Endothelium-dependent mesenteric vasorelaxant effects and systemic actions of endothelin (16-21) and other endothelin-related peptides in the rat

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1 The rat isolated superior mesenteric bed, perfused with Krebs-Henseleit solution containing 10 μ M indomethacin and precontracted with 100 μ M methoxamine, was used to study the vasorelaxation produced by some fragments of endothelin-1, by two alanyl-substituted analogues, and by human and porcine proendothelins. The systemic cardiovascular effects of some of these peptides were also studied in anaesthetized rats.

2 Endothelin (16-21) was an endothelium-dependent vasodilator about 10 times less potent than acetylcholine and its amide was about 500 times less potent than the free acid. Human proendothelin-1 and porcine proendothelin-1 also caused endothelium-dependent relaxations but neither [Ala^{3,11}]endothelin-1 nor [Ala^{1,3,11,15}]endothelin-1 produced significant reductions in the methoxamine-induced tone. Sodium nitroprusside was approximately 200 times less potent than acetylcholine in the presence of the endothelium and was the only vasorelaxant to be active after destruction of the endothelium by perfusion with 0.3% CHAPS; in the absence of the endothelium it was 3.7 times more potent as a vasodilator than in its presence.

3 Porcine proendothelin-1 had weak contractile activity in the isolated mesenteric bed but porcine endothelin (22-39), endothelin (16-21) and endothelin (16-21) amide did not have vasoconstrictor actions.

4 Endothelin-3, [Ala^{3,11}]endothelin-1, [Ala^{1,3,11,15}]endothelin-1 and endothelin (16-21) all had systemic blood pressure effects in the anaesthetized rat. Although endothelin (16-21) did not cause vasoconstriction *in vitro*, it increased mean arterial pressure *in vivo* but was at least 100 times less potent than endothelin-3. Despite causing no vasorelaxation *in vitro*, [Ala^{1,3,11,15}]endothelin-1 and [Ala^{3,11}]endothelin-1 induced short-lived systemic depressor responses which were followed by pressor responses. Endothelin-3 was more potent as a systemic depressor agent than as a pressor agonist and, as a vasodepressor agent, it was 3–4 times more potent than either of the alanyl-substituted analogues. Endothelin-3, [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 were equipotent vasopressor agents. Porcine endothelin (22-39) had no systemic blood pressure effects.

5 This study shows directly that the presence of both disulphide bonds is required in the endothelins for them to be able to cause endothelium-dependent relaxation in the mesenteric vascular bed and that this response is mediated by different receptors from those causing contraction of the vascular smooth muscle in the mesentery. Vasorelaxation caused by endothelin (16-21) and its amide suggests that there is a receptor on the endothelium similar to one reported in the guinea-pig trachea. Finally, the ability of endothelin peptides to cause systemic vasodepressor responses is not related to their ability to cause endothelium-dependent relaxation, at least in the mesenteric circulation, and, for endothelin (16-21), its systemic pressor response is not likely to be the result of vasoconstriction in the mesenteric bed.

Keywords: Endothelin; endothelin (16-21); proendothelin-1; endothelin-1 analogues; acetylcholine; sodium nitroprusside; rat mesenteric bed; vasodilatation; endothelium; systemic depressor response

Introduction

Most members of the endothelin/sarafotoxin family of peptides are potent systemic pressor agents (Inoue *et al.*, 1989; Gardiner *et al.*, 1990a,b) and have vasoconstrictor actions at low concentrations in many blood vessels and vascular beds (Lovenberg & Miller, 1990). They consist of 21 amino acids linked by two cystine disulphide bridges between positions 1–15 and 3–11 (Yanagisawa *et al.*, 1988; Kloog *et al.*, 1988; Inoue *et al.*, 1989) and their vasoconstrictor activity is characterized by requiring the terminal tryptophan residue to be the free acid (Nakajima *et al.*, 1989) and, preferably, in the L-conformation (Kimura *et al.*, 1988). The overall systemic response to these peptides, however, includes a characteristic, short-lived vasodepressor phase before the long-lasting pressor effect develops (Wright & Fozard, 1988; Gardiner *et al.*, 1989a) although the precise mechanism underlying this initial systemic depressor response is currently unknown.

Despite the controversy surrounding the mechanism involved in mediating the systemic vasodepressor responses observed to the endothelin/sarafotoxin peptides, endothelium-dependent relaxation is known to occur *in vitro* in response to endothelin-3 (Warner *et al.*, 1989; Douglas & Hiley, 1990), endothelin-1, endothelin-2, mouse vasoactive intestinal contractor and sarafotoxin S6b (Douglas & Hiley, 1990). The order of potency of these peptides in causing endothelium-dependent relaxation appears to be different from that for their vasoconstrictor effect; endothelin-3 is the least potent at causing vascular smooth muscle contraction but is the most potent as a vasorelaxant (Douglas & Hiley, 1990). This suggests that different receptors may be mediating the two responses. This proposition receives further support from the observation that, although all the peptides shown to cause endothelium-dependent relaxation in the rat isolated mesenteric arterial bed also showed modulation by the endothelium of their pressor responses (Douglas & Hiley, 1990), two peptides, [Ala^{1,15}]endothelin-1 and [Ala^{3,11}]endothelin-1, in which one or other of the two disulphide bridges was absent

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showed no endothelium-dependence in their vasoconstrictor effects (Randall *et al.*, 1989). However, whether or not these two alanyl-substituted analogues possess vasodilator activity has not yet been directly assessed.

Confirmation of the existence of more than one receptor for the endothelins has come with the cloning of two separate receptor proteins (Arai *et al.*, 1990; Sakurai *et al.*, 1990) one of which, termed the ET_A receptor, has a much greater affinity for endothelin-1 than endothelin-3 whilst the other, the ET_B receptor, shows no great differences in affinity for endothelin-1 and other members of this peptide family (Vane, 1990). A receptor cloned from the A10 rat vascular smooth muscle cell line shows 90% homology with the ET_A receptor and binds endothelin-1 about 100 times better than it does endothelin-3 (Lin *et al.*, 1991). Other interesting, functional, evidence for receptor heterogeneity in the responses to the endothelins has been obtained by Maggi *et al.* (1989a,b). They showed that the C-terminal hexapeptide of the endothelins was inactive in the rat aorta but was a full agonist in the guinea-pig bronchus although endothelin-1, endothelin-2, endothelin-3 and sarafotoxin S6b were all potent full agonists in both tissues. However, the absence of an antagonist to the actions of the endothelins has prevented the certain identification of the receptors at which endothelin (16-21) acts as being identical with those for the parent 21 amino acid peptides.

