

Endothelium Derived Relaxant Factor

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The phenomenon of endothelium mediated vasodilatation has become apparent only in the last few years. It is likely to be of considerable physiological importance, though there is much that is yet unknown about it.

We first became aware of it when we were developing an isolated coronary artery preparation, as a more appropriate model than conventional strip preparations, with which to investigate vasomotor control mechanisms in large coronary arteries. It is, of course, the large coronary arteries that are particularly relevant to the pathogenesis of coronary artery disease in man. The preparation consisted of an isolated intact left coronary artery of the rabbit, perfused at constant flow, with monitoring of pressure as a measure of constrictor tone. Initially we observed the expected increase in perfusion pressure upon infusion of a variety of vasoconstrictor agents, but as we gained experience with the preparation we became less able to elicit these conventional constrictor responses. We then noted that localised damage to the arterial wall in an otherwise unresponsive artery would result in a localised response[1]. The nature of this phenomenon became clear when in 1980 Furchgott published his evidence that endothelium possesses vasodilator properties[2]: he showed that acetylcholine, generally regarded as an arterial constrictor, exerted a paradoxical dilator effect if the vessel wall endothelium was carefully preserved during preparation (Fig. 1). We therefore developed ways of preserving or removing endothelium in our preparation, validated always by *en face* silver staining, and proceeded to study its contribution to vasomotor control.

Using the perfused coronary artery preparation of the rabbit we compared concentration-response curves, with and without endothelium, to a number of physiologically relevant constrictors—histamine, acetylcholine, 5-hydroxytryptamine and phenylephrine. The influence of the endothelium was striking: it markedly suppressed, to the point of almost abolishing, the constrictor responses (Fig. 2). We also found that deliberate localised damage to the artery made it susceptible to localised constriction—reversible, reproducible and non-specific. Local endothe-

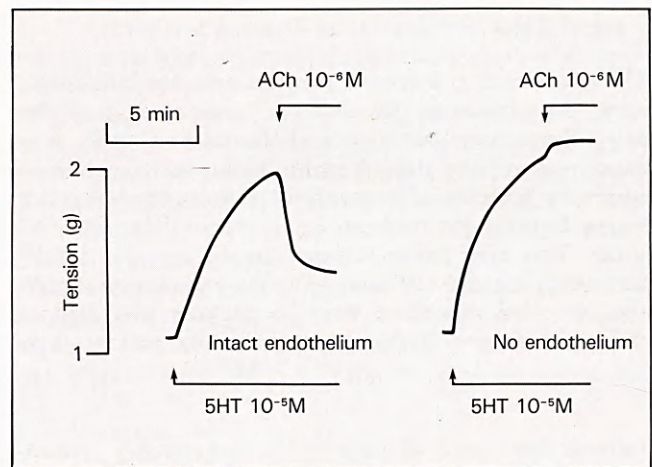


Fig. 1. Effect of acetylcholine (ACh) ($10^{-6}M$) in aortic strips precontracted by 5-hydroxytryptamine (5HT). In preparations with an intact endothelium (left panel) relaxation occurs: in preparations denuded of endothelium (right panel) further constriction occurs.

lial damage had converted one dose-response into the other and, in effect, had reproduced a model of 'coronary spasm'.

Parallel experiments were performed in conventional isometrically mounted aortic strip preparations of the rabbit—again with and without endothelium (Fig. 3). In this preparation, by contrast to the perfused coronary preparation, the endothelium exerted relatively little influence on the responses. Statistical comparison showed that there was nevertheless a significant difference between the responses with and without endothelium, albeit in the opposite direction with all the constrictors except acetylcholine which was known from Furchgott's work to stimulate the endothelium-dependent dilator response. The paradoxically greater constrictor response to the other three agents in the presence of intact endothelium was subsequently explained by the fact that the response starts from a lower baseline in the presence of endothe-

