The Interaction of Sodium Nitroprusside with Human Endothelial Cells and Platelets: Nitroprusside and Prostacyclin Synergistically Inhibit Platelet Function

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SUMMARY Sodium nitroprusside (NP) is a potent vasodilator that also inhibits platelet aggregation. To test the hypothesis that NP causes both of these effects by altering the balance between prostacyclin (PGI₁) produced by endothelial cells and thromboxane A₂ (TXA₂) produced by platelets, we incubated each of these cell types with NP for 5 minutes and assayed the PGI₁ and TXA₂ produced. NP at pharmacologically achieved doses (0.01–30 μg/ml) inhibited platelet aggregation and resultant TXA₂ synthesis in a dose- and time-dependent manner (p < 0.001). The inhibition was not dependent on cAMP production, external calcium concentration, or suppression of TXA₂ synthesis. NP did not alter the production of PGI₁ by cultured human endothelial cells as measured by radioimmunoassay for 6-Keto-PGF₁α, the stable hydrolysis product of PGI₁. However, supernates of NP-treated endothelial cells containing low, noninhibitory concentrations of NP unexpectedly inhibited platelet aggregation. This inhibition of platelet aggregation was due to synergy between PGI₁ (0.1–3 nM) and NP (p interaction < 0.003). The synergistic inhibition by NP and PGI₁ of platelet aggregation and TXA₂ synthesis in vivo may explain some of the beneficial actions of NP in the treatment of hypertension and congestive heart failure.

SODIUM NITROPRUSSIDE (NP) is a vasodilator widely used to treat hypertensive crises and congestive heart failure complicating myocardial ischemia. It is one of a group of vasodilators that includes nitroglycerin, dipyridamole, verapamil and hydralazine, which also inhibit platelet aggregation in vitro. NP also inhibits the formation of platelet aggregates in vitro, and part of its beneficial effect may result from this inhibition.

We recently demonstrated that nitroglycerin induces human endothelial cells in tissue culture to synthesize prostacyclin (PGI₁), a potent, naturally occurring vasodilator and platelet inhibitor. The PGI₁ produced by these endothelial cells inhibits platelet aggregation in vitro. To explore the hypothesis that NP modulates vascular tone and relieves ischemia by shifting the ratio of PGI₁ to thromboxane A₂ (TXA₂) in the circulation toward PGI₁ excess, we studied the effect of NP on endothelial cells and platelets.

Methods

Platelet Aggregation and Thromboxane B₂ Production

Platelet-rich plasma (PRP) was prepared from venous blood and platelet counts were determined on a model ZBI Coulter counter by methods described. Aggregation was induced with the following aggregating agents (expressed as final concentration in the cuvette): sodium arachidonate (Nu Chek), 0.1–3 mM; fibrillar bovine collagen (Hormon-Chemie), 0.6–1.6 μg/ml; and epinephrine (Parke Davis), 2–4.3 μM. Platelet aggregation was performed in a dual-channel
aggregometer (Payton Associates) as previously described. In brief, light transmission through PRP was set at 0% and through platelet-poor plasma at 100%. The threshold aggregating concentration of each agent was determined after incubating the PRP for 1–5 minutes with buffer I such that the final volume after all additions was 0.5 ml. Threshold aggregation was then defined as the lowest concentration of an agent that caused at least a 90% increase in light transmission at 5 minutes when added to PRP maintained at 37°C and stirred at a constant rate of 1000 rpm.

Aggregation of platelets may be quantitated by measuring any of the characteristics of the curve of increasing light transmission generated as the platelets aggregate. These include measurement of the lag phase after addition of the stimulus and before the onset of aggregation, the initial slope of the curve, the maximal increase in light transmission, or the area under the curve. We have found that measuring the area under the curve of light transmission 5 minutes after addition of the stimulus to be a very sensitive and reproducible technique that reflects all of the three other variables of the curve listed above. The area under the curve is determined by tracing the curve with a planimeter (no. 123A, Dietzgen Corp.) three times and calculating the average.

