RESEARCH COMMUNICATION
Novel activity of endothelin-converting enzyme: hydrolysis of bradykinin

Mien V. HOANG and Anthony J. TURNER
School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Endothelin-converting enzyme (ECE) is the key enzyme in the production of the potent vasoconstrictor endothelin from its inactive precursor big endothelin. To date, no other physiological peptide substrate has been identified for ECE. Here, by using Chinese hamster ovary (CHO) cells transfected with rat ECE-1 cDNA, we have established that ECE can hydrolyse the vaso-dilator bradykinin. The hydrolysis of bradykinin by ECE is exclusively at the Pro^2–Phe^3 bond, producing bradykinin-(1–7) and bradykinin-(8–9). Hydrolysis is completely inhibited by 100 µM phosphoramidon and 200 µM EDTA, but only slightly by the specific neprilysin inhibitor thiorphan (100 µM). The ability of ECE to act as a peptidyl dipeptidase rather than an endopeptidase in hydrolysing bradykinin suggests a much broader specificity for the enzyme than previously recognized, which may lead to the design of new and specific inhibitors of ECE and to the identification of other potential physiological substrates.

INTRODUCTION

Endothelin-1 (ET-1) is a 21-amino-acid peptide originally identified as the most potent vasoconstrictor peptide produced by endothelial cells [1]. Three separate endothelin genes have been identified encoding three distinct endothelin peptides (ET-1, -2 and -3) [2]. Elevation of endothelin levels has been implicated in the pathogenesis of several diseases, including atherosclerosis [3], heart failure [4], fibrotic diseases [5] and pulmonary hypertension [6]. ET-1 is initially synthesized as a large precursor polypeptide (proendothelin-1), the processing of which occurs in two steps: firstly, cleavage in the constitutive secretory pathway by furin [7], with cleavage at the Pro^21–Val^22 bond by endothelin-converting enzyme (ECE) to generate ET-1 and its C-terminal fragment (see [8] for a review). Two ECE genes have been cloned (ECE-1 and ECE-2): both of the proteins show sequence similarity to neutral endopeptidase-24.11 (NEP; neprilysin, EC. 3.4.24.11) and with KELL and PEX proteins [9]. All contain the zinc-binding motif HExxH and are members of a subfamily of the M13 gluzincin peptidase family.

No other peptide substrate has yet been identified for ECE, except for some truncated sequences of big ET itself [10]. In the present study, we have examined various vasoregulatory peptides as possible ECE substrates, including ET-1, atrial natriuretic peptide (ANP), angiotensins I and II, and bradykinin (BK). Using Chinese hamster ovary (CHO) cells transfected with rat ECE-1 cDNA, we have shown that ECE-1 can hydrolyse BK with cleavage at the Pro^2–Phe^3 bond. ECE does not hydrolyse ET-1, consistent with previous observations [11]. No significant hydrolysis of ANP and angiotensins I and II, over background levels, was detected.

EXPERIMENTAL

Materials

CHO cells were from the European Collection of Cell Culture (Salisbury, Wiltshire, U.K.) Trypsin/EDTA, penicillin, streptomycin, non-essential amino acids, foetal-calf serum and transfection reagent (lipofectAmine) were purchased from Gibco–BRL (Paisley, U.K.). Phosphoramidon was obtained from the Peptide Institute (Osaka, Japan). The thimet oligopeptidase inhibitor N-[1-(R,S)-carboxy-3-phenylpropyl]Ala-Ala-Phe-4-aminobenzoate (Cpp-AAF-4ab) was donated by Dr. A. I. Smith (Baker Medical Research Institute, Melbourne, Australia). The plasmid pcDL-SR296/ETE (containing rat ECE-1 cDNA) and monoclonal antibody AEC27-121 were gifts from Dr. K. Tanzawa (Sankyo, Tokyo, Japan) [12]. The peptides big ET-1, ET-1 and the C-terminal fragment of big ET-1 were obtained from Cambridge Research Biochemicals (Northwich, Cheshire, U.K.). The synthetic peptide substrate for ECE, [Phe^9][big ET-1-(18–34)] [13], was synthesized by the Multiple Sclerosis Peptide Laboratory (Oxford Brookes University, Oxford, U.K.). The enhanced chemiluminescence (ECL) detection kit was from Amersham International (Amersham, Bucks., U.K.). BK, BK-(1–7), ANP, angiotensin I, angiotensin II, Glasgow minimal essential medium, nucleosides, sodium pyruvate, phenylalanine and the neprilysin inhibitor thiorphan were purchased from Sigma (Poole, Dorset, U.K.). Phe-Arg was synthesized by Dr. J. Keen (University of Leeds, Leeds, U.K.). All other reagents were of analytical grade.

