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Enzymatic pathways involved in the generation of endothelin-1(1–31) from exogenous big endothelin-1 in the rabbit aorta

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1 We investigated whether blood vessels contribute to the production of ET-1(1–31) from exogenous big endothelin-1 (BigET-1) in the rabbit and assessed which enzymes are involved in this process.

2 Vascular reactivity experiments, using standard muscle bath procedures, showed that BigET-1 induces contraction in endothelium-intact rabbit aortic rings. Preincubation of the rings with phosphoramidon, CGS35066 or thiorphan reduced BigET-1-induced contraction. Conversely, chymostatin did not affect BigET-1-induced contraction.

3 Thiorphan and phosphoramidon, but not CGS35066 or chymostatin, reduced ET-1(1–31)-induced contraction. None of the enzymatic inhibitors affected the contraction afforded by ET-1.

4 BQ123-, but not BQ788-, selective antagonists for ET_A and ET_B receptors, respectively, produced concentration-dependent rightward displacements of the ET-1(1–31) and ET-1 concentration–response curves.

5 By the use of enzymatic assays, we found that the aorta, as well as the heart, lung, kidney and liver, possess a chymase-like activity.

6 Enzyme immunoassays detected significant levels of Ir-ET-1(1–31) in bathing medium of aortas after the addition of BigET-1 (30 nM). Neither thiorphan nor chymostatin altered the levels of Ir-ET-1(1–31). Conversely, the levels of Ir-ET-1(1–31) were increased in the presence of phosphoramidon. This marked increase of the 31-amino-acid peptide was abolished when phosphoramidon and chymostatin were added simultaneously.

7 The major new finding of the present work is that the rabbit aorta generates ET-1(1–31) from exogenously administered BigET-1. Additionally, by measuring the production of ET-1(1–31), we showed that a chymase-like enzyme is involved in this process when ECE and NEP are inhibited by phosphoramidon. Our results also suggest that ET-1(1–31) is an alternate intermediate in the production of ET-1 following BigET-1 administration. Finally, we showed that NEP is the predominant enzymatic pathway involved in the cleavage of ET-1(1–31) to a bioactive metabolite that will act on ET_A receptors to induce contraction in the rabbit aorta.
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Abbreviations: BigET-1, big endothelin-1; BQ123, c(DTrp–Dasp–Pro–Dval–Leu); BQ788, *N-cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl1-D-1methoxycarbonyltryptophanyl-D-norleucine; ET-1, endothelin-1; ET-1(1–31), endothelin-1(1–31)

Introduction

The potent vasoconstrictor endothelin-1 (ET-1), a-21 amino-acid peptide, was initially isolated from porcine aortic endothelial cells by Yanagisawa *et al.* (1988). This peptide is generated from the 38-amino-acid precursor, BigET-1, through cleavage of the Trp^{21}–Val^{22} bond by an ET-converting enzyme (ECE) and produces its effects *via* stimulation of two specific G-protein-coupled receptors, namely ET_A and ET_B. Additionally, other metalloproteases have been postulated to catalyze the formation of ET-1 from BigET-1, such as the neutral endopeptidase 24.11 (NEP 24.11) (Turner & Murphy, 1996).

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An alternative synthetic pathway towards the production of the vasoconstrictor ET peptides was first suggested by Patterson *et al.* (1990), who showed that cathepsin G cleaved BigET-1 to produce a novel vasoconstrictor peptide between 27 and 31 amino acids. This peptide was subsequently confirmed as BigET-1(1–31) by electrospray-mass spectrometry (Kaw *et al.*, 1992). Furthermore, it was demonstrated that incubation of BigET-1 with membrane fractions of human lungs leads to the production of ET-1(1–31) as a major hydrolysis product (Hanson *et al.*, 1997). In this tissue, the peptide was generated by chymostatin but not phosphoramidon-sensitive conversion of BigET-1 to ET-1. At the same time, it was reported that incubation of BigET-1 with purified

human connective tissue mast cell chymase resulted in the formation of ET-1(1–31) (Nakano *et al*., 1997; Kido *et al*., 1998). 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). ET-1(1–31) was also reported to selectively activate ET_A receptors (Mazzocchi *et al*., 2000; Rebuffat *et al*., 2001).

On the other hand, it was more recently shown that ET-1(1–31) must be converted to ET-1 *via* the NEP 24.11, in order to induce its pharmacological effects in the human bronchial smooth muscle *in vitro* (Hayasaki-Kajiwara *et al*., 1999). Furthermore, our laboratory showed that NEP 24.11 is the enzyme involved in the cleavage of ET-1(1–31) to a bioactive metabolite (possibly ET-1) that will act on both ET_A and ET_B receptors to induce its physiological effects on the systemic and pulmonary vasculatures of the guinea-pig (Honoré *et al*., 2002; Plante *et al*., 2002).

Interestingly, we have also recently established the pharmacological profile of the 31-amino-acid peptide *in vivo* in the rabbit (Fecteau *et al*., 2005). This model was initially used to identify an ECE-dependent production of ET-1, following intracardiac administration of BigET-1 (D'Orleans-Juste *et al*., 1990; Gratton *et al*., 2000).

We have shown that following exogenous BigET-1 administration, ET-1(1–31) is an alternate intermediate in the production of ET-1 in the rabbit *in vivo* (Fecteau *et al*., 2005). Moreover, we provided evidence that in contrast to BigET-1, the NEP, and to a lesser extent the ECE, are involved in the production of ET-1 from the 31-amino-acid intermediate, suggesting that BigET-1 and ET-1(1–31) are converted by distinct enzymatic processes *in vivo*.

