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Platelet inhibition by endothelium-derived relaxing factor from the rabbit perfused aorta

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1 The platelet inhibiting activity of endothelium-derived relaxing factor (EDRF) released by the perfused thoracic aorta of the rabbit was investigated.

2 The aortic effluent superfused a ring of the abdominal aorta without endothelium in order to bioassay EDRF. Aliquots of effluent were collected on rabbit washed platelets and aggregation induced by U-46619 was measured after 1 min. Prostacyclin (PGI_2) was monitored by radioimmunoassay of 6-oxo-prostaglandin $F_{1\alpha}$.

3 Acetylcholine (ACh) caused a dose-dependent secretion of EDRF, PGI_2 and anti-aggregating activity. Plasma and methylene blue suppressed the platelet inhibition by the effluent.

4 The PGI_2 content of the effluent was not sufficient to account for all the anti-aggregating activity. However, the platelet inhibition disappeared when PGI_2 formation was blocked with indomethacin.

5 Compression of the thoracic aorta increased the EDRF content in the effluent. A transient secretion of anti-aggregating activity was then observed in aortic effluent in the absence of PGI_2. This activity coincided with the presumed EDRF peak in the effluent.

6 Superoxide dismutase enhanced the ACh-induced EDRF content and revealed secretion of an anti-aggregating substance when PGI_2 formation was blocked. Pretreatment of the platelets with subthreshold concentrations of PGI_2, or the cyclic GMP phosphodiesterase inhibitor RX-RE 56, also revealed the release of a labile platelet inhibitor in response to ACh.

7 The results indicate that EDRF released by fresh aortic endothelium may suppress platelet aggregation, particularly when PGI_2 is present.

Introduction

Endothelium-derived relaxing factor (EDRF) is a labile mediator responsible for the vasodilator activity of acetylcholine (ACh) and several other agents (Furchgott, 1984). Chemiluminescence and pharmacological techniques (Palmer *et al.*, 1987) supported earlier proposals that nitric oxide (NO) accounts for the biological activity of EDRF. Based on studies with rabbit perfused aortae (Azuma *et al.*, 1986) and isolated aortic segments of the rabbit (Furlong *et al.*, 1987), it has been suggested that EDRF also inhibits blood platelets. However, the involvement of prostacyclin (PGI_2) or products released by vascular smooth muscle cells was not fully excluded in these experiments. Indeed, we were unable to detect the release of a humoral, anti-aggregating activity by fresh aortic endothelium when PGI_2 was eliminated (Bult *et al.*, 1987). On the other hand, it has been suggested that EDRF released by endothelial cells in culture may cause disaggregation (Murray *et al.*, 1986) and may inhibit aggregation through a cyclic GMP-mediated mechanism (Busse *et al.*, 1987; Moncada *et al.*, 1987; Radomski *et al.*, 1987a,b). The aim of the present study was to investigate this apparent discrepancy. One possibility could be that EDRF of fresh endothelium and EDRF of cultured cells are different. Another explanation could be that aortic endothelium released insufficient EDRF for platelet inhibition. Reduction of the aortic volume as well as the aortic transit time raised the EDRF content in the aortic effluent. This permitted the detection of an anti-aggregating activity with characteristics of EDRF under certain conditions.

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Methods

Platelet preparation and aggregation

New Zealand White rabbits were anaesthetized (pentobarbitone, $30\,mg\,kg^{-1}$) and blood was

obtained from a cannulated carotid artery. Platelet-rich plasma (PRP, 4 ml) was prepared by centrifugation (300 *g*, 10 min) of 10 ml citrated (12.9 mM) blood. For preparation of a platelet suspension (PS), PRP was made from blood collected on 5.8 mM EDTA. Plasma was removed by centrifugation (1000 *g*, 10 min), the platelets were washed with buffer (135 mM NaCl, 12 mM Tris-HCl pH 7.4, 1.54 mM EDTA, 0.2% gelatin), gently resuspended in 2 ml Ca^{2+}-free Krebs solution and kept under 95% O_2 and 5% CO_2.

