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Molecular characterization of human and bovine endothelin converting enzyme (ECE-1)

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Abstract A membrane-bound protease activity that specifically converts Big endothelin-1 has been purified from bovine endothelial cells (FBHE). The enzyme was cleaved with trypsin and the peptide sequencing analysis confirmed it to be a zinc chelating metalloprotease containing the typical HEXXH (HELTH) motif. RT-PCR and cDNA screens were employed to isolate the complete cDNAs of the bovine and human enzymes. This human metalloprotease was expressed heterologously in cell culture and oocytes. The catalytic activity of the recombinant enzyme is the same as that determined for the natural enzyme. The data suggest that the characterized enzyme represents the functional human endothelin converting enzyme ECE-1.

Key words: Endothelin-1; Endothelin converting enzyme

1. Introduction

Endothelin, ET, was first characterized as an endothelium-derived contracting factor (EDCF) [1] and subsequently purified and sequenced [2]. Endothelin-1 (ET-1) is regarded as the most important vasoconstrictor of the three identified isopeptides ET-1, -2, -3 [3], is synthesized by vascular endothelial cells [2] and is found in plasma. There are a number of diseases in which ET-1 is thought to play a role [4]. By means of specific ET-1 receptor antagonists and endothelin specific antibodies it has been possible to demonstrate in animal models an involvement of ET-1 in pathological situations such as acute renal failure and subarrachnoid hemorrhage [5–7]. Besides its potential role in cardiovascular diseases, ET-1 has been shown to be essential for normal development [8].

ET-1 is released from an inactive intermediate form called Big-ET-1. This step is catalyzed by a putative endothelin converting enzyme (ECE), as has been hypothesized by Yanagisawa and coworkers [2], and much effort has been directed towards the molecular characterisation of this enzyme. Various proteases have been implicated in the processing of Big-ET-1 to ET-1 [9] but recent literature has focused on a metalloprotease as the physiologically relevant enzyme [10–14] which is found mainly in endothelial cells. ECE activity and the secretion of ET-1 can be inhibited unselectively by phosphoramidon which is a more powerful inhibitor of neutral endopeptidase (NEP 3.4.24.11). However, ECE-activity can be differentiated from that of NEP 3.4.24.11 by means of specific NEP 3.4.24.11 inhibitors which do not interfere with the effects of Big-ET-1 in vivo and which do not inhibit the secretion of ET-1 from endothelial cells [15–18]. ECE-1 can be defined as a phosphoramidon inhibitable endopeptidase which cleaves Big-ET-1 between Trp^{21} and Val^{22}. Using this definition several groups have characterized this enzyme. With respect to the enzymatic activity of ECE, there has been more agreement in the literature (K_m Big-ET-1 = 10^{-5} M; IC_{50} Phosphoramidon = 3–10 μM) than on the molecular characterization of ECE, e.g. the molecular weight of the putative ECE has been estimated to be 100–350 kDa [10,12–14]. Although ECE has been repeatedly characterized as a membrane-bound enzyme, there have also been reports suggesting its location in the cytosol [9]. In the absence of specific ECE-inhibitors, the characterization of ECE and its distinction from other endoproteinases is a prerequisite in the evaluation of its role in physiological and pathological conditions.

2. Materials and methods

2.1. Cell culture

FBHE bovine heart endothelial cells were obtained from the ATCC (CRL 1395) and cultured in DMEM +10% FCS +25 ng/ml bFGF (Intergen). Cells were passaged twice a week. Two days before harvest, 10^{-4} M phosphoramidon (Peptide Inst.) was added to the cell culture medium. Phosphoramidon had no influence on the growth of the cells. Cells were harvested by scraping and concentrated by centrifugation. The resulting cell suspension was washed with PBS.

CHO-K1 cells were obtained from the ATCC (CCL61) and cultured in DMEM/F12 + 10% FCS.

