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The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide

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1 The interactions between endothelium-derived nitric oxide (NO) and prostacyclin as inhibitors of platelet aggregation were examined.

2 Porcine aortic endothelial cells treated with indomethacin and stimulated with bradykinin (10–100 nM) released NO in quantities sufficient to account for the inhibition of platelet aggregation attributed to endothelium-derived relaxing factor (EDRF).

3 In the absence of indomethacin, stimulation of the cells with bradykinin (1–3 nM) released small amounts of prostacyclin and EDRF which synergistically inhibited platelet aggregation.

4 EDRF and authentic NO also caused disaggregation of platelets aggregated either with collagen or with U46619.

5 A reciprocal potentiation of both the anti- and the dis-aggregating activity was also observed between low concentrations of prostacyclin and authentic NO or EDRF released from endothelial cells.

6 It is likely that interactions between prostacyclin and NO released by the endothelium play a role in the homeostatic regulation of platelet-vessel wall interactions.

Introduction

The vascular endothelium generates factors which modulate the interactions between platelets and the vessel wall. One of the most potent of these factors is prostacyclin, which inhibits platelet aggregation and induces disaggregation of aggregated platelets (for review see Moncada, 1982).

Endothelium-derived relaxing factor (EDRF) is a labile humoral agent released by vascular endothelium which is responsible for the vascular relaxant properties of some vasodilators (Furchgott, 1984). EDRF also inhibits platelet aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987). We have recently demonstrated that nitric oxide (NO) is released by vascular endothelial cells in amounts sufficient to account for the vasodilator properties of EDRF (Palmer *et al.*, 1987). We have further shown that NO inhibits platelet aggregation in a manner which is indistinguishable from that of EDRF. The inhibition of aggregation induced by both compounds is potentiated by M&B 22948, a selective inhibitor of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) phosphodiesterase and by superoxide dismutase (SOD) and is inhibited by Fe^{2+} and haemoglobin (Hb; Radomski *et al.*, 1987).

We have now examined the release of NO from endothelial cells to determine whether its release accounts for the inhibition of platelet aggregation attributed to EDRF. Furthermore, we have studied the interactions between authentic and endogenous NO and prostacyclin as inhibitors of platelet aggregation. We have also studied the disaggregation of aggregated platelets induced by these agents.

Methods

Release of EDRF and nitric oxide from endothelial cells

EDRF was released by bradykinin (Bk) from porcine aortic endothelial cells cultured on microcarriers and bioassayed either on spiral strips of rabbit thoracic aorta or on platelet aggregation as described previously (Gryglewski *et al.*, 1986a; Radomski *et al.*, 1987). Briefly, between 0.5 and 1.5 ml of microcarriers ($0.8–5.0 \times 10^7$ cells) in a total volume of 2 ml were stimulated with Bk (1–100 nM). In some experiments the cells were treated either with indomethacin (10 μM)

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or with Hb (100 nM) 1 min before the addition of Bk. After incubation at 37°C for 15 s, 0.5 ml of incubate was removed with a syringe and filtered rapidly (< 5 s) through a millipore filter (0.22 μm) so that 0.1 ml of cell-free incubate was added to 0.5 ml of the platelet suspension or to the cascade bioassay.

Platelet aggregation

Human blood was obtained and washed platelet suspensions prepared as described previously (Radomski & Moncada, 1983). Platelet aggregation was recorded in a Payton dual-channel aggregometer by the method of Born & Cross (1963). In studies of anti-aggregating activity, EDRF, authentic NO and/or prostacyclin were added to the platelet suspension 1 min before the addition of collagen (4 μg ml^{-1}), 9,11-dideoxy 9α, 11α-methano epoxy-prostaglandin $F_{2\alpha}$ (U46619; 1 nM), ADP (10 μM) or thrombin (0.02 u ml^{-1}). These concentrations caused maximal aggregation of the platelets. Platelet aggregation was then monitored for 6 min. When the interaction between anti-aggregating agents was studied, subthreshold concentrations of prostacyclin, EDRF or authentic NO were added to the platelets 1 min before the agent to be potentiated and incubation continued for a further 1 min before addition of the aggregating agent.

Disaggregation of platelets was studied by addition of authentic NO, EDRF or prostacyclin 3 min after initiating aggregation with collagen or U46619 (1 nM). At this time more than 75% of maximal aggregation had usually occurred. The inhibition of platelet aggregation was expressed as a percentage of maximal aggregation, and disaggregation as a percentage of the disaggregation produced by a maximally effective concentration of NO (1.25 μM).

