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Vascular activities of endothelin-1 and some alanyl substituted analogues in resistance beds of the rat

Michael D. Randall, Stephen A. Douglas & [1]C. Robin Hiley

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 The effects of endothelin-1 and of three analogues in which alanyl residues had been substituted in place of cysteinyl residues were studied in the rat, isolated, Krebs-Henseleit-perfused mesenteric bed and the *in situ*, blood-perfused, mesenteric and hindquarters circulations of the rat. The effects on the vascular responses to these peptides of removing the endothelium by detergent perfusion or of cyclo-oxygenase inhibition by indomethacin were also determined.

2 In all three preparations, endothelin-1 was a potent vasoconstrictor (ED_{50} values ranged from 40 to 400 pmol) although it was rather less potent in the hindquarters than in the mesentery. Also, the maximum response was very much smaller in the isolated mesentery (24.7 ± 2.1 mmHg) than in the *in situ* mesentery (81.8 ± 2.6 mmHg) or hindquarters (107 ± 10 mmHg).

3 Removal of the endothelium by perfusion with detergent significantly enhanced the potency of endothelin as a vasoconstrictor in the *in situ* messentery, but reduced the maximum response obtained, whereas removal of the endothelium *in vitro* significantly increased the maximum response without changing the ED_{50}. The presence or absence of indomethacin had no significant effects in the blood-perfused hindquarters preparation or the isolated mesentery but, after administration of 5 mg kg^{-1} indomethacin to the *in situ* mesenteric preparation, the maximal response to endothelin-1 was enhanced.

4 When the preparations were preconstricted with α_1-adrenoceptor agonists, endothelin-1 had modest vasodilator effects. These vasodilator effects were abolished when the endothelium was destroyed by detergent perfusion.

5 Both [$Ala^{3,11}$]endothelin-1 and [$Ala^{1,15}$]endothelin-1 were also vasoconstrictor agents in the mesenteric preparations but they were less potent than endothelin-1 itself; [$Ala^{3,11}$]endothelin-1 was intermediate in potency between endothelin-1 and [$Ala^{1,15}$]endothelin-1. In the *in situ* preparation these analogues gave similar maximal responses to the parent peptide but, *in vitro*, they gave maximal responses that were much greater than that of endothelin-1 and which were similar to those found with all 3 peptides in the *in situ* mesentery. Destruction of the endothelium *in vitro* had no effect on the responses to these 2 analogues and the log dose-response curve for [$Ala^{1,15}$]endothelin-1 in the isolated mesentery was biphasic.

6 A third analogue possessing no disulphide bridges ([$Ala^{1,3,11,15}$]endothelin-1) was a partial agonist in the *in situ* preparations but had no vasoconstrictor effect in the *in vitro* mesentery. It had no vasorelaxant effect in the hindquarters preparation but it enhanced the responses to endothelin-1 when the 2 peptides were administered together in all 3 preparations.

7 It is concluded that it is not essential for the endothelin family of peptides to possess 2 disulphide bridges for them to be vasoconstrictor agents. However, only endothelin-1, of the 4 peptides studied, showed either endothelium-dependent vasorelaxant activity or modulation by the endothelium of its pressor effects. This, together with some differences in the vasoconstrictor log dose-response curves to the peptides and the results of co-administration of [$Ala^{1,3,11,15}$]endothelin-1 and endothelin-1, suggests that there may be more than one receptor type mediating vascular responses to the peptides studied.

[1] Author for correspondence.

Introduction

The endothelins are a class of recently discovered 21 amino acid peptides which are amongst the most potent vasoconstrictor agents known. The first characterized of the family, endothelin-1, was detected in culture medium conditioned by porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). It produces prolonged pressor responses in the ganglion-blocked rat which are preceded by transient depressor responses. Endothelin-1 has also been shown to have actions in a very wide range of tissues, not only those associated with the cardiovascular system. Thus, it contracts isolated arterial and venous segments (D'Orleans-Juste *et al.*, 1988), releases endothelium-derived relaxing factor (Warner *et al.*, 1989), causes bronchoconstriction (Turner *et al.*, 1989), inhibits the release of renin from juxtaglomerular cells (Takagi *et al.*, 1988), inhibits twitch responses in the field-stimulated guinea-pig ileum but augments those of the rat vas deferens (Hiley *et al.*, 1989b), inhibits platelet aggregation (Thiemermann *et al.*, 1988), produces ventral root depolarization in newborn rat spinal cord preparations (Yoshizawa *et al.*, 1989), possesses mitogenic activity on cultured smooth muscle cells (Komuro *et al.*, 1988) and is ulcerogenic in the stomach (Wallace *et al.*, 1989; Whittle *et al.*, 1989; MacNaughton *et al.*, 1989).

Since the original description of endothelin-1, two other sequences have been detected in the human genome and these encode very similar 21 amino acid peptide structures to the parent compound (Inoue *et al.*, 1989); these have been designated endothelin-2 and endothelin-3. Human endothelin-3 has an identical sequence to the peptide originally found to be coded in the rat genome. The endothelins contain disulphide bridges between positions 1–15 and 3–11, forming an intramolecular loop structure which is not only conserved between the three forms of endothelin that have currently been characterized, but is also found in a family of snake toxins, the sarafotoxins S6. Not only are the positions of the two disulphide bridges conserved between the endothelins and sarafotoxins, but there is also considerable conservation in the remainder of the amino acid sequence and sarafotoxin S6b, which is the best characterized of the toxins, is a potent vasoconstrictor (Kloog *et al.*, 1988). The relatively unusual possession of two disulphide bridges in this family of vasoactive peptides made it of particular interest to examine the effect of removing them from endothelin-1 by chemical substitution of the bridge-forming cysteinyl residues with alanyl residues. This yielded a series of analogues: one with a disulphide bridge at the 3-11 position ([$Ala^{1,15}$]endothelin-1), one with a bridge between positions 1 and 15 ([$Ala^{3,11}$]endothelin-1) and an analogue without either bridge ([$Ala^{1,3,11,15}$]endothelin-1). The vascular actions of this series were assessed, and compared to those of endothelin-1, in the superior mesenteric bed of the rat both *in vivo*, perfused with blood, and *in vitro*, perfused with modified Krebs-Henseleit solution. The blood-perfused hindquarters of the rat were also studied. These beds are of particular interest as haemodynamic studies have revealed that, in them, endothelin-1 can have either constrictor or relaxant actions, or both (Thomas *et al.*, 1989; Gardiner *et al.*, 1989).

A preliminary account of some of these results was presented at the First William Harvey Workshop on Endothelin, London, December 1988 (Hiley *et al.*, 1989a).

Methods

The isolated, Krebs-Henseleit-perfused superior mesenteric arterial bed of the rat

Male Wistar rats (250–350 g; Bantin & Kingman, Hull) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.: Sagatal; Rhône Poulenc, Dagenham, U.K.) and heparinized (1000 u kg^{-1}, i.p.). The superior mesenteric artery was cannulated and the vascular bed perfused, by a Harvard Type 1203A peristaltic perfusion pump, according to the method of McGregor (1965), at 2 ml min^{-1} with Krebs-Henseleit solution containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 5.5. In addition, the buffer contained 10 μM indomethacin. The perfusing fluid was saturated with 95% O_2/5% CO_2 and, together with the tissue, was maintained at 37°C. Perfusion pressure was recorded by a Bell & Howell Type 4-422-0001 pressure transducer connected to a T-piece placed in the perfusion circuit close to the point of insertion into the superior mesenteric artery. Recordings were made on a Grass Model 79D polygraph.

After a 30 min equilibration period, peptides were administered into the perfusion circuit in volumes not exceeding 100 μl. Administration of peptides was repeated 5 min after the tissue response to the previous dose of peptide had returned to the resting basal perfusion pressure. In co-administration studies, the peptides were mixed and immediately injected into the perfusion circuit in volumes of 200 μl or less.

