Nitroprusside Inhibition of Platelet Function Is Transient and Reversible by Catecholamine Priming

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Background: The time course and reversibility of sodium nitroprusside's in vivo inhibition of platelet function are unclear.

Methods: Platelet aggregation and P-selectin expression as measures of platelet dense and α-granule release, respectively, were examined before and after administration of sodium nitroprusside (18 mg) to human volunteers and in in vitro studies. Hypotension occurring with sodium nitroprusside administration was treated with intravenous crystalloid and/or phenylephrine.

Results: Compared with preinfusion studies, platelet aggregation to epinephrine was significantly inhibited immediately and 4 min after discontinuation of the sodium nitroprusside infusion but returned to baseline at 8 and 12 min after discontinuing sodium nitroprusside. However, both dense and α-granule release to adenosine diphosphate after in vitro sodium nitroprusside were never significantly inhibited even at the time when sodium nitroprusside infusion was maximal. In contrast to our in vivo findings, in vitro incubation of platelet-rich plasma with sodium nitroprusside resulted in significant inhibition of dense and α-granule release to adenosine diphosphate. These in vitro inhibitory effects of sodium nitroprusside were reversed by pretreatment with epinephrine but not phenylephrine.

Conclusions: In normal volunteers, sodium nitroprusside inhibits platelet aggregation to epinephrine but not adenosine diphosphate; inhibition was reversed within 8–12 min after discontinuing sodium nitroprusside. Sodium nitroprusside in vitro inhibition of platelet function to adenosine diphosphate was reversed by epinephrine pretreatment. Because of the rapid reversibility of its antiplatelet effect, sodium nitroprusside may be clinically useful even when there is the potential for impaired coagulation. (Key words: Blood: coagulation. Catecholamines: epinephrine. Platelets: CD62P; P-selectin; platelet aggregation. Nervous system: sympathetic. Pharmacology: sodium nitroprusside.)

SODIUM nitroprusside is a nitrovasodilator with relaxant properties thought to result from metabolism of sodium nitroprusside to nitric oxide in vascular smooth muscle. Sodium nitroprusside has also been shown to directly produce a dose-dependent inhibition of platelet aggregation in vitro and a similar degree of inhibition when administered to patients with congestive heart failure or patients about to undergo coronary artery bypass grafting. Inhibition of platelet function by sodium nitroprusside may be due to an increase in platelet cyclic guanosine monophosphate (cGMP) and cGMP-dependent protein kinases, which are similarly induced by nitric oxide. While nitric oxide's platelet inhibitory effects are known to decay rapidly such that full platelet function returns within minutes after discontinuation of nitric oxide exposure, the time course and reversibility of sodium nitroprusside's inhibition of human platelets have not been clearly defined.

In a canine coronary artery model using cyclic flow reduction as a measure of platelet function, sodium nitroprusside's inhibitory effects were abolished within 5–25 min after discontinuing the drug. If sodium nitroprusside's effects on human platelets were similarly short-lived in accordance with its hemodynamic effects, then sodium nitroprusside might provide a uniquely "reversible" form of platelet inhibition; such use of sodium nitroprusside would be advantageous in clinical situations where temporary platelet inhibition might be desirable, i.e., the period after coronary artery bypass
grafting when there is a risk for early graft occlusion. In addition, concerns over exacerbation of bleeding with sodium nitroprusside administration for hemodynamic control might be allayed if sodium nitroprusside’s antiplatelet effects were clearly transient.

Platelet function may be evaluated by assessing platelet dense granule and α-granule release. Aggregometry is the classic method by which stirred platelets in the presence of an agonist are induced to release their dense granule contents, including adenosine diphosphate (ADP), serotonin, and calcium. These released dense granule constituents then recruit additional platelets to undergo dense granule release, cause fibrinogen to bind to the platelet receptor glycoprotein IIb/IIIa, and induce clot formation.

By contrast, platelet α-granule release may occur without clot development. Furthermore, it is possible that a drug may differentially affect platelet dense and α-granule release; for example, we found that α-granule release to some platelet agonists was not inhibited by aspirin at doses that did prevent dense granule release and aggregation. The α-granule releases platelet-derived growth factor and transforming growth factor β, which have been implicated in the promotion of atherogenesis. When the platelet undergoes α-granule release, the α-granule membrane fuses with the external platelet membrane, and the granule membrane protein P-selectin (also termed PADGEM, GMP-140, or CD62P) becomes an integral platelet membrane protein.

