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Vasoconstrictor activity of novel endothelin peptide, ET-$1_{(1-31)}$, in human mammary and coronary arteries *in vitro*

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1 The ability of the putative chymase product of big endothelin-1 (big ET-1), ET-$1_{(1-31)}$, to constrict isolated endothelium-denuded preparations of human coronary and internal mammary artery was determined.

2 pD_2 values in coronary and mammary artery respectively were 8.21 ± 0.12 ($n=14$) and 8.55 ± 0.11 ($n=12$) for ET-1, 6.74 ± 0.11 ($n=16$) and 7.10 ± 0.08 ($n=16$) for ET-$1_{(1-31)}$ and 6.92 ± 0.10 ($n=15$) and 7.23 ± 0.11 ($n=12$) for big ET-1. ET-$1_{(1-31)}$ was significantly less potent than ET-1 ($P<0.001$, Student's *t*-test) and equipotent with big ET-1.

3 Vasoconstrictor responses to 100–700 nM ET-$1_{(1-31)}$ were significantly ($P<0.05$, Student's paired *t*-test) attenuated by the ET_A antagonist PD156707 (100 nM).

4 There was no effect of the ECE inhibitor PD159790 (30 μM), the ECE/NEP inhibitor phosphoramidon (100 μM) or the serine protease inhibitor chymostatin (100 μM) on ET-$1_{(1-31)}$ responses in either artery.

5 Radioimmunoassay detected significant levels of mature ET in the bathing medium of coronary (1.6 ± 0.5 nM, $n=14$) and mammary (2.1 ± 0.6 nM, $n=14$) arteries, suggesting that conversion of ET-$1_{(1-31)}$ to ET-1 contributed to the observed vasoconstriction.

6 ET-$1_{(1-31)}$ competed for specific [^{125}I]-ET-1 binding to ET_A and ET_B receptors in human left ventricle with a pooled K_D of 71.6 ± 7.0 nM ($n=3$).

7 Therefore, in human arteries the novel peptide ET-$1_{(1-31)}$ mediated vasoconstriction *via* activation of the ET_A receptor. The conversion of ET-$1_{(1-31)}$ to ET-1, by an as yet unidentified protease, must contribute wholly or partly to the observed constrictor response. Chymase generated ET-$1_{(1-31)}$ may therefore represent an alternative precursor for ET-1 production in the human vasculature.

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Abbreviations: ECE, endothelin-converting enzyme; ET, endothelin; NEP, neutral endopeptidase

Introduction

The potent vasoconstrictor peptide endothelin-1 (ET-1) (Yanagisawa *et al*., 1988) is synthesized in human endothelial cells by cleavage of its precursor, big ET-1, by unique endothelin converting enzyme(s) (ECE) (for review see Russell & Davenport, 1999). Endothelial cells secrete both ET-1 and big ET-1 (Plumpton *et al*., 1994) *via* the constitutive and stimulated synthetic pathways and we hypothesize that released big ET-1 may undergo further processing by the underlying smooth muscle cells or may act as a circulating hormone in plasma, being converted to a biologically active form at its target organ. We have demonstrated that human blood vessels, denuded of their endothelium, are capable of converting big ET-1 to vasoactive ET-1, implying the presence of ECE activity on vascular smooth muscle cells (Maguire *et al*., 1997a; Maguire & Davenport, 1998). Indeed the observation that the pro-inflammatory cytokines tumour necrosis factor-α and γ-interferon induce ET-1 production by cultured human vascular smooth muscle cells supports the hypothesis that these cells may become an important site of ET-1 production in disease (Woods *et al*., 1999).

