Br. J. Pharmac. (1986), **88**, 411–415

Endothelium-dependent inhibition of platelet aggregation

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1 In cascade perfusion and superfusion experiments on rabbit tissues, when acetylcholine (ACh) was introduced into the circuit so as to perfuse the aorta under perfusion with noradrenaline (NA), the effluent relaxed the transverse aortic strip which had been denuded of endothelium.

2 The effluent from the perfused aorta which was capable of relaxing the transverse aortic strip also significantly inhibited platelet aggregation induced by arachidonic acid (AA) in a volume-related manner. The inhibitory activity was decreased by the prolongation of transit time before addition of the effluent to platelet-rich plasma.

3 Neither the inhibition of AA-induced aggregation nor the relaxation of the transverse strip by the effluent could be observed after the removal of endothelium from the aorta, or after pretreatment of aorta with mepacrine or nordihydroguaiaretic acid (NDGA).

4 The AA-induced platelet aggregation was unaffected by pretreatment of platelets with mepacrine or NDGA at the concentration tested.

5 Pretreatment of aorta with indomethacin failed to modify the relaxation of the transverse strip induced by the effluent.

6 These results strongly suggest that endothelium-derived vascular relaxant factor (EDRF) possesses inhibitory activity on AA-induced aggregation in addition to its vasodilator activity.

Introduction

The relaxation of many vascular smooth muscle preparations induced by various pharmacological agents has been shown to be dependent upon the presence of a functional endothelium (Furchgott & Zawadzki, 1980; Furchgott, 1981; De Mey & Vanhoutte, 1981; Altura & Chand, 1981; Chand & Altura, 1981; Cherry *et al.*, 1982; De Mey *et al.*, 1982; Rapoport & Murad, 1983). The substance released from endothelium is thought to be arachidonic acid (AA) or some other unsaturated fatty acid, since an inhibitor of phospholipase A_2 inhibited the endothelium-dependent, agonist-induced relaxation (Furchgott & Zawadzki, 1980). It has also been postulated that endothelium-derived vascular relaxant factor (EDRF) may be a lipoxygenase product(s) of AA, since anoxia and inhibitors of lipoxygenase inhibited the relaxation, but cyclo-oxygenase inhibitors did not (Furchgott & Zawadzki, 1980; Furchgott, 1981). Recent evidence suggests that relaxation induced by EDRF occurs concomitantly with a rise in the levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the vascular smooth muscle cells, whereas cyclic AMP levels were unaltered (Holzmann, 1982; Rapoport & Murad, 1983; Diamond & Chu, 1983; Ignarro *et al.*, 1984; Martin *et al.*, 1985). As mentioned above, although properties of the relaxation response mediated by EDRF are well documented, it is unknown whether or not EDRF affects platelet function. The present experiments were, therefore, undertaken to investigate the effects of EDRF on platelet aggregation *in vitro*. Here, we propose a hypothesis that EDRF plays a role as one of the physiological inhibitors of platelet aggregation in addition to its vasodilator activity.

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Methods

Cascade perfusion and superfusion experiments

Descending thoracic aortae were excised from male albino rabbits weighing 2.2 to 2.6 kg, and trimmed free

of adhering fat and connective tissue. Special care was taken to avoid unintentional rubbing of the intraluminal surface. A transverse ring, width 2.5 mm, was cut off with a razor blade and a transverse strip made by cutting across the ring. Cascade perfusion and superfusion experiments were performed as follows: 7 cm of the descending thoracic aorta (with side branches tied) was perfused intraluminally and separately superfused with oxygenated (95% O_2 plus 5% CO_2) Krebs solution at 37°C at a constant flow rate of 6 ml min^{-1} (Varioperpex II pump, LKB, Sweden), and the transverse strip was superfused with the intraluminal effluent. Isometric changes in tension of the transverse strip were recorded through a force-displacement transducer (SB-1T, Nihon Kohden Kogyo Co.). In these experiments, the transverse strips were denuded of endothelium (Furchgott & Zawadzki, 1980; De Mey & Vanhoutte, 1981) to avoid any complicating effects of endothelium and used to bioassay EDRF released from aorta. In some experiments, the endothelium of the aorta was mechanically removed by rubbing the intraluminal surface of the aorta with a small cotton ball wetted with Krebs solution. Electron microscopy performed on such treated tissues clearly showed that only the endothelial cells were removed by this technique; the underlying structures, including the vascular smooth muscle cells, were not damaged. Removal of endothelium by the above method resulted in a complete loss of the relaxation response of the transverse strips to a perfusion of acetylcholine (ACh) and often (at the same concentration of 10^{-6}M which formerly produced relaxation) a contractile response was noted. In order to inhibit the release and/or production of EDRF, the aorta was perfused with Krebs solution containing 10^{-5}M mepacrine or 10^{-5}M nordihydroguaiaretic acid (NDGA) in addition to 10^{-6}M noradrenaline (NA) for 5 min before stimulation with 10^{-6}M ACh. The composition (in mM) of the Krebs solution was: NaCl 115.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0 (pH 7.4).

