Effects of basal and acetylcholine-induced release of endothelium-derived relaxing factor on contraction to α -adrenoceptor agonists in a rabbit artery and corresponding veins

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- 1 The effects of an endothelium-dependent (acetylcholine) and an endothelium-independent (sodium nitroprusside) relaxant against noradrenaline-induced contractions were compared in three isolated superficial blood vessels of the rabbit, the lateral saphenous vein, plantaris vein and distal saphenous artery. Both produced concentration-related relaxations of all three vessels and were more effective against sub-maximal than maximal contractions to noradrenaline. Transient contractions to high concentration of acetylcholine occurred only in endothelium-intact preparations of saphenous vein and were inhibited by flurbiprofen.
- 2 In endothelium-denuded preparations sodium nitroprusside was 3 times more effective than in endothelium-intact preparations, while acetylcholine ($<3\ \mu\text{M}$) was inactive. Sensitivity was similar for each relaxant: lateral saphenous vein $\geq$ plantaris vein $>$ distal saphenous artery. The similar profile of sodium nitroprusside and acetylcholine suggests that differences in susceptibility to endothelium-derived relaxing factor (EDRF) are caused by inter-vessel variations in the excitation-coupling process for noradrenaline.
- 3 Haemoglobin inhibited acetylcholine-induced relaxations in the endothelium-intact preparation of the lateral saphenous vein and distal saphenous artery, which suggests a similar EDRF in each preparation and the likelihood that this is a single substance, presumably nitric oxide.
- 4 The influence of basal, spontaneously released EDRF on α -adrenoceptor function was tested either by mechanical disruption of the endothelium or by adding haemoglobin to endothelium-intact segments. Endothelial disruption slightly reduced contractions to noradrenaline (NA) in distal saphenous artery but increased response size of lateral saphenous and plantaris veins, in the latter also increasing sensitivity to NA: haemoglobin mimicked endothelial disruption. Thus, basal release of EDRF like acetylcholine and nitroprusside was more effective in the veins than in the corresponding artery.
- 5 In lateral saphenous vein responses to phenylephrine were enhanced by endothelial disruption, but without change in sensitivity: responses to UK-14304, B-HT 920 and cirazoline, which had a relatively slow speed of onset of contraction were not affected. There was no correlation between enhancement and α -adrenoceptor sub-type although the agonists which were enhanced all activate α_1 -adrenoceptors. Competitive antagonists failed to reveal an α -adrenoceptor subtype enhanced by endothelial disruption. However, effects of phenoxybenzamine suggest that α_1 -adrenoceptors are necessary for the influence of basal EDRF.

Introduction

In addition to mediating the effects of various vasodilator hormones, the endothelium spontaneously releases endothelium-derived relaxing factor (EDRF) which modulates the responsiveness of vascular smooth muscle to constrictor stimuli. Mechanical removal of the endothelium in the rat isolated thoracic aorta is associated with an increase in the maximum contraction to phenylephrine and an increase in both the maximum response and potency of various partial agonists at α -adrenoceptors (e.g. clonidine, B-HT 920) (Lues & Schümann, 1984; Godfraind *et al.*, 1985). Although part of this effect has been attributed to α_2 -adrenoceptors on the endothelium which mediate the release of EDRF (Bullock *et al.*, 1987), it is clear that there is basal release of EDRF from the endothelial cells. This is supported by the observation that haemoglobin, which binds EDRF (Martin *et al.*, 1986a; Ignarro *et al.*, 1987a), augments α -adrenoceptor-mediated contractions in endothelium-intact preparations and mimics the effect of mechanical removal of the endothelium (Martin *et al.*, 1986b).

However, observations in other large blood vessels provide conflicting evidence regarding the functional significance of the spontaneous release of EDRF. For example, removal of the endothelium from bovine intrapulmonary arteries and veins decreased the size of responses to phenylephrine but increased sensitivity, in canine isolated arteries and veins

vascular responses were again reduced, although sensitivity to noradrenaline was unchanged (De Mey & Vanhoutte, 1982; Ignarro *et al.*, 1987a) and in several rabbit isolated arteries neither responsiveness of the preparations nor the potency of α -adrenoceptor agonists was altered (Oriowo *et al.*, 1987).

There are few reports of a powerful influence of EDRF in veins. This is surprising since EDRF is believed to be nitric oxide (Palmer *et al.*, 1987), and nitrogen-containing vasodilators, e.g. sodium nitroprusside and glyceryl trinitrate, which effect vasodilatation by a common mechanism (stimulation of smooth muscle soluble guanylate cyclase; Ignarro *et al.*, 1987a), have clinical activity generally attributed to a preferential action on venous smooth muscle. The majority of studies to date have concentrated on the action of EDRF on the arterial side of the circulation. Clearly, part of the problem is that many of the endothelium-dependent arterial relaxants generally do not relax, and in some cases contract, endothelium-intact venous preparations. For example, acetylcholine has been reported to be inactive in pre-contracted endothelium-intact segments of the bovine isolated intrapulmonary vein (Ignarro *et al.*, 1987b) and, at high concentrations, to elicit contractions of canine isolated veins by an action on muscarinic receptors present on the smooth muscle (DeMey & Vanhoutte, 1982).

We have recently found that acetylcholine produced endothelium-dependent relaxation of noradrenaline-induced contractions in the rabbit isolated lateral saphenous vein

(Daly *et al.*, 1987) and, in preliminary experiments, observed that this effect was more pronounced than in the corresponding endothelium-intact artery. Quantitative differences in the effectiveness of endothelium-dependent relaxants in various isolated blood vessels can arise from a variety of factors, e.g. a concomitant stimulatory action on the smooth muscle, the release of an endothelium-dependent constrictor substance, differences in the coupling mechanism for the contractile agent, differences in the effectiveness of the coupling between the endothelial receptor and EDRF production or even the release of different EDRFs (Rubanyi & Vanhoutte, 1987; 1988; Siedel & La Rochelle, 1987).