CHO cells were cultured in Glasgow minimal essential medium, supplemented with 10% (v/v) foetal-calf serum (heat inactivated), 2 mM nucleosides, 10 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine/asparagine at 37°C in 5% CO₂. The expression vector pcDL-SR296/ETE was used to transfect CHO cells. For transient expression, CHO cells were plated in 150 cm² flasks at approx. 2 x 10⁶ cells. After 24 h growth, the cells were washed twice with Opti Mem and transfected (5 µg of DNA/flask) by using lipofectAmine as cationic lipid (DNA/lipid, 1:10, w/w). The cells were incubated for 3 h before adding Glasgow minimal essential medium containing 10% (v/v) foetal calf serum. At a time of

Abbreviations used: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; BK, bradykinin; CHO, Chinese hamster ovary; Cpp-AAF-4ab, N-[1-(R,S)-carboxy-3-phenylpropyl]Ala-Ala-Phe-4-aminobenzoate; ECE, endothelin-converting enzyme; ECL, enhanced chemiluminescence; ET, endothelin; NEP, neutral endopeptidase-24.11 (neprilysin).

¹ To whom correspondence should be addressed.
24 h after transfection, the medium was aspirated and the cells were replaced with fresh medium. After a further 24 h, the cells were harvested and membranes were prepared as previously described [14]. Isolated membranes were solubilized with 1% (w/v) Triton X-100, then subjected to Western blotting or enzyme assay. Protein concentrations were determined using the bicinchoninic acid method [15].

**SDS/PAGE and immunoblotting**

PAGE was performed as described in [16], using a 5% stacking and a 7.5% separating gel. The gels were blotted [17] using a semi-dry blot, and the detection of protein was performed by using ECL (ECL kit, Amersham). Pre-stained molecular-mass standards were run in parallel.

**Hydrolysis of vasoregulatory peptides by ECE-1**

For the detection of peptide hydrolysis by rat ECE-1, solubilized transfected and non-transfected CHO cell membranes (10 µg of total protein) were preincubated in a total volume of 100 µl for 30 min in 100 mM Tris/HCl buffer, pH 7.0, containing CppAAF-4ab to inhibit low levels of endogenous thimet oligopeptidase activity. Appropriate reaction mixtures contained 100 µM phosphoramidon to check the specificity of hydrolysis. The reaction was started by adding BK to 250 µM final concentration. After incubation for 30 min at 37 °C, samples were heated to 100 °C for 4 min and then centrifuged at 2000 g for 20 min at 4 °C. A similar procedure was employed for investigating the possible hydrolysis of angiotensin I, II and ANP. For the determination of $K_m$ and $V_{max}$, the BK concentration was in the range 0.1–2.0 mM, and for determining the $I_{50}$ of various inhibitors, the BK concentration was 0.25 mM. Reaction conditions were maintained such that hydrolysis was linear with respect to time and protein concentration. The assay of ECE activity when using big ET-1 or [Phe$^5$]big ET-1-(18–34) as substrate, and their product quantification, was as described in [13].

**HPLC quantification of BK, BK-(1–7) and BK-(8–9)**

BK, BK-(1–7) and BK-(8–9) (Phe-Arg) were separated on a reverse-phase µBondapak C$_{18}$ column, using trifluoroacetic acid/acetonitrile/water as the mobile phase. All the separations were carried out at room temperature at a flow rate of 1.5 ml/min. Solvent A consisted of 0.02% (v/v) trifluoroacetic acid in water, and solvent B consisted of 0.02% (v/v) trifluoroacetic acid and 45% (v/v) acetonitrile in water. Both solvents were filtered and degassed before use. BK, BK-(1–7) and BK-(8–9) were resolved and quantified by HPLC using a linear gradient of acetonitrile from 9 to 91% in 0.02% (v/v) trifluoroacetic acid, pH 2.5, for 20 min, followed by a 5 min wash at final conditions. Product detection was at 214 nm. The BK-(1–7) was used for kinetic quantification and was calibrated against an authentic standard. Similar procedures were used for the separation of ANP, angiotensins I and II, ET-1 and their products.

