Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets

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1   The pharmacological effects of endothelium-derived relaxing factor (EDRF), nitric oxide (NO) and prostacyclin on human and rabbit platelets were examined.

2   EDRF is released from porcine aortic endothelial cells, cultured on microcarriers and treated with indomethacin, in sufficient quantities to inhibit platelet aggregation induced by 9,11-dideoxy-9α,11α-methano epoxy-prostaglandin $F_{2\alpha}$ (U46619) and collagen.

3   The anti-aggregating activity of EDRF was potentiated by M&B 22948, a selective inhibitor of cyclic GMP phosphodiesterase, and by superoxide dismutase (SOD) and was inhibited by haemoglobin and Fe^{2+}.

4   Both NO and prostacyclin inhibited platelet aggregation.

5   The anti-aggregatory activity of NO, but not that of prostacyclin, was potentiated by M&B 22948 and by SOD and was inhibited by haemoglobin and Fe^{2+}. Thus NO is a potent inhibitor of platelet aggregation whose activity on platelets mimics that of EDRF.

6   It is likely that the inhibitory effect of NO on platelets represents the action of endogenous EDRF and therefore this substance, together with prostacyclin, is a regulator of platelet-vessel wall interactions.

Introduction

The vascular endothelium generates factors which modulate the homeostatic interactions between platelets and the vessel wall. One of the most potent of these factors is prostacyclin, a product of the metabolism of arachidonic acid by cyclo-oxygenase (Moncada *et al.*, 1976). Prostacyclin inhibits platelet aggregation and induces vasodilatation by stimulating adenylate cyclase and inducing a rise in adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Gorman *et al.*, 1977; Tateson *et al.*, 1977). Recently, another product of the vascular endothelium, endothelium-derived relaxing factor (EDRF), has been shown to account for some of the vasodilator properties of several pharmacological agents (Furchgott & Zawadzki, 1980). The vascular relaxation induced by EDRF is mediated by the stimulation of soluble guanylate cyclase and the consequent rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Rapoport *et al.*, 1983). EDRF has also been reported to inhibit platelet aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987).

Nitrovasodilators, such as sodium nitroprusside, inhibit platelet aggregation by increasing cyclic GMP levels. The biologically active species of the nitrovasodilators is thought to be nitric oxide (NO)

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which has also been shown to inhibit platelet aggregation (Schafer *et al.*, 1980; Mellion *et al.*, 1981). Furchgott (1987) has recently suggested that EDRF might be NO. In view of this, we have studied the pharmacological effects of NO and EDRF as inhibitors of platelet aggregation and compared their activity with that of prostacyclin.

Methods

Preparation of platelets

Platelet-rich plasma (PRP) was obtained by centrifugation of citrated (3.15% trisodium citrate, 1:9 v:v) human or rabbit blood at 240 g for 20 min at room temperature. Rabbit or human washed platelets were prepared from the corresponding PRP by the method of Radomski & Moncada (1983).

The generation and assay of EDRF

Porcine aortic endothelial cells were cultured on microcarriers as previously described (Gryglewski *et al.*, 1986a). Between 0.5 and 1.5 ml of microcarriers

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(0.3–5 × 10⁷ cells) were washed 3 times in Tyrode solution containing indomethacin (10 μM). The microcarriers were then resuspended in a total volume of 2 ml of the above solution and were maintained in Eppendorf tubes in a water bath at 37°C, before addition of bradykinin (10–100 nM). After 15 s incubation, 0.5 ml was removed with a syringe and filtered rapidly through a millipore filter (0.45 μm pore size) so that 100 μl was added to the washed platelets (500 μl) in the cuvette. After 1 min, 9,11-dideoxy-9α, 11α-methano epoxy-prostaglandin $F_{2\alpha}$ (U46619; 1 nM) or collagen (4 μg ml⁻¹) was added to the platelets and aggregation monitored for a further 5 min. Confirmation of the release of EDRF by the endothelial cells was obtained by administration of 100 μl of the above supernatant to a bioassay of spiral strips of rabbit aorta superfused in a cascade as described before (Gryglewski *et al.*, 1986a). The generation of prostacyclin in the endothelial cell incubate was monitored by measuring the release of 6-keto-prostaglandin ($F_{1\alpha}$ 6-keto-$PGF_{1\alpha}$) by specific radioimmunoassay (Salmon, 1978).