We have now assessed some of these factors in three vessels from rabbit hind-limb, chosen to allow the following comparisons: (i) artery and vein, (ii) influence of EDRF versus responses mediated by different α -adrenoceptor sub-types, viz. lateral saphenous vein, plantaris vein and distal saphenous artery. We have characterized the α -adrenoceptor populations in these vessels (Daly *et al.*, 1988a). Previous comparisons between arteries and vein have used large hind-limb vessels from other species (Siedel & La Rochelle, 1987; Rubanyi & Vanhoutte, 1988). First, in order to chart the basic properties of the preparations, we compared the effectiveness, versus the physiological contractile agonist, noradrenaline, of an endothelium-dependent relaxant (acetylcholine) and an endothelium-independent relaxant (sodium nitroprusside) known to activate smooth muscle guanylate cyclase activity.

We then went on to study the influence of the basal release of EDRF, first by comparing the effect of two procedures known to abolish or limit the effect of the spontaneous release of EDRF on α -adrenoceptor-mediated contractions; mechanical removal of the endothelium and the addition of haemoglobin to endothelium-intact preparations. Our findings indicate that there is qualitative agreement between the effects of the two manoeuvres, with the veins exhibiting greater reactivity following removal of the basal EDRF.

Finally, by the use of 'selective' agonists and antagonists, we examined whether the relative contribution of α_1 - and α_2 -adrenoceptors to contractions in the lateral saphenous vein is altered following removal of the endothelium. A preliminary report suggested that basal release of EDRF in this preparation may exert a preferential inhibition of the function of post-junctional α_1 -adrenoceptors (Daly *et al.*, 1987).

Methods

White albino New Zealand rabbits of either sex weighing 2.3–3.0 kg were killed by stunning followed by exsanguination. Segments of the distal saphenous artery, the lateral saphenous vein and the plantaris vein were cleaned of fat and connective tissue *in situ* and then placed in ice-cold modified Krebs-Henseleit saline. The plantaris vein was taken as the 15 mm distal segment of the continuation of the lateral saphenous vein measured from the ankle and the distal saphenous artery was taken as a 20 mm segment of the saphenous artery (medial side) between the knee and ankle. From each vein or artery 3 mm length segments were prepared and suspended between two 0.2 mm thick wire supports as previously described (Daly *et al.*, 1988a). These preparations are referred to as endothelium-intact segments. An adjacent 3 mm segment of each endothelium-intact preparation was also taken and the endothelial cells mechanically removed. These are referred to as endothelium-denuded segments. This was achieved by inserting one smooth-edged arm of a pair of watchmaker forceps into the lumen of the vessel and gently rolling the moistened segment between the surface of a forefinger and the forceps for 30 s without undue stretching. The upper support was connected by surgical silk to a Grass FT03 isometric transducer while the lower support was connected to a glass tissue holder. Preparations were then mounted in 30 ml isolated organ baths under an initial resting tension of either 1.5 gwt. (distal saphenous artery), or 1.0 gwt. (plantaris vein

and lateral saphenous vein) and allowed to relax. The final resting tension for the artery and veins were 0.7–0.8 gwt. and 0.2–0.3 gwt. respectively. Preliminary experiments established that these were optimal for contractile responses. Each segment was bathed in Krebs saline maintained at 37°C and gassed with 5% CO₂ in O₂.

After a 90 min equilibration period, during which a steady resting tension was achieved, each preparation was exposed to noradrenaline (NA) 3 μ M and allowed to contract until a stable response was attained. Acetylcholine 3 μ M was then added to determine whether the segment was endothelium-intact (relaxation) or denuded (no effect). In approximately 15% of 'endothelium-denuded' segments of the lateral saphenous vein, acetylcholine produced a small relaxation (5–10% of the induced tone) indicating that complete mechanical removal of the endothelial cells had not been effected. However, in view of the pronounced effect of acetylcholine in 'endothelium-intact' preparations (70–80% relaxation of induced tone) these preparations were still employed as 'endothelium-denuded' segments. Abolition of acetylcholine-induced relaxations in the lateral saphenous vein could be effected by more vigorous mechanical rubbing, but this was only achieved at the expense of damage to the underlying smooth muscle, indicated by smaller responses to agonists. Following complete washout, an additional 1 h equilibration period was allowed before starting the experiment. This procedure was found to minimize changes in the sensitivity of the preparation to further addition of NA and the relaxants. Basal tension following the sighting response remained stable for the rest of the experiment; 0.5–0.6 gwt. for the distal saphenous artery and 0.2–0.3 gwt. for the plantaris vein and lateral saphenous vein. Isometric contractions were recorded by a Grass FT03 transducer connected to a Linseis 6025 pen recorder.

Acetylcholine- and sodium nitroprusside-response curves

Segments of the three blood vessels were paired as endothelium-intact and endothelium-denuded and then exposed to 3–10 μ M NA to produce a stable maximal contraction. Cumulative concentrations of acetylcholine (1 nM–3 μ M) or sodium nitroprusside (1 nM–30 μ M) were then added, at a minimum of 3 min intervals or until a stable response was observed, and a concentration-response curve constructed. Preparations were then washed thoroughly with Krebs saline over a 30 min period and a further 30 min was allowed before the next concentration-response curve. A submaximal concentration of NA (0.1–0.2 μ M) was added to each organ bath and the contraction allowed to proceed to a stable response (55–70% of the maximum). The acetylcholine or sodium nitroprusside concentration-response curves were then repeated. Only one relaxant was employed in each preparation and only two response curves constructed. In another series of experiments, the acetylcholine-response curve in endothelium-intact preparations of the blood vessels was constructed both in the absence and presence of either flurbiprofen 1 μ M or haemoglobin 1 μ M.

Removal of the endothelium: comparison with the effect of haemoglobin

A cumulative concentration-response curve (CRC) to NA was constructed in paired endothelium-intact and endothelium-denuded preparations of each blood vessel. The concentration of NA was increased by approximately a half log unit only after the preceding response had peaked or reached an equilibrium. Following attainment of the maximum response, preparations were washed repeatedly with Krebs saline to effect complete relaxation and a 40 min re-equilibration period allowed. Preparations were then exposed to haemoglobin 3 μ M 15 min before repeating the NA CRC.

Removal of the endothelium: correlation of increased response with α -adrenoceptor subtype

Selective agonists A CRC to the selective α_1 -adrenoceptor agonist phenylephrine (McGrath, 1982) and another to the selective α_2 -adrenoceptor agonist UK-14304 (Cambridge, 1981) were constructed in endothelium-intact and endothelium-denuded segments of the three blood vessels, following completion of the control CRC to NA. The order of the synthetic agonists was alternated between experimental days. A similar protocol was also adopted to examine together the contractile responses to the selective α_2 -adrenoceptor agonist B-HT 920 (Kobinger & Pichler, 1981) and the selective α_1 -adrenoceptor agonist cirazoline (Ruffolo & Waddell, 1982) in endothelium-denuded and endothelium-intact segments of the lateral saphenous vein.