Notwithstanding the above-mentioned observations, which organs/tissues are predominantly implicated in the generation of ET-1(1–31) from exogenous BigET-1 in the rabbit (or in any other species for that matter) still remains to be elucidated. Functional experiments in a variety of vascular tissues, such as the human umbilical vein (Maguire *et al*., 1997), the human placental artery (Hanson *et al*., 1998) and the rat basilar artery (Zimmermann *et al*., 2002), previously showed that the contraction induced by BigET-1 was sensitive to phosphoramidon, thus supporting a role for the vascular system in the generation of ET peptides. Interestingly, a similar role for chymase has been proposed in the processing of angiotensin I to angiotensin II in the rabbit aorta (Akasu *et al*., 1998). However, it still remains unclear as to whether chymase-generated ET peptides have significant physiological actions in the rabbit aorta.

In view of the above-mentioned considerations, we have investigated, in the present study, whether the rabbit aorta produces ET-1(1–31) from exogenously administered BigET-1. We aimed to clarify the enzymatic pathways involved in this process by using inhibitors of ECE, NEP and chymase. Also, we examined whether ET-1(1–31) directly exerts its vasoconstrictor activity and characterizes the receptors involved in the constrictor properties of this peptide in the isolated rabbit aorta.

Methods

Ethics

The care of animals and all research protocols conformed to the guiding principles for animal experimentation as enunciated by the Canadian Council on Animal Care and approved by the Ethical Committee on Animal Research of the Université de Sherbrooke Medical School.

Vascular reactivity studies

New Zealand White rabbits (1.5–2.5 kg) (Réjean Brisebois, Fleurimont, Canada) of either sex were anesthetized with ketamine/xylazine (40/10 mg kg^{-1}, intramuscularly (i.m.) and killed by spinal transection. The thoracic aorta artery was quickly removed, cleaned of adherent connective tissues and cut into 6-mm-length rings. Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (Grass Model 7) to measure tension in the vessels. The rings were placed in 5 ml organ chambers containing Krebs solution, pH 7.4, gassed with 95% O_2/5% CO_2 and maintained at 37°C. The composition of Krebs solution was as follows (mM): NaCl, 117.5; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 25.0; glucose, 5.5; $CaCl_2 \cdot 6H_2O$, 2.5. The rings were stretched until an optimal basal tension of 2.0 g was reached and were allowed to equilibrate for 90 min with the bath fluid being changed every 15–20 min, as described previously (Shetty *et al*., 1995). Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μM), in the presence of contractile tone induced by phenylephrine (0.1 μM). The ring was discarded if relaxation with acetylcholine was not 70% or greater. Following the acetylcholine test, the rings were exposed to KCl (90 mM). Agonist data were expressed as the percentage of contraction induced by KCl (%KCl).

Effects of phosphoramidon, thiorphan, CGS35066 and chymostatin on the contraction induced by BigET-1, ET-1(1–31) and ET-1

Concentration–response curves for BigET-1 (10^{-12}–10^{-7} M) were obtained in the absence (control) or presence of phosphoramidon (0.1 and 1 mM), a nonselective ECE/NEP inhibitor, thiorphan (10 and 30 μM), a selective NEP inhibitor, CGS35066 (10 and 30 μM), a selective ECE inhibitor, and chymostatin (100 μM), a nonselective inhibitor of chymase or the combination of chymostatin (100 μM) and phosphoramidon (0.1 mM). The concentration–response curves for ET-1(1–31) (10^{-12}–10^{-7} M) and ET-1 (10^{-12}–10^{-7} M) were obtained in the absence or presence of phosphoramidon (0.1 mM), thiorphan (10 μM), CGS35066 (10 μM) and chymostatin (100 μM). The inhibitors were added 30 min prior to the construction of the curve and the responses were compared with those observed in the absence of the inhibitors.

Effects of BQ123 and BQ788 on ET-1 and ET-1(1–31)-induced contraction

Both the selective ET_A (BQ123; Ihara *et al*., 1992) and ET_B (BQ788; Ishikawa *et al*., 1994) receptor antagonists were tested. BQ123 (0.3 and 1 μM) or BQ788 (0.3, 1 and 3 μM) were added 30 min prior to the construction of the concentration–response curves for ET-1 (10^{-12}–10^{-7} M) or ET-1(1–31) (10^{-12}–10^{-7} M). The responses were compared with those observed in time-matched control experiments in the absence of the antagonists.

Enzymatic assays for the determination of MCA-forming activity and chymase-like activity

Chymase-like activity was determined in the rabbit aorta, heart, lung, kidney and liver using the fluorometric assay previously described by Nakano *et al*. (1997). The organs were dissected in small pieces and placed in a potassium phosphate buffer (0.1 g/400 μl), pH 8, at 4°C and homogenized using a Polytron homogenizer at 6000 r.p.m. The homogenates were centrifuged at 14,000 × *g* for 20 min at 4°C. The pellets were discarded and the supernatant was used for the assay. The chymase activity was measured at 37°C in a 1.5 ml reaction mixture comprising 100 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-MCA, in 100 μM potassium phosphate buffer, pH 8. The reaction was terminated by adding 1 ml of stopping solution reagent (100 mM sodium monochloroacetate, 30 mM sodium acetate and 70 mM acetic acid, pH 4.3). The amount of 7-amino-4-methylcoumarin liberated from the substrate was determined fluorometrically with excitation and emission wavelengths of 370 and 460 nm, respectively, with a Cary Eclipse Varian fluorescence spectrophotometer. MCA activity was expressed as nanomoles of MCA formed per minute per milligram of protein. The chymase-like activity was calculated by subtracting the MCA formed in the absence of chymostatin from the MCA formed in the presence of chymostatin. The Bradford assay was used to determine protein concentration. In another series of experiments, the assays were carried out in the presence of phosphoramidon (100 μM) or BigET-1 (1, 10 or 100 nM). Results were analyzed as compared to a standard curve of MCA ranging from 0 to 10 μM.