The possibility that an alternative synthetic pathway for vasoconstrictor ET peptides exists was first suggested by the finding that cathepsin G cleaved big ET-1 to produce a novel vasoconstrictor peptide of between 27 and 31 amino acids (Patterson *et al*., 1990). This was subsequently confirmed as ET-$1_{(1-31)}$ by electrospray-mass spectrometry (Kaw *et al*., 1992). More recently, incubation of big ET-1 with membrane fractions of human lung led to the identification of ET-$1_{(1-31)}$ as a major hydrolysis product (Hanson *et al*., 1997). In this tissue, the peptide was generated by chymostatin- and soybean trypsin inhibitor-sensitive enzymatic activity that appeared to be independent of the phosphoramidon-sensitive conversion of big ET-1 to ET-1. Importantly ET-$1_{(1-31)}$ was shown to have vasoconstrictor activity, although it was approximately 100 times less potent than ET-1 in isolated rabbit pulmonary arteries. At the same time it was reported that incubation of big ET-1 with purified human connective tissue mast cell chymase resulted in the formation of

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ET-$1_{(1-31)}$ (Nakano *et al*., 1997; Kido *et al*., 1998) implicating this enzyme as the unidentified serine protease activity in human lung. In this instance ET-$1_{(1-31)}$ reportedly exhibited comparable constrictor activity to ET-1 without further degradation (Nakano *et al*., 1997; Kishi *et al*., 1998) and these authors also reported that tissue concentrations of ET-$1_{(1-31)}$ were higher than those of ET-1 suggesting that the alternative processing of big ET-1 to ET-$1_{(1-31)}$ may be physiologically important. ET-$1_{(1-31)}$ generation may also contribute significantly to ET-1 mediated pathophysiological processes as human chymase is known to be up-regulated in, for example, coronary artery disease (Kaartinen *et al*., 1994).

Therefore our aim was to assess the importance of ET-$1_{(1-31)}$ as a vasoconstrictor peptide in human arteries and to clarify whether ET-$1_{(1-31)}$ acts directly on smooth muscle to elicit vasoconstriction or acts as an intermediate which is subsequently broken down to ET-1 for biological activity. We compared the response of human epicardial coronary and internal mammary arteries to ET-1, its precursor big ET-1 (which must be converted to ET-1 for activity) and to ET-$1_{(1-31)}$. In these vessels (including atherosclerotic coronary artery) ET_A receptors prodominate on the smooth muscle cells (Dashwood *et al*., 1994; Davenport *et al*., 1995; Bacon *et al*., 1996) and are responsible for the majority of the ET-mediated vasoconstrictor response observed both *in vitro* (Bax *et al*., 1994; Maguire & Davenport, 1995; Holm & Franco-Cereceda, 1996; Opgaard *et al*., 1996) and *in vivo* (Kyriakides *et al*., 2000). This is consistent with the observation that infusion of the ET_A receptor antagonist BQ123 will completely block the reduction in forearm blood flow produced by ET-1 and indeed BQ123 alone elicits vasodilatation (Haynes & Webb, 1994). Infusion of the ET_B antagonist BQ788 produces vasoconstriction in human forearm (Verhaar *et al*., 1998; Strachan *et al*., 1999). This occurs in both healthy volunteers and heart failure patients (Love *et al*., 2000). The conclusion from these studies is that the ET_A receptor is important for vasoconstriction in human arteries and the main function of vascular ET_B receptors is to produce vasodilatation *via* release of endothelium-derived vasodilators such as nitric oxide. From our functional studies it would not be possible to determine if ET-$1_{(1-31)}$ had significant effects on smooth muscle ET_B receptors. Therefore to determine if ET-$1_{(1-31)}$ also has affinity for this subtype, competition binding assays were carried out in sections of human left ventricle, a tissue chosen as it contains both ET_A and ET_B receptors. Preliminary data have been presented to the British Pharmacological Society (Maguire & Davenport, 1999).

Methods

Tissue collection

Human tissue was collected with local ethical approval. Human coronary arteries were from recipient hearts of patients transplanted for cardiomyopathy (four male, one female, 40–57 years of age), ischaemic heart disease (five male, one female, 52–58 years of age) or donor hearts for which there were no suitable recipients (three male, two female, 18–61 years of age). Arteries from cardiomyopathy patients were histologically normal (Dec & Fuster, 1994) whereas those from patients with ischaemic heart disease had visible atheroma. Mammary arteries were from 16 patients (15 male, one female, 45–89 years of age) receiving coronary artery bypass grafts. Blood vessels were transported to the laboratory in cold, Krebs' solution. Human heart left ventricle was from three patients transplanted for dilated cardiomyopathy (two male, one female, 25–53 years of age). Drugs included nitrates, angiotensin converting enzyme inhibitors, calcium antagonists, anti-arrhythmics, cardiac glycoside, diuretics and anticoagulants.