Platelet aggregation

Platelet aggregation was recorded in a Payton Dual Channel Aggregometer according to the method of Born & Cross (1963). The anti-aggregatory effect of EDRF was studied in human washed platelets and those of NO and prostacyclin in human PRP, and in human and rabbit washed platelets. The interactions between NO, EDRF, prostacyclin and other pharmacological agents were studied in human washed platelets. Inhibitors or potentiators of aggregation were incubated for periods varying from 15 s to 7 min before the addition of aggregating agents. Platelet aggregation was then monitored for 3 min, or for 5 min in experiments carried out in the presence of indomethacin. Inhibition of platelet aggregation was expressed as a percentage of the extent of maximal aggregation at the appropriate time.

Reagents

Stock solutions of adenosine 5' diphosphate (ADP), superoxide dismutase (SOD), catalase (all Sigma), human thrombin (Ortho Diagnostic Systems Inc), $NaNO_2$, $NaNO_3$ (BDH) and HL 725 (9,10-bimethy-oxy-3-methyl-2-mesityl-imino-3,4,6,7-tetrahydro-2-4-pyrimido (6,1-A)-isochinoline-4-on-hydrochloride) (Hoechst AG) were prepared and diluted in distilled water. Bradykinin (Sigma) was prepared and diluted in 0.85% NaCl. Calcium ionophore A23187 (Calbiochem) and U46619 (Cayman Chemical) were dissolved in ethanol and diluted in distilled water. L-α-Phosphatidylcholine-β-acetyl-γ-O-alkyl (Paf) was supplied in chloroform solution (Sigma). This was dried under

nitrogen and the residue dissolved and diluted in 0.25% bovine serum albumin (Sigma) in 0.85% NaCl. Collagen (Hormon-Chemie) was supplied and diluted in the manufacturer's buffer. Nitric oxide gas (British Oxygen Corporation) was dissolved in He-deoxygenated water at concentrations of 0.1% or 0.3% (v:v) as described previously (Palmer *et al.*, 1987). Stock solutions of prostacyclin sodium salt (Wellcome) were prepared in 1 M Tris, pH 9 at 4°C and kept on ice until discarded at the end of the experiment. Dilutions were made as required in 0.05 M Tris, pH 9 at 4°C. Stock solutions of M & B 22948 (2-0-propoxyphenyl-8-azapurin-6-one; May & Baker) and 3-isobutyl-1-methyl-xanthine (IBMX; Aldrich Chemical Co. Ltd), in triethanolamine (20% v:v) and dimethyl sulphoxide (DMSO) respectively, were diluted in distilled water. The concentrations of triethanolamine and DMSO in platelet incubations did not exceed 0.07 and 0.02% respectively. Purified human haemoglobin was prepared according to the method of Paterson *et al.* (1976) and diluted in 0.85% NaCl. Stock solutions of indomethacin (Sigma) were prepared in 5% $NaHCO_3$ and diluted in 0.85% NaCl.

Results

The effect of EDRF on platelet aggregation

Incubation of 1.5 ml of microcarriers (2–5 × 10⁷ endothelial cells) with bradykinin (100 nM) in the presence of indomethacin (10 μM) generated an unstable vasorelaxant substance whose pharmacological effects on the cascade bioassay were indistinguishable from those of EDRF (Gryglewski *et al.*, 1986b; data not shown). When an aliquot (100 μl) of this incubate was added to the platelet suspension it also inhibited platelet aggregation induced by U46619 (1 nM; Figure 1) and by collagen (4 μg ml⁻¹; n = 5). The anti-aggregatory effect was rapid in onset (maximum effect within 1 min of adding the incubate to the platelets), decayed with a half-life of approximately 2 min and disappeared completely after 4 min incubation (Figure 2). 6-keto-$PGF_{1\alpha}$ was not detectable in these endothelial cell incubates (<0.03 nM; n = 3).

Release of anti-aggregating activity from the endothelial cells was dependent on the concentrations of bradykinin. Thus, when 1.5 ml of endothelial cells were stimulated with 100 nM bradykinin, inhibition of platelet aggregation was 100% (Figure 1) but with 30 nM bradykinin, inhibition was only 15–20% (n = 4). Anti-aggregating activity was not detectable in incubates of 1.5 ml of endothelial cells stimulated with 10 nM bradykinin (n = 4).