Production of ET-1(1–31) by the rabbit aorta

The rings were kept in an organ chamber and stimulated with BigET-1 at 30 nM (40 min) in the absence or presence of phosphoramidon (0.1 mM), chymostatin (100 μM), thiorphan (10 μM) or the association of phosphoramidon (0.1 mM) and chymostatin (100 μM). The stimulation with BigET-1 was performed for 40 min, since we observed that at the concentration of 30 nM, this was the period necessary for the peptide to induce its maximal contractile effect. The bath contents were collected and stored at −80°C for the subsequent measurement of ET-1(1–31) levels. In another set of experiments, the rings were not incubated with BigET-1 and the bath contents were collected for the determination of the basal levels of ET-1(1–31) and passed through a syringe-driven filter 0.22 μm (Millex GP). Eluates were finally evaporated using a Savant Speed Vac Concentrator before further analysis.

High-performance liquid chromatography (HPLC) and enzyme immunoassay analysis

The HPLC analysis was performed as described previously (Fecteau *et al*., 2005). In brief, desiccated residues were reconstituted in 1 ml of 10% acetonitrile/1% TFA for HPLC analysis. A reversed-phase HPLC was performed using a Zorbax 3000SB-C18 column (Agilent Technologies). Samples were eluted with a 35-min linear gradient of acetonitrile (from 28 to 40%) in 0.1% TFA at a flow rate of 1 ml min^{-1}, using a pump (Waters, Model 510). Thirty-second fractions (0.5 ml) were separated in two for subsequent determination of ET-1(1–31) levels, evaporated to dryness and analyzed with an EIA kit (Immuno-Biological Laboratories, Japan). The fraction collected at 37 min of elution time was used to determine ET-1(1–31) contents, since it had been previously shown to correspond to the optimal retention time of the authentic ET-1(1–31) standard (Fecteau *et al*., 2005).

Drugs and solutions

ET-1, ET-1(1–31), BigET-1, phosphoramidon and Suc-Leu-Leu-Val-Tyr-MCA were purchased from Peptide International (Louisville, KY, U.S.A.); chymostatin, phenylephrine, acetylcholine and thiorphan from Sigma Co. (Oakville, Ontario, Canada). BQ123 and BQ788 were synthesized by Dr Witold Neugebauer, as described previously (Brosseau *et al*., 2005). CGS35066 was supplied by Dr Arco Jeng (Novartis, Nuttley, NJ, U.S.A.). Drugs were dissolved in phosphate-buffered saline (PBS) (pH 7.4), except for BQ123, BQ788, thiorphan, chymostatin and ET-1(1–31), which were diluted in 10% dimethyl sulfoxide (DMSO) and subsequently in PBS; CGS35066 was diluted in 0.25 M of $NaHCO_3$/PBS. The bath concentration of DMSO did not exceed 0.5%, which was shown to have no effect *per se* on the basal tonus of the preparations or on the agonist-mediated contraction.

Data analysis

Contractions were recorded as changes in the displacement (in grams) from baseline and expressed as a percentage of contraction induced by KCl (90 mM) (%KCl). Agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 2.01; GraphPad Software Inc., San Diego, CA, U.S.A.). Agonist potencies and maximum response are expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max} (maximum effect elicited by the agonist), respectively. Statistically significant differences were calculated by one-way analysis of variance (ANOVA followed by Bonferroni's multiple comparison test), as indicated in the text, tables and legends. $P<0.05$ was considered as statistically significant.

Results

Vascular reactivity studies

BigET-1 and ET-1(1–31) concentration-dependently constricted rabbit aortas with similar pD_2 values of 7.92 ± 0.13 ($n=7$) and 7.70 ± 0.16 ($n=8$), respectively. However, these peptides were significantly less potent than ET-1 (pD_2: 9.07 ± 0.15; $n=5$) ($P<0.05$; ANOVA) (Figures 1 and 2). Furthermore, maximal responses afforded by these three peptides in rabbit aortas were not significantly different (BigET-1: 106.26 ± 7.61%; ET-1(1–31): 109.48 ± 7.32%; ET-1: 107.41 ± 5.78%).