Destruction of the endothelium was achieved by perfusion of the vascular bed with a 0.3% (w/v) solution of the detergent CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; Sigma Chemical Co., Poole, Dorset) in distilled water for

120–150 s followed by 45 min re-equilibration. This procedure has been shown by histology to lead to loss of the endothelium (Hiley *et al.*, 1987). In each case functional loss of the endothelium was confirmed by showing that acetylcholine was unable to oppose noradrenaline-induced vasoconstriction before construction of dose-response curves to the peptide.

The vasorelaxant properties of endothelin-1 were assessed by recording reductions in tone that had been established by infusion of methoxamine into the perfusate at a concentration of 10^{-4} M. It was found necessary to include bovine serum albumen (Sigma) in the perfusate at a concentration of $5\,g\,l^{-1}$ and the flow rate used was $4\,ml\,min^{-1}$. In some control experiments, indomethacin was omitted from the perfusion fluid and, where this was done, it is specifically stated in the text.

In situ *blood perfusion of the superior mesenteric arterial bed*

Male Wistar rats (250–330 g; Bantin & Kingman, Hull) were anaesthetized with a mixture of $60\,mg\,kg^{-1}$ sodium thiopentone (Trapanal; BYK Gulden, Konstanz, F.R.G.) and $30\,mg\,kg^{-1}$ sodium pentobarbitone (Sagatal) given i.p. A tracheal cannula was inserted to allow them to breath air spontaneously and the left common carotid artery cannulated for administration of drugs and infusion of 0.9% saline at $6\,ml\,h^{-1}$ (to prevent volume depletion). The right common carotid artery was cannulated and connected to a Bell & Howell Type 4-422-0001 pressure transducer coupled to a Grass Model 79D polygraph to record central arterial pressure. Heart rate was derived from the pressure wave by means of a Grass 7P44 tachograph preamplifier. Rectal temperature was maintained at 37°C by means of a homeothermic blanket system (BioScience, Sheerness, Kent).

Preparation for the *in situ* blood perfusion of the superior mesenteric arterial bed was similar to that described by Jackson & Campbell (1980) and has been described in detail elsewhere (Hiley *et al.*, 1985). Briefly a mid-line abdominal incision was made and ligatures placed around the aorta distal to the renal arteries and around the superior mesenteric artery close to the aorta. Following a 20 min period to allow haemostasis, the animal received $1000\,u\,kg^{-1}$ heparin into the jugular vein. The abdominal aorta was cannulated with PP60 tubing which led to a Harvard Type 2903 servo-controlled pump and then an air compliance chamber which was inserted into the circuit to act as a bubble trap. The blood was reheated to 37°C by means of a heat exchanger and returned to the animal through the superior mesenteric artery via a PP50 inflow cannula. The ischaemic period experienced by the bed during cannulation did not exceed 1 min. Perfusion pressure in the mesentery was measured by means of a second Bell & Howell pressure transducer placed a fixed distance from the end of the inflow cannula. Following a 20 min equilibration period drugs were administered into the extracorporeal circuit proximal to the heat exchanger in volumes not exceeding $100\,\mu l$, except in the co-administration experiments when the volume did not exceed $200\,\mu l$. In the relaxation experiments the volume of injection did not exceed $10\,\mu l$.

Destruction of the endothelium *in vivo* was achieved by perfusing the mesenteric vascular bed with a 0.3% solution (w/v) of CHAPS in 0.9% saline at $2\,ml\,min^{-1}$ for 150 s in place of blood. In order to limit the contact of the detergent with the blood the bed was initially loaded with 2–3 ml of 0.9% saline. The use of CHAPS in this way has been shown by electron microscopy to lead to destruction of the endothelium without morphological changes in the vascular smooth muscle (Randall, 1989). Following such treatment the vasorelaxation to acetylcholine is lost, while dilatation to sodium nitroprusside and constriction to noradrenaline remain intact (Randall & Hiley, 1988).

Administration of endothelin-1 into the portal vein was achieved by injecting it directly into the splenic pulp by means of a steel needle. Haemostasis was achieved by cyanoacrylate adhesive. All rats used in these experiments were pretreated with $5\,mg\,kg^{-1}$ indomethacin to eliminate the vascular actions of prostaglandins released from the spleen by endothelin (Rae *et al.*, 1989).

Blood perfusion of the hindquarters of the rat in situ

The preparation is essentially as described for the blood perfusion of the mesenteric bed except that blood from the extracorporeal circuit is returned to the animal through a cannula (PP50) located proximal to the iliac bifurcation in the abdominal aorta.

In both blood-perfused preparations, when the relaxant properties of the peptides were examined, tone was established by a close arterial infusion of a $50\,\mu g\,ml^{-1}$ solution of (−)-phenylephrine at $5\,\mu g\,min^{-1}$. Pretreatment of either blood-perfused preparation with indomethacin was by administration of $5\,mg\,kg^{-1}$ into the jugular vein before perfusion was commenced through the extracorporeal circuit. The injection volume was $2\,ml\,kg^{-1}$.

Statistical and data analysis

All values are given as the mean ± s.e.mean and the number of animals in each group is represented by *n*.

Comparison between means was carried out by Student's unpaired t test.

Dose-response curves were analysed by fitting the logistic equation:

$$R = \frac{R_{max} A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the increase in perfusion pressure, A the dose of peptide, R_{max} the maximum pressor response, n_H the slope function and ED_{50} the dose of peptide giving the half-maximal response. Where appropriate, the data were fitted to a double hyperbola according to the following equation:

$$R = \frac{R_{max_1} \cdot A^{n_{H_1}}}{ED_{50_1}^{n_{H_1}} + A^{n_{H_1}}} + \frac{R_{max_2} \cdot A^{n_{H_2}}}{ED_{50_2}^{n_{H_2}} + A^{n_{H_2}}}$$

in which the subscripts 1 and 2 serve to denote the values of R_{max}, n_H and ED_{50} for each of the two sites. A modified Marquardt procedure, as implemented in the Harwell routine VB01A on the Cambridge University IBM 3081 mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

Drugs and peptides

Apart from the peptides, all drug solutions were made freshly on the day of the experiment. (−)-Phenylephrine hydrochloride and (−)-noradrenaline bitartate (Sigma Chemical Co., Poole, Dorset) were both dissolved in saline (0.9% NaCl w/v), which contained 1 mg ml^{-1} of ascorbic acid (Fisons, Loughborough, U.K.), to give stock solutions of 1 mg ml^{-1} and 0.5 mg ml^{-1} respectively. Methoxamine hydrochloride (Sigma) was dissolved in saline as was acetylcholine bromide (Sigma); serial dilutions were prepared for the latter drug. Indomethacin (Sigma) was dissolved to give a 1 mg ml^{-1} solution in 5% sodium bicarbonate and then diluted to the required concentration in saline or Krebs-Henseleit solution. Solutions (0.3% w/v) of the non-denaturing, zwitterionic detergent CHAPS (Sigma) were also prepared either in distilled water (*in vitro* studies) or saline (*in situ* studies). Endothelin-1 (originally designated as human/porcine endothelin) was obtained from Novabiochem (Läufelfingen, Switzerland). The three alanyl substituted analogues of endothelin-1, [Ala^{1,15}]endothelin-1, [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1, were synthesized by Dr J.T. Pelton of the Merrell Dow Research Institute, Strasbourg. The peptides were dissolved in distilled water to give 100 μM solutions (except for [Ala^{1,3,11,15}]endothelin-1; 10 μM) and frozen until used.

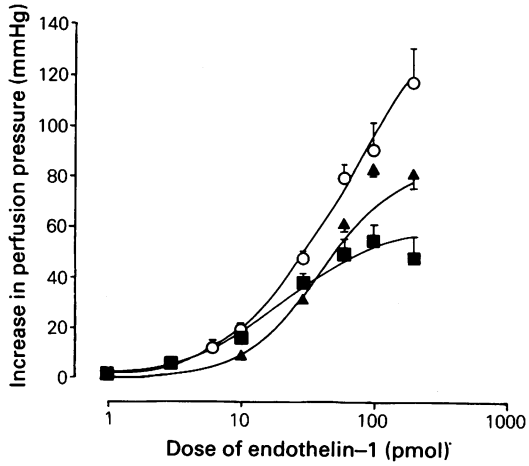

Figure 1 Pressor responses to endothelin-1 in the blood-perfused mesenteric arterial bed of the rat. (▲) shows the control responses to endothelin-1 ($n = 5$); (■) represents the responses to the peptide after destruction of the endothelium by perfusion with CHAPS ($n = 5$–6); and (○) gives the responses obtained with endothelin-1 in animals pretreated with 5 mg kg^{-1} indomethacin ($n = 8$). The points show the mean and the vertical lines show s.e.mean.

