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Endothelium-derived relaxing factor inhibits *in vitro* platelet aggregation

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1 We studied the effects of endothelium-derived relaxing factor (EDRF), bovine retractor penis muscle inhibitory factor and sodium nitroprusside, three stimulants of guanylate cyclase, on the *in vitro* aggregation of washed human platelets.

2 Platelet aggregation induced either by collagen or by the thromboxane A_2 analogue U46619 was inhibited by all three agents.

3 The anti-aggregatory effect of each agent was inhibited by haemoglobin.

4 The anti-aggregatory effect of EDRF was potentiated by superoxide dismutase.

5 These findings are discussed in relation to a potential role for EDRF in haemostasis.

Introduction

Endothelial cells play an important role in the regulation of coagulation. The endothelium is the major vascular site of production of prostacyclin (MacIntyre *et al.*, 1978; Weksler *et al.*, 1982), which is the most potent inhibitor of platelet aggregation known (Whittle & Moncada, 1983). Prostacyclin inhibits platelet aggregation by stimulating adenyl cyclase and increasing cyclic adenosine monophosphate (cyclic AMP) levels (Gorman *et al.*, 1977; Tateson *et al.*, 1977). Nitrovasodilators such as sodium nitroprusside also inhibit platelet aggregation, but act by stimulating guanylate cyclase, increasing cyclic guanosine monophosphate (cyclic GMP) levels (Schultz *et al.*, 1977; Takai *et al.*, 1981). Prostacyclin and sodium nitoprusside have been found to act synergistically to inhibit platelet aggregation (Levin *et al.*, 1982). The inhibitory factor (IF) isolated from the bovine retractor penis muscle (Gillespie & Martin, 1980) like the nitrovasodilators also induces vascular smooth muscle relaxation by elevation of intracellular cyclic GMP levels (Bowman & Drummond, 1984).

Endothelial cells produce a vascular smooth muscle relaxant known as endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980a; Griffith *et al.*, 1984). The relaxant effect of EDRF on vascular smooth muscle like that of IF is analogous to that of the nitrovasodilators in that it too stimulates guanylate cyclase and increases cyclic GMP levels (Holzmann, 1982; Furchgott & Jothianandan, 1983). In the present study we have investigated the platelet anti-aggregatory properties of EDRF and compared it with sodium nitroprusside and IF. A preliminary account of some of these findings has been reported to the British Pharmacological Society (Furlong *et al.*, 1986).

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Methods

Preparation of washed human platelets

Washed human platelets were prepared by the method of Blackwell *et al.*, (1982). Briefly, venous blood was collected into 3.2% trisodium citrate (4.5 ml blood per 0.5 ml citrate), centrifuged at 200 *g* for 20 min at 20°C, and the platelet-rich plasma (PRP) removed. Prostacyclin (final concentration 2ng ml^{-1}) was added to the PRP, which was then centrifuged at 100 *g* for 10 min at 20°C to remove any remaining red or white cells. A further 50–150 ng ml^{-1} (final concentration) prostacyclin was added to the PRP, which was then centrifuged at 600 *g* for 10 min at 20°C. The pellet was resuspended in 15 ml Tyrode solution (composition (mM): Na^+ 149.2, K^+ 2.7, Ca^{2+} 3.6, Mg^{2+} 2.1, Cl^- 145.3, $H_2PO_4^-$ 0.4, HCO_3^- 11.9, glucose 5.5) containing 0.03% bovine serum albumin (BSA) and prostacyclin 50–150 ng ml^{-1}, and centrifuged at 600 *g* for 10 min at 20°C. The pellet was again resuspended in Tyrode solution containing BSA (0.03%) and prostacyclin

(50–150 ng ml^{-1}), and centrifuged. The pellet was finally resuspended in Tyrode solution containing BSA (0.03%), gassed for 1–2 min with 95% O_2:5% CO_2 and placed in a capped perspex container. A manual platelet count was performed, and the resuspended platelets diluted as necessary to give a platelet count of 100 or 200 × 10^9 l^{-1}. Whenever the container was opened to withdraw an aliquot of washed platelets, the remaining platelets were gassed briefly (3–4 min) with 95% O_2:5% CO_2 to maintain the pH of the Tyrode solution at 7.4.

