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A SIMPLE METHOD FOR ISOLATING HUMAN ENDOTHELIN CONVERTING ENZYME FREE FROM CONTAMINATION BY NEUTRAL ENDOPEPTIDASE 24.11

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SUMMARY: Subcellular fractionation of the phosphoramidon sensitive membrane-bound endothelin converting enzyme (ECE-1) activity from homogenates of bovine aortic endothelial cells and the human endothelial cell line EA.hy 926, combined with studies of intact cells, shows ECE-1 to be localised primarily to the plasma membrane with the topology of an ectoenzyme. To overcome the problem of neutral endopeptidase 24.11 contaminating the human ECE-1 activity solubilised from the plasma membrane fractions of EA.hy 926, we have used isoelectric focusing to simultaneously solubilise and separate these activities. The metallopeptidase ECE-1 obtained displayed a neutral pH optimum, a molecular weight of 250 kDa on gel filtration chromatography and was inhibited by phosphoramidon with an IC_{50} of 0.8 μM.

The hypothesis that conversion of big endothelin-1 (big ET-1) to active ET-1 requires a novel endopeptidase referred to as endothelin converting enzyme (ECE) has become widely accepted (1,2). Recent work has focused on the purification of a membrane-bound phosphoramidon-sensitive metallopeptidase from endothelial cells (3-6), and the cloning of the rat and bovine enzymes have recently been described (7-9). This has shown the enzyme, now referred to as ECE-1, to be structurally related to neutral endopeptidase 24.11 (E-24.11) (7-9). Human umbilical vein endothelial cells (HUVEC) and the human endothelial cell line EA.hy 926 express ECE activity, which is in part due to high levels of E-24.11 on these cells (6,10,11). Because E-24.11 not only generates ET-1 from big ET-1 but also degrades the ET-1 formed (12), it interferes with the purification and evaluation of ECE. Hence, the isolation of ECE activity which is not contaminated by E-24.11 is important for the characterisation of the human ECE-1 homologue. Purification of ECE from porcine endothelial cells yielded an enzyme with an isoelectric point (pI) of 4.1 (3). Here we have used this information to develop an isoelectric focusing (IEF) method for obtaining human ECE-1 from the plasma membranes of EA.hy 926 which is free from contamination by E-24.11. The properties of this human enzyme are described.

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In addition, we have investigated the distribution of membrane-bound ECE activity in subcellular fractions obtained from sucrose density gradient centrifugation of homogenates of bovine aortic endothelial cells (BAEC) and EA.hy 926. The distribution of ECE activity was compared to the well characterised plasma membrane ectoenzyme angiotensin converting enzyme (ACE). To determine the sensitivity to phosphoramidon (PHA) of the plasma membrane ECE activities of BAEC, EA.hy 926 and HUVEC, and the contribution of E-24.11 to the total activity of each cell type, the conversion of exogenous big ET-1 to ET-1 by intact cultured cells was studied. For comparison with the conversion of exogenous big ET-1, we have characterised the inhibitory effects of PHA on endogenous ET-1 synthesis in human cells using EA.hy 926, HUVEC and the epithelial cell line A549, which by fusion with HUVEC was used to established the EA.hy 926 cell line (12).

METHODS

Cell culture

BAEC and EA.hy 926 were cultured as previously described (10). A549 were cultured under the same conditions. BAEC up to passage 4, and EA.hy 926 cells from passage 30 to 50 were used in these studies. Subcultures were prepared by treating confluent cultures with trypsin (0.05%) and either seeded onto 35 mm well plates for studies of intact cells, or onto large plates (24.5 x 24.5 cm; Gibco-Nunc) for subcellular fractionation studies. HUVEC were isolated from human umbilical cords by the widely used enzymatic method. Briefly, the umbilical vein was cannulated, flushed with Hanks balanced salt solution, and then filled with Dispase II (2.4 U/ml, Boehringer Mannheim). After incubation at 37°C for 30 min, detached cells were washed out with MCDB 104 medium (Gibco) containing 20% foetal calf serum (FCS). After a short centrifugation (5 min, 250 g), cells were resuspended in MCDB 104 medium supplemented with 20% FCS, human recombinant epithelial growth factor (10 ng/ml; Sigma) and endothelial cell growth supplement (30 µg/ml; Sigma), and seeded onto plates pre-coated with gelatin (0.5%, overnight at 37°C). HUVEC up to passage 4 were used. Treatment of cultures with PHA for 24 h (Peptide Institute) (2 ml/well), or for short periods with big ET-1 (1 µM, 1 ml/well), was carried out in serum free DMEM.