For the measurement of platelet TXB₂ (the stable hydration product of TXA₂) production, platelet samples were prepared by a modification of the method of Fitzpatrick and Gorman. Five minutes after addition of the stimulus to each aggregation cuvette, a 50–100-μl sample was removed, immediately added to 900–950 μl of a buffer containing 10 mM Heps (pH 7.35 at 37°C), 137 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5.5 mM glucose (buffer I) that contained 56 μM indomethacin (Sigma Chemical Co.). The sample was then vortexed and frozen in a dry ice-acetone bath at −70°C. Samples were subsequently thawed and centrifuged at 8500 g for 3 minutes; the supernates were removed and stored at −70°C for testing in the radioimmunoassay (RIA) for TXB₂.

In each experiment, platelet-poor plasma was prepared by centrifuging blood, from which PRP had been removed, at 8500 g for 5 minutes. Controls for TXB₂ measurement were prepared by incubating platelet-poor plasma in aggregation cuvettes in a manner identical to that for PRP. Aliquots of each aggregating agent and inhibitor alone and in combination were then incubated in the cuvette containing platelet-poor plasma for 5 minutes and processed as described above. These controls were used to verify that none of the agents added to PRP interfered with the RIA for TXB₂. Samples of PRP incubated for 5 minutes without additions other than buffer were also prepared to determine background TXB₂ levels.

Platelet Inhibitory Effect of Nitroprusside and Nitroprusside-Prostacyclin Mixtures

Inhibition of platelet aggregation by NP (Roche Laboratories), PGI₂, and combinations of NP and PGI₂ was studied using the aggregating agents noted above. NP, 0.01–30 μg/ml, i.e., 38 nM to 115 μM, was incubated with aliquots of PRP for 1–5 minutes, and aggregation was then induced. The area under the curve was compared with the area under control curves generated in the absence of NP. Control aggregations were performed throughout each experiment to verify the stability of the system. Aggregations containing test materials were performed in triplicate.

Synergy between NP and synthetic PGI₂ was studied as follows. The extent of inhibition of platelet aggregation induced by various concentrations of each agent alone was determined. NP and PGI₂ at concentrations that alone caused minimal inhibition of platelet aggregation were then added to the PRP in rapid sequence and incubated for 1–5 minutes. The stimulus was then added and aggregation recorded. The order of addition did not affect the extent of inhibition. Because of the length of experiments, control aggregation studies and responses to single inhibitors were performed frequently to verify the stability of the platelet response.

Cell Culture Technique

Human endothelial cells were obtained from umbilical cord veins and cultured in 24-well (16-mm diameter) cluster plates (Costar), as previously described. Cultures were used at confluency in the second or third serial passage and were fed with fresh medium within 24 hours of use to ensure maximal PGI₂ production. Cell counts were performed as described. Any well in which the cell count was not within 10% of the mean cell count was rejected from further evaluation.

Treatment of Endothelial Cells with Nitroprusside

A stock solution of NP containing 1.5 mg/ml in a sterile aqueous solution of 5% glucose was prepared on the day of use. Serial dilutions in buffer I were prepared in light-shielded, polypropylene tubes (Falcon) immediately before use.

Each monolayer of endothelial cells was prepared and treated as previously described. Ten microliters of NP solution were mixed in 490 μl of buffer I to yield final concentrations of 0.01–30 μg/ml in the well. Endothelial cell monolayers were incubated with these solutions for 5 minutes. The supernates were removed, rapidly frozen and stored at −70°C until use.

In each experiment, the following control incubations with endothelial cells were performed: buffer I alone; buffer I containing 20 μM sodium arachidonate; and buffer I containing highly purified human thrombin, 0.1 U/ml. In each experiment NP, sodium arachidonate and thrombin were each incubated with endothelial cells pretreated with aspirin (Sigma Chemical Co.) or 15-hydroperoxy arachidonic acid, as previously described.

Radioimmunoassay for 6-Keto-PGF₁α and Thromboxane B₂

The development and performance of the RIAs has been described. In brief, the RIA for 6-Keto-PGF₁α was performed as follows. Samples containing the test material, 6-Keto-PGF₁α (100 Ci/mmol, New England Nuclear) containing 10,000 dpm, and the anti-6-
Keto-PGF$_{1a}$ antisera diluted 1:3200 were incubated for 24 hours at room temperature. A suspension of formalin-fixed Staphylococcus aureus Cowan type I (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp.) was then added and incubated at room temperature for 30 minutes. The mixture was centrifuged at 1300 g for 30 minutes, and the supernate was removed and counted in a liquid scintillation counter. Standard curves were run with each assay. Standards were assayed in triplicate and unknowns in duplicate at two dilutions. The data were analyzed using the four-parameter logistic method of Rodbard and Hutt.