Preparation of bovine retractor penis inhibitory factor

The inhibitory factor (IF) was prepared by the method described by Gillespie & Martin (1980). Briefly, frozen (−20°C for up to 2 months) bovine retractor penis muscles were chopped in a food processor and extracted overnight in methanol (5 ml g^{-1}) at 30°C. The methanol was filtered and passed through 3.5 × 0.5 cm columns of Bio Rad AG1-X8 anion exchange resin (formate form, 50 ml methanol per column). The columns were washed with 2 × 5 ml of twice distilled water and the inhibitory factor eluted with 6 ml 300 mM sodium chloride. Adenine nucleotides were removed by passage through 3.5 × 0.5 cm columns of alumina at pH 9 (Bowman *et al.*, 1979). When extracted by this method, IF is obtained in an inactive form which can be converted to the active form by reducing the pH to an optimum of 2 (Gillespie & Martin, 1980). The mechanism of the acid-activation, however, is unknown. When required, IF was acid-activated by reducing the pH of the extract to 2 with 1 N HCl, leaving for 10 min, then neutralising with 1 N NaOH.

Preparation of rabbit aorta

Aortae were taken from New Zealand White rabbits (2–2.5 kg, killed by a blow to the neck), dissected free of fat and connective tissue, and cut into 1 cm lengths. The endothelium was either left intact, or removed by gentle abrasion with a wooden stick. Random vessels were examined histologically for the presence or absence of endothelium by the method of Poole *et al.*, (1958). Vessels were considered to be without endothelium when > 95% had been removed. The endothelium-containing or endothelium-denuded vessels were then tied singly to fine stainless steel rods so that the lumen of the aorta remained open at both ends. They were incubated at 37°C in Tyrode solution gassed with 95% O_2:5% CO_2 containing flurbiprofen 10^{-5} M for at least 1 h before use. Immediately before an experiment, the vessel was washed three times for 10 min each in fresh Tyrode solution at 37°C to remove excess flurbiprofen. In those experiments where superoxide dismutase was used, it was present in the Tyrode solution at a concentration of 60 units ml^{-1}.

Experimental protocol

Platelet aggregometry A Born-type turbidometric aggregometer was used to measure platelet aggregation. Washed platelets, (500 or 750 µl) were used. Collagen (4 µg ml^{-1}) or the thromboxane A_2 analogue, U46619 (500 ng ml^{-1}), was used as the aggregating agent. In preliminary experiments, collagen-induced platelet aggregation was found to be greatly reduced after contact with blood vessels, whether or not they contained intact endothelium. The reason for this observation is unknown. Only U46619 was therefore used as the *in vitro* aggregating agent in the EDRF experiments. Platelet aggregation was assessed by measurement of rate and extent. The rate is expressed as the slope of the aggregation record over the first 3 min. To calculate this value a straight line was drawn on the aggregation record from the point at which the aggregating agent was added to the point that aggregation had reached after 3 min; the tangent of the angle between this line and the time axis was taken to represent the rate of aggregation and accordingly since the length of each line was used to calculate the tangent, the rates expressed in the tables have no units. Extent of aggregation was measured when the light transmission reached a plateau, and was calculated from the formula

$$\frac{\text{Increase in light transmission by intervention}}{\text{Maximum light transmission}} \times 100$$

Sodium nitroprusside and inhibitory factor Sodium nitroprusside or inhibitory factor were added to 500 µl of washed platelets (200 × 10^9 l^{-1}) to give final concentrations of 10^{-5} M and 0.1 ml ml^{-1} (0.1 ml IF = 200 mg tissue) respectively and incubated for 4 min at 37°C before the addition of collagen or U46619. The studies were repeated in the presence of haemoglobin 10^{-5} M (added 4 min before sodium nitroprusside or IF) to inhibit stimulation of guanylate cyclase.

Endothelium-derived relaxing factor An aliquot (750 µl) of washed human platelets (100 × 10^9 l^{-1}) was incubated for 10 min at 37°C with either an endothelium-intact or endothelium-denuded vessel in the presence of acetylcholine (10^{-6} M) to stimulate EDRF release. This was achieved by placing the 1 cm length of aorta supported by the stainless steel rod in the cuvette holding the suspension of washed platelets. A small magnetic stirrer ensured mixing of the tube contents and access to the aortic lumen. After removal of the vessel, U46619 (500 ng ml^{-1}), was immediately added (i.e. within 5 s) to induce aggregation. The

studies were repeated in the presence of haemoglobin (10^{-5} M) (added 4 min before the vessel incubation) and in the presence of superoxide dismutase (60 u ml^{-1}) present throughout experiment). A 10 min incubation period was arbitrarily chosen to allow sufficient time for generation of platelet intracellular cyclic GMP levels since no data are available concerning the temporal effects of EDRF on platelet function.