Preparation of subcellular fractions

Four plates (24.5 x 24.5 cm) were used for each experiment (~7.2 x 10^7 cells per plate). Cells were homogenised in 0.25 M sucrose containing 10 mM NaHEPES pH 7.4 with a tight-fitting Dounce glass homogeniser. After centrifugation (1,000 g, 5 min) to remove cell debris, the supernatant (2 ml) was overlaid on discontinuous sucrose gradients consisting of 0.5, 0.8, 1.0, 1.2, 1.4 and 1.6 M sucrose layers, and centrifuged at 100,000 g for 3 h at 4°C. Fractions (1 ml) were collected, and membrane-bound material was precipitated by centrifugation at 120,000 g for 1h. Precipitates were sonicated with 50 mM Tris-HCl buffer (pH 7.0) for subsequent determination of ACE and ECE activity.

Biochemical analyses

ET-1 was measured by RIA (crossreactivity with big ET-1 <0.015%) (10). For ET-1 release from HUVEC, EA.hy 926 or A549 cells, medium was extracted prior to RIA (10). Human big ET-1 and big$ET_{[22-38]}$ (C-terminal fragment; CTF) released from EA.hy 926 and HUVEC were measured after extraction of the medium, or after HPLC, using a RIA specific for human big$ET_{[22-38]}$ (10). HPLC identification of ET-1, big ET-1 and CTF was performed as described (12). ECE activity was determined by incubating human big ET-1 (1 µM; Peptide Institute) at 37°C with particulate material in 50 mM Tris-HCl pH 7.0, or with intact cells in DMEM in an atmosphere of 5% CO_2 in air. Incubations were carried out for 2 - 4 h, followed by 10 min heat-treatment at 80°C. ECE activity was quantified by ET-1 RIA and is shown as pmol ET-1 generated/h/ml of sample unless otherwise indicated. Peptidase inhibitors were from Sigma.

Angiotensin converting enzyme (ACE) activity was determined using Benzoyl-Gly-Arg-Pro (BzGRP; Peptide Institute) as the substrate (14). Particulate material from sucrose density gradient fractions (100 - 200 µl) was incubated with 5 mM BzGRP in 0.1 M sodium phosphate buffer pH 7.8 at 37°C. After 30 min, the incubation mixture was diluted with an equal volume of 1 M HCl, and the hippuric acid (HA; Benzoyl-Gly) generated was extracted with 2 ml ethyl acetate and dried down at 50°C. HA was reconstituted in 0.1% TFA and subjected to HPLC using a column of phenyl-silica (5 µm, 4.6 x 250 mm, HPLC Technology) eluted at 1 ml/min with a linear gradient of 0-9% acetonitrile containing 0.1% TFA over 15 min. Absorbance was measured at 230 nm, and HA was quantified by reference to the peak area of HA standards (Sigma), elution time 9.5 min.