Fifty percent inhibition of binding was reached at 50–60 pg and the range of sensitivity was 3–1000 pg. Cross reactivities were as follows: PGI$_1$, 100%; 6, 15-diketo-PGF$_{1a}$, 1.3%; 6-Keto-PGF$_{1a}$, 1.1%; 13, 14-dihydro-6, 15 diketo-PGF$_{1a}$, 1%; PGF$_{2\alpha}$, 0.1%. The cross-reactivities of PGF$_{1a}$, PGE$_1$, PGF$_{1a}$, PGE$_2$, PGD$_2$ and TXB$_2$ were all less than 0.1%.

Because of the presence of human serum in samples assayed for TXB$_2$ by radioimmunoassay, a double-antibody technique using goat antirabbit IgG bound to beads (Immunobead second antibody, Bio-Rad Laboratories) was used. The TXB$_2$, RIA using $^3$H-TXB$_2$ (New England Nuclear, 150 Ci/mmol) and antisera at 1:4800 was otherwise performed in the same way as the 6-Keto-PGF$_{1a}$, RIA described above. The range of sensitivity for TXB$_2$ was 3–1000 pg with 50% binding at 30–40 pg. Cross reactivities were as follows: PGF$_2\alpha$, 0.6%; PGE$_{1\alpha}$, 0.06%; PGF$_{2\alpha}$, 0.5%; PGI$_1$, 0.2%; 6-Keto-PGF$_{1a}$ and 6-Keto-PGE$_{1\alpha}$, < 0.02%; 6, 15-diketo-PGF$_{1a}$ and 13, 14 dihydro 6, 15-diketo-PGF$_{1a}$, < 0.02%.

**Mechanism of Nitroprusside Inhibition of Platelet Aggregation**

A series of platelet aggregation experiments was performed to determine the role of several platelet systems or constituents in the mediation of NP inhibition of platelet aggregation.

**Thromboxane A$_2$**

PRP was prepared from donors who had ingested 650 mg of aspirin within 24 hours before the experiment. Complete inhibition of platelet TXA$_2$ synthesis was inferred by demonstrating the absence of aggregation in response to sodium arachidonate at any concentration. PRP was stimulated with 11–$\mu$M epinephrine, which yielded a stable primary wave of aggregation with a 20% increase in light transmission. The output to the recorder was then adjusted such that this 20% increase yielded a deflection of 10 cm. Thus, aggregation could then be quantitated as described above. The dose-response to NP was then determined and compared with the same donor’s response to NP in a control state when no aspirin had been ingested.

**Calcium**

Experiments were performed in which the ionized calcium concentration in PRP was varied. Blood from single donors was drawn either into 1/10 volume of Hepes-buffered saline containing sodium heparin, 50 U/ml (Upjohn Co.), which does not alter the ionized calcium concentration of plasma, or into 1/10 volume of 3.2% sodium citrate containing sodium heparin, 50 U/ml. Sodium citrate at a final concentration of 0.32% as obtained in these experiments lowers plasma ionized calcium concentration from the normal level of approximately 1 mM to 0.04 mM, a 25-fold decrease. PRP was then prepared as described above. Aggregation was simultaneously induced by collagen (0.8–1.6 $\mu$g/ml) in both heparin and heparin-citrate PRP in an autostandardizing, dual-channel aggregometer (model 600, Payton Associates) after a 1-minute incubation with either buffer I alone or NP (0–3 $\mu$g/ml). Inhibition of aggregation by NP as a function of calcium concentration was then determined by comparing the amount of inhibition induced by the varying concentrations of NP in both the heparin (normal calcium) and heparin-citrate (low calcium) PRP.

**cAMP**

Platelet adenylyl cyclase was inhibited by incubating PRP with 2',5’-dideoxyadenosine (DDA) (P-L Biochemicals), final concentration 100 $\mu$M, for 1–2 minutes at 37°C. The activity of DDA was verified by demonstrating that 100 $\mu$M DDA abolished the inhibition of aggregation induced by PGI$_1$ (1–10 nM), which is known to inhibit platelet aggregation by inducing a rise in platelet cAMP. After incubation with DDA, threshold aggregation was induced as described above, and the inhibition of aggregation by NP (0.1–10 $\mu$g/ml) was determined.