Drugs

Acetylcholine chloride, bovine serum albumin, sodium nitroprusside and superoxide dismutase were obtained from Sigma Chemical Co. U46619 (15S)-hydroxy-11 α, 9 α-(epoxymethano) prosta-5Z, 13E-dienoic acid was a gift from Upjohn Ltd., U.S.A. The collagen was obtained from Semmelweis S.r.l., Italy and the prostacyclin from the Wellcome Foundation Ltd. Haemoglobin was prepared as follows: 1 ml of packed red cells were haemolysed with 9 ml distilled water, centrifuged at 600 *g* for 15 min; 3 ml of the supernatant were applied to a 2 × 40 cm Sephadex G25 column and the haemoglobin eluted with Tyrode solution. Spectrophotometric analysis showed the concentration to be ca. 10^{-4} M.

Statistical analysis

Results are expressed as mean ± s.e.mean, compared by Student's *t* test for unpaired data and considered significantly different when $P < 0.05$.

Results

Endothelium-derived relaxing factor

The effects of EDRF are shown in Tables 1 and 2. The rate of U46619-induced platelet aggregation was slowed in the presence of acetylcholine-stimulated vessels with endothelium, compared with the rate of aggregation in the presence of acetylcholine-stimulated vessels denuded of endothelium. This endothelium-dependent effect was enhanced by superoxide dismutase, and reversed by haemoglobin in the presence or absence of superoxide dismutase.

The extent of aggregation was also influenced by endothelium but to a smaller degree than the rate of aggregation. The effect of endothelium on the extent of aggregation reached significance only in the presence of superoxide dismutase.

Acetylcholine alone, either in the presence or absence of haemoglobin, had no direct effect on the rate or extent of aggregation.

The presence of denuded vessels stimulated by acetylcholine likewise had no effect on the rate or extent of aggregation except in the presence of superoxide dismutase.

Sodium nitroprusside and inhibitory factor

The effects of sodium nitroprusside and of inhibitory factor both on collagen- and on U46619-induced platelet aggregation are shown in Table 3. Both

Table 1 Change in light transmission (rate and extent) induced by U46619 (500 ng ml^{-1}) in the presence of acetylcholine (10^{-6} M) alone, following preincubation (10 min) with segments of rabbit aorta, with (+ Endo) or without endothelium (− Endo), with and without haemoglobin 10^{-5} M

Columns	1 *Control*	2 *ACh*	3 *ACh + Hb*	4 *+ Endo + ACh*	5 *− Endo + ACh*	6 *+ Endo + ACh + Hb*	7 *− Endo + ACh + Hb*
Rate (mean + s.e.)	1.59 ± 0.09	1.51 ± 0.06	1.45 ± 0.13	0.42 ± 0.08	1.27 ± 0.08	1.5 ± 0.18	1.56 ± 0.18
n	6	3	3	7	6	6	6
P value		NS cf. Column 1	NS cf. Column 2		< 0.001 cf. Column 4	< 0.001 cf. Column 4	NS cf. Column 5
Extent (mean ± s.e.)	82.7 ± 0.5	75 ± 2	78 ± 3	53 ± 10	70 ± 4	82 ± 5	85 ± 3
n	6	3	3	7	6	6	6
P value		NS cf. Column 1	NS cf. Column 2		NS cf. Column 4	NS cf. Column 4	NS cf. Column 5

Rate is expressed as slope of the aggregation curve over the first 3 min (see Methods). Extent of aggregation is expressed as percentage increase in light transmission at point of maximum aggregation. ACh = acetylcholine; Hb = haemoglobin; NS = not significant.

Table 2 Change in light transmission (rate and extent) induced by U46619 (500 ng ml^{-1}) in the presence of acetylcholine (10^{-6} M) and superoxide dismutase (60 u l^{-1}), following preincubation (10 min) with segments of rabbit aorta, with (+ Endo) or without endothelium (− Endo), with and without haemoglobin (10^{-5} M)

	1 *ACh*	2 *ACh* *+ SOD*	3 *+ Endo* *+ ACh* *+ SOD*	4 *− Endo* *+ ACh* *+ SOD*	5 *+ Endo* *+ ACh* *+ SOD* *+ Hb*	6 *− Endo* *+ ACh* *+ SOD* *+ Hb*
Rate (mean ± s.e.)	1.5 ± 0.06	1.68 ± 0.01	0.056 ± 0.02	1.24 ± 0.17	1.16 ± 0.3	1.61 ± 0.09
n	3	3	5	4	5	5
P value		< 0.05 cf. Column 1	< 0.005 cf. Column 4 Table 1	< 0.001 cf. Column 3	< 0.001 cf. Column 3	NS cf. Column 4
Extent (mean ± s.e.)	75 ± 2	82.4 ± 3.5	3.8 ± 1.7	70 ± 5.5	73 ± 6.6	81 ± 3.5
n	3	3	5	5	5	5
P value		NS cf. Column 1	< 0.005 cf. Column 4 Table 1	< 0.001 cf. Column 3	< 0.001 cf. Column 3	NS cf. Column 4

Conventions as in Table 1. SOD = superoxide dismutase; NS = not significant.

nitroprusside and activated inhibitory factor reduced collagen- and U46619-induced aggregation. Their effects were reduced by haemoglobin. Unactivated and boiled activated inhibitory factor were without effect. Haemoglobin alone had a small depressant effect on collagen-induced aggregation but had no demonstrable effect on U46619-induced aggregation.