Thus, it is possible to quantitate the percentage of circulating platelets that have undergone α-granule release by determining the percentage of platelets that express P-selectin. Recent studies have shown that platelet P-selectin is a functional ligand for binding leukocytes, and these leukocyte-platelet conjugates may have delayed procoagulant functions. Thus, inhibition of platelet dense granule release by sodium nitroprusside prevents the formation of acute platelet aggregates, whereas any inhibition of α-granule release would affect atherogenesis and late thrombotic events.

This study was designed to evaluate the degree and duration of the in vivo inhibitory effect of sodium nitroprusside on human platelets in normal volunteers; we studied both dense granule function (aggregometry) and the ability to release α-granules (P-selectin expression). To determine whether any secondary effects of the hypotensive response to sodium nitroprusside in vivo might affect platelet function, we also correlated these in vivo findings with sodium nitroprusside’s in vitro ability to inhibit platelet dense and α-granule release.

Materials and Methods

Materials

Sodium nitroprusside solution for in vivo and in vitro studies was freshly prepared using 50 g of anhydrous sodium nitroprusside powder (Gensia Laboratories, Irvine, CA) diluted with 5% dextrose in water to a final concentration of 200 μg/ml. All sodium nitroprusside solutions were protected from light and used within 1 h of preparation. Platelet agonists included phenylephrine HCl (American Regent Lab, Shirley, NY), epi- nephrine HCl (Parke-Davis, Morris Plains, NJ), and adenosine diphosphate (ADP, Sigma, St. Louis, MO).

Human Studies

After obtaining approval by the Yale University Human Investigation Committee and informed consent, 19 healthy volunteers were studied. All subjects were interviewed before the study; any subject with a history of hypertension, bleeding tendency, or ingestion of any medication known to interfere with platelet function within the previous 10 days was excluded. Subjects were placed supine, and an intravenous infusion of 0.9% normal saline was begun at a rate of 80 ml/h. A radial artery catheter was placed (using local anesthesia) for direct and continuous blood pressure measurement. In addition, pulse oximetry and electrocardiogram were monitored continuously. Sodium nitroprusside was infused at an initial rate of 2 μg·kg⁻¹·min⁻¹ and subsequently increased by 2 μg·kg⁻¹·min⁻¹ every 10 min until a total dose of 18 mg sodium nitroprusside was delivered (MAX SNP). During the sodium nitroprusside infusion, any decrease in mean arterial blood pressure to ≤70 mmHg was treated by the administration of additional normal saline and/or intravenous phenylephrine at the discretion of the attending physician, and the next sodium nitroprusside dose escalation was delayed for 10 min. On reaching MAX SNP, the sodium nitroprusside infusion was discontinued, but normal saline administration was continued at ≥40 ml/h for the next 30 min to maintain catheter patency.

Blood Sampling

All samples for platelet function were obtained from the arterial catheter. Citrated blood for platelet aggre-
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gation was obtained before the start of the sodium nitroprusside infusion (baseline), at MAX SNP, and then at 4, 8, and 12 min after discontinuation of the sodium nitroprusside infusion. Whole blood samples for flow cytometry were drawn at baseline and at MAX SNP and were immediately fixed in paraformaldehyde (1% final concentration, Baker, Phillipsburg, NJ) in phosphate-buffered saline. Additional whole blood for flow cytometry agonist studies was drawn into 5 mm EDTA, immediately incubated with ADP (2 μM and 5 μM) for 5 min at 22°C, and fixed in parafomaldehyde. All fixed samples were stored for 60 min at 4°C before washing and labeling to measure platelet P-selectin expression. Platelet Aggregometry
Whole blood drawn into sodium citrate (0.38% final concentration) was immediately spun to prepare platelet-rich plasma and platelet-poor plasma. The platelet count in the platelet-rich plasma was adjusted to 2.5 × 10⁹/ml. Standard platelet aggregometry at 37°C was performed using a dual-sample DP-247E aggregometer (Sienco, Morrison, CO). All aggregation studies were completed within 60 min of blood collection. Baseline samples (pre-sodium nitroprusside infusion) were stimulated with ADP (2 and 5 μM) and epinephrine (5, 10, and 25 μM). To increase the sensitivity of the assay to inhibitory effects of sodium nitroprusside, the smallest dose of each agonist that produced second wave aggregation was used for the post-infusion aggregation studies. The aggregation value measured was the maximum change in light transmittance after agonist. All postinfusion aggregation values were then reported as a percentage of the preinfusion value to compare studies between subjects. Only subjects with normal preinfusion second wave platelet aggregation to at least one dose of both epinephrine and ADP were included in the data analysis.

