Modulation by endothelium of the responses induced by endothelin-1 and by some of its analogues in rat isolated aorta

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1 The contractile effects of endothelin-1 and various analogues were studied in rat isolated aortic rings. The potency order of the peptides studied was endothelin-1 > sarafotoxin S6b > [Ala^{3,11}]endothelin-1 > [Ala^{1,15}]endothelin-1. [Ala^{1,3,11,15}]endothelin-1 was neither agonist nor antagonist.

2 The concentration of endothelin-1 required to induce contractions equal to 50% of those induced by 1 μ M noradrenaline was reduced from 5.8 nM when the vascular endothelium was present to 1.4 nM after it had been mechanically removed.

3 Contractions elicited by [Ala^{3,11}]endothelin-1, [Ala^{1,15}]endothelin-1 and sarafotoxin S6b were not modulated by the endothelium.

4 Endothelin-1 increased the tissue content of guanosine 3',5'-cyclic monophosphate (cyclic GMP) in rat aortic segments with endothelium by about 4 fold, suggesting that it increased the release of endothelium-derived relaxing factor. Sarafotoxin S6b did not reduce, or significantly increase, tissue cyclic GMP levels and therefore had little effect on EDRF release.

5 The concentration of sodium nitroprusside required to relax half-maximally aortae denuded of endothelium was 430 nM if the aortae had been precontracted with 10 nM endothelin-1 and 35 nM if 10 nM sarafotoxin S6b was used as the spasmogen. This indicates that differential sensitivities of the smooth muscle to cyclic GMP cannot explain differences between responses to endothelin-1 and sarafotoxin S6b in the presence of endothelium.

6 It is concluded that endothelin-1 contractions of rat aorta are modified by the endothelium, probably by enhancing the release of endothelium-derived relaxing factor (EDRF) and not by affecting the sensitivity of the smooth muscle to EDRF. This suggests that a stimulated release of an adequate amount of EDRF is necessary to modulate contractile responses to these peptides.

Introduction

The existence of a contracting factor derived from endothelial cells was first demonstrated about 5 years ago (O'Brien & McMurtry, 1984; Agricola *et al.*, 1984; Hickey *et al.*, 1985) and in 1988 a 21-amino acid peptide, endothelin, was purified from the medium of porcine endothelial cells in culture (Yanagisawa *et al.*, 1988). This peptide has been shown to be one of the most potent vasoconstrictors known (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988). The endothelin originally characterized was that produced by human and porcine endothelial cells in culture. It is a member of a family of at least three closely related mammalian peptides and has been renamed endothelin-1 (Inoue *et al.*, 1989). Sarafotoxin S6b, a constituent of the venom of the burrowing snake *Atractaspis engadensis*, is homologous with the endothelins and is also a vasoconstrictor (Lee & Chiapinelli, 1988; Takasaki *et al.*, 1988). Sarafotoxin S6b and endothelin-1 inhibit the specific binding of each other to heart and brain membranes (Ambar *et al.*, 1989) and other active analogues of endothelin-1, such as [Ala^{3,11}]- and [Ala^{1,15}]-endothelin-1, inhibit [¹²⁵I]-endothelin-1 specific binding (Hiley *et al.*, 1990) but do not have an identical pharmacological profile to that of endothelin-1 (Randall *et al.*, 1989; Topouzis *et al.*, 1989b; Miller *et al.*, 1990). Endothelin-1 contains two disulphide bonds between residues 3–11 and 1–15, generating a peptide containing two almost equal sized rings (Yanagisawa *et al.*, 1988). In the analogues [Ala^{3,11}]- and [Ala^{1,15}]-endothelin-1, the cysteines at positions 3–11 and 1–15 respectively have been removed creating one large ring, the [Ala^{3,11}] analogue, or one small ring, the [Ala^{1,15}] analogue. The cystine residues were replaced with alanines because this substitution retains a chiral amino acid, it is also a neutral residue and does not

introduce any new charge at the substituted position and is pseudo-isosteric with cystine.

The vasoconstrictor activity of many compounds is modulated by the presence of endothelium (Miller *et al.*, 1988). In rat aorta the modulatory effect of endothelium is thought to be due to either basal (Martin *et al.*, 1986; MacLeod *et al.*, 1987) or both basal and stimulated (Miller *et al.*, 1984; Bigaud *et al.*, 1984; Malta *et al.*, 1986), release of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980). In any case this modulation in the presence of endothelium is associated with an increase in tissue levels of guanosine 3',5'-cyclic monophosphate (cyclic GMP) compared to tissues without endothelium.