Aortic effluent (3 to 5 drops, number depending on drop size, volume equivalent to 200 μl) was collected on 100 μl prewarmed PS or 200 μl PRP. Aggregation was induced immediately with 0.2 or 0.3 μM U-46619 in a Payton Dual Channel Aggregometer and measured 1, 3 and 5 min later. Only the values obtained after 1 min are shown. Occasionally the effluent was stored for 4 min before testing. The aggregation was always expressed as percentage of the aggregation in the presence of the corresponding effluent taken just before that particular ACh injection.

Perfusion of the thoracic aorta and bioassay of EDRF

Heparin (100 units kg^{-1}, i.v.) was given after blood collection and 2 min later the rabbit was killed by an overdose of pentobarbitone. The chest was opened, the aorta exposed and the intercostal arteries were coagulated and cut (Bircher Model 755 Blendtome). The segment between diaphragm and aortic arch was removed and placed in Krebs solution. One cannula was inserted in the rostral opening, red blood cells were gently flushed away with 10 ml Krebs solution and the second cannula (50 μl internal volume) was placed in the caudal outlet. The aorta was positioned on a perspex support in an organ chamber filled with 400 ml Krebs solution and perfused with Krebs (3 ml min^{-1}). Its intraluminal volume could be reduced by placing another perspex plate 1.5 mm above the supporting plate.

The aortic effluent superfused a horizontally suspended ring (3 mm) of the abdominal aorta without endothelium (Verbeuren *et al.*, 1986). Its isometric tension was recorded and the tissue was mounted under 9.5 $\pm$ 0.6 g (n = 24) resting tension. It was contracted (6.5 $\pm$ 0.5 g, n = 24) by an infusion of noradrenaline (0.1 μM), which contained 0.1 μM atropine to block contractile effects of ACh. The minimum tension after a bolus injection of ACh (30 μl) was expressed as percentage of the initial tension.

Experimental protocol

ACh was injected at 20 min intervals and perfusate for aggregation studies was collected before and exactly 60 s after ACh injection. PGI_2 was measured in samples taken before and from 45 to 75 s after ACh. In one experiment, the endothelium was randomly exposed to 0.003, 0.03, 0.3, 3 and 30 nmol ACh. Indomethacin (3 μM) was then added to the Krebs solution and 30 min later 30 nmol ACh was tested again. Thereafter the aorta was compressed by placing a piece of plexiglass 1.5 mm above the supporting plate and the series of ACh bolus injections was repeated in the presence of indomethacin.

The kinetics of mediator release were studied in compressed aortae. Anti-aggregating activity was tested 15, 30, 45, 60, 90 and 120 s after injection of 30 nmol ACh, whereas from 7, 22, 37, 52, 82 and 112 s perfusate was collected for 15 s to assess PGI_2. In an attempt to quantify the EDRF responses, NO (1 to 32 nmol) was injected directly in 1 μl into the drop of Krebs above the aortic detector.

Radioimmunoassay

PGI_2 was assessed by specific and sensitive radioimmunoassay (RIA) of 6-oxo-prostaglandin $F_{1\alpha}$ (Bult *et al.*, 1985). Indomethacin, noradrenaline, atropine, ACh and 1 μM methylene blue did not interfere with the assay. Dose-interpolation was done with the four parameter logistic function (Dudley *et al.*, 1985) using the IBM-PC RIA data reduction package provided by M.L. Jaffe (Silver Spring, MD, U.S.A.).

Materials

The Krebs solution (Krebs) contained (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 25, glucose 5.5 and was gassed with 80% N_2, 15% O_2 and 5% CO_2. The Ca^{2+}-free Krebs solution contained 0.2% gelatin, but $CaCl_2$ was omitted. Acetylcholine, superoxide dismutase (bovine erythrocytes, 3500 units mg^{-1}) and methylene blue were obtained from Sigma (St Louis, MO, U.S.A.). Indomethacin was a gift from Merck, Sharp & Dohme (Brussels, Belgium), prostacyclin was given by the Wellcome Research Laboratories (Beckenham, U.K.), RX-RE 56 (7-benzylamino-6-chloro-4-morpholino-2-piperazinopteridin) was a gift from Dr K. Thomae GmbH (Biberach an der Riss, F.R.G.) and (15S)-hydroxy-11 α,9 α-(epoxymethano) prosta-5Z,13E-dienoic acid (U-46619) was a gift from The Upjohn Company (Kalamazoo, MI, U.S.A.). Nitric oxide gas (NO) (Air Liquide) was dissolved in, and diluted with He-deoxygenated 0.9% NaCl (Palmer *et al.*, 1987), or injected directly into the Krebs solution.