Determination of nitric oxide and prostacyclin

Authentic NO and NO released from endothelial cells were determined as the chemiluminescent product of their reaction with ozone as described previously (Palmer *et al.*, 1987). Briefly, authentic NO or the cell-free incubate from endothelial cells treated with indomethacin and stimulated with Bk was injected into a reaction vessel containing 75 ml 1.0% sodium iodide in glacial acetic acid under reflux. Nitric oxide was removed from the reflux vessel in a stream of N_2 under reduced pressure, mixed with ozone and the chemiluminescent product measured with a photomultiplier. The areas under the peaks were converted to nmol of NO by reference to a NO_2^- standard curve.

Prostacyclin was determined as its stable breakdown product, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), by specific radioimmunoassay (Salmon, 1978).

Materials

Authentic NO (> 99.89% pure; British Oxygen Corporation) was prepared as 0.03 and 0.1% (v/v) solutions in He-deoxygenated H_2O as described previously (Palmer *et al.*, 1987) and added to the platelets or to the bioassay cascade in 2–10 μl aliquots.

Adenosine-5'-diphosphate, Bk, indomethacin, SOD (all Sigma), collagen (Hormon-Chemie), human thrombin (Ortho Diagnostic Systems Inc.), U46619 (Cayman Chemical), prostacyclin sodium salt (Wellcome) and M&B 22948 (2-0-propoxyphenyl-8-azapurin-6-one; May & Baker) were obtained from the sources indicated. Purified human Hb was prepared as described by Paterson *et al.* (1976). The compounds were dissolved and diluted as described previously (Radomski *et al.*, 1987).

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. Student's unpaired t test was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant.

Results

Release of EDRF

Supernatants from 1.5 ml of unstimulated endothelial cells on microcarriers did not contain EDRF-like activity. However, when the cells were stimulated with Bk (100 nM) for 15 s, in the presence of indomethacin, they released a biological activity which was identified as EDRF since it relaxed spiral strips of rabbit aorta superfused in a cascade and had a half-life of < 5 s (data not shown). Supernatants from unstimulated cells or cells stimulated in the presence of indomethacin did not contain detectable amounts of 6-keto-$PGF_{1\alpha}$ (< 0.03 nM).

When an aliquot (0.1 ml) of the supernatant of stimulated cells containing EDRF was added to the platelet suspension, it inhibited aggregation induced by U46619 (1 nM), ADP (10 μM), thrombin (0.02 u ml^{-1}) and by collagen (4 μg ml^{-1}). The amounts of EDRF released by Bk (100 nM) were proportional to the volume of cells in the incubate (Figure 1a). Incubates from 1.5 ml of cells inhibited aggregation completely while incubates from 1.0 ml of cells inhibited platelet aggregation by 54 ± 8% (n = 7). The anti-aggregating activity of EDRF in incubates of 0.5 ml of cells was not detectable.

The release of EDRF was also dependent on the concentration of Bk used as a stimulus. Supernatants