The anti-aggregating activity of incubates of endothelial cells stimulated with bradykinin (100 nM) was also dependent upon the number of cells in the incubate. Inhibition of aggregation by incubates from
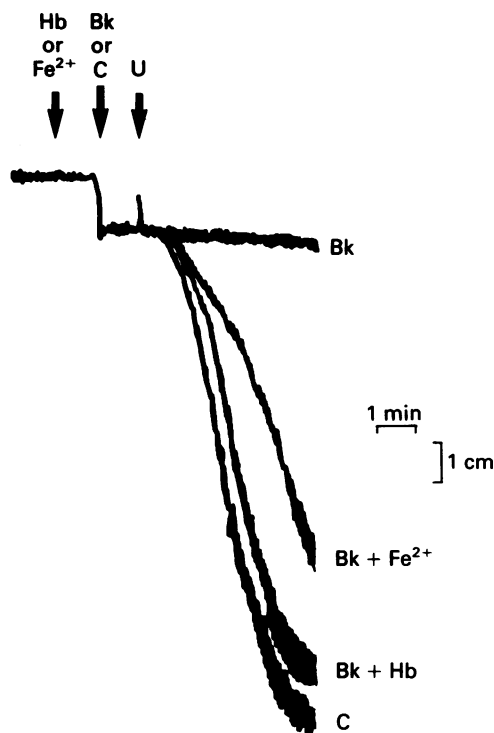
Figure 1 Platelet aggregation induced by U46619 (1 nM) is inhibited by 100 μl of incubate from 1.5 ml of microcarriers (2–5 × 10⁷ endothelial cells) stimulated with bradykinin (100 nM; Bk). This effect is inhibited by haemoglobin (100 nM; Bk + Hb) and by Fe^{2+} (8 μM; Bk + Fe^{2+}). C = control incubate from the same volume of unstimulated cells. These results are representative of 4 similar experiments.

Figure 2 The time-dependent decay of the anti-aggregating activity of endothelium-derived relaxing factor (EDRF). Collagen (4 μg ml⁻¹)-induced aggregation was completely inhibited by incubation of platelets for 15 s with 100 μl of incubate from 1.5 ml of bradykinin-stimulated porcine endothelial cells. This anti-aggregating activity decayed during incubation with the platelet suspension (●). This decay was decreased by preincubation of platelets with superoxide dismutase (20 u ml⁻¹; ▲) or with M & B 22948 (1 μM; ■) for 1 min prior to addition of EDRF. Each point is the mean of at least 4 separate experiments; vertical lines show s.e.mean.

1.5 ml endothelial cells was complete and by incubates from 1.0 ml endothelial cells was approximately 40–50% (n = 5). Anti-aggregating activity was not detectable in incubates of 0.5 ml endothelial cells (n = 4).

The addition of haemoglobin (40–100 nM) to the platelet suspension 1 min before addition of the incubate from 1.5 ml of endothelial cells, stimulated with bradykinin (100 nM), caused a concentration-dependent reduction in the anti-aggregating activity (Figure 1). The anti-aggregating activity was also inhibited, in a concentration-dependent manner, by Fe^{2+} (1–20 μM) under these conditions (Figure 1).

The anti-aggregating activity was potentiated when SOD (20 uml⁻¹), but not catalase (20 u ml⁻¹; n = 3), was added to the platelet suspension before addition of the incubate. In four experiments, aliquots (100 μl) from incubates of 1.0 ml of endothelial cells, which caused 40–50% inhibition of platelet aggregation under control conditions, caused complete inhibition
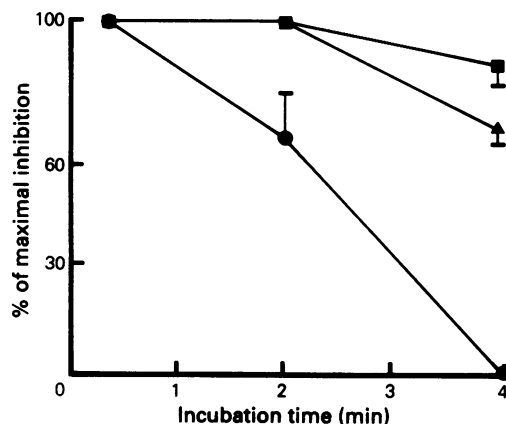
in the presence of SOD (Figure 3). Furthermore, aliquots from 0.5 ml of endothelial cells, which did not have any effect in control experiments, induced 40–50% inhibition of platelet aggregation in the presence of SOD (n = 3).

