Interactions of iloprost and sodium nitroprusside on vascular smooth muscle and platelet aggregation

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1 The aim of the study was to assess and quantify any synergism occurring between the stable analogues of prostacyclin (iloprost) and nitric oxide (sodium nitroprusside) with respect to both relaxation of vascular smooth muscle and inhibition of platelet aggregation in the rabbit.

2 Iloprost ($0.3\text{--}3\text{ ng ml}^{-1}$) and sodium nitroprusside ($0.3\text{--}3\text{ ng ml}^{-1}$) caused dose-dependent relaxation of the rabbit mesenteric and coeliac arteries.

3 Iloprost ($0.3\text{--}30\text{ ng ml}^{-1}$) and sodium nitroprusside ($0.03\text{--}30\text{ }\mu\text{g ml}^{-1}$) caused dose-dependent inhibition of rabbit platelet aggregation induced by adenosine diphosphate or collagen.

4 In combination, iloprost and sodium nitroprusside caused an inhibition of platelet aggregation that was 2–3 fold greater than would be expected by summation, while no such potentiation was observed on vascular smooth muscle.

5 Thus, our results indicate that under physiological conditions the mediators prostacyclin and endothelium-derived relaxing factor (NO) can exert a synergistic action on platelets, but have only an additive effect on vascular smooth muscle.

Introduction

Prostacyclin (PGI_2 ; Moncada *et al.*, 1976) activates adenylate cyclase (Tateson *et al.*, 1977; Gorman *et al.*, 1977) and the adenosine 3':5'-cyclic monophosphate (cyclic AMP) formed results in vascular smooth muscle relaxation and inhibition of platelet aggregation. Endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) has been identified as nitric oxide (NO; Palmer *et al.*, 1987; Ignarro *et al.*, 1987) and activates guanylate cyclase (Mellion *et al.*, 1980). The guanosine 3':5'-cyclic monophosphates (cyclic GMP) formed also leads to relaxation of vascular smooth muscle (Rapoport *et al.*, 1983) and inhibition of platelet aggregation (Radomski *et al.*, 1987a). Activators of these two secondary messenger systems act synergistically to inhibit platelet aggregation (Levin *et al.*, 1982; Radomski *et al.*, 1987a; MacDonald *et al.*, 1988); an important finding since the receptor-mediated release of PGI_2 and NO from endothelial cells is coupled (de Nucci *et al.*, 1988). However, synergism of these mediators on the relaxation of vascular

smooth muscle has not been demonstrated. Here we show that the stable analogues of PGI_2 and NO, iloprost and sodium nitroprusside (NP), do not act synergistically as relaxants of vascular smooth muscle. In addition, we have determined the degree of synergism between these drugs on rabbit platelets. Some of these results were presented to the British Pharmacological Society (Antunes *et al.*, 1988).

Methods

Vascular smooth muscle

Spiral strips of rabbit mesenteric artery (RbMesA) and rabbit coeliac artery (RbCA) were denuded of endothelium and set up in a cascade (Vane, 1964). The tissues were superfused with oxygenated (95% $\text{O}_2/5\% \text{ CO}_2$) and warmed (37°C) Krebs solution containing indomethacin ($5.6\text{ }\mu\text{M}$) at a flow rate of 5 ml min^{-1} . In some experiments, U46619 (30 nM) was infused (0.05 ml min^{-1}) to contract the tissues. Iloprost and NP were infused (0.05 ml min^{-1}) for 5 min.

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Platelet aggregation

Male New Zealand White rabbits (2–2.5 kg) were treated with sodium pentobarbitone (Sagatal, 30 mg kg⁻¹, i.v.) for general anaesthesia and lignocaine (xylocaine, 2%) for local anaesthesia. The carotid artery was cannulated and blood collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1. The blood was centrifuged in a Petalfuge at low speed (200 g) for 8 min to produce platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by further centrifugation of the PRP (Hermle centrifuge Z 230 M, FRG) at high speed (12,000 g) for 1 min.

Platelet aggregation was studied by use of a dual channel Payton aggregometer calibrated with PRP (0%) and PPP (100%) with respect to the degree of light transmission. Aliquots (0.5 ml) of PRP were added to siliconised cuvettes (37°C) and stirred (1000 rev min⁻¹). Platelets were incubated for approximately 1 min to establish a baseline and incubated with iloprost, NP or both for 1 or 5 min before the addition of a dose of adenosine diphosphate (ADP) or collagen which caused near maximal aggregation (approximately 90%).