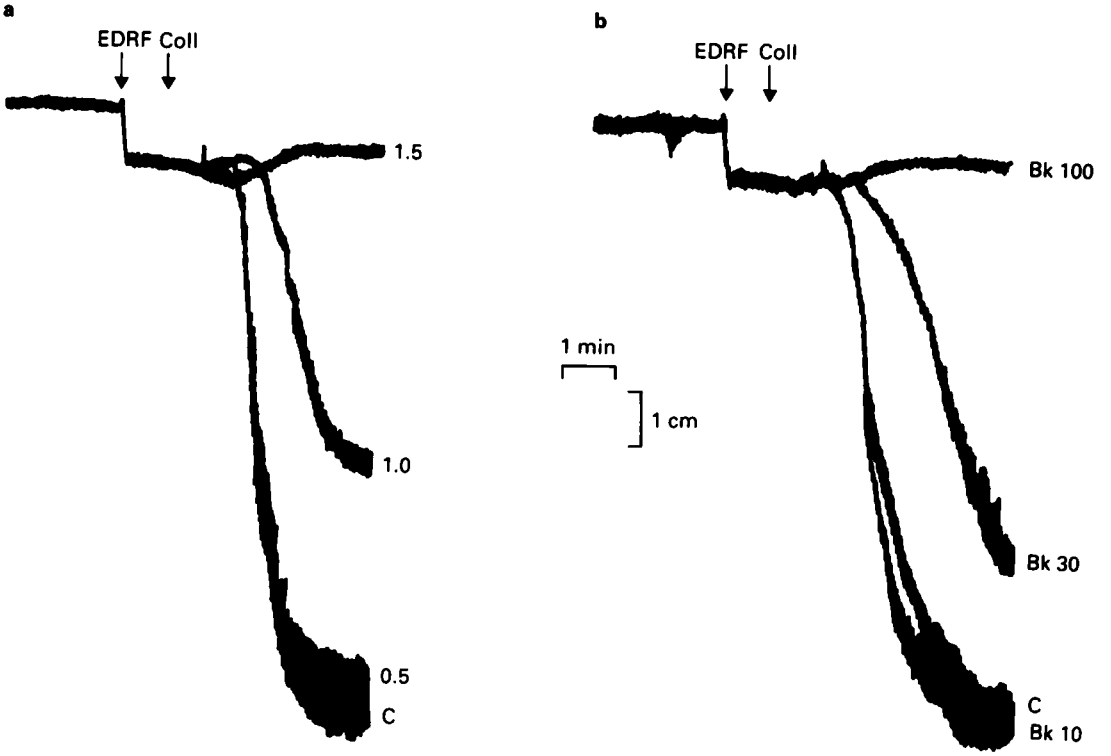

Figure 1 The anti-aggregating activity of EDRF. (a) Inhibition of aggregation induced by aliquots (0.1 ml) of 2 ml incubates containing 0.5, 1.0 and 1.5 ml of endothelial cells treated with indomethacin (10 μM) and stimulated with bradykinin (Bk, 100 nM). Control aggregation (C) induced by collagen (Coll, 4 $\mu g\ ml^{-1}$) in the presence of an aliquot of 1.5 ml unstimulated cells. (b) Inhibition of aggregation induced by aliquots (0.1 ml) from 2 ml incubates containing 1.5 ml of endothelial cells treated with indomethacin as above and stimulated with 10, 30 and 100 nM of Bk. Control (C) as in (a). Tracings are representative of 6–8 experiments.

from 1.5 ml of cells stimulated with 30 nM Bk inhibited platelet aggregation by $26 \pm 4\%$ ($n = 6$), while no release could be detected when this volume of cells was stimulated with 10 nM Bk (Figure 1b).

Release of nitric oxide

When endothelial cells were stimulated with Bk they released NO into the supernatant in a concentration- and cell number-dependent manner. The release induced by Bk (30 and 100 nM) from 1.5 ml of endothelial cells was 0.08 ± 0.01 and 0.24 ± 0.02 nmol, respectively ($n = 3$). The release induced by 100 nM Bk from 1.0 ml of endothelial cells was 0.12 ± 0.02 nmol ($n = 3$). The release of NO was not detectable (< 0.05 nmol) with lower concentrations of Bk and smaller volumes of cells. The concentrations of NO and prostacyclin achieved when the amounts present in 0.1 ml of the incubate were added to the platelet suspension are shown in Table 1.

Table 1 Release of nitric oxide (NO) and prostacyclin (PGI_2) and inhibition of platelet aggregation

Bk (nM)	*PGI_2* (nM)	*NO* (μM)	Inhibition (%)
100	18.5 ± 1.7	0.47 ± 0.05	100 ± 0
30	7.7 ± 1.0	0.16 ± 0.03	100 ± 0
10	2.3 ± 0.3	ND	100 ± 0
3	1.4 ± 0.2	—	83 ± 5
1	0.08 ± 0.02	—	38 ± 3

Endothelial cells (1.5 ml) stimulated with bradykinin (Bk, 1–100 nM) released prostacyclin (PGI_2) and nitric oxide (NO) in a concentration-dependent manner. The inhibition of platelet aggregation (induced by collagen 4 $\mu g\ ml^{-1}$) caused by an aliquot (0.1 ml) of the incubate is also shown. Results are mean ± s.e.mean of 3–6 experiments. The concentrations of authentic NO and of prostacyclin that inhibited platelet aggregation were 0.12–0.45 μM (IC_{50} 0.25 ± 0.03 μM) and 0.3–6.0 nM (IC_{50} 2.3 ± 0.4 nM), respectively ($n = 10$).
ND, not detected (< 0.1 μM).