The present study aimed to investigate further the question of heterogeneity in functional endothelin/sarafotoxin receptors by studying the relaxant actions of the C-terminal hexapeptide and of two 21 amino acid analogues in which one ([Ala^{3,11}]endothelin-1) or both ([Ala^{1,3,11,15}]endothelin-1) of the disulphide bridges were absent; these studies were performed on the isolated mesentery and in anaesthetized rats. Since the putative precursors of endothelin-1 in the pig and man, i.e. porcine proendothelin-1 (of 39 amino acids) and human proendothelin-1 (with 38 amino acid residues), have pressor activity it was also decided to determine if these precursors, as well as the proendothelin fragment, porcine proendothelin (22-39), had vasorelaxant activity (using acetylcholine and sodium nitroprusside for comparison). The relationship between the vasorelaxant actions of these peptides and the systemic depressor response that occurs following the i.v. administration of these peptides in the anaesthetized rat was also examined.

A preliminary account of some of these findings was given to the joint meeting of the British Pharmacological Society and Association Française des Pharmacologistes in Lyon during April 1991 (Douglas & Hiley, 1991b).

Methods

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

Adult male Wistar rats (250–400 g; Bantin & Kingman, Hull) were given a single i.p. injection of sodium pentobarbitone (60 mg kg⁻¹; Sagatal; RMB Animal Health Ltd, Dagenham) and heparin (1000 u kg⁻¹; Paines & Byrne, Greenford). After laparotomy, the superior mesenteric artery was identified and cleaned of adherent tissue to allow perfusion of the mesenteric arterial bed essentially according to the method of McGregor (1965). The artery was ligated proximal to the abdominal aorta and cannulated with polythene PP50 tubing (Portex, Hythe). The mesenteric bed was perfused at 37 ± 1°C and 4 ml min⁻¹ with oxygenated (95% O₂:5% CO₂) Krebs-Henseleit solution of the following composition (mM): NaCl 118.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, CaCl₂ 2.5 and D-glucose 5.5. The Krebs-Henseleit solution contained 10 µM indomethacin and 5 g l⁻¹ bovine serum albumin. The perfused mesentery was separated from the gastrointestinal tract, placed on a Perspex block maintained at 37 ± 1°C and covered with Nescofilm (Nippon Shoji Kaishi Ltd, Osaka, Japan) to avoid surface damage.

The pressure in the mesentery was recorded from a T-piece, placed in the perfusion circuit near the point at which the cannula was inserted into the artery, by means of a Bell & Howell Type 4-422-0001 pressure transducer coupled to a Grass model 7D polygraph. Zero pressure was set at zero flow and, since the flow rate was kept constant, the changes in perfusion pressure were directly proportional to changes in vascular resistance.

When required, the endothelium was removed by perfusion of the vascular bed with a 0.3% (w/v) solution in distilled water of the detergent CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate) for 120–150 s (Hiley *et al.*, 1987). For this procedure the flow rate was reduced to 2 ml min⁻¹ and, afterwards, the tissue was allowed 45 min to re-equilibrate with the Krebs-Henseleit solution. In all preparations, the presence or absence of a functional endothelium was assessed by determining whether or not acetylcholine opposed noradrenaline-induced vasoconstriction (Randall *et al.*, 1989). The flow rate was returned to 4 ml min⁻¹ before the construction of a dose-response curve for the dilatation caused by a peptide, acetylcholine or sodium nitroprusside. The vasorelaxant actions of the drugs were assessed by recording reductions in the tone established by infusion of 100 µM methoxamine (final perfusate concentration) into the preparation. Agonist administration was in bolus doses of 40 µl or less.

Experiments to determine the pressor activity of peptides in the mesenteric bed were carried out at a flow rate 2 ml min⁻¹ using Krebs-Henseleit solution containing indomethacin but in the absence of methoxamine and bovine serum albumin (Randall *et al.*, 1989). After a 30 min equilibration period, peptides were administered into the perfusion circuit in volumes of 100 µl or less. Doses were given 5 min apart or 5 min after the tissue perfusion pressure had returned to the resting basal value following the previous dose of peptide, which ever was the longer.

Systemic blood pressure responses

Male Wistar rats (200–400 g) were anaesthetized with 60 mg kg⁻¹ sodium thiopentone (Trapanal; BYK Gulden, Konstanz, Germany) and 30 mg kg⁻¹ sodium pentobarbitone (Sagatal; RMB, Dagenham) given i.p. Cannulae were placed in the trachea, to allow artificial respiration (20 ml kg⁻¹ at 54 breaths min⁻¹), and the left jugular vein. The right common carotid artery was cannulated for measurement of arterial pressure with a Bell & Howell transducer (Type 4-422-0001) connected to a Grass Model 79D polygraph. Heart rate was derived from the ECG using subcutaneous needle electrodes and a Grass 7P4 ECG preamplifier. Body temperature was maintained at 37 ± 1°C with a homeothermic blanket (BioScience, Sheerness). A 20 min equilibration period was allowed before peptides were given through the jugular vein cannula as bolus doses in volumes of 100 µl or less. All doses were washed through the cannula with 250 µl saline (0.9% NaCl, w/v) and each animal was used only to examine the effects of a single peptide.

Statistical and data analysis

Values are given as the mean ± s.e.mean. The values of *n* show the number of preparations in which the individual doses used to construct a dose-response curve were examined; since some doses of an agonist were used on all the preparations, the greatest value of *n* shows the total number of preparations used. Means were compared with Student's unpaired *t* test.

Where possible, dose-response curves were analysed by fitting the logistic equation:

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

Figure 1 Traces showing representative relaxation responses in the isolated perfused mesenteric bed precontracted with 100 μ M methoxamine to (a and b) 3 nmol sodium nitroprusside, (c) 150 pmol acetylcholine, (d) 200 pmol [Ala^{3,11}]endothelin-1, (e) 4 nmol [Ala^{1,3,11,15}]endothelin-1, (f) 3 nmol endothelin (16-21) and (g) 100 nmol endothelin (16-21) amide. +EC indicates that a functional endothelium was present and -EC shows an experiment in which the endothelium had been removed with CHAPS.

where R is the response, A the dose of drug, R_{max} the maximum response, n_H the slope function and ED₅₀ the dose giving the half maximal response. A modified Marquardt procedure, implemented in Harwell routine VB01A on an IBM 3084Q mainframe computer, was used to carry out the curve fitting (Randall *et al.*, 1989).

Drugs and peptides

All solutions were made fresh daily. (–)-Noradrenaline bitartrate (Koch-Light, Haverhill) was dissolved in distilled water containing 1 mg ml⁻¹ ascorbic acid (Fisons, Loughborough) to give a solution of 5 mg ml⁻¹; subsequent dilutions were made in saline. A 1 mg ml⁻¹ solution of indomethacin (Sigma Chemical Company, Poole) was made in 5% (w/v) sodium bicarbonate and this was added to the Krebs-Henseleit solution to give the required concentration. Sodium nitroprusside (Koch-Light), methoxamine hydrochloride (Sigma) and acetylcholine iodide (Sigma) were dissolved in saline and solutions of sodium nitroprusside were protected from light. Fraction V bovine serum albumin and CHAPS were from Sigma.