Results

Pressor activity of endothelin-1 in the in situ *blood-perfused, superior mesenteric arterial bed and the isolated, Krebs-Henseleit-perfused mesenteric preparation*

Endothelin-1 (1–200 pmol, *in situ*, results shown in Figure 1; 1–1000 pmol, *in vitro*, data shown in Figure 2) when injected into either preparation produced dose-dependent increases in perfusion pressure which reached a maximum 1–2 min after injection and returned to baseline within 20–25 min in the *in situ* preparation and within 30–45 min in the *in vitro* preparation.

The ED_{50} for vasoconstrictor activity of the peptide *in situ* was 40.3 ± 4.5 pmol, the calculated maximum increase in perfusion pressure (R_{max}) was 81.8 ± 2.6 mmHg and the slope function (n_H) was 1.58 ± 0.03 ($n = 5$). Figure 1 also shows that endothelial cell destruction *in situ* resulted in an increase in the potency of the peptide as a pressor agent; the ED_{50} was significantly ($P < 0.05$) lower at 22.2 ± 5.5 pmol. However, the R_{max} was reduced significantly ($P < 0.05$) to 60.9 ± 6.7 mmHg ($n = 5$–6).

In the isolated, Krebs-Henseleit-perfused mesenteric bed endothelin-1 had a similar ED_{50} (66.2 ± 16.3 pmol) to that *in vivo*, but the calculated

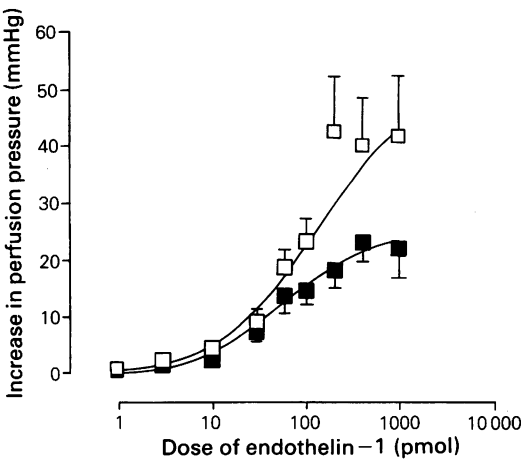

Figure 2 Pressor responses to endothelin-1 in the mesenteric arterial bed of the rat perfused *in vitro* with Krebs-Henseleit solution containing 10 μM indomethacin. (■) show the control responses ($n = 9$) and (□) the responses obtained in preparations in which the endothelium had been destroyed with CHAPS ($n = 7$). The points show the mean and the vertical lines show s.e.mean.

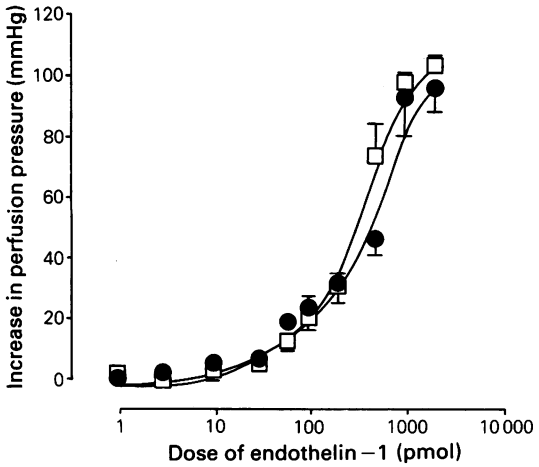

Figure 3 Pressor responses to endothelin-1 in the blood-perfused hindquarters preparation of the rat. (●) shows the control responses to endothelin-1 ($n = 4$) and (□) represents the responses to the peptide obtained in animals pretreated with 5 mg kg^{-1} indomethacin ($n = 4$). The points show the mean and the vertical lines show s.e.mean.

maximum pressor response was significantly ($P < 0.001$) lower than that in the *in situ* preparation with a value of 24.7 ± 2.1 mmHg ($n = 9$). Destruction of the vascular endothelium *in vitro* significantly ($P < 0.01$) increased the R_{max} of the peptide to 50.3 ± 7.4 mmHg without affecting the ED_{50} value (Figure 2) which was 133.2 ± 54.6 pmol ($n = 7$). The slope functions, 1.03 ± 0.10 and 0.84 ± 0.08 for the control curve and that obtained following CHAPS perfusion respectively, showed no statistical differences. Omission of indomethacin from the Krebs-Henseleit solution did not significantly alter the dose-response relationship for vasoconstriction by endothelin-1 (1–200 pmol) in the presence of endothelium; the R_{max} was 18.6 ± 4.1 mmHg, the ED_{50} was 25.6 ± 12.3 pmol and n_H was 1.34 ± 0.34 ($n = 6$).

Pressor effects of endothelin-1 in the blood-perfused hindquarters of the rat

Endothelin-1 (1 pmol–2 nmol) produced dose-related increases in the perfusion pressure in the blood-perfused hindquarters ($n = 4$), similar in time course to those in the mesenteric circulation. The ED_{50} was 419 ± 136 pmol, the calculated maximum pressor response was 107 ± 10 mmHg and the slope function was 0.85 ± 0.10 (Figure 3).

Effect of indomethacin on pressor activity of endothelin-1 in the blood-perfused superior mesenteric arterial bed and hindquarters of the rat

Pretreatment of the rats with indomethacin (5 mg kg^{-1}, i.v.) had no significant effects on either mean systemic arterial pressure, heart rate or basal perfusion pressure in either the mesenteric or hindquarters preparations. However, Figure 1 shows that the treatment significantly ($P < 0.05$) increased the maximum constrictor response of the peptide in the mesentery ($R_{max} = 133.4 \pm 16.2$ mmHg; $n = 8$) as compared to the control value (see above). The ED_{50} (47.1 ± 10.0 pmol) did not differ significantly from the control value but n_H (1.19 ± 0.08) was significantly lower than the control ($P < 0.05$). In the blood perfused hindquarters indomethacin treatment had no significant effects (Figure 3); after its administration the ED_{50} was 322 ± 64 pmol, the R_{max} was 117 ± 7 mmHg and n_H was 1.29 ± 0.13 ($n = 4$).

Effects of intrasplenically administered endothelin-1 on mesenteric perfusion pressure and mean arterial pressure

Injections of endothelin-1 (100–600 pmol) intrasplenically into rats pretreated with indomethacin had limited effects on mesenteric perfusion pressure; the maximum dose used, 600 pmol, produced an increase of 6.96 ± 0.99 mmHg ($n = 3$). The systemic effects of

this dose of the peptide given intrasplenically consisted of a transient depressor response of 22.7 ± 1.6 mmHg and subsequently mean arterial pressure was only 3.33 ± 1.67 mmHg greater than the pressure before administration of the endothelin-1 (104 ± 15 mmHg).

Effect of endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 on phenylephrine-induced tone in the blood-perfused mesentery and hindquarters of the rat

Infusion of 5 μg min^{-1} (−)-phenylephrine increased perfusion pressure by 69.2 ± 9.3 mmHg (n = 7) and 93.9 ± 8.5 mmHg (n = 9) respectively in the mesenteric and hindquarters vascular beds. In the latter preparation, endothelin-1 (0.01–10 pmol) caused a modest dilatation, which was maximal, 12.2 ± 2.4% (n = 7) of the induced tone, at 0.3 pmol (Figure 4a). The vasodilatation in the hindquarters was unaffected by pretreatment of the animals with 5 mg kg^{-1} indomethacin; the maximum effect observed was a reduction in established tone of 8.4 ± 1.8% which was obtained at 0.3 pmol (n = 5).