**Microtubule Agents**

The effect of NP on platelet aggregation was determined in the presence of the microtubule stabilizer deuterium oxide (D$_2$O, Sigma Chemical Co.) and the microtubule disruptor colchicine (Sigma Chemical Co.). For experiments with D$_2$O, PRP was prepared and then diluted with buffer I enriched with D$_2$O (0–100%) such that final concentrations of D$_2$O in PRP were 0–60%. Platelet counts after all dilutions were not less than 150,000/μl. In experiments using colchicine, PRP was incubated in the aggregation cuvette for 1–2 minutes with colchicine (0.15–1 mM) before addition of a threshold stimulus. The effects of NP on platelet aggregation in PRP alone and in the presence of D$_2$O or colchicine were then compared.

**Statistical Analysis**

Statistical analyses were performed on a Hewlett-Packard 9815A computer using prepared programs of the Hewlett-Packard statistics library (09815–15001) for one- and two-way analysis of variance (ANOVA). Although the data are plotted graphically or displayed in tables as a percentage of control, analyses of variance were performed using the nontransformed data.

**Results**

**Nitroprusside Inhibition of Platelet Aggregation and Thromboxane A$_2$ Synthesis**

NP inhibited platelet aggregation induced by sodium arachidonate, collagen, and epinephrine in a dose-
The inhibition RIA in dependent, and reached experiments (fig. 1). Platelet aggregation induced by sodium arachidonate was most sensitive to the inhibitory effect of NP (concentration yielding 50% inhibition \(IC_{50}\) = 0.04 \(\mu g/ml\) NP); collagen-induced (\(IC_{50}\) = 0.28 \(\mu g/ml\) NP) and epinephrine-induced (\(IC_{50}\) = 2 \(\mu g/ml\) NP) aggregation were less sensitive. The inhibition of platelet aggregation by NP was time dependent, and reached a maximum at 1–3 minutes depending on dose (\(p < 0.0004\), one-way ANOVA, data not shown).

NP also inhibited TXA\(_2\) synthesis measured as TXB\(_2\) by RIA in a time-dependent (\(p < 0.001\), one-way ANOVA) and dose-dependent manner (\(p < 0.001\), one-way ANOVA) in these same experiments. TXA\(_2\) production induced by sodium arachidonate was also the most sensitive to the inhibitory effect of NP (\(IC_{50}\) = 0.07 \(\mu g/ml\) NP). However, in contrast to inhibition of aggregation, epinephrine induced TXB\(_2\) production (\(IC_{50}\) = 0.2 \(\mu g/ml\) NP) was more sensitive to NP than was collagen (\(IC_{50}\) = 1.4 \(\mu g/ml\) NP). Thus, the patterns of inhibition of platelet aggregation and TXA\(_2\) synthesis differ depending on the aggregating agent used; this finding suggests that the inhibition of platelet aggregation induced by NP does not result from a direct inhibition of TXA\(_2\) synthesis.

**Nitroprusside Fails to Stimulate Endothelial Cell Prostacyclin Production**

To determine whether NP induces endothelial cells to synthesize PGI\(_2\), we incubated monolayer cultures of human umbilical vein endothelial cells with NP (0.01–30 \(\mu g/ml\)) for 5 minutes and analyzed the supernates by RIA for 6-Keto-PGF\(_{1\alpha}\), the stable hydrolysis product of PGI\(_2\). There were no significant differences in the amounts of PGI\(_2\) produced by endothelial cells whether incubated with NP or buffer alone (\(p > 0.20\), one-way ANOVA, data not shown). Sodium arachidonate and thrombin, both of which stimulate endothelial cell PGI\(_2\) production, were used in wells of each plate as positive controls. Both agents induced a fourfold to 20-fold increase in PGI\(_2\) production compared with the buffer control, which verified that the cells could metabolize exogenous or endogenous arachidonic acid to PGI\(_1\).
To determine whether NP might alter the production of endothelial cell PGI₂ in response to other agents, endothelial cells were treated with either thrombin (0.1 U/ml) or sodium arachidonate (20 μM) alone or in combination with NP. NP neither enhanced nor interfered with the synthesis of PGI₂ induced by thrombin ($p_{synergy} > 0.58$, two-way ANOVA) or sodium arachidonate ($p_{synergy} > 0.69$, two-way ANOVA, data not shown). Thus, NP neither induces endothelial cell PGI₂ synthesis itself nor alters PGI₂ synthesis induced by other agents.