[Ala^{3,11}]endothelin-1, porcine proendothelin (22-39) and endothelin (16-21) were from Peninsula Laboratories (Europe) Ltd, St. Helens. Human proendothelin-1 and porcine proendothelin-1 were from the Peptide Institute, Osaka, Japan. [Ala^{1,3,11,15}]endothelin-1 and endothelin (16-21) amide were synthesized at the Marion Merrell Dow Research

Institute, Strasbourg. All the peptides were dissolved in distilled water to give 100 μ M stock solutions. The concentrations of these solutions were checked by absorbance spectrophotometry at 280 nm using the following molar extinction coefficients (for a 10 mm path length): porcine proendothelin (22-39), 1400; endothelin (16-21) and endothelin (16-21) amide, 5600; [Ala^{1,3,11,15}]endothelin-1, 7000; [Ala^{3,11}]endothelin-1, 7125; human proendothelin-1 and porcine proendothelin-1, 8650.

Results

Vasorelaxant actions of acetylcholine and sodium nitroprusside

In the presence of endothelium, both acetylcholine and sodium nitroprusside decreased methoxamine-induced tone in the mesenteric bed (Figure 1) in a dose-related manner (Figure 2). Sodium nitroprusside was about 200 fold less potent than acetylcholine as a vasodilator but it caused a significantly greater maximal degree of reversal of the methoxamine-induced tone (Table 1). Figure 1 shows that the duration of the relaxant response to acetylcholine (1–2 min) was less than that to sodium nitroprusside (5–6 min).

Perfusion of the mesentery with 0.3% CHAPS abolished the functional integrity of the endothelium without affecting the

Table 1 Comparison of the potency and maximal responses for the vasodepressor effects of endothelin (16-21) and porcine proendothelin-1 with those of acetylcholine and sodium nitroprusside

	R _{max} (% methoxamine induced tone)	ED ₅₀ (pmol)	n _H	n
Acetylcholine	60.8 ± 2.1	7.3 ± 1.4	1.0 ± 0.1	5–6
Sodium nitroprusside + EC	86.8 ± 8.7**/†††	1560 ± 690*/†	0.6 ± 0.1	5–6
Sodium nitroprusside –EC	95.0 ± 1.3***/†††	414 ± 49***/†††	1.1 ± 0.1	7–11
Endothelin (16-21)	48.9 ± 2.8**	84.9 ± 20.0**	1.1 ± 0.1	4–8
Porcine proendothelin-1	29.1 ± 1.4**/††	109 ± 9***	3.0 ± 0.4	4

These values were obtained from fitting a logistic function to the dose-response data. All experiments were carried out in the presence of a functional endothelium (except for sodium nitroprusside –EC) and of 10 μ M indomethacin. The concentration of methoxamine in the perfusate was 100 μ M. Statistical significance: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 from the corresponding values for acetylcholine; † *P* < 0.05, †† *P* < 0.01, ††† *P* < 0.001 from those for endothelin (16-21).

Figure 2 Log dose-response curves for the relaxant responses to acetylcholine (●) in the presence of a functional endothelium and to sodium nitroprusside in the presence (■) and absence (□) of the endothelium. The points show the mean and the vertical bars $\pm$ s.e.mean. The lines drawn are those obtained from the curve-fitting procedure.

ability of the vascular smooth muscle to relax. Prior to CHAPS perfusion, 30 nmol noradrenaline increased perfusion pressure by 35.6 ± 4.6 mmHg ($n = 11$) whereas the same dose, co-administered in a bolus dose with 0.4 nmol acetylcholine, induced a significantly smaller pressor response (15.3 ± 2.0 mmHg, $n = 11$; $P < 0.001$). After perfusion of the mesentery with 0.3% CHAPS, 30 nmol noradrenaline increased the perfusion pressure by 39.3 ± 4.6 mmHg and 39.7 ± 3.8 mmHg ($n = 11$ for both) when administered either alone or with 0.4 nmol acetylcholine, respectively. However, after endothelial destruction, sodium nitroprusside, unlike acetylcholine, still caused dose-dependent reductions in methoxamine-induced tone in the mesentery (Figure 2; Table 1). Endothelial cell destruction enhanced the potency of this nitrovasodilator ($P < 0.05$) without altering the maximum degree of vasodilatation obtained (Figures 1 and 2; Table 1).

Relaxant effects of endothelin (16-21) and its amide

Endothelin (16-21) caused dose-related relaxation in the pre-constricted mesenteric bed with an intact endothelium (Figure 3; Table 1) without inducing any secondary increases in tone (Figure 1). It was less potent than acetylcholine but more potent than sodium nitroprusside (Table 1). Figure 3 shows that this peptide did not cause vasorelaxation in mesenteries pretreated with CHAPS ($n = 4$).

Substitution of the free acid to give endothelin (16-21) amide significantly reduced the potency of the hexapeptide as a vasodilator with the result that, despite using a wide dose-range (0.1–100 nmol; $n = 3-7$), a full dose-response relationship could not be obtained. Nevertheless, it may be seen from Figure 3 that, since its ED_{50} was in excess of 40 nmol, the amide was at least 500 times less potent than the free acid.

In contrast to acetylcholine and sodium nitroprusside, the relaxant responses to both of these peptides were very transient, lasting for a maximum of 15–30 s (Figure 1).

Vasoconstrictor actions of endothelin (16-21) and its amide in the mesenteric arterial bed

Neither endothelin (16-21) (0.5–20 nmol; $n = 3$) nor endothelin (16-21) amide (0.1–20 nmol; $n = 3$) had any vasoconstrictor activity in preparations exposed to CHAPS and which were not precontracted with methoxamine.

Effects of [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 in the mesenteric arterial bed

Between 0.1 and 2000 pmol, neither of the alanyl-substituted analogues of endothelin-1 reduced the methoxamine-induced

Figure 3 Dose-response relations for the relaxation responses to (●, ○) endothelin (16-21) and (■, □) endothelin (16-21) amide in the presence (●, ■) and absence (○, □) of the endothelium in the methoxamine-precontracted mesenteric arterial bed. The points are the mean response and vertical bars show $\pm$ s.e.mean except where the error is contained within the symbol. The curve shown for endothelin (16-21) is that obtained from the curve-fitting procedure.

tone in mesenteric beds with an intact endothelium ($n = 4$ for both). However, [Ala^{3,11}]endothelin-1 caused vasoconstrictor responses, over and above the tone induced by methoxamine, at doses of 100 pmol or more (Figure 1) although [Ala^{1,3,11,15}]endothelin-1 produced no pressor responses over the dose-range studied.

Effects in the mesenteric bed of human proendothelin-1, porcine proendothelin-1 and porcine proendothelin-1 (22-39)

Both the human and porcine proendothelins relaxed methoxamine-induced tone in the mesenteric vascular bed (Figure 4). In the case of porcine proendothelin-1 it was possible to obtain a full dose-response curve (Table 1) since it only gave very small secondary vasoconstrictor responses; perfusion pressure was increased by 0.6 ± 0.6 , 1.2 ± 1.2 , 3.1 ± 1.2 , 1.1 ± 1.1 and 6.2 ± 1.6 mmHg respectively after doses of 40, 100, 200, 400 and 1000 pmol. In mesenteric beds which were not precontracted with methoxamine, porcine proendothelin (1-39) was only a very weak vasoconstrictor agonist in either the presence or absence of the endothelium and subsequently it was not possible to show any modulation of these responses by the endothelium; the responses obtained at the highest doses used were 3.2 ± 0.9 mmHg ($n = 4$; 4 nmol; with endothelium) and 5.9 ± 1.4 mmHg ($n = 3$; 10 nmol; without endothelium).

