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Endothelin converting enzyme (ECE) activity in human vascular smooth muscle

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1 We have characterized the human smooth muscle endothelin converting enzyme (ECE) present in the media of the endothelium-denuded human umbilical vein preparation.

2 Endothelin-1 (ET-1) and ET-2 were potent constrictors of umbilical vein with EC_{50} values of 9.2 nM and 29.6 nM, respectively. ET-1 was at least 30 times more potent than ET-3 suggesting the presence of constrictor ET_A receptors. Little or no response was obtained to the ET_B-selective agonist sarafotoxin 6c. These data suggest that endothelin-mediated vasoconstriction is via ET_A receptors in this preparation.

3 Autoradiographical visualization of endothelin receptors with subtype selective ligands confirmed the predominance of the ET_A receptor in the media of umbilical vein. High density of binding was obtained with the ET_A selective [^{125}I]-PD151242, with much lower levels detected with the ET_B selective [^{125}I]-BQ3020.

4 Big ET-1 (EC_{50} = 42.7 nM) and big ET-$2_{(1\text{-}38)}$ (EC_{50} = 99.0 nM) were less potent than ET-1 and ET-2, respectively. Big ET-$2_{(1\text{-}38)}$ was more potent than its isoform big ET-$2_{(1\text{-}37)}$ with concentration–response curves to big ET-$2_{(1\text{-}37)}$ incomplete at 300 nM. No response was obtained to big ET-3 at concentrations up to 700 nM. The C-terminal fragments, big ET-$1_{(22\text{-}38)}$ and big ET-$2_{(22\text{-}38)}$ were inactive.

5 Responses to ET-1 were unaffected by either the neutral endopeptidase (NEP) inhibitor thiorphan (10^{-5} M) or by the dual NEP/ECE inhibitor phosphoramidon (10^{-5} M). Big ET-1 was also unaffected by thiorphan but antagonized in a concentration-dependent manner by phosphoramidon (10^{-5} M and 10^{-4} M).

6 Addition of all four big endothelin peptides to human umbilical vein preparations resulted in detectable amounts of ET-IR in the bathing medium. Therefore, although big ET-3 was functionally inactive this reflects the low potency of ET-3 at the ET_A receptor rather than the lack of ability of this smooth muscle ECE to convert big ET-3 to ET-3.

7 To conclude we have demonstrated the presence of a phosphoramidon-sensitive ECE on the smooth muscle layer of the human umbilical vein which can convert big ET-1, big ET-$2_{(1\text{-}37)}$, big ET-$2_{(1\text{-}38)}$ and big ET-3 to their mature biologically active forms. The precise subcellular localization of this enzyme and its physiological relevance remains to be determined.

Keywords: Endothelin; big endothelin; endothelin converting enzyme (ECE); phosphoramidon; endothelin receptor subtypes; human umbilical vein; vascular smooth muscle; *in vitro* pharmacology; radioimmunoassay; autoradiography

Introduction

The dual endothelin converting enzyme (ECE) and neutral endopeptidase (NEP, neprolysin) inhibitor phosphoramidon, when infused into the human forearm, elicits a vasodilator response, whereas administration of the NEP inhibitor thiorphan results in vasoconstriction (Webb, 1995). This observation provided compelling evidence that the endogenous production of endothelin contributes significantly to the physiological maintenance of human vascular tone. Over-expression of endothelin is implicated in the development and progression of several cardiovascular diseases (for review see Huggins *et al*., 1993). Considerable interest has been focused on the potential of endothelin antagonists as therapeutic agents (Tamirisa *et al*., 1995; Doherty, 1996), resulting in the development of a large number of selective compounds, some of which are currently being evaluated for clinical efficacy. Alternatively, limiting the unwanted effects of endothelin in disease may be achieved more precisely by restricting its production.

The endothelin-1 (ET-1) peptide is cleaved from its precursor, big ET-1, by the action of one or more ECEs. ECE activity is blocked by phosphoramidon which is also an effective inhibitor of NEP. However, ECE and NEP can be distinguished by use of thiorphan, which is ineffective against ECE but a potent inhibitor of NEP (see Turner & Murphy, 1996). Two distinct ECEs have so far been cloned and sequenced. ECE-1, first described in bovine adrenal cortex (Xu *et al*., 1994), exists in two isoforms. In man (Schmidt *et al*., 1994; Shimada *et al*., 1995; Valdenaire *et al*., 1995; Yorimitsu *et al*., 1995), as in other species (Ikura *et al*., 1994; Schmidt *et al*., 1994; Shimada *et al*., 1994; 1995), these isoforms differ in the N-terminal sequence and are generated by alternative splicing of the same gene product. A second enzyme, ECE-2, possesses approximately 60% sequence homology with ECE-1 but differs markedly in its pH optimum (Emoto & Yanagisawa, 1995). Evidence now unequivocally points to ECE being a membrane-bound zinc metalloprotease (Okada *et al*., 1990; Ohnaka *et al*., 1992; Schmidt *et al*., 1994; Xu *et al*., 1994) belonging to the same family as NEP and the Kell blood group proteins.