Potentiation of the anti-aggregating activity was also observed when M & B 22948 (1 μM) was added instead of SOD (Figure 3). The potentiating effect of M & B 22948 was more pronounced than that of SOD. Incubates of 0.5 ml of endothelial cells in the presence of M & B 22948 caused complete, rather than only partial, inhibition of aggregation (n = 3). The anti-aggregating activity of incubates was not affected by the specific cyclic AMP phosphodiesterase inhibitor, HL 725 (1 fM; n = 2).

These concentrations of both SOD and M & B 22948 reduced the decay in the anti-aggregating activity of incubates from 1.5 ml of endothelial cells (Figure 2). No loss of anti-aggregating activity was observed after 4 min with M & B 22948 and only 20% loss was observed with SOD after this time.

The effect of nitric oxide and prostacyclin on platelet aggregation

The incubation of human PRP with NO (0.18–1.50 μM) resulted in a concentration-dependent inhibi-
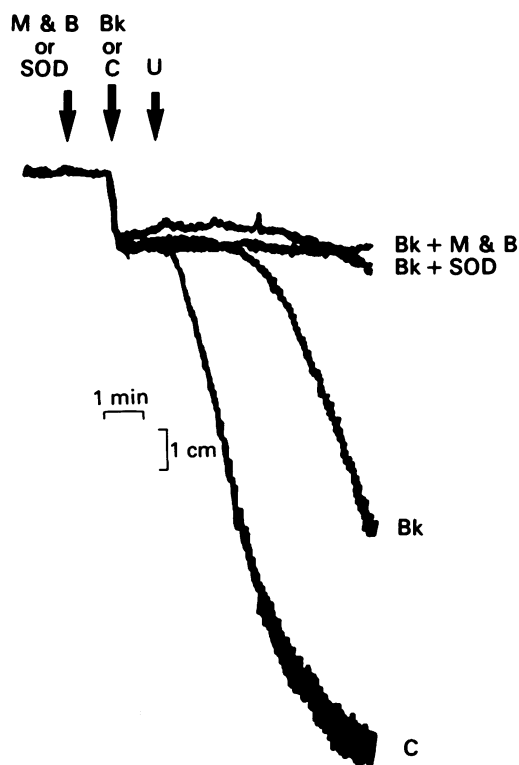
M & B   Bk
or   or
SOD   C   U

Bk + M & B
Bk + SOD

1 min

1 cm

Bk

C

Figure 3 Platelet aggregation induced by U46619 (1 nM) is inhibited by 100 μl of incubate from 1.0 ml of microcarriers stimulated with bradykinin (100 nM; Bk). This effect is potentiated by M & B 22948 (1 μM; Bk + M & B) and by superoxide dismutase (20 u ml^{-1}; Bk + SOD). C = control incubate from the same volume of unstimulated cells. These results are representative of 4 similar experiments.

Table 1 Comparison of anti-aggregating activity of nitric oxide (NO) and prostacyclin in human washed platelets

Agonists	IC_{50}	
	NO (μM)	PGI$_2$ (nM)
ADP	0.30 ± 0.04	1.5 ± 0.4
Collagen	0.25 ± 0.03	2.1 ± 0.6
Thrombin	0.32 ± 0.05	3.7 ± 1.2
U46619	0.24 ± 0.03	1.6 ± 0.4
A23187	1.08 ± 0.20	12.1 ± 5.4

Platelet aggregation was induced by concentrations of ADP (8–10 μM), collagen (1 μg ml^{-1}), thrombin (0.02 u ml^{-1}), U46619 (1 nM), and A23187 (10 nM) which resulted in the maximal aggregatory response. Each value is the mean ± s.e.mean of 10 separate determinations.