Selective antagonists In endothelium-intact and endothelium-denuded segments of the lateral saphenous vein, NA CRCs were repeated in the presence of competitive antagonism by either prazosin $0.1 \mu\text{M}$ (thus leaving a residual α_2 -adrenoceptor mediated response), rauwolsine $2.5 \mu\text{M}$ (residual α_1), or following irreversible inactivation of post-junctional α_1 -adrenoceptors with phenoxybenzamine $0.3 \mu\text{M}$ in combination with rauwolsine $1 \mu\text{M}$ for 'receptor protection' and hence pharmacological isolation of the population of post-junctional α_2 -adrenoceptors (see Daly *et al.*, 1988b).

Analysis of results

All responses have been given as the mean $\pm$ s.e.mean of a minimum of 5 observations. Responses to contractile agonists are expressed either in absolute tension developed (g wt.) or as a percentage of the maximum response in the control CRC: the negative logarithm of the concentration of the agonist required to produce 50% of the maximum contractile response ($-\log \text{EC}_{50}$ or pD_2) was also determined. In this study the term 'responsiveness' has been used to discuss changes in the absolute force generated by agonists (g wt.), while the term 'sensitivity' refers to changes in the pD_2 value for agonists. Responses to inhibitory or 'relaxant' stimuli are expressed in relation to either the NA-induced tone or the maximum relaxation effected. The sensitivity of each preparation to the relaxants has been determined as the negative logarithm of the molar concentration required to produce 50% of the maximum relaxation observed ($-\log \text{EC}_{50}$ or pD_2), by straight line interpolation between bracketing concentrations. The significance of differences between mean values was determined with either a paired or unpaired Student's *t* test as appropriate. Unless otherwise stated, $P < 0.05$ is considered significant.

Solutions and drugs

The composition of the modified Krebs-Hensleit saline was (mm): NaCl 118.4, KCl 4.7, CaCl_2 2.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, NaHCO_3 24.9, KH_2PO_4 1.2 and glucose 11.1. Na_2EDTA ($23 \mu\text{M}$) was included in all experiments to prevent oxidative degradation of NA. Propranolol ($1 \mu\text{M}$) and cocaine ($10 \mu\text{M}$) were also included to inhibit β -adrenoceptors and uptake₁, respectively. The following drugs were used: acetylcholine hydrochloride (Sigma), B-HT 920 (6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo-[4,5-d]azepin-dihydrochloride, Boehringer Ingelheim), cocaine HCl (Macarthys), cirazoline hydrochloride (Synthelabo), sodium flurbiprofen dihydrate (Boots), bovine haemoglobin Type 1 (Sigma), sodium nitroprusside ('Nipride', Roche), (–)-noradrenaline bitartrate (Sigma), phenylephrine hydrochloride (Sigma), (±)-propranolol HCl (Sigma), UK-14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer). Stock solutions of NA were prepared in $23 \mu\text{M}$ Na_2EDTA . Sodium flurbiprofen dihydrate was dissolved in 10% absolute ethanol and subsequent dilutions were made in distilled water. All other drugs

were dissolved in distilled water and added to the organ baths in 0.1 ml volumes. Sodium nitroprusside was prepared on a daily basis and kept on ice in foil-wrapped containers during the course of the experiment. Haemoglobin was prepared by adding to a 1 mM solution of bovine haemoglobin Type 1 (a mixture of haemoglobin and methoglobin) in distilled water a 10 fold excess of sodium dithionite which was then removed by dialysis against 100 volumes of distilled water for 2 h. The solution was then frozen and stored in aliquots at -20°C for up to 14 days.

Results

Acetylcholine and sodium nitroprusside

Figure 1 shows the effect of both acetylcholine and sodium nitroprusside on endothelium-intact and endothelium-denuded segments of the rabbit isolated distal saphenous artery, plantaris vein and lateral saphenous vein activated by both maximal and sub-maximal concentrations of NA. Acetylcholine produced a concentration-dependent relaxation of both maximal and sub-maximal contractions to NA in all three endothelium-intact blood vessels (Figures 1a, b and c). In endothelium-denuded segments of the plantaris vein and distal saphenous artery acetylcholine was without effect on the NA-induced tone at concentrations up to $3 \mu\text{M}$. Based upon the pD_2 for acetylcholine in submaximally activated preparations and the maximum relaxation effected against the high concentration of NA, the rank order of sensitivity of these

Figure 1 A comparison of the effects of acetylcholine (ACh) (left) and sodium nitroprusside (SNP) (right) against maximal and submaximal contractions to noradrenaline (NA) in three isolated superficial blood vessels from the rabbit: (a, d) distal saphenous artery; (b, e) plantaris vein; (c, f) lateral saphenous vein. Endothelium-intact segments are represented by closed symbols and endothelium-denuded segments are represented by open symbols. The degree of muscle activation of each segment are represented by (■ □) for submaximal tone (approximately 55–70% of the maximum contractile response) and (● ○) for maximal tone. For the sake of clarity the effects of acetylcholine in endothelium-denuded segments have been excluded.

Table 1 pD_2 values for the vasodilator action of acetylcholine and sodium nitroprusside against maximal and sub-maximal contractions to noradrenaline in three isolated superficial blood vessels from the rabbit

		<i>Distal saphenous artery</i>		<i>Plantaris vein</i>		<i>Lateral saphenous vein</i>	
		<i>Maximal tone</i>	<i>Sub-maximal tone</i>	<i>Maximal tone</i>	<i>Sub-maximal tone</i>	<i>Maximal tone</i>	<i>Sub-maximal tone</i>
Acetylcholine	Intact	6.82 ± 0.07	6.80 ± 0.13	6.99 ± 0.14	7.23 ± 0.11*	8.16 ± 0.05	8.12 ± 0.07
Sodium nitroprusside	Intact	5.19 ± 0.23	6.47 ± 0.13*	6.65 ± 0.10	7.69 ± 0.19*	7.10 ± 0.15	7.70 ± 0.12*
	Denuded	5.47 ± 0.14	6.85 ± 0.15*	6.95 ± 0.10	8.13 ± 0.19*	7.70 ± 0.15	8.11 ± 0.17*

Acetylcholine failed to produce relaxations in endothelium-denuded segments of the distal saphenous artery and plantaris vein, and small responses (<10%) in lateral saphenous vein.