This study was undertaken to assess the effects of endothelium on responses of rat isolated aorta elicited by endothelin-1, sarafotoxin S6b and three vasoactive analogues of endothelin-1; [Ala^{3,11}]-, [Ala^{1,15}]- and [Ala^{1,3,11,15}]-endothelin-1 (Randall *et al.*, 1989; Topouzis *et al.*, 1989b). The results show that while responses elicited by endothelin-1 were modulated by the presence of endothelium, responses to the other peptides were not affected. From measurements of tissue cyclic GMP content, it is concluded that a stimulation of EDRF release, produced only by endothelin-1, is required to modulate the contractile effects of these peptides in the rat isolated aorta. Preliminary reports of some of these results have been made (Topouzis *et al.*, 1989a; Huggins *et al.*, 1990).

Methods

Male Sprague-Dawley rats weighing 200–250 g were killed and the thoracic aortae removed taking care not to damage the endothelium. Four rings were taken from each aorta and mounted, under a tension of 2 g, in organ baths containing a physiological solution (composition in mM: NaCl 112, KCl 5,

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NaHCO₃ 25, KH₂PO₄ 1, MgSO₄ 1.2, CaCl₂ 1.25, glucose 11.5) equilibrated to pH 7.4 and gassed with a mixture of 95% O₂ and 5% CO₂. Half of the segments were stripped of their endothelium by rubbing with a wooden match stick or with forceps.

Contractile experiments

Artery segments were allowed to equilibrate for 60 min and were then contracted with noradrenaline, 1 μ M, a concentration which elicits a maximal response (Godfraind & Miller, 1983). After the response had reached a plateau, acetylcholine, 1 μ M, was added to the organ bath to test for the absence or presence of endothelium (Furchgott & Zawadzki, 1980). Tissues that responded to acetylcholine with a relaxation of greater than 50% of the initial noradrenaline-induced tone were considered to be intact, whereas tissues in which acetylcholine elicited no effect were regarded as endothelium-denuded. Tissues were then washed and left to equilibrate for 60 min, during which period the medium was changed 4 times. In some experiments tissues were further incubated for 30 min in the presence of indomethacin, 10 μ M, or for 45 min in the presence of [Ala^{1,3,11,15}]endothelin-1, 50 nM. After the equilibration period, concentrations of peptides were added to the baths of artery segments in a cumulative fashion. In all cases pairs of rings were studied in parallel, one with and one without endothelium. Only one concentration-effect curve to a single peptide was obtained in each artery ring in the absence or presence of indomethacin or of [Ala^{1,3,11,15}]endothelin-1. In other experiments, aortic rings without endothelium were precontracted with endothelin-1, 10 nM, or sarafotoxin S6b and then concentration-effect curves were obtained for the relaxation of the rings by cumulative addition of sodium nitroprusside.

Cyclic GMP determinations

Aortic segments were equilibrated in physiological solution for 60 min at 37°C and then treated with acetylcholine, 1 μ M, endothelin-1, 10 nM, or sarafotoxin S6b 10 nM. At various times thereafter the tissues were frozen with clamps cooled in liquid nitrogen and stored at -70°C. Without thawing, each ring was homogenized in 1 ml trichloroacetic acid 0.6 M in a Polytron and subsequently centrifuged at 40 000 g for 15 min. The pellet was resuspended in NaOH 1 M and assayed for protein content (Lowry *et al.*, 1951). The supernatants were washed four times with 5 volumes of water-saturated diethyl ether and assayed for cyclic GMP with an Amerlex radioimmunoassay kit. Tissue cyclic GMP was expressed relative to protein content in order to normalise for variations in amount of tissue.

Materials

Noradrenaline bitartrate (Boyer, France), acetylcholine HCl (Sigma, France) and sodium nitroprusside (Prolabo, France) solutions were prepared daily in deionised water. Indomethacin (Sigma, France) was dissolved in 0.5% NaHCO₃ solution to a concentration of 10 mM and diluted with deionised water as necessary. Endothelin-1, sarafotoxin S6b (both from Peptide Institute, Japan), [Ala^{3,11}]endothelin-1, [Ala^{1,15}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 (synthesized at Merrell Dow Research Institute, Strasbourg, France) were stored at 4°C as 0.1 mM solutions in deionised water and further dilutions were prepared daily. The Amerlex cyclic GMP assay kit was from Amersham International, United Kingdom.