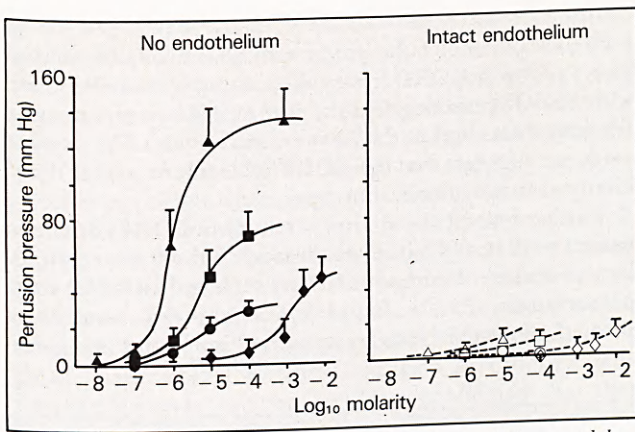


Fig. 2. Coronary artery responses to histamine (\blacktriangle), acetylcholine (\blacksquare), 5-hydroxytryptamine (\bullet) and the α_1 -agonist phenylephrine (\blacklozenge) are markedly depressed by the presence of intact endothelium (right panel), compared to responses in preparations denuded of endothelium (left panel).

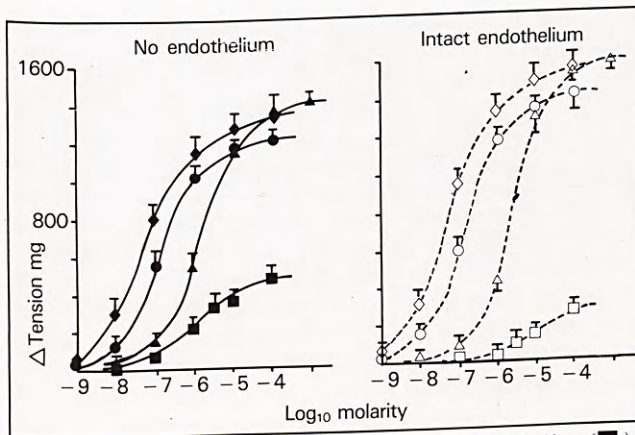


Fig. 3. Aortic responses to histamine (\blacktriangle), acetylcholine (\blacksquare), 5-hydroxytryptamine (\bullet) and phenylephrine (\blacklozenge) are relatively similar in the presence of endothelium (right panel) and in its absence (left panel). Responses to acetylcholine are depressed in the presence of endothelium, but those to the other agents are (paradoxically) enhanced by the presence of endothelium.

um, due to basal activity of the endothelium-dependent dilating effect, and that the ceiling of the constrictor response is the same with or without endothelium, as the constrictor response over-rides the basal endothelium derived relaxant factor (EDRF) activity[1].

The phenomenon of endothelium-dependent vasodilatation is likely to be of general physiological relevance: it has been demonstrated in all mammalian species so far studied, including man, and in all types of blood vessel. Our data with the coronary arteries show furthermore that it can be very potent. Its underlying mechanism has become a subject of intensive investigation.

The Nature of EDRF

Furchgott postulated that the phenomenon was mediated by a humoral agent released from endothelial cells. To