* Denotes a significant difference ($P < 0.05$) in the potency of the relaxant against maximal and submaximal contractions.

blood vessels to endothelium-dependent relaxation is lateral saphenous vein > plantaris vein > distal saphenous artery (Figure 1, Table 1).

Sodium nitroprusside produced concentration-dependent relaxation of maximal and sub-maximal NA contractions in both endothelium-intact and endothelium-denuded segments of all three blood vessels (Figures 1d, e and f). In general, the endothelium-denuded segments were more sensitive (approximately 3 fold) to sodium nitroprusside than were the endothelium-intact preparations; a feature observed under both maximal and submaximal activation by NA (Table 1). Based upon the pD_2 for sodium nitroprusside in submaximally-activated segments and the maximum relaxation produced against a high concentration of NA, the rank order of the sensitivity of these blood vessels to endothelium-independent relaxation is lateral saphenous vein ≥ plantaris vein > distal saphenous artery (Figure 1, Table 1). Within each vessel the maximum relaxations effected by acetylcholine and sodium nitroprusside against high concentrations of NA in endothelium-intact segments are very similar: lateral saphenous

vein – $83.8 \pm 2.7\%$ ($n = 7$) and 100% ($n = 6$), respectively; plantaris vein – $50.8 \pm 7.8\%$ ($n = 5$) and $-74.2 \pm 7.8\%$ ($n = 7$), respectively; distal saphenous artery – -17.3 ± 7.2 ($n = 5$) and $-17.3 \pm 5.5\%$ ($n = 5$), respectively.

The effects of haemoglobin and flurbiprofen against acetylcholine-induced relaxations

Figure 2a shows the effect of haemoglobin $3 \mu M$ against acetylcholine-induced relaxations in endothelium-intact segments of the lateral saphenous vein. Haemoglobin ($3 \mu M$) produced a slowly developing contraction, approximately 10% of the maximum response, which returned to baseline in 3 out of 6 preparations over 15 min. In the presence of haemoglobin $3 \mu M$, the maximum contraction of the lateral saphenous vein to NA was significantly increased by $64.6 \pm 16.3\%$ ($n = 6$) and this was associated with a significant reduction in both the maximum relaxation ($82.9 \pm 5.0\%$ to $34.7 \pm 5.7\%$, $n = 6$) and the sensitivity of the preparation to acetylcholine (pD_2

Figure 2 Representative trace recordings from segments of the isolated lateral saphenous vein of the rabbit. (a–b) The effect of acetylcholine-induced relaxations of maximally-activated (by noradrenaline $10 \mu M$) endothelium-intact segment in (a) the absence and (b) the presence of flurbiprofen $1 \mu M$. Dashed lines indicate the baseline tension prior to activation with noradrenaline. The first marker shown represents the addition of $3 nM$ acetylcholine: increments of 0.5 log units. (c–d) The cumulative concentration-response curves to (c) noradrenaline and (d) cirazoline in endothelium-intact (E+) and endothelium-denuded (E–) segments. The concentration of the agonists, starting at $3 nM$ (noradrenaline) or $30 nM$ (cirazoline), was increased in approximately 0.5 log unit increments following attainment of either a peak or equilibrium response.

Figure 3 The effect of (a) haemoglobin $1 \mu M$ and (b) flurbiprofen $1 \mu M$ on acetylcholine-induced relaxations of maximally-activated endothelium-intact segments of the rabbit isolated saphenous vein. The control concentration-response curve to acetylcholine is represented by (●) and that in the presence of either haemoglobin or flurbiprofen by (○). Responses have been expressed as a percentage of the contractile responses prior to addition of the acetylcholine. The values shown are the mean of 5–8 observations and the vertical lines denote the s.e. mean.

-8.03 ± 0.12 to 7.43 ± 0.1 , $n = 6$). Haemoglobin abolished acetylcholine-induced relaxations in the distal saphenous artery ($n = 3$).

In endothelium-intact segments of the lateral saphenous vein, high concentrations of acetylcholine were observed to produce small contractions superimposed on the remaining NA-induced tone (Figures 2b and 3a). This effect was not observed in the other two blood vessels or with endothelium-denuded segments of the lateral saphenous vein. A 30 min exposure to flurbiprofen $1 \mu\text{M}$ did not alter baseline tension or the response to NA, but increased the maximum relaxation produced by acetylcholine and blocked the contractions (Figures 2b and 3a). In the distal saphenous artery and plantaris vein, however, flurbiprofen did not affect acetylcholine-induced relaxations ($n = 2$ of each).

Comparison of mechanical removal of the endothelium and the effect of haemoglobin

Figure 4 shows the effect of mechanical removal of the endothelium and of haemoglobin $3 \mu\text{M}$ on noradrenaline-induced contractions of the rabbit isolated distal saphenous artery, plantaris vein and lateral saphenous vein. In the arterial preparation responses to NA were slightly, though not significantly, smaller in endothelium-denuded segments (Figure 4a). In contrast, in both veins the maximum responses to NA were significantly greater in endothelium-denuded segments (Figure 4b and c). For the lateral saphenous vein, this was more pronounced at high concentrations of NA. An increase in the sensitivity (pD_2) of the preparation to NA following removal of the endothelium was observed only in the plantaris vein (Table 2).

