Nitric Oxide–mediated Cyclooxygenase Activation

A Key Event in the Antiplatelet Effects of Nitrovasodilators

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Abstract

We have evaluated the contributions of nitric oxide (NO) and prostacyclin (PGI_2) in the in vivo antiplatelet effects of clinically useful nitrovasodilators. In rats, intravenous infusion of three NO donors, glyceryl trinitrate, sodium nitroprusside, or 3′-morpholinosydnonimine, the stable metabolite of molsidomine, released 6-keto $PGF1\alpha$ (the stable metabolite of PGI_2) and inhibited ex vivo human platelet aggregation to adenosine diphosphate by at least 80%. In in vitro studies, glyceryl trinitrate, sodium nitroprusside, and 3′-morpholinosydnonimine, at clinically attainable concentrations, increased cyclooxygenase activity in endothelial cells (EC), which resulted in a four- to sixfold release of 6-keto $PGF1\alpha$. Pretreatment of the EC with hemoglobin which binds to and inactivates the biological actions of NO, but not by methylene blue (MeB), attenuated the NO-mediated PGI_2 from the EC by at least 70%. Release of 6-keto $PGF1\alpha$ by the NO donors increased the ability of these compounds to inhibit thrombin-induced human platelet aggregation by at least 10 times; this potentiation was inhibited by hemoglobin but not by MeB. MeB blocked the direct antiplatelet effect of the NO donors in the absence of EC. In summary, we have demonstrated that NO, directly as well as together with an NO-driven cyclooxygenase activation (and hence PGI_2), release contributes to the marked antiplatelet effects observed after the in vivo administration of clinically used nitrovasodilators. (*J. Clin. Invest.* 1996. 97: 2562–2568.) Key words: nitric oxide • prostacyclin • cyclooxygenase • platelet aggregation

Introduction

The L-arginine pathway within endothelial cells (EC)[1] in blood vessels generates the endogenous nitrovasodilator, nitric oxide (NO) (1, 2). Within the cardiovascular system NO plays a fundamental role in keeping blood vessels in a dilated state and in maintaining the surface of the endothelium nonthrombogenic (for review see reference 3). NO stimulates the soluble guanylate cyclase (sGC) by interacting with the ferroheme center of the enzyme resulting in the generation of guanosine 3′:5′-cyclic monophosphate (cGMP) (4, 5). Increased cGMP levels subsequently lead to vasodilation (4) and inhibition of platelet aggregation (6, 7). The vasorelaxant and antiplatelet effects of NO are abolished by methylene blue (MeB), an inhibitor of the sGC (4, 5), and by hemoglobin (Hb) which binds to and inactivates NO (8). Thus, endothelium-derived NO, through its vasodilator and antiplatelet properties, prevents vasospasm and thrombus formation in the circulation and thereby helps to maintain blood flow to vital organs such as the heart.

The discovery of the L-arginine to NO pathway revealed that NO was the active component of a group of drugs known as the nitrovasodilators (for review see reference 9) which had been used for many years to lower blood pressure and relieve anginal attacks. This warrants their use as NO replacement therapy in diseases such as myocardial ischemia, thrombosis, and atherosclerosis that are associated with a failing endogenous NO pathway (for review see reference 10). For instance, since NO donors do not require an intact endothelium to be effective (11), they should be able to restore the desired vascular dilation and suppress the tendency to platelet aggregation despite advanced atherosclerosis (12, 13).

The cardiovascular effects exerted by endogenously produced NO are often mediated in conjunction with another important vasodilator and platelet inhibitory eicosanoid released from the endothelium, namely, prostacyclin (PGI_2). PGI_2 formed during the bioconversion of arachidonic acid (AA) by the heme-containing enzyme, cyclooxygenase (COX), exerts its vasodilator and antithrombotic effects by activating adenylate cyclase and increasing the levels of adenosine 3′:5′:cyclic monophosphate (cAMP) (14). Two forms of COX have been identified; one is constitutively expressed (COX-1), whereas the other is induced (COX-2) during an inflammatory insult (for reviews see references 15 and 16). COX-1 activation accounts for the physiological and beneficial roles attributed so far to PGI_2 (e.g., cytoprotection in the stomach and kidney, vasodilation, and antiplatelet activity) (15, 16).

Although it is becoming recognized that inhibition of platelet reactivity may be an important property pertinent to the therapeutic effects of the NO donors in cardiovascular diseases, their mechanism of action in vivo remains to be defined. We have reported recently that NO stimulates COX activity, leading to an augmented production of COX metabolites, and we proposed that this may be a widespread mechanism through which NO regulates both physiological and pathological events (17–19).

The finding that COX activity was regulated by NO led us to investigate the exciting possibility that PGI_2 released upon

1. *Abbreviations used in this paper:* COX, cyclooxygenase; EC, endothelial cells; GTN, glyceryl trinitrate; Hb, hemoglobin; MeB, methylene blue; NO, nitric oxide; PGI_2, prostacyclin; sGC, soluble guanylate cyclase; SIN-1, 3′ morpholinosydnonimine; SNP, sodium nitroprusside.

activation of COX contribute to the in vivo antiplatelet effects of therapeutically useful nitrovasodilators.

