, at the end of a series of secretion experiments, repeat control secretions were performed to assure that the platelets were still viable and that the control secretory response was unchanged over the course of the experiment.

Cyclic Nucleotide Assays and Determination of Intraplatelet cGMP

Samples for determination of cGMP and cAMP were prepared in parallel in selected aggregation and secretion experiments by removal of aliquots into cold 10% trichloroacetic acid (1:5 [vol/vol]). The assays for each cyclic nucleotide were performed, and recoveries were monitored as previously described.13 Recoveries ranged from 31% to 52% and averaged 41%.

In selected experiments, [3H]cGMP was used as a tracer to determine the intraplatelet cGMP levels in the presence of exogenous cGMP. Platelets were preincubated with exogenous cGMP (final concentration, 10 nM)\textsuperscript{16,17} to which [3H]cGMP was added as a tracer to a specific activity of 4.7 × 10\textsuperscript{6} cpm/μmol. At time points identical to parallel secretion experiments, the platelets were activated by the addition of 0.5 unit/ml thrombin and subsequently centrifuged through a silicone oil cushion, as previously described,\textsuperscript{13} to separate platelet-associated cGMP from the cGMP remaining in solution. The tubes were spun for 3 minutes and inverted, the tips were amputated, and then platelet-associated [3H]cGMP was determined with a β counter (model 10/600 Plus, ICN Biomedicals, Cleveland, Ohio).

Preparation of S-Nitrosocysteine

SNOC was prepared at 25°C by reacting equimolar concentrations of L-cysteine with NaNO\textsubscript{2} at acidic pH, as previously described.18 Solutions turned from
clear to rose-colored after completion of the reaction. SNOC was prepared within 1 hour of use and was kept in 0.5N acetic acid at 4°C. The compound was diluted into aqueous buffer immediately before its use in a given experiment. Care was taken to expose platelets to SNOC that had been diluted into aqueous buffer for identical periods of time in all experiments (90 seconds).

Statistical Analyses
Data were analyzed using a two-tailed Student’s t test, one-way analysis of variance, and Newman-Keuls subgroup comparison with a commercially available computer program (PC ANOVA, Human Systems Dynamics, Northridge, Calif.).

Results
Inhibition of Platelet Aggregation in Human Platelets by S-Nitrosocysteine
Exposure of human platelets to SNOC for 2 minutes led to a marked, dose-dependent inhibition of platelet aggregation by 10 μM ADP (Figure 1). This is consistent with previous reports regarding the inhibition of platelet function by related nitrosothiol compounds.13,18-20 In our studies, the IC50 for inhibition of aggregation by SNOC was 2 × 10−5 M and near-complete inhibition of aggregation was noted at 10−4 M SNOC, in agreement with previous reports.13,18 Control studies of the individual constituents used to synthesize SNOC had no effect on platelet aggregation at the concentrations used (data not shown).

Inhibition of Platelet Secretion in Human Platelets by S-Nitrosocysteine
Next, we analyzed the effects of SNOC on platelet secretion by using [14C]serotonin-labeled platelets, as detailed in “Materials and Methods.” Platelets were loaded with [14C]serotonin and then preincubated with 10−6 M SNOC for 1 minute before thrombin activation. Platelet secretion at 2 minutes after thrombin averaged 45 ± 6% of control (n = 3, p = 0.001 compared with control; see Figure 2). Similar secretion experiments were performed in platelets preincubated with either the specific cGMP phosphodiesterase inhibitor M&B 22,94821 or with methylene blue, a known inhibitor of soluble guanylate cyclase,22 and then exposed to SNOC for 1 minute before thrombin addition. Platelets were pretreated with a final concentration of either 2 μM methylene blue or 500 μM M&B 22,948 for 15 minutes at room temperature and then exposed to thrombin for 2 minutes, as in the experiments above. The presence of M&B 22,948 augmented SNOC-induced inhibition of secretion (from 55% inhibition for SNOC alone to 80% by M&B 22,948 and SNOC, p = 0.02) (see Figure 2). Preincubation of platelets with 2 μM methylene blue for 15 minutes at room temperature reversed the inhibition of secretion by 10−6 M SNOC toward normal, resulting in an increase in thrombin-induced secretion to 71% of control (p = 0.03 from SNOC alone, p = 0.001 from SNOC+M&B 22,948) (see Figure 2). Control experiments with each of the solvents and buffers used in the preparation of the M&B 22,948, methylene blue, and SNOC again had no effect on platelet secretion (data not shown).