2.2. Enzyme assay

1 μl of individual fractions was diluted into 21 μl of 50 mM Tris-HCl pH 7.4; 50 mM imidazole; 250 mM NaCl and added to 2.5 μl human Big-ET-1 (2 mg/ml in 0.1% acetic acid). After a 4 h incubation at 37°C the reaction was stopped by adding 72 μl 0.5% trifluoro-acetic acid and samples were analyzed for ET-1 by reversed-phase HPLC on an Aquapore C8 column. ET-1 was quantified (1 $\mu U = 10^{-6}$ μM ET-1 × min^{-1}) from the HPLC areas ($UV_{208\ nm}$) by calibration with standard ET-1. A similar analysis was performed for the other peptide substrates (Big-ET-2 and Big-ET-3). Protein concentrations were determined after Bradford [19].

2.3. Enzyme purification

A pellet of 5×10^8 FBHE cells, which had been incubated for two days in the presence of 10^{-4} M phosphoramidon, was resuspended in PBS, supplemented with 0.5 mM DIFP and sonicated (20 min, 0°C).

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Abbreviations: ET-1, endothelin-1; big ET-1, big endothelin-1; ECE-1, endothelin-1 converting enzyme; RT-PCR, reverse transcript PCR; SDS-PAGE, sodium dodecylsulphate polyacrylamide gelelectrophoresis, DIFP, diisopropyl fluorophosphate; kDA, kilo dalton; kb, kilo base(pair); DIG, digoxigenin; CHO, chinese hamster ovary; RACE, rapid amplification of cDNA ends.

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After a 20 min, 1000 × g centrifugation, the membrane fraction was isolated from the supernatant by 1 h ultracentrifugation at 100,000 × g. Membranes were solubilized in 0.1 M Tris-HCl pH 8.0; 1% Triton X-100 and chromatographed on a MonoQ-HR 5/5 column (Pharmacia) equilibrated with buffer A (0.5 M Tris-HCl pH 8.0; 0.1% Triton X-100). ECE-1 was eluted with a linear gradient from buffer A to buffer A + 1 M NaCl. Fractions containing ECE-1 activity were pooled and chromatographed on a Superose-12 HR 10/36 column (Pharmacia) in 20 mM sodium phosphate buffer pH 7.4; 250 mM NaCl; 0.05% Triton X-100.

2.4. SDS-PAGE and tryptic digestion in the gel

Fractions of Superose-12 column separations were run on an 8% SDS-PAGE under non-reducing conditions and the gel was stained with Coomassie brilliant blue. Gel slices containing about 100 pmol of the 250 kDa protein were excised, destained in water and equilibrated with 200 μl of 0.1 M NH_4HCO_3. 0.5 μg trypsin was added and after an overnight incubation at room temperature the supernatant was harvested. The procedure was repeated and the combined supernatants were freeze-dried, dissolved in 40 μl water, 4 μl 40 mM DTT; after 30 min 4 μl 100 mM iodine acetamide in PBS was added and stirred for a further 30 min. The samples were freeze-dried and redissolved in 0.5% trifluoro acetic acid (TFA). Tryptic peptides were chromatographed on a RP 8 column (Brownlee, 1 mm × 15 cm) with a linear gradient from 0.5% TFA in H_2O to 0.5% TFA in acetonitrile/H_2O (9:1). A total of 25 fractions were collected and each fraction of 50 μl was analyzed with a gas-phase sequencer (477A, Applied Biosystems).

2.5. ECE-1 cDNA cloning

Tryptic peptide 4 was backtranslated to synthesize degenerate primers A, (5'-GARATYGTSTTYCCYGCYGG-3') and B, (5'-ATYCTSCAGGCYCCYTTYTAYAC-3'). For RT-PCR, total RNA was recovered from phosphoramidon-treated FBHE cells and reverse transcribed with primer (5'-CGAGGGGGATGGTCGACGGAAGCGACCT$_{18}$-3'). First strand cDNA was amplified in a nested set RACE-PCR [20] reaction as described previously [21] using primer A and primer (5'-CGAGGGGGATGGTCGACGG-3'). The products were separated on agarose gels and DNA fragments from fractionated slices were amplified in a second PCR with primer B and primer (5'-GATGGTCGACGGAAGCGACC-3'). A 1 kb fragment, which was generated, was non-isotopically labelled according to DIG DNA labelling kit (Boehringer Mannheim) and used to screen a FBHE cDNA library (prepared according to Stratagene UniZAP cloning kit) as well as a human placenta λgt11 cDNA library (Clontech). The resulting clones were sequenced and yielded the coding regions from aa 47–754 (bovine cDNA) and aa 51–753 (human cDNA). To complete the cDNAs, PCR reactions were performed on a bovine lung cDNA in λgt11 and an internally primed human placenta cDNA in λUniZAP with nested internal antisense primers and λ vector primers. All sequences were determined on both strands. The sequences have been deposited with the EMBL/Genbank/DDBJ databases and assigned the accession numbers Z35306 and Z35307.