In the mesenteric arterial preparation, endothelin-1 (0.01–10 pmol) produced small decreases in this established tone (Figure 4b); the maximum observed inhibition of tone was 14.8 ± 4.3% at 0.3 pmol (n = 7). At doses above 6 pmol vasodilatation was no longer observed and a pressor response was obtained.

The only one of the alanyl-substituted analogues of endothelin-1 that was tested for direct vasorelaxant actions was [Ala^{1,3,11,15}]endothelin-1 and it had no significant activity in the hindquarters over the dose range 7 fmol–6.5 pmol (Figure 4c; n = 5).

Relaxation of methoxamine-induced tone in the isolated, Krebs-Henseleit-perfused superior mesenteric arterial bed

In the isolated mesenteric arterial bed, endothelin-1 (0.5–500 pmol) also relaxed tone established by infusion of methoxamine (Figure 5); 25 pmol endothelin-1 gave the maximum effect which was a reduction of 32.9 ± 7.2% of the established tone (n = 6). Figure 5 also shows that the inclusion of indomethacin in the perfusion fluid changed the concentration at which the maximum relaxation occurred to 50 pmol although the maximum degree of relaxation (22.2 ± 3.4%; n = 7) was not significantly different from the reduction of induced tone observed in the absence of indomethacin. In the experiments in which indomethacin was absent from the perfusion fluid, the tone established by methoxamine infusion was 63.1 ± 7.6 mmHg, whereas in the presence of indomethacin it was 91.6 ± 5.5 mmHg. Experiments carried out in the presence of indo-

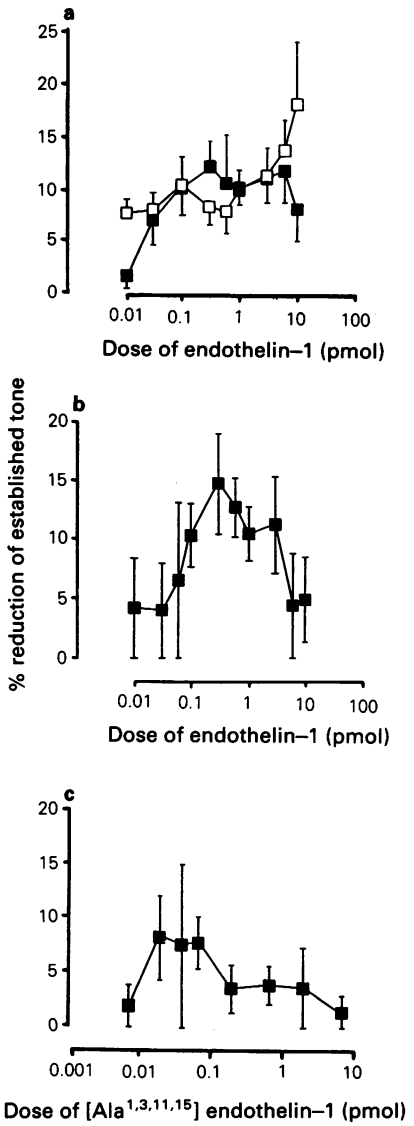

Figure 4 Relaxation by endothelin-1 or [Ala^{1,3,11,15}]endothelin-1 of vascular tone induced by infusion of 5 μM phenylephrine into either the *in situ* blood-perfused mesenteric bed (b) or the blood-perfused hindquarters preparation (a and c) of the rat. (a) Reductions in established tone by endothelin-1 in the hindquarters in control preparations (■; n = 9) and in preparations pretreated with 5 mg kg^{-1} indomethacin (□; n = 6). (b) Vasorelaxation in response to endothelin-1 in the *in situ* mesenteric arterial bed in the absence of indomethacin (n = 7). (c) Reductions in established tone by [Ala^{1,3,11,15}]endothelin-1 in the blood perfused hindquarters preparation (n = 5). The points show the mean and vertical lines show s.e.mean.

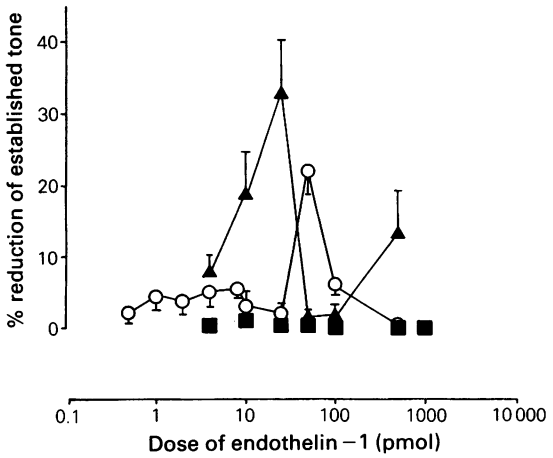

Figure 5 Relaxation by endothelin-1 of vascular tone established by infusion of 100 μM methoxamine into the *in vitro*, Krebs-Henseleit-perfused mesenteric arterial bed preparation of the rat. (■) shows the responses obtained in the presence of the endothelium and 10 μM indomethacin (n = 6–7); (○) represents the responses obtained in the presence of 10 μM indomethacin after destruction of the endothelium with CHAPS (n = 4–5); and (▲) gives the results obtained in the absence of indomethacin but in the presence of the endothelium (n = 6). The points show the mean and vertical lines show s.e.mean.

methacin showed that the relaxation in response to endothelin-1 was abolished by removal of the endothelium (n = 4–5).

Pressor actions of alanyl substituted analogues of endothelin-1 in the in situ, *blood-perfused, and in the isolated, Krebs-Henseleit-perfused, mesenteric arterial beds*

[Ala^{3,11}]endothelin-1 (0.75 pmol–2.25 nmol) increased perfusion pressure in the *in situ* mesentery (Figure 6a), but was significantly ($P < 0.01$) less potent than endothelin-1, with an ED_{50} of 270 ± 52 pmol, while the calculated R_{max}, 94.0 ± 6.2 mmHg (n = 7), was similar to that of endothelin-1 (Figure 6a). *In vitro*, [Ala^{3,11}]endothelin-1 produced a maximum increase in perfusion pressure (128 ± 10 mmHg; n = 3) comparable to that *in vivo* (Figure 7), but significantly ($P < 0.01$) greater than that for endothelin-1 *in vitro* (24.7 ± 2.1 mmHg; Figure 2); the ED_{50}, 0.94 ± 0.17 nmol (n = 3), was also significantly ($P < 0.001$) greater than that found for endothelin-1 in the same preparation. After the endothelium had been removed with CHAPS *in vitro* neither the R_{max} (110 ± 13 mmHg) nor the ED_{50} (0.62 ± 0.08 nmol) for this analogue were changed significantly from those found in the preparations with endothelium (n = 3; Figure 7).

[Ala^{1,15}]endothelin-1 (1.36 pmol–6.80 nmol) also produced dose-related vasoconstrictor responses in the blood-perfused mesentery; the dose-response curve for these responses had two components and was best fitted to a double hyperbola (Figure 6a). The component describing its action at the lower doses (1.36–1360 pmol) had an R_{max} of 19.1 ± 1.7 mmHg and an ED_{50} of 40.7 ± 19.6 pmol (n = 8). Two higher doses, 4.08 nmol and 6.80 nmol, produced pressor responses of 70.4 ± 4.9 mmHg (n = 6) and 89.3 ± 8.5 mmHg (n = 3) respectively. The actual maximum response could not be obtained owing to the acute systemic effects of the peptide (see below). Figure 7 shows that, *in vitro*, this analogue was significantly less potent than either endothelin-1 ($P < 0.001$) or [Ala^{3,11}]endothelin-1 ($P < 0.01$); the ED_{50} was found to be 3.5 ± 0.5 nmol (n = 4). The R_{max} for [Ala^{1,15}]endothelin-1 was 107 ± 14 mmHg which is comparable to that for [Ala^{3,11}]endothelin-1, but significantly ($P < 0.001$) greater than that of endothelin-1. Destruction of the vascular endothelium *in vitro* had no significant effect on either the ED_{50} or R_{max}, with values of 3.9 ± 0.2 nmol and 71.0 ± 7.5 mmHg, respectively (n = 5).