It was not possible to carry out curve-fitting on the vaso-relaxant responses given by human proendothelin-1. The maximum response occurred at 250 pmol (Figure 4) and, at this and higher doses, secondary vasoconstriction also occurred. These secondary responses were much higher than those seen with the porcine precursor; for example, although at 250 pmol the response was 1.6 ± 0.7 mmHg ($n = 7$), the responses rose in a dose-dependent manner to reach 24.7 ± 5.4 mmHg ($n = 5$) at 5 nmol.

Porcine proendothelin-1 (22-39) had no vasodilator effects between 2 pmol and 4 nmol in endothelium-intact, methoxamine precontracted, preparations ($n = 3$) nor did it cause vasoconstriction in de-endothelialized preparations between 400 pmol and 20 nmol ($n = 3$).

Effects on systemic blood pressure

When administered i.v., the most striking feature of the systemic response to endothelin-3 was a fall in blood pressure which was relatively long lasting, not returning to the basal value for

Figure 4 Dose-relaxation response relations for (●, ○) human proendothelin-1 (a) and (■, □) porcine proendothelin-1 (b) in the isolated, precontracted mesenteric arterial bed of the rat. The closed symbols show the mean responses obtained in the presence, and the open symbols those obtained in the absence, of a functional endothelium. Vertical bars show $\pm$ s.e.mean except where the error lies within the symbol.

around 5 min (Figure 5), and which was dose-related (Figure 6). In contrast, both $[Ala^{3,11}]$ endothelin-1 (Figure 7) and $[Ala^{1,3,11,15}]$ endothelin-1 (Figure 8) produced depressor responses which were only transient (lasting for only 30–60 s; Figure 5) and which therefore more closely resembled the responses previously reported for endothelin-1 and the proen-

dothelins (Douglas & Hiley, 1991a) than those to endothelin-3. Table 2 shows that endothelin-3 gave the same maximum systemic depressor response as endothelin-1 whereas both $[Ala^{3,11}]$ endothelin-1 and $[Ala^{1,3,11,15}]$ endothelin-1 gave maxima which were about 70% less. Also, endothelin-3 did not significantly differ in potency from endothelin-1 whilst the two alanyl-substituted analogues were significantly less potent agonists.

Neither endothelin (16–21) (0.004 – 100 nmol kg $^{-1}$; Figure 9) nor porcine endothelin (22–39) (1 – 20 nmol kg $^{-1}$) had any significant systemic vasodepressor activity. With 100 nmol kg $^{-1}$ endothelin (16–21), 2 out of 4 animals showed no depressor response, one responded with a decrease of 2 mmHg and the fourth (shown in Figure 5d) gave a depressor response of 20 mmHg; thus 100 nmol kg $^{-1}$ may be a threshold dose for a systemic depressor effect.

Figure 5 shows that the systemic depressor actions of endothelin-3, $[Ala^{3,11}]$ endothelin-1 and $[Ala^{1,3,11,15}]$ endothelin-1 were followed by longer lasting vasopressor responses which reached their maxima in 5–10 min and took approximately 30–45 min to return to the basal resting values. The responses were dose-related and endothelin-3 (Figure 6) and $[Ala^{3,11}]$ endothelin-1 (Figure 7) gave similar maximum pressor effects which were not significantly different from that previously found for endothelin-1 (Table 2). In contrast, $[Ala^{1,3,11,15}]$ endothelin-1 (Figure 8) gave a maximum response which was 20–30% lower. Endothelin-3, $[Ala^{3,11}]$ endothelin-1 and $[Ala^{1,3,11,15}]$ endothelin-1 were significantly less potent systemic pressor agonists than endothelin-1 (Table 2).

Endothelin-3 was approximately 2–3 times more potent as a systemic vasodepressor agent than it was as a pressor agonist but the alanyl-substituted analogues $[Ala^{3,11}]$ endothelin-1 and $[Ala^{1,3,11,15}]$ endothelin-1 were equipotent as depressor and pressor agents. These results contrast with those for endothelin-1 which was about 2–3 fold more potent as a systemic pressor agent rather than a depressor agent (Douglas & Hiley, 1991a).

Whereas endothelin (16–21) had no consistent depressor activity, it caused significant increases in systemic arterial blood pressure (Figure 9). Due to limitations in solubility, it was not possible to construct a full dose-response curve but it was clearly the least potent of the peptides examined (Table 2).

Between 1 – 20 nmol kg $^{-1}$, porcine endothelin (22–39) had no vasopressor activity.

At the higher doses examined, the changes in mean arterial pressure obtained with some of these peptides were associated with pronounced reductions in heart rate; e.g. 400 pmol kg $^{-1}$ endothelin-3 reduced heart rate by 31 ± 5 beats min $^{-1}$ ($n = 7$), 2 nmol kg $^{-1}$ $[Ala^{3,11}]$ endothelin-1 reduced it by 30 ± 7 beats min $^{-1}$ ($n = 11$) and $[Ala^{1,3,11,15}]$ endothelin-1 caused a

Table 2 Comparison of the systemic potency and maximal responses for the initial vasodepressor and subsequent vasoconstrictor effects of some endothelin family peptides following intravenous injection in the anaesthetized rat

	Endothelin-1 <i>n</i> = 6–12	Endothelin-3 <i>n</i> = 4–7	$[Ala^{3,11}]$ endothelin-1 <i>n</i> = 5–13	$[Ala^{1,3,11,15}]$ endothelin-1 <i>n</i> = 6–10	Endothelin (16–21) <i>n</i> = 3–6
<i>Basal parameters</i>					
Heart rate (min $^{-1}$)	390 ± 13	415 ± 18	405 ± 12	425 ± 16	393 ± 14
Mean arterial pressure (mmHg)	110 ± 7	95 ± 10	115 ± 7	118 ± 8	107 ± 10
<i>Systemic depressor response</i>					
R_{max} (mmHg)	35.8 ± 2.5	35.2 ± 5.2	$24.3 \pm 1.0^{***}$	$24.2 \pm 1.6^{**}$	No consistent
ED_{50} (pmol kg $^{-1}$)	141 ± 45	88.3 ± 36.9	$267 \pm 31^*$	$344 \pm 62^{**}$	depressor
n_H	0.7 ± 0.1	1.4 ± 0.5	1.8 ± 0.2	1.6 ± 0.2	response
<i>Systemic pressor response</i>					
R_{max} (mmHg)	28.7 ± 2.1	28.9 ± 3.2	31.9 ± 2.2	$23.3 \pm 1.2^*$	> 22.7
ED_{50} (pmol kg $^{-1}$)	$48.0 \pm 11.0^\dagger$	$216 \pm 60^{**/\dagger}$	$326 \pm 84^{**}$	$265 \pm 47^{***}$	> 40000
n_H	1.0 ± 0.1	1.3 ± 0.3	0.9 ± 0.1	1.3 ± 0.1	

The values obtained for endothelin-1 are from Douglas & Hiley (1991a) and are reproduced in this table for ease of comparison. The data shown represent the mean $\pm$ s.e.mean of *n* observations. The values given are those obtained from fitting a logistic function. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from the corresponding value for endothelin-1 and $^\dagger P < 0.05$ between the ED_{50} values for the systemic depressor and pressor responses for an individual peptide.