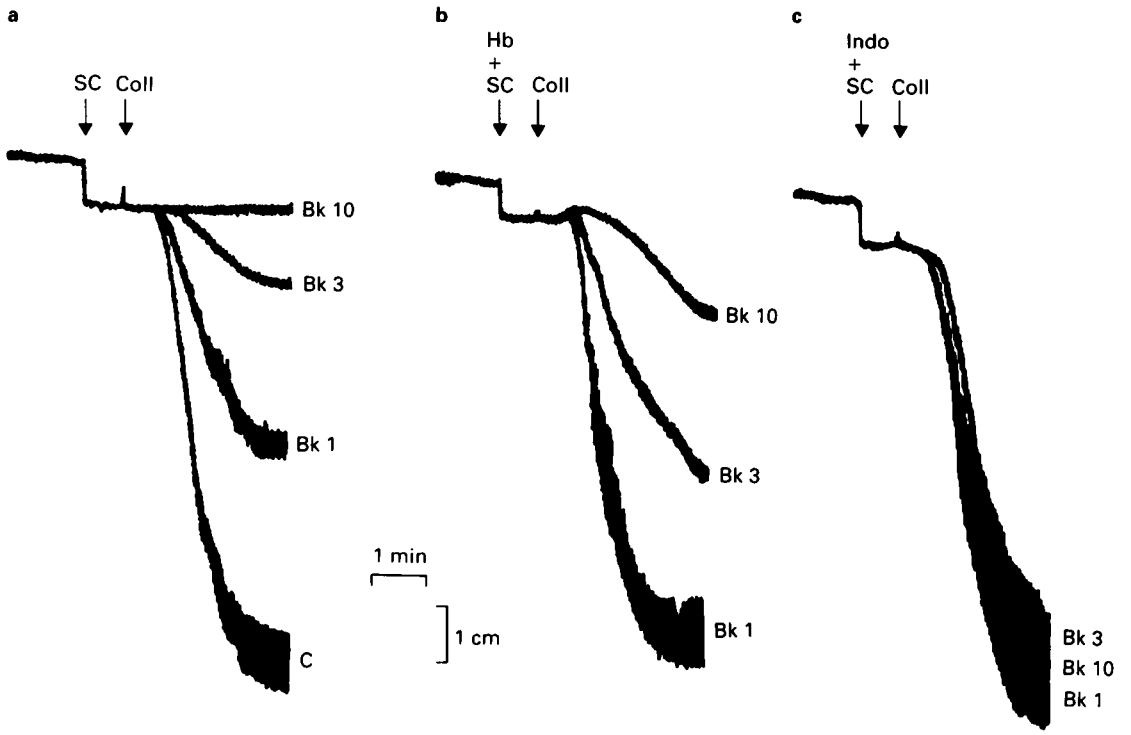

Figure 2 Endogenous prostacyclin and EDRF synergize to inhibit platelet aggregation. (a) An aliquot (0.1 ml) of supernatant from 2 ml incubates containing 1.5 ml of unstimulated endothelial cells does not inhibit platelet aggregation (C). Aliquots of the same volume of stimulated cells (SC) with 1, 3 and 10 nM of bradykinin (Bk) inhibit aggregation in a concentration-dependent manner. (b) The inhibition of aggregation observed with supernatants of cells stimulated with 1, 3 and 10 nM of Bk (as in (a)) is reduced by treating the cells with haemoglobin (Hb, 100 nM) 1 min before stimulation with Bk. (c) The inhibition of aggregation observed with supernatants of cells stimulated with 1, 3 and 10 nM of Bk (as in (a) and (b)) is abolished by treating the cells with indomethacin (Indo, 10 μM) 1 min before stimulation with Bk. Tracings are representative of 3 experiments.

The anti-aggregating activity of vascular endothelium

The amounts of prostacyclin and NO released by low concentrations of Bk (1–3 nM; Table 1) were not sufficient to explain the anti-aggregating activity observed when the incubate was added to the platelet suspension. This was most noticeable with cells stimulated with 1 nM Bk which did not release detectable amounts of NO (<0.1 μM) and released amounts of prostacyclin below those that caused inhibition of aggregation (<0.3 nM). Nevertheless, when these supernatants were added to the platelets, inhibition of aggregation was observed (Figure 2a, Table 1).