The dose–response for inhibition of secretion by SNOC in the absence or presence of M&B 22,948 was determined next and is illustrated in Figure 3. In our system the IC50 for the inhibition of platelet secretion by SNOC alone was 10−6 M. Dose–response experiments were performed in the presence of the M&B 22,948, at concentrations of the phosphodiesterase in-
Inhibitor similar to those reported to potentiate EDRF-mediated relaxation in vascular smooth muscle cells. The dose–response curves for platelets pretreated with either 300 or 500 μM M&B 22,948 were significantly shifted leftward as compared with SNOC alone (p=0.07 and 0.005, respectively, by analysis of variance). Figure 3 also illustrates that pretreatment with M&B 22,948 resulted in a dose-dependent decrease in the IC₅₀, from 10⁻⁶ M by SNOC alone to 2×10⁻⁷ M in the presence of 500 μM M&B 22,948.

Inhibition of Platelet Secretion by cGMP Analogues

To further confirm the role of cGMP in the inhibition of platelet secretion in our system, platelets were exposed to 10 mM of either dibutyryl-cGMP or 8-bromo-cGMP for 15 minutes at 22°C before the initiation of secretion. As shown in Figure 4, both cGMP analogues were capable of significantly inhibiting platelet secretion to 0.5 unit/ml thrombin. 8-Bromo-cGMP (10 mM) inhibited secretion by 61% (p=0.04), whereas dibutyryl-cGMP (10 mM) inhibited secretion by 60% (p=0.002), consistent with previous studies demonstrating inhibition of platelet activation at similar concentrations.

cGMP Levels With Inhibition of Platelet Secretion by S-Nitrosocysteine

Cyclic nucleotide levels were determined in separate, parallel experiments in which platelet secretion was inhibited by SNOC or combinations of SNOC and M&B 22,948. At 2 minutes after exposure of the platelets to SNOC, significant inhibition of secretion and simultaneous increases in intracellular cGMP levels were noted (Figure 5). Intraplatelet cGMP increased from 2.8 pmol/10⁸ platelets in the resting (control) state to a maximum of 22.9 pmol/10⁸ platelets after guanylate cyclase stimulation with 10⁻⁶ M SNOC for 2 minutes in the presence of 500 μM M&B 22,948. Importantly, simultaneous measurements of cAMP levels after exposure to SNOC or combinations of SNOC and M&B 22,948 were unchanged from resting levels, as demonstrated previously, cAMP levels averaged 1.4–1.8 pmol/10⁸ platelets, consistent with resting levels reported by other investigators.

Inhibition of platelet secretion by S-nitrosocysteine (SNOC) and platelet cGMP levels. Platelet secretion (mean±SEM) to 0.5 unit/ml thrombin after exposure to 10 μM SNOC alone or after pretreatment with 100 μM (M₁₀₀) or 500 μM (M₅₀₀) M&B 22,948. Simultaneous measurement of platelet cGMP levels from parallel experimental points are displayed on the right-hand y axis. Three separate experiments, in duplicate, are represented. *p=0.001, **p=0.008, ***p=0.0001 from control secretion. Simultaneous cAMP levels were unchanged from baseline in all groups.

Figure 3. S-Nitrosocysteine–induced inhibition of platelet secretion in the presence of M&B 22,948. Dose–response curves for 2-minute exposure to S-nitrosocysteine alone or after pretreatment with 300 or 500 μM M&B 22,948. Platelet secretion was measured to 0.5 unit/ml thrombin in each case. Each point represents the mean±SEM of duplicate determinations for three separate experiments. *p=0.07, **p=0.005 compared with S-nitrosocysteine alone by one-way analysis of variance.

Figure 4. Inhibition of platelet secretion by cGMP analogues. Platelets were preincubated with 10 mM dibutyryl-cGMP (DB-cGMP) or 8-bromo-cGMP (8Br-cGMP) for 15 minutes and secretion to 0.5 unit/ml thrombin was measured. Normalized secretion ± SEM for three experiments in duplicate is displayed. *p=0.002. **p=0.04 compared with control.

Figure 5. Inhibition of platelet secretion by S-nitrosocysteine (SNOC) and platelet cGMP levels. Platelet secretion (mean±SEM) to 0.5 unit/ml thrombin after exposure to 10 μM SNOC alone or after pretreatment with 100 μM (M₁₀₀) or 500 μM (M₅₀₀) M&B 22,948. Simultaneous measurement of platelet cGMP levels from parallel experimental points are displayed on the right-hand y axis. Three separate experiments, in duplicate, are represented. *p=0.001, **p=0.008, ***p=0.0001 from control secretion. Simultaneous cAMP levels were unchanged from baseline in all groups.
To analyze further the correlation between inhibition of platelet secretion and changes in cGMP levels after SNOC, platelet secretion was plotted as a function of cGMP levels. A direct, highly significant correlation between the logarithmic intracellular cGMP levels attained and the inhibition of platelet secretion was evident (Figure 6). This is consistent with similar correlations noted between the logarithmic cGMP levels and inhibition of fibrinogen binding reported recently.13