In sections of human vascular tissues immunoreactive endothelin and big ET are localized to endothelial cells (Howard *et al*., 1992; Marciniak *et al*., 1992; Davenport *et al*., 1996; Plumpton *et al*., 1996), with little evidence for the presence of these peptides in smooth muscle cells. ET-1 is the principle isoform that is released by human endothelial cells. As it is co-released with big ET-1 *in vitro* (Plumpton *et al*., 1994; Ashby *et al*., 1995) this suggests that conversion of big ET-1 to ET-1 occurs, at least in part, intracellularly. This is supported by studies showing that ECE-1 is localized to the membranes of some intracellular organelles, particularly the Golgi apparatus (Gui *et al*., 1993; Xu *et al*., 1994). However, the rapid conversion of exogenous big ET-1 to ET-1 both *in vitro* and *in vivo*

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also suggests a role for ECE as an ecto-enzyme. Indeed the presence of ECE-1 on the endothelial cell surface has been suggested by several groups (for example Harrison *et al*., 1993; Barnes *et al*., 1995; Takahashi *et al*., 1995).

What remains unclear is whether ECE is also present in the vascular smooth muscle cells underlying the endothelium. Functional experiments in isolated vascular preparations do suggest that conversion of exogenously applied big ET-1 is unaffected by the removal of the endothelium (Fukuroda *et al*., 1990; Hisaki *et al*., 1993; Mombouli *et al*., 1993), implying the presence of a smooth muscle enzyme. However, this functional data does contrast with the apparent lack of immunoreactive ECE observed in smooth muscle cells (Takahashi *et al*., 1995), although we have recently identified immunoreactive ECE in the foetal smooth muscle cells of the human umbilical vein. The physiological role of this putative smooth muscle ECE remains to be determined. One possibility is that this enzyme is responsible for the local conversion of big ET-1, which is released from the endothelial cells, and of big ET-$2_{(1\text{-}37)}$, big ET-$2_{(1\text{-}38)}$ and big ET-3 which have been detected in human plasma (Matsumoto *et al*., 1994).

We have therefore functionally characterized the ECE present in human umbilical vein vascular smooth muscle and determined its sensitivity to thiorphan and phosphoramidon. We have made use of the endothelium-denuded human umbilical vein preparation, since vasoconstriction in this blood vessel is suggested to be mediated mainly through the ET_B receptor (Bogoni *et al*., 1996). All three endothelin isoforms are equipotent at the ET_B receptor and therefore this preparation should allow us to determine the substrate specificity of the enzyme. To confirm that conversion had taken place and that the big ETs do not themselves possess significant constrictor activity, we have analysed the bathing medium for the presence of mature endothelin by use of a selective radioimmunoassay (RIA). Preliminary results have been presented to the British Pharmacological Society (Maguire *et al*., 1997).

Methods

Tissue collection

Umbilical cords were obtained following normal and caesarean deliveries and kept refrigerated in sterile phosphate buffered saline (PBS) until required, typically with 8–12 h. For autoradiographical experiments blocks of whole cord, 2–3 cm in length, were snap frozen in liquid nitrogen and stored at −70°C. For immunocytochemistry and *in vitro* experiments cords were cut into 4 mm lengths from which the umbilical vein was dissected.

Immunocytochemistry

For functional experiments rings of umbilical vein were denuded of their endothelium by gently rubbing the luminal surface with the rounded tip of blunt metal forceps. Removal of the endothelial cell layer was confirmed by the absence of staining with rabbit antiserum to human von Willebrand factor as previously described (Davenport *et al*., 1996).

Autoradiography

The endothelin receptor subtypes present on the smooth muscle cells of umbilical vein were visualized by use of previously published methods (Davenport *et al*., 1988). Briefly, consecutive 10 μm sections of umbilical cord ($n=6$) were thaw mounted onto gelatine coated slides and pre-incubated in HEPES buffer (50 mM HEPES containing 5 mM $MgCl_2$ and bovine serum albumin (BSA), 0.3% w/v, pH 7.4) for 15 min at room temperature (23°C). The sections were incubated for a further two hours with the same buffer to which had been added either 0.1 nM [^{125}I]-ET-1 to label both ET_A and ET_B receptors or the ET_A-selective ligand [^{125}I]-PD151242 (0.1 nM) (Davenport *et al*., 1994) or 0.3 nM [^{125}I]-BQ3020 (Molenaar *et al*., 1992) to label selectively the ET_B receptors. The concentration of each ligand was calculated to bind to approximately 30% of the ET_A and ET_B receptors, respectively, according to the dissociation constant of each radioligand. Non-specific binding was determined by the inclusion of 1 μM of the corresponding unlabelled peptides. Finally, sections were rinsed in three successive washes of ice cold Tris buffer (50 mM, pH 7.4), dried under a stream of cold air and apposed, along with ^{125}I microscale standards, to radiation sensitive film (Hyperfilm βmax, Amersham International plc, Amersham, U.K.) for 5 days. Specific binding was visualized by means of computer-assisted image analysis (Quantimet 970, Cambridge Instruments).