The inhibitory activity obtained with low concentrations of Bk (1–10 nM) was partially blocked by pretreatment of the cells with Hb (100 nM; Figure 2b; Table 2). Furthermore, the inhibitory activity was abolished when the cells were treated with indomethacin (10 μM) 1 min before the addition of Bk (Figure 2c; Table 2).

Table 2 Inhibition of aggregation by incubates of endothelial cells: reversal by indomethacin and haemoglobin

	Inhibition (%)		
Bk (nM)	*a*	*b*	*c*
100	100 ± 0	100 ± 0	100 ± 0
30	100 ± 0	100 ± 0	26 ± 4
10	100 ± 0	75 ± 9	0
3	83 ± 5	45 ± 7	0
1	38 ± 3	0	0

Inhibition of platelet aggregation by aliquots (0.1 ml) from incubates of 1.5 ml endothelial cells stimulated with bradykinin (Bk, 1–100 nM), (a) control; (b) cells treated with haemoglobin (100 nM), (c) cells treated with indomethacin (10 μM). Results are mean ± s.e.mean of 3 experiments.

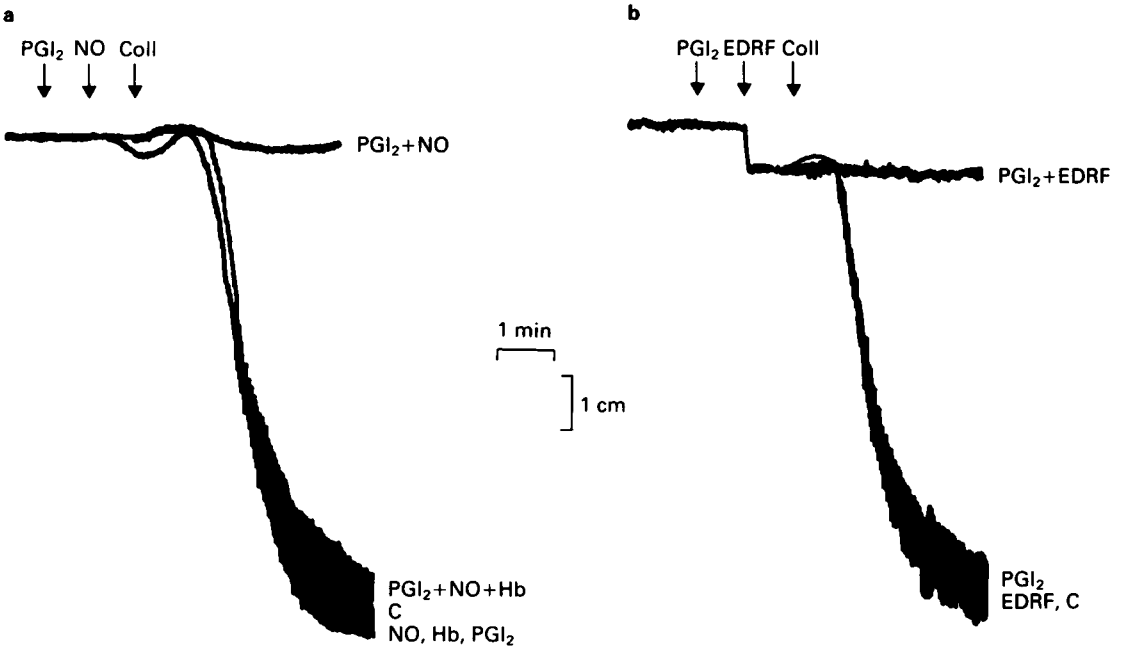

Figure 3 Prostacyclin potentiates the anti-aggregating activity of nitric oxide (NO) and EDRF. Subthreshold concentrations of NO (0.1 μM, a) and amounts of EDRF released from 0.5 ml of endothelial cells treated with indomethacin (10 μM) and stimulated with bradykinin (Bk, 100 nM; b) are both potentiated by a subthreshold concentration of prostacyclin (PGI_2, 0.1 nM). The inhibition of aggregation induced by a combination of these subthreshold concentrations of prostacyclin and NO was reversed by haemoglobin (Hb, 100 nM). (C) Control aggregation induced by collagen (Coll, 4 μg ml^{-1}). Tracings are representative of 3–6 experiments.