Nitroprusside and Prostacyclin Synergistically Inhibit Platelet Aggregation and Thromboxane A₂ Synthesis

Although NP does not increase endothelial cell PGI₂ synthesis, supernates of NP-treated endothelial cells containing low, noninhibitory concentrations of NP inhibited platelet aggregation (fig. 2). This was unexpected because NP does not stimulate endothelial cell PGI₂ production. We considered two alternate hypotheses to explain this phenomenon. Either NP induced endothelial cells to synthesize an inhibitor of platelet aggregation other than PGI₂, or NP and PGI₂ acted synergistically to inhibit platelet aggregation.

The first possibility was studied by pretreating endothelial cells with the cyclooxygenase inhibitor aspirin, 50 μM, for 2 hours, or the prostacyclin synthetase inhibitor 15-hydroperoxyarachidonic acid, 5–20 μg/ml, for 30 minutes. Both of these agents almost completely inhibited PGI₂ production by endothelial cells as verified by RIA; supernates of NP-treated endothelial cells pretreated with either of these agents did not inhibit platelet aggregation (data not shown). Furthermore, when an active supernate was incubated at 37°C for 5 minutes, the activity was abolished. This decay rate is consistent with the decay of the biologic activity of PGI₂. Therefore, the presence of PGI₂ in the supernate was necessary, but insufficient alone, to inhibit platelet aggregation (fig. 2). This finding suggested that both PGI₂ and NP were necessary to inhibit platelet aggregation at the concentrations tested.

To demonstrate that NP and PGI₂ synergistically inhibit platelet aggregation and TXA₂ synthesis, we preincubated various concentrations of NP alone, synthetic PGI₂ alone, and combinations of NP and PGI₂ with PRP for 1 minute before stimulation with threshold doses of epinephrine. The data in tables 1 and 2 demonstrate that NP and PGI₂ synergistically inhibit platelet aggregation.

**Figure 2.** Inhibition of collagen-induced platelet aggregation by supernates from nitroprusside (NP)-treated endothelial cells (EC). Platelet-rich plasma was incubated for 1 minute with supernates from endothelial cells or empty wells (gelatin-coated, medium-conditioned, cell-free wells) previously treated for 5 minutes with either NP, 0.3 μg/ml, or buffer I. A threshold stimulus of collagen was then added. The curve labeled NP represents the effect of the supernate from an empty well incubated with NP. This curve is identical to the control curve (empty well incubated with buffer I). The curve labeled Buffer + EC shows slight inhibition of aggregation induced by the supernate from endothelial cells incubated with buffer I alone. The curve labeled NP + EC shows complete inhibition of aggregation induced by the supernate from endothelial cells incubated with buffer containing NP, 0.3 μg/ml. Samples of the supernates from all the wells were also analyzed for 6-Keto-PGF₁α by radioimmunoassay. The results represent the concentration of 6-Keto-PGF₁α in the supernates and show no difference between the two supernates obtained from endothelial cells, although the effect on platelet aggregation is markedly different.

**Table 1.** Synergy Between Nitroprusside and Prostacyclin in the Inhibition of Platelet Aggregation

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<thead>
<tr>
<th>NP (μg/ml)</th>
<th>PGI₂ (nM)</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1</td>
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<tr>
<td>0</td>
<td>0 ± 2</td>
<td>12 ± 2</td>
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<tr>
<td>2</td>
<td>7 ± 11</td>
<td>37 ± 8</td>
</tr>
<tr>
<td>3</td>
<td>32 ± 9</td>
<td>68 ± 5</td>
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Platelet-rich plasma was preincubated with buffer I alone or the concentrations of NP and PGI₂ listed for 1 minute. Aggregation was then initiated with a threshold stimulus of epinephrine. The results are expressed as a percentage of inhibition of controls in which neither NP nor PGI₂ was added (mean ± SD, n = 3). Statistical analysis was performed by two-way analysis of variance to assess the significance of the effects of each agent alone and their combination, with the following results: $p_{NP}$, $p_{PGI₂}$, $p_{synergy}$ all < 0.0001. Abbreviations: PGI₂ = prostacyclin; NP = nitroprusside.