Antibodies
Monoclonal antibody 1E326 (donated by Dr. K. Ault, Maine Medical Center Research Institute, South Portland, ME) is directed against the extracellular domain of the platelet α-granule protein P-selectin. The P2 monoclonal antibody (AMAC, Westbrook, ME) recognizes GPIIb/IIIa on platelets.

Fluorescence Labeling for P-selectin. As noted earlier, when a platelet undergoes α-granule release, P-selectin present in the granule membrane becomes an integral platelet membrane protein with an extra-cellular domain. Expression of P-selectin has been shown to correlate with other measures of α-granule release. The flow-cytometric detection of the percentage of platelets expressing P-selectin was performed as previously described using fluorescein-isothiocyanate-conjugated P2 monoclonal antibody to confirm platelet identity and biotin-conjugated 1E3 monoclonal antibody and phycoerythrin-streptavidin to detect P-selectin. Leukocytes were excluded from analysis using a combination of cell size and fluorescein isothiocyanate fluorescence. The threshold for P-selectin positivity was set using an irrelevant, isotype-specific biotin-conjugated monoclonal antibody and phycoerythrin-streptavidin; the baseline (preinfusion) percentage of platelets expressing P-selectin was uniformly less than 7%. The final value reported for agonist studies was the percentage of platelets expressing P-selectin minus the baseline percentage for each patient.

In Vitro Studies
Platelet-rich plasma from four additional healthy subjects (receiving no medications) was prepared as described earlier and incubated with sodium nitroprusside at final concentrations of 2.5 and 25 μg/ml or diluent for 10 min at 37°C. Because in vivo sodium nitroprusside concentrations were not measured, these in vitro concentrations were chosen to bracket theoretically achievable sodium nitroprusside concentrations for this study and are based on a total sodium nitroprusside dose of 18 mg distributed in a plasma volume of 2800 ml, which yields a final sodium nitroprusside concentration of 6–7 μg/ml. Platelet-rich plasma samples were then subjected to aggregometry with phenylephrine, epinephrine, or ADP, or combinations of agonists (phenylephrine or epinephrine incubation for 2 min followed by ADP). Similarly, aliquots of sodium nitroprusside- or diluent-incubated platelet-rich plasma were examined for ADP-, phenylephrine/ADP-, or epinephrine/ADP-induced P-selectin expression as above. Probable in vivo phenylephrine concentrations were calculated similarly using a maximum total dose of 300 μg, yielding a final phenylephrine concentration of 100 ng/ml for in vitro studies.

Statistics
Statistical analysis included repeated-measures analysis of variance and Student’s paired t test. A P value < 0.05 was considered statistically significant.

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Results

**Human Studies**
All 19 volunteers completed the study without complications. Three subjects did not exhibit second-wave aggregation to any epinephrine dose in the preinfusion sample, and their data were excluded from further analysis. The time of sodium nitroprusside administration ranged from 44 to 67 min, with a mean of 55 ± 6 min (SD). All 16 evaluable subjects had a decrease in the mean arterial blood pressure to less than 70 mmHg (mean nadir 63 ± 5 mmHg; SD). The subjects also had an increase in heart rate from 72 ± 12 bpm preinfusion to a peak rate of 107 ± 8 bpm (mean ± SD). The subjects received an average of 1800 ± 220 ml (mean ± SD) normal saline; 12 subjects received 146 ± 66 µg (mean ± SD) phenylephrine (range 100–350 µg) with the last dose occurring 26 ± 16 min (mean ± SD) before the MAX SNP time point.

