Evidence for Intracellular Endothelin-Converting Enzyme-2 Expression in Cultured Human Vascular Endothelial Cells

Fraser D. Russell, Anthony P. Davenport

Abstract—We have previously reported the intracellular localization of the endothelin-converting enzyme-1 (ECE-1) in human umbilical vein endothelial cells. In the present study, we provide the first immunocytochemical and biochemical evidence for the presence of ECE-2 in human cells. ECE activity was determined by conversion of exogenously added big endothelin-1 (big ET-1) to ET-1 in subcellular fractions obtained by sucrose density gradient centrifugation of human umbilical vein endothelial cell homogenates. ECE-1 and ECE-2 can be differentiated by pH dependence for optimal activity and by sensitivity to phosphoramidon, which shows selectivity for ECE-2 over ECE-1 and PD159790, a novel ECE-1 selective inhibitor. Optimal ECE activity was measured at pH 6.0, a value intermediate between that reported for ECE-1 (pH 6.8) and ECE-2 (pH 5.5), indicating expression of both enzymes. At pH 6.9, conversion of big ET-1 was inhibited markedly by 30 μmol/L PD159790 and by 100 μmol/L phosphoramidon but not by 0.1 μmol/L phosphoramidon. In contrast, ECE activity was unaffected by 30 μmol/L PD159790 but was inhibited markedly by 0.1 and 100 μmol/L phosphoramidon at pH 5.4 (IC₅₀ 1.5 nmol/L), consistent with ECE-2 activity. Confocal microscopy revealed a punctate pattern of ECE-2–like immunoreactive staining in the cell cytosol, suggesting localization to secretory vesicles with a possible role in processing big ET-1 while in transit to the cell surface via the constitutive secretory pathway. (Circ Res. 1999;84:891-896.)

Key Words: endothelin ■ endothelin-converting enzyme ■ endothelium

Endothelin (ET) is a potent vasoconstrictor that is cleaved from a low-activity precursor, big ET by endothelin-converting enzymes (ECEs). Although ET has been shown to contribute to maintenance of vascular tone in humans, a pathogenic role has been implicated by its overproduction in patients with diseases including atherosclerosis, primary pulmonary hypertension, coronary vasospasm, and congestive heart failure. Inhibitors of the pathway leading to production of ET may therefore be of therapeutic benefit, and it is suggested that examination of ECEs in human cells will be useful in drug development.

Sequence identity of two ECEs (ECE-1 and ECE-2) has been determined using molecular techniques. The enzymes have 59% overall homology and both are membrane-bound, phosphoramidon-sensitive metalloproteases. Two isoforms of ECE-1 (ECE-1α and ECE-1β) are encoded by a single gene and differ only in their cytoplasmic N-terminal domains. Studies on a soluble construct of ECE-1 and molecular modeling experiments reveal that the putative extracellular domain contains the catalytic site for ECE activity.

Gene knockout studies have underlined the importance of ET in normal fetal development. Targeted null mutation in the mouse ECE-1 gene produced embryos that exhibited marked craniofacial and cardiac defects as well as an absence of epidermal melanocytes and enteric neurons of the distal gut. High levels of mature ET peptide were measured in homozygous ECE-1 knockout embryos, suggesting expression of a non–ECE-1 protease, such as ECE-2. Whereas both ECE-1 and ECE-2 are sensitive to phosphoramidon but insensitive to thiorphan treatment, the two enzymes can be differentiated by their pH optimum for converting enzyme activity (ECE-2 has pH optimum of 5.5 compared with 6.8 for ECE-1) and by their sensitivity to phosphoramidon (ECE-2 is 250-fold more sensitive to the metalloprotease inhibitor than ECE-1). The enzymes may also be differentiated by sensitivity to newly developed ECE inhibitors that have selectivity for ECE-1 over ECE-2.

In previous studies, we have reported the localization of ECE-1α and ECE-1β in human umbilical vein endothelial cells (HUVECs). However, to date, there has been little evidence for expression of other ECEs in nonbovine mammalian tissues. In the present study, we investigated ECEs expressed in human endothelial cells using biochemical and immunocytochemical techniques and reveal evidence for the intracellular localization of ECE-2.

Materials and Methods

Site-directed antisera were raised against a synthetic peptide corresponding to a deduced amino acid sequence of bovine ECE-2.
(bECE-2α/β, γ). This sequence was distinct from ECE-1 isoforms, and no other closely related sequences were detected in SWISS-PROT and TrEMBL databases. Antisera were affinity-purified using the immobilized immunizing peptide. Antisera were tested as previously described. Briefly, the immune sera cross-reacted in ELISA with 20 nmol/L of the immobilized immunizing peptide with a titer of 1×10^6. Preabsorbing the antisera with 1 μmol/L of bECE-2α/β, γ before immunocytochemistry abolished staining within HUVECs. A monoclonal mouse antibody raised against human von Willebrand factor (clone F8/86) was obtained from Dako. Affinity-purified fluorescent-conjugated anti-rabbit IgG (H+L) and Texas Red anti-mouse IgG (H+L) were from Vector Laboratories. Citifluor glycerol solution was from Agar Scientific Ltd. Synthetic culture medium 199 was obtained from Life Technologies. Nonenzymatic cell-dissociation solution, phosphoramidon, and thiorphan were from Sigma-Aldrich Co Ltd. PD159790 was obtained from Parke-Davis Pharmaceutical Research Division. All other reagents were of analytical or electron microscopy grade.