Methods

In vivo measurements. Male Sprague Dawley rats (300–350 grams) were purchased from Charles River Laboratories (Wilmington, MA) and used throughout these studies. All animal protocols were approved by the Institutional Animal Care and Use Committee. Animals were anesthetized with methoxyflurane before surgical preparation. Polyethylene catheters (PE50) were inserted into a femoral artery and vein. The urinary bladder was cannulated using a flared PE90 polyethylene catheter. The animals were placed into individual restraining cages and allowed to regain consciousness. Mean arterial blood pressure and heart rate were measured continuously via the femoral artery using a pressure transducer (type 041-500-503; Cobe, Lakewood, CO) connected to a polygraph (model 7E; Grass Instrument Co., Quincy, MA). Mean arterial pressure and heart rate were recorded at 5-min intervals on a computerized data acquisition system. Glyceryl trinitrate (GTN; 1.1 µmol/kg/min), sodium nitroprusside (SNP; 0.5 µmol/kg/min), 3′ morpholinosydnonimine (SIN-1; 0.2 µmol/kg/min), or verapamil (0.5 µmol/kg/min) was infused via the femoral vein for a total of 1 h. Control rats received an infusion of 0.9% sodium chloride via the femoral vein (0.07 ml/kg/min) for the duration of the study. When required, MeB or indomethacin (or an equivalent volume of the vehicle; phosphate-buffered saline, 1% Tween 80, pH 7.4) was given intravenously at a dose of 10 or 3 mg/kg, respectively, 1 h before the start of drug infusion. 0.5-ml blood samples were taken immediately before and at 1 h after saline or drug infusion for ex vivo platelet aggregation and for the determination of plasma NO_2^-/NO_3^- and 6-keto PGF1α. For ex vivo platelet aggregation, blood was centrifuged so as to obtain platelet rich plasma; platelets were aggregated by stimulation with the aggregating agent, ADP, at a dose (10 µM) that caused submaximal platelet aggregation (80–90%) (20).

Determination of plasma nitrite and 6-keto PGF1α. Arterial blood was collected in Microtainer tubes containing lithium heparin (Becton Dickinson, Rutherford, NJ) and centrifuged to collect plasma samples. Plasma samples were passed through an Ultra-MC filter (10,000 MWCO; Millipore Corp., Bedford, MA). NO reacts with oxygen to yield nitrate and nitrite. To obtain the net amount of NO generated in vivo from the various NO donors, values for nitrite (A) and nitrite plus nitrate (B) were measured in the plasma samples. Levels of nitrite in the samples (5 µl) were measured in the absence of nitrate reductase and using sodium nitrite as a standard. Nitrate in plasma aliquots (5 µl) was converted to nitrite by the addition of nitrate reductase (14 mU) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH, 1 nmol) with incubation for 10 min at room temperature. The reaction was terminated by dilution with water and addition of the diaminonapthalene reagent, and nitrite concentrations in these samples were determined fluorimetrically (21) using sodium nitrate as a standard. All determinations were performed in duplicate. The difference between A and B is taken to represent net NO released in vivo from the NO donors. 6-keto PGF1α was measured in the unextracted plasma by ELISA (Cayman Chemical Co., Inc., Ann Arbor, MI) as described (22).

Preparation of cultured EC. EC were obtained from bovine aortae and grown as monolayer cultures in T75 tissue culture flasks. When confluent, the EC were treated briefly (10–20 s) with 0.05% trypsin 1 h before use and centrifuged to form a pellet at 1,000 rpm in a Biofuge A rotor for 5 min. The pellet containing the EC was then resuspended in Hanks' balanced salt solution containing 10 mM Hepes (HHBSS) at pH 7.4; the final EC count was adjusted to 2×10^6 cells/ml. 10-µl aliquots of this stock were used for the platelet assay. For determination of cGMP and 6-keto PGF1α release, the EC were then plated in 48-well plates at $\sim 1 \times 10^6$/well in HHBSS. The total incubation volume in the wells was 0.5 ml. When confluent, cells were used for measuring cGMP and 6-keto PGF1α. Protein concentrations

in each well were determined by the Bradford method (23) using bovine serum albumin as a standard and results were expressed as femtomoles of cGMP per milligram of protein and picograms of 6-keto PGF1α per milligram of protein. All determinations were performed in duplicate.

Activation of sGC activity in EC. cGMP were determined by radioimmunoassay (24) after acetylation of the samples with acetic anhydride (25). EC were plated in 48-well plates, and when confluent cells in each well were stimulated at 37°C for 30 min with GTN, SNP, or SIN-1 (1–10 µg/ml) in the absence or presence of Hb (10 µM) or MeB (30 µM). Isobuthylmethylxanthine (1 mM) was present in the incubation mixture to inhibit phosphodiesterase activity. The incubation medium was aspirated and the cGMP was extracted by the addition of ice-cold HCl (0.1 M). After 10 min, the samples were transferred to a new plate, dried, and reconstituted in 5 mM sodium acetate (pH 4.75) for cGMP determination.