Prostacyclin inhibited the aggregation of washed human platelets induced by ADP, collagen, thrombin and U46619 with IC$_{50}$s ranging from 1.5 to 3.7 nM ($n = 6$). The anti-aggregating activity of prostacyclin decayed with a half-life of 4–5 min ($n = 6$). Both NO and prostacyclin were less effective as inhibitors of platelet aggregation induced by A23187, with IC$_{50}$s of 1.08 μM and 12.1 nM respectively (Table 1).
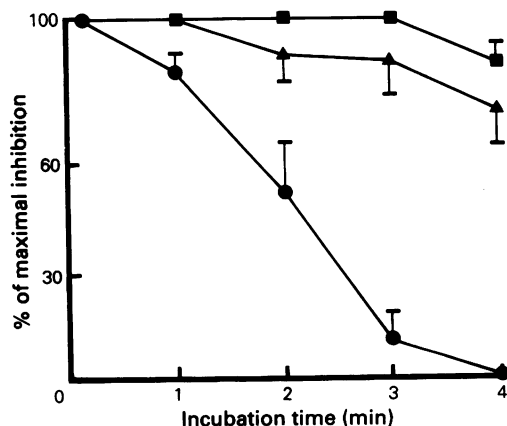
tion of platelet aggregation induced by ADP, collagen, thrombin and U46619, with IC$_{50}$s ranging from 0.54 to 0.87 μM ($n = 6$). Nitric oxide was 2–3 fold more potent in human washed platelets (IC$_{50}$s for the above agonists ranging from 0.24 to 0.32 μM; Table 1). The potency of NO in rabbit washed platelets was 3–4 fold less than that in human washed platelets; IC$_{50}$s ranged from 1.02 to 1.30 μM for inhibition of aggregation induced by ADP, Paf, collagen, thrombin or U46619 ($n = 8$). The anti-aggregating activity of NO decayed during incubation with human washed platelets (half-life approximately 2 min) and disappeared completely after 4 min incubation (Figure 4). Platelet aggregation was not affected by NO$_2^-$ or NO$_3^-$ (as their sodium salts) at concentrations up to 100 μM ($n = 5$).

Figure 4 The time-dependent decay of the anti-aggregating activity of nitric oxide (NO). Collagen (1 μg ml^{-1})-induced aggregation was completely inhibited by incubation of platelets for 15 s with NO (0.45 μM). The anti-aggregating activity decayed during incubation with the platelet suspension (●). This decay was decreased by preincubation of platelets with superoxide dismutase (20 u ml^{-1}; ▲) or with M & B 22948 (1 μM; ■) for 1 min before addition of NO. Each point is the mean of 6 separate experiments; vertical lines show s.e.mean.
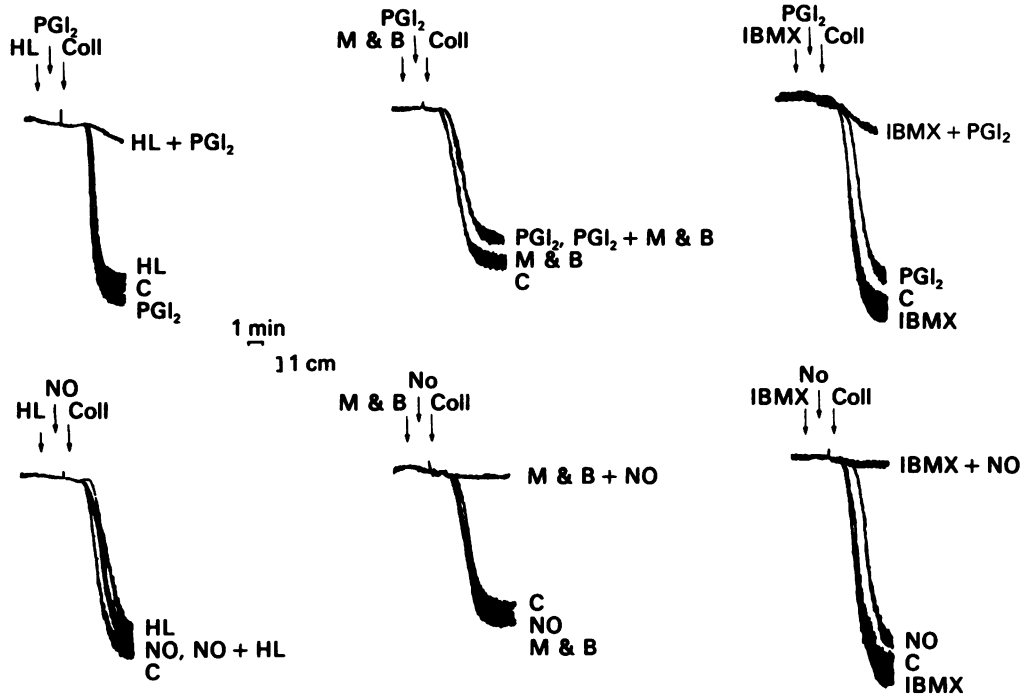
Figure 5 The effect of HL 725 (1 fM; HL), M & B 22948 (1 μM; M & B) and 3-isobutyl-1-methylxanthine (5 μM, IBMX) on prostacyclin (0.4 nM, PGI$_2$)- or nitric oxide (0.18 μM, NO)-induced inhibition of platelet aggregation. Control platelet aggregation (C) was induced by collagen (1 μg ml^{-1}; Coll). These results are representative of 6 similar experiments.