Figure 5 Traces of representative systemic blood pressure and heart rate responses obtained after i.v. administration to anaesthetized rats of (a) 200 pmol kg^{-1} endothelin-3, (b) 4 nmol kg^{-1} $[\text{Ala}^{3,11}]$ endothelin-1, (c) 4 nmol kg^{-1} $[\text{Ala}^{1,3,11,15}]$ endothelin-1, (d) 100 nmol kg^{-1} endothelin (16-21) and (e) 20 nmol kg^{-1} endothelin (22-39) followed by 250 pmol kg^{-1} endothelin-1.

bradycardia of $30 \pm 5 \text{ beats min}^{-1}$ ($n = 9$). The C-terminal hexapeptide endothelin (16-21) had no significant effects on heart rate at the doses examined.

Discussion

This study shows that, whether or not the endothelium is present, the C-terminal hexapeptide endothelin (16-21) does not elicit pressor responses in the isolated superior mesenteric arterial bed of the rat. It has previously been reported to be a full agonist (relative to endothelin-1) in the guinea-pig isolated bronchus and rat isolated vas deferens but to have relatively little activity, if any, in the rat isolated thoracic aorta, guinea-pig ileum, human urinary bladder, renal pelvis or renal artery (Maggi *et al.*, 1989a,b). On the basis of these studies it has been proposed that endothelin (16-21) distinguishes between endothelin receptor subtypes, acting as an agonist in the guinea-pig isolated bronchus but not in the rat thoracic aorta (Maggi *et al.*, 1989a,b). The lack of contractile activity in the rat isolated mesentery accords with the observations made by Maggi *et al.* (1989a,b) in another arterial preparation, the rat thoracic aorta, and suggests that there are no functional receptors for this hexapeptide in mesenteric arterial smooth muscle. However, this does not mean that endothelin (16-21) lacks contractile activity in all vascular smooth muscle preparations since it does contract isolated rat jugular veins and rabbit pulmonary arteries. Therefore, it would appear that both regional and species differences exist in the sensitivity of vascular smooth muscle to this peptide (Maggi *et al.*, 1989a).

Despite lacking contractile activity in the isolated mesentery, endothelin (16-21) does act in this preparation to cause endothelium-dependent vasorelaxation. Indeed, it is a potent

agonist, being about one order of magnitude less potent than acetylcholine but around 20 times more potent than the endothelium-independent vasorelaxant, sodium nitroprusside. Amongst the preparations they studied, Maggi *et al.* (1989a) found the most sensitive to endothelin (16-21) to be the guinea-pig isolated bronchus where it was 30–35 times less potent than endothelin-1 as a spasmogen. However, endothelin (16-21) is approximately equipotent with endothelin-1 as a vasorelaxant in the precontracted mesentery since the threshold dose of endothelin-1 for relaxation is 50 pmol (Randall *et al.*, 1989).

Incidentally, it is interesting to note that the potency of sodium nitroprusside was enhanced after endothelium destruction whilst the maximum degree of relaxation remained unaltered. A similar potentiation, not only to sodium nitroprusside but also to glyceryl trinitrate, has been reported in rat aortic rings by Moncada *et al.* (1991). The reason for this supersensitivity is unclear but may reflect changes at the level of the cytosolic guanylate cyclase with which the nitrovasodilators interact. It is also possible that this enhanced potency results from the removal of a diffusion barrier to nitroprusside.

We have previously reported that members of the endothelin/sarafotoxin family are not only potent pressor agents in the isolated mesentery but are also endothelium-dependent vasodilators. Thus, endothelin (16-21) shares the latter of these actions. However, it is not possible to say whether or not this endothelin fragment shares a common receptor with the full 21 amino acid peptides as has been suggested for guinea-pig bronchial smooth muscle by Maggi *et al.* (1989a,b). At this stage, there is no evidence to refute the suggestion that endothelin (16-21) might act at other peptide receptors mediating relaxation, neither is it easy to discount

Figure 6 Log dose-response curves for the systemic (a) pressor and (b) depressor responses to endothelin-3 administered i.v. to anaesthetized rats. The points show the mean responses, the curves shown are those obtained from the curve-fitting procedure and the vertical bars show $\pm$ s.e.mean.

Figure 7 Log dose-response relationships for the systemic (a) pressor and (b) depressor responses to $[\text{Ala}^{3,11}]$ endothelin-1 administered i.v. to anaesthetized rats. The points show the mean responses, the curves shown are those obtained from the curve-fitting procedure and the vertical bars show $\pm$ s.e.mean.

the possibility that this peptide is an agonist at a previously uncharacterized class of receptors.

It is clear that it is important that the C-terminal Trp residue is in the free acid form in order to see the activity of endothelin (16-21) as an endothelium-dependent vasodilator and a similar requirement has been reported for the vasoconstrictor actions of endothelin-1 (Nakajima *et al.*, 1989; Kimura *et al.*, 1988). Amidation of the C-terminal Trp of endothelin (16-21) lowers vasodilator potency in the isolated mesentery by 2-3 orders of magnitude and this accords with the findings that the amide was much less potent than the free acid in a number of isolated tissues including the guinea-pig bronchus, rabbit pulmonary artery and rat vas deferens (Maggi *et al.*, 1989a,b).

Evidence for the presence of two different endothelin/sarafotoxin receptor subtypes in the mesentery, one type on the vascular smooth muscle and another on the endothelium, has come from examining the rank order of potency of the peptides in inducing vasoconstriction and vasorelaxation. Thus, endothelin-1 is more potent than endothelin-3 in causing constriction whilst endothelin-3 is the more potent of the two in causing endothelium-dependent relaxation (Douglas & Hiley, 1990). Additional evidence for subtypes of endothelin receptor in the isolated mesenteric bed has come from our observations that, unlike the endogenous members of this peptide family such as endothelin-1, the pressor actions of $[\text{Ala}^{3,11}]$ endothelin-1 and $[\text{Ala}^{1,15}]$ endothelin-1 are not modulated in any way by the endothelium (Hiley *et al.*, 1989; Randall *et al.*, 1989; Douglas & Hiley, 1990). This has been interpreted as showing that both disulphide bridges are

required to cause EDRF release from the endothelium. This inference now has direct support from the finding that $[\text{Ala}^{3,11}]$ endothelin-1 and $[\text{Ala}^{1,3,11,15}]$ endothelin-1 cannot relax precontracted mesenteries; both disulphide bonds, whilst not being a prerequisite for smooth muscle receptor activation, are required for agonist activity at the endothelial cell receptor.