[Ala^{1,3,11,15}]endothelin-1 produced modest vasoconstrictor responses in both the blood-perfused mesenteric (dose range 0.65–390 pmol; Figure 6b) and hindquarters (dose range 6.5–650 pmol; Figure 6c) preparations. In the intact, blood-perfused mesentery this analogue had an R_{max} of 20.9 ± 3.0 mmHg (n = 7), which was significantly ($P < 0.001$) lower than that for endothelin-1 in this vascular bed over a similar dose range. Its ED_{50} was 45.3 ± 22.6 pmol, comparable to that of endothelin-1. In the isolated mesentery [Ala^{1,3,11,15}]endothelin-1 had no pressor activity in the dose range 0.65–1300 pmol (n = 3); endothelial cell destruction did not reveal any pressor activity of this analogue. In the blood-perfused hindquarters preparation [Ala^{1,3,11,15}]endothelin-1 had similar activity to the *in situ* mesentery; the R_{max} was 17.6 ± 1.7 mmHg, which is significantly ($P < 0.001$) lower than that for endothelin-1 in this bed and the ED_{50} was 38.2 ± 5.4 pmol, which is significantly ($P < 0.001$) less than the corresponding value for endothelin-1 (n = 5).

Effects on mean systemic blood pressure of close arterial administration of endothelin-1 and its alanyl-substituted analogues into the mesenteric or hindquarters circulations

Administration of endothelin-1 (1–200 pmol) into the superior mesenteric artery had no significant effects on mean arterial blood pressure. On administration into the lower abdominal aorta perfusing the hind-

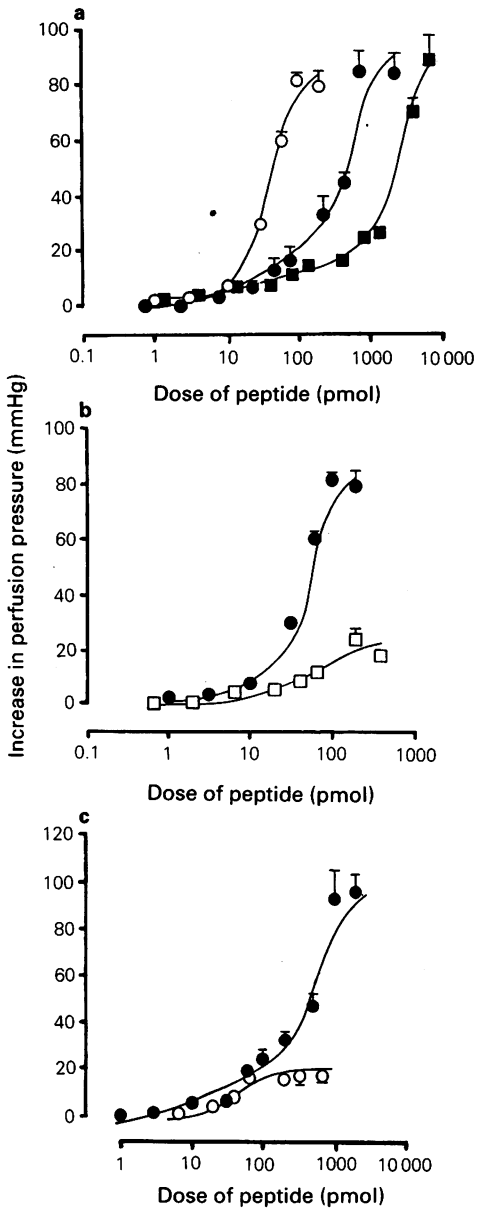

Figure 6 Pressor responses to alanyl-substituted analogues of endothelin-1 in the blood-perfused mesenteric or hindquarters preparations of the rat. (a) Responses to endothelin-1 (○; $n = 5$), [Ala^{1,15}]endothelin-1 (■; $n = 3$–8) and [Ala^{3,11}]endothelin-1 (●; $n = 7$) in the mesenteric arterial bed. (b) Responses to endothelin-1 (●; $n = 5$) and [Ala^{1,3,11,15}]endothelin-1 (□; $n = 7$) in the mesenteric bed. (c) Responses to endothelin-1 (●; $n = 4$) and [Ala^{1,3,11,15}]endothelin-1 (○; $n = 5$) in the hindquarters circulation. In (a) and (b) the curve for endothelin-1 is shown for comparison and the data are the control results for endothelin-1 in Figure 1. Similarly, the data for endothelin-1 in the hindquarters are the control data shown in Figure 3. The points are the mean and vertical lines show s.e.mean.

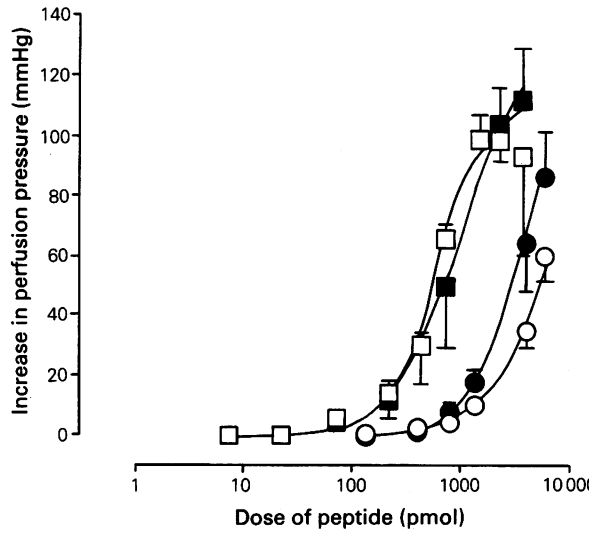

Figure 7 Pressor responses to [Ala^{1,15}]endothelin-1 (○, ●) and [Ala^{3,11}]endothelin-1 (□, ■) in the isolated mesenteric arterial bed of the rat perfused with Krebs-Henseleit solution containing 10 μM indomethacin. The closed symbols (●, ■) show results obtained in preparations with an intact endothelium and the open symbols (○, □) give results from preparations in which the endothelium had been destroyed with CHAPS. The points are the mean and the vertical lines show 1 s.e.mean. For [Ala^{3,11}]endothelin-1, $n = 3$ for both intact and endothelium-denuded preparations, but for [Ala^{1,15}]endothelin-1, $n = 4$ for the intact preparations and $n = 5$ for the preparations without endothelium.

quarters, endothelin-1 (1–2000 pmol) produced a modest elevation of mean arterial blood pressure commencing at 60 pmol, at 2000 pmol the pressor response was 28.3 ± 4.4 mmHg ($n = 3$).

On mesenteric administration, both [Ala^{3,11}]-endothelin-1 (45 pmol–2.25 nmol) and [Ala^{1,3,11,15}]-endothelin-1 (19.5–390 pmol) produced relatively long lasting (5–20 min) falls in mean systemic arterial blood pressure which were not followed by pressor responses. [Ala^{3,11}]endothelin-1 injected close arterially into the mesenteric circulation produced a systemic depressor response at doses above 45 pmol; at the maximum dose used (2250 pmol), the decrease in mean arterial blood pressure was 21.7 ± 6.7 mmHg ($n = 7$). [Ala^{1,3,11,15}]endothelin-1 also caused depressor responses at doses greater than 19.5 pmol; at the maximum dose used

(390 pmol) the decrease in mean arterial pressure was 10.5 ± 4.5 mmHg ($n = 7$).

[Ala^{1,15}]endothelin-1, on administration into the mesenteric arterial bed, produced systemic depressor responses at the lower doses (81.6–816 pmol; $n = 8$), with a maximum fall of 16.1 ± 1.8 mmHg at 816 pmol of the peptide. Higher doses (1–6.8 nmol) produced pressor responses, with 6.8 nmol increasing mean systemic arterial pressure by 25.0 ± 1.0 mmHg.