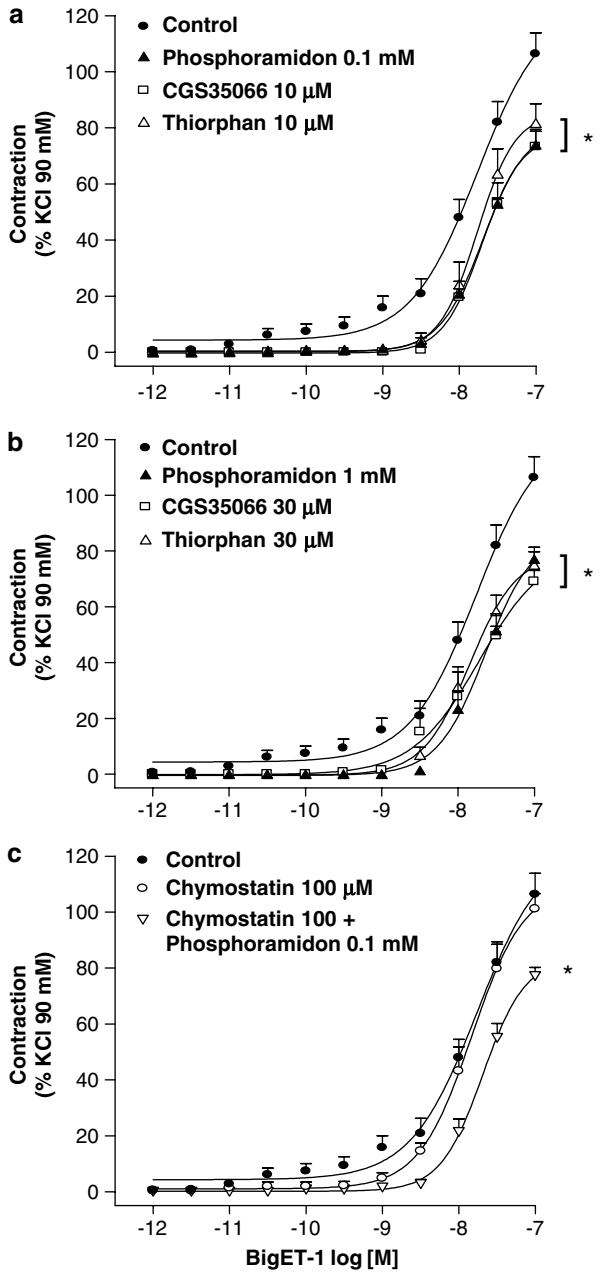

Figure 1 Concentration–response curves for BigET-1 obtained in endothelium-intact rabbit aortic rings. (a) Effect of phosphoramidon (0.1 mM), thiorphan (10 μM) or CGS35066 (10 μM) on BigET-1-induced contraction. (b) Effect of phosphoramidon (1 mM), thiorphan (30 μM) or CGS35066 (30 μM) on BigET-1-induced contraction. (c) Effect of chymostatin (100 μM) or the association of phosphoramidon (0.1 mM) and chymostatin (100 μM) on BigET-1-induced contraction. The rings were preincubated with the drugs for 30 min. Values are means $\pm$ s.e.m. of five to eight independent preparations. *$P<0.05$ when compared to control curves of agonists + vehicle or in c) agonist + chymostatin (ANOVA followed by Bonferroni's comparison test).

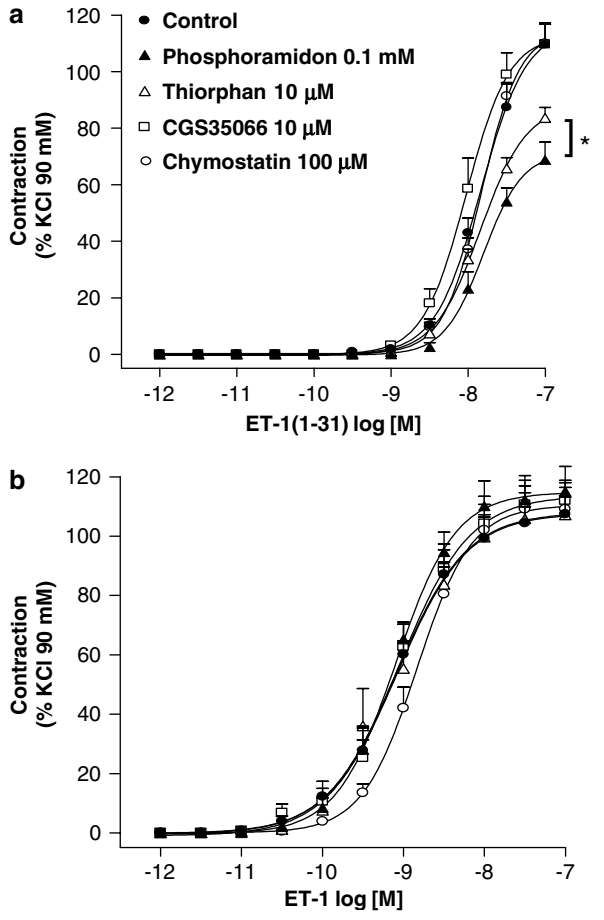

Figure 2 Concentration–response curves for ET-1(1–31) (a) and ET-1 (b) obtained in endothelium-intact rabbit aortic rings. The concentration–response curves were obtained in the absence (control) or in the presence of phosphoramidon (0.1 mM), thiorphan (10 μM), CGS35066 (10 μM) or chymostatin (100 μM). The rings were preincubated with the drugs for 30 min. Values are means $\pm$ s.e.m. of five to eight independent preparations. *$P<0.05$ when compared to control curves of agonists + vehicle (ANOVA followed by Bonferroni's comparison test).

Contribution of NEP, ECE or chymase in the aortic responses to ET peptides

Table 1 shows that phosphoramidon at 0.1 mM produced a three-fold rightward displacement of the apparent affinity of BigET-1 and reduced its maximal contractile response ($P<0.05$; ANOVA). A further increase in the concentration of phosporamidon (1 mM) did not have additional inhibitory effect on the response to BigET-1 (+phosphoramidon: E_{max}, 73.10 $\pm$ 1.88%; pD_2, 7.38 $\pm$ 0.20, $n=5$).

The E_{max} but not the pD_2 values for BigET-1 were reduced by the ECE inhibitor CGS35066 by 25% at 10 (Table 1) and more than 30% at 30 μM (E_{max}: 69.11 $\pm$ 3.97%; pD_2: 7.74 $\pm$ 0.30, $n=5$). Furthermore, the NEP inhibitor thiorphan reduced by close to 20% at 10 (Table 1) and 25% at 30 μM (E_{max}: 75.05 $\pm$ 4.71%; pD_2: 7.65 $\pm$ 0.12, $n=6$), the maximal response to the same agonist ($P<0.05$; ANOVA).