**RESULTS AND DISCUSSION**

**Hydrolysis of BK by ECE**

Immunoblotting of a membrane preparation (Figure 1) from ECE-1-transfected (lane 1) and mock-transfected (lane 2) CHO cells indicated that ECE-1 protein is only detected in the ECE-1-transfected cells. Incubation of ECE-1-transfected membranes with 250 µM BK for 30 min, followed by HPLC separation, revealed extensive hydrolysis of the peptide (Figure 2b). The primary attack on this peptide was at the Pro$^5$–Phe$^6$ bond,
producing BK-(1–7) and BK-(8–9). The products were identified by co-elution with synthetic peptide, MS and amino acid composition (results not shown). No BK hydrolysis was detected in membranes from mock-transfected cells (Figure 2a). The hydrolysis of BK was almost completely inhibited by 100 µM phosphoramidon (Figure 2c) but only slightly by 100 µM thiorphan (Figure 2d). Using BK as substrate, phosphoramidon exhibits an I₅₀ of 2.5 µM and thiorphan of > 200 µM (Figure 3), consistent with previous studies of ECE using big ET-1 as substrate [12]. Similar inhibition curves are observed when using big ET-1 or the big ET-1 analogue, [Phe²²]big ET-1-(18–34), as substrate (results not shown). Thus ECE hydrolyses BK at the Pro²²–Phe²³ bond, and therefore acts as a peptidyl dipeptidase in addition to its endopeptidase action on big ET-1. No hydrolysis of ET-1 was observed with ECE-1-transfected membranes on prolonged incubation up to 16 h, which is consistent with previous observations [11]. No significant hydrolysis of ANP or angiotensin I or II over background levels was detected (results not shown).

Hydrolysis of BK-(8–9) (Phe-Arg) by CHO cells

The BK-(8–9) peak (Figure 2b, peak 3) was also found to contain some free Phe, due to further hydrolysis of the Phe-Arg. This was not due to ECE activity, since it was not inhibitable by 100 µM phosphoramidon. Non-transfected CHO cells hydrolysed 100 µM Phe-Arg to the same extent as transfected cells, indicating an endogenous Phe-Arg-hydrolysing activity. This activity was inhibited by 100 µM EDTA but not by amastatin, bestatin, cilastatin or guanidinoethylmercaptoacetic acid (inhibitors, respectively, of aminopeptidases, membrane dipeptidase and carboxypeptidase B).

Kinetic analysis of peptide hydrolysis by ECE-1

Table 1 shows kinetic comparisons of ECE, using three different substrates. The Kₘ for BK approaches 1 mM, several orders of magnitude higher than for circulating concentrations of the peptide. However, the Vₘₐₓ/Kₘ values for big ET-1 and BK are similar. The big ET-1 analogue ([Phe²²]big ET-1-(18–34)) is hydrolysed at approximately a 5-fold greater efficiency than the natural substrate, consistent with previous studies [10]. The I₅₀ values for inhibition of ECE hydrolysis by phosphoramidon and thiorphan are broadly similar for all three substrates.

Previous studies on ECE have revealed some distinctive features of its specificity that contrast markedly with the very broad specificity of its homologue, NEP (see [9] for review). These include mutagenesis and expression of preproendothelin in heterologous cell lines [18], study of truncated sequences of big ET-1 [10], chemical modification [11] and molecular modelling of big ET-1 [19]. Thus conversion of big ET substrate is limited to the production of ET, and no further degradation occurs. There are even marked differences in the ability of ECE to process the three different big ET isoforms, the velocity of hydrolysis being: big ET-1 > big ET-2 > big ET-3. The secondary structure of big ET appears to be important for determining the rate of hydrolysis [11], and the N-terminal disulphide-loop region of big ET-1 (amino acids 1–15) appears to hinder conversion, since truncated forms lacking this loop region exhibit higher specific activities. For example, big ET-1-(16–37) shows approx. 3-fold greater rate of hydrolysis than big ET-1 itself [10]. The sequence of Ile₁⁶–Ile₁₈–Trp²¹ on the amino side of the scissile bond (Trp¹⁵–Val²²) is the minimum requirement for efficient cleavage of a truncated big ET analogue. Replacement of the valine residue in the P' position by phenylalanine enhances cleavage by an order of magnitude [10]. Based on these studies is our use of the analogue [Phe²²]big ET-1-(18–34) as a substrate for rapid and sensitive HPLC assay of ECE [13]. The expression of mutant preproendothelins in Xenopus oocytes has also shown that there is no strict requirement for Trp-Val at the processing site [18]. C-terminal extension of the substrate sequence appears to be essential, and comparison of C-terminally truncated peptides [10] has shown that the C-terminal region at residues 32–37 of big ET-1 plays an important part in recognition of big ET-1 by ECE, since big ET-1-(1–31) is not cleaved. In the light of these studies, therefore, it is remarkable that ECE cleaves the unrelated BK at a significant rate, thereby acting as a peptidyl dipeptidase rather than a true endopeptidase.