Of these alanyl-substituted analogues, only [Ala^{1,3,11,15}]endothelin was administered into the hindquarters and it had no significant systemic actions up to the maximum dose given (650 pmol).

Effect of [Ala^{1,3,11,15}]endothelin-1 on the pressor activity of endothelin-1 in the mesentery in vivo *and* in vitro

[Ala^{1,3,11,15}]endothelin-1 (130 pmol), which produced a pressor response of 11.3 ± 0.9 mmHg ($n = 4$) in the blood-perfused mesenteric arterial bed augmented the pressor activity of endothelin-1 when the two peptides were co-administered (Figure 8a). A higher dose of 260 pmol [Ala^{1,3,11,15}]endothelin-1 also caused an increase in mesenteric perfusion pressure (15.5 ± 2.2 mmHg; $n = 4$), while on co-administration with endothelin-1 it produced a leftward shift in the log dose-response curve for endothelin-1 compared to that for endothelin-1 alone (Figure 8b). Figure 8a also shows that the positions of the curves for endothelin-1 obtained in the presence of either dose of [Ala^{1,3,11,15}]-endothelin-1 were very similar. Further, the mean responses at each dose of endothelin-1 obtained in the absence of [Ala^{1,3,11,15}]endothelin-1 were significantly different ($P < 0.05$) from those obtained in the presence of either 130 or 260 pmol [Ala^{1,3,11,15}]endothelin-1 with only two exceptions, 400 pmol endothelin-1 with 260 pmol analogue and 10 pmol endothelin-1 with 130 pmol analogue.

Figure 8b shows that, in the blood-perfused hindquarters preparation, the pressor activity of endothelin-1 co-administered with 260 pmol [Ala^{1,3,11,15}]endothelin-1 was greater than that of endothelin-1 alone in the lower dose range (up to 100 pmol), while from 300 pmol to 2 nmol endothelin-1 there was no significant difference between the two groups. In the absence of the analogue the dose-response relationship for endothelin-1 was described by an ED_{50} of 436 ± 90 pmol, an R_{max} of 99.7 ± 3.5 mmHg and a slope function of 1.72 ± 0.37 but, on co-administration with the analogue, the dose-response curve for endothelin-1 became distinctly biphasic; significant enhancement of the response to the parent peptide occurred with the 4 lowest doses tested, that is, 10 pmol ($P < 0.05$), 30 pmol, 60 pmol and 100 pmol (all $P < 0.01$; Student's unpaired t test; $n = 6$ for both the control and co-administration groups).

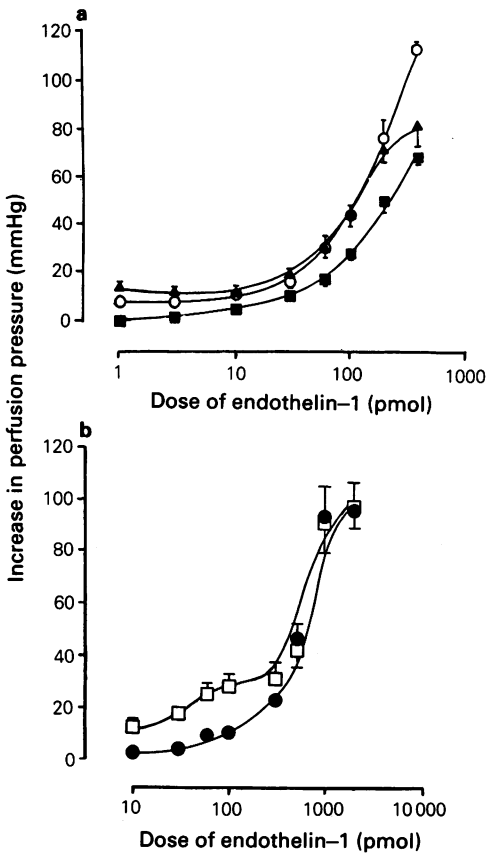

Figure 8 Pressor responses obtained either for endothelin-1 alone or for the parent peptide co-administered with [Ala^{1,3,11,15}]endothelin-1 in the blood-perfused superior mesenteric or hindlimb preparations. (a) Results obtained in the superior mesenteric arterial bed. (■) shows the responses obtained with endothelin-1 alone ($n = 4$); (○) represents the responses obtained with endothelin-1 administered together with 130 pmol [Ala^{1,3,11,15}]endothelin-1 ($n = 4$); and (▲) shows the responses obtained when endothelin-1 was co-administered with 260 pmol endothelin-1 ($n = 5$). (b) Pressor responses to endothelin-1 alone (●; $n = 3$–6) or endothelin-1 co-administered with 260 pmol [Ala^{1,3,11,15}]endothelin-1 (□; $n = 3$–6) in the hindquarters circulation. The points are the mean and vertical lines show s.e.mean.

In vitro, co-administration of 130 pmol [Ala^{1,3,11,15}]endothelin-1 with endothelin-1 (60–400 pmol) in the mesenteric bed did not alter the pressor activity of endothelin-1 significantly (Figure 9a). However, Figure 9b shows that co-administration of 260 pmol [Ala^{1,3,11,15}]endothelin-1

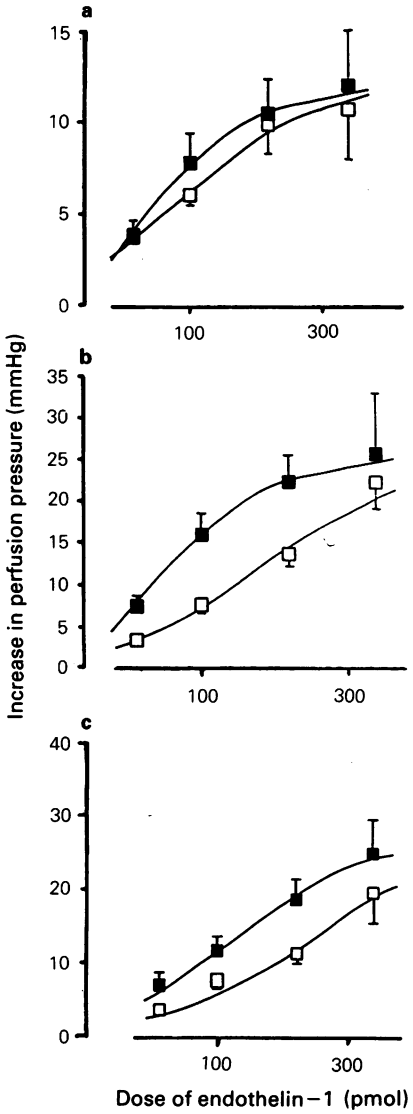

Figure 9 Increases in perfusion pressure in response to endothelin-1 either administered alone or in conjunction with [Ala^{1,3,11,15}]endothelin-1 in the rat isolated superior mesenteric arterial bed preparation. (a) Responses to endothelin-1 either alone (□; $n = 7$) or when administered with 130 pmol [Ala^{1,3,11,15}]-endothelin-1 (■; $n = 5$–7) in preparations with intact endothelium. (b) Pressor responses to endothelin-1 administered either alone (□; $n = 8$) or together with 260 pmol [Ala^{1,3,11,15}]endothelin-1 (■; $n = 8$) in preparations with intact endothelium. (c) Effects of endothelin-1 given either separately (□; $n = 6$) or together with 260 pmol [Ala^{1,3,11,15}]endothelin-1 (■; $n = 5$–7) in preparations denuded of endothelium by perfusion with CHAPS. Values are the mean with vertical lines showing s.e.mean.

with the parent peptide ($n = 8$) significantly augmented the pressor responses relative to those observed in response to endothelin-1 alone ($n = 8$) at doses of 60 pmol ($P < 0.01$), 100 pmol ($P < 0.01$) and 200 pmol ($P < 0.05$). This potentiating action of the alanyl-substituted analogue on the pressor responses produced by endothelin-1 was shown to be an endothelium-independent action. After endothelial cell destruction by perfusion of the mesenteric bed with CHAPS, co-administration of 260 pmol [Ala^{1,3,11,15}]endothelin-1 with endothelin-1 resulted in significant increases in the pressor responses to doses of 100 pmol ($P < 0.05$) and 200 pmol ($P < 0.05$) of endothelin-1 (Figure 9c).