2.6. Northern analysis

A human tissue Northern blot, containing approx. 2 μg of polyA$^+$ RNA per tract, was hybridized under standard conditions with a ^{32}P-labelled DNA-probe (specific activity ≥ 10^7 Bq/μg) representing the region between amino acids 610–702 of human ECE-1 cDNA. The blot was washed in 0.1 × SSC, 0.5% SDS at 65°C and exposed to X-ray film at −70°C overnight.

Table 1
Purification of ECE-1 from bovine endothelial cells (FBHE)

Purification step	Protein (mg)	Activity (μU)	Yield (%)	Specific activity (μU/mg)
Membranes	87	35,600	100	410
Solubilisate	88	31,460	88	360
MonoQ	6.6	24,800	69	3,760
Superose-12	0.35	5,280	15	15,100

For experimental details see section 2. The determination of the enzymatic activity is described in section 2.2.

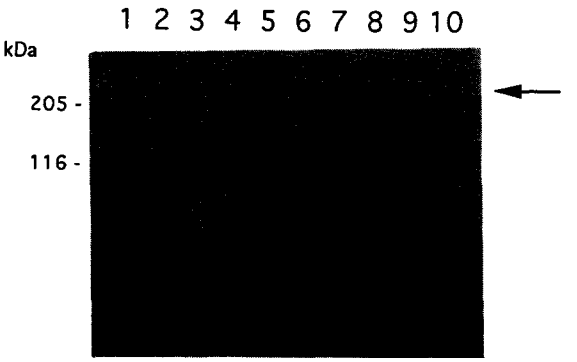


Fig. 1. SDS-PAGE and tryptic digestion in the gel. Fractions of Superose-12 columns separations were run on an 8% SDS-PAGE under non-reducing conditions and the gel was stained with Coomassie brilliant blue. The arrow denotes the 250 kDa band identified as ECE-1.

2.7. Heterologous expression of recombinant ECE-1

Recombinant human ECE was expressed in CHO-cells using pcDNA3neo (Invitrogen, Prod. #V790–20) as expression vector and the entire coding region of human ECE-1 cDNA including 420 bp of 3'-untranslated region. A similar construction was performed for oocyte injection with the in vitro transcription vector pSP73 (Promega Corp., Prod. #P2221). In this case a synthetic polyA-stretch and a unique *MluI* site were added to the 3' region. The cDNA was transcribed in vitro with SP6 polymerase after linearization of the plasmid with *MluI*.

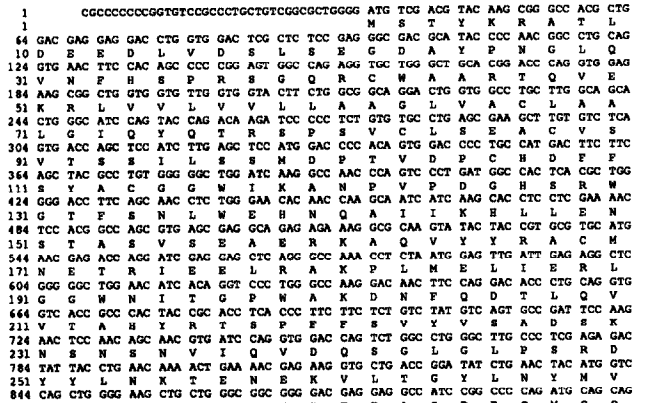
CHO-K1 cells were transfected using lipofectamine (Gibco) and selected with G418. Resistant colonies were pooled, expanded and harvested. Solubilization and enrichment was performed using the MonoQ-HR 5/5 column described in section 2.3. ECE-acitivity was measured as described in section 2.2. IC_{50} is the phosphoramidon concentration at which ECE activity is inhibited by 50%.