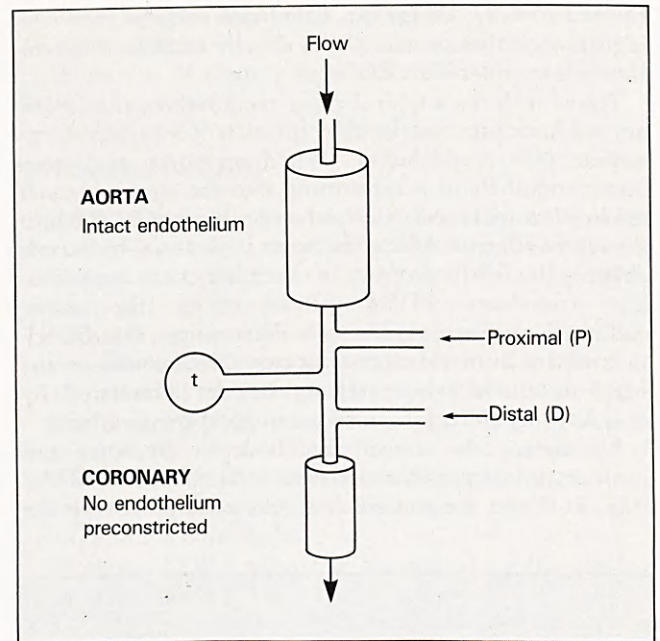
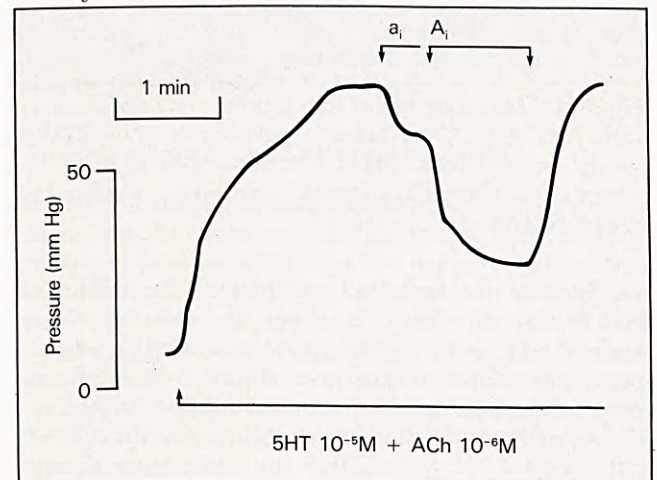


Fig. 4. Diagram of bioassay system. t = pressure transducer.

test such a hypothesis we developed a bioassay for the putative vasodilator substance (or EDRF)[3]. Figure 4 shows the bioassay system schematically. An intact aorta possessing endothelium is perfused in series with a coronary artery which has been denuded of endothelium and precontracted by the infusion of a constrictor agonist. The pressure response of the coronary artery allows detection of vasomotor substances in the coronary perfusate. The length of the intervening tubing can be altered to give a range of transit times between aorta and

Fig. 5. Representative trace of a bioassay experiment. The coronary artery is precontracted by infusion of 5HT ($10^{-5}M$) and acetylcholine (ACh) ($10^{-6}M$). a_i = introduction of unstimulated aorta into circuit. A_i = stimulation of aorta by ACh, infusion of which is transposed from post- to pre-aortic (see Fig. 4). Basal release from an unstimulated aorta and enhanced release from stimulated aorta are shown.



coronary artery. Drugs can be infused into the intervening tubing either proximally or distally to allow different interaction times with EDRF in transit.

Figure 5 shows a typical experiment, where the coronary has been precontracted by infusion of 5-hydroxytryptamine plus acetylcholine. When an aorta possessing intact endothelium is introduced into the circuit a small fall in pressure is seen, due to basal release of EDRF into the aortic effluent. When the aorta is perfused by acetylcholine, the fall in pressure is much larger, as acetylcholine stimulates EDRF release from the aortic endothelium. Such experiments demonstrate that EDRF is indeed a humoral agent released continuously in the basal state and whose release can be stimulated by acetylcholine (for up to an hour in good preparations).

By altering the transit time between the aorta and coronary it was possible to measure the half-life of EDRF (Fig. 6). From the straight line relationship between the