Interaction between prostacyclin, EDRF and authentic nitric oxide

The anti-aggregating activity of EDRF and of authentic NO was potentiated by a subthreshold concentration of prostacyclin (Figure 3). In the absence of prostacyclin the IC_{50}s for authentic NO against prostacyclin the IC_{50}s for authentic NO against collagen, ADP, thrombin and U46619 were 0.25 ± 0.03, 0.28 ± 0.04, 0.31 ± 0.04 and 0.34 ± 0.05 μM, respectively (n = 10) and were not significantly different from each other. In the presence of a subthreshold concentration of prostacyclin (0.1 nM) these IC_{50}s were significantly reduced to 0.06 ± 0.02, 0.07 ± 0.02, 0.08 ± 0.03 and 0.05 ± 0.01 μM, respectively (n = 10); these again were not significantly different from each other.

The anti-aggregating activity of prostacyclin was also increased by subthreshold concentrations of authentic NO and of EDRF (Figure 3). The IC_{50}s for prostacyclin against collagen, ADP, thrombin and U46619 were 2.3 ± 0.4, 2.0 ± 0.5, 3.7 ± 1.2 and 2.4 ± 0.3 nM (n = 10) and were not significantly different from each other. In the presence of a subthreshold concentration of NO (0.1 μM) these IC_{50}s were significantly reduced to 0.8 ± 0.2, 0.6 ± 0.1, 1.3 ± 0.2 and 0.9 ± 0.3 nM respectively (n = 10) and were not significantly different from each other.

When subthreshold concentrations of NO and prostacyclin were added together to the platelets, aggregation was inhibited by 85 ± 5% (n = 3; Figure 3). The inhibitory effect of this combination of subthreshold concentrations of prostacyclin and NO was completely abolished by addition of Hb (100 nM).

Platelet disaggregation

Authentic NO (0.15–1.25 μM) caused disaggregation of platelets aggregated with collagen (Figure 4a) or U46619, with EC_{50}s of 0.42 ± 0.07 and 0.44 ± 0.08 μM, respectively (n = 6). When platelets were aggregated with collagen the maximal disaggregation observed with NO (1.25 μM) and prostacyclin (6 nM) was 68.0 ± 1.8% and 70.0 ± 2.6% (n = 6), respectively. EDRF induced disaggregation of platelets but this could only be detected with incubates of 1.0 or 1.5 ml of endothelial cells stimulated with Bk (100 nM; Figure