3. Results and discussion

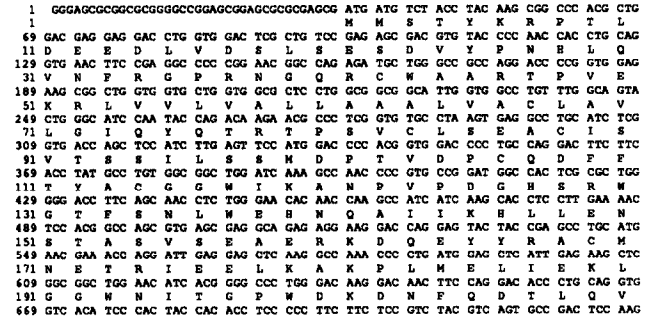
Membrane preparations of permanent bovine endothelial cells (FBHE) contain a Big-ET-1 specific converting activity that can be inhibited by phosphoramidon. Interestingly, we found that membranes prepared from FBHE cells which were previously treated with phosphoramidon, showed an up to ten-fold increase in specific activity for Big-ET-1 conversion (manuscript in preparation). A similar effect was also seen with other cell lines, although to a lesser extent (3- to 5-fold, data not shown). Phosphoramidon is a competitive inhibitor of low binding affinity for ECE. It easily dissociates from ECE during the first purification steps. This activity was solubilized, purified and differentiated against Big-ET-2/-3 conversion (Table 1). ECE-1 activity from the gel filtration step was separated on SDS-PAGE gels under non-reducing conditions (Fig. 1). A prominent 250 kDa band was correlated with ECE-1 by three different means: (1) the 250 kDa band showed up only after purification from phosphoramidon-treated cells but not from untreated cells; (2) the intensity of the stained band matched exactly the activity measured in different fractions; (3) digestion with chymotrypsin, which affects the activity of the enzyme, led to the disappearance of the 250 kDa band; whereas, under limited conditions, papain and trypsin, that affected only unrelated proteins, had neither an effect on the activity nor on the disappearance of this band (data not shown). Therefore, we concluded that this band contained ECE-1. Another report has

described ECE-activity for a protein of approx. 250–280 kDa [14] which might correspond to the ECE described in this paper. The putative ECE-1 from FBHE-membrane fractions displays an apparent molecular weight of 120 kDa as determined in polyacrylamide gels under reducing conditions (data not shown). ECE-1 appears to be glycosylated, as the slightly diffuse 120 kDa band was transformed into a sharp band of about 85 kDa after deglycosylation with a mixture of peptide-*N*-glycosidase F and endoglycosidase F (data not shown).

To prove these hypotheses, we cloned and expressed the cDNA coding for ECE-1. To this end, the protein band of 250 kDa under non-reducing conditions was excised and the protein in the gel was digested with trypsin. The amino acid sequences of the peptide products were analyzed on a gas-phase sequencer after separation on reversed phase HPLC. The amino acid sequences of several tryptic peptides are shown in Fig. 2. Among those, SSPNALNFGGIGVVVG**HELT**HAF was indicative of the presence of a metalloprotease, since it contained the putative active site, zinc-chelating HEXXH-motif. The peptide sequence NEIVFPAGILQAPFYT (4 in Fig. 2) was used to deduce two degenerate oligonucleotide primers (see section 2) in order to to follow a nested set RT-PCR strategy. First strand cDNA was synthesized from total RNA from phosphoramidon-treated FBHE cells with the help of a composite oligo-dT-primer (see Fig. 2) and used as a template. During the nested set PCR a 1 kb product was amplified. After subcloning, this DNA fragment was sequenced and the presence of other tryptic peptides (5, 6 and 7 in Fig. 2) in the open reading frame confirmed it to be a partial cDNA coding for the enzymatic activity described above. The same



a



b

Fig. 2 (a and b). Nucleotide and amino acid sequences of human and bovine ECE-1. (c) Comparison of the amino acid sequences of human and bovine ECE-1. Underlined residues correspond to sequenced peptides, and are numbered at the N-terminus. The resulting open reading frames of the cDNA clones from both species are aligned and amino acids in lower case denote differences between the species. Amino acids which have been shown for NEP 3.4.24.11 to be involved in ligand binding and have been conserved in ECE are indicated with bold letters. Potential glycosylation sites are denoted by asterisks.