**Table 2.** Synergy Between Nitroprusside and Prostacyclin in the Inhibition of Thromboxane B₂ Synthesis

<table>
<thead>
<tr>
<th>NP (μg/ml)</th>
<th>PGI₂ (nM)</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.1</td>
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<tr>
<td>0</td>
<td>0 ± 2</td>
<td>39 ± 9</td>
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<tr>
<td>2</td>
<td>−5 ± 2</td>
<td>69 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>−8 ± 9</td>
<td>100 ± 0</td>
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Platelet-rich plasma was preincubated with buffer I alone or the concentrations of NP and PGI₂ listed for 1 minute. Aggregation was then initiated with a threshold stimulus of epinephrine. After 5 minutes, an aliquot of platelet-rich plasma was removed and analyzed by radioimmunoassay for thromboxane B₂. The results are expressed as percent inhibition of controls in which neither NP nor PGI₂ was added (mean ± SD, n = 3). Statistical analysis was performed by two-way analysis of variance to assess the significance of the effects of each agent alone and their combination, with the following results: $p_{NP}$ < 0.001, $p_{PGI₂}$, $p_{synergy}$ all < 0.0004. Abbreviations: PGI₂ = prostacyclin; NP = nitroprusside.
show that when NP and PGI₂ are combined, the resultant inhibition of platelet aggregation and TXA₂ synthesis is much greater than the sum of the inhibitions generated by each agent alone; this type of result defines synergistic inhibition. These interactions (synergies) are highly significant (p < 0.0001 and p < 0.0004, respectively, two-way ANOVA). Similar results were obtained with collagen and arachidonate as aggregating agents (data not shown).

Mechanism of Nitroprusside's Inhibition of Platelet Aggregation

To explore the mechanism of NP-PGI₂ synergy in inhibiting platelet aggregation, we examined four possible mechanisms of NP inhibition of platelet aggregation: direct inhibition of TXA₂ synthesis, inhibition of transmembrane calcium movement, elevation of platelet cAMP, and interaction with platelet microtubules.

Thromboxane A₂ Inhibition

The experiments shown in figure 1 suggested that NP did not inhibit platelet aggregation by directly inhibiting TXA₂ synthesis. Depending on the aggregating agent, similar inhibitions of aggregation occurred despite markedly different extents of inhibition of TXA₂ synthesis. To study this further, NP was incubated with aspirin-treated platelets and primary-wave aggregation was induced with epinephrine. This TXA₂-independent aggregation was significantly inhibited by NP at concentrations identical to those used in normal platelets (IC₅₀ = 3 μg/ml NP, p < 0.0001). RIA for TXB₂ confirmed that no TXA₂ had been synthesized. Thus, in the absence of TXA₂, NP inhibited primary aggregation in aspirin-treated platelets in a manner equivalent to the inhibition of full aggregation in non-aspirin-treated platelets. Therefore, it is unlikely that NP inhibits aggregation by suppressing platelet TXA₂ synthesis directly.

Calcium

Calcium-45 flux experiments in other systems have suggested that NP does not act as a muscle relaxant by inhibiting transmembrane calcium movement. We explored the role of calcium indirectly by studying the inhibitory effect of varying doses of NP on platelet aggregation induced by collagen (0.8–1.6 μg/ml) in both normocalcemic, heparinized PRP and hypocalcemic, heparinized-citrated PRP. As in previous experiments, NP inhibited platelet aggregation (P₅₀ < 0.0001, two-way ANOVA). Lowering the ionized calcium concentration from the normal level of approximately 1 mM to approximately 0.04 mM with 0.32% sodium citrate caused a significant decrease in the extent of aggregation (P₅₀ < 0.0001, two-way ANOVA). However, there was no interaction between calcium and NP in inhibiting aggregation (data not shown, P₅₀<sub>calc</sub> < 0.40, two-way ANOVA), which suggests that NP does not inhibit platelet aggregation by altering calcium flux through the platelet membrane.

cAMP

Inhibitors of platelet function such as PGI₂ act by causing an elevation of platelet cAMP, which in turn inhibits aggregation. The adenosine analog 2',5'-dideoxyadenosine is a specific inhibitor of adenylate cyclase, which abolishes both the elevation of platelet cAMP induced by PGI₂, and the subsequent inhibition of platelet aggregation. Incubation of PRP with 100 μM dideoxyadenosine almost completely abolishes PGI₂-mediated inhibition of epinephrine-induced platelet aggregation, but does not change the NP-mediated inhibition of platelet aggregation (fig. 3). This lack of effect was demonstrated at multiple concentrations of NP. Similar results were obtained when collagen was the aggregating agent. Thus, NP does not inhibit platelet aggregation by causing an elevation in platelet cAMP.