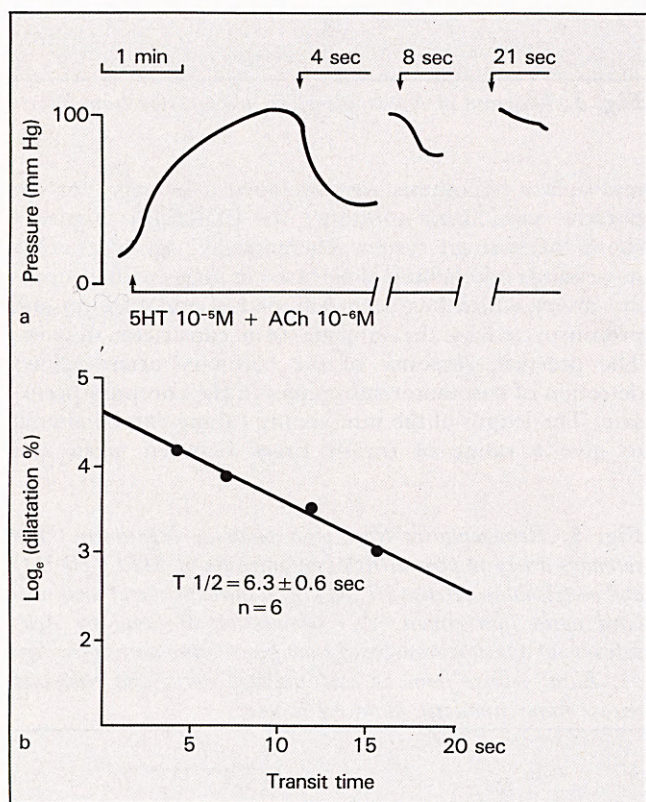


Fig. 6(a). Increasing transit time between aorta and coronary artery from 4 to 21 sec causes a marked fall off in EDRF induced vasodilatation. **(b)** A logarithmic plot of dilatation against transit time yields a straight line allowing calculation of EDRF half-life as 6.3 ± 0.3 sec.

logarithm of dilatation and transit time, the half-life of EDRF was calculated as about six seconds. Other workers have subsequently performed similar experiments using different experimental models and different mammalian species, and found an almost identical half-life: 5.4 ± 0.4 sec for EDRF from cultured bovine cells[4], and 6.3 ± 1.2 sec with EDRF from dog femoral arte-

ries[5]. Recent data indicate that the exact half-life is influenced by molecular oxygen and has been obtained as 24 ± 3 sec with EDRF from rabbit aorta[6] and 49 ± 5 sec with EDRF from dog femoral arteries[6] at lower oxygen tensions than used in the above experiments. The present evidence suggests that the EDRF molecule is similar if not identical in all mammalian species.

To characterise the chemical nature of EDRF, different agents were tested to see which might inhibit its action. A large number of compounds was screened in aortic strip preparations. Those found to be effective were then studied using the bioassay system (Fig. 7) to distinguish

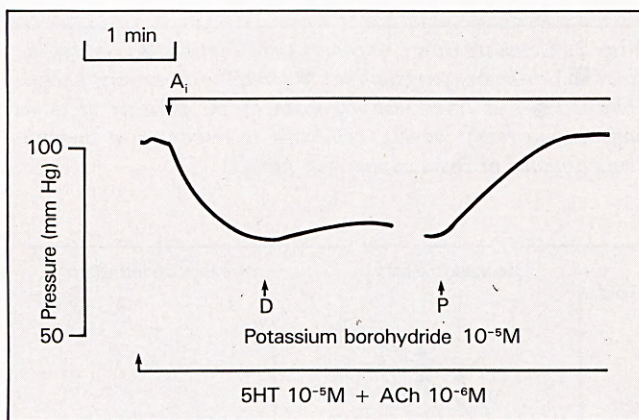


Fig. 7. Bioassay experiment, showing dilator response to EDRF released from stimulated aorta (A_i) and the effect of infusing the EDRF inhibitor, potassium borohydride, into the intervening tubing. Distal infusion (D) to allow 0.5 sec interaction time has little effect. Proximal infusion (P) to allow a 4 sec interaction time results in complete inhibition. Phenylhydrazine and antioxidant inhibitors gave similar results.

those which interacted chemically with EDRF in transit from those which influenced its production or its action on smooth muscle. The two chemical properties that emerge as common to agents which inhibited endothelium-mediated relaxation by direct chemical interaction with EDRF are that they are either antioxidants or combine with carbonyl groups—properties that are mutually consistent. Analysis of concentration-inhibition curves provides evidence that the interaction of EDRF with the four inhibitors—phenylhydrazine, potassium borohydride, dithiothreitol and phenidone—obeys first-order kinetics[7]. We were also able pharmacologically to exclude the possibility that EDRF is a cyclo-oxygenase product, such as prostacyclin, or a lipoxygenase product, such as a leucotriene. We conclude that EDRF probably possesses a carbonyl group at or near its active site[3]. However, the chemical identity of this unstable agent remains to be defined.