Isolation of human ECE-1 from EA.hy 926 cells

Plasma membrane fractions from sucrose density gradient centrifugation of ~5 x 10^8 cells were mixed with glycerol (20%), triton X-100 (TX-100, 0.5%) and Bio-Lyte ampholyte (1%) prefocused between pH 4 to 7. This was loaded into a Rotofor IEF cell (BioRad) and run at 4°C with 12 W constant power for 4 h. ECE activity was determined in each of the harvested fractions. PHA-sensitive ET-1 degrading activity (pmol/h/ml), as a measure of E-24.11, was determined by comparing ET-1 degradation in the presence and absence of 0.1 µM PHA. To obtain sufficient ECE-1 for characterisation, fractions focused between pH 3.9 and 4.2 from four separate IEF runs were diluted four fold with 10 mM Tris-HCl pH 9 containing 50 µM $ZnCl_2$ (to restore fully ECE activity), loaded onto a column of DEAE Sephacel (1.5 x 15 cm), and eluted with NaCl containing 0.1% TX-100. The molecular weight (M_r) of the ECE-1 obtained from DEAE chromatography was determined by gel filtration on a column of Sephacryl S300 HR (6.6 x 500 mm) eluted with 10 mM Tris-HCl pH 9 containing 0.15 M NaCl, 0.1% TX-100 and 0.01% azide.

RESULTS AND DISCUSSION

Sucrose density gradient separation of subcellular fractions of BAEC or EA.hy 926 cells showed the peak of membrane-bound ECE activity to be in fraction 4 (0.8-1.0 M sucrose) (Fig. 1). Consistent with the majority of membrane-bound ECE activity in BAEC being a PHA-sensitive enzyme (4,8), PHA (100 µM) reduced the peak activity by 62.2 ± 4.9% (n = 4). In BAEC, ACE activity had a parallel distribution to the ECE activity. In fractions from EA.hy 926, ACE activity was not detectable. In contrast to BAEC, the ECE activity from EA.hy 926 fractions was inhibited by 35 to 65 % with 0.1 µM PHA, and by 87% with 100 µM PHA (Fig. 1b). The activity inhibited by 0.1µM PHA is the result of high levels

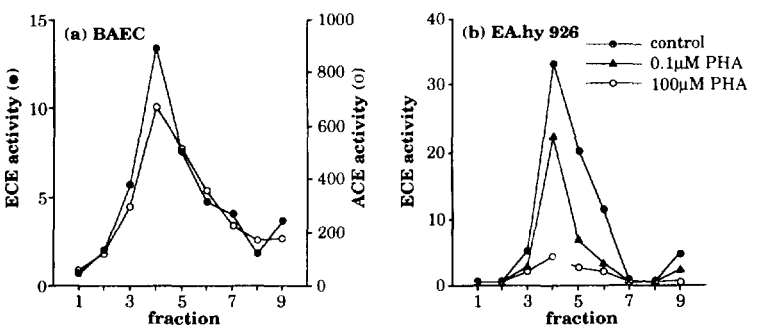

Figure 1. **(a)** Distribution of ACE and ECE activity (both pmol/h/10^6 cells) in subcellular fractions of BAEC, mean results from 3 experiments. Fractions (1 ml) were collected from the top of the gradient (1 corresponds to 0.25 M sucrose, and 9 to 1.4 M sucrose). **(b)** ECE activity (pmol/h/10^6 cells) in subcellular fractions of EA.hy 926; comparison of control activity with that obtained in the presence of PHA (0.1 or 100 µM).

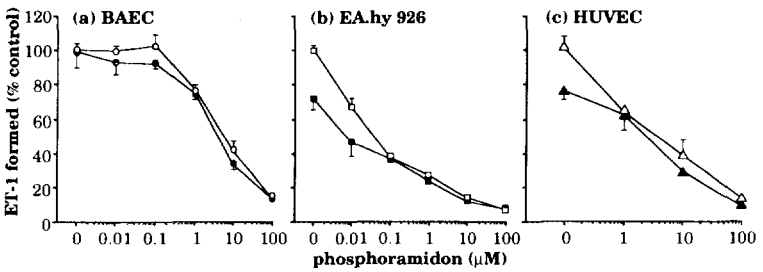

Figure 2. Effect of PHA on the conversion of exogenous big ET-1 (1 μM) to ET-1 by intact cells. Mean data from two experiments (n = 4 - 6 for each point) (open symbols, PHA alone; closed symbols, PHA with 1 μM thiorphan).