The anti-aggregating activity of prostacyclin was potentiated selectively by HL 725 (1 fM). In contrast, M & B 22948 (1 μM) selectively potentiated the inhibitory effect of NO. The anti-aggregating activity of both prostacyclin and NO was potentiated by IBMX (5 μM; Figure 5). M & B 22948 also reduced the decay of the anti-aggregatory activity of NO (Figure 4) so that only 10% of the activity was lost after 4 min. The decay of the anti-aggregating activity of prostacyclin was reduced by HL 725, but not by M & B 22948, so that no significant loss of activity was observed after 6 min of incubation (n = 6). None of the phosphodiesterase inhibitors affected platelet aggregation directly at the concentrations used (n = 6).

The anti-aggregating activity of NO was potentiated by incubating platelets for 1 min with SOD (20 u ml^{-1}, n = 6) before addition of NO (Figure 6). Superoxide dismutase also reduced the decay of the anti-aggregating activity of NO (Figure 4). Catalase (20 u ml^{-1}; n = 3) did not affect either the potency or the decay of NO. Preincubation of platelets with haemoglobin (30–150 nM) reduced the anti-aggregating activity of NO (IC$_{50}$ = 76.0 ± 11.0 nM, mean ± s.e.mean, n = 6), but not that of prostacyclin (n = 3).
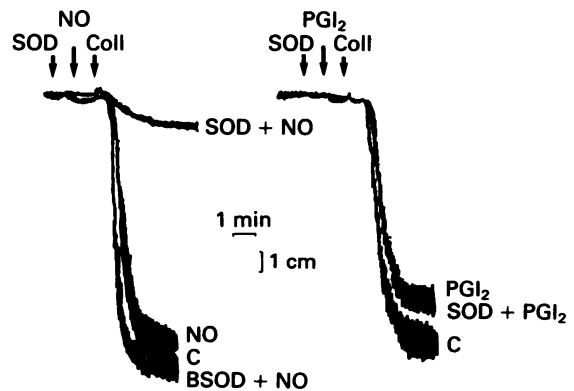
Figure 6 Platelet aggregation induced by collagen (1 μg ml^{-1}; Coll) is inhibited by nitric oxide (0.18 μM, NO) and by prostacyclin (0.4 nM; PGI$_2$). Superoxide dismutase (20 u ml^{-1}, SOD) potentiates the inhibitory effect of NO (SOD + NO) but not that of prostacyclin (SOD + PGI$_2$). Boiled SOD (BSOD) had no effect (BSOD + NO). Control aggregation (C) was induced by collagen. These results are representative of 6 similar experiments.

The anti-aggregating activity of NO was also inhibited by Fe^{2+} (1–20 μM) in a concentration-dependent manner with an IC_{50} of 4.5 $\pm$ 1.5 μM ($n = 4$). None of these compounds affected either the anti-aggregating activity of prostacyclin or platelet aggregation directly at the concentrations used ($n = 4$).