Haemoglobin ($3 \mu\text{M}$) produced small transient contractions in endothelium-intact segments of all three blood vessels; distal saphenous artery, $0.15 \pm 0.03 \text{ g wt.}$ ($n = 5$); plantaris vein, $0.32 \pm 0.08 \text{ g wt.}$ ($n = 5$); lateral saphenous vein $0.35 \pm 0.08 \text{ g wt.}$ ($n = 6$). These responses usually declined towards baseline over 15 min. Similar contractions were observed in 2 out of 6 endothelium-denuded segments of the lateral saphenous vein, probably resulting from the difficulty associated with ensuring complete removal of the endothelium without damaging the smooth muscle (see Methods), and in 1 out of 5 preparations of endothelium-denuded segments in each of plantaris vein and distal saphenous artery. As shown in Figure 4 (d, e and f) and Table 2, the effect of haemoglobin ($3 \mu\text{M}$) on α -adrenoceptor-mediated responses in endothelium-intact segments of the three blood vessels was similar to that observed with mechanical removal of the endothelium (Figures 4a–c), i.e. no effect in the distal saphenous artery, increased sensitivity and responsiveness in the plantaris vein and increased responsiveness of the lateral saphenous vein. These changes were not additive with the effects of physical

Figure 4 A comparison of contractile responses to noradrenaline (NA) in endothelium-intact and endothelium-denuded segments (left) and the effect of haemoglobin on NA-induced contractions in endothelium-intact segments (right) in three isolated superficial blood vessels from the rabbit: (a, d) distal saphenous artery; (b, e) plantaris vein; (c, f) lateral saphenous vein. Control responses to NA in endothelium-intact segments are represented by open symbols. Responses in endothelium-denuded segments or endothelium-intact segments exposed to haemoglobin $3 \mu\text{M}$ are represented by closed symbols. The values shown are the mean of 5–8 observations and the vertical lines denote the s.e.mean.

disruption of the endothelium since haemoglobin ($3 \mu\text{M}$) did not produce such changes in endothelium-denuded segments (Table 2).

The effect of removal of the endothelium on responses to α -adrenoceptor agonists

Following the removal of the endothelium, neither the sensitivity (pD_2) nor the responsiveness (E_{max} relative to NA) of the distal saphenous artery or plantaris vein to the selective α_1 -adrenoceptor agonist phenylephrine and the selective

Table 2 The effect of haemoglobin $3 \mu\text{M}$ on noradrenaline-induced contractions of 'endothelium-intact' and 'endothelium-denuded' segments of rabbit isolated blood vessels

		Control	pD_2 Haemoglobin	% change/difference in maximum responses	
				Control	Haemoglobin
Distal saphenous artery	Intact	7.45 ± 0.09	7.57 ± 0.07	—	4.0 ± 2.0
	Denuded	7.38 ± 0.12	†	12.5 ± 2.5	†
Plantaris vein	Intact	7.39 ± 0.11	$7.96 \pm 0.2^*$	—	$18 \pm 6.9^*$
	Denuded	$7.80 \pm 0.16^*$	7.85 ± 0.11	27.5 ± 4.5	2.1 ± 3.2
Lateral saphenous vein	Intact	7.29 ± 0.17	7.31 ± 0.12	—	$64.6 \pm 16.3^*$
	Denuded	7.36 ± 0.15	7.11 ± 0.09	$27\text{--}47^b$	3.2 ± 4.2

* Indicates a significant change ($P < 0.05$, paired Student's t test) following exposure to haemoglobin $3 \mu\text{M}$ in endothelium-intact segments.

† Indicates a significant difference ($P < 0.05$, paired Student's t test) between endothelium-intact and endothelium-denuded segments.

^b Indicates that this is the range of % difference between intact and denuded preparations from four separate experiments.

† The effect of haemoglobin $3 \mu\text{M}$ was examined in only two 'endothelium-denuded' segments of the distal saphenous artery, no effect was observed.

The results shown represent the mean $\pm$ s.e.mean of a minimum of 5 observations.

Table 3 Comparison of the potency and intrinsic activity of several α -adrenoceptor agonists in endothelium-intact and endothelium-denuded segments of isolated blood vessels from the rabbit

		Phenylephrine		UK-14304		B-HT 920		Cirazoline	
		pD ₂	E _{max}	pD ₂	E _{max}	pD ₂	E _{max}	pD ₂	E _{max}
Distal saphenous artery	Intact	6.77 ± 0.15	0.95 ± 0.06	6.29 ± 0.15	0.70 ± 0.09	ND	ND	ND	ND
	Denuded	6.57 ± 0.13	0.90 ± 0.07	6.13 ± 0.13	0.72 ± 0.07	ND	ND	ND	ND
Plantaris vein	Intact	6.24 ± 0.10	0.97 ± 0.02	7.84 ± 0.19	0.63 ± 0.02	ND	ND	ND	ND
	Denuded	6.32 ± 0.13	0.97 ± 0.03	7.96 ± 0.18	0.62 ± 0.05	ND	ND	ND	ND
Lateral saphenous vein	Intact	5.79 ± 0.10	0.96 ± 0.03	7.59 ± 0.10	0.86 ± 0.03	6.42 ± 0.11	0.59 ± 0.04	6.00 ± 0.10	0.76 ± 0.04
	Denuded	5.86 ± 0.09	0.90 ± 0.04	7.52 ± 0.13	0.75 ± 0.04*	6.41 ± 0.11	0.48 ± 0.04*	6.05 ± 0.06	0.64 ± 0.01*

Values shown are the mean ± s.e.mean of 5–9 experiments. ND indicates experiment not performed.

* Significant difference ($P < 0.05$) between endothelium-intact and endothelium-denuded values (Student's paired t test).

α_2 -adrenoceptor agonist UK-14304 were altered (Table 3). In contrast, the responsiveness of the lateral saphenous vein to phenylephrine was increased while that to UK-14304 was slightly, though not significantly, reduced (Figure 5a and b). The difference between the two agonists is highlighted by the significant reduction in E_{max} for UK-14304 associated with removal of the endothelium (Table 3). These observations are similar to those previously reported for the selective α_1 -adrenoceptor agonists amidephrine (increased respon-

siveness) and the selective α_2 -adrenoceptor agonist xylazine (no change) following mechanical removal of the endothelium in the lateral saphenous vein. On its own, this reinforces the suggestion that the basal release of EDRF preferentially modulates the function of α_1 -adrenoceptors in this preparation (Daly *et al.*, 1987) (but see below).

Figure 3b shows representative recordings of contractile responses to the selective α_1 -adrenoceptor agonist cirazoline in endothelium-intact and endothelium-denuded segments of the lateral saphenous vein. Unlike NA, cirazoline produced slowly developing contractions (time to equilibrium > 3 min). Contractile responses to the selective α_2 -adrenoceptor agonist B-HT 920 were qualitatively similar to those elicited by cirazoline. Removal of the endothelium did not significantly affect responses to either agonist in contrast to the increase found with NA: consequently the E_{max} values (relative to NA) in endothelium-denuded segments are less than in endothelium-intact segments (Table 3, Figures 5c and d).