Activation of COX activity in EC. 6-keto PGF1α, the stable metabolite of PGI$_2$, was measured as an index of COX activity after activation of the EC with AA. EC in each well were washed twice with fresh HHBSS and allowed to equilibrate in the absence or presence of various compounds (indomethacin, MeB, Hb) for 30 min. The cells were then incubated with AA (30 µM) for 10 min. All incubations were terminated by placing the plates on ice. The supernatants were removed and assayed for 6-keto PGF1α by ELISA (Cayman Chemical Co., Inc.). The lower limit of detection of 6-keto PGF1α was 0.2 ng/ml.

Platelet aggregation in the presence and absence of EC. Human washed platelets treated with indomethacin (10 µM) were prepared as described previously (26) and the platelet count was adjusted to $1–1.5 \times 10^8$/ml. A 500-µl suspension was incubated at 37°C for 4 min in a dual channel aggregometer (Payton Scientific Inc., Buffalo, NY) with continuous stirring at 1,000 rpm and then stimulated with thrombin (40 mU/ml) to give a submaximal aggregation (80–90%). The decrease in optical density was recorded for 5 min. After a 3-min incubation with platelets, the inhibitory effects of GTN or SNP on thrombin-induced platelet aggregation were measured either alone or in the presence of EC (10 µl of stock = 0.2×10^5 cells). Experiments were performed with EC that were either not treated or treated with indomethacin (10 µM, 1 h). When required, oxyHb (10 µM) was added to the platelet mixture for the 3-min incubation period. When using EC or oxyHb, the calibrations were performed in the presence of these agents to compensate for possible changes in light transmission; inhibition of platelet aggregation was calculated as described (20).

Materials. oxyHb was prepared by reduction of bovine Hb with sodium hydrosulfite as described (20). GTN (Nitronal) was obtained from Lipha Pharmaceuticals (West Drayton, Middlesex, United Kingdom). SIN-1, the stable metabolite of the antianginal molsidomine, was a gift from Hoeschst (Frankfurt, Germany). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Indomethacin was dissolved in phosphate-buffered saline, pH 7.4, 1% (vol vol^{-1}) Tween 80; all other compounds were dissolved in saline.

Statistical analysis. Statistical significance between groups was determined by two-way ANOVA followed by unpaired Student's *t* test. A probability (*P*) value of 0.05 or less was taken to indicate statistical significance. Results are expressed as mean±SEM for (*n*) experiments.

Results

In vivo hemodynamic and antiplatelet changes evoked by NO donors. In conscious restrained rats, the intravenous infusion (for 1 h) of GTN (1.1 µmol/kg/min, *n* = 8), SNP (0.5 µmol/kg/min, *n* = 8), or SIN-1 (0.2 µmol/kg/min, *n* = 8) released equivalent amounts of NO from their molecule as detected by an increase in plasma levels of NO_2^-/NO_3^- from a basal of 14±1 to 145±10 µM, 165±15 and 157±13 µM, respectively (*n* = 8), and inhibited by at least 80% ex vivo platelet aggregation to ADP (10 µM, *n* = 8) (Fig. 1).
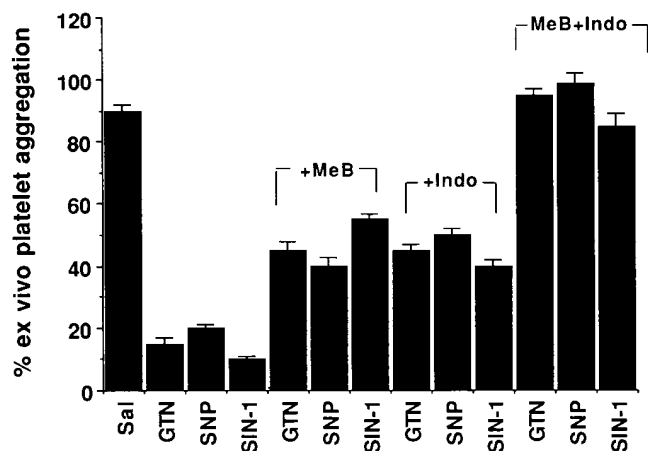
Figure 1. Effects of in vivo infusions of NO donors in conscious restrained rats on ex vivo platelet aggregation. ADP (10 μM)-induced platelet aggregation in plasma prepared from blood taken from saline (*Sal*)-infused rats was significantly attenuated in rats that received GTN, SNP, or SIN-1. This effect was attenuated by MeB (10 mg/kg) or indomethacin (*Indo*, 3 mg/kg). Results are expressed as the percentage of platelet aggregation. Each point is the mean±SEM for eight experiments.