Platelet aggregation study

Platelet aggregation was studied as described previously (Azuma *et al.*, 1984). In brief, rabbit blood was collected into 1/10 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 90 *g* for 15 min at room temperature, and platelet-poor plasma (PPP) was obtained by further centrifugation at 1000 *g* for 15 min. Platelet aggregation was studied by the turbidometric method of Born (Born, 1962) utilizing a dual sample aggregometer (Sienco, DP-247E) which was modified to provide continuous stirring and maintenance of a constant temperature (37°C). The standard reaction mixtures consisted of 200 µl of citrated PRP, 200 µl (unless otherwise stated) of the effluent (or 200 µl of the Krebs solution containing 10^{-6}M NA and 10^{-6}M ACh for the control) and 4 µl of arachidonic acid (AA). The effluent (200 µl) was collected for 2 s into the cuvette containing 200 µl of the citrated PRP, which was preincubated for 3 min at 37°C, after ACh had been introduced into the circuit so as to perfuse aorta, stimulate aortic production of EDRF and relax the transverse strip. AA at the minimum effective concentration (40 to 130 µM, mean = 70.0 ± 3.1 µM, $n = 59$), which was determined for each experiment, was added immediately (within 1 s) after the addition of the effluent or the control medium. The extent of aggregation was evaluated by the maximum change of light transmission and expressed as a percentage; the difference between light transmission for PRP and PPP was taken as 100%. Percentage inhibition of aggregation by the effluent was calculated by dividing the percentage aggregation with effluent by the aggregation observed in the control run, then multiplying by 100. All individual tests were carried out in rapid succession (within 3 h of preparation) to avoid potential errors which might occur due to alterations in the sensitivity of the platelets towards AA during storage at room temperature. The number of platelets was determined with the aid of a Coulter Counter (Coulter Electronics Inc., Hialeah, Florida, USA).

Chemicals

The following chemicals were used: acetylcholine chloride (ACh, Nakarai Chemicals Co.), indomethacin (Merck), (−)-noradrenaline bitartrate (NA), mepacrine, nordihydroguaiaretic acid (NDGA) and arachidonic acid (AA, all from Sigma). AA was dissolved in ethanol and diluted with an aqueous solution of Na_2CO_3 (2 mol of Na_2CO_3 corresponded to 1 mol AA), stirred on ice and kept at −20°C until use. Indomethacin was dissolved in dimethylsulphoxide (DMSO, Merck), which was present in a final concentration of 0.5% or less in experiments with this agent; this concentration of DMSO had no effect on any of the parameters studied.

Statistical analysis

Results shown in the text, Figures and Tables are expressed as the mean ± s.e. For statistical evaluation, data were analysed by Student's *t* test.

Results

When 10^{-6}M ACh was introduced into the circuit so as to perfuse the aorta already under perfusion with 10^{-6}M NA, the effluent had a relaxant effect of

Table 1 Responses of aortic transverse strips and arachidonic acid (AA)-induced platelet aggregation in the absence or presence of a functional endothelium in aortae, and effects of mepacrine and nordihydroguaiaretic acid

Treatment of aortae	n	*Responses of transverse strip*[a]		*AA-induced aggregation* (%)	
		Contraction (mg)[b]	*Relaxation* (mg)[c]	*Control*[d]	*Effluent*[e]
Unrubbed	20	2846.0 ± 221.4	488.5 ± 77.7	71.7 ± 2.3	3.6 ± 1.4**
Rubbed	20	3102.0 ± 212.3	−221.8 ± 68.2	71.6 ± 1.6	71.4 ± 1.4
Unrubbed MC[f]	9	3178.9 ± 215.3	−191.1 ± 35.5	66.1 ± 2.5	66.5 ± 3.2
Unrubbed NDGA[g]	10	2734.0 ± 342.3	−38.0 ± 17.6	70.8 ± 4.1	65.7 ± 5.1