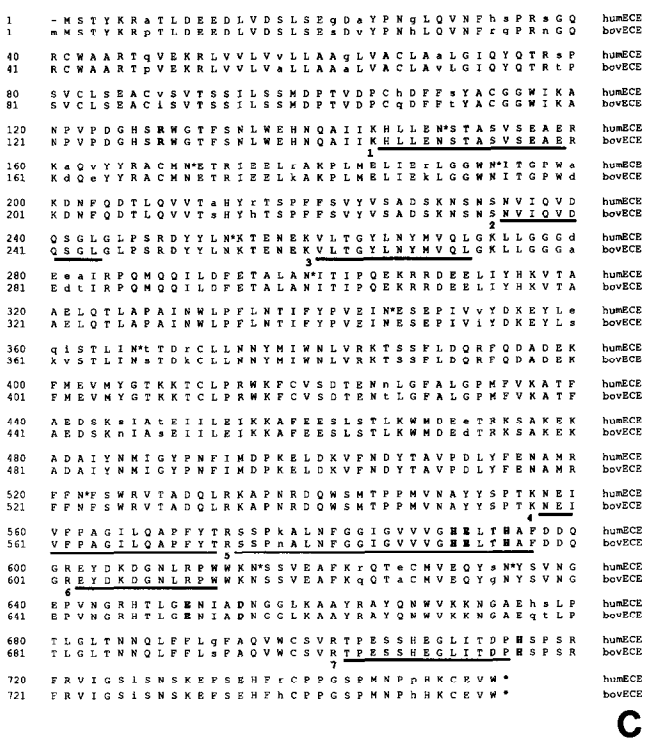


Fig. 2 (*continued*).

1 kb fragment was used to screen an FBHE- and a human placenta cDNA library. Since the isolated clones from both screens did not contain the entire coding region, a combination of 5′-RACE and additional screens were employed.

The resulting open reading frames are displayed in Fig. 2. The human and bovine cDNAs encode proteins of 753 and 754 amino acids, respectively, corresponding to approx. 85 kDa. This size is identical to that of natural ECE-1 after deglycosylation and analyzed under reducing conditions. Since all sequenced peptides derived from the 250 kDa band by tryptic digestion were found in the coding sequence, we concluded that homodimerisation of ECE-1 was a reasonable assumption. In addition, immunoprecipitated recombinant ECE-1 from membranes of metabolically labeled *Xenopus* oocytes displays the same 250 kDa band under non-reducing conditions and a 120 kDa band under reducing conditions that we observed with the protein from natural sources (data not shown). Therefore, we assume that human ECE-1 is a homodimeric membrane protein.

With the exception of an additional amino-terminal methionine in the bovine ECE-1 coding sequence, both sequences are colinear with a high degree of conservation (94% identity). In a comparison of the amino acid sequence of the rat endothelin converting enzyme published by Shimada and coworkers [22] with the human amino acid sequence presented in this study, we observed a similar high degree of conservation (95% identity).

Recently, Yanagisawa and coworkers [23] published the sequence of an endothelin converting enzyme isolated from bovine adrenal cortex. Although there is identity with our bovine sequence over almost the entire coding region, there is a complete lack of homology in the N-terminal 32 amino acid residues of the sequence.

By carefully analyzing the corresponding regions of ours and the published sequences, we observed the following: (1) the corresponding coding region between our bovine, and the human and rat sequences are highly conserved and differ by only 4 and 2 amino acid residues, respectively; (2) the nucleotide sequence published by Yanagisawa and coworkers [23] contains a perfect consensus 3′ splice junction between positions 205 and 219 (T/C > 11 N C/T AG); (3) the nucleotide sequence between positions 1 and 219 is in excellent agreement (>80% identity) with intronic sequences that we generated with human genomic clones of the endothelin converting enzyme (data not shown).

We therefore conclude, that this region of the sequence published by Yanagisawa is due to a misinterpretation of incorporated intronic sequences and does not reflect the authentic amino acid sequence.