This was associated with an $\sim$ 20-fold increase in the release of 6-keto PGF1α and was attenuated by at least 50% in rats pretreated with indomethacin (3 mg/kg, $n = 8$) (Fig. 2). Pretreatment of the rats with MeB (10 mg/kg, $n = 8$), an inhibitor of the sGC and hence of NO-mediated platelet effects, blocked as expected the antiplatelet effects of the NO donors by at least 50% (Fig. 1) but not their capacity to release in vivo PGI$_2$ (Fig. 2). Complete reversal of the antiplatelet effects of the NO donors on ex vivo platelet aggregation was obtained in
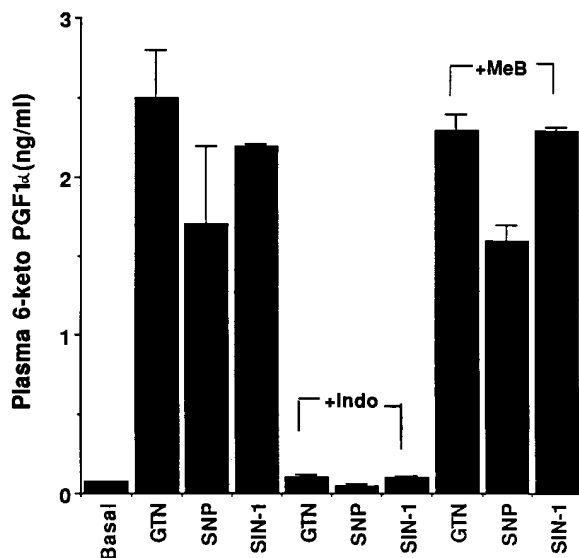

Figure 2. Infusion of GTN, SNP, or SIN-1 in conscious restrained rats increased the plasma levels of 6-keto PGF1α; this increase was prevented in rats pretreated with indomethacin (*Indo*, 3 mg/kg) but not MeB (10 mg/kg). Each point is the mean±SEM for eight experiments.

plasma prepared from blood taken from rats that received the dual combination of MeB and indomethacin (Fig. 1). Ex vivo platelet aggregation to ADP in platelets prepared from rats infused with saline was not modified by MeB or indomethacin ($n = 8$, not shown).

Verapamil, a vasodilator which does not release NO, was used as control agent to show that any effect observed on PGI$_2$ release by the nitrovasodilators cannot be accounted for by changes in hemodynamic parameters. Thus, verapamil, when infused for 1 h at a dose (0.5 μmol/kg/min, $n = 8$) that elicited a similar fall in blood pressure (from a basal level of 120±2 to 61±4 mmHg) as the one evoked by the NO donors (Table I, $n = 8$) did not increase the levels of 6-keto PGF1α (0.08±0.001 to 0.1±0.05 ng/ml, $n = 8$), indicating that hypotension was not the stimulus of PGI$_2$ release. The effects of various drugs on blood pressure changes are shown in Table I.

Effects of NO donors on the release of PGI$_2$ from EC. Stimulation of EC for 10 min with AA (30 μM) led to a fivefold increase of 6-keto PGF1α release in the culture medium (from 30±2 to 150±2 pg/mg protein, $n = 10$). A 30-min pretreatment of EC before the addition of AA with GTN, SNP, or SIN-1 (0.1–10 μg/ml, $n = 10$) significantly increased the total capacity of the EC to synthesize 6-keto PGF1α in response to exogenous AA (Fig. 3). The effects of GTN, SNP, or SIN-1 were abolished by coincubation with 10 μM Hb or indomethacin (Table II).

NO decomposes in aqueous solutions to nitrite and nitrate (for review see reference 9). Incubation of the EC for 30 min with high concentrations of sodium nitrite or nitrate (200 μM, $n = 10$) did not potentiate the production of 6-keto PGF1α in response to AA (30 μM, 10 min) (not shown). Therefore, these findings exclude a possible role for nitrite/nitrate in the actions of NO.

These results suggest that NO, the active principle released from the molecule of the NO donors, activates COX activity in the EC, resulting in an augmented release of PGI$_2$.

Role of sGC on the release of PGI$_2$ from EC by NO donors. Elevation of intracellular cGMP levels after stimulation

Table I. Effects of MeB or Indomethacin (Indo) on Mean Arterial Blood Pressure (MAP) Changes Elicited by Infusion of NO Donors or Verapamil in Conscious Restrained Rats

Rats infused with	Treatments		
	None	MeB	Indo
Saline	120±2	115±5	112±3
GTN	70±2*	125±5‡	90±5§
SNP	70±4*	115±5‡	95±2§
SIN-1	62±2*	120±2‡	95±2§
Verapamil	61±4*	70±2	69±5