In vitro *pharmacology*

Human coronary and mammary arteries were cleaned of connective tissue and cut into 4 mm rings. The luminal side of each ring was gently rubbed with a pair of blunt metal forceps to disrupt the endothelium and this was verified histologically as previously described (Maguire *et al*., 1997a). Rings were transferred to 5 ml organ baths containing oxygenated Krebs' solution maintained at 37°C and set up for isometric force recordings using F30 transducers (Hugo Sachs Elektronik, Freiburg, Germany). Output was to a chart recorder (Graphtec Corporation, Yokohama, Japan) and data was also recorded using the MP100 data acquisition system (Biopac Systems Inc., CA, U.S.A.) for subsequent analysis. Arteries were allowed to equilibrate for 60 min and optimum resting tension for each ring was determined by recording the response to 100 mM KCl at incrementally increasing levels of basal tension. When subsequent KCl responses were of similar magnitude tissues were allowed to re-equilibrate for a further 60 min. One vessel ring from each patient was used for each agonist response curve. Cumulative concentration-response curves were constructed to ET-1 ($1\times10^{-10}-3\times10^{-7}$ M), ET-$1_{(1-31)}$ ($1\times10^{-10}-7\times10^{-7}$ M) and big ET-1 (1×10^{-10} M-7×10^{-7} M) and when no further increase in response was obtained the experiment was terminated by the addition of 100 mM KCl, to determine the maximum possible constrictor response of tissue from each patient. Agonist contractions were expressed as a percentage of this terminal KCl response (%KCl). In additional experiments cumulative concentration-response curves to ET-$1_{(1-31)}$ were repeated in the presence of the ECE/neutral endopeptidase (NEP) inhibitor phosphoramidon (100 μM), the ECE inhibitor PD159790 (30 μM) (Ahn *et al*., 1998), the serine-protease inhibitor chymostatin (100 μM) or the ET_A receptor antagonist PD156707 (100 nM) (Doherty *et al*., 1995; Reynolds *et al*., 1995).

Bath contents from the control experiments were collected for subsequent analysis by radioimmunoassay.

Radioimmunoassay

Bathing medium was assayed for the presence of mature ET using a specific radioimmunoassay that detects the common C-terminal sequence of the three endogenous endothelin peptides but does not cross react with the ET-1 precursor peptide big ET-1 (Plumpton *et al*., 1996). Cross reactivity of the assay was determined for ET-$1_{(1-31)}$. Briefly, aliquots (100 μl) of ET-1 (4 pM–10 nM) and ET-$1_{(1-31)}$ (1 nM–10 μM) standards and bath contents from the *in vitro* experiments were incubated in duplicate with 100 μl of antiserum (final dilution 1 : 30,000) for 16–24 h at 4°C. [^{125}I]-ET-1 (15,000 c.p.m.) was then added and the tubes

incubated for a further 16–24 h at 4°C. Bound [^{125}I]-ET-1 was separated from free using 250 μl Amerlex-M reagent (Amersham Pharmacia Biotech., Bucks., U.K.) and counted in a Cobra II gamma counter (Canberra Packard, Berks., U.K.). The concentration of mature ET in ET-$1_{(1-31)}$ standards and bathing medium samples was determined from the resulting ET-1 standard curve. Comparison of curves for ET-1 and ET-$1_{(1-31)}$ determined the extent of cross-reactivity of ET-$1_{(1-31)}$ for the ET-1 radioimmunoassay.