Discussion

This study shows that endothelin-1 is a potent vasoconstrictor of both the mesenteric and the hindquarters circulations of the rat. Furthermore, analogues of endothelin-1, in which one or other of the disulphide bridges was absent, also caused vasoconstriction of the mesenteric arterial bed and an analogue in which there was no disulphide bridge was a partial agonist, relative to the parent peptide, in the two blood-perfused preparations investigated.

Endothelin-1 was 10 fold less potent in the blood perfused hindquarters preparation than in the *in situ* mesenteric arterial bed. This difference is interesting since it has been shown that profound changes in mesenteric blood flow, associated with systemic pressor responses to endothelin-1, are not accompanied by reductions in hindlimb blood flow in conscious, spontaneously hypertensive (Wright & Fozard, 1988) or normotensive (Gardiner *et al.*, 1989) rats. Also, in pithed rats we have found that a modest increase in systemic blood pressure caused by endothelin-1 is associated with a large rise in mesenteric vascular resistance, which is not accompanied by an increase in skeletal muscle vascular resistance (MacLean *et al.*, 1989).

It is of interest that the peptide has a higher maximal pressor activity in the *in situ* blood-perfused mesentery compared to the isolated, buffer-perfused preparation. The difference in activity probably reflects the anatomical differences between the two different preparations; the *in situ* bed is essentially intact and contains the smaller arterioles, capillaries, venules and hepatic portal vein which are absent in the isolated bed. The increased maximal pressor activity *in situ* may thus reflect a substantial constrictor action on the venous side of the bed. This hypothesis would accord with the finding that endothelin-1 appears to be a more potent vasoconstrictor in venous tissues than it is on arterial preparations (D'Orleans-Juste *et al.*, 1988). However, this

anatomical difference in potency of endothelin-1 may only apply to the larger blood vessels on the venous side of the bed as Brain *et al.* (1988) have found that, in the hamster cheek pouch, endothelin-1 was only an arteriolar vasoconstrictor and had no apparent actions on the venules. In the present study the administration of endothelin-1 into the hepatic portal venous system via the spleen had only very small effects on the arterial perfusion pressure in the mesentery. Since systemic effects were apparent after intrasplenic administration of endothelin-1, the peptide must have entered the portal circulation, as the spleen drains into this vein. Therefore the small rises in mesenteric perfusion pressure found in this study may be the result of portal venous constriction, recirculation of the peptide through the systemic circulation or both. However, a substantial contribution by portal venoconstriction to the pressor activity of endothelin-1 in the mesentery seems very unlikely. Alternatively, the increased constrictor activity *in vivo* may be due to the fact that the intact bed is a closed circuit and thus constrictor activity generally may be enhanced. This would seem unlikely in view of the comparable maximum pressor responses to [$Ala^{3,11}$]endothelin-1 *in vivo* and *in vitro* obtained in this study. Thus it would seem that the isolated bed has a diminished number of responsive sites for endothelin-1 compared to the intact bed and that such a difference does not exist for [$Ala^{3,11}$]endothelin-1.

Endothelin-1 releases EDRF in the isolated Krebs-Henseleit perfused mesentery (De Nucci *et al.*, 1988; Warner *et al.*, 1989) which leads to a modulation of the pressor activity of the peptide. Enhanced pressor activity for endothelin-1 was also found in this study after endothelial cell destruction in the isolated preparation and this supports a role for the endothelium in the modulation of the pressor actions of endothelin-1 by the release of EDRF. Release of EDRF by endothelin-1 probably also accounts for the relaxation of induced tone that the peptide produced in both the isolated and *in situ* mesenteric beds and in the hindquarters circulation. Alternatively, the enhancement of the pressor responses to endothelin-1 in the isolated mesenteric bed may reflect the loss of a diffusional barrier, facilitating the access of the peptide to the vascular smooth muscle (Pohl & Busse, 1989). However, selective destruction of the endothelium *in vivo* resulted in decreased vascular reactivity towards the peptide. The same method of endothelial destruction *in vivo* had no effect on the maximum pressor responses to noradrenaline (Randall *et al.*, 1988), suggesting that the change observed with endothelin-1 is not general for vasoconstrictor agents. It is possible that, during blood perfusion, some of the pressor activity of endothelin-1 may be due to release of endothelium-derived vasoconstrictors, although the reduction in maximum response may be due to an action of the detergent on the processes underlying endothelin-induced vasoconstriction while leaving the pressor activity to noradrenaline intact.

Cyclo-oxygenase inhibition by indomethacin potentiates the pressor activity of endothelin-1 in the pithed rat (De Nucci *et al.*, 1988) and in the isolated kidney and spleen (Rae *et al.*, 1989). In the current study, cyclo-oxygenase products appeared to be able to influence the activity of endothelin-1 in the *in situ* mesentery, but not the hindquarters, since indomethacin, at a dose which inhibits peripheral cyclo-oxygenases (Jackson & Campbell, 1980), increased the maximal response to the peptide in the mesentery but not the hindquarters. This suggests that eicosanoids, which are either tonically released, released by stimulation or produced by the two processes together, may modulate the activity of this peptide in the mesenteric circulation but not the hindquarters. The prostanoids are unlikely to be of endothelial origin as destruction of the vascular endothelium *in vivo* does not have the same effect as indomethacin pretreatment. Modulation of pressor responses to endothelin-1 has been shown to be due to eicosanoid activity in the intact rat, since indomethacin or piroxicam pretreatment increased responses to second and subsequent doses of endothelin-1 (De Nucci *et al.*, 1988); this potentiation is abolished by adrenal ligation (Walder *et al.*, 1989). Thus it was proposed that eicosanoids derived from the adrenal glands modulate the constrictor actions of endothelin-1. Adrenal-derived eicosanoids are unlikely to account for the modulation found in the current study, due to the lack of effect of the cyclo-oxygenase inhibitor on endothelin-1-stimulated constriction in the hindquarters. Further, there were no systemic effects observed after endothelin-1 administration into the mesentery *in situ*. Thus the modulation observed *in situ* is probably due to non-endothelial cyclo-oxygenase activity in the mesentery.

A slight effect of cyclo-oxygenase products in the isolated mesentery was also found; the vasodilator effect of the peptide could be observed at lower doses in the absence of indomethacin than in its presence. Thus, in these circumstances, modulation is apparently primarily by vasoconstrictor products of the enzyme. However, it should be noted that there were no significant changes in the pressor responses to endothelin-1 when indomethacin was omitted from the perfusing fluid and thus it would seem that there is not a very great capability for modification of the effects of endothelin-1 by cyclo-oxygenase products in this isolated preparation.

The observation that endothelin-1 caused systemic vasoconstriction when administered close arterially

into the hindquarters, but not when given into the superior mesenteric artery, probably reflects greater overspill of the peptide from the hindquarters to the systemic circulation. Reduced access from the mesenteric bed is probably due to extensive binding of the peptide in the hepatosplanchnic circulation. This may possibly be in the liver as ^{125}I-labelled endothelin-1 has been shown to bind extensively in this organ (Shiba *et al.*, 1989; Neuser *et al.*, 1989). Similar studies performed by Ånggård *et al.* (1989) have led to the conclusion that the liver, along with the lungs and kidneys, may be an important organ in the removal of circulating endothelin-1 from the blood stream.