The % inhibition of platelet aggregation was calculated from the maximum increase in light transmission observed over a 4 min period after addition of the aggregating agent, as compared to that of a control.

Preparation of washed platelets

Prostacyclin (300 ng ml⁻¹) was added to PRP and centrifuged (900 g for 18 min) in a Hereus Minifuge T centrifuge to sediment the platelets. The resulting supernatant was removed and replaced with an equal volume of Krebs solution (Ca²⁺-free), warmed to 37°C. The pellet was gently resuspended and a further dose of prostacyclin (300 ng ml⁻¹) was added. The platelets were sedimented again by centrifugation (900 g, 18 min) and the platelet pellet resuspended in fresh Krebs solution (Ca²⁺-free) at 37°C. CaCl₂ (1 mM) was added and the washed platelet suspension (WPS) kept at room temperature (Radomski & Moncada, 1983).

Materials

The composition of the Krebs solution was (mM): NaCl 118, NaHCO₃ 25, glucose 5.6, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ · 7H₂O 1.17 and CaCl₂ 2.5. Collagen (fibril suspension) was obtained from Hormon-Chemie (Munchen GMBH). ADP, indomethacin and NP were bought from Sigma Chemical Co (Poole, UK). Iloprost was a gift from Dr L. Sprazagala, Schering Co, (FRG) and PGI₂ was a gift

from Dr B.J.R. Whittle, Wellcome Research Laboratories, U.K. U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxyprostaglandin F_{2a}) was a gift from Dr J. Pike, Upjohn, Kalamazoo, MI.

Statistical comparison

All dose-response curves were analysed by use of a two-way analysis of variance followed by a least significant difference procedure (LSD) to determine the nature of the response (SPSS Inc., Chicago, IL, USA). A *P* value of <0.05 was considered statistically significant. ED₅₀ values (dose responsible for 50% maximum relaxation or 50% inhibition of platelet aggregation) were calculated for comparison.

Results

Interactions of threshold doses of iloprost and sodium nitroprusside

Vascular smooth muscle Iloprost (0.3–30 ng ml⁻¹) or NP (0.3–30 ng ml⁻¹) caused dose-dependent relaxations of the RbMesA and RbCA precontracted with U46619. Co-infusion of a threshold dose of NP (0.3 ng ml⁻¹) did not significantly alter the iloprost dose-response curve on the RbMesA or the RbCA (Figure 1). Similarly, co-infusion of a threshold dose of iloprost (0.3 ng ml⁻¹) did not alter the NP dose-response curve on either vascular tissue. These data are included in Table 1, expressed as ED₅₀ values.

Table 1 The effect of threshold concentrations of sodium nitroprusside (NP) and iloprost (Ilo) on iloprost and NP-induced relaxation of vascular smooth muscle

Tissue	Drug (ng ml ⁻¹)	ED ₅₀ (ng ml ⁻¹)
RbMesA	Ilo	7.4 ± 2.2
	Ilo + NP (0.3)	9.9 ± 2.2
	NP	13.9 ± 2.8
	NP + Ilo (0.3)	11.7 ± 2.7
RbCA	Ilo	8.4 ± 1.7
	Ilo + NP (0.3)	7.0 ± 1.5
	NP	16.1 ± 4.5
	NP + Ilo (0.3)	10.3 ± 2.3

Threshold concentrations of NP (0.3 ng ml⁻¹) or iloprost (0.3 ng ml⁻¹) did not significantly alter the ED₅₀ values for iloprost or NP-induced relaxation of rabbit mesenteric artery (RbMesA) and rabbit coeliac artery (RbCA) precontracted with U-46619 (30 nM). Data are expressed as mean ± s.e.mean of at least four observations. No significant difference was detected as calculated by a two-way analysis of variance.

Figure 1 A threshold concentration of sodium nitroprusside (NP) did not act synergistically with iloprost on rabbit mesenteric (a) or rabbit coeliac (b) arteries precontracted with U-46619 (30 nM). The dose-response curve to iloprost alone (●) was not significantly different from iloprost in the presence of 0.3 ng ml^{-1} NP (○). Data are expressed as mean of four observations. Vertical lines show s.e.mean.