Competition binding experiments

Cryostat-cut sections (10 μm thick) of human heart left ventricle were incubated for 2 h at room temperature (23°C) with 0.1 nM [^{125}I]-ET-1 and increasing concentrations of ET-$1_{(1-31)}$ ($2\times10^{-12}-1\times10^{-5}$ M). Non-specific binding was defined by inclusion of 1 μM ET-1. Sections were washed briefly and counted for radioactive content. Data were analysed using the iterative curve-fitting suite of programmes KELL (Biosoft, Cambridge, U.K.) to determine values of K_D.

Materials

ET-1, ET-$1_{(1-31)}$ and big ET-1 were from the Peptide Institute (Osaka, Japan) and were stored as aliquots of 10^{-4} M stock solution (in 0.1% acetic acid) at −20°C until required. [^{125}I]-ET-1 (2000 Ci mmol^{-1}) was from Amersham Pharmacia Biotech. (Bucks., U.K.). PD156707 and PD159790 were gifts from Parke-Davis Pharmaceutical Research, U.S.A. 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, L-glutamic acid, 5.

Results

Vasoconstrictor response to ET peptides in human arteries

For the arterial rings used to compare ET-1, ET-$1_{(1-31)}$ and big ET-1, responses to 100 mM KCl were 67±8, 81±8 and 77±8 mN ($F=0.83$, $P>0.05$, one-way analysis of variance) respectively in coronary artery and 31±5, 53±7 and 52±9 mN ($F=2.84$, $P>0.05$, one-way analysis of variance) respectively in mammary artery. For each agonist, in both arteries, these terminal KCl responses were not significantly different from the KCl responses determined before the cumulative concentration response curves to the ET peptides (data not shown). There was, therefore, no evidence either of toxicity or of sensitization of the contractile proteins by any of the three test peptides.

ET-1 constricted human coronary and mammary arteries with comparable pD_2 values of 8.21±0.12 ($n=14$) and 8.55±0.11 ($n=12$), respectively (Figure 1). In both arteries, ET-$1_{(1-31)}$ and big ET-1 were significantly ($P<0.001$, Student's two-tailed t-test) less potent than ET-1 but not significantly ($P>0.05$) different from each other. ET-$1_{(1-31)}$ contracted coronary artery with a pD_2 value of 6.74±0.11 ($n=16$) and big ET-1 with a pD_2 value of 6.92±0.10 ($n=15$). In mammary artery the pD_2 value for ET-$1_{(1-31)}$ was 7.10±0.08 ($n=16$) and for big ET-1 was 7.23±0.11 ($n=12$) (Figure 1). Maximum responses for the three peptides were not different in mammary artery (ET-1 85.4±4.7%; big ET-1 80.9±5.2%; ET-$1_{(1-31)}$ 73.5±4.0%), however in coronary artery the maximum response to ET-$1_{(1-31)}$ (58.7±6.0%) was significantly ($P<0.05$, Student's two-tailed t-test) lower than that to ET-1 (78.8±5.7%) but not different from that to big ET-1 (75.2±5.9%).

Effect of ET_A antagonist PD156707 on ET-$1_{(1-31)}$ vasoconstriction

In the presence of 100 nM PD156707 the response to higher concentrations of ET-$1_{(1-31)}$ ($\geq$100 nM) were significantly attenuated ($P<0.05$, Student's paired t-test) in both coronary artery (Figure 2A) and mammary artery (Figure 2B) such that the curves were incomplete at 700 nM, the maximum concentration obtainable in the 5 ml organ bath from the 10^{-4} M stock solution of peptide.