**Platelet Aggregometry.** The threshold doses for eliciting preinfusion second-wave aggregation to epinephrine were: 5 µM (n = 8), 10 µM (n = 3), and 25 µM (n = 5). For ADP-induced second-wave aggregation, these doses were 2 µM (n = 12) and 5 µM (n = 4). As shown in figure 1A, the aggregation response to epinephrine at MAX SNP was significantly decreased (P < .01) when compared with the preinfusion aggregation response (44 ± 8% of baseline, mean ± SEM). Aggregation to epinephrine was still significantly decreased (P < 0.05) 4 min after termination of the sodium nitroprusside infusion (86 ± 30%). However, epinephrine-induced aggregation returned to 97 ± 15% and 127 ± 28% of baseline at 8 and 12 min postinfusion, respectively (P > 0.5 for both values compared with preinfusion).

By contrast, the platelet aggregation response to ADP (fig. 1B) was not significantly decreased at MAX SNP (87 ± 9% of baseline, P = 0.15). Adenosine diphosphate aggregation values at 4, 8, and 12 min after discontinuation of the sodium nitroprusside infusion were 99 ± 11%, 128 ± 20%, and 128 ± 15% of baseline, respectively; none of these aggregation values were significantly different from the preinfusion response (P > 0.13 for all comparisons).

Of the 16 subjects, only one had aggregation responses to both ADP and epinephrine that had not returned to baseline by 12 min postinfusion (61% and 83% of baseline values, respectively). One other subject had decreased epinephrine aggregation at 12 min (63% of baseline) but did have restoration of the ADP response (134% of baseline). When the subjects who received phenylephrine (n = 12) were analyzed separately from those who did not receive phenylephrine (n = 4), there was no difference between the groups with respect to recovery of the aggregation response to ADP. The platelet aggregation response to ADP in the phenylephrine group at MAX SNP, and at 4, 8, and
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Fig. 2. Platelet aggregation after in vitro incubation with diluent control or sodium nitroprusside (representative tracings from a single donor). Platelet-rich plasma was incubated with diluent (A) or 2.5 μg/ml sodium nitroprusside (B) and (C) as described in methods for 10 min at 37°C. Samples of platelet-rich plasma were then stimulated separately with adenosine diphosphate 5 μM and epinephrine 25 μM (A) and (B) or epinephrine 25 μM followed 2 min later by adenosine diphosphate 5 μM (C).

12 min after discontinuation of sodium nitroprusside was 90 ± 10%, 101 ± 11%, 133 ± 21%, and 128 ± 19%, respectively (mean ± SEM). The corresponding values in the group that did not receive phenylephrine were 76 ± 15%, 94 ± 31%, 113 ± 15%, and 127 ± 19%, respectively (P > 0.1 for all comparisons).

P-selectin Expression. There was no significant inhibition of platelet α-granule release to ADP at MAX SNP compared with the preinfusion value. The percentage of platelets expressing P-selectin in response to 2 μM ADP was 59 ± 6% (mean ± SEM) and 60 ± 4% at the preinfusion and MAX SNP time points, respectively (P = 0.98). In response to 5 μM ADP, 70 ± 4% of platelets expressed P-selectin preinfusion compared with 66 ± 6% at MAX SNP (P = 0.59).

In Vitro Studies
Platelet Aggregometry. Platelet-rich plasma incubation with sodium nitroprusside at either 2.5 or 25 μg/ml completely inhibited second-wave platelet aggregation to ADP 5 μM and epinephrine 25 μM in all donors (figs. 2A and 2B). When sodium-nitroprusside-incubated platelet-rich plasma was pretreated with epinephrine 25 μM for 2 min followed by addition of ADP 5 μM, full second-wave aggregation was restored in all four donors (fig. 2C).