Data analysis

Data are given as mean $\pm$ s.e.mean of 6 to 7 rabbits. Comparisons were made by use of Student's t test

Table 1 Luminal volume, transit time and lag time before and after compression of the aorta

Parameter	*Normal*	*Compressed*
Volume	474 ± 54 μl	134 ± 38 μl*
Transit time	9.5 ± 1.1 s	2.7 ± 0.8 s*
Lag time	37.9 ± 2.0 s	22.3 ± 1.0 s*

Lag time is the delay between the injection of acetylcholine (ACh) and the start of the relaxation of the aortic ring. The aortic transit was calculated as aortic volume/flow. Results are mean ± s.e.mean of the six aortae shown in Figure 1.
* $P < 0.05$, Student's paired t test.

for paired observations. The PGI_2 data were transformed to logarithms in order to obtain normal distributions.

Results

ACh caused a dose-dependent release of EDRF, anti-aggregating activity and PGI_2 (Figure 1). Release of the former was indicated by the relaxation of a ring of the abdominal aorta without endothelium. The detector did not relax in response to ACh when the thoracic aorta was bypassed. The stimulation of EDRF and PGI_2 release was absent when atropine was included in the Krebs solution, or when the aortic lumen had been exposed to distilled water for 2.5 min (results not shown).

Compression of the aorta reduced the intraluminal volume, the calculated aortic transit time and the lag time between ACh injection and start of relaxation of the detector ring (Table 1). About 10 fold less ACh was then required to induce EDRF release and the maximum relaxation was slightly increased (Figure 1). Indomethacin abolished the release of PGI_2 in normal and compressed aortae, without influencing EDRF release. Even in compressed aortae anti-aggregating activity was no longer detectable 60 s after ACh injection when indomethacin was present in the Krebs solution (Figure 1). However, a labile platelet inhibitor was observed in effluent of compressed aortae when the PS was pretreated with 10^{-9} M PGI_2 (74 ± 14% inhibition, $n = 6$) or the cyclic GMP phosphodiesterase inhibitor RX-RE 56 (10^{-7} M, 42 ± 9% inhibition, $n = 6$). The anti-aggregating activity disappeared when the effluent was stored for 4 min (Figure 2). These concentrations of PGI_2 (4 ± 7% inhibition, $n = 6$) and RX-RE 56 (7 ± 5% inhibition) did not affect aggregation by themselves (cf Figure 2). Infusion of superoxide dismutase (20 units ml^{-1}) into compressed aortae resulted occasionally in 'spontaneous' relaxation of the detector tissue. It enhanced the relaxation in