Finally, albeit chymostatin (100 μM) did not affect BigET-1-induced contraction *per se,* the combination of the chymase inhibitor with phosphoramidon (0.1 mM) reduced the response of the 38-amino-acid precursor to the same extent as when the later inhibitor is administered alone (Table 1).

On the other hand, the E_{max} but not pD_2 values for ET-1(1–31) were reduced by 32% by phosphoramidon at 0.1 mM and 17% by thiorphan at 10 μM. Conversely, neither E_{max} nor the

Table 1 E_{max} (%KCl) and pD_2 values for BigET-1 and ET-1(1–31) in endothelium intact rabbit aorta

Groups	*BigET-1*		*ET-1(1–31)*	
	E_{max}	pD_2	E_{max}	pD_2
Control	106.26±7.61	7.92±0.13	109.48±7.32	7.70±0.16
Phosphoramidon 0.1 mM	74.00±5.38[a]	7.46±0.10[a]	68.82±6.26[a]	7.80±0.08
Thiorphan 10 μM	81.67±6.89[a]	7.62±0.09	83.66±3.72[a]	7.84±0.07
CGS35066 10 μM	73.20±5.54[a]	7.77±0.08	109.67±7.67	8.00±0.09
Chymostatin 100 μM	96.28±6.54	7.75±0.12	110.00±6.88	7.84±0.05
Phosphoramidon 0.1 mM + chymostatin 100 μM	77.13±3.04[a]	7.44±0.12[a]	—	—

Values are means±s.e.m. of five to eight independent experiments.
[a]Compared to control group ($P<0.05$; ANOVA followed by Bonferroni's comparison test).

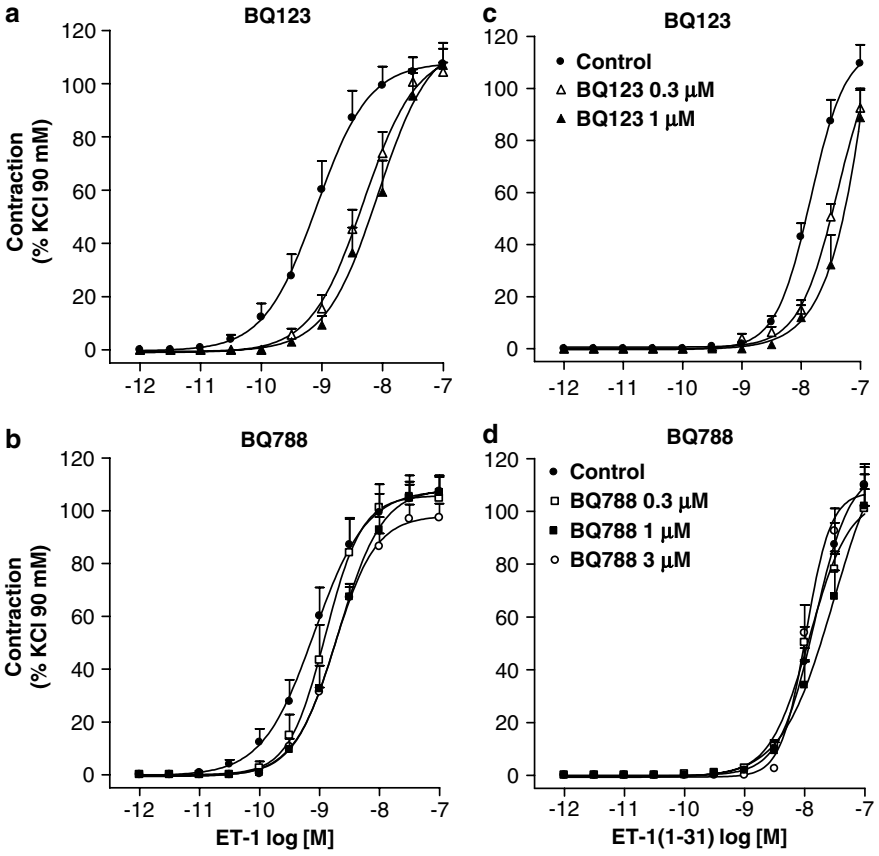

Figure 3 Effect of BQ123 and BQ788 on ET-1 and ET-1(1–31)-induced contraction of endothelium-intact rabbit aortic rings. The curves for ET-1 and ET-1(1–31) were obtained in the absence (control) or presence of BQ123 (a, c) or BQ788 (b, d). Values are means±s.e.m. of five to six independent preparations.

pD_2 values for ET-1(1–31) were altered by CGS35066 at 10 μM or chymostatin at 100 μM (Figure 2, Table 1).

In last series of control experiments, ET-1-induced responses were not affected significantly by phosphoramidon at 0.1 mM, thiorphan at 10 μM, CGS35066 at 10 μM nor by chymostatin at 100 μM (Figure 2).

Effect of ET_A and ET_B antagonists on ET-1 and ET-1(1–31)-induced contraction

The ET_A antagonist BQ123 at 0.3 and 1 μM produced rightward displacements (four- and nine-fold, respectively) of the ET-1(1–31) concentration–response curves, with no reduction of the maximal response. On the other hand, BQ788 at 0.3, 1 or 3 μM did not affect the contraction induced by ET-1(1–31) (Figure 3, Table 2).

In addition, BQ123 concentration-dependently shifted to the right (by 0.75 and 0.9 log units, respectively), the apparent affinity of ET-1 (+vehicle: 9.05±0.15; +BQ-123 (0.3 μM): 8.30±0.13; +BQ-123 (1 μM): 8.15±0.12, $n=5$–6, $P<0.05$ when compared to control; ANOVA), with no reduction of the maximal response (Figure 3). Conversely, BQ788 at concentrations up to 3 μM did not affect the ET-1 apparent affinity or maximal effect on the rabbit aortic strips (Figure 3, Table 2).