Discussion

The results show that EDRF, inhibitory factor and sodium nitroprusside each inhibit platelet aggregation as measured by *in vitro* tests. Each of these three agents elevates intracellular cyclic GMP by stimulation of guanylate cyclase (Schultz *et al.*, 1977; Bowman *et al.*,

Table 3 Change in light transmission (extent) induced by collagen (4 μg ml^{-1}) or U46619 (500 ng ml^{-1}) alone (control); in the presence of haemoglobin (10^{-5} M); sodium nitroprusside (10^{-5} M); activated inhibitory factor (Act IF, 0.1 ml ml^{-1}); unactivated IF (Unact IF, 0.1 ml ml^{-1}); boiled activated IF (Boiled IF, 0.1 ml ml^{-1})

Collagen	1 *Control*	2 *Hb*	3 *NP*	4 *NP + Hb*	5 *Act IF*	6 *Unact IF*	7 *Act IF* *+ Hb*	8 *Boiled IF*
Mean	73.2	53.4	33.4	61.2	16.8	89.8	33.5	86.5
+ s.e.	± 20	± 14	± 13	± 4.5	± 14	± 8	± 10	± 12
n	13	8	8	6	13	6	6	6
P values		< 0.05 cf. Column 1	< 0.001 cf. Column 1	< 0.001 cf. Column 3	< 0.001 cf. Column 1	NS cf. Column 1	< 0.05 cf. Column 5	NS cf. Column 1
U46619	1 *Control*	2 *Hb*	3 *NP*	4 *NP + Hb*	5 *Act IF*	6 *Unact IF*	7 *Act IF* *+ Hb*	
Mean	82.6	77.6	10.4	85.1	24.9	97.6	93.6	
± s.e.	± 4.08	± 2.9	± 7.56	± 5.21	± 9.77	± 1.86	± 3.25	
n	8	3	8	5	7	3	8	
P values		NS cf. Column 1	< 0.001 cf. Column 1	< 0.001 cf. Column 3	< 0.001 cf. Column 1	NS cf. Column 1	< 0.001 cf. Column 6	

Conventions as in Table 1. NP = sodium nitroprusside; IF = inhibitory factor of bovine retractor penis muscles; NS = not significant.

1982; Rapoport & Murad, 1983; Bowman & Drummond, 1984). The anti-aggregatory effects of each agent were inhibited by haemoglobin, which inhibits stimulants of soluble guanylate cylase (Murad *et al.*, 1978; Martin *et al.*, 1985). Endothelium-mediated reduction of *in vitro* platelet aggregation has now been reported also by Ayuma and colleagues, although their experiments did not exclude the possibility that the phenomenon may have been attributable to prostacyclin (Ayuma *et al.*, 1986).

The antiaggregatory effects of EDRF were much more marked with respect to rate than extent of aggregation, as might be expected from consideration of the short half-life of EDRF. In the presence of superoxide dismutase, which prolongs the half-life of EDRF (Rubanyi & Vanhoutte, 1985; Gryglewski *et al.*, 1986), EDRF also reduced the extent of aggregation. In the presence of superoxide dismutase, even 'denuded' vessels had a slight antiaggregatory effect, attributable possibly to some residual endothelial cells.

Platelet aggregation releases ATP and ADP, which stimulate both prostacyclin and EDRF release from endothelial cells (DeMey & Vanhoutte, 1980, 1981; Furchgott & Zawadzki, 1980b; Pearson *et al.*, 1983; Gordon & Martin, 1983). The present study shows that EDRF acts like nitroprusside to inhibit platelet aggregation. The anti-aggregatory properties of prostacyclin have been shown previously to be synergistic with those of nitroprusside (Levin *et al.*, 1982). EDRF may therefore act synergistically with prostacyclin to limit thrombus formation *in vivo*, thus preventing its extension to areas of intact endothelium. These effects are likely to be limited to those platelets which are in intimate contact with endothelium since with the short half life of EDRF, it probably has negligible 'downstream' effects in the intact circulation (Angus *et al.*, 1983; Edwards *et al.*, 1985).

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