Cell Culture

Human umbilical cords were collected from the Rosie Maternity Hospital, Cambridge, UK. Endothelial cells were obtained from the umbilical vein by collagenase digestion as previously described and grown and maintained at 37°C in a CO2 incubator (95% air/5% CO2) and grown and maintained at 37°C in a CO2 incubator (95% air/5% CO2). Endothelial cells were obtained from Life Technologies. Nonenzymatic cell-dissociation solution, phosphoramidon, and thiorphan were from Sigma-Aldrich Co Ltd. PD159790 was obtained from Parke-Davis Pharmaceutical Research Division. All other reagents were of analytical or electron microscopy grade.

Subcellular Fractionation by Sucrose Density Gradient Centrifugation

Stored cells were thawed, diluted with medium 199, pelleted in a Denley centrifuge (400g, 5 minutes, 23°C), and then resuspended in homogenate buffer (0.25 mol/L sucrose, 10 mmol/L HEPES, 1 mmol/L N-ethylmaleimide, 10 μmol/L diginiton; pH 6.9). The cells were homogenized by 4 strokes of a glass plunger homogenizer and then spun in an Ole Dich microcentrifuge (2×500g, 5 minutes, 4°C) to remove cell debris. The homogenate was layered onto a sucrose gradient (ρ = 1.04 to 1.30 g/mL) and spun in an SW 41 Ti Beckman preparative swinging bucket rotor (120 000g, 3 hours, 4°C). Fractions (0.75 mL) were removed sequentially from the top of the tube, and density was measured directly by weighing.

ECE Activity and the Effect of pH in Subcellular Endothelial Fractions

Fractions were preincubated in labeling solution (10 mmol/L HEPES, 1 mmol/L N-ethylmaleimide, 0.1% Triton X-100; pH 6.9) in the presence of enzyme inhibitors for 1 hour at 37°C before addition of big ET-1 (final concentration 20 nmol/L). After an additional 2 hours of incubation at 37°C, 0.1-mL aliquots were obtained, and ET was measured by radioimmunoassay. The effect of pH (range 3.0 to 8.3) on ECE activity was determined in fractions 5 through 10 (density 1.07 to 1.19 g/mL; n = 4). The effect of the enzyme inhibitors 0.1 and 100 mmol/L phosphoramidon, 30 μmol/L PD159790, and 10 mmol/L thiorphan was investigated under conditions designed to favor ECE-1 activity (pH 6.9) or under conditions designed to favor ECE-2 activity (pH 5.4). The IC50 values for inhibition of ECE activity by phosphoramidon were determined by combining fractions 5 and 6 and preincubating aliquots with the inhibitor (0.01 to 30 mmol/L; n = 3). Maximal inhibition was determined using 100 mmol/L phosphoramidon, and results were analyzed using Fig P (Biosoft). The pH conditions (pH 5.0 to 7.0) had no effect on ET-1 stability. Fractions were incubated with ET peptide (6.7 nmol/L) for 2 hours at 37°C, and the levels of ET detected in the culture medium were 5.9, 5.5, and 6.3 nmol/L (mean n = 2) at pH 5, 6, and 7, respectively.

Measurement of ET, von Willebrand Factor, and 5'-Nucleotidase Activity

ET was measured in fractions by radioimmunoassay. A 200 μL sample of ET standard (range 0.5 to 500 fmol per tube) was incubated overnight at 4°C with 100 μL of antibody raised against ET at 1:10 000-fold dilution. [125I]-ET-1 (~13 000 cpm/100 μL) was added to the supernatants, and these were incubated overnight at 4°C. Amerlex-M reagent was added (1 hour, 20°C), and the tubes were placed in a magnetic rack. Supernatants were discarded, and bound radioactivity in the precipitates was counted. von Willebrand factor was measured by using an enzyme-linked immunosassay (Innubind) (n = 3) with absorbance readings at 450 nm. 5'-Nucleotidase activity was measured spectrophotometrically at 340 nm by using a 5'-nucleotidase kit (Sigma Diagnostics) (n = 3).

Localization of ECE-2-Like Immunoreactivity by Using Confocal Microscopy

HUVECs were grown on glass coverslips in synthetic culture medium 199 (n = 3). The cells were rinsed in 0.1 mol/L PBS, fixed, permeabilized by incubation with methanol/acetone (1:1; 10 minutes, −20°C), rinsed in PBS, and blocked in 10% FCS for 30 minutes at 23°C. The cells were labeled with antisera raised against ECE-2 (10 μg/mL), von Willebrand factor (1:25), or both (18 hours, 4°C). Cells were washed in PBS (6×5 minutes, 4°C). Immunoreactivity was detected by using fluoresceinated anti-rabbit IgG (20 μg/mL), Texas Red anti-mouse IgG (20 μg/mL), or both together. Coverslips were washed in PBS (6×5 minutes, 4°C), dipped in distilled water, and mounted onto glass microscope slides using glycerol. Cells were viewed using a Leica TCS 4D confocal laser scanning microscope (Leica) equipped with an argon krypton laser source, Leica 63× oil immersion objective, and dual-channel photodetectors. Optical sections were collected at excitation wave-lengths of 488 and 568 nm to image the distribution of FITC- and Texas Red–labeled antisera, respectively. Colocalization of double-labeled antisera was determined by electronic overlay of signals obtained from channels 1 and 2.