Statistics

Tests of significance were made by Student's *t* test on a Compac 286e computer with RS/1 software (Version 12.10E,

BBN Software Products Co). *P* values less than 0.05 were taken to indicate significant differences.

Results

Endothelin-1 (0.3 to 30 nM), [Ala^{3,11}]endothelin-1 (2.25 to 225 nM), [Ala^{1,15}]endothelin-1 (41 nM to 1.36 μ M) and sarafotoxin S6b (0.1 to 30 nM) all contracted rat aortic preparations without endothelium in a concentration-dependent manner (Figure 1). At the highest concentrations tested all peptides induced contractile effects similar to the maximal responses to noradrenaline 1 μ M obtained in the same tissues, but the concentrations eliciting 50% of the maximal responses to noradrenaline were different. The potency order was endothelin-1 > sarafotoxin S6b > [Ala^{3,11}]endothelin-1 > [Ala^{1,15}]endothelin-1 (Table 1). Higher concentrations of peptides than indicated in Figure 1 were not tested due to their limited availability. In the presence of endothelium, endothelin-1 was significantly less potent than in tissues without endothelium, but maximal responses were not significantly changed (Figure 1a). Indomethacin (10 μ M) had no significant effect on contractions to endothelin-1 in the presence or absence of endothelium.

Maximal responses elicited in the presence and absence of endothelium by the highest concentrations tested of [Ala^{3,11}]endothelin-1 and [Ala^{1,15}]endothelin-1 were also not significantly different (Figure 1b, c) and nor were their potencies as assessed by comparison of concentrations eliciting responses equal to 50% of the effect of noradrenaline 1 μ M (Table 1). Maximal contractions were elicited by sarafotoxin S6b and these were not significantly different in the presence or absence of endothelium (Figure 1d). [Ala^{1,3,11,15}]endothelin-1 failed to elicit detectable contractions at concentrations of 10 nM to 1 μ M in either intact or endothelium-denuded tissues. A 50 nM concentration of this peptide also failed to modulate responses to endothelin-1 or sarafotoxin S6b.

The modulation of vasoconstrictor responses of rat aorta to endothelin-1, but not to [Ala^{3,11}]endothelin-1, [Ala^{1,15}]endothelin-1 or sarafotoxin S6b in the presence of the endothelium was surprising and was investigated further.

Modulation of agonist responses in intact vessels is often due to release of EDRF by the endothelium and an index of its release is changes in tissue cyclic GMP levels (Miller *et al.*, 1988). When tissues with endothelium were incubated with 10 nM endothelin-1, a significant increase in tissue cyclic GMP, of about 4 fold basal levels, was observed after 1 min. On prolonged incubation, tissue cyclic GMP levels returned to basal values (Figure 2b). Incubation with sarafotoxin S6b 10 nM did not significantly elevate cyclic GMP levels. In tissues without endothelium, neither endothelin-1 nor sarafotoxin S6b had any significant effect on cyclic GMP levels (Figure 2b). One min incubation of endothelium-containing tissues with 1 μ M acetylcholine increased tissue content by about 13 fold (Figure 2a).

It has been postulated that the presence of endothelium can be enough to alter agonist-induced responses (Malta *et al.*, 1986; Martin *et al.*, 1986). A reason, therefore, for the lack of effect of endothelium on responses to sarafotoxin S6b, is that sarafotoxin S6b desensitizes aortic smooth muscle to the effects of EDRF and hence of intracellular cyclic GMP. The effects of direct stimulation of soluble guanylate cyclase were assessed in the presence of peptides. In aortic rings without endothelium precontracted with either endothelin-1 (10 nM) or sarafotoxin S6b (10 nM), concentrations that elicited similar responses, sodium nitroprusside elicited concentration-dependent relaxations amounting to about 90% of the initial contraction (Figure 3). In tissues precontracted with endothelin-1, the EC₅₀ value for sodium nitroprusside was 430 $\pm$ 180 nM (*n* = 7) but when sarafotoxin S6b was the spasmogen the EC₅₀ value of 34.6 $\pm$ 18.8 nM (*n* = 8) was significantly lower (*P* < 0.05).