The NO donors, GTN, SNP, or SIN-1 caused a fall in mean arterial pressure (expressed in mmHg) when infused for 1 h; this hypotensive effect was restored to near control values in rats treated with MeB (10 mg/kg) and to a lesser extent in rats treated with Indo (3 mg/kg). MeB or Indo had no effect on the hypotensive effect elicited by verapamil. Results are expressed as MAP (mmHg) with each value representing the mean±SEM for eight experiments. *$P < 0.01$ when compared with MAP in control saline-treated rats. §$P < 0.05$ and ‡$P < 0.001$ when compared with values obtained with the NO donor in the absence of MeB or Indo.
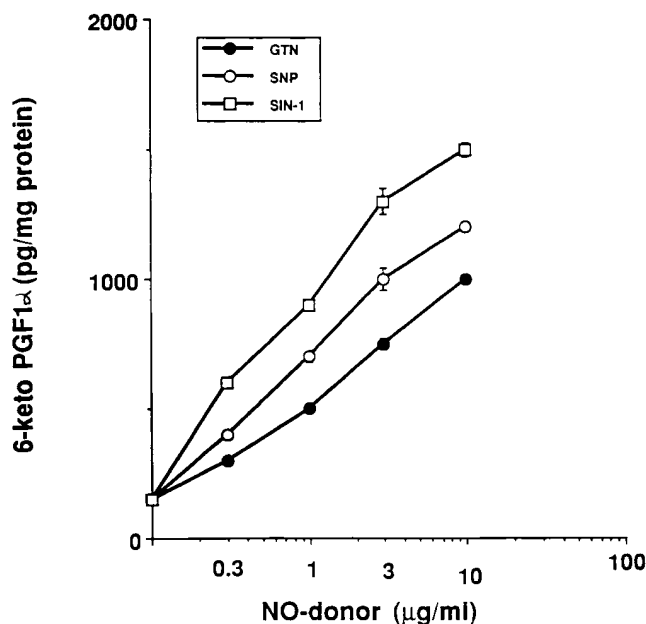
Figure 3. Effects of NO donors on the production of 6-keto PGF1α by AA in EC. AA (30 μM)-stimulated 6-keto PGF1α release from EC was markedly increased by GTN, SNP, or SIN-1 (0.3–10 μg/ml). Data are the mean±SEM for 10 experiments.

Table II. Effects of Indomethacin (Indo) or Hb on the Release of 6-keto PGF 1α by EC Evoked by GTN, SNP, or SIN-1

Treatments	NO donor (μg/ml)				
	0	0.3	1	3	10
AA + GTN	150±2	300±10*	500±15‡	750±20‡	1000±30‡
+ Indo	22±1§	20±2‖	25±2‖	25±2‖	15±3‖
+ Hb	140±3	130±2§	135±5‖	140±3‖	100±2‖
AA + SNP	150±2	400±10*	700±20‡	1000±40‡	1200±10‡
+ Indo	20±2§	15±7‖	17±2‖	19±3‖	20±5‖
+ Hb	120±7	130±5§	130±5‖	110±10‖	100±3‖
AA + SIN-1	150±2	600±20‡	900±10‡	1300±10‡	1500±10‡
+ Indo	20±2‖	10±5‖	16±2‖	15±2‖	30±5‖
+ Hb	120±7	120±10‖	140±10‖	130±20‖	150±50‖

The basal release of 6-keto PGF 1α from EC in the absence of AA was 30±2 pg/mg protein and this was increased fivefold to 150±2 pg/mg protein ($n = 10$) by AA. GTN, SNP, or SIN-1 (0.3–10 μg/ml, $n = 10$) when incubated with EC for 30 min enhanced even further the production of 6-keto PGF1α by AA. The effects of the NO donors were inhibited by Indo or Hb (10 μM). Data are expressed in picograms per milligram of protein and each value is the mean±SEM for 10 experiments. $*P < 0.05$ and $‡P < 0.001$ when compared with control values (in the absence of NO donors) and $§P < 0.05$ and $‖P < 0.001$ when compared with values obtained with the NO donors in the absence of Indo or Hb.

of sGC is responsible for a number of NO-mediated effects (for review see reference 3). To determine whether cGMP played a role in the NO-driven PGI₂ release, we evaluated the effects of MeB on cGMP and 6-keto PGF1α levels in EC exposed to the NO donors. Fig. 4 *A* shows that a 30-min stimulation of the EC with SIN-1 (1–10 μg/ml, $n = 10$) increased the levels of cGMP in EC and this increase was inhibited by MeB (30 μM, $n = 10$) or Hb (10 μM, $n = 10$). As found in the in vivo studies, MeB had no effect on SIN-1–mediated 6-keto PGF1α release, whereas Hb inhibited its release (Fig. 4 *B*). Similar results were obtained with GTN or SNP (10 μg/ml, $n = 10$) (Table III).

These results exclude the role of cGMP in the increased production of PGI₂ in EC mediated by the NO donors.