Each value is given as mean ± s.e. $^{**}P < 0.005$ vs. control. [a]Transverse strips were denuded of endothelium. [b]Contraction induced by the perfusion of 10^{-6}M noradrenaline (NA). [c]Relaxation induced by the perfusion of 10^{-6}M acetylcholine (ACh). Negative values show contraction. [d]200 μl of Krebs solution containing 10^{-6}M NA and 10^{-6}M ACh was used as control. [e]200 μl of the effluent from the stimulated and perfused aorta. [f,g]Aorta was perfused with 10^{-6}M NA in combination with 10^{-5}M mepacrine (MC) or 10^{-5}M nordihydroguaiaretic acid (NDGA) for 5 min before stimulation of EDRF with 10^{-6}M ACh (see text).

17.2 ± 2.4% ($n = 20$) against the NA-induced contraction of the transverse strips which had been denuded of endothelium. The relaxation was seen only when the endothelium of the aorta was intact. It was unaffected by a continuous perfusion with 10^{-5}M indomethacin (data not shown) and could be inhibited by adding 10^{-5}M mepacrine or 10^{-5}M NDGA to the perfusion medium (Table 1). Removal of endothelium of the aorta and addition of mepacrine or NDGA to the perfusion medium resulted in a complete loss of the relaxation response of the transverse strips to ACh, and often a contractile response was noted.

The effluent from the perfused aorta which was capable of relaxing the transverse strip significantly inhibited the platelet aggregation induced by AA. The magnitude of the inhibitory activity of the effluent was changed when the transit time between the end of the aorta and the tip of the outlet was altered by changing the length of the intervening tube. As shown in Figure 1a, the inhibitory activity of 96.1 ± 1.5% ($n = 20$) at

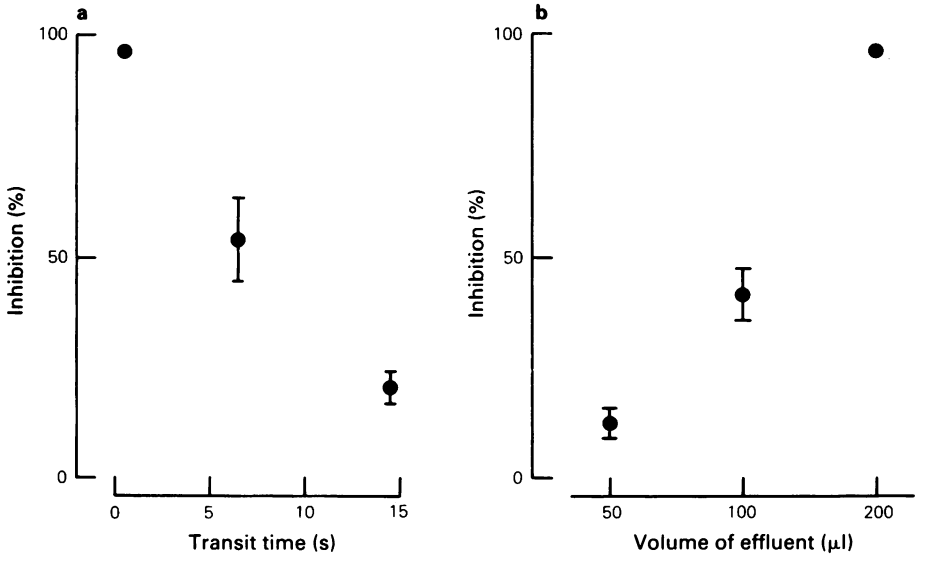

Figure 1 (a) Relationship between inhibitory activity on platelet aggregation and (a) transit time and (b) volume of effluent. Transit time between the end of aorta and the tip of outlet was altered by changing the length of the intervening tube (see text). To determine the inhibitory effect on arachidonic acid (AA)-induced platelet aggregation 200 μl of the effluent from the perfused aorta was used. In (b) transit time was fixed at 0.5 s. Vertical bars show s.e.