Figure 1 Concentration-effect curves elicited by (a) endothelin-1, (b) $[\text{Ala}^{3,11}]$ endothelin-1, (c) $[\text{Ala}^{1,15}]$ endothelin-1 and (d) sarafotoxin S6b in rat isolated aorta in the presence (●) and absence (○) of endothelium. Responses are expressed in g developed tension. At the highest concentrations tested, all peptides induced contractile effects similar to the maximal responses to noradrenaline $1\text{ }\mu\text{M}$ obtained in the same tissues. Each point is the mean of 7 to 10 observations. Vertical bars represent s.e.mean.

Discussion

Endothelin-1 as well as two of its analogues, $[\text{Ala}^{3,11}]$ - and $[\text{Ala}^{1,15}]$ -endothelin-1, that have been shown to compete with $[\text{I}^{125}]$ -endothelin-1 for a specific binding site (Hiley *et al.*, 1990), contracted rat isolated aorta in a concentration-dependent manner. Sarafotoxin S6b interacts with specific binding sites for $[\text{I}^{125}]$ -endothelin-1 (Ambar *et al.*, 1989) and elicits contractions (Figure 1). The order of potency of the peptides is the same as that reported for rat mesenteric vessels and guinea-pig trachea (Randall *et al.*, 1989; Pelton & Miller, 1990) and is the same as the order of binding affinities to rat cerebellar membranes (Hiley *et al.*, 1990). A third analogue, $[\text{Ala}^{1,3,11,15}]$ endothelin-1, also competes with $[\text{I}^{125}]$ -endothelin-1 for specific binding sites in central nervous tissue (Hiley *et al.*, 1990). This analogue constricts the blood perfused rat mesenteric vasculature (Randall *et al.*, 1989) and guinea-pig isolated trachea (Pelton & Miller, 1990), but failed

Figure 2 Cyclic GMP levels measured in isolated rat aortic rings. (a) Effect of 1 min exposure to acetylcholine $1\text{ }\mu\text{M}$ with (hatched column) and without (open column) endothelium. (b) Time course of the effects of endothelin-1 (10 nM) (●, ○) and of sarafotoxin S6b (10 nM) (■, □) with (filled symbols) and without (open symbols) endothelium. Each point is the mean of 11 or 12 observations. Vertical lines represent s.e.mean. * indicates significantly different from tissues without endothelium (a) or from time 0 (b).

either to contract rat aorta or to antagonize contractions elicited by endothelin-1 or sarafotoxin S6b. These observations, and other evidence from binding (Badr *et al.*, 1989) and functional studies (Randall *et al.*, 1989) seem to indicate the existence of more than one endothelin receptor.

Contractile responses to endothelin-1 in rat isolated aortic segments were modulated in the presence of endothelium, as compared to responses in its absence, by a displacement to the right of concentration-effect curves by a factor of about 4, without a significant effect on maximal responses (Figure 1a). This sort of modulation is similar to that of many other contractile agonists such as 5-hydroxytryptamine, prostaglandin $\text{F}_{2\alpha}$ and full α -adrenoceptor agonists in this and other arterial preparations (Miller *et al.*, 1988). However, all contractile agonists whose responses are modulated by the endothelium do not stimulate the release of EDRF (Miller *et al.*, 1988). The modulation of endothelin-1 responses by endothelium was not mediated by cyclo-oxygenase products since they were unaf-

Table 1 Concentrations of peptides eliciting 50% of the responses to noradrenaline (NA), $1\text{ }\mu\text{M}$, ($\text{EC}_{\text{NA}50}$) in the absence (–endo) and presence (+endo) of endothelium in rat isolated aorta and maximal responses of peptides in the absence of endothelium (E_{max} , expressed as % of responses to noradrenaline $1\text{ }\mu\text{M}$) obtained at the maximal concentrations tested (EC_{max})

Agonist	$\text{EC}_{\text{NA}50}(\text{nM})$		$\text{E}_{\text{max}}(\%)$	$\text{EC}_{\text{max}}(\text{nM})$
	–endo	+endo		
Endothelin-1	1.4 ± 0.4	$5.8 \pm 1.0^*$	109.2 ± 22.3	10
$[\text{Ala}^{3,11}]$ endothelin-1	18.7 ± 4.6	14.1 ± 4.2	101.1 ± 18.6	225
$[\text{Ala}^{1,15}]$ endothelin-1	269.0 ± 64.0	418.0 ± 50.0	125.2 ± 7.4	1360
Sarafotoxin S6b	3.9 ± 0.6	3.8 ± 0.3	114.8 ± 8.0	30

Each value is the mean ($\pm$ s.e.mean) of 7–10 observations.