EC enhance the in vitro antiplatelet effects of the NO donors: roles of NO and PGI₂. In human washed platelets, GTN (0.1–100 μg/ml, $n = 10$), SNP (0.1–10 μg/ml, $n = 10$), or SIN-1

(0.03–10 μg/ml, $n = 10$) inhibited thrombin (40 mU/ml)-induced platelet aggregation (Figs. 5 and 6). The concentration required to inhibit platelet aggregation by 50% (IC₅₀) was the lowest for SIN-1 (IC₅₀ = 0.36 μg/ml) followed by SNP (IC₅₀ = 0.9 μg/ml) and GTN (IC₅₀ = 12.4 μg/ml). As expected, these effects were prevented by coincubation with Hb and MeB (not shown) supporting a role for NO as the active antiplatelet principle of these NO donors.

Figs. 5 and 6 reveal that the antiplatelet effects of GTN, SNP, or SIN-1 were magnified at least 10-fold by those EC (0.2×10^5 cells) previously shown in Fig. 3 to release large quantities of PGI₂ upon exposure to NO donors. In this scenario, the IC₅₀ values for GTN, SNP, and SIN-1 were, respectively, 0.9, 0.09, and 0.03 μg/ml ($n = 10$). This potentiation was attenuated by coincubation with Hb (10 μM, $n = 10$) or by treating the EC with indomethacin (10 μM, 30 min) (Figs. 5 and 6).
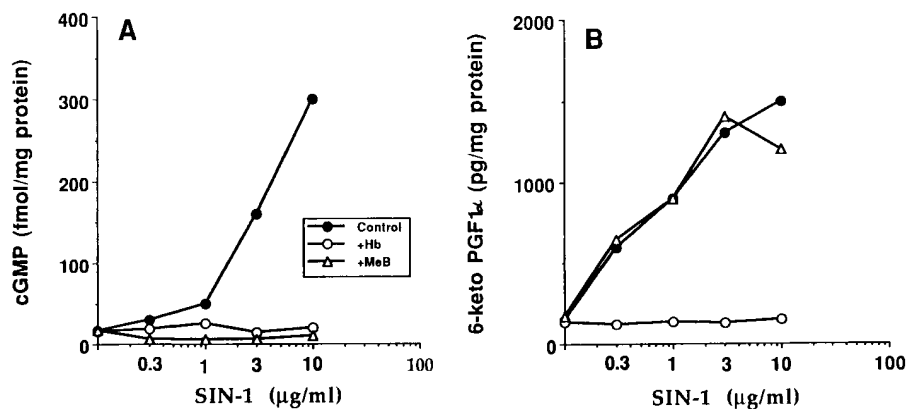

Figure 4. SIN-1 (1–10 μg/ml) increased the levels of cGMP in EC and this increase was inhibited by coincubation with Hb or MeB (10 μM) (*A*). On the other hand, increased production of AA-induced 6-keto PGF1α release was blocked by Hb but not by MeB (*B*). Each point is the mean±SEM for 10 experiments.

Table III. Effects of MeB on cGMP and 6-keto PGF1α Levels in EC

Treatments	cGMP	6-keto PGF1α
	fmol/mg protein	*pg/mg protein*
Saline	17±2	150±3
Saline + MeB	15±2	145±7
GTN	113±5*	1000±30*
GTN + MeB	10±4‡	975±10
SNP	145±7*	1200±30*
SNP + MeB	8±2‡	980±50

GTN or SNP (10 μg/ml, 30 min) increased the levels of cGMP in EC and this increase was inhibited by coincubation with MeB (10 μM). On the other hand, increased production of 6-keto PGF1α after a 10-min incubation with 30 μM AA in the presence of GTN or SNP was not blocked by MeB. Each point is the mean±SEM for 10 experiments. cGMP values are expressed in femtomoles per milligram of protein and 6-keto PGF1α values in picograms per milligram of protein. *$P < 0.001$ when compared with values obtained in the absence of GTN or SNP and ‡$P < 0.001$ when compared with values in the presence of GTN or SNP but in the absence of MeB.

EC alone (0.2×10^5) whether not treated or treated with indomethacin did not inhibit thrombin-induced platelet aggregation ($n = 10$, not shown).

Discussion

The experiments performed in this study demonstrated that NO released exogenously from three clinically used NO donors, namely GTN, SNP, or SIN-1, activates COX leading to the release of PGI$_2$; subsequently, NO in concert with PGI$_2$ contributes to the antiplatelet effects of these drugs.
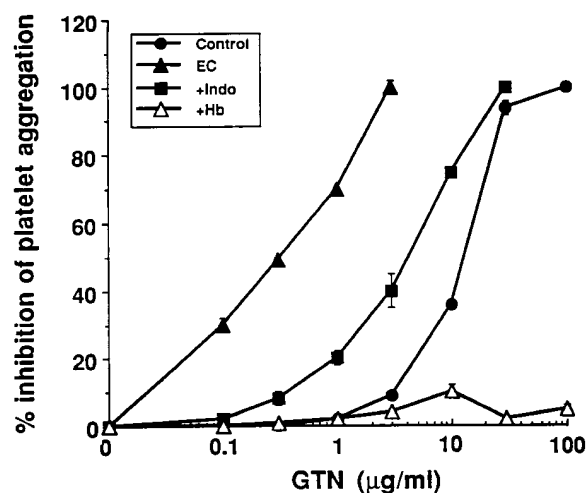

Figure 5. Inhibition of thrombin-induced human platelet aggregation by GTN (0.1–100 μg/ml) is magnified by small number of EC (0.2×10^5). This effect is blocked by Hb (10 μM) and by pretreating the EC for 30 min with indomethacin (10 μM). Results are expressed as percent inhibition of platelet aggregation. Each point is the mean±SEM for 10 experiments.