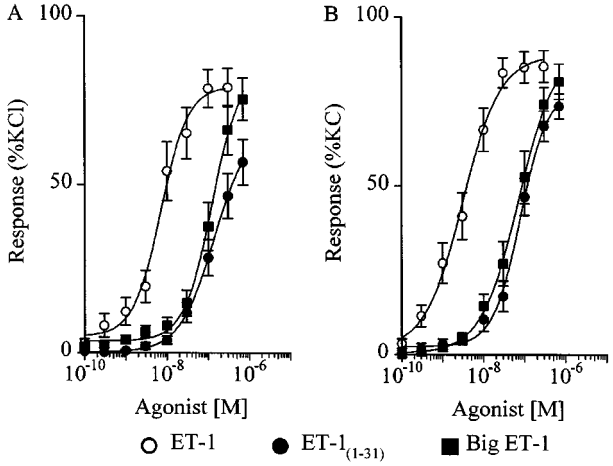

Figure 1 Concentration-response curves to ET-1, ET-$1_{(1-31)}$ and big ET-1 in (A) human coronary artery ($n=14-15$) and (B) human internal mammary artery ($n=12-14$) *in vitro*. Values are mean± s.e.mean and n values are the number of patients from whom blood vessels were obtained.

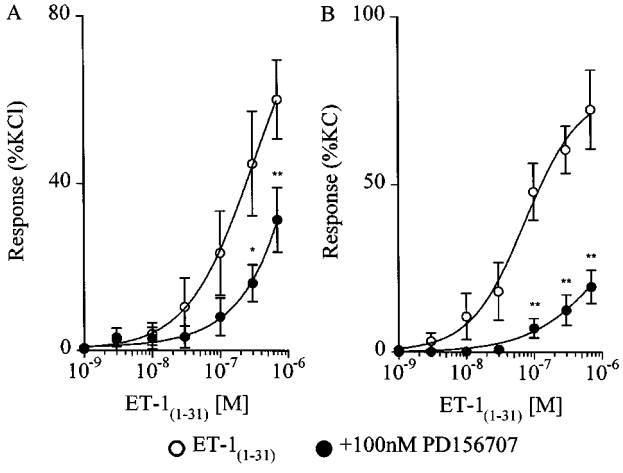

Figure 2 Concentration-response curves to ET-$1_{(1-31)}$ in the absence and presence of 100 nM PD156707 in (A) human coronary artery ($n=6$) and (B) human internal mammary artery ($n=4$). Values are mean± s.e.mean and n values are the number of patients from whom arteries were obtained. *$P<0.05$; **$P<0.01$ Student's paired t-test.

Effect of enzyme inhibitors

Vasoconstrictor responses to ET-$1_{(1-31)}$ in both arteries were unaffected by the ECE inhibitor PD159790 (30 μM), which we have previously shown blocks ECE conversion of big ET-1 to ET-1 in human coronary artery (Maguire *et al*., 1999). In coronary artery pD_2 values for ET-$1_{(1-31)}$ were 6.49 ± 0.22 in the absence and 6.90 ± 0.17 in the presence of PD159790 ($n=4$, $P>0.05$, Student's 2-tailed *t*-test). For mammary artery pD_2 values for ET-$1_{(1-31)}$ were 7.02 ± 0.15 in the absence and 6.94 ± 0.12 in the presence of PD159790 ($n=5$, $P>0.05$, Student's 2-tailed *t*-test) (Figure 3A,C).

There was also no effect of the ECE/NEP inhibitor phosphoramidon (100 μM) on ET-$1_{(1-31)}$ constrictor responses either in coronary artery (control $pD_2=6.67 \pm 0.29$, with phosphoramidon $pD_2=6.34 \pm 0.49$, $n=5$ $P>0.05$ Student's 2-tailed *t*-test) or in mammary artery (control $pD_2=6.99 \pm 0.19$, with phosphoramidon $pD_2=6.86 \pm 0.25$, $n=4$, $P>0.05$, Student's 2-tailed *t*-test) (Figure 3B,D). In coronary artery, additional experiments were carried out with the serine protease inhibitor chymostatin. The pD_2 value for ET-$1_{(1-31)}$, 7.00 ± 0.24, $n=5$, was not significantly different in the presence of 100 μM chymostatin, 7.08 ± 0.08, $n=5$.

Radioimmunoassay

Using the specific radioimmunoassay that recognizes the C-terminal hexapeptide sequence of ET-1, ET-2 and ET-3, we determined the cross reactivity of the antibody for ET-$1_{(1-31)}$. The competition curves for ET-1 and ET-$1_{(1-31)}$ were parallel with the ET-$1_{(1-31)}$ curve displaced to the right (Figure 4).