We are not surprised, that the recombinant enzyme generated by Yanagisawa [23] displaying unrelated N-terminal intronic sequences is nevertheless an active and specific endothelin converting enzyme. During the course of our studies we generated a secreted version of the recombinant enzyme lacking the entire N-terminal region including the membrane anchor domain and still obtained an active and specific enzyme (manuscript in preparation).

The zinc-chelating motif HEXXH, typical for metalloproteases and the putative active site, starts at amino acid position 590 (bovine: 591). The amino acid sequence between position 52 (53) and 72 (73) contains a very hydrophobic stretch which most probably represents the membrane anchor of ECE-1 and identifies it as a class II transmembrane protein [24]. Significant homologies were detected with NEP 3.4.24.11 [25] (37% identity) and Kell blood group antigen [26] (25%). This homology places ECE in the evolutionary subfamily M13 [27] of the 'zincin' [28] metalloproteases. Several residues which have been shown to be involved in zinc and ligand-binding by NEP 3.4.24.11 [29–36] have been conserved in the ECEs and are indicated in Fig. 2 with bold letters. There are ten potential N-linked glycosylation sites in ECE, three of which appear at equivalent positions compared with NEP 3.4.24.11 [29] (sites 1, 5 and 10). The existence of ten potential glycosylation sites in

Table 2
Enzymatic comparison of recombinant and natural ECE

	Natural (bovine)	Recombinant (human)
IC_{50} (phosphoramidon) in μM	6	8
K_m (Big-ET-1) in μM	18	23
Big-ET-1	100%	100%
Big-ET-2	10%	10%
Big-ET-3	0%	0%
(Conversion of Big-ET-1 was set to 100%)		

Natural ECE was purified from FBHE cells. Recombinant human ECE was expressed in CHO-cells. Results from at least 2 independent sets of experiments are shown. ECE-activity was measured as described in section 2. IC_{50} is the phosphoramidon concentration at which ECE activity is inhibited by 50%. K_m, Michaelis–Menten constant.

the ECE-1 sequence is compatible with a strong glycosylation of the native protein so that a molecular weight for the glycosylated protease monomer of 120 kDa appears reasonable. Of the fourteen cysteine-residues, ten occur at equivalent positions compared with NEP 3.4.24.11 [29] and are probably involved in disulfide bridges.

Recombinant human ECE-1 (rh-ECE-1) was expressed in *Xenopus laevis* oocytes as well as in Chinese hamster ovary (CHO) cells. SP6 polymerase derived cRNA and a mock control were each injected into 100 oocytes . After 24, 48 and 72 h, 30 oocytes were homogenized and pelleted by centrifugation at 10,000 × *g*. ECE-1 activity in mock-injected oocytes was determined to be 12, 22 and 28 μU/mg for the three time points and 75, 132 and 137 for the cRNA-injected oocytes respectively. The increase in ECE-1 activity was more than fivefold and clearly demonstrates that cRNA from the cloned cDNA leads to active recombinant enzyme in this system. The ECE-1 activity found in mock-injected oocytes may be important since in mice it has been recently shown that ET-1 is a morphogen [8] and the presence of an ET-receptor subtype has been demonstrated in *Xenopus* [37]. CHO cells were transfected with a plasmid construct which expressed ECE-1 under the control of the strong CMV intermediate early gene promoter. Stable cell lines were established and a pool of several clones was grown to 10^9 cells. Cells were harvested and ECE-1 was recovered from MonoQ (as described in Table 1). We could not detect any ECE-1 activity in parallel experiments with untransfected CHO-cells. The specific enzymatic activity of rh-ECE-1 and its K_m value were determined (see Table 2). The data suggest that the characterized enzyme represents the functional human endothelin converting enzyme ECE-1 with a very similar catalytic activity. These preparations show ECE-1-activity in membraneous fractions with no detectable activity in the supernatant of the cells or in the cytosol. This activity distribution is consistent with ECE-1 being an integral membrane protein class II as deduced from its sequence.