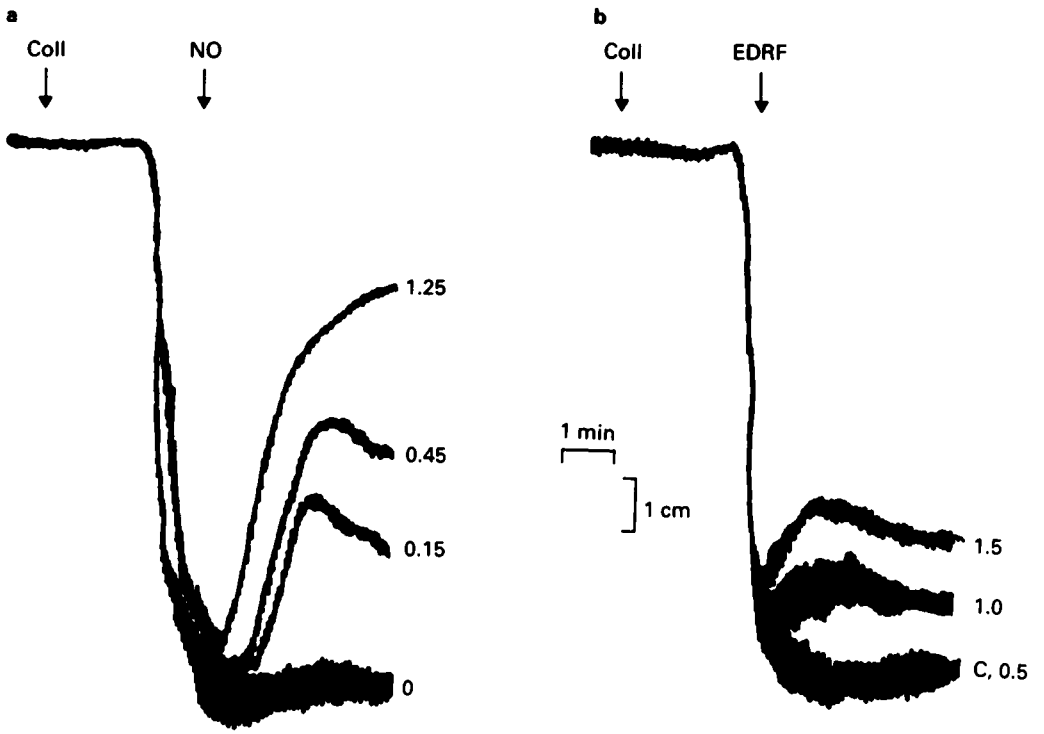

Figure 4 Disaggregating activity of (a) authentic nitric oxide (NO) and (b) EDRF released from endothelial cells (0.5, 1.0 and 1.5 ml) treated with indomethacin (10 μM) and stimulated with bradykinin (100 nM). Control aggregation in the presence of supernatant from 1.5 ml unstimulated endothelial cells (C). Tracings are representative of 6 experiments.

4b). These incubates caused 21 ± 6 and 43 ± 10% (n = 6) disaggregation, respectively. The amounts of NO released from endothelial cells (Table 1) resulted in concentrations equivalent to those of authentic NO which disaggregated platelets. Subthreshold concentrations of NO caused disaggregation when combined with subthreshold concentrations of prostacyclin (Figure 5a). However, no synergism was observed between maximally effective concentrations of prostacyclin (6 nM) and maximally effective concentrations of NO (1.25 μM; n = 5).

The disaggregating activity of subthreshold concentrations of NO (0.1 μM) and of EDRF released from 0.5 ml of endothelial cells by Bk (100 nM) could only be observed in the presence of subthreshold concentrations of prostacyclin (0.1 nM), or of SOD (20 u ml^{-1}), or of a selective cyclic GMP phosphodiesterase inhibitor M&B 22948 (1 μM; Figure 5a, b).

Discussion

We have shown that stimulation of porcine aortic endothelial cells with Bk results in a concomitant release of prostacyclin and EDRF (Gryglewski *et al.*, 1986a). In addition, when the production of prostacyclin is abolished by indomethacin, stimulation of the endothelium induces the release of EDRF which inhibits platelet aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987; Radomski *et al.*, 1987). We have recently shown that the release of NO accounts for the vascular relaxation attributed to EDRF (Palmer *et al.*, 1987). Moreover, the pharmacological profile of EDRF on platelets is identical to that of authentic NO (Radomski *et al.*, 1987). We have now demonstrated that the inhibitory effect on platelet aggregation attributed to EDRF is also accounted for by the release of NO. This release is dependent on the number of endothelial cells and on the concentration of Bk utilized to stimulate them.

Furthermore, prostacyclin and NO released from vascular endothelial cells synergize with each other to inhibit platelet aggregation. At the lowest concentration of Bk used to stimulate the cells there was no detectable release of NO and the concentrations of prostacyclin detected were not sufficient to explain the