Mechanisms of Action of EDRF

There is now a substantial body of evidence that nitro-dilators such as glyceryltrinitrate and nitroprusside act by elevating smooth muscle cyclic guanosine monophos-

phate (cGMP) levels[8, 9]. Their physiological and biochemical effects can be inhibited by methylene blue (which inhibits the enzyme guanylate cyclase)[8, 9] and potentiated by MB22948 (which prevents cGMP hydrolysis by inhibiting the enzyme cGMP phosphodiesterase)[9, 10]. Evidence that EDRF might act in an analogous manner appeared when Austrian and American workers demonstrated elevation of arterial cGMP when endothelium was stimulated to produce EDRF[11, 12]. Furthermore, EDRF and nitro-dilator-induced relaxation are associated with identical alterations in the phosphorylation[13] of proteins, including contractile proteins. Using these pharmacological probes infused at different sites into the bioassay system, we have confirmed that they influence the action of EDRF at the level of the smooth muscle response (Fig. 8) and shown that they do not exert

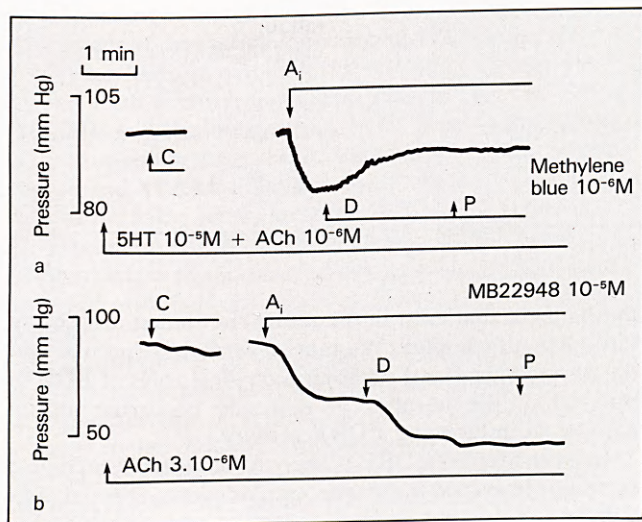


Fig. 8. Bioassay experiment showing effect of (a) methylene blue and (b) MB22948. Left traces show negligible effects of adding these agents solely to the coronary perfusate (control, C). Right traces show EDRF mediated dilatation by effluent from a stimulated aorta (A_i), with addition of agents distally (D) or proximally (P) into the intervening tubing. In each case the effects of proximal and distal infusion are identical (unlike those of direct EDRF inhibitors, Fig. 7).

an additional effect on EDRF release or by interacting with EDRF in transit[14]. It thus appears that EDRF behaves functionally as an endogenous 'nitrite'. Relevant to our hypothesis that EDRF contains a carbonyl group is the finding that carbonyl agents stimulate guanylate cyclase[15].

The intracellular control of tension development in smooth muscle is inadequately understood. cGMP is thought to mediate one mechanism of relaxation through altered phosphorylation of contractile proteins. We have recently shown that EDRF-mediated dilatation is also associated with a reduction of net influx of calcium, which might contribute to its mode of action[16]. This finding may explain the apparently greater sensitivity of the coronary artery than the aorta to the dilator action of EDRF[1]: vasoconstriction in the rabbit coronary artery

is very largely dependent on influx of extracellular Ca^{++} , whereas in the aorta it is more dependent on mobilisation of Ca^{++} from intracellular sites (unpublished observations).

Production of EDRF

Basal release can be demonstrated from the rabbit aortic preparation, and its stimulated release can likewise be maintained for up to an hour. Endothelium-dependent relaxation has been found to be stimulated by a large number of agents, with some differences between different vessels and different species (Table 1). Consideration of the naturally occurring substances which appear in this list must provide pointers to the possible physiological role of EDRF. It should perhaps be noted that not all observed endothelium-dependent dilatation has yet been confirmed by bioassay as indeed being due to EDRF, though this seems likely.