of E-24.11 in these cells (6,11) acting as an 'ECE' under these incubation conditions (12). Hence, approximately half of the total ECE activity in EA.hy 926 may be accounted for by E-24.11. These subcellular fractionation studies show that the majority of ECE activity in endothelial cells has a parallel distribution to the well characterised plasma membrane ectoenzymes ACE and E-24.11. An earlier study also concluded that ECE activity in EA.hy 926 cells was localised predominantly in plasma membrane fractions (6).

To characterise this activity on intact cells we measured the conversion of exogenous big ET-1 to ET-1, and determined the degree of inhibition obtained with PHA alone and in the presence of the E-24.11 inhibitor thiorphan (1 μM) (Fig. 2). Control levels of ECE activity were 1596 ± 144, 3697 ± 132, and 4064 ± 356 fmol ET-1 formed/h/10^6 cells for BAEC, EA.hy 926 cells and HUVEC. With BAEC, PHA inhibited the conversion of exogenous big ET-1 with an IC_{50} of 4.6 μM. Thiorphan had no effect on the ECE activity of BAEC. With EA.hy 926, thiorphan alone reduced the ECE activity by 30% and in combination with low concentrations of PHA (up to 0.1 μM) reduced activity by 63% to 1366 ± 102 fmol ET-1/10^6 cells/h. Thus, in agreement with the subcellular fractionation studies, more than half the ECE activity is due to E-24.11. Inhibition by >0.1 μM PHA, alone or with thiorphan, was superimposable indicating that this was due to the plasma membrane ECE activity (IC_{50} 4.5 μM) (Fig. 2b). In comparison with EA.hy 926, approximately 25% of the ECE activity of HUVEC was attributable to E-24.11. For HUVEC the IC_{50} for the ECE activity, excluding that due to E-24.11, was also ~5 μM PHA (Fig. 2c). The predicted amino acid sequences for the cloned ECE-1 show the active site is in the extracellular domain (7-9); hence these results from intact cells confirm that this plasma membrane peptidase has the topology of an ectoenzyme.

HPLC characterisation of ET-1 immunoreactivity formed from exogenous big ET-1 by the three cell types showed the majority (>90%) to elute in the position of authentic ET-1. Moreover, measurement of big $ET\text{-}1_{[22\text{-}38]}$ immunoreactivity in HPLC fractions showed CTF to elute in the correct position, hence confirming that ET-1 was formed by hydrolysis of the Trp^{21}-Val^{22} bond in big ET-1. With EA.hy 926 there was a second minor peak of immunoreactivity, which eluted in the position of $ET\text{-}1_{[17\text{-}21]}$. This pentapeptide has been observed as a hydrolysis product in incubations of big ET-1 with E-24.11 (12).

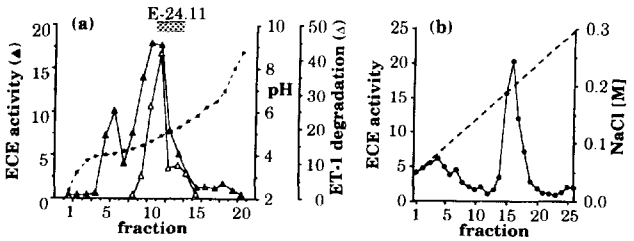

Figure 3. (**a**) Solubilised ECE activity and PHA sensitive ET-1 degrading activity from plasma membranes of EA.hy 926 cells (broken line, pH gradient; pI of porcine E-24.11 is indicated). (**b**) DEAE Sephacel chromatography of solubilised ECE.

Consistent with it being due to E-24.11, 0.1 μM PHA reduced by 50% the ET-1 identified by HPLC and eliminated this pentapeptide fragment.