Table 2 Effect of BQ123 or BQ788 on the E_{max} (%KCl) and pD_2 values for ET-1(1–31) in endothelium intact rabbit aorta

Antagonist	E_{max}	pD_2	n
Vehicle	109.48±7.32	7.70±0.16	6
BQ123 (0.3 μM)	92.44±6.95	7.10±0.07[a]	5
BQ123 (1 μM)	88.77±11.23	6.92±0.20[a]	6
BQ788 (0.3 μM)	100.94±13.13	7.90±0.07	5
BQ788 (1 μM)	103.62±8.20	7.58±0.17	6
BQ788 (3 μM)	110.01±7.87	7.74±0.10	5

Values are means±s.e.m. of *n* independent experiments.
[a]Compared to control group ($P<0.05$; ANOVA followed by Bonferroni's comparison test).

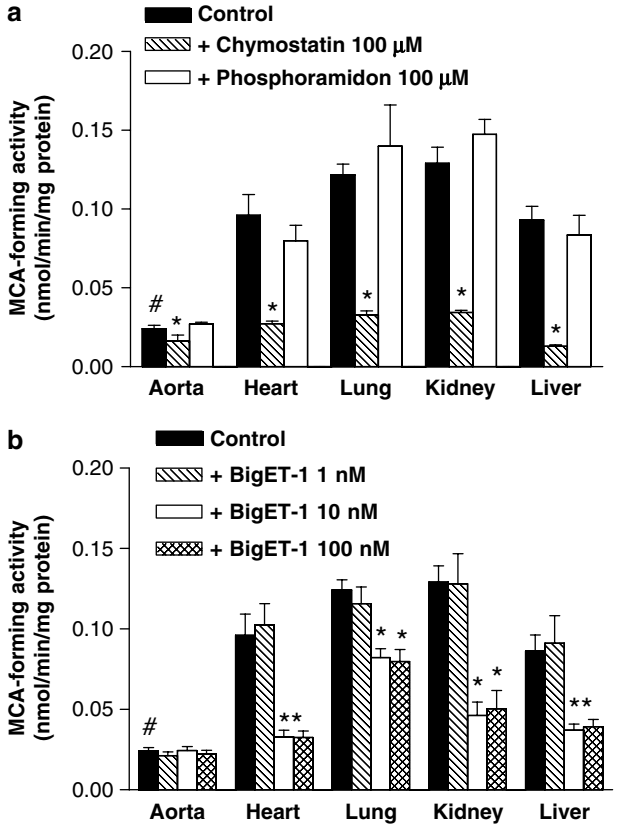

Figure 4 Total MCA-forming activities in the rabbit aorta, heart, lung, kidney and liver. The MCA-forming activity was obtained in the absence (control) or presence of chymostatin or phosphoramidon (a) and BigET-1 (1, 10 and 100 nM) (b). *Compared to respective control group; #compared to heart, lung, kidney and liver ($P<0.05$; ANOVA followed by Bonferroni's comparison test). Values are means±s.e.m. of five to eight independent experiments.

Comparison of MCA-forming activity and chymase-like activity

The rabbit aorta, heart, lung, kidney and liver were analyzed for their MCA-forming activity. Chymostatin, but not phosphoramidon, significantly reduced MCA-forming activity in all tissues (Figure 4a). Interestingly, BigET-1 at 10 and 100 nM significantly reduced MCA-forming activity in the heart, lung, kidney and liver, but not in the aorta (Figure 4b). In another series of experiments, we observed that the rabbit aorta, heart, lung, kidney and liver presented chymase-like activity. However, this activity was reduced in the aorta when compared to the other tissues (Figure 5).

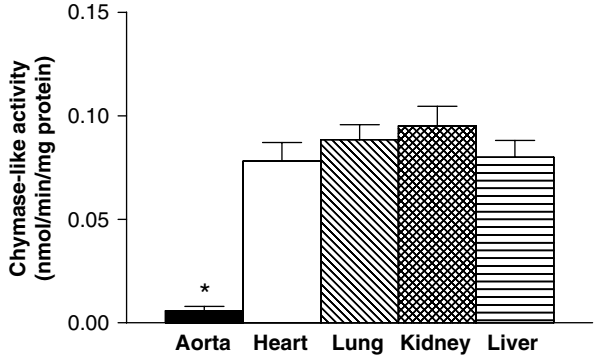

Figure 5 Comparison of chymase-like activity in the rabbit aorta, heart, lung, kidney and liver. *Compared to heart, lung, kidney and liver ($P<0.05$; ANOVA followed by Bonferroni's comparison test). Values are means±s.e.m. of six to eight independent experiments.

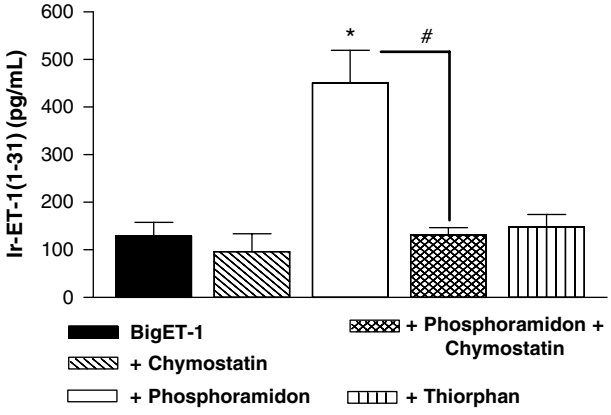

Figure 6 Bath content of Ir-ET-1(1–31) after the administration of BigET-1 (30 nM). Ir-ET-1(1–31) was determined in the organ bath content after the administration of BigET-1 in the absence (control) or presence of phosphoramidon (0.1 mM), chymostatin (100 μM), thiorphan (10 μM) or the association of phosphoramidon (0.1 mM) and chymostatin (100 μM). *Compared to BigET-1 ($P<0.05$; ANOVA followed by Bonferroni's comparison test). #$P<0.05$. Values are means±s.e.m. of at least six independent experiments.