Figure 5 (e–h) shows the effects of prazosin 0.1 μ M or rauwolsine 2.5 μ M on responses to phenylephrine, UK-14304, cirazoline and B-HT 920 in endothelium-intact segments of lateral saphenous vein. Prazosin (0.1 μ M) produced a significant inhibition of responses to all four agonists. Rauwolsine (2.5 μ M) produced a substantial rightward displacement of the curves for B-HT 920 and UK-14304. But, in the case of phenylephrine and cirazoline, inhibition by rauwolsine (2.5 μ M) was associated with the presence of a 'resistant' component which was greater for cirazoline. This latter observation shows that mediation by 'rauwolsine-resistant' α_1 -adrenoceptors is not, on its own, a sufficient criterion for augmentation of a response following removal of the endothelium.

Subtypes of α -adrenoceptors stimulated by NA in endothelium-intact and endothelium-denuded segments of the lateral saphenous vein

In this series of experiments, the maximum responses to NA in endothelium-denuded preparations (4.38 ± 0.45 g wt., $n = 10$) were significantly greater than were those observed in paired endothelium-intact segments (3.06 ± 0.39 g wt.). Prazosin (0.1 μ M) produced a similar rightward displacement of the NA CRC in both endothelium-intact and endothelium-denuded preparations (Figure 6a); log shifts at 50% of the maximum response were 1.20 ± 0.11 and 1.26 ± 0.22 ($n = 5$), respectively. Similarly, there was no significant difference in the effect of rauwolsine (2.5 μ M) in endothelium-intact and endothelium-denuded segments, with the exception of the response elicited by NA 300 μ M (Figure 6b). Thus, selective competitive antagonists failed to reveal any alteration in the relative contribution of the two α -adrenoceptor subtypes to responses to NA following mechanical removal of the endothelium.

Following isolation of post-junctional α_2 -adrenoceptors by prior exposure to rauwolsine (1 μ M) and phenoxybenzamine (0.3 μ M), a small but significant fall in the sensitivity to NA was found on removal of the endothelium (pD_2 6.73 ± 0.09

Figure 5 Effects of the endothelium and selective antagonists on contractions to α -adrenoceptor agonists in the rabbit isolated lateral saphenous vein. Responses to (a) phenylephrine, (b) UK-14304, (c) B-HT 920 and (d) cirazoline, in endothelium-intact (○) and endothelium-denuded segments (●). The effect of prazosin 0.1 μ M (●) and rauwolsine 2.5 μ M (□) on contractile responses to (e) phenylephrine, (f) UK-14304, (g) B-HT 920 and (h) cirazoline in endothelium-intact segments. Control responses to the agonists are represented by the (○). The values shown are the mean of 5–9 observations and the vertical lines denote the s.e.mean. * indicates a significant difference between responses in paired endothelium-intact and endothelium-denuded segments ($P < 0.05$).

Figure 6 The effects of α -adrenoceptor antagonists on contractile responses to noradrenaline in endothelium-intact (open symbols) and endothelium-denuded (closed symbols) segments of the rabbit isolated lateral saphenous vein. The cumulative concentration-response curves in the presence and absence of (a) prazosin $0.1 \mu\text{M}$, (b) rauwolscine $2.5 \mu\text{M}$ and (c) following exposure to rauwolscine $1 \mu\text{M}$ (35 min) and phenoxybenzamine $0.1 \mu\text{M}$ (30 min) followed by repeated washing over 60 min to remove the antagonist. Responses in the presence and absence of the competitive antagonists (a and b) or before and after exposure to the irreversible antagonist (c) are represented by ($\circ$ $\bullet$) and ($\square$ $\blacksquare$), respectively. Responses have been expressed as a percentage of the maximum response of the preparation in the absence of the antagonists (a and b) or as the absolute size of responses (c). The values shown are the mean of 5–9 observations and the vertical lines denote the s.e.mean.

shifted to 6.43 ± 0.14 , $n = 8$). Furthermore, the maximum response was not significantly altered, in contrast to the control, in which the maximum is larger without endothelium (Figure 5c). Thus, the effect of phenoxybenzamine on the maximum response is greater in endothelium-denuded segments ($49.4 \pm 2.3\%$ reduction, $n = 8$) than in endothelium-intact preparations ($25.7 \pm 2.5\%$, $P < 0.01$), suggesting a greater contribution of α_1 -adrenoceptors in the absence of endothelium.

Discussion

Acetylcholine produced a pronounced endothelium-dependent relaxation against maximal and sub-maximal contractions to NA in two isolated superficial veins which was considerably

greater than in a corresponding artery. This stands in sharp contrast to the general view that acetylcholine is not a particularly effective endothelium-dependent relaxant of isolated veins (DeMey & Vanhoutte, 1982; Furchgott, 1983; Ignarro *et al.*, 1987b). On the other hand, our observations are consistent with the known propensity of nitrogen-containing vasodilators, with which a common mechanism of action is assumed, to exert a preferential effect on veins (Ignarro & Kadowitz, 1985). Similarly the basal release of EDRF constrained the function of α -adrenoceptors in the veins more than in the artery. This is based on two different manoeuvres designed to remove or inhibit the effect of spontaneously released EDRF: mechanical removal of the endothelium (functionally assessed by the absence of a vasodilator response to acetylcholine) or the inclusion of haemoglobin.

No evidence of a direct stimulatory action of acetylcholine ($< 3 \mu\text{M}$) on the smooth muscle was observed in the three blood vessels studied, as has been reported for other preparations (see: Furchgott, 1983; Rubanyi & Vanhoutte, 1988). Thus, the basis of the differences found between this superficial artery and veins must be primarily related either to the nature or quantity of the EDRF or to the nature of the contractile response. It seems unlikely that this is attributable to the α -adrenoceptor subtype activated by NA in a simple way (see below). Another possibility that seems to be discounted is that the greater media thickness of the arterial preparation hinders the diffusion of EDRF through the blood vessel wall, thereby rendering the contractile responses more resistant to the relaxant (see Bassenge *et al.*, 1987). If this were correct, large quantitative discrepancies would be evident in the effect of endothelium-independent and endothelium-dependent relaxations in the distal saphenous artery and this was not the case.