There was no primary or secondary wave aggregation in response to phenylephrine at doses ranging from 10 to 100 ng/ml (data not shown). When platelet-rich plasma was pretreated with phenylephrine 100 ng/ml for 2 min followed by ADP 5 μM, the aggregation response was similar to pretreatment with diluent for 2 min followed by ADP 5 μM (59 ± 7% light transmittance vs. 54 ± 6%, respectively, P = 0.53). When platelet-rich plasma was incubated with sodium nitroprusside such that second-wave aggregation was abolished (identical to fig. 2B), pretreatment with phenylephrine 100 ng/ml for 2 min did not restore second-wave aggregation to ADP 5 μM in any of the four donors (data not shown).

P-selectin Expression. Similar to the in vitro aggregation studies, platelet-rich plasma incubated with sodium nitroprusside for 10 min at 37°C resulted in significant inhibition of ADP-induced P-selectin expression (table 1); this inhibition was reversed by priming with epinephrine for 2 min before addition of ADP. Unlike epinephrine, sodium nitroprusside’s inhibition of P-selectin expression to ADP was not restored by pretreatment with phenylephrine 100 ng/ml for 2 min before the addition of ADP (table 1).

Discussion

This study examined the characteristics and duration of nitroprusside’s platelet inhibitory effects in healthy human volunteers. Our in vitro data confirm the observation that sodium nitroprusside administration inhibits second-wave platelet aggregation to epinephrine. Our data also provide new information that demonstrates that platelet aggregation to epinephrine recovers within 8–12 min after cessation of the sodium nitroprusside infusion. Thus, at a clinically relevant dose of sodium nitroprusside (18 mg), a partial antiplatelet effect (approximately 50% inhibition of platelet aggregation to epinephrine) can be achieved. Moreover,

Table 1. Sodium Nitroprusside (SNP) Inhibition of Platelet α-Granule Release In Vitro

<table>
<thead>
<tr>
<th>Agonist Incubation</th>
<th>Pre-incubation</th>
<th>Diluent</th>
<th>Phenylephrine</th>
<th>Epinephrine</th>
</tr>
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<tbody>
<tr>
<td>Diluent</td>
<td>66 ± 3</td>
<td>69 ± 5</td>
<td>88 ± 2</td>
<td></td>
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<tr>
<td>SNP (2.5 μg/ml)</td>
<td>46 ± 8*</td>
<td>41 ± 2*</td>
<td>80 ± 6</td>
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PPR = platelet-rich plasma, ADP = adenosine diphosphate.

PPR was pre-incubated with diluent or SNP for 10 min at 37°C, followed by incubation with diluent, phenylephrine 100 ng/ml, or epinephrine 10 μM for 2 min at 22°C. All samples were then treated with ADP 2 μM for 5 min at 22°C, fixed, and examined for the percentage of platelets (mean ± SEM) expressing P-selectin (n = 4 for all values).

*P < 0.05 versus diluent pre-incubation.
the antiplatelet effect of sodium nitroprusside can be reversed within minutes of stopping the drug. The extent of in vivo platelet inhibition by sodium nitroprusside in this study is consistent with previous investigations. In a study of low-dose (<1 μg·kg⁻¹·min⁻¹) sodium nitroprusside administration to congestive heart failure patients, Mehta et al. determined that sodium nitroprusside inhibited maximal platelet aggregation to epinephrine and ADP by 20–40%. Hines and Barash found that platelet aggregation to either epinephrine or ADP was inhibited by ≤50% in bypass patients receiving sodium nitroprusside in a dose (>1 μg·kg⁻¹·min⁻¹) similar to the current study. Our findings are also consistent with animal models of sodium nitroprusside’s inhibitory effects on platelets. Rovin tested a similar dose of sodium nitroprusside (2–6 μg·kg⁻¹·min⁻¹) in a dog model of coronary artery stenosis and found that the return of platelet function occurred from 5 to 25 min after the sodium nitroprusside infusion was discontinued. This dog model used cyclic flow reductions as an indirect measure of platelet function; thus, vascular factors may have contributed to the variability that was observed.

In contrast to both our in vitro data and the studies in patients with cardiovascular disease reported earlier, our in vivo study of normal volunteers did not demonstrate significant sodium nitroprusside inhibition of ADP-induced platelet aggregation. Several factors could account for this finding. Although we used a threshold dose of ADP to increase our ability to detect sodium nitroprusside inhibition, it is possible that a higher dose of ADP for platelet aggregation would have demonstrated inhibition. However, this is unlikely because 12 of our 16 subjects were tested with 2 μM ADP, the exact dose employed in two previous studies.