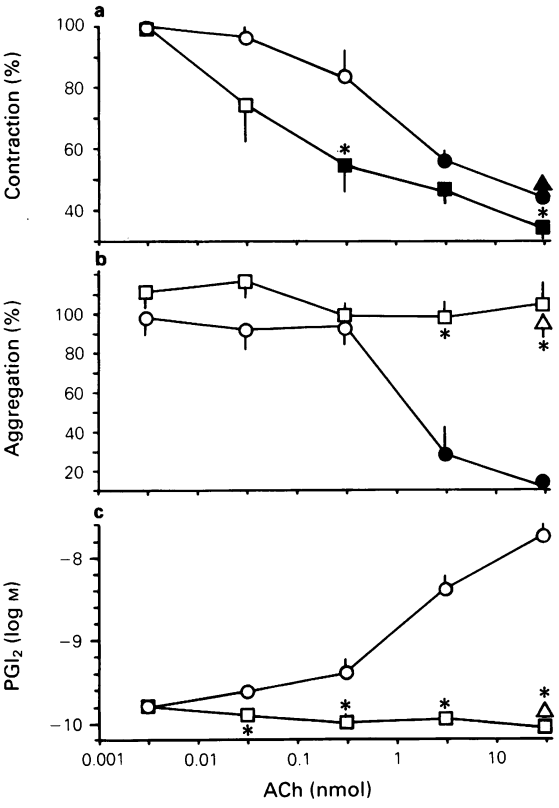

Figure 1 Effect of indomethacin and aortic compression on the acetylcholine (ACh)-induced release of endothelium-derived relaxing factor (EDRF) (a), anti-aggregating activity (b) and prostacyclin (PGI_2, c, logarithmic scale) by rabbit perfused aortae. A dose-response curve was obtained from normal aorta (○, ●), 3 μM indomethacin was added to the Krebs solution and 30 nmol ACh was tested again (△, ▲). Finally, the aorta was compressed and the dose-response curve for ACh was repeated in the presence of indomethacin (□, ■). The decreased contraction of the detector tissue served as an index of EDRF and was expressed as % of the initial tension. Aggregation was induced by 0.3 μM U-46619 in the presence of 200 μl effluent collected 60 s after ACh injection. It was measured after 1 min and expressed as % of the aggregation with effluent obtained before ACh injection. PGI_2 was assessed in effluent obtained between 45 and 75 s after ACh injection. Solid symbols are different from the baseline; *significantly different from normal aorta ($P < 0.05$, Student's paired t test).

response to 30 nmol ACh (from 50 ± 7% to 69 ± 5%) and the effluent then contained platelet suppressive activity (24 ± 5% inhibition, $n = 6$).

Methylene blue (1 μM) did not raise the tension of the detector tissue, but abolished ACh-induced relaxations and suppressed the anti-aggregating activity

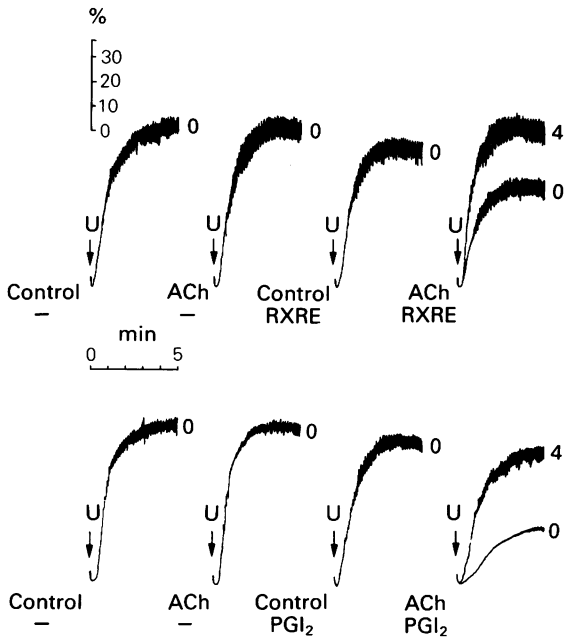

Figure 2 Addition of prostacyclin (PGI_2; 10^{-9} M) or the cyclic GMP phosphodiesterase inhibitor RX-RE 56 (10^{-7} M) to a platelet suspension (PS) reveals the release of a labile anti-aggregating activity when aortic endothelium is stimulated with acetylcholine (ACh). Compressed aortae were used and 3 μM indomethacin was present in the perfusate. Effluent was collected directly into the PS (0) or added after 4 min storage (4). U, injection of 0.3 μM U-46619; control, aggregation with 200 μl effluent obtained before ACh injection; ACh, aggregation with effluent obtained 60 s after injection of 30 nmol ACh; —, buffer (10 μl) added to PS.