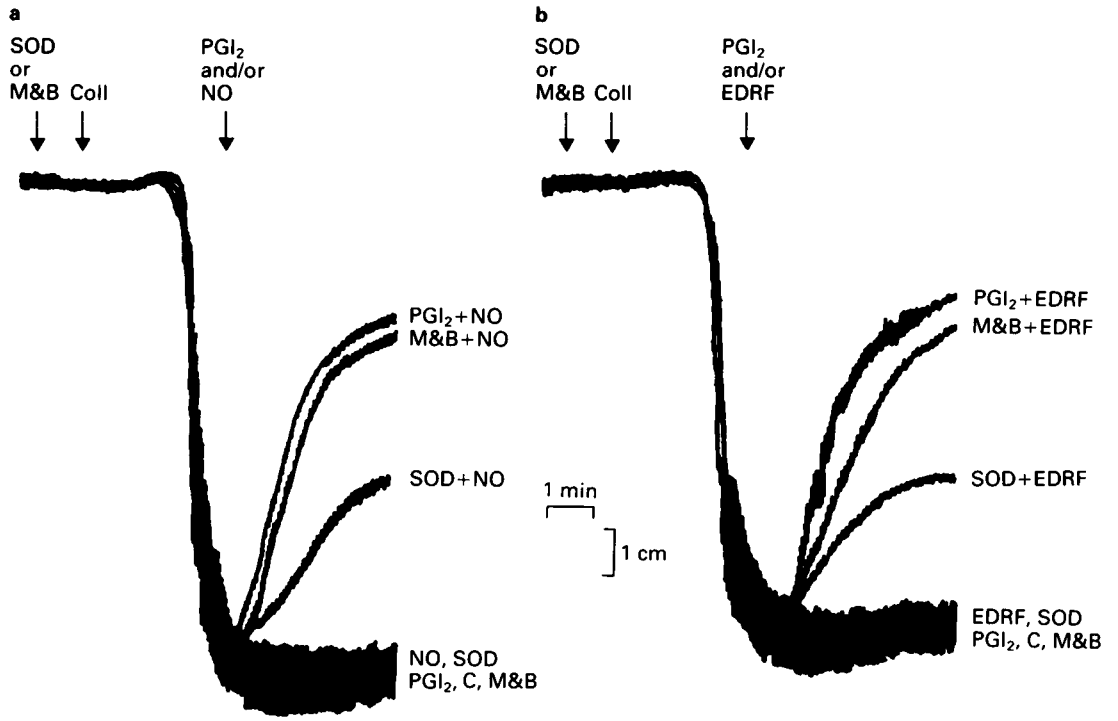

Figure 5 Potentiation of disaggregating activity of (a) nitric oxide (NO) and (b) EDRF by prostacyclin (PGI_2), superoxide dismutase (SOD) and M&B 22948 (M&B). Disaggregating activity of NO (0.1 μM) or EDRF released from 0.5 ml of endothelial cells treated with indomethacin (10 μM) and stimulated with bradykinin (100 nM) is potentiated by M&B 22948 (1 μM), SOD (20 u ml^{-1}) and by a subthreshold concentration of prostacyclin (0.1 nM). Tracings are representative of 6 experiments.

anti-aggregating activity present in the supernatant. Yet, the anti-aggregating activity could be abolished by treatment of the cells with indomethacin. This could be explained by the synergy of prostacyclin with concentrations of NO which on their own were too low to inhibit aggregation. This interpretation is strongly supported by the experiments in which the anti-aggregating activity of the incubate was partially reversed by Hb, a known inhibitor of NO. Indeed, Hb, by inactivating NO, unmasked its presence in the cell incubate. Futher evidence is provided by the experiments in which the synergy between subthreshold concentrations of authentic NO and prostacyclin is also reversed by similar concentrations of Hb.

All these findings taken together clearly demonstrate how bioassay reveals the presence of an active substance in a situation where chemical methods do not. More importantly, they suggest that prostacyclin and NO may regulate platelet-vessel wall interactions *in vivo*, at far smaller concentrations than those detectable by non-biological means (Blair *et al.*, 1982). Prostacyclin may indeed have a physiological homeostatic role in controlling the ability of platelets to aggregate if it acts on a background of NO close to the endothelial surface. Basal release of EDRF from vascular endothelial cells has been demonstrated *in vitro* (Martin *et al.*, 1985; Gryglewski *et al.*, 1986b). Whether there is a basal release *in vivo* remains to be investigated.

Such an interactive system between prostacyclin and NO should also be amenable to pharmacological manipulation, for as we and others have shown, the stability of EDRF (Gryglewski *et al.*, 1986b; Rubanyi & Vanhoutte, 1986) and that of authentic NO (Palmer *et al.*, 1987) is increased by SOD. Furthermore, the anti-aggregating activity of EDRF and NO is enhanced by SOD and by M&B 22948 (Radomski *et al.*, 1987).

Both EDRF and authentic NO, in common with prostacyclin, induce disaggregation of aggregated platelets. Furthermore, their actions are synergistic. Interestingly, none of the compounds induced more than 70% disaggregation and a combination of maximally effective concentrations of prostacyclin and

NO did not cause any further disaggregation.

Although there is a reciprocal potentiation between prostacyclin and NO, in our experiments prostacyclin seems to be more active in potentiating NO. This, however, could be due to the instability of NO and to its shorter biological half-life (Radomski *et al.*, 1987).

In conclusion, prostacyclin and NO are released by vascular endothelium and synergize with each other to inhibit platelet function. These interactions between NO and prostacyclin may represent a regulating system for maintenance of platelet homeostasis in physiological conditions. In addition, injurious stimuli will activate this anti-aggregating system to protect further the integrity of the vascular wall. Impairment of the production of or interactions between these two substances may play a role in the pathophysiology of conditions such as thrombosis, vasospasm and atherosclerosis.

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