Recently, much interest has been focused on the mechanisms underlying the systemic actions of the endothelin/sarafotoxin family peptides in a number of species of animals. We, and many others, have reported that the systemic response in the anaesthetized rat to i.v. administration of endothelin-1 is biphasic; initially, a transient depressor response is observed after which mean arterial pressure is raised for a prolonged period of time (Douglas & Hiley, 1991a). The depressor response has received much attention and is not thought to involve reflex mechanisms since it occurs in rats chemically denervated with mecamylamine (Wright & Fozard, 1989) or a combination of bunazosin, propranolol and atropine (Yanagisawa *et al.*, 1988) and in pithed animals (MacLean *et al.*, 1989). Also, reductions in heart rate or cardiac output do not occur (Le Monnier de Gouville *et al.*, 1989). Indeed, cardiac output is increased during the depressor phase in conscious animals (Gardiner *et al.*, 1990c) and the reduction in blood pressure appears to be the result of a short lived decrease in total peripheral resistance (Gardiner *et al.*, 1990c,e). Thus, i.v. administration of endothelin-1 initially increases carotid and hindquarter vascular conductances and these are followed by more sustained reductions in conductance (the secondary conductance change

Figure 8 Log dose-response curves for the systemic (a) pressor and (b) depressor responses to [Ala^{1,3,11,15}]endothelin-1 administered i.v. to anaesthetized rats. The points show the mean responses, the curves shown are those obtained from the curve-fitting procedure and the vertical bars show $\pm$ s.e.mean.

being associated with the prolonged systemic hypertension; Wright & Fozard, 1988; Gardiner *et al.*, 1989a). Since these peptides are known to cause the release of EDRF (Randall *et al.*, 1989; Warner *et al.*, 1989; Douglas & Hiley, 1990), it was originally proposed that it is this action which underlies the initial systemic depressor response to i.v. administration of peptide.

This study found that, in the anaesthetized rat, endothelin-3 caused a prolonged depressor response lasting for about 5 min. This contrasts both with the short-lasting depressor response found in the same conditions for endothelin-1 (Douglas & Hiley, 1991a) and for endothelin-3 itself in conscious rats (Gardiner *et al.*, 1990c). The difference between the results with endothelin-3 in conscious and anaesthetized animals may represent a more rapid correction taking place due to reflex actions in the conscious animals. In the anaesthetized rats, the difference between endothelin-3 and endothelin-1 may be due to the fact that endothelin-3 is more potent in causing a systemic depressor response than the secondary increase in blood pressure. The reverse is true for endothelin-1 (Douglas & Hiley, 1991a). Since endothelin-3 is more potent as a vasorelaxant than a vasoconstrictor *in vitro*, and the opposite is true for endothelin-1 (Randall *et al.*, 1989; Douglas & Hiley, 1990), these findings would not conflict with the hypothesis that the initial hypotension observed with these peptides on i.v. administration is a reflection of their ability to cause endothelium-dependent relaxation in peripheral vascular beds. Such a mechanism was proposed by Whittle *et al.* (1989) who were able to attenuate the initial systemic depres-

Figure 9 Dose-response relations for the systemic (a) pressor and (b) depressor responses to endothelin (16-21) administered i.v. to anaesthetized rats. The points show the means and the vertical bars show the s.e.mean except where the error lies within the symbol.

sor response to endothelin-1 using N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase. However, the role of EDRF/nitric oxide in mediating the initial hypotensive response observed with these peptides *in vivo* has been questioned by other groups. Gardiner *et al.* (1989b) could not reproduce the findings of Whittle *et al.* (1989); they found that N^G-monomethyl-L-arginine had no effect upon the hypotensive action of endothelin-1. However, N^G-nitro-L-arginine methyl ester, a more potent nitric oxide synthase inhibitor, attenuated some of the regional increases in vascular conductance but not the systemic depressor response, although the latter was attenuated relative to that of nitroglycerin (Gardiner *et al.*, 1990e). In contrast, Hoffman *et al.* (1989) reported that the depressor response to endothelin-1 was unaffected by methylene blue. Thus, the evidence for the hypothesis that the systemic depressor response to the endothelin/sarafotoxin family peptides is the result of a peptide-induced release of EDRF/nitric oxide is mixed. On the other hand, the systemic vasodepressor effect could be the result of the action of eicosanoids, which are released from rat lung by endothelin-1 (de Nucci *et al.*, 1988), but this seems unlikely since indomethacin has no effect on this response (Gardiner *et al.*, 1990d).

If the systemic depressor response were due to the release of EDRF/nitric oxide, it would follow that [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 should only increase mean arterial blood pressure on systemic administration since this study shows that they do not release EDRF, at least in the isolated mesentery. However, these analogues induced biphasic changes in mean arterial blood pressure after i.v. administration. Therefore it would appear that the initial systemic hypotensive response induced by the endothelin/sarafotoxin family peptides does not result from their ability to induce EDRF release in the mesentery. This is particularly surprising in the case of endothelin-3 which is more potent in releasing

EDRF in the isolated mesentery than it is in causing vasoconstriction since, *in vivo*, it increases mesenteric vascular resistance, which suggests sufficient peptide reaches this bed to activate its smooth muscle receptors, but does not cause any decrease in mesenteric conductance (Gardiner *et al.*, 1990b,c). Nevertheless, it has to be acknowledged that the systemic vascular actions of these peptides might involve EDRF release at other sites in order to bring about the systemic depressor response. Whether or not EDRF is involved elsewhere, it seems that the mesenteric vascular bed does not contribute to this response.

It is of interest to note that a monocyclic analogue of endothelin-1, where the inner intramolecular disulphide bridge has been prevented from forming using blocking acetamidomethyl groups at residues Cys³ and Cys¹¹, has no systemic actions in the anaesthetized cat at a dose of 30 nmol kg⁻¹ (Minkes & Kadowitz, 1991). The contrast between the lack of activity of this monocyclic analogue and the clear systemic actions of the [Ala^{3,11}]endothelin-1 in the anaesthetized rat may result from the presence of blocking groups on Cys³ and Cys¹¹ causing a greater alteration in the peptide conformation than is seen by the pseudoisosteric substitution of Cys residues with Ala residues in positions 3 and 11; indeed, a similar effect has been noted in a ligand binding study with [¹²⁵I]endothelin-1 (Hiley *et al.*, 1990).

Although endothelin (16-21) is a potent endothelium-dependent relaxant in the isolated mesentery, even in doses of up to 100 nmol kg⁻¹ no reproducible depressor response was observed on systemic administration. Paradoxically, the only clear systemic effects observed with the hexapeptide were pressor responses and, for these, it was about 3 orders of magnitude less potent than endothelin-1. This finding agrees with that of Giuliani *et al.* (1991) and supports the hypothesis that the systemic depressor response observed to the endothelin/sarafotoxin family peptides does not involve the release of EDRF.