Production of ET-1(1–31) by the rabbit aorta

Low Ir-ET-1(1–31) levels were detected in the bathing medium when the tissues were not exposed to BigET-1 in four out of six vessels (17.22±8.36 pg ml^{-1}). The addition of BigET-1 (30 nM) to the organ bath induced an increase in the levels of Ir-ET-1(1–31) (Figure 6). Phosphoramidon (100 μM), administered prior to BigET-1, induced a four-fold increase in Ir-ET-1(1–31) levels when compared to tissues incubated with BigET-1 alone. On the other hand, neither chymostatin (100 μM) nor thiorphan (10 μM) altered Ir-ET-1(1–31) levels after the administration of BigET-1. However, when added in association with phosphoramidon, chymostatin prevented the increase in Ir-ET-1(1–31) levels induced by phosphoramidon (Figure 6).

Discussion

The present study shows for the first time that the vascular system of the rabbit can generate ET-1(1–31) from exogenous-administered BigET-1. We also confirm a chymase-like activity in the rabbit aorta. Additionally, by measuring the production of ET-1(1–31), we provided evidence that the generation of the 31-amino-acid peptide involves a chymase-dependent process when ECE and NEP are blocked by phosphoramidon.

Our results show that BigET-1 was a less potent constrictor than ET-1 in the rabbit aorta, as previously described in other vascular tissues (Howard *et al*., 1992; Maguire *et al*., 1997). It is well established that BigET-1 must be converted to ET-1 to exert its biological activity. Accordingly, phosphoramidon, a nonselective ECE/NEP inhibitor, reduces the contraction induced by BigET-1 in several vascular tissues (Maguire *et al*., 1997; Hanson *et al*., 1998) as well as the levels of mature ET-1 in the bathing medium after addition of the 38 amino acids precursor (Maguire *et al*., 1997). Our results show that phosphoramidon produced rightward displacements of the BigET-1 concentration–response curves with reduction of the maximal response, further indicating that this peptide must be converted to exert its biological activity. In addition, both CGS35066, a selective ECE inhibitor, and thiorphan, a selective NEP inhibitor, reduced the BigET-1-induced contraction, thus confirming a role for both ECE and NEP in the generation of biologically active ET-1 from BigET-1 in the rabbit aorta. Our data obtained *in vitro* with phosphoramidon, CGS35066 and thiorphan are in line with results obtained in our laboratory in the rabbit *in vivo*, in which the pressor response afforded by BigET-1 was significantly attenuated by these inhibitors (Fecteau *et al*., 2005). Indeed, we have suggested, in this later study, that BigET-1 is converted directly, predominantly by the ECE and to a lesser extent by the NEP through the processing of the intermediate ET-1(1–31), following intracardiac administration of the 38-amino-acid precursor in the rabbit *in vivo*.

The current findings also show that ET-1(1–31) and BigET-1 induce contraction of the rabbit aorta with similar potency, albeit both less potently than ET-1. The relative potencies of ET-1(1–31) and ET-1 appear to vary, depending on the smooth muscle preparation studied. ET-1(1–31) has comparable potency to ET-1 in the rat trachea (Nakano *et al*., 1997) and the human umbilical artery (Takeji *et al*., 2000). However, this peptide is approximately 10 times less potent than ET-1 in the porcine coronary artery (Kishi *et al*., 1998) and almost 100 times less potent in the rabbit pulmonary artery (Hanson *et al*., 1997). These variations in potency of ET-1(1–31) and BigET-1 could be correlated to differences in the activity or expression of ECE or NEP in the various tissues.

On the other hand, earlier studies suggested that the biological activity of ET-1(1–31) was not dependent on further cleavage to ET-1 (Nakano *et al*., 1997; Kido *et al*., 1998; Inui *et al*., 1999). Later, it was found that the ECE/NEP inhibitor, phosphoramidon and the selective NEP inhibitor, thiorphan, attenuated the biological responses induced by ET-1(1–31) (Hayasaki-Kajiwara *et al*., 1999; De Campo *et al*., 2002; Honoré *et al*., 2002; Plante *et al*., 2002), therefore implying a role for NEP in the conversion of ET-1(1–31) to ET-1. In agreement with the later reports, the present study shows that ET-1(1–31)-induced contraction was inhibited by phosphoramidon and thiorphan, but not by CGS35066 or chymostatin.