In vitro *pharmacology*

Rings (4 mm) of endothelium-denuded human umbilical vein were transferred to 5 ml tissue baths (Linton Instrumentation, Diss, Norfolk, U.K.) containing modified Krebs solution, maintained at 37°C and continuously oxygenated with 95% O_2/5% CO_2. The blood vessel rings were set up between two L-shaped wires for isometric force measurements (F30 isometric force transducers, Hugo Sachs Elektronik, March-Hugstetten, Germany) and allowed to equilibrate for 60 min. Responses were elicited to 50 mM KCl at increasing levels of resting tension until no additional increase in the magnitude of the KCl response was obtained. The preparations were left for a further 30 min until a stable baseline was established. Cumulative concentration-response curves were constructed to ET-1, ET-2, ET-3, sarafotoxin 6c (S6c), big ET-1, big ET-$2_{(1\text{-}37)}$, big ET-$2_{(1\text{-}38)}$, big ET-3 and the C-terminal fragments big ET-$1_{(22\text{-}38)}$ and big ET-$2_{(22\text{-}38)}$ (10^{-10}–10^{-6} M). Only one agonist was tested on each vein ring. Concentration-response curves to ET-1 and big ET-1 were repeated in the presence of thiorphan (10^{-5}M) and phosphoramidon (10^{-5} M and 10^{-4} M) which were added to the bathing medium 20 min before the agonists. Experiments were terminated by the addition of 50 mM KCl to the bath to determine the maximum contractile response for each preparation. Agonist reponses were subsequently expressed as a percentage of this maximum. Values of EC_{50} (concentration producing 50% of the maximum response) were obtained from the graphs of $\log_{10}$ agonist concentration plotted against response (% of KCl). At the end of the experiments the bath contents were collected and stored at −20°C for the subsequent measurement of endothelin-like immunoreactivity (ET-IR) by radioimmunoassay (RIA).

Radioimmunoassay

The method used is essentially that previously described (Plumpton *et al*., 1996) and employed an antiserum directed against the C-terminal sequence that is common to the three mature endothelin peptides. This assay detects all three isoforms equally well, but does not cross-react with the big endothelins or truncated forms of the endothelins. Dilutions of ET-1 standards and bath content samples were made in assay buffer (50 mM sodium phosphate, 0.25% BSA, 0.01% Tween 20, 0.05% sodium azide, pH 7.4) and 100 μl aliquots were incubated for 16–24 h at 4°C with an equal volume of the antiserum (final dilution 1 : 30,000). The tracer ([^{125}I]-ET-1 2000 Ci mmol^{-1}) was added at 10,000 c.p.m. per tube and incubated for a further 16–24 h at 4°C. Bound and free tracer were separated with Amerlex-M reagent (Amersham International plc, Amersham, U.K.) and radioactive content of the samples measured by use of a Cobra 5010 gamma counter (Canberra Packard, Pangbourne, Berks., U.K.). Levels of ET-IR in the samples were determined from the graph of counts bound as a % of total plotted against $\log_{10}$ concentration of ET-1 for the standards.

Statistical analysis

Throughout, *n* values refer to the number of umbilical cords from which veins were obtained. Values for EC_{50} for each agonist, in the absence and presence of inhibitors, were expressed as geometric mean and 95% confidence interval. All other data were expressed as mean ± s.e.mean. The maximum contractile responses to KCl and agonists were compared by use of Student's one-tailed *t* test with a significance level of 5%.

Materials

Iodinated peptides, [^{125}I]-ET-1, [^{125}I]-PD151242 and [^{125}I]-BQ3020, all with a specific activity of 2000 Ci mmol^{-1} were obtained from Amersham International plc (Amersham, U.K.). Unlabelled ET-1, ET-2, ET-3, big ET-1, big ET-$2_{(1\text{-}37)}$, big ET-$2_{(1\text{-}38)}$, big ET-3, big ET-$1_{(22\text{-}38)}$, big ET-$2_{(22\text{-}38)}$, sarafotoxin 6c (S6c) and phosphoramidon were purchased from the Peptide Institute (Osaka, Japan) and thiorphan was from Sigma Chemical Co. (St. Louis, MO., U.S.A.). Stock solutions