One important difference that could explain the failure of sodium nitroprusside to inhibit ADP-induced platelet aggregation is that the current study examined normal volunteers. As a result, sodium nitroprusside administration in this study resulted in significant hypotension and tachycardia requiring treatment with crystalloid (16 of 16 subjects) and phenylephrine (12 of 16 subjects). In those in vivo studies that found that sodium nitroprusside significantly inhibited ADP aggregation, study patients did not become hypotensive after sodium nitroprusside administration; in addition, bypass patients were anesthetized (fentanyl ≥ 30 μg/kg), which may have abrogated catecholamine release. As previously reported, it is likely that sodium nitroprusside-induced hypotension in our volunteers resulted in significant catecholamine release; we hypothesize that the in vivo platelet aggregation response to subsequent ADP may have been primed by such catecholamine release. Our in vitro demonstration that epinephrine pretreatment restored full second-wave aggregation to ADP after incubation with sodium nitroprusside might then explain the apparent in vivo failure of sodium nitroprusside to inhibit ADP aggregation.

A third explanation is that phenylephrine treatment in 12 of our subjects may have directly primed platelets to ADP aggregation in a manner similar to the epinephrine priming demonstrated in vitro; however, we found no difference in ADP aggregation between patients who received phenylephrine and those who did not. In addition, in vitro pretreatment of platelets with phenylephrine did not restore ADP-induced aggregation after sodium nitroprusside inhibition, unlike epinephrine pretreatment.

In addition to platelet aggregation that measures platelet–platelet interactions dependent on shear forces and dense granule release, this study also examined sodium nitroprusside’s ability to inhibit platelet α-granule release by measuring expression of P-selectin. Administration of sodium nitroprusside in vitro did not significantly inhibit platelet P-selectin expression to ADP, similar to the in vivo ADP aggregation response. By contrast, in vitro incubation with sodium nitroprusside resulted in inhibition of P-selectin release to ADP; this inhibition was similarly overcome by preincubation with epinephrine. Thus, epinephrine priming of both dense and α-granule responses to ADP reverses the in vitro inhibitory effects of sodium nitroprusside.

The platelet dense granule releases substances that act immediately on other platelets in the microenvironment; by contrast, the platelet α-granule products transforming growth factor-β and platelet-derived growth factor act on leukocytes and smooth muscle cells to induce chronic and potentially atherogenic changes. In addition, P-selectin mediates binding of activated platelets to neutrophils and monocytes; this adhesion has been postulated to subsequently induce increased leukocyte expression of CD11b and tissue factor. Because these surface molecules promote fibrin formation and activation of the extrinsic coagulation pathway, respectively, it is likely that platelet α-granule release and formation of platelet–leukocyte conjugates are relatively procoagulant events. We have previously reported that aspirin does not inhibit P-selectin expression or formation of leukocyte–platelet

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conjugates after ADP stimulation, even at aspirin doses that fully inhibit dense granule release. The current study has found that, unlike aspirin, sodium nitroprusside can inhibit P-selectin expression, further studies are required to determine if sodium nitroprusside might prevent subsequent procoagulant or atherogenic sequelae through its inhibition of platelet α-granule release.

Platelets play a critical role in cardiovascular disorders due to their prothrombotic effects and their ability to modulate leukocyte and endothelial function. A recent study demonstrating that platelet inhibition by aspirin decreases the incidence of myocardial infarction but increases bleeding complications points out the desirability of antiplatelet agents, the actions of which are quickly reversible. Sodium nitroprusside reversibly inhibits platelet–platelet adhesion, probably in part due to time-dependent decay of sodium nitroprusside’s effects and possibly as a result of catecholamine priming of the platelet response to ADP. Sodium nitroprusside has traditionally been employed for its vasodilatory properties, and this study suggests that sodium nitroprusside can be used for hemodynamic control even when there is abnormal hemostatic function. Further studies are necessary to determine if sodium nitroprusside will be a clinically useful agent for providing controlled, transient platelet inhibition.

References