Notably, EDRF release is stimulated by the calcium ionophore A23187. We have confirmed that it is dependent on the presence of extracellular calcium[33]: in bioassay experiments its production can be abruptly stopped by removal of extracellular calcium and equally rapidly restored by its replacement (Fig. 9). It is not known if calcium is required for *de novo* biosynthesis or for release of stored EDRF from intracellular vesicles. The presence of some oxygen also appears to be a necessary requirement[2].

Duration of Effect

The half-life of EDRF in well oxygenated aqueous buffer at 37°C is 6 sec. The duration of its effect in the vascular compartment *in vivo* is almost certainly much shorter. We have shown that a heat labile component of plasma blocks EDRF activity in aortic strips (unpublished observations). Endothelial permeability is increased under these unphysiological *in vitro* conditions so that plasma proteins probably penetrate the endothelial barrier[34]. The implication is that EDRF will be rapidly inactivated in the intravascular compartment *in vivo*, so localising its effect to adjacent smooth muscle, with no downstream effect. Therefore EDRF may be regarded as an autocoid.

It has recently been observed that the vascular effects of EDRF can be inhibited by $>10^{-7}M$ free haemoglobin[35], an observation which may be relevant in a number of pathological conditions.

Possible Endogenous Analogues of EDRF

It is of considerable interest that an inhibitory neurotransmitter isolated from retractor penis and anococcygeous muscle of a number of species has many characteristics in common with EDRF[36]. It relaxes both vascular and non-vascular smooth muscle[37], and is thought to do so by elevating smooth muscle cGMP levels[38]. It is also known to be unstable and appears to be a carbonyl compound[39]. EDRF may therefore represent one of a family of physiologically important and previously undescribed substances.

Table 1. EDRF stimulation in different species.

EDRF Stimulant	Receptor type/ mechanism	Species
A23187 (calcium ionophore)	? increased calcium influx	man[17], rabbit[3, 18, 19]
acetylcholine	muscarinic	man[17], rabbit[2], guinea-pig[2], cat[2], rat[2], dog[20]
adenosine diphosphate (ADP)	P ₂	rabbit[3, 21], dog[20], pig[22]
adenosine triphosphate (ATP)	P ₂	rabbit[3, 21], dog[20], pig [22]
clonidine	α ₂	dog[23]
ergometrine	not 5HT or α	rabbit[24]
ergometrine	?	man[17]
histamine	H ₁	dog[25], rat[26], NOT rabbit[1]
hydrallazine	?	rabbit[27]
5-hydroxytryptamine	?5HT ₁	dog[24, 28], NOT rabbit[1]
noradrenaline	α ₂	dog[24], pig[24]
thrombin	heparin-sensitive receptor	dog[29]
Peptides:		
vasoactive intestinal polypeptide	VIP	rat[30]
bradykinin	bradykinin	man[31], dog[31], NOT cat[31]
substance P	substance P	rabbit[21], dog[21], cat[21]
cholecystokinin	? substance P	rabbit[21]
Electrical stimulation	? increased calcium influx	rabbit[32], cat[32], monkey[32]
Non-specific:		
saturated/unsaturated fatty acids (may stimulate or block)	? membrane fluidity	rabbit[2], dog[21]

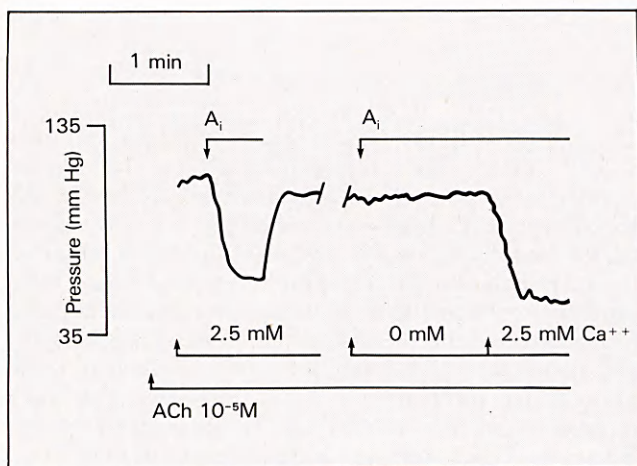


Fig. 9. Bioassay experiment demonstrating dependence of EDRF production on extracellular calcium: left trace shows a control experiment demonstrating EDRF mediated vasodilatation by effluent from the stimulated aorta (A_i) while this is in circuit (horizontal bar). Right trace shows loss of vasodilator response when calcium is omitted from the aortic perfusate (but infused into intervening tubing to maintain normal calcium in coronary perfusate) and demonstrates the immediate recovery of dilator responses (with overshoot) on re-introduction of calcium into aortic perfusate.