Inhibition of ex vivo platelet aggregation by in vivo administration of three NO donors was due to the release of exogenous NO (detected in plasma as its breakdown products, nitrite/nitrate) acting through a cGMP-dependent mechanism since it was suppressed, as expected by the sGC inhibitor, MeB (4, 5).

However, the new and exciting finding was that these in vivo antiplatelet effects were associated with a marked increase in plasma PGI$_2$, a potent antiplatelet endothelium-derived COX metabolite (27), and were inhibitable by at least 50% by pretreating the rats with the COX inhibitor indomethacin. Taken together these results indicated to us that besides the well known mechanism of action of the NO donors (e.g., stimulation of the sGC), these drugs also exerted part of their effects through an additional pathway that involves PGI$_2$ release possibly from the endothelium since this is the most likely source of PGI$_2$ in the vasculature. Furthermore, direct application of NO donors at clinically attainable concentrations released PGI$_2$ from cultured EC. Subsequent release of PGI$_2$ by the NO donors increased at least 10 times the ability of these compounds to inhibit thrombin-induced human platelet aggregation. These results clearly show how powerful of an antiplatelet agent NO becomes in the presence of endothelium-derived PGI$_2$. Release of PGI$_2$ is cGMP independent (MeB insensitive) and may reflect direct NO-mediated activation of COX (17–19) after an *S*-nitrosation of cysteine residues in the catalytic domain of the enzyme (28). This nitrovasodilator-mediated COX activation within the vascular endothelium suggests that in diseases such as ischemia-reperfusion injury, atherosclerosis, and hypertension that are associated with a failing endogenous L-arginine to NO pathway or a dysfunctional sGC activity (for reviews see references 29–31), the regulation of COX activity by NO is an alternative pathway used by exogenous nitrovasodilators to elicit vasodilation and inhibition of platelet-dependent thrombotic events. Although in this study we have focused primarily on the platelet actions of the NO donors, similar effects may occur at the levels of the vasculature. Indeed, the hypotensive effects of GTN, SNP, or SIN-1 were not only blocked by MeB but also partially reduced by indomethacin, suggesting the role of a COX metabolite.

The importance of our findings is highlighted in the model shown in Fig. 7. NO is the active principle responsible for the therapeutic actions of clinically used nitrovasodilators including GTN, SNP, and SIN-1. The mechanisms by which NO is formed from the NO donors vary. Thus, GTN releases NO only upon metabolic bioconversion. In smooth muscle cells and EC this bioconversion is mainly enzymatic (32, 33) although some occurs nonenzymatically in the presence of thiols (34). Platelets lack the enzyme responsible for the bioconversion of GTN to NO and thus metabolize it poorly (35), explaining the weak in vitro inhibitory action of GTN. On the other hand, SNP or SIN-1 releases NO spontaneously (for review see reference 9) explaining why lower concentrations (with respect to GTN) are required to inhibit platelet function in vitro. Regardless of how NO is formed from the NO donor, inhibition of platelet aggregation will occur via two steps: direct NO-stimulated sGC activation and cGMP elevation, and NO-mediated activation of COX in the EC leading to PGI$_2$ release and cAMP elevation. With respect to the mechanism of antiplatelet action of organic nitrates such as GTN, this finding is an important one because it provides the key for under-
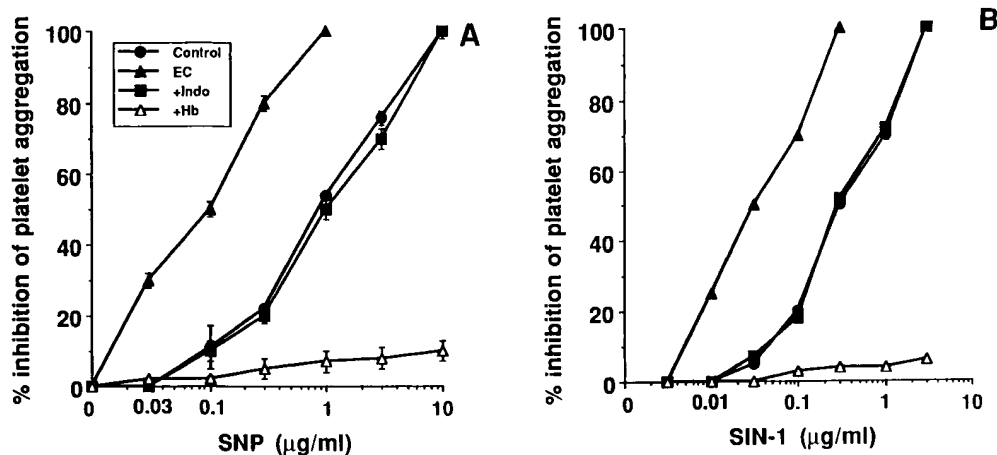
Figure 6. Inhibition of thrombin-induced human platelet aggregation by SNP (*A*) or SIN-1 (*B*) (0.01–10 μg/ml) is magnified by small number of EC (0.2×10^5) (*A* and *B*). This effect is blocked by Hb (10 μM) and by pretreating the EC for 30 min with indomethacin (10 μM). Results are expressed as percent inhibition of platelet aggregation. Each point is the mean±SEM for 10 experiments.