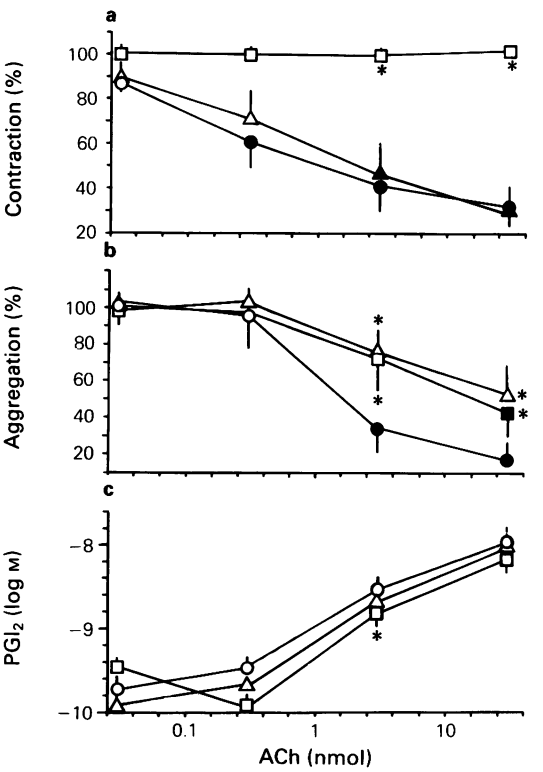

Figure 3 Effect of 1 μM methylene blue (□, ■) and plasma (△, ▲) on the ACh-induced release of EDRF (a), anti-aggregating activity (b) and prostacyclin (PGI_2, c, logarithmic scale) by normal rabbit aortae. The anti-aggregating activity was assessed in a platelet suspension (○, ●), then in the corresponding platelet-rich plasma (PRP; △, ▲), and after addition of methylene blue to the perfusate the experiment was repeated with the washed platelets. Further details are given in the legend of Figure 1 and Methods. Solid symbols are different from the baseline; *significantly different from the control ($P < 0.05$, Student's paired t test).

of the effluent (Figure 3). This concentration of methylene blue did not reduce or shift the dose-relaxation response curve for glyceryltrinitrate (results not shown). A similar decrease of anti-aggregating activity was seen when effluent was tested in PRP (Figure 3).

Finally, the kinetics of the release of EDRF, PGI_2 and platelet inhibitory activity by compressed aortae were studied (Figure 4). A dose-relaxation curve was made for NO in order to calibrate the detector ring. After NO injection into the drop above this tissue, relaxation started about 6 s later and another 10 to 30 s were required before maximum relaxation was attained. In view of this sluggish response, the relaxation in response to ACh was not only expressed as percentage of the initial contraction, but also on the basis of the relaxation reached in the subsequent 15 s. Bracketing assay was used to assess the equivalent amount of NO at both time points, and in Figure 4 the EDRF in the effluent is also expressed in NO units ($nmol_{t+15} - nmol_t$). The peak concentration of EDRF was reached 45 s after injection of 30 nmol ACh, assuming that the detector responded with 15 s delay. From 90 s onwards it was no longer detectable. Neither curve was influenced by indomethacin. Effluent collected between 45 and 90 s caused almost complete platelet inhibition, whereas the PGI_2 concentration reached a plateau between 60 and 90 s. The half-life of the anti-aggregating activity in effluent obtained 45 s after ACh was less than 4 min (results not shown). Indomethacin abolished PGI_2 formation, but some anti-platelet activity was still present in effluent collected at 45 s (Figure 4). The inhibition disappeared when this effluent was stored for 4 min (results not shown).