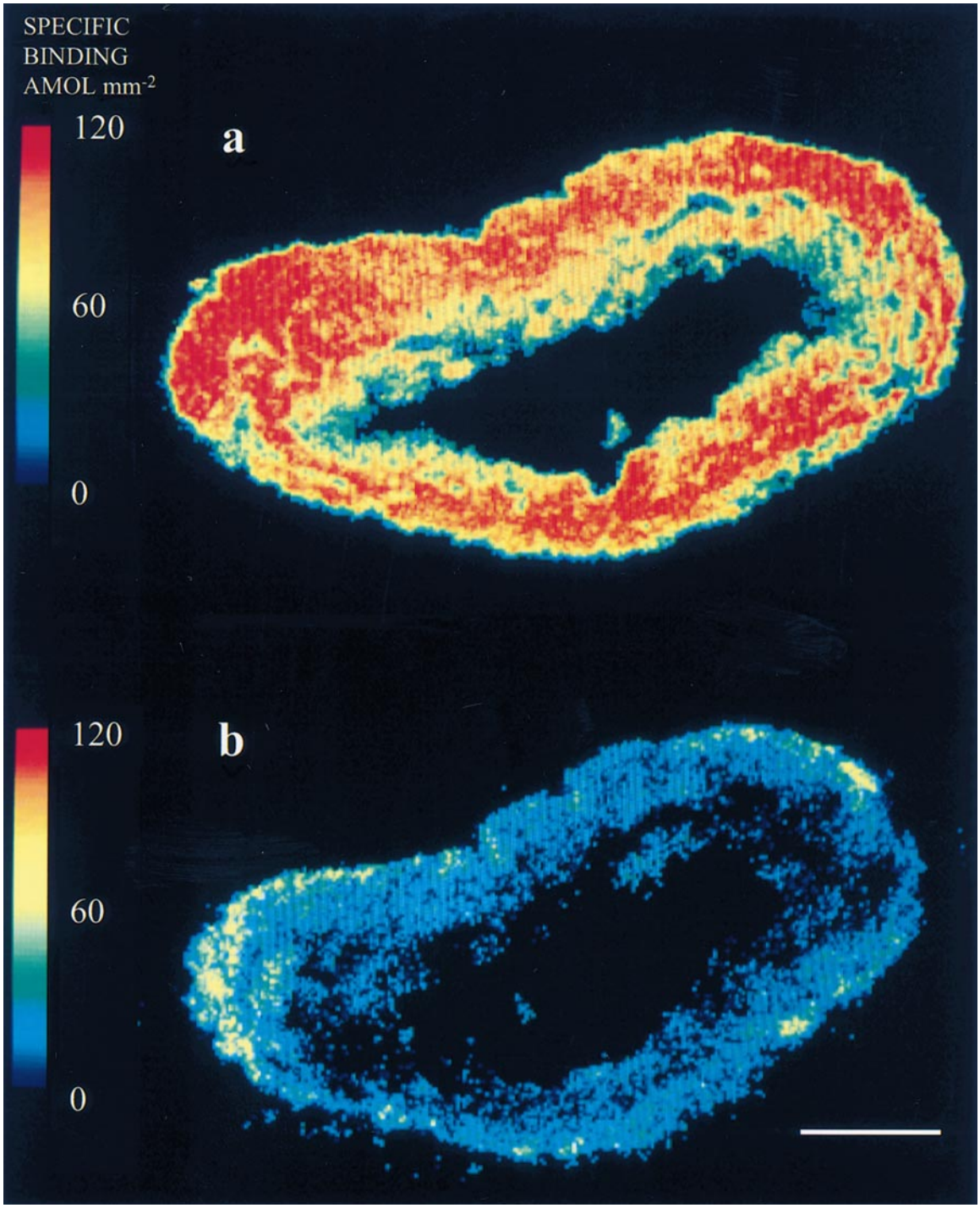

Figure 1 Colour coded images of the distribution of endothelin receptors in transverse sections of human umbilical vein. For each ligand the autoradiographic image of the non-specific binding was digitally subtracted from the total to produce a computer-generated image showing the amount of specific binding of [^{125}I]-PD151242 to ET_A receptors (a) and [^{125}I]-BQ3020 to ET_B receptors (b). The image of specific binding was colour-coded to show the density of receptors by interpolation from the standard curve generated by coexposing sections of radiolabelled tissue with ^{125}I standards. Scale bar = 1 mm.

(10^{-2}–10^{-4} M) were prepared in 0.1% acetic acid and stored at −20°C. Unlabelled PD151242 (N-[hexahydro-1-azepinyl)-carbonyl]L-Leu(1-me)D-Trp-D-Tyr) was a gift from Dr Annette Doherty, (Park Davis, Pharmaceutical Research, Ann Arbour, MI., U.S.A.) and BQ3020 ([$Ala^{11,15}$]Ac-ET-$1_{(6-21)}$) was synthesized by solid phase t-Boc chemistry. Stock solutions of PD151242 (10^{-2} M) were prepared in dimethylsulphoxide and those of BQ3020 (10^{-4} M) was prepared in 0.01 M ammonia, also stored at −20°C. Immunocytochemistry reagents were from Dako A/S (Denmark). All other reagents were from Sigma Chemical Co. (Poole, Dorset) or BDH (Lutterworth, Leics., U.K.) and were of analar grade or better.