Despite lacking contractile activity in the isolated mesentery at doses of up to 20 nmol, endothelin (16-21) produced quite marked increases in mean arterial pressure. However, Minkes & Kadowitz (1991) have recently reported that it is inactive on the systemic blood pressure over a similar dose-range in the anaesthetized cat. The reason for this disparity is unclear but may reflect species differences in the sensitivity of vascular smooth muscle to this peptide. However, since it contracts rat jugular vein preparations *in vitro* (Maggi *et al.*, 1989a) an action on venous tissue may explain why it causes systemic pressor responses. Certainly, the systemic pressor response would not be expected to be due to any change in mesenteric arteriolar resistance since the hexapeptide had no contractile effects in the isolated mesentery.

[Ala^{1,3,11,15}]endothelin-1 has previously been shown to have no vasoconstrictor activity in the isolated mesentery (Randall *et al.*, 1989) and here we have shown that it lacks vasodilator activity in the same preparation. However, on i.v. administration, it induced a hypotensive response which was followed by a prolonged pressor response. On the basis of the findings that [Ala^{1,3,11,15}]endothelin-1 lacks activity in the isolated mesentery, one would expect this peptide to have no effect on mean arterial pressure when given systemically. However, the isolated mesenteric bed contains only arteriolar smooth muscle and studies performed in the *in situ* blood perfused mesentery, which also contains the venous side of the resistance bed, have shown that [Ala^{1,3,11,15}]endothelin-1 is a weak partial agonist (Randall *et al.*, 1989). Since rabbit and dog mesenteric veins are approximately an order of magnitude more sensitive to endothelin-1 than the corresponding mesenteric arteries (De Nucci *et al.*, 1988; Cocks *et al.*, 1989), and since the duration of the contractile response observed to endothelin-1 is more prolonged in the venous side of the rat isolated mesenteric bed (Warner, 1990), it is not surprising that the contractile actions of this weak agonist are difficult to observe in the isolated mesentery. However, when administered systemically, [Ala^{1,3,11,15}]endothelin-1 is able to gain

access to act on the veins. It is also unwise to assume that the mesenteric bed is typical of all vascular beds with respect to the way in which it responds to these peptides. For example, during the initial period following i.v. administration of endothelin-1, the predominant effect observed in the mesentery is an increase in vascular resistance whereas the hind-quarters show an increase in vascular conductance (Wright & Fozard, 1988; Gardiner *et al.*, 1989a). It is just such a regional variation, and the absence of the venous side of the circulation in isolated arterial beds, which requires caution to be exercised when trying to draw comparisons between *in vitro* observations and responses recorded in the intact cardiovascular system.

Regional differences in vascular sensitivity may help explain the differences in the relative potency of [Ala^{1,3,11,15}]endothelin-1 to that of endothelin-1 in isolated vascular preparations and following systemic administration. However, there is a good correlation between the relative potencies of [Ala^{3,11}]endothelin-1 and endothelin-1 *in vitro* and *in vivo*; [Ala^{3,11}]endothelin-1 is approximately 6 times less potent than endothelin-1 as a vasoconstrictor in the isolated mesenteric preparation (Randall *et al.*, 1989) and about 7 times less potent as a systemic pressor agent. These similar differences in functional potency correspond well with [Ala^{3,11}]endothelin-1 having an apparent K_i approximately 9 times higher than the parent peptide in the kidney (Jones *et al.*, 1989).

We have previously reported that human proendothelin-1 is a vasoconstrictor in the isolated mesenteric arterial bed, and that both human and porcine proendothelin are active not only in the *in situ* blood-perfused mesenteric bed but also when administered systemically (Douglas & Hiley, 1990; Douglas & Hiley, 1991a). Here we found that porcine proendothelin-1 was a weak vasoconstrictor *in vitro* and the responses obtained were too small for it to be shown unambiguously whether or not they were modulated by the endothelium. However, both proendothelins caused endothelium-dependent relaxation in the isolated mesentery and this explains why the vasoconstrictor actions of human proendothelin-1 are augmented following endothelium removal (Douglas & Hiley, 1990). Indeed, porcine proendothelin-1 is equipotent with endothelin (16-21) as a vasodilator in the isolated mesentery.

Endothelin-1 gives a significantly smaller maximum pressor response than porcine proendothelin-1 on i.v. administration (Douglas & Hiley, 1991a). On the basis of this, it would appear that either porcine proendothelin-1 does not require conversion *in vivo* to release endothelin-1 to be vasoactive or that the peptide fragment that was produced together with endothelin-1 following cleavage at residues Trp²¹-Val²² was vasoactive itself. However, since this study has shown that endothelin (22-39) has no vasoactivity either *in vitro* or *in vivo*, it would appear that porcine proendothelin-1 is vasoactive in itself and does not require cleavage at Trp²¹-Val²² in order to possess full biological activity.

In summary, the endothelins need both disulphide bonds to induce endothelium-dependent relaxation in the mesenteric vascular bed and this response is mediated by different receptors from those which mediate contraction of the mesenteric vascular smooth muscle. Vasorelaxation caused by endothelin (16-21) and its amide suggests that there is a receptor on the endothelium similar to one reported in the guinea-pig trachea. In the case of endothelin (16-21) it appears that its systemic pressor response does not arise from mesenteric arteriolar constriction and, finally, the ability of endothelin peptides to cause systemic vasodepressor responses is not simply related to the ability to cause endothelium-dependent relaxation.

S.A.D. is a Medical Research Council Research Student. We are grateful to Dr J.T. Pelton and Dr J. Drijfhout of the Marion Merrell Dow Research Institute, Strasbourg for generous gifts of some of the peptides used. This study was supported by a grant from the SmithKline (1982) Foundation.