Physiological and Pathophysiological Role for EDRF

The physiological role of this newly recognised and potent dilator substance remains to be established, as does its potential role in disease states. The vasomotor control

mechanisms that exist in the intact circulation are clearly multiple and complex. We may nevertheless speculate on the physiological and pathophysiological roles of EDRF, mindful of the number of naturally occurring agents capable of influencing EDRF activity.

In large arteries EDRF is likely to act as a physiological regulator in response to intravascular events. Thrombosis will expose the endothelium to agents such as adenosine diphosphate (ADP) and 5-hydroxytryptamine (5HT) which are released from platelets and are known to stimulate EDRF. This would represent a short-term negative feedback. That such a phenomenon may be important *in vivo* is suggested by tissue bath experiments showing that arterial smooth muscle relaxes in response to aggregating platelets when intact endothelium is present and contracts when the endothelium is removed[40]. The possibility of local spasm where endothelium is functionally impaired, particularly in arteries susceptible to its action such as the coronary, may be relevant to pathogenetic mechanisms in variant angina. We have shown that ergometrine maleate, an agent used to provoke coronary artery spasm in susceptible patients, is able to stimulate EDRF release[25]; functional impairment of endothelium and consequent lack of EDRF effect may therefore explain its usefulness in this test. Inactivation of EDRF by haemoglobin and plasma proteins may be relevant to the prolonged arterial spasm which is sometimes seen after subarachnoid haemorrhage.

Endothelium-dependent dilatation has been described in relation to flow rate[41], acting presumably through shear stress on the endothelium, though it has not yet been proved that this phenomenon is mediated by EDRF itself. Flow-dependent endothelium-mediated dilatation

may be an important homeostatic mechanism coupling large artery calibre to changes in microvascular resistance, thus optimally distributing shear stress at the blood-intimal interface throughout the vascular tree.

The influence of EDRF activity on resistance vessels has not been adequately investigated. Peptides such as vasoactive intestinal polypeptide (VIP), whose action is endothelium-dependent in large arteries at low concentrations[30], have been demonstrated histochemically in nerve endings found in the adventitia around small vessels[42]. It is possible therefore that there is neurogenically-mediated control of EDRF in the microvasculature, the stimulus in this case arriving from outside these small vessels where diffusion distance is small. Concentrations of VIP known to stimulate EDRF release *in vitro* can be detected in the effluent from some isolated organ preparations when their nerve supply is stimulated and there is accompanying vasodilation[43]. This action may be a component of the complex neurogenically mediated vasomotor mechanism of penile erection[44]. Substance P, derived from sensory nerve terminals, is also a stimulant of EDRF and has been shown in rat hind limb preparations to mediate neurogenic vasodilatation[45]. Acetylcholine and VIP have been shown histochemically to co-exist in the same nerve terminals[42] and of course both are potent stimulants of EDRF. Does EDRF participate in the ultimate mechanism of the neurogenic vasodilatation that results from vagal activity, as in vasovagal syncope?

It is intriguing to speculate whether alterations in EDRF activity play any part in atherogenesis. Experimental atheroma is associated with increased calcium influx, can be induced experimentally by alteration of calcium metabolism and prevented by calcium antagonists[46, 47]. We have shown that EDRF reduces calcium influx *in vitro* as measured with ⁴⁵Ca flux studies. Conversely, the presence of atheroma, an intimal disease, may itself affect EDRF activity, either by altering its production or by interposing a physical barrier between the endothelium and the smooth muscle. Indeed, it has recently been reported that endothelium-dependent relaxation is diminished by atheroma in both human coronary arteries[17] and in rabbits fed a cholesterol-supplemented diet[48].

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