Modified Krebs solution had the following composition (mM): NaCl 90, KCl 5, $MgSO_4$. $7H_2O$ 0.5, Na_2HPO_4 1, $NaHCO_3$ 45, $CaCl_2$ 2.25, glucose 10, Na pyruvate 5, fumaric acid 5 and L-glutamic acid 5.

Results

Endothelium removal procedure

In sections of intact umbilical vein ($n=3$) it was possible to visualize individual endothelial cells stained with the von Willebrand factor antiserum. These cells were absent in sections of vein ($n=3$) whose luminal surface had been rubbed verifying the success of the procedure (data not shown).

Autoradiography

High specific binding was observed in the media (smooth muscle layer) of umbilical vein ($n=6$) to the non-selective ligand [^{125}I]-ET-1. Similarly, significant binding was seen with the ET_A-selective compound [^{125}I]-PD151242 (Figure 1a) suggesting that the majority of the receptors labelled by [^{125}I]-ET-1 are ET_A receptors. This was confirmed by the much lower density of ET_B receptors visualized with [^{125}I]-BQ3020 (Figure 1b).

In vitro *pharmacology*

The majority of the isolated umbilical vein preparations exhibited spontaneous, phasic contractile activity. This appeared immediately after they were placed in the tissue bath or developed following the normalization procedure. The remaining preparations were quiescent throughout the experiments. In all tissues the responses to the agonists were determined as the increase in baseline tension upon which these phasic contractions may be superimposed.

ET-1 and ET-2 potently contracted isolated umbilical vein with EC_{50} values of 9.2 nM (6.0–14.0, $n=17$) and 29.6 nM (16.5–53.2, $n=10$) (geometric mean with 95% confidence intervals), respectively. The response of umbilical vein ($n=11$) to ET-3 was much more variable. ET-3 was less potent than either ET-1 or ET-2 with the concentration–response curves either incomplete at 700 nM, or in a minority of vessels no response was obtained even at this concentration (Figure 2). Vessels which contracted poorly following addition of high concentrations of ET-3 showed normal responsiveness to 50 mM KCl, with a mean maximum contraction of 9.9±1.3 g

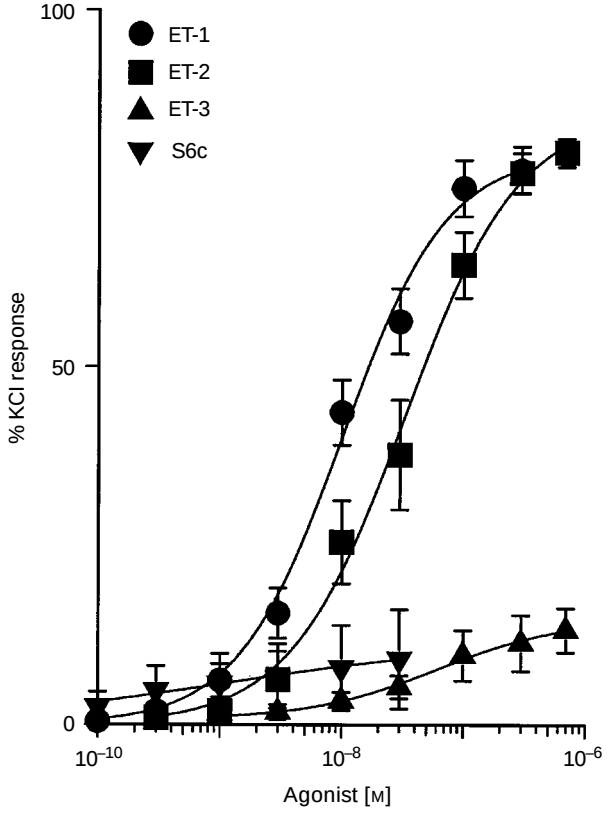

Figure 2 Cumulative concentration–response curves to ET-1, ET-2, ET-3 and S6c in endothelium-denuded human umbilical vein. Data points are the mean of 8–17 experiments; vertical lines show s.e.mean.

Table 1 Comparison of the vasoconstrictor potencies of the endothelin peptides and their precursors in human umbilical vein

Peptide	*EC_{50}* (nM) *(95% CI)*	*Resp. to KCl* (g)	*Maximum agonist response* (%KCl)	n
ET-1	9.2 (6.0–14.0)	7.9±0.9	77.5±3.3	17
ET-2	29.6 (16.5–53.2)	7.7±1.0	77.1±2.8	10
ET-3	>300	9.9±1.3	CRC incomplete at 700 nM	11
S6c	No response up to 100 nM in 6/8	7.9±1.2	–	8
Big ET-1	42.7 (26.6–68.7)	8.75±1.2	84.2±2.0	23
Big ET-$2_{(1-37)}$	>300	8.1±1.0	CRC incomplete at 700 nM	9
Big ET-$2_{(1-38)}$	99.0 (61.4–159.6)	9.6±1.2	74.0±5.1	11
Big ET-3	No response up to 700 nM	8.4±1.9	–	5