The two disulphide bridges present in endothelin-1 represent a relatively unusual structure in vasoactive peptides. Since this 'double loop' structure is conserved not only between the various forms of endothelin encoded in the human genome, but is also found in the sarafotoxin group of snake venoms which possess vasoconstrictor activity (Kloog *et al.*, 1988), it was thought that this structure might be important for biological activity. Selective substitution of the bridge cysteinyl with alanyl residues, leading to removal of the bonds, has led to a series of vasoactive analogues, suggesting that the disulphide bonds are not an absolute requirement for activity. Kimura *et al.* (1988) have also shown that vasoconstrictor activity is retained when the disulphide bridges are removed from the parent peptide, but their analogues included blocked cysteinyl residues and they were much less potent than the alanyl substituted analogues used here. However, the 1–16 fragment of endothelin-1, containing the intramolecular loops but not the 5 C-terminal amino-acids, was without constrictor activity on the porcine coronary artery (Kimura *et al.*, 1988). From these structure-activity relationships they concluded that the activity of the peptide resides in the hydrophobic C-terminus and that this is enhanced by the intramolecular loop. They proposed that enzymic degradation of either the loop structure or C-terminus could represent *in vivo* mechanisms for inactivation of the peptide. However, the present data suggest that metabolic products with disrupted disulphide bridges may have appreciable biological activity.

[Ala^{3,11}]endothelin-1, which lacks the 3-11 bridge forming the smaller loop, was less potent than endothelin-1 as a vasoconstrictor both *in vivo* and *in vitro*. *In vivo* this analogue was less potent than endothelin-1 by a factor of about seven and *in vitro* by a factor of about twenty-four; but these potency ratios are comparable to the 12 fold lower potency obtained for [Ala^{3,11}]endothelin-1 in the rat isolated aorta (Topouzis *et al.*, 1989) and the 8 fold lower potency, found for the analogue compared to endothelin-1, at increasing the twitch response in the electrically-stimulated vas deferens of the rat (Hiley *et al.*, 1989b). The greater maximum responses to this analogue and to [Ala^{1,15}]endothelin-1 *in vitro* compared to that of endothelin-1 are particularly interesting and show that endothelin-1 is a partial agonist relative to its analogues on the arterial side of the bed. This has also been observed with respect to [Ala^{3,11}]endothelin-1 in the rat aorta (Topouzis *et al.*, 1989). However, in the intact, *in situ* mesenteric circulation there was no apparent difference in the maximal reactivities of [Ala^{3,11}]endothelin-1 and endothelin-1, thus endothelin-1 is behaving as a full agonist here. Therefore, it is likely that the reduced reactivity in the isolated bed is due to a limited receptor population for endothelin-1 relative to the analogues, but the data do not allow differentiation of whether this is due to endothelin-1 having a lower efficacy than the analogues at the same receptor or to its having a different receptor. It is also of interest that endothelial cell destruction led to enhanced reactivity for endothelin-1 but not the analogues *in vitro*, this is in accord with the contractile responses of the rat isolated aorta being enhanced to endothelin-1 but not to [Ala^{3,11}]endothelin-1 on removal of the endothelium (Topouzis *et al.*, 1989). However, the data presented here do not suggest that endothelium-dependent modulation of the pressor response occurs for either [Ala^{1,15}]-endothelin-1 or [Ala^{3,11}]endothelin-1; with neither of these peptides *in vitro* was there either an enhancement of the maximum response or an increase in potency. These observations suggest that the alanyl analogues and endothelin-1 interact with receptors in different ways, such that the putative ability to release EDRF is dependent on the possession of both disulphide bridges, and add further weight to the proposition that these alanyl-substituted peptides are not acting on exactly the same population of receptors as endothelin-1 itself. However, previous work has shown that both [Ala^{1,15}]endothelin-1 and [Ala^{3,11}]endothelin-1 inhibit the binding of [^{125}I]endothelin-1 from homogenates of rat cerebellum and hindbrain (Jones *et al.*, 1989b,c), and that [Ala^{3,11}]endothelin-1 inhibits iodinated endothelin-1 binding to rat kidney cortex (Jones *et al.*, 1989a) indicating that there are some sites at which they interact directly.

Removal of the 1–15 disulphide bridge, which forms the outer ring structure, in [Ala^{1,15}]-endothelin-1 leads to a greater loss of pressor activity in the isolated mesentery, suggesting that for actions on the arterial side of the bed the possession of the 1–15 bridge is more important for activity than the 3–11 bridge. In the intact bed the dose-response relationship for the pressor activity of this analogue was best fitted to a double hyperbola, indicating that the actions of this analogue may well be

the result of it interacting with two independent sites. The higher of the two ED_{50}s for [$Ala^{1,15}$]endothelin-1 is close to that of this analogue in the isolated bed. Thus it is likely that the sites with the lower ED_{50} (and perhaps higher affinity) are located on the smaller arterioles or on the venous side of the bed. Hence, the vascular actions of these two analogues, when compared to those of endothelin-1, make it probable that there is more than one receptor type for endothelin-like peptides.

Removal of both of the disulphide bridges resulted in an analogue ([$Ala^{1,3,11,15}$]endothelin-1) with partial agonist activity in both the blood-perfused mesentery and hindquarters, while in the isolated mesentery this tetra-alanyl analogue had no pressor activity at the doses examined. This lack of pressor activity is not due to a concomitant induction of release of endothelium-derived vasorelaxants since destruction of the vascular endothelium did not reveal any pressor activity. Thus, the lack of effect of the analogue in the isolated mesentery, whilst it possesses activity in the intact bed, may be due to differences in either receptor reserve or types of receptor. These findings would be compatible with those of Spokes *et al.* (1989) who have compared the activities of endothelin-1 and endothelin-3 on rat stomach strips, guinea-pig ileum and guinea-pig trachea. These workers have also produced data which would appear to suggest the existence of multiple receptor subtypes for endothelin. The results of Warner *et al.* (1989) also suggest different receptor types on endothelium and vascular smooth muscle, since endothelin-3 was equipotent with endothelin-1 as a vasodilator but less potent as a vasoconstrictor agent.

The systemic depressor actions of [$Ala^{3,11}$]-endothelin-1 and [$Ala^{1,3,11,15}$]endothelin-1 following mesenteric administration are interesting in view of the observations that these analogues did not have their pressor activity changed by removal of the vascular endothelium. The depressor actions at the lower doses and the pressor activity of the higher doses of [$Ala^{1,15}$]endothelin-1 mirror the two-site model for the action of this analogue on the mesentery. The systemic pressor activity occurs at doses which form the component of the dose-response curve with the higher ED_{50} in the mesentery. This supports the proposition that the peptides may act at two receptors in the periphery. The systemic vasodepressor actions of these three analogues might reflect a specific activation by the peptides of receptors which bring about vasodilatation in vascular beds not investigated here, or it might be due to changes in cardiac performance. Alternatively, there might be competitive interactions with endogenous endothelin. However, the co-administration experiments with [$Ala^{1,3,11,15}$]endothelin-1 and endothelin-1 did not show any antagonism of the pressor responses to the parent peptide by the analogue but rather an enhancement.

The partial agonist activity of [$Ala^{1,3,11,15}$]-endothelin-1 in the blood-perfused vascular beds led us to examine the effect of this analogue on the activity of endothelin-1 in all the preparations used. The enhancement of the pressor activity of endothelin-1 in the isolated mesentery is of particular interest as the tetra-alanyl analogue had no pressor activity in this bed and suggests a pharmacological action of the analogue in this preparation. Its action may be to enhance the activity of endothelin-1 at the smooth muscle level or possibly the analogue might antagonize the action of endothelin-1 on endothelial receptors, so attenuating the release of humoral modulators. However, this latter explanation appears to be unlikely as the *in vitro* studies have shown this effect to be endothelium-independent. In both of the blood-perfused beds there is a clear-cut summation of the activities of the analogue with those of endothelin-1 at the lower doses of endothelin-1, and no evidence of antagonism at higher dose levels as would be expected with a partial agonist. This lends further support to the contention that there are two independent receptor classes and that there is selectivity between endothelin-1 and its analogues.

The results presented here show that endothelin-1 is a potent vasoconstrictor of the two vascular beds studied. It may cause release of EDRF which modulates its pressor activity. The vasoconstrictor actions of the endothelin family of peptides are not dependent on the retention of the two disulphide bridges within the molecule, but there is evidence that there may be more than one subtype of receptor for these agents.

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