However, cytosolic ECE-activities not further identified so far have also been described [9]. Since we find that in bovine endothelial cells the only reproducible ECE-1 activity has been in the membraneous fraction, questions arise as to whether other, possibly isoenzymes with ECE-1-activity, may exist and to which extent they may contribute to the release of ET-1. With the knowledge of the DNA-sequence of ECE, questions relating to the physiological and pathophysiological role of ECE might be approached by oligonucleotide directed antisense experiments or knock-out of the ECE gene in mice.

The rat [22], bovine ([23] and our work) and human ECEs have been cloned and expressed. However, the substrate specificity of the respective recombinant enzymes is controversial. Whereas the activity of the cloned rat enzyme is identical to the activity of our cloned human enzyme (poor conversion of Big-ET-2; Big-ET-3) the cloned bovine enzyme does have high conversion activity towards Big-ET-2 and Big-ET-3. It seems possible that species differences are responsible for this difference in ECE-activity. However, bovine ECE from endothelial cells does not show high activity towards Big-ET-2 and Big-ET-3 ([39] and our unpublished data). We speculated that differences in membrane anchoring between the recombinant human and rat ECE on one hand and the recombinant bovine ECE of Xu and coworkers [23] on the other, resulting from the different amino termini, might account for the substrate specificity. This

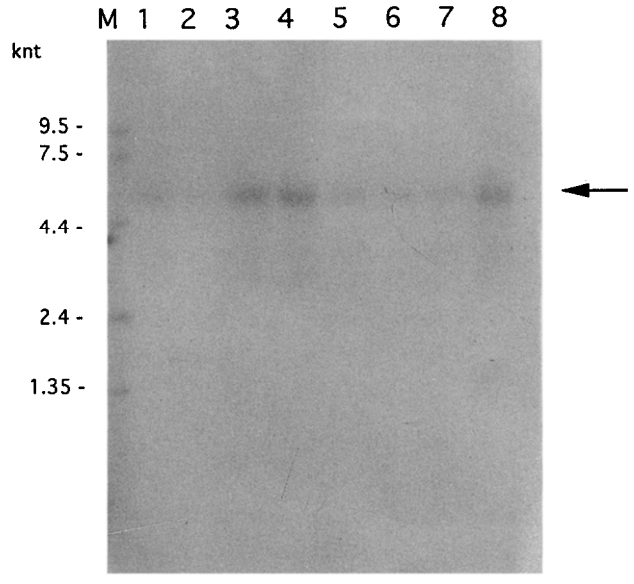


Fig. 3. RNA levels in different tissues determined by Northern blot analysis. A human tissue Northern blot was hybridized under standard conditions with a ^{32}P-labelled human ECE-1 cDNA probe as specified in section 2. The lanes represent: (M) molecular weight marker, (1) heart, (2) brain, (3) placenta, (4) lung, (5) liver, (6) skeletal muscle, (7) kidney and (8) pancreas; knt: kilo nucleotides.

view is corroborated by our expressing the extracellular domain of human ECE without any membrane anchor (manuscript in preparation). We could demonstrate that this truncated ECE had the same behaviour towards Big-ET-2 and Big-ET-3 as the full size human ECE. We also analyzed whether purification and assay conditions might account for these differences in activity of the recombinant enzymes. Under all circumstances, we have been unable to demonstrate efficient cleavage of Big-ET-2 and Big-ET-3 compared to Big-ET-1 with the recombinant human ECE-1. The processing of these two peptides under physiological conditions is therefore probably carried out by other, yet unidentified enzymes.

A 4.6 kb ECE-1 mRNA was detected by Northern blot analysis in various human tissues (Fig. 3), the strongest expression being seen in lung, pancreas and placenta. Almost no transcription was detected in brain. The strong expression of ECE-1 message in lung and placenta can be rationalized for these endothelium-rich organs. The observed RNA level in pancreas is in accordance with the high concentration of other peptidases and prohormone converting proteases found in this organ; however, its potential role in the pancreas requires clarification. Reports from other groups extend these findings with respect to the relative abundance of ET-receptor mRNA in different tissues. mRNA specific for the smooth muscle cell receptor type ET_A was found to be strongly represented in human lung, whereas the endothelial type ET_B-mRNA was also found in human lung and in placenta, but only in low amounts in pancreas [27]. This makes an autocrine or paracrine mode of ET-action in these tissues, with the possible exception of the pancreas, likely.

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