0.5 s transit time decreased to 54.0 ± 9.2% ($n = 6$) at 6.5 s and 20.5 ± 3.2% ($n = 6$) at 14.5 s. Figure 1b shows the relationship between the volume of the effluent and the inhibitory activity on platelet aggregation. It was found that AA-induced platelet aggregation was inhibited by the effluent in a volume-related manner (50–200 μl). The inhibition induced by 50, 100 and 200 μl of the effluent was determined to be 12.4 ± 3.1% ($n = 10$), 41.8 ± 5.7% ($n = 9$) and 96.1 ± 1.5% ($n = 20$), respectively. However, the aggregation was little affected by volumes of effluent of 20 μl or less. In addition, 200 μl of the effluent from the unstimulated perfused aorta did not produce a detectable change in AA-induced aggregation. The inhibition of aggregation induced by the effluent was not observed after removal of endothelium from the aorta, or by pretreatment of aorta with 10^{-5}M mepacrine or 10^{-5}M NDGA (Table 1). Pretreatment of platelets with mepacrine or NDGA at the concentration tested failed to modify AA-induced aggregation (Table 2).

Table 2 Effects of mepacrine and nordihydroguaiaretic acid (NDGA) on arachidonic acid (AA)-induced aggregation

Treatment	n	*Aggregation (%) Without agent*	*With agent*
Mepacrine 10^{-5}M	9	64.8 ± 4.2	63.4 ± 4.5
NDGA 10^{-5}M	10	70.5 ± 3.3	70.2 ± 3.6

Results are given as mean ± s.e.

Discussion

The existence of an endothelium-derived vascular relaxant factor (EDRF) was postulated by Furchgott and colleagues (1980, 1981) when they observed that ACh paradoxically relaxed preconstricted aortic strip preparations by an endothelium-dependent mechanism. In the present cascade perfusion and superfusion experiments, the fact that rubbing the intraluminal surface of the aorta resulted in a complete loss of the relaxation response of the transverse strip strongly suggests that the endothelial cells of the aorta are necessary for the relaxation of the superfused transverse strip induced by the effluent (see Griffith *et al.*, 1984).

The effluent from the perfused aorta which was capable of relaxing the transverse strips significantly inhibited AA-induced platelet aggregation. However, this inhibition of aggregation, as well as the relaxation of the transverse strip with the effluent, were not observed after removal of endothelium from the aorta or after pretreatment of aorta with mepacrine, an inhibitor of phospholipase A_2 (Flower & Blackwell, 1976) or NDGA, an inhibitor of lipoxygenase (Hamberg, 1976). These findings led us to postulate that not only the relaxation of the transverse strips but also the inhibition of AA-induced platelet aggregation by the effluent are produced by an endothelium-dependent mechanism and might be mediated by EDRF, a lipoxygenase product(s) of AA (or some other unsaturated fatty acid). This hypothesis appears to be supported in part by the proposal that ACh, acting on the muscarinic receptors of the endothelial cells, somehow activates a reaction sequence in which AA (or some other unsaturated fatty acid) is liberated and then oxidized by lipoxygenase to a product that is responsible for the relaxation of the vascular smooth muscle cells as anoxia, lipoxygenase inhibitors and phospholipase A_2 inhibitors all produce inhibitory effects (Furchgott & Zawadzki, 1980; Furchgott, 1981).

It is well known that the endothelium plays a key role in the production of prostacyclin in the arterial wall (Moncada *et al.*, 1977; MacIntyre *et al.*, 1978) and that prostacyclin is the most potent endogenous inhibitor of platelet aggregation yet discovered (Moncada *et al.*, 1976). However, the generation of prostacyclin is unlikely to be involved in the inhibitory effect of the effluent on AA-induced platelet aggregation as indomethacin, a cyclo-oxygenase inhibitor, has no effect on the ACh stimulated relaxation response (Furchgott & Zawadzki, 1980; Furchgott, 1981; De Mey & Vanhoutte, 1981). In addition, this speculation is also corroborated by the finding that ADP-induced aggregation was still significantly inhibited by effluent from the perfused and stimulated aorta in the presence of 10^{-5}M indomethacin (Azuma, unpublished observation) and that pretreatment of aorta with NDGA, a lipoxygenase inhibitor (Hamberg, 1976), abolished the anti-aggregatory effect of the effluent.

Recent evidence suggests that when relaxation induced by EDRF occurs the levels of cyclic GMP in the vascular smooth muscle cells increase; whereas cyclic AMP levels are unaltered (Holzmann, 1982; Rapoport & Murad, 1983; Ignarro *et al.*, 1984; Martin *et al.*, 1985). The detailed mechanisms of the inhibitory action of EDRF on platelet aggregation remain to be clarified, especially, in relation to cyclic GMP levels in platelets.

Finally, we propose that endothelium-derived vascular relaxant factor (EDRF) plays a role as one of the physiological inhibitors of platelet aggregation in addition to its vasodilator activity.

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(Received October 30, 1985.
Revised January 9, 1986.
Accepted January 29, 1986.)