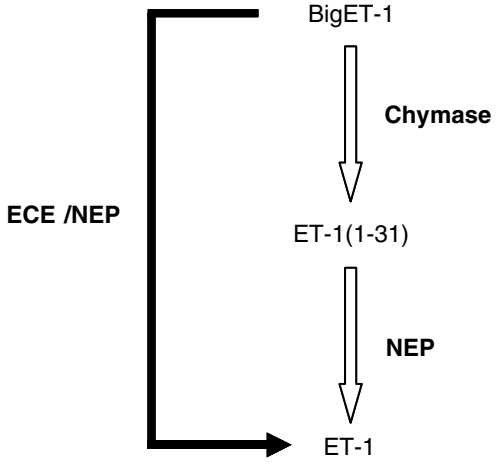

Figure 7 Schematic representation of the enzymatic pathways involved in the production of ET peptides. The closed arrow indicates the main pathway implicated in the production of ET-1 from BigET-1 by ECE and NEP. The opened arrows indicate the alternative pathway involved in the production of ET-1. ET-1(1–31) is an intermediate peptide in the production of ET-1 from BigET-1. The inhibition of the main pathways (i.e. ECE and NEP) with phosphoramidon unmasks the production of ET-1(1–31). ET-1, endothelin-1; BigET-1, big endothelin-1; ET-1(1–31), endothelin-1(1–31); ECE, endothelin-converting enzyme; NEP, neutral endopeptidase.

Furthermore, we found that the selective antagonist for ET_A receptors, BQ123, but not the selective antagonist for ET_B receptors, BQ788, produced a rightward displacement of ET-1(1–31) and ET-1 curves. Taken together, these results indicate that NEP is the predominant enzymatic pathway involved in the cleavage of ET-1(1–31) to ET-1, which subsequently acts on ET_A receptors to induce contraction in the rabbit aorta.

We have also investigated whether a chymostatin-sensitive enzyme, possibly present in the rabbit aorta, converts exogenous BigET-1 to biologically active fragments. In the present study, a chymostatin-sensitive contractile response induced by BigET-1 was not observed. Our data corroborate previous findings in the murine (De Campo *et al*., 2002) and primate (Takai *et al*., 1998) tracheas, in which chymostatin did not affect BigET-1-induced contraction. Additionally, we found that the association of phosphoramidon and chymostatin did not produce any additional effect when compared to phosphoramidon alone. A possible explanation for this observation is that the conversion of BigET-1 to ET-1 by ECE and NEP is the main pathway involved in the production of ET-1 in the rabbit aorta (Figure 7).

Interestingly, by using enzymatic assays, we identified a chymase-like in the rabbit aorta. The MCA-forming activity, which is the result of the hydrolysis of the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-MCA, was significantly inhibited by chymostatin in this tissue. On the contrary, phosphoramidon did not affect MCA-forming activity, further confirming the selectivity of a chymotrypsin-type protease (probably chymase) for this substrate. Additionally, we found that MCA-forming activity was reduced in the presence of BigET-1, indicating that chymase can also cleave BigET-1, as previously described in human tissues (Hanson *et al*., 1997; Nakano *et al*., 1997). However, it is important to note that the chymase-like enzymatic activity in the aorta was lower than in

the heart, lung, kidney and liver, thus suggesting that this particular serine protease may have a lesser physiological role in this tissue. Our data thus corroborate previous findings, namely that chymase-like angiotensin II-forming activity is low in the rabbit aorta when compared to the heart and lung (Akasu *et al*., 1998). In support of this later hypothesis, Craig & Schwartz (1989) described that there are considerable differences in the density and subclass of mast cells in the human lungs, heart and alimentary tract. Thus, the distribution or subclass of mast cells in each tissue could be a possible explanation for the differences in tissue chymase-like activity observed. Unfortunately, there has been no systematic study of the tissue distribution of chymase mRNA and immunoreactivity in rabbits.

Low yet detectable Ir-ET-1(1–31) levels were measured in the bathing medium when the tissues were incubated without BigET-1, suggesting that the former peptide is produced by the rabbit aorta. We also observed an increased production of ET-1(1–31) after administration of BigET-1 to the organ bath, indicating that the rabbit aorta also produces ET-1(1–31) from exogenously administered BigET-1. On the other hand, the levels of Ir-ET-1(1–31) in the bathing medium were increased when BigET-1 was added after incubation of the tissues with phosphoramidon. These results show that the inhibition of ECE/NEP facilitates the production of ET-1(1–31), further suggesting that the direct production of ET-1 from the 38-amino-acid precursor, BigET-1 is the predominant pathway (Figure 7). These results are in line with our *in vivo* study, where a dynamic increase of plasma ET-1(1–31) levels following administration of BigET-1 was observed only under conditions of phosphoramidon treatment (Fecteau *et al*., 2005). Taken together, these results suggest that ET-1(1–31) is an alternate intermediate in the production of ET-1 following BigET-1 administration. Our data also support a role for chymase in this mechanism. In physiological situations however, the production of ET-1(1–31) by chymase in the aorta is not the main pathway involved in the generation of ET. In support of this notion, the present study also showed that BigET-1 triggers a chymostatin-insensitive contraction of aortas. This state of event suggests that chymase-containing rabbit aorta does not generate sufficiently high levels of ET-1(1–31) to trigger contraction, notwithstanding the fact that detectable levels of this peptide were measured in our biochemical assay. Whether the same postulate holds true in conditions where the number of mast cells and chymase activity are increased, such as those found in human abdominal aortic aneurysms (Nishimoto *et al*., 2002; Tsunemi *et al*., 2002), remains to be determined. Also, it is important to remember that the chymase-like enzymatic activity in the aorta was lower than in the heart, lung, kidney and liver. This fact suggests that this enzyme has a greater importance in the production of ET-1(1–31) in these latter organs.

In conclusion, the current findings show that the rabbit aorta contributes to the conversion of exogenous-applied BigET-1 to ET-1(1–31), which is generated in the aorta by steps that involve the participation of a chymase-like enzyme when ECE and NEP, the main enzymes involved in the production of ET-1 from BigET-1, are inhibited. In addition, the NEP is the predominant enzymatic pathway involved in the cleavage of ET-1(1–31) to ET-1, which subsequently acts on ET_A receptors to induce contraction in the rabbit aorta.

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