Figure 2 A threshold concentration of sodium nitroprusside (NP) acts synergistically with iloprost on inhibition of platelet aggregation induced by adenosine diphosphate (a) but not by collagen (b). The dose-response curve to iloprost alone (●) was significantly different from iloprost in the presence of 30 ng ml^{-1} NP (○) when using adenosine diphosphate, but not significantly different in the presence of 100 ng ml^{-1} NP when using collagen as the aggregating agent. Data are expressed as mean of four observations; vertical lines show s.e.mean. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Platelet aggregation

In PRP iloprost ($0.3\text{--}30 \text{ ng ml}^{-1}$) or NP ($0.03\text{--}30 \mu\text{g ml}^{-1}$) caused dose-dependent inhibition of platelet aggregation induced by ADP ($4 \mu\text{M}$) or collagen ($4 \mu\text{g ml}^{-1}$). A threshold dose of NP enhanced slightly the iloprost-induced inhibition of platelet aggregation (Figure 2), although only the dose-response curve obtained with ADP reached a statistically significant difference. Similar results were obtained with a threshold dose of iloprost on the inhibition of platelet aggregation induced by NP. These data are included in Table 2, expressed as ED_{50} values.

An increase in the incubation time from 1 to 5 min before challenge with collagen did not enhance the activity of NP. When collagen-induced aggregation

of PRP was studied the potency-ratio of iloprost ($\text{ED}_{50} = 6.3 \pm 0.9 \text{ ng ml}^{-1}$) and NP ($\text{ED}_{50} = 6000 \pm 2300 \text{ ng ml}^{-1}$) was 1:952, while in WPS the potency ratio of iloprost ($\text{ED}_{50} = 2.7 \pm 0.9 \text{ ng ml}^{-1}$) and NP ($\text{ED}_{50} = 1410 \pm 710 \text{ ng ml}^{-1}$) was 1:517. The activity of NP as compared to iloprost was only slightly increased following the platelet washing procedure.

Interaction of effective doses of iloprost and sodium nitroprusside

Vascular smooth muscle Co-infusion of effective doses of iloprost (3 ng ml^{-1}) and NP (3 ng ml^{-1})

Table 2 The effect of threshold concentrations of sodium nitroprusside (NP) and iloprost (Ilo) on iloprost and NP-induced inhibition of platelet aggregation

Agonist	Drug (ng ml ⁻¹)	ED ₅₀ (ng ml ⁻¹)
ADP	Ilo	7.5 ± 0.4
	Ilo + NP (30)	5.2 ± 0.3*
	NP	1600 ± 100
	NP + Ilo (0.3)	1100 ± 200
Collagen	Ilo	6.3 ± 0.9
	Ilo + NP (100)	4.9 ± 0.1
	NP	6000 ± 2300
	NP + Ilo (0.3)	2500 ± 1000

Threshold concentrations of NP (30 ng ml⁻¹ for adenosine diphosphate, 100 ng ml⁻¹ for collagen) or iloprost (0.3 ng ml⁻¹) caused a reduction in the ED₅₀ values for iloprost or NP-induced inhibition of platelet aggregation. Data are expressed as mean ± s.e.mean of four observations. Statistical significance was calculated by a two-way analysis of variance (* *P* < 0.05).

caused smooth muscle relaxation which was equivalent to the expected 'additive' result. A co-infusion of higher concentrations of iloprost and NP (10 ng ml⁻¹) similarly indicated a lack of synergism (Table 3).

Platelet aggregation Incubation of 2 or 4 ng ml⁻¹ iloprost with PRP induced a small inhibition of collagen-induced platelet aggregation, similar to that induced by 1 or 2 µg ml⁻¹ NP. However, co-incubation of 2 ng ml⁻¹ iloprost and 1 µg ml⁻¹ NP with PRP caused an inhibition of platelet aggregation equivalent to either 8 ng ml⁻¹ iloprost or 4 µg ml⁻¹ NP, exhibiting synergism (Figure 3).

Table 3 The effect of co-infusion of iloprost (Ilo) and sodium nitroprusside (NP) on vascular smooth muscle

Drug (ng ml ⁻¹)	% maximum relaxation	
	RbMesA	RbCA
Ilo (3)	22.8 ± 5.0	23.2 ± 6.8
NP (3)	17.8 ± 2.1	22.2 ± 5.4
Ilo (3) + NP (3)	41.2 ± 2.8	49.6 ± 11.7
Ilo (10)	56.8 ± 6.3	52.7 ± 11.3
NP (10)	42.2 ± 3.5	42.2 ± 8.2
Ilo (10) + NP (10)	91.4 ± 2.7	78.1 ± 10.0

This illustrates the summation of the relaxant action of iloprost and NP on rabbit mesenteric arteries (RbMesA) and rabbit coeliac arteries (RbCA) precontracted with U-46619 (30 nM). Data are expressed as mean ± s.e.mean of at least five experiments.