This interference of E-24.11 has hindered the characterisation of human ECE-1. Therefore, to overcome this problem we have used IEF as a means of separating E-24.11 from ECE-1. In agreement with studies of porcine endothelial cells (3), human ECE-1 solubilised from plasma membrane fractions displayed a pI of ~4 (Fig. 3a). This was well separated from the second "ECE activity" which, in terms of its pI, ability to degrade ET-1 and sensitivity to PHA, had the characteristics of E-24.11. To further purify human ECE-1, the activity with a pI of 4 was subjected to DEAE chromatography (Fig. 3b). Consistent with this being human ECE-1, a peak of ECE activity free of contaminating ET-1 degrading activity, and therefore free of E-24.11, was obtained. Using 1 μM big ET-1 as substrate the activity of the peak fraction was 700 pmol/h/mg protein. This fraction was used for the further characterisation of the properties of human ECE-1.

The deduced amino acid sequences for the rat and bovine ECE-1 predict a M_r of 85 kDa (7,8). But in practice this enzyme has been found to have a M_r of 120-130 kDa by SDS-PAGE probably as a result of extensive glycosylation (3,5,8). By gel filtration chromatography, the M_r for human ECE-1 was estimated to be ~250 kDa (Fig. 4a). Similar results (280 kDa) have been obtained by electrophoresis under non-denaturing conditions (6). Under the same gel filtration conditions the structurally related peptidase, porcine E-24.11, eluted as expected with a M_r of 100kDa (Fig 4a). Hence, the anomalously high M_r observed for native human ECE-1 may indicate that it exists as a dimer.

In agreement with published data for bovine ECE-1, the purified enzyme is a metallopeptidase inhibited by 98% with EDTA (100 μM) or *o*-phenanthroline (100 μM). PMSF (100 μM), DCI (10 μM), NEM (100 μM), E64 (10 μM) and pepstatin A (1 μM) had no effect on the activity. The IC_{50} for PHA was somewhat lower than with intact cells (0.8 μM, Fig. 4b) possibly due to the effects of the isolation procedure. Thiorphan up to concentrations of 10 μM had no effect on the ECE activity even though in parallel incubations E-24.11 was completely inhibited at this concentration. The pH optimum, 6.7-6.9 depending on the buffer system (Fig. 4c), is very similar to the bovine enzyme (8).

It has become widely accepted that PHA inhibits the physiologically relevant ECE in cultured endothelial cells leading to a decrease in ET-1 secretion and an accumulation

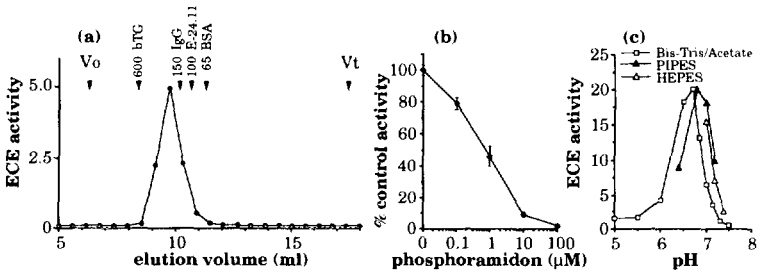

Figure 4. Characterization of human ECE-1. (**a**) Sephacryl S300 gel filtration chromatography. The elution positions of the *Mr* markers (kDa) are indicated: bovine thyroglobulin, rabbit IgG, porcine E-24.11 and bovine serum albumin. (**b**) Inhibition of purified human ECE-1 by PHA. (**c**) pH optimum of human ECE-1.