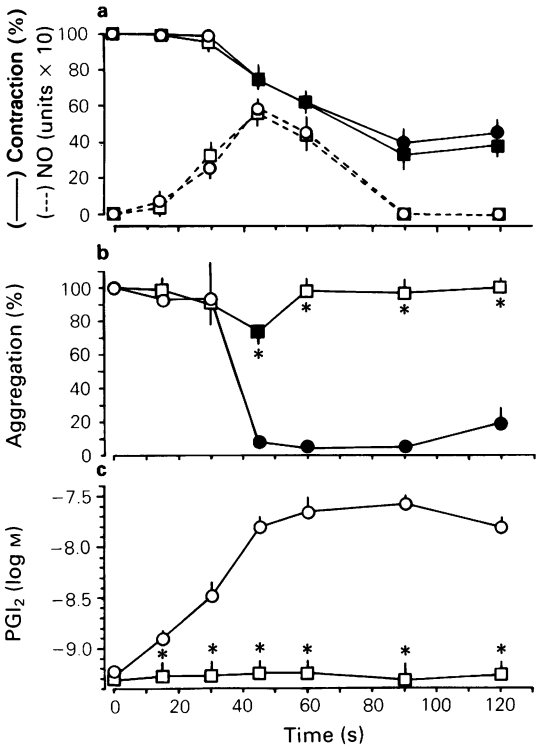

Figure 4 Time course curves for the release of EDRF (a), anti-aggregating activity (b) and prostacyclin (PGI_2, c, logarithmic scale) by compressed aortae in the absence (○, ●) or presence of 3 μM indomethacin (□, ■). The endothelium was stimulated with 30 nmol acetylcholine (ACh). The contraction of the abdominal detector was expressed as % of the baseline tension as an index of EDRF release (a, solid lines). In addition the relaxations were compared with a dose-response curve for NO (bracketing assay). The broken lines give the relaxation in the subsequent 15 s in NO units ($[\text{nmol NO}_{t+15} - \text{nmol}_t]_* 10$). Further details are given in the legend of Figure 1 and Methods. Solid symbols are different from the baseline; * significantly different from control without indomethacin ($P < 0.05$, Student's paired t test).

When aggregation was plotted against the PGI_2 concentration in the PS, it appeared that PGI_2 could not fully explain the platelet inhibition: the effluent was more effective than authentic PGI_2 (results not shown). The additional inhibition was not observed in PRP. Aggregation induced by U-46619 in a PS was dose-dependently suppressed by NO. The effect was most pronounced in the first min (IC_{50} 0.84 μM, 95% confidence limits 0.18–4.24 μM) and decreased rapidly (e.g. at 3 min IC_{50} 8.7 μM, 95% confidence limits 3.9–19 μM). NO was less active in PRP, but more active as a dilator of rabbit isolated aortic segments (Verbeuren *et al.*, unpublished observations).

Discussion

The presence of EDRF in aortic effluent was assessed by bioassay. The assay tissue responded with 6 s delay on the passage of a drop with NO and a further 15 s were required before maximum relaxation was reached (cf Gruetter *et al.*, 1981). The bioassay thus recorded EDRF that passed the detector at least 15 s earlier. A first derivative of the EDRF curve may therefore provide a better assessment of EDRF in the effluent at any time. It suggested that the peak EDRF concentration was reached about 45 s after injection of ACh into the compressed aorta. The maximum PGI_2 concentration was reached 15 to 45 s later.

Compression of the aorta reduced the volume of its lumen, thus concentrating the endothelial products in the effluent. This was apparent from the two fold increase of PGI_2 in effluent from compressed (29 ± 7 nM, $n = 6$) when compared with normal aortae (14 ± 2 nM, $n = 14$) 60 s after stimulation with 30 nmol ACh. In addition, the aortic transit time was three fold shorter. Both factors would have favoured an increased content of the very labile EDRF in the effluent. Furthermore, EDRF release is flow-dependent (Rubanyi *et al.*, 1986). Flow was kept constant in our experiments, but enhanced shearforces could have formed an additional stimulus for EDRF release after compression. As the dose-relaxation response curve of the detector was shifted ten fold to the left, in compressed aortae, it is assumed that the amount of EDRF leaving the perfusion circuit was about ten times higher when compared with the normal aorta.

The release of anti-aggregating activity was blocked by indomethacin, confirming findings obtained with ACh infusions in normal aortae (Bult *et al.*, 1987). This again is at variance with the unpublished observations by Azuma *et al.* (1986), and indicates that PGI_2 is the principal humoral anti-aggregating substance produced by endothelial cells (cf. Moncada *et al.*, 1987). However, when the EDRF content of the effluent was raised by compression of the aorta, a transient anti-platelet activity was observed in the absence of PGI_2. It coincided with the presumed EDRF peak in the effluent. Indirect evidence further supported the idea that the endothelium secreted a labile anti-aggregating factor with characteristics of EDRF in addition to the PGI_2.