Potency values are the geometric mean with 95% confidence intervals in parentheses; *n* values are the number of umbilical cords from which veins were obtained.

weight. This was not significantly different from the maximum contractile response observed with KCl following either the ET-1 (7.9±0.9 g weight) or the ET-2 (7.7±1.0 g weight) concentration–response curves ($P>0.05$, Student's one-tailed t test). The ET_B agonist S6c (10^{-11}–10^{-7} M) was inactive in six of the eight veins in which it was tested. In the two veins in which responses were obtained, contractions were obtained to low concentrations of S6c, with EC_{50} values of less than 1 nM. However, the maximum responses were considerably less (<50%) than those obtained to ET-1 in the same experiment (Figure 2). The maximum KCl contraction following addition of S6c was 7.9±1.2 g weight, again comparable to that for the other agonists (Table 1).

Big ET-1 was approximately five times less potent than ET-1 with an EC_{50} value of 42.7 nM (26.6–68.7 nM, $n=23$). Big ET-1 was at least twice as potent as big ET-$2_{(1\text{-}38)}$ (EC_{50} 99.0 nM (61.4–160 nM, $n=11$)), which was more potent again than its isoform big ET-$2_{(1\text{-}37)}$ ($n=9$). The peak responses to big ET-1 and big ET-$2_{(1\text{-}38)}$ were not different from that to ET-1, the concentration–response curve to big ET-$2_{(1\text{-}37)}$ was incomplete at 700 nM (Figure 3). Big ET-3 ($n=5$) did not elicit any response at concentrations up to 700 nM. C-terminal fragments, big ET-$1_{(22\text{-}38)}$ and big ET-$2_{(22\text{-}38)}$ were inactive and did not antagonize the contractile responses to ET-1.

The concentration–response curve of ET-1 was unaffected by the presence of the NEP inhibitor thiorphan (10^{-5} M) or by the dual NEP/ECE inhibitor phosphoramidon (10^{-5} M) (Figure 4a, Table 2). Although there was no alteration of the big ET-1 curve following the addition of thiorphan (10^{-5} M) there was a small shift of the big ET-1 curve in the presence of phosphoramidon (10^{-5} M) (Figure 4b). This effect of phosphoramidon was concentration-dependent with a further degree of shift obtained with the higher concentration (10^{-4} M) of the inhibitor (Figure 4b, Table 2).

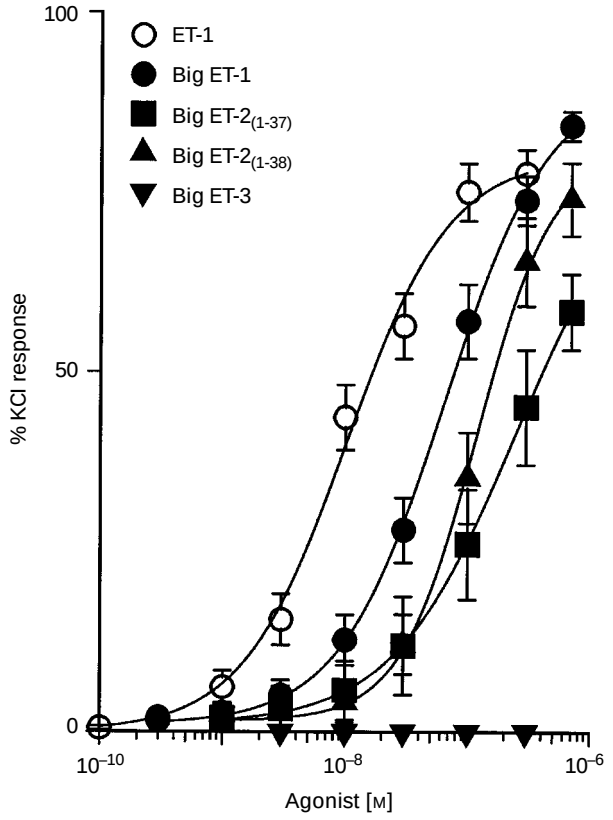

Figure 3 Cumulative concentration–response curves to ET-1, big ET-1, big ET-$2_{(1\text{-}37)}$, big ET-$2_{(1\text{-}38)}$ and big ET-3 in endothelium-denuded human umbilical vein. Data points are the mean of 5–23 experiments; vertical lines show s.e.mean.