of big ET-1 in the medium without any change in the rate of synthesis (2,15). Although PHA has been shown to suppress ET-1 levels in HUVEC (16), HPLC characterisation of the relative changes in ET-1 and big ET-1 secretion have not been reported previously for human cells. Earlier studies of EA.hy 926 showed no reduction of ET-1 release up to 100 μM PHA (10). Here we have performed a more extensive investigation and compared the sensitivity of human cells with BAEC. The mean amounts of ET-1 accumulating over 24 h in control wells were 343 ± 27 (n = 15), 565 ± 33 (n = 19), 392 ± 40 (n = 9) and 2590 ± 302 (n = 18) fmol/well for EA.hy 926, HUVEC, A549 cells and BAEC respectively. The control release for HUVEC and EA.hy 926 was determined in the presence of 1 μM PHA to inhibit E-24.11 dependent degradation. This concentration had no effect on ET-1 secretion in either the epithelial cell line A549 or BAEC. Inhibition of endogenous ET-1 synthesis by PHA showed a marked difference in the IC_{50} value for cultured human cells (~200 μM) compared to the corresponding value for BAEC (40 μM) (Fig. 5a). Measurement of total big ET-$1_{[22\text{-}38]}$ immunoreactivity showed the effect of PHA was not due to a non-specific suppression of proendothelin-1 synthesis. For example big ET-$1_{[22\text{-}38]}$ values for EA.hy 926 and HUVEC in the presence of 1 mM PHA were 112 ± 7% (n = 12) and 106 ± 2% (n = 9) of the corresponding reference values. HPLC of control medium from HUVEC showed 95% of ET-1 to elute in the position of the authentic peptide (Fig. 5b), and 94% of big ET-$1_{[22\text{-}38]}$ immunoreactivity to elute as CTF and only 6% as big ET-1. After 1 mM PHA, the ET-1 peak area was only 16% of control and big ET-1 became the major product. Thus confirming for human cells that 1 mM PHA inhibits the hydrolysis of big ET-1 by the physiologically relevant ECE.

In conclusion, we describe here a simple method for preparing human ECE-1 for further characterisation or inhibitor development. The human ECE-1 obtained has very similar characteristics to the rat, porcine and bovine enzymes (3,5,8). The IC_{50} for inhibiting the conversion of exogenous big ET-1 by intact bovine and human cells is ~5 μM PHA. However, inhibition of endogenous synthesis required 8 and 40 fold higher concentrations for bovine and human cells. The fact that such high concentrations are required to suppress endogenous ET-1 production indicates that the conversion of big ET-1 occurs intracellularly. Differences in the rate of PHA uptake may account for the

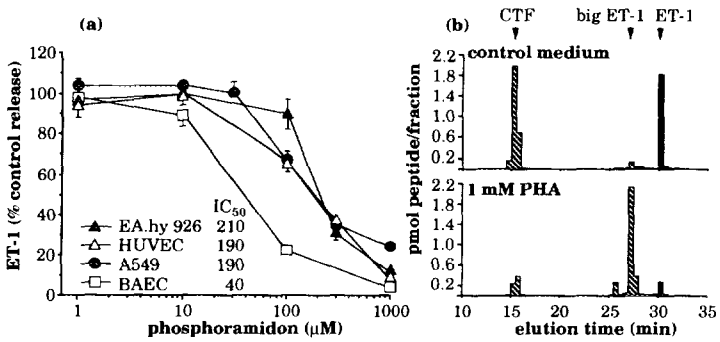

Figure 5. (**a**) Effect of 24 h treatment with PHA on endogenous ET-1 secretion. (**b**) HPLC characterisation of ET-1, big ET-1 and CTF in conditioned medium from HUVEC. ET-1, solid columns; big ET-1[22-38], hatched columns. Elution positions of peptide standards are indicated. To prevent E-24.11 hydrolysis of secreted peptides, 1 μM SQ 28603 was included in all wells. For HPLC, medium from 4 control or PHA wells (8 ml) was extracted.

difference in sensitivity of bovine and human cells to PHA. Subcellular fractionation studies show the majority of ECE activity to be associated with the plasma membrane. ECE-1 is the first peptidase to be characterised which satisfies the criteria of a true ECE (8,9), however for it to fulfil the role of the intracellular ECE, its colocalisation with ET-1 in endothelial cells needs to be demonstrated.

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