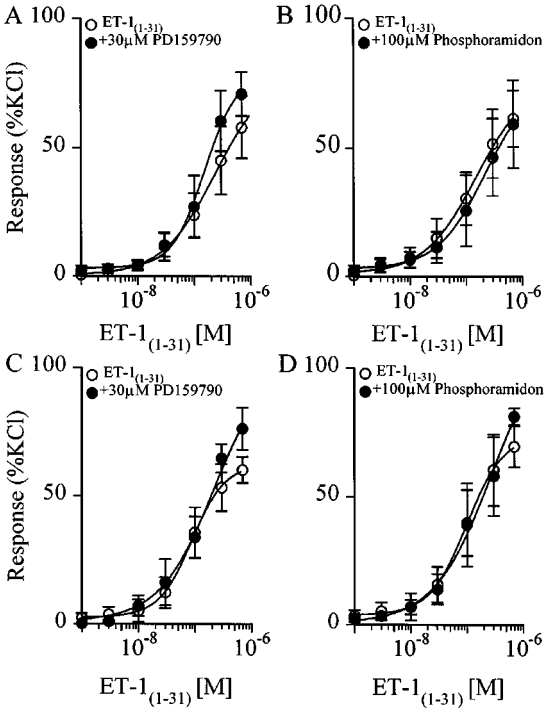

Figure 3 Cumulative concentration-response curves to ET-$1_{(1-31)}$ in the absence and presence of either 30 μM PD159790 or 100 μM phosphoramidon in human coronary artery (A, $n=4$; B, $n=5$) or internal mammary artery (C, $n=5$; D, $n=4$). Values are mean$\pm$s.e.mean. *n*-values are the number of patients from whom blood vessels were obtained.

The concentration of ET-1 required to reduce the binding of [^{125}I]-ET-1 to the antibody by 50% was 92.8 pM (95% C.I. 5.5 pM – 1.6 nM) ($n=3$) whilst that for ET-$1_{(1-31)}$ was 307.9 nM (95% C.I. 1.1 – 8.7 nM) ($n=3$) indicating cross-reactivity of ET-$1_{(1-31)}$ with the mature ET-1 radioimmunoassay of $<0.04\%$. These data indicate that at the highest concentration of ET-$1_{(1-31)}$ added to the bathing medium for the *in vitro* experiments (700 nM) the radioimmunoassay would report 0.3 ± 0.1 nM ($n=3$) mature ET present in the sample due to cross-reactivity. However levels of mature ET

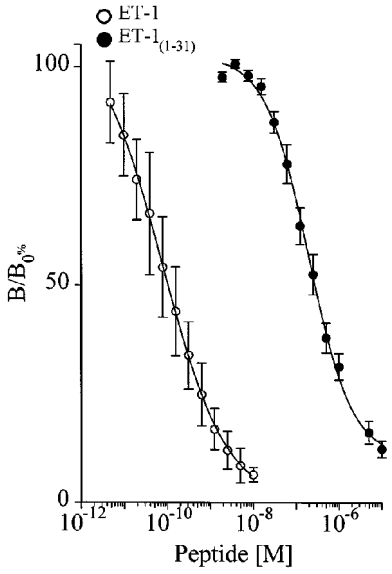

Figure 4 Competition curves for ET-1 and ET-$1_{(1-31)}$ standards in the mature ET radioimmunoassay. Cross-reactivity of ET-$1_{(1-31)}$ was $<0.04\%$. Points are mean$\pm$s.e.mean from three separate assays.