standing why organic nitrates inhibit platelet function in vivo, whereas they generally display weak antiplatelet effects in vitro (35, 36). Subsequently, increased levels of cGMP or cAMP in smooth muscle cells or in platelets elicit vasodilation and platelet inhibition restoring altered vascular and platelet reactivity, two phenomena central to the occlusion of blood vessels as seen during the incidence of myocardial ischemia and atherosclerosis (for review see reference 37).

The discovery that the therapeutic effects of the NO donors are the result of the NO-mediated activation of two endogenous transduction mechanisms namely the sGC and the COX raises the possibility that increased platelet aggregation (37), vasoconstriction, and elevation of systemic blood pressure (for reviews see references 29 and 30) induced by inhibition of endogenous NO by nitric oxide synthase inhibitors could be due not only to removal of endogenous NO but also to a consequent reduced production of antiplatelet and vasodilator COX products. This last mechanism might be of interest for a clearer understanding of the beneficial effects of nitric

oxide synthase inhibitors observed in animals and humans in the treatment of vascular dysfunctions occurring in cytokine-induced hypotension or LPS-induced shock.

In conclusion, we propose that dual formation of NO and PGI$_2$ and their subsequent interactions at the levels of the vasculature and circulating cells such as platelets may represent a powerful mechanism used by therapeutically administered nitrovasodilators to restore abnormal vascular tone and platelet reactivity in pathological states associated with an altered endothelium.

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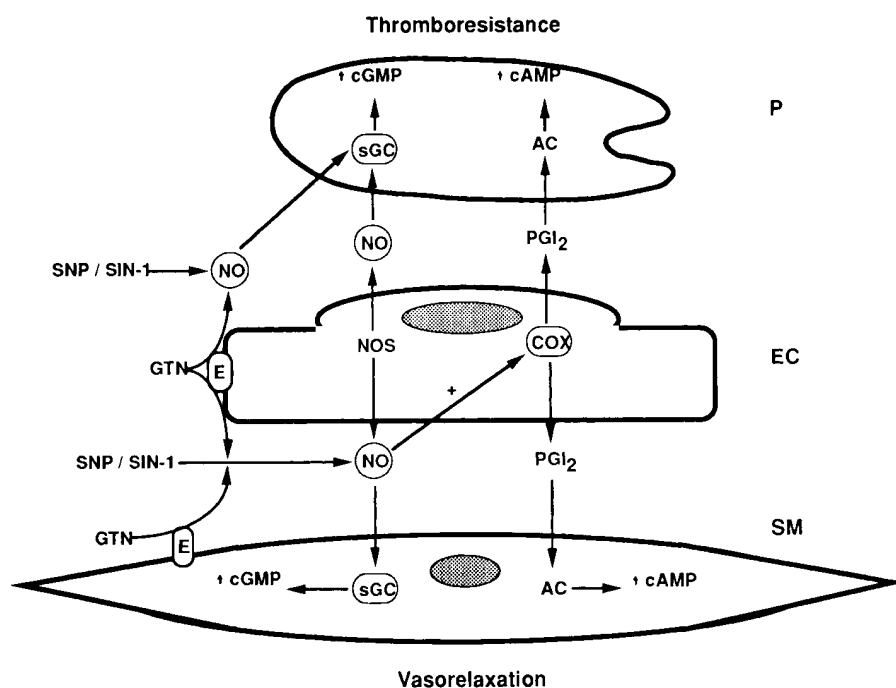
Figure 7. NO-mediated activation of sGC and COX: a proposed model of the in vivo effects for therapeutically administered nitrovasodilators. NO released spontaneously from SNP or SIN-1 or enzymatically (*E*) from GTN in EC and smooth muscle cells (*SM*) stimulates sGC in smooth muscle and platelets (*P*). In addition, NO activates COX in EC leading to PGI$_2$ release. PGI$_2$ activates adenylate cyclase (*AC*) in SMC and P leading to an increase in cAMP. Elevations in cGMP and cAMP levels in SMC and P will evoke vasodilation and confer thromboresistance, respectively.

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