Table 1 Comparison of ECE specificity, using BK, [Phe²²]big ET-1-(18–34) or big ET-1 as substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nmol·min⁻¹·mg⁻¹)</th>
<th>Vₘₐₓ (nmol·min⁻¹·mg⁻¹)</th>
<th>Kₘ (µM)</th>
<th>Vₘₐₓ/Kₘ (min⁻¹·mg⁻¹·µM⁻¹)</th>
<th>I₅₀ (µM)</th>
</tr>
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<tbody>
<tr>
<td>Bradykinin</td>
<td>21.2 ± 1.4</td>
<td>38.8 ± 1.5</td>
<td>978 ± 18</td>
<td>0.04 ± 0.002</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>[Phe²²]big ET-1-(18–34)</td>
<td>17.2 ± 1.3</td>
<td>37.6 ± 1.2</td>
<td>203 ± 13</td>
<td>0.19 ± 0.01</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Big ET-1</td>
<td>0.17 ± 0.02</td>
<td>0.30 ± 0.01</td>
<td>7.6 ± 0.2</td>
<td>0.04 ± 0.002</td>
<td>8.5 ± 0.3</td>
</tr>
</tbody>
</table>
than an endopeptidase. The bond cleaved in BK, Pro–Phe, also implies that ECE can act as a post-proline cleaving enzyme and emphasizes the importance of a large hydrophobic group in the P_1' position of the substrate.

**Hydrolysis of the Pro_7–Phe_8 bond of BK by ECE and other membrane metallopeptidases**

Inactivation of BK by cleavage of the Pro_7–Phe_8 bond has been identified for at least three mammalian ectopeptidases, angiotensin-converting enzyme (ACE), NEP and now ECE [20]. Thus this further reinforces the fact that ECE and NEP are highly similar in their active sites [21]. The main differences between these enzymes in their processing of BK lies in the affinity of BK for each enzyme (approx. K_m values: ACE, 0.4 μM; NEP, 90 μM; ECE, 970 μM) and the occurrence of secondary hydrolysis sites. ECE hydrolyses exclusively the Pro_7–Phe_8 bond, whereas ACE cleaves consecutively the Pro_7–Phe_8 bond and the Phe_9–Ser_10 bond. NEP hydrolyses either at the Gly_7–Phe_8 or the Pro_7–Phe_8 bond. Although ACE (EC 3.4.15.1) is the principal contributor to BK metabolism in vitro [22], it will be interesting to determine, by using specific ECE inhibitors in vitro or in vivo, whether ECE may contribute locally to BK metabolism at the surface of endothelial cells where it is predominantly located.

**General conclusions**

The present study has established that ECE can hydrolyse the vasodilator peptide BK, in addition to its presumed substrate, big ET. The lack of sequence similarity in these peptides and the different nature of the hydrolysis (peptidyl dipeptidase compared with endopeptidase action) suggests that ECE may have a far broader specificity than previously recognized and may have additional physiological roles. This may relate to the subcellular location of ECE, which is found both at the plasma membrane as an ectoenzyme and intracellularly in a vesicle population and in the trans-Golgi network [23]. Intracellular ECE may play the predominant role in processing of big ET through the constitutive secretory pathway, whereas cell-surface ECE may additionally contribute to the metabolism of distinct circulating peptides yet to be identified. This observation has important consequences in terms of the design and application of ECE inhibitors in therapy. The precise structural requirements for substrate binding and catalysis by ECE remain to be defined fully. A primary requirement would appear to be a large hydrophilic residue in the S_2' site but with rather greater flexibility at the S_1’ site.

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**REFERENCES**