**Inhibition of Platelet Secretion by Exogenous cGMP**

We also sought to assess the ability of exogenous cGMP itself to inhibit human platelet secretion. Pilot experiments were performed to quantitate the amount of platelet-associated cGMP present at various concentrations of exogenously added cGMP by using \[^{3}H\]cGMP as a tracer (total cGMP concentration, 10 mM; specific activity, 4.7 \times 10^{6} \text{cpm/\mu mol})

The platelet-associated \[^{3}H\]cGMP was subsequently quantitated by separating platelets through a silicone oil cushion, as described in "Materials and Methods" and reported previously.13 Platelet-associated cGMP averaged 0.89% of the total cGMP added, corresponding to a maximum platelet cGMP concentration of 45 \(\mu\text{M}\) (8.9–10.4 pmol/10\(^{8}\) platelets based on a mean platelet volume of 6–7 fl).24 As shown in Figure 7, this produced a statistically significant inhibition of platelet secretion at a level of platelet-associated cGMP comparable to that achieved by guanylate cyclase activation with SNOC (compare with Figure 6). Figure 7 also demonstrates the ability of the cGMP phosphodiesterase inhibitor M&B 22,948 to augment inhibition of platelet secretion after exposure of platelets to exogenous cGMP, with a progressive, significant decrement in secretion after treatment with exogenous cGMP in combination with M&B 22,948.

**Discussion**

Previous studies have suggested a positive correlation between the platelet inhibitory effects of exogenous and endogenous nitrate derivatives, and cGMP. In prior studies, however, no direct, systematic evaluation of the inhibitory effects of cGMP have been presented.4-11 With the use of potent guanylate cyclase stimulators such as SNOC and the availability of cGMP analogues and the specific cGMP phosphodiesterase inhibitor M&B 22,948, pharmacological manipulation of intraplatelet cGMP levels is now straightforward and the direct effect of cGMP on specific platelet processes such as secretion may be examined.

In initial experiments, we confirmed the ability of SNOC to inhibit platelet aggregation by ADP, results that were expected based on our previous work and that from other investigators.13,19,20 We sought to study platelet function in a physiological (plasma) milieu and therefore used the classic thrombin-induced secretion model15 for subsequent experiments. The initial aggregation studies were performed with ADP, however, because this is an important physiological agonist released by platelet secretion and because dose–response studies of thrombin-induced aggregation are not feasible in plasma because of marked fibrin generation and platelet clumping.15 It is not possible to fairly compare the IC\(_{50}\) values for
aggregation and secretion in SNOC-treated platelets given the known differences in the sensitivities of these two experimental techniques.\textsuperscript{15}

Using the EDRF-like \textit{S}-nitrosothiol SNOC,\textsuperscript{12} we observed dose-dependent inhibition of platelet secretion, accompanied by and significantly correlated with dose-dependent increases in intraplatelet cGMP levels. This inhibition is potentiated by the specific cGMP phosphodiesterase inhibitor M&B 22,948 and is accompanied by increases in intraplatelet cGMP levels without any change in cAMP levels. In addition, pharmacological manipulation of the intraplatelet cGMP level with M&B 22,948, cGMP analogues, or exogenous cGMP itself each provides additional evidence for the role of cGMP in the inhibition of platelet secretion.

The role of cGMP in mediating platelet function has been controversial for two decades.\textsuperscript{25} Although the present study provides evidence that cGMP can mediate the inhibition of platelet secretion by nitrovasodilator compounds, the mechanism by which cGMP influences intracellular events and leads to these inhibitory effects remains to be defined. Signal transduction events involving the phosphoinositide pathway have been shown to be sensitive to inhibition by cGMP-elevating agents.\textsuperscript{13,17,26–31} In addition, several groups have demonstrated distinct protein phosphorylation events dependent on and regulated by cGMP.\textsuperscript{27,29,30,32} that are candidates for regulatory steps in the cGMP-mediated effects of nitrovasodilators. It is also plausible that cGMP has direct effects on platelet function that are independent of cGMP-dependent protein kinase and its protein substrates.\textsuperscript{33}

In conclusion, we have demonstrated that 1) SNOC inhibits serotonin secretion in a dose-dependent fashion that is potentiated by the specific cGMP phosphodiesterase inhibitor M&B 22,948 and reversed by the guanylate cyclase inhibitor methylene blue, 2) this inhibition is significantly correlated with increases in intraplatelet cGMP levels, and 3) these inhibitory effects of the EDRF-like nitrovasodilator SNOC on platelet secretion are similarly produced by exposure of platelets to cGMP analogues or exogenous cGMP. These experiments provide direct evidence for the role of cGMP in mediating the inhibitory effects of exogenous nitrate derivatives and the endogenous nitrovasodilator EDRF on platelet secretion.

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