Firstly, the anti-aggregating activity of PGI_2 is not influenced or even prolonged in the presence of plasma, whereas the inhibition by aortic effluent and NO was less in PRP. Plasma curtails the efficacy of

EDRF, possibly due to traces of haemoglobin (Edwards *et al.*, 1986) which inhibits the activity of EDRF and binds NO avidly (Martin *et al.*, 1985; Moncada *et al.*, 1987; Palmer *et al.*, 1987; Radomski *et al.*, 1987a). Secondly, the anti-aggregating activity of aortic effluent was suppressed by methylene blue, which did not affect the guanylate cyclase activity of the detector tissue, but either inactivated EDRF (cf. Martin *et al.*, 1985) or inhibited its release. Thirdly, the PGI_2 released in response to increasing amounts of ACh effluent was not sufficient to account for all anti-aggregating activity, when compared with the activity of PGI_2 in the same platelet suspensions. Finally, PGI_2 is relatively stable in Krebs solution or PRP, whereas the platelet inhibition by perfusate collected 45 s after ACh injection disappeared within 5 min.

The vasodilating (Gruetter *et al.*, 1981; Martin *et al.*, 1985) and anti-aggregating activities of NO (Mellion *et al.*, 1981) and EDRF from cultured endothelial cells (Busse *et al.*, 1987) are mediated through stimulation of soluble guanylate cyclase. Cyclic GMP exerts synergism with cyclic AMP as platelet inhibitor (Radomski *et al.*, 1987b), possibly by suppressing cyclic AMP breakdown (Maurice & Haslam, 1987). Secretion of a labile anti-platelet activity in response to ACh was seen in the presence of subthreshold concentrations of PGI_2 or the selective cyclic GMP phosphodiesterase inhibitor RX-RE 56. Both results confirm the findings obtained with EDRF released by cultured endothelial cells as well as NO (Moncada *et al.*, 1987; Radomski *et al.*, 1987a). Finally, superoxide anion destroys EDRF and superoxide dismutase prolongs its half-life (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986). Indeed, superoxide dismutase enhanced the output of both EDRF and anti-aggregating activity from compressed aortae. Stimulation of PGI_2 release by hydrogen peroxide generated by the superoxide dismutase (Ager & Gordon, 1984; Rampart *et al.*, 1985) was eliminated by indomethacin, as confirmed by the RIA.

In previous studies on anti-aggregating activity of EDRF, the platelets were either pretreated with μM concentrations of PGI_2 (Busse *et al.*, 1987; Furlong *et al.*, 1987; Moncada *et al.*, 1987; Radomski *et al.*, 1987a,b) or PGI_2 formation during the experiment was not excluded (Azuma *et al.*, 1986; Furlong *et al.*, 1987). The prolonged platelet suppression by PGI_2 probably favoured subsequent inhibition by agents that act via cyclic GMP such as EDRF. The present study, in which both the long-lasting effects of PGI_2 and its formation were excluded, indicated that fresh aortic endothelium from the rabbit released a non-prostanoid inhibitor of platelet aggregation with the characteristics of EDRF. However, EDRF generation by fresh endothelium was often not sufficient for platelet inhibition when PGI_2 was absent (Bult *et al.*, 1987). The geometry of the blood vessel, i.e. its volume to surface ratio, is important for the humoral effects of EDRF. It implies that the ability of humoral EDRF to have an effect increases towards the microcirculation. Even then EDRF can only affect cells in close proximity to endothelium, since plasma curtailed its activity.

In conclusion, EDRF is a local vasodilator (Edwards *et al.*, 1986) that may suppress activation of platelets adhering to endothelium (cf. Furlong *et al.*, 1987). The synergy between EDRF and PGI_2 is interesting in this respect. It should be noted, however, that the arterial PGI_2 concentration in rabbits ($< 5 \times 10^{-11}$ M, Bult *et al.*, 1985) is normally below the threshold for this synergy. Release of PGI_2 is presumably a sign of endothelial distress that may help to maintain blood flow through the affected blood vessel (Bult *et al.*, 1988). An obstruction of blood flow will favour the anti-platelet effects of EDRF, which in combination with the PGI_2 formed as a result of endothelial distress, may then prevent growth of mural thrombi or help to dissolve existing platelet emboli.

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