Radioimmunoassay

RIA of the tissue bath contents, to which ET-1, ET-2 or ET-3 had been added, revealed free concentrations of ET-IR of

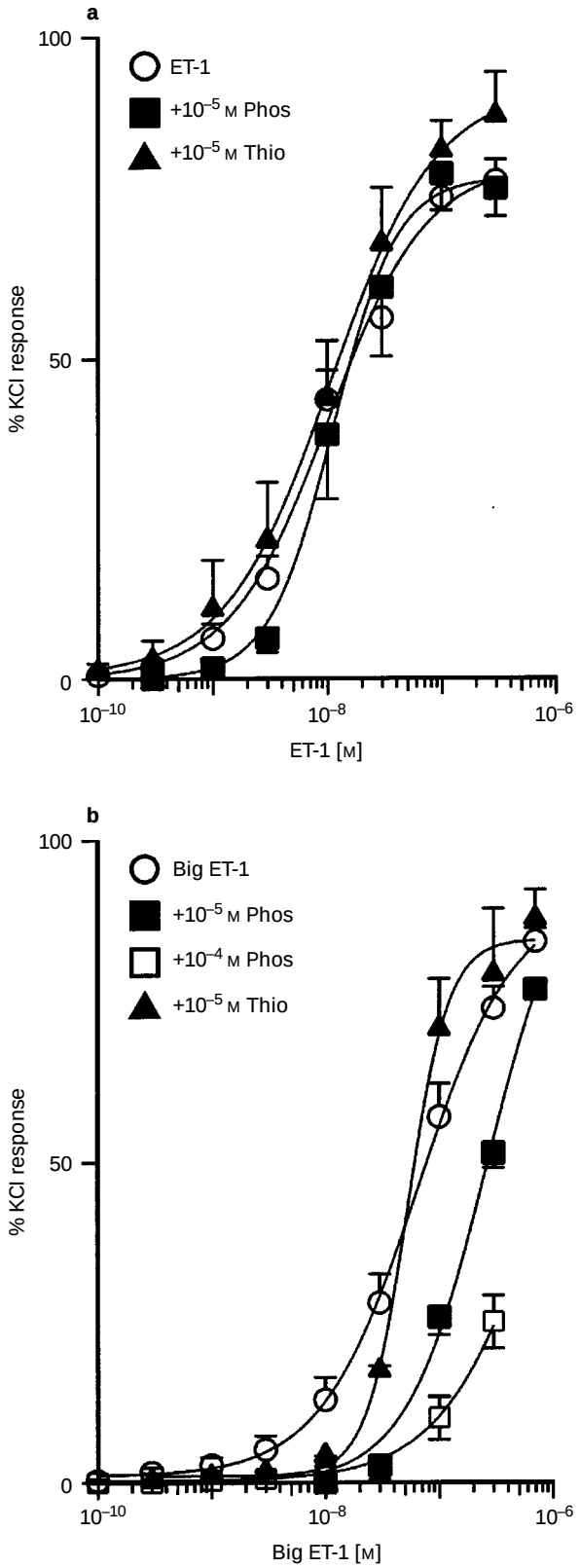

Figure 4 Concentration–response curves to (a) ET-1 and (b) big ET-1 in the absence and presence of 10^{-5} M and 10^{-4}M phosphoramidon (Phos) and 10^{-5} M thiorphan (Thio) in endothelium-denuded human umbilical vein. The inhibitors were added to the bath 20 min before the agonist response was determined. Data points are the mean and vertical lines show s.e.mean, $n=5$–23.

Table 2 The effect of the metalloprotease inhibitors, phosphoramidon and thiorphan on the responses to ET-1 and big ET-1 in human umbilical vein

Peptide	*EC_{50}* (nM) *(95% CI)*	*Resp. to KCl* (g)	*Maximum agonist response* (%KCl)	n
ET-1	9.2 (6.0–14.0)	7.9±0.9	77.5±3.3	17
ET-1 + 10^{-5} M phosphoramidon	13.8 (7.7–24.8)	7.8±1.3	78.7±5.7	5
ET-1 + 10^{-5} M thiorphan	11.0 (3.5–34.5)	6.6±1.1	88.0±5.2	5
Big ET-1	42.7 (26.6–68.7)	8.75±1.2	84.2±2.0	23
Big ET-1 + 10^{-5} M phosphoramidon	188.2 (78.4–451.6)	9.7±1.8	76.7±4.5	7
Big ET-1 + 10^{-4} M phosphoramidon	>300 nM	8.1±1.4	CRC incomplete at 700 nM	7
Big ET-1 + 10^{-5} M thiorphan	45.6 (29.6–70.1)	8.1±0.5	87.7±1.2	12

Potency values are the geometric mean with 95% confidence intervals in parentheses; *n* values are the number of umbilical cords from which veins were obtained.

Table 3 The concentration (nM) of immunoreactive endothelin (ET-IR) present following the incubation of endothelin peptides and their precursors with preparations of human umbilical vein *in vitro*

Peptide	*ET-IR* (nM)	n
ET-1	38±8	13
ET-2	8±1	7
ET-3	25±8	11
Big ET-1	0.9±0.3	21
Big ET-$2_{(1-37)}$	1.4±0.5	9
Big ET-$2_{(1-38)}$	0.9±0.4	9
Big ET-3	0.9±0.5	5

Values are the mean±s.e.mean; *n* values are the number of experiments from which bath contents were analysed.