Colchicine and Deuterium Oxide

Colchicine disrupts the equilibrium between tubulin dimers and assembled microtubules and favors microtubule disruption, which in turn inhibits platelet aggregation. D₂O stabilizes microtubule formation and enhances platelet aggregation. If NP interacts with microtubules, the inhibition of platelet aggregation induced by NP should be synergistically altered by preincubation of PRP with either colchicine or D₂O. Figure 4 shows that D₂O and colchicine alter the inhibition by NP of collagen-induced platelet ag-

![Figure 3. Effect of dideoxyadenosine (DDA) on prostacyclin (PGI₂) and nitroprusside (NP) inhibition of aggregation. Platelet-rich plasma (PRP) was preincubated with either DDA (100 μM) for 2 minutes, PGI₂ (10 nM for 1 minute), NP (3 μg/ml) for 1 minute or combinations of these as noted. Aggregation was then initiated with a threshold dose of epinephrine. (A) The curve labeled DDA shows the effect of DDA alone on aggregation and is identical to the control curve, which shows the effect of buffer I alone. The curve labeled PGI₂ demonstrates inhibition of aggregation induced by incubation of PGI₂ with PRP. The curve labeled DDA + PGI₂ demonstrates the abolition of PGI₂-induced inhibition of platelet aggregation when DDA is added to PRP before addition of PGI₂. (B) The curve labeled DDA is described above. The curve labeled NP shows the inhibition of aggregation induced by incubation of NP with PRP. The curve labeled NP + DDA shows that DDA did not change the NP-induced inhibition of platelet aggregation.](http://circ.ahajournals.org/lookup/doi/10.1161/01.CIR.66.6.1304)
The results showed that colchicine and D_2O synergistically inhibited platelet aggregation induced by PRP. The curve labeled D_2O (□ - □) shows the effect of preincubating PRP with colchicine (0.5 mM) for 2 minutes before addition of D_2O. The results in each curve are expressed as a percentage of inhibition compared with its own control containing no D_2O (i.e., for the control curve, control samples contained buffer 1; for D_2O curve, controls contained 60% PRP; for colchicine curve, controls contained 0.5 mM colchicine; values are mean ± SEM, n = 6 for control, n = 3 for D_2O and colchicine). Statistical analyses comparing the decreases in aggregation induced by NP in the three conditions were performed by two-way analysis of variance. The effects of NP, D_2O, colchicine, and the interactions of NP + D_2O and NP + colchicine were all significant (p < 0.0001).

Discussion

In this study, NP inhibited both platelet aggregation and the resultant TXA_2 synthesis in a dose- and time-dependent manner. Further, while NP did not stimulate endothelial cells to synthesize PGI_2, NP and PGI_2 synergistically inhibited both platelet aggregation and TXA_2 synthesis. The inhibition of platelet aggregation induced by supernates of NP-treated endothelial cells was dependent on this synergy, because pretreating the endothelial cells with either aspirin or 15-hydroperoxy arachidonic acid blocked both the background production of PGI_2 as measured by RIA and the inhibition of platelet aggregation. This finding does not absolutely rule out the presence of additional endothelial cell metabolites, such as adenosine, that inhibit platelet aggregation. However, adenosine release is not affected by aspirin treatment, and adenosine is only slowly produced from ATP released by endothelial cells. Thus, only small amounts of adenosine would be present after the 5-minute incubation used in our experiments. Furthermore, the activity of the supernate is heat labile and adenosine is not. Thus, although other metabolites may contribute to the inhibition of platelet aggregation by supernates of NP-treated cells, our studies demonstrate that synergy is absolutely dependent on PGI_2 and NP and can be produced in vitro using only synthetic PGI_2 and NP alone.