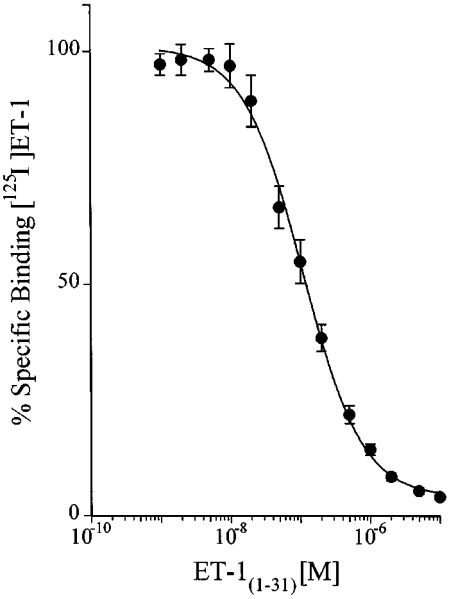

Figure 5 Inhibition of specific [^{125}I]-ET-1 binding to human heart left ventricle by increasing concentrations of ET-$1_{(1-31)}$ ($n=3$). Values are mean$\pm$s.e.mean, *n*-values are the number of patients from whom tissue was obtained.

actually detected in the bathing medium following addition of ET-$1_{(1-31)}$ were significantly greater than this at 2.1 ± 0.6 nM ($n=14$) for mammary artery and 1.6 ± 0.5 nM ($n=14$) for coronary artery ($P<0.01$ Student's 2-tailed t-test). These were comparable to levels of mature ET in the bathing medium following addition of up to 700 nM big ET-1 that were 1.9 ± 0.6 nM ($n=12$) and 2.5 ± 0.7 nM ($n=15$) respectively.

Competition experiments

The data were analysed for one, two or three site fit both individually and by pooling data files. A one-site fit model was preferred. ET-$1_{(1-31)}$ competed for specific [^{125}I]-ET-1 binding in sections of human left ventricle with a pooled K_D value of 71.6 ± 7.0 nM ($n=3$), indicating that ET-$1_{(1-31)}$ does not distinguish between the ET_A and ET_B receptors present in this tissue (Figure 5).

Discussion

We have shown that ET-$1_{(1-31)}$ has vasoconstrictor activity in human coronary and mammary artery that is comparable to that of big ET-1 but 30–40 times less than that of ET-1. The relative potency of ET-$1_{(1-31)}$ and ET-1 appears to vary with the smooth muscle preparation studied. ET-$1_{(1-31)}$ has comparable potency to ET-1 in rat trachea (Nakano *et al*., 1997; Goldie *et al*., 2000) and human umbilical artery (Takeji *et al*., 2000); is approximately 10 times less potent than ET-1 in porcine coronary artery (Kishi *et al*., 1998, Niwa *et al*., 2000) and rat aorta (Kishi *et al*., 1998) and almost 100 times less potent in rabbit pulmonary artery (Hanson *et al*., 1997). If the effect of ET-$1_{(1-31)}$ is direct, these variations may imply differences in the receptors for the two agonists. If ET-$1_{(1-31)}$ is cleaved to ET-1 for biological activity the relative potency of ET-$1_{(1-31)}$ may correlate to the activity of the converting enzyme in the different tissues. Our additional experiments attempted to clarify this observed variability.

We have previously demonstrated that the ET-mediated constrictor response in human coronary and mammary arteries is mediated predominantly *via* the ET_A receptor (Maguire & Davenport, 1995). We found that the response to ET-$1_{(1-31)}$ was blocked by the ET_A selective antagonist PD156707 (Doherty *et al*., 1995; Maguire *et al*., 1997b) implying that ET-$1_{(1-31)}$ also mediates vasoconstriction *via* this receptor subtype. In vascular smooth muscle cells ET_A receptor antagonists also inhibit ET-$1_{(1-31)}$-mediated increases in intracellular calcium signalling (Yoshizumi *et al*., 1998a; Inui *et al*., 1999; Nagata *et al*., 2000) and cell proliferation (Yoshizumi *et al*., 1998b; Nagata *et al*., 2000). To determine if ET-$1_{(1-31)}$ has selectivity for the ET_A receptor compared to the ET_B receptor we carried out competition binding assays in sections of human left ventricle that contain both receptor subtypes. ET-$1_{(1-31)}$ competed in a monophasic manner for [^{125}I]-ET-1 binding, therefore ET-$1_{(1-31)}$ was unable to distinguish between the two subtypes. This is in agreement with the recent observation that [^{125}I]-ET-$1_{(1-31)}$ binds to both BQ123-sensitive (ET_A) and sarafotoxin 6c-sensitive (ET_B) sites in rat lung (Goldie *et al*., 2000) in the presence of the protease inhibitor phenylsulphonyl fluoride. ET_B mediated functional effects of ET-$1_{(1-31)}$ have also been reported including nitric oxide production and increased intracellular Ca^{2+} levels in cultured porcine coronary artery endothelial cells (Niwa *et al*., 2000) and vasoconstrictor responses in rat aorta (Kishi *et al*., 1998).