10–40 nM. Lower levels of ET-IR were also present in all bath samples to which the big endothelin peptides had been added (Table 3). ET-IR was not detected when big ET-1 was incubated without tissue, or when tissue was incubated without big ET-1 (data not shown). Therefore big ET-1 was not reverting to ET-1 spontaneously, nor was ET-1 being produced in measurable amounts by the umbilical vein rings *in vitro*.

Discussion

Inhibition of the ECE activity in vascular endothelial cells may prove to be an effective therapeutic strategy to limit the unwanted effects of over-production of endothelins in disease. However, endothelial cells produce exclusively ET-1 (Plumpton *et al*., 1996) and therefore the other two isoforms, ET-2 and ET-3, must be processed from their precursors at other sites. Big ET-2 and big ET-3 are present in significant amounts in the plasma and, as there is little evidence for ECE activity in human blood (Watanabe *et al*., 1991), it is likely that they are converted to their mature forms at their target organs. One possibility is that circulating big ET-2 and big ET-3, together with big ET-1 that is co-released with ET-1 from the endothelium, are converted to the active peptides by an ECE present on vascular smooth muscle cells. The existence of such a smooth muscle ECE has previously been suggested. Homogenates of bovine carotid artery converted big ET-1 to ET-1 in a pH-sensitive manner; activity at neutral pH was phosphoramidon-sensitive and predominantly membrane bound (Hioki *et al*., 1991). A more recent study also localized ECE-1 to the extracellular surface of cultured vascular smooth muscle cells (Emoto *et al*., 1996) and described the conversion of exogenuously applied big ET-1.

In the present study, we have investigated the nature of the smooth muscle ECE in human umbilical vein preparations. Bogoni and colleagues (1996) have recently published functional data which suggests that the endothelin peptides mediate their constrictor effects mainly via the ET_B receptor. However, we found that ET-1 and ET-2 were much more potent than ET-3 suggesting the presence of constrictor ET_A receptors. This was confirmed by the lack of significant response obtained with the selective ET_B agonist sarafotoxin 6c. These data were also supported by the autoradiographic visualization of endothelin receptor subtypes by use of receptor-specific radioligands that showed the predominance of the ET_A receptor in the vascular smooth muscle layer. It is difficult to explain the discrepancies between these two investigations. The only striking difference in experimental method was the use of a vein strip preparation by Bogoni and colleagues (1996) and a ring preparation in the present study. The use of agonists and antagonists that have better selectivity for human endothelin receptor subtypes may be required for this anomaly to be resolved.

Big ET-1 was a less potent constrictor than ET-1, of umbilical vein, although the difference in potency of the two peptides was much less than that found for other blood vessels including human coronary arteries (Howard *et al*., 1992; Bax *et al*., 1994). If the EC_{50} values obtained for the two peptides are compared then big ET-1 appears to have 20% of the activity of ET-1. This suggests that either big ET-1 itself has affinity for endothelin receptors or it must be converted to ET-1 for its biological activity. To exclude the possibility that big ET-1 possesses intrinsic biological activity and to confirm that conversion to ET-1 must occur for its biological activity, we used RIA to show the presence of ET-IR in the bathing medium following addition of big ET-1. Therefore our data obtained *in vitro* agree with results determined in the human forearm *in vivo*, where the haemodynamic effects of infused big ET-1 are due, at least in part, to its conversion to ET-1 (Plumpton *et al*., 1995).

The ECE present in this particular human smooth muscle preparation appears to convert all of the big endothelin precursors. Thus big ET-$2_{(1-37)}$ and big ET-$2_{(1-38)}$ had some vasoconstrictor activity, though less than big ET-1, and given the low potency of ET-3 it was unsurprising that big ET-3 was inactive at the concentrations tested. However, in each case we detected similar levels of ET-IR in the bathing medium. It is

not possible to determine the absolute substrate specificity of the enzyme in the present study, as the amounts of ET-IR reflect only free peptide concentration and not receptor bound and non-specifically bound peptide. The enzyme responsible for this conversion differs from NEP since the vasoconstrictor response of big ET-1 was blocked by phosphoramidon but unaffected by the NEP inhibitor thiorphan. These data also suggest that the smooth muscle ECE is different from the endothelial cell ECE which does not convert big ET-2 or big ET-3 as efficiently as big ET-1 (Schmidt *et al*., 1994; Xu *et al*., 1994; Shimada *et al*., 1995).

Therefore, we have demonstrated the presence of a phosphoramidon-sensitive ECE present on human vascular smooth muscle cells that does not distinguish between the endothelin peptide precursors. The precise subcellular localization of this enzyme and its physiological relevance remains to be determined.

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