References

- ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.
- COCKS, T.M., FAULKNER, N.L., SUDHIR, K. & ANGUS, J. (1989). Reactivity of endothelin-1 on human and canine large veins compared with large arteries in vitro. *Eur. J. Pharmacol.*, **171**, 17–24.
- DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci., U.S.A.*, **85**, 9797–9800.
- DOUGLAS, S.A. & HILEY, C.R. (1990). Endothelium-dependent vascular activities of endothelin-like peptides in the isolated superior mesenteric arterial bed of the rat. *Br. J. Pharmacol.*, **101**, 81–88.
- DOUGLAS, S.A. & HILEY, C.R. (1991a). Responses to endothelin-1, human proendothelin (1-38) and porcine proendothelin (1-39) in the rat on intravenous administration and in the blood perfused mesentery. *Neurochem. Int.*, **18**, 445–454.
- DOUGLAS, S.A. & HILEY, C.R. (1991b). The C-terminal hexapeptide of endothelin (ET₁₆₋₂₁) is an endothelium-dependent vasodilator in the rat. *Fundamental Clin. Pharmacol.*, (in press).
- GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1989a). Regional hemodynamic effects of endothelin-1 in conscious, unrestrained, Wistar rats. *J. Cardiovasc. Pharmacol.*, **13** (Suppl. 5), S202–S204.
- GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1989b). N^G-monomethyl-L-arginine does not inhibit the hindquarters vasodilator action of endothelin-1 in conscious rats. *Eur. J. Pharmacol.*, **171**, 237–240.
- GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990a). Regional hemodynamic effects of endothelin-2 and sarafotoxin-S6b in conscious rats. *Am. J. Physiol.*, **258**, R912–R917.
- GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990b). Regional haemodynamic effects of endothelin-1 and endothelin-3 in conscious Long Evans and Brattleboro rats. *Br. J. Pharmacol.*, **99**, 107–112.
- GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990c). Cardiac output effects of endothelin-1, -2 and -3 and sarafotoxin S6b in conscious rats. *J. Auton. Nerv. System*, **30**, 143–148.
- GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990d). Effects of indomethacin on the regional haemodynamic responses to low doses of endothelins and sarafotoxin. *Br. J. Pharmacol.*, **100**, 158–162.
- GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990e). Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101**, 632–639.
- GIULIANI, S., LECCI, A., MAGGI, C.A., ROVERO, P. & GIACHETTI, A. (1991). Effect of intrathecal administration of ET-1, ET-3 and ET(16–21) on blood pressure and micturition reflex in anaesthetized rats. *Neurochem. Int.*, **18**, 565–570.
- HILEY, C.R., DOUGLAS, S.A. & RANDALL, M.D. (1989). Pressor effects of endothelin-1 and some analogs in the perfused mesenteric arterial bed of the rat. *J. Cardiovasc. Pharmacol.*, **13** (Suppl. 5), S197–S199.
- HILEY, C.R., JONES, C.R., PELTON, J.T. & MILLER, R.C. (1990). Binding of [¹²⁵I]-endothelin-1 to rat cerebellar homogenates and its interactions with some analogues. *Br. J. Pharmacol.*, **101**, 319–324.
- HILEY, C.R., PHOON, C.K.L. & THOMAS, G.R. (1987). Acetylcholine relaxation in superior mesenteric arterial bed of the rat is endothelium-dependent and sensitive to antioxidants. *Br. J. Pharmacol.*, **91**, 378P.
- HOFFMAN, A., GROSSMAN, E., ÖHMAN, K.P., MARKS, E. & KEISER, H.R. (1989). Endothelin induces an initial increase in cardiac output associated with selective vasodilation in rats. *Life Sci.*, **45**, 249–255.
- INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci., U.S.A.*, **86**, 2863–2867.
- JONES, C.R., HILEY, C.R., PELTON, J.T. & MILLER, R.C. (1989). Autoradiographic localisation of endothelin binding sites in kidney. *Eur. J. Pharmacol.*, **163**, 379–382.
- KIMURA, S., KASUYA, Y., SAWAMURA, T., SHINMI, O., SUGITA, Y., YANAGISAWA, M., GOTO, K. & MASAKI, T. (1988). Structure-activity relationships of endothelin: importance of the C-terminal moiety. *Biochem. Biophys. Res. Commun.*, **156**, 1182–1186.
- KLOOG, Y., AMBAR, I., SOKOLOVSKY, M., KOCHVA, E., WOLLBERG, Z. & BDOLAH, A. (1988). Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain. *Science*, **242**, 268–270.
- LE MONNIER DE GOUVILLE, A.-C., LIPPTON, H.L., CAVERO, I., SUMMER, W.R. & HYMAN, A.L. (1989). Endothelin – a new family of endothelium-derived peptides with widespread biological properties. *Life Sci.*, **45**, 1499–1513.
- LIN, H.Y., KAJI, E.H., WINKEL, G.K., IVES, H.E. & LODISH, H.F. (1991). Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *Proc. Natl. Acad. Sci., U.S.A.*, **88**, 3185–3189.
- LOVENBERG, W. & MILLER, R.C. (1990). Endothelin: a review of its effects and possible mechanisms of action. *Neurochem. Res.*, **15**, 407–417.
- MCGREGOR, D.D. (1965). The effect of sympathetic nerve stimulation on vasoconstrictor responses in perfused mesenteric blood vessels of the rat. *J. Physiol.*, **177**, 21–30.
- MACLEAN, M.R., RANDALL, M.D. & HILEY, C.R. (1989). Effects of moderate hypoxia, hypercapnia and acidosis on haemodynamic changes induced by endothelin-1 in the pithed rat. *Br. J. Pharmacol.*, **98**, 1055–1065.
- MAGGI, C.A., GIULIANI, S., PATACCHINI, R., ROVERO, P., GIACHETTI, A. & MELI, A. (1989a). The activity of peptides of the endothelin family in various mammalian smooth muscle preparations. *Eur. J. Pharmacol.*, **174**, 23–31.
- MAGGI, C.A., GIULIANI, S., PATACCHINI, R., SANTICOLI, P., ROVERO, P., GIACHETTI, A. & MELI, A. (1989b). The C-terminal hexapeptide, endothelin-(16–21), discriminates between different endothelin receptors. *Eur. J. Pharmacol.*, **166**, 121–122.
- MINKES, R.K. & KADOWITZ, P.J. (1991). Differential effects of endothelin peptides in the systemic and pulmonary vascular beds of the cat. *Neurochem. Int.*, **18**, 547–552.
- MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J. (1991). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. *Proc. Natl. Acad. Sci., U.S.A.*, **88**, 2166–2170.
- NAKAJIMA, K., KUBO, S., KUMAGAYE, S.-I., NISHIO, H., TSUNEMI, N., INUI, T., KURODA, H., CHINO, N., WATANABE, T.X., KIMURA, T. & SAKAKIBARA, S. (1989). Structure-activity relationship of endothelin: importance of charged groups. *Biochem. Biophys. Res. Commun.*, **163**, 424–429.
- RANDALL, M.D., DOUGLAS, S.A. & HILEY, C.R. (1989). Vascular activities of endothelin-1 and some alanyl substituted analogues in resistance beds of the rat. *Br. J. Pharmacol.*, **98**, 685–699.
- SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.
- VANE, J. (1990). Endothelins come home to roost. *Nature*, **348**, 673.
- WARNER, T.D. (1990). Simultaneous perfusion of rat isolated superior mesenteric arterial and venous beds: comparison of their vasoconstrictor and vasodilator responses to agonists. *Br. J. Pharmacol.*, **99**, 427–433.
- WARNER, T.D., DE NUCCI, G. & VANE, J.R. (1989). Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. *Eur. J. Pharmacol.*, **159**, 325–326.
- WRIGHT, C.E. & FOZARD, J.R. (1988). Regional vasodilation is a prominent feature of the haemodynamic response to endothelin in anaesthetized, spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **155**, 201–203.
- WHITTLE, B.J.R., LOPEZ-BELMONTE, J. & REES, D.D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **98**, 646–652.
- YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

(Received May 15, 1991
Revised June 3, 1991
Accepted June 6, 1991)