However, the strikingly similar profiles of the endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) relaxants in the three preparations examined in the present study, suggest that the observed heterogeneity between vessels is primarily a product of the smooth muscle. In particular, the quantitative agreement between the maximum relaxation produced by both agents in maximally-activated endothelium-intact segments of the distal saphenous artery indicates that soluble guanylate cyclase in the smooth muscle is fully activated by acetylcholine (Figures 1a and 1d).

Several other observations also appear to eliminate the possibility that it is substantial differences between the properties of the endothelium of different vessels which contribute to the observed differences in EDRF-related vascular inhibition. First, evidence for a countervailing influence of an endothelium-dependent constrictor factor, whose effect might cause an underestimate of the difference between artery and vein, was found only with a high concentration of acetylcholine and only in the lateral saphenous vein. Furthermore, this phenomenon, apparently due to a consequence of cyclooxygenase activation, occurred at concentrations clearly above the concentration-response curve for EDRF release. Secondly, the ability of haemoglobin to modify endothelium-dependent relaxations in the distal saphenous artery and lateral saphenous vein indicates that nitric oxide is most probably the mediator in both preparations (Ignarro *et al.*, 1987b). Finally, the greater effectiveness of nitrovasodilators in endothelium-denuded segments provides further evidence for the basal release of EDRF, the removal of which allows greater scope for the effect of endothelium-independent relaxants on soluble guanylate cyclase (Alheid *et al.*, 1987). Thus, the approximately 3 fold increase in the sensitivity of all three vessels to sodium nitroprusside following removal of the endothelium (Table 1), suggests that there was no substantial difference in the basal release of EDRF and, therefore, in the integrity of the endothelium in each 'intact' preparation. Overall, the evidence in these vessels suggests a single EDRF, probably nitric oxide, capable of matching maximal exogenous activation of soluble guanylate cyclase.

It has long been recognized that contractions elicited by agents that depolarize the smooth muscle membrane and open voltage-operated calcium channels are more resistant to the nitrovasodilators (see: Ignarro & Kadowitz, 1985) and that this is a property shared by endothelium-dependent relaxants (Furchgott, 1983). Thus, the relative insensitivity of NA-induced contractions of the distal saphenous artery to both sodium nitroprusside and acetylcholine, may be attributable to the greater utilization of voltage-operated calcium channels resulting from depolarization of the smooth muscle, while responses in veins depend to a greater extent on receptor-operated calcium channels. In support of this possibility, Schümann & Lues (1983) reported that the calcium entry blocker, nitrendipine, produced little inhibition of contractile responses to phenylephrine in the rabbit isolated lateral saphenous vein. Similarly, we find that equilibrium responses to NA in this blood vessel are dependent upon the presence of extracellular calcium (Daly *et al.*, 1990), yet resistant to nifedipine (McGrath & Wilson, unpublished observations). Thus, it appears that a primary determinant of the effectiveness of endothelium-dependent relaxants in isolated blood vessels from the rabbit may be the nature of the coupling process utilized by the contractile agents.

If the major influence of the endothelium on the adjacent smooth muscle is the continuous and spontaneous release of nitric oxide, then 'removal' of the endothelium or blocking the effect of EDRF pharmacologically with haemoglobin should produce the same effect; an enhancement of responses to contractile agonists. As noted in the Introduction, even when part of this is tested, the hypothesis is not always confirmed, and this sequence of tests is not often carried out. The present study shows a good degree of correlation between the increases in contractile activity caused by endothelial disruption and by haemoglobin. The logic is confirmed in that removal of the endothelium enhances the potency of nitroprusside, and the pattern for the extent of these effects is reflected across the three preparations. It could be argued that in future assessments of the influence of the endothelium in any given preparation these tests should be applied and any deviations explained. For example, in circumstances which permit the continuous release of an endothelium-derived contractile factor such as endothelin (Yanagisawa *et al.*, 1988), it should be expected that a countervailing excitatory effect of the endothelium should be lost by disruption but that this should not be mimicked by haemoglobin (i.e. the increase in muscle responsiveness should be greater in haemoglobin-treated endothelium intact preparations than in endothelium-denuded preparations). Although it has been reported that venous smooth muscle is extremely sensitive to the exogenous application of endothelin (D'Orleans Juste *et al.*, 1989), no indirect evidence for the exogenous production of this peptide was found in the present study.

The observation that contractile responses in isolated superficial veins from the rabbit are more sensitive to both endothelium-dependent and endothelium-independent relaxants than the corresponding artery, poses the question of whether this is true for the rest of the circulation. The general requirement for submaximal concentrations of a contractile agent (producing approximately 60% of the maximum response) when examining the role of EDRF in isolated arteries from the rabbit would appear to support this (see: Furchgott, 1983; Martin *et al.*, 1985; Bassenge *et al.*, 1987; Oriowo *et al.*, 1987), i.e. arteries show poor effectiveness of EDRF versus a strong vasoconstrictor stimulus, whereas in veins EDRF remains effective even against 'maximal' stimuli. Furthermore, we have noted that maximal contractile responses to NA in the rabbit isolated ear vein are also very sensitive to acetylcholine (McGrath and Wilson, unpublished observation).

The relative lack of influence of the endothelium on α -adrenoceptor-mediated responses in large isolated arteries from the rabbit need not be a general property of arterial vessels in this species. An effect may be more apparent in

vessels of smaller calibre. For example, pressor responses to extraluminally administered NA in the rabbit isolated perfused ear artery were augmented following mechanical disruption of the endothelium (Verrecchia *et al.*, 1985). Furthermore, an inverse relationship between the vasodilator potency of acetylcholine (stimulated EDRF release) and vessel diameter has been reported in both the rabbit ear (Owen & Bevan, 1985) and the rat mesenteric bed (De Mey & Gray, 1985). However, if the arterio-venous difference in susceptibility to EDRF applies throughout the vasculature, the α -adrenoceptor function in venules may still be subject to more influence by the basal release of EDRF than the corresponding arterioles. Thus, in addition to its important role in controlling arterial blood flow (Rubanyi & Vanhoutte, 1986; Griffiths *et al.*, 1987; Randall & Hiley, 1988), the endothelium may also regulate venous capacitance. Such an effect is also consistent with the known pharmacology of the endothelium-independent nitrovasodilators, with which EDRF is thought to share a similar mechanism of action (Martin *et al.*, 1986; Ignarro *et al.*, 1987a). In preliminary experiments we have shown this more directly and convincingly than is possible with isometric ring preparations, using video image analysis techniques on isolated, perfused segments of rabbit distal saphenous vein: at constant intraluminal pressure, noradrenaline produces venoconstriction which is reversed by acetylcholine (J.C. McGrath, V.A. Moss & M. Wight, unpublished). Finally, it is noteworthy that a significant role for EDRF in the regulation of human venous tone has been demonstrated *in vivo* (Collier & Vallance, 1989).