Discussion

Bradykinin induces the release of an anti-aggregating factor from porcine aortic endothelial cells cultured on microcarriers and treated with indomethacin. This substance is not prostacyclin, as the release of 6-keto $PGF_{1\alpha}$ from these cells was not detectable. Furthermore, its effect was shorter lasting than that of prostacyclin and was not affected by HL 725, a selective inhibitor of cyclic AMP phosphodiesterase (Ruppert & Weithmann, 1982). In addition, the activity of this substance was potentiated by SOD and M & B 22948, a selective inhibitor of cyclic GMP phosphodiesterase (Lugnier *et al.*, 1986), and inhibited by haemoglobin and Fe^{2+}. These compounds have similar effects on the vascular activity of EDRF (Gryglewski *et al.*, 1986b; Martin *et al.*, 1985; 1986). All these data therefore clearly indicate that EDRF is the inhibitory factor released by bradykinin.

We have also shown that NO is a potent inhibitor of platelet aggregation induced by a variety of agonists in PRP, confirming previous reports (Mellion *et al.*, 1981; 1983). This activity of NO is not attributable to the presence of its breakdown products, NO_2^- and NO_3^-, as they were inactive at concentrations up to 100 μM. Unlike prostacyclin, whose potency is not changed in washed platelets (Vargas *et al.*, 1982), that of NO is significantly increased. The binding of NO to plasma proteins, in particular to ferrous haemoproteins (Keilin & Hartree, 1937), may account for its reduced potency in PRP.

The potency of prostacyclin against ADP-, collagen-, U46619- and thrombin-induced platelet aggregation was very similar. Nitric oxide also had a similar potency against all these agonists, suggesting that both agents inhibit a step in the aggregation process which is common to many agents. Prostacyclin and NO-generating compounds inhibit platelet aggregation by raising intra-platelet levels of cyclic AMP (Gorman *et al.*, 1977; Tateson *et al.*, 1977) and cyclic GMP (Mellion *et al.*, 1981) respectively. The anti-aggregating actions of prostacyclin and NO are potentiated by HL 725 and M & B 22948 respectively. Cyclic AMP is thought to regulate calcium levels by promoting its uptake into the dense tubular system (Käser-Glanzmann *et al.*, 1977) and cyclic GMP by inhibiting calcium influx and mobilization from intracellular stores (Henderson *et al.*, 1987). Therefore the final common step in the inhibition of platelet aggregation by prostacyclin and NO is likely to be calcium availability. The present finding that both prostacyclin and NO are less potent against A23187-induced aggregation supports this concept.

In our experiments NO appears to be less potent than prostacyclin. However, NO is extremely unstable especially when stirred in solution (Furchgott, 1987) as in the aggregometer cuvette. Thus, the concentrations reaching the platelets may be much lower than those calculated from the mass of NO added to the cuvette. It is, therefore, impossible to determine the exact potency of NO and, as a consequence, its potency relative to prostacyclin.

The inhibitory effect of NO, but not that of prostacyclin, is potentiated by SOD. In addition, SOD increases the duration of action of NO. Since catalase did not affect the activity of NO or of EDRF, it is likely that superoxide anions (O_2^-), rather than another oxygen-derived radical, play a role in the destruction of these compounds (Gryglewski *et al.*, 1986b). Superoxide anions are present in the medium of platelet suspensions (Marcus *et al.*, 1977), although the amount of O_2^- present does not increase during aggregation and SOD does not affect platelet function directly (Marcus *et al.*, 1977; Clemmons *et al.*, 1985). These findings suggest that although O_2^- are not involved in platelet aggregation *per se* they may contribute to the inactivation of NO.

As with EDRF, the anti-aggregating activity of NO, but not that of prostacyclin, was reduced by haemoglobin and Fe^{2+}. Haemoglobin probably acts by binding NO (Keilin & Hartree, 1937) and Fe^{2+} by either reacting with NO or via the formation of O_2^- anions, which in turn inactivate NO (Gryglewski *et al.*, 1986b). Therefore two compounds that inactivate EDRF by different mechanisms also inactivate NO.

In summary, EDRF and NO have identical pharmacological activity on platelets, supporting the hypothesis that EDRF may be NO.* If this is the case, then NO is a potent endogenous vasodilator and inhibitor of platelet aggregation whose biological actions are similar to those of prostacyclin. The study of the interactions of these two compounds during physiological and pathophysiological events in the vessel wall is likely to increase our understanding of conditions such as atherosclerosis, thrombosis or vasospasm.

*Note added in proof
We have recently demonstrated that NO released from endothelial cells accounts for the biological activity of EDRF (Palmer *et al.*, 1987).

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