The concentration of NP in which these phenomena were noted was 0.01–30 μg/ml. Although the lowest concentration of NP that directly inhibited arachidonate-induced platelet aggregation and TXA_2 synthesis was 0.1 μg/ml, the lowest concentration at which synergy for these inhibitions was detected was 0.01 μg/ml. These concentrations of NP (0.01–30 μg/ml) can be clinically attained in vivo. Rodkey and Collison demonstrated a plasma NP concentration in baboons of 15 μg/ml during a rapid, concentrated infusion of NP, 4.8 μg/kg/min. Desprez and colleagues noted levels of 8.5 to 28 μg/ml in five human patients in severe congestive heart failure or hypertensive crisis who had received infusions of NP for varying times at usual clinical infusion rates for these conditions. Therefore, on the basis of concentration alone, it would be anticipated that NP as used clinically would directly inhibit both platelet aggregation and TXA_2 synthesis. An inhibition of platelet aggregation induced by NP in vivo has in fact been demonstrated by several investigators using various assay methods.

Whether the synergy between NP and PGI_2 in inhibiting platelet aggregation and TXA_2 synthesis is present in vivo and therefore responsible in part for the clinical observations on platelet function noted above will depend on the local concentration of PGI_2. Measurements of circulating levels of PGI_2 have varied widely depending on technique, but the most recent studies in humans under physiologic conditions have shown concentrations to be quite low, i.e., 100–350 pM. However, since exposure of endothelial cells to thrombin and other substances that might be present during formation of an occlusive thrombus in an atherosclerotic artery induces marked elevations in PGI_2 synthesis, local concentrations of PGI_2 in pathologic states may be much higher. The lowest concen-
tration of PGI₂, at which synergy with NP was observed in our studies was 100 μM, with both collagen- and arachidonate-induced aggregation. Therefore, synergy could be present in vivo with “physiologic” levels of capillary PGI₂, and very likely is present with the theoretically elevated levels of PGI seen in local circulations in pathologic conditions.

The mechanism of the synergistic interaction between NP and PGI₂ in inhibiting platelet function is not known. NP did not inhibit aggregation through a direct inhibition of TXA₂ synthesis; rather, TXA₂ synthesis appeared decreased secondary to the inhibition of platelet function at some other level. Further, NP inhibition of aggregation was not altered by raising the external calcium concentration, which suggests that NP does not act by inhibiting calcium flux, a finding reported for vascular smooth muscle.31–33 We could not detect a decrease in NP action when platelet cAMP production was inhibited with dideoxyadenosine. Further, although recent investigators have shown that NP induces marked elevations of cGMP, it is not yet certain that these elevations directly mediate the inhibition of both vascular smooth muscle and platelet aggregation by NP.47–50 Since an inhibitory guanosine analog similar to dideoxyadenosine is not available, we could not directly test the role of cGMP in NP-mediated inhibition of platelet function.

We demonstrated a significant interaction between NP and the microtubule agents colchicine and D₂O. Microtubule function has an important role in platelet activation.35–37, 51–54 D₂O, a microtubule stabilizer,27–29 synergistically diminished the effect of NP, whereas colchicine, a microtubule disrupter,28 synergistically enhanced NP inhibition of platelet aggregation. The combination of colchicine and PGI₂, however, also synergistically inhibited platelet aggregation; therefore, our studies do not prove that NP inhibits microtubule function. Further studies will be needed to determine whether these synergistic interactions are due to a direct effect on microtubules or indicate simply that microtubule function is a common element in the effector pathway for platelet aggregation.

The clinical relevance of the interaction between vasodilators and platelet and endothelial cell prostaglandin production lies in the growing recognition that platelet aggregation may contribute to such ischemic events as angina and myocardial infarction.55 Both experimental and clinical observations, indicate that platelet aggregates form at narrowed and eroded areas of atherosclerotic plaques and result in transient obstruction of the circulation.55–57 If unrelied, such obstruction may result in an occlusive thrombosis and concomitant infarction.58 The platelet aggregates may also release TXA₂ and cause local vasoconstriction, which will further limit flow, and may also cause endothelial disruption,57 which will lead to increased platelet activation. If this hypothetical framework is correct, vasodilators may be beneficial not only because they reduce preload and afterload, but also because they interrupt the cycle of platelet aggregation, TXA₂ synthesis and vascular spasm.

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References

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