Our attempts to examine a possible differential influence of the endothelium on post-junctional α_1 - compared with α_2 -adrenoceptors in the lateral saphenous vein did not show a straightforward α_1/α_2 split. Contractile responses to noradrenaline, phenylephrine and amidephrine were enhanced in endothelium-denuded segments but those to BHT920, UK14304, xylazine and cirazoline were not (Daly *et al.*, 1987; present study). Since noradrenaline, phenylephrine and amidephrine all activate α_1 -adrenoceptors here and previously (Butler & Jenkinson, 1978; McGrath, 1982), an involvement of α_1 -adrenoceptors appears likely: supporting this, most of the agonists in the unaffected group, i.e. B-HT 920, UK-14304 and xylazine, did not elicit 'rauwolscine-resistant' contractions over the major part of the concentration-response curve, confirming that they act mainly via α_2 . On the other hand, cirazoline seemed quite selective for α_1 -adrenoceptors, and like NA, exhibited partial resistance to rauwolscine. However, it may be relevant that responses to cirazoline were qualitatively similar to those elicited by the selective α_2 -adrenoceptor agonists (slow in onset). An analogous correlation is found in the pressor responses to infusion of α -adrenoceptor agonists in the pithed rat: responses to cirazoline showed similarities in both time-course (slow but maintained) and susceptibility to the Ca^{2+} entry blocker, nifedipine (highly sensitive), to those of 'selective' α_2 -adrenoceptor agonists as distinct from noradrenaline and other phenethylamine-based α_1 -adrenoceptor agonists (O'Brien *et al.*, 1985; O'Brien & McGrath, 1987). This seems to be another example of a split between imidazolines and structural analogues, such as cirazoline, and phenethylamines on the other, again raising the possibility that these groups might have different capacities either for activating different α_1 -adrenoceptor sub-populations or activating different transduction processes through a single recognition site (McGrath, 1982; McGrath *et al.*, 1989). These were earlier described as α_{1a} and α_{1b} adrenoceptor-mediated responses, respectively, to keep these options open (McGrath, 1982). These effects, analysed in detail in the lateral saphenous vein, were reinforced by the finding in plantaris vein that the sensitivity to NA was increased by both the removal of the endothelium and the addition of haemoglobin to endothelium-intact segments (Table 3). Indeed, these observations represent the only example in the present study, where removal of the spontaneous release of EDRF enhanced the 'potency' (pD_2) of an agonist.

Observations with antagonists again showed no simple correlation. The competitive antagonists prazosin and rauwolscine revealed no role for any receptor subtype in the difference between endothelium-intact and endothelium-denuded segments. This was not altogether surprising, since in an earlier study we demonstrated that the use of reversible selective antagonists in the lateral saphenous vein does not guarantee functional isolation of one receptor subtype (Daly *et al.*, 1988c). In the present study, responses to all agonists were sensitive to 0.1 μM prazosin (Figures 5e–h and 6a). Thus, irreversible inactivation of post-junctional α_1 -adrenoceptors (with protection and subsequent isolation of α_2 -adrenoceptors) by prior exposure to phenoxybenzamine and rauwolscine was attempted, and this revealed that the responsiveness of endothelium-intact and endothelium-denuded segments to NA (α_2 -only) were not significantly different. In this case the α_1 response cannot break through since the number of receptors available is less than that required to allow sufficient biological stimulus for a threshold effect (Daly *et al.*, 1988c). This confirms that the additional response of vascular smooth muscle which is normally held suppressed by basal EDRF has an α_1 -adrenoceptor basis.

There was no specific evidence from our study that α_1 -adrenoceptors can mediate the release of EDRF, as has been reported for the α_2 -subtype (Miller & Vanhoutte, 1985; Bullock *et al.*, 1987). This would cause functional antagonism of the contractile response: however, that possibility cannot entirely be excluded.

If EDRF does preferentially oppose α_1 as compared to α_2 then the effects of cirazoline need to be accounted for. Responses to cirazoline were unaffected by the removal of the endothelium. Furthermore, since the E_{max} for cirazoline is intermediate between that for phenylephrine and amidephrine (see Table 3 and Daly *et al.*, 1987), agonists potentiated by removal of the endothelium, the effect of removal of the endothelium in the lateral saphenous vein is not a simple inverse

function of the intrinsic activity of the agonist, as has been advanced to account for observations in other preparations (Lues & Schümann, 1984; Godfraind *et al.*, 1985). Finally, the difference between cirazoline and other selective α_1 -adrenoceptor agonists also eliminates the possibility that post-junctional α_1 -adrenoceptors appear more susceptible to basal release of EDRF because of closer proximity to the intimal surface of the blood vessel than post-junctional α_2 -adrenoceptors. The existence of a subtype of receptor or 'response' not accessible to activation by cirazoline (α_{1b} ; McGrath, 1982) seems a plausible explanation.

In conclusion, we have demonstrated that α -adrenoceptor function in two isolated superficial veins from the rabbit is constrained to a greater degree by the basal release of EDRF than in a corresponding artery. This is paralleled by the effects of agonist-stimulated EDRF release and an endothelium-independent nitrovasodilator in these preparations, demonstrating that it is the capacity of the smooth muscle to relax which is the critical factor. Furthermore, the effects of removing the endothelium parallel the effects of haemoglobin, demonstrating that there is likely to be a single EDRF, in all likelihood nitric oxide. Experiments with 'selective' antagonists for α -adrenoceptor sub-types suggest that removal of the endothelium is associated with a preferential increase in the function of α_1 -adrenoceptors stimulated by NA. However, observations with 'selective' synthetic agonists indicate that the nature of the contractile response rather than the receptor sub-type *per se* is a factor in determining the functional consequences of spontaneously released EDRF.

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