Figure 3 Synergism between effective doses of iloprost (Ilo) and sodium nitroprusside (NP) in inhibiting platelet aggregation induced by collagen (Col). The superimposed traces illustrate the dose-dependent effect of iloprost (1–16 ng ml⁻¹) and NP (0.5–8 µg ml⁻¹) on platelet aggregation. Incubation of 2 ng ml⁻¹ iloprost in combination with 1 µg ml⁻¹ NP had a greater than additive effect.

Similar results were obtained with ADP-induced platelet aggregation (Figure 4). The degree of inhibition obtained on incubation of PRP with iloprost and NP clearly had a greater than additive effect (Table 4).

Discussion

The demonstration of synergism between PGI₂ and EDRF in platelets has relied upon representation of dramatic inhibition of aggregation with combinations of low concentrations of these substances (Radomski *et al.*, 1987a; McDonald *et al.*, 1988) without presenting a full dose-response curve for quantitative analysis. Such data are essential in order to establish whether these agonists have a real

Figure 4 Synergism between effective doses of iloprost (Ilo) and sodium nitroprusside (NP) in inhibiting platelet aggregation induced by adenosine diphosphate (ADP). The superimposed traces illustrate the dose-dependent effect of iloprost (0.5–32 ng ml⁻¹) and NP (0.25–16 µg ml⁻¹) on platelet aggregation. Incubation of 2 ng ml⁻¹ iloprost in combination with 1 µg ml⁻¹ NP had a greater than additive effect.

Table 4 The effect of co-incubation of iloprost (Ilo) and sodium nitroprusside (NP) on inhibition of platelet aggregation

Drug (ng ml ⁻¹)	% inhibition	
	ADP	Collagen
Ilo (1)	3 ± 0	4 ± 2
NP (500)	14 ± 2	4 ± 2
Ilo (1) + NP (500)	35 ± 4	28 ± 9
Ilo (2)	11 ± 2	6 ± 2
NP (1000)	22 ± 0	12 ± 2
Ilo (2) ± NP (1000)	61 ± 5	74 ± 6

This illustrates the synergism between the inhibitory actions of iloprost and NP on adenosine diphosphate (ADP)- and collagen-induced platelet aggregation. Data are expressed as mean ± s.e.mean of three experiments.

synergistic action or merely an additive effect. In this work, we used NP and iloprost as stable analogues of nitric oxide and prostacyclin, in order to compare their interaction in platelets with that in smooth muscle.

With respect to inhibition of platelet aggregation, co-incubation of iloprost and NP showed a 2–3 fold increase in activity, above the expected additive effect. Interestingly, the synergism was most clearly observed when active concentrations were combined, presumably because increases in both cyclic AMP and cyclic GMP were needed. However, no synergism was observed on the relaxation of vascular smooth muscle, when using either threshold or active concentrations of iloprost or NP.

The finding that iloprost and NP act synergistically in platelets but not in smooth muscle may have therapeutic implications. In theory, the ability to potentiate the inhibition of platelets, while not potentiating the concomitant vasodilatation and fall in blood pressure would be beneficial in treatments

directed at preventing aggregate formation without causing hypotension. For such a synergism to be exploited, a nitrovasodilator which has a similar potency on vascular smooth muscle and on platelets would be needed.

Although identical concentrations of iloprost and NP were required to relax vascular smooth muscle, 1000 times more NP than iloprost was needed to inhibit platelet aggregation. Since this difference was also observed with washed platelets, the difference in potency cannot be attributed to binding of NP to plasma proteins. Furthermore, the inhibitory activity of NP on platelets was not enhanced by increasing the incubation time, suggesting that a balance exists between release of NO and its breakdown to nitrite and nitrate. NP is thought to liberate NO spontaneously (see Waldman & Murad 1987), but authentic NO, when used exogenously, is approximately equipotent on vascular smooth muscle and on platelets (Hutchinson *et al.*, 1987; Radomski *et al.*, 1987b). We must assume, therefore, that the liberation of NO from NP is greatly accelerated by a mechanism present in vascular smooth muscle but absent from platelets. This mechanism may be similar to that proposed for the action of organic nitrates, such as glyceryl trinitrate, which are converted to NO enzymically (see Waldman & Murad 1987) or stimulated to release NO by thiol-containing compounds, such as cysteine (Feelisch & Noack, 1987). Indeed, like NP, glyceryl trinitrate has a weak inhibitory effect on platelet aggregation compared to its action on vascular smooth muscle (Schafer, 1980); a strong indication that platelets lack an efficient mechanism to generate NO from either organic nitrates or NP.

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