Where the effects of peptidase inhibitors on ET-$1_{(1-31)}$ effects have been investigated it was concluded that the peptide's biological activity was not dependent on further cleavage to ET-1 (Nakano *et al*., 1997; Kido *et al*., 1998; Inui *et al*., 1999). Indeed extracts of human granulocytes show the presence of immunoreactive ET-$1_{(1-31)}$, ET-$2_{(1-31)}$ and ET-$3_{(1-31)}$ (Okishima *et al*., 1999). Only one recent investigation has shown attenuation of ET-$1_{(1-31)}$ responses by the ECE/NEP inhibitor phosphoramidon and the selective NEP inhibitor thiorphan (Hayasaki-Kajiwara *et al*., 1999) implying a role for NEP in conversion of ET-$1_{(1-31)}$ to ET-1. In agreement with initial reports, we were unable to detect any effect on ET-$1_{(1-31)}$-mediated vasoconstriction by the ECE inhibitor PD159790, the ECE/NEP inhibitor phosphoramidon or the serine protease inhibitor chymostatin. The lack of effect of PD159790 supports the observation that purified ECE from porcine aortic endothelium requires Trp21 and the carboxy-terminal sequence His27-Gly34 for enzyme activity (Ohnaka *et al*., 1993). Therefore our functional data suggested that either ET-$1_{(1-31)}$ does act directly on ET_A receptors to elicit human vascular smooth muscle contraction or it was metabolized to ET-1 by an enzyme other that ECE, NEP or serine proteases such as chymase. To confirm that ET-$1_{(1-31)}$ was acting directly without further degradation to ET-1 we determined whether mature ET was present in the bathing medium following addition of ET-$1_{(1-31)}$. We detected significant amounts of mature ET in the bathing medium that was present at comparable concentration to that obtained following addition of big ET-1. Since we know that big ET-1 must be converted to ET-1 for biological activity, this suggests that this may also be the case for ET-$1_{(1-31)}$ in these tissues. The enzyme responsible for this conversion is yet to be determined. Interestingly a recent report suggests that an endopeptidase other than ECE-1 is responsible for the conversion of big ET-1 to ET-1 in bovine pulmonary artery smooth muscle cells (Barker *et al*., 2001) and it is known that measurable levels of ET-1 remain in ECE-1 and ECE-2 knockout animals (Yanagisawa *et al*., 1998; 2000). Therefore there is likely to be a significant role for, as yet, unidentified proteases in the processing of big ET-1, and potentially ET-$1_{(1-31)}$, to ET-1.

In conclusion, we have demonstrated the ability of the novel peptide ET-$1_{(1-31)}$ to potently contract human coronary and mammary artery *via* activation of the ET_A receptor. Competition binding experiments confirm that, like ET-1, this peptide does not distinguish between human ET_A and ET_B receptors. Contrary to earlier reports we suggest that the vasoconstrictor activity of ET-$1_{(1-31)}$ in human arteries is due, at least in part, to cleavage to ET-1 by an as yet unidentified protease activity. The presence of ET-$1_{(1-31)}$ in human granulocytes may suggest that chymase generated ET-$1_{(1-31)}$ may act as an alternative paracrine precursor for ET-1 in humans.

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