* significantly different from value obtained –endo (t test, $P = 0.002$).

Figure 3 Concentration-effect curves elicited by sodium nitroprusside in tissues precontracted with endothelin-1 (10 nM) (○) or with sarafotoxin S6b (10 nM) (●) in rat isolated aortic segments without endothelium. Each point is the mean of 7 or 8 observations. Vertical bars represent s.e.mean.

ected by indomethacin. Endothelin-1 responses have also been shown to be modulated by endothelium in the perfused mesenteric bed of the rat *in vitro* (Randall *et al.*, 1989).

Acetylcholine stimulated an increase in tissue cyclic GMP levels of about 13 fold in tissues with endothelium compared to those without (Figure 2a). This degree of stimulation is at least comparable with that already reported in vascular tissues with endothelium (Holzmann, 1982; Martin *et al.*, 1986; Schoeffter & Miller, 1986). Endothelin-1 stimulated an increase of cyclic GMP levels of about 4 fold only in tissues with endothelium, demonstrating a stimulation of EDRF release. Such a stimulation of EDRF release has been reported in the rat mesenteric bed (De Nucci *et al.*, 1988). The magnitude of the stimulation of cyclic GMP levels in rat aortic tissues with endothelium is very similar to that observed with contractile agonists such as phenylephrine, B-HT 920 and clonidine, that elicit responses sensitive to the presence of the endothelium (Miller *et al.*, 1984; Bigaud *et al.*, 1984). An alter-

nate explanation, that endothelin-1 raised cyclic GMP levels within endothelial cells is unlikely, given the very small proportion which these cells contribute to aortic tissue volume (Schini *et al.*, 1989).

Contractile responses to [Ala^{3,11}]endothelin-1, [Ala^{1,15}]endothelin-1 and sarafotoxin S6b were not modulated by the presence of an intact endothelium (Figures 1b-d). This surprising result differentiates these three compounds from all other contractile agonists so far studied in this preparation (Miller *et al.*, 1988). The stimulation by sarafotoxin S6b of cyclic GMP levels was small (Figure 2) with an effect at 1 min bordering on significance ($P = 0.059$). Three testable explanations for the differences between these agonists and endothelin-1 involving the influence of EDRF are: that the analogues, but not endothelin-1, might inhibit basal EDRF release; or that sarafotoxin S6b but not endothelin-1 desensitizes aortic smooth muscle to EDRF; alternatively, that basal release of EDRF is not sufficient to modify contractile responses to any of the peptides and that only endothelin-1 stimulates release of EDRF. Basal release of EDRF does not seem to be inhibited by sarafotoxin S6b. In fact it seemed, if anything, to increase tissue cyclic GMP content (Figure 2). Desensitization does not seem to be an explanation either, since responses to sodium nitroprusside, an activator of the same guanylate cyclase stimulated by EDRF (Waldman & Murad, 1987), were potentiated in the presence of sarafotoxin S6b (Figure 3). This interesting observation remains to be explained. The remaining possibility, that only endothelin-1 stimulates EDRF release and tissue cyclic GMP levels sufficiently to modify contractile responses, seems to be a reasonable explanation for the lack of effect of the endothelium on responses to sarafotoxin S6b (Figure 1). This could indicate the presence of different receptors for endothelins on endothelium and smooth muscle, or that sarafotoxin S6b is less efficacious than endothelin-1 in the endothelium.

Overall, this work shows differences in the effects of endothelium, depending on whether endothelin-1 or various analogues were used to contract the aortic smooth muscle. Some of the results suggest that for endothelin-1 and sarafotoxin S6b these differences are likely to be caused by agonist-specific differences in altering EDRF release and not by differences in smooth muscle sensitivity to cyclic GMP. It has been suggested that modulation of contractile responses to spasmogens in vascular tissue may be due to basal (Martin *et al.*, 1986; MacLeod *et al.*, 1987) or agonist-stimulated release of EDRF (Miller *et al.*, 1984) or both (Malta *et al.*, 1986). However the differences between endothelin-1 and its analogues suggests that a stimulated release of an adequate amount of EDRF is necessary to modulate contractile responses to these peptides.

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