Results

ECE activity was determined by conversion of exogenously added big ET-1 to ET-1 in subcellular fractions obtained by sucrose density gradient centrifugation of HUVEC homogenates. In the absence of big ET-1, the concentration of ET was <150 pmol/L, reflecting low-level basal release of peptide over the course of the experiment (Figure 1). When big ET-1 was added to the incubation solution, the concentration of ET was highest in dense sucrose fractions (1.10 to 1.14 g/mL).

Conversion of big ET-1 was almost abolished by 100 μmol/L phosphoramidon, a dual inhibitor of ECE and neutral endopeptidase 24.11 (NEP), but was unaffected by 30 μmol/L PD159790, the ECE-1 selective inhibitor, at pH 6.9 (Figure 2). Little or no activity was observed in fractions 1 through 4 and fraction 11. A sharp peak of 5'-nucleotidase activity, a plasma membrane marker, was detected in fractions 6 through 8. Only low-level activity was detected in fractions 1 through 5 and 9 through 11. von Willebrand factor, a storage granule glycoprotein, was mainly associated with fractions 1 through 4 and fractions 8 through 10. Only
low concentrations of von Willebrand factor were detected in fractions 5 through 7 and fraction 11.

Because predominant ECE activity was detected in fractions 5 through 10, these were used in subsequent experiments. The pH for optimal ECE activity was found to be 6.0 in each of these samples (Figure 3), a value intermediate between that reported for ECE-1 and ECE-2. Experiments were carried out at pH 6.9, which favors ECE-1 activity, and at pH 5.4, which favors ECE-2 activity, to characterize enzymes that contribute to conversion of exogenously added big ET-1 in HUVECs. ECE activity was markedly inhibited using 30 μmol/L PD159790 at pH 6.9 (mean for fractions 5 through 10 55.2%; range 45.9% to 61.5%; n = 3), indicating expression of ECE-1 (Figure 4). However, ECE activity was largely unaffected by 30 μmol/L PD159790 at pH 5.4 (mean

Figure 1. Representative plot showing ECE activity in subcellular fractions of human umbilical vein homogenates isolated by sucrose density gradient centrifugation. Enzymatic activity, measured by conversion of 20 nmol/L big ET to ET in each fraction (●), was almost abolished by 100 μmol/L phosphoramidon (▲) but was unaffected by 10 μmol/L thiorphan (●). Basal release of ET-1, measured in fractions to which big ET-1 was not added, was <150 pmol/L (●). I.R. indicates immunoreactivity.

Figure 2. Representative plots showing comparison of ECE activity (A), 5′-nucleotidase activity (5′-ND) (B), and von Willebrand factor (vWF) (C) in subcellular endothelial fractions obtained by sucrose density gradient centrifugation. ECE activity was primarily associated with fractions 5 through 10 and was inhibited by 30 μmol/L PD159790, the ECE-1 selective inhibitor, at pH 6.9. A sharp peak of 5′-nucleotidase activity, a plasma membrane marker, was detected in fractions 6 through 8. von Willebrand factor was mainly associated with fractions 1 through 4 and fractions 8 through 10.

Figure 3. Effect of pH on ECE activity in endothelial cell fractions obtained by sucrose density gradient centrifugation. Optimum ECE activity was obtained at pH 6.0 in all fractions examined. Values are mean ± SEM, n = 4.

Figure 4. Effect of pH on sensitivity of ECE activity to 30 μmol/L PD159790 and 0.1 μmol/L phosphoramidon. ECE activity was inhibited by 30 μmol/L PD159790 at pH 6.9 but was insensitive to 30 μmol/L PD159790 at pH 5.4 (A). ECE activity was only weakly affected by 0.1 μmol/L phosphoramidon treatment at pH 6.9 but was inhibited markedly at pH 5.4 (B). No further inhibition in ECE activity was observed when fractions were incubated with 100 μmol/L phosphoramidon at pH 5.4 (not shown). Values are mean ± SEM with significance defined by P < 0.05 using an unpaired t test. The number of experiments for each treatment is shown in a box at the base of each bar.
inhibition for fractions 5 through 10 4.3%; range 0% to 14.7%; n=4). The detectable activity measured in the presence of the ECE-1 inhibitor at pH 5.4 is consistent with expression of a converting enzyme that is distinct from ECE-1.

To confirm expression of ECE-2 in HUVECs, the sensitivity of ECE activity to 0.1 μmol/L phosphoramidon was examined at pH 5.4 and 6.9. At pH 7.9, ECE activity was only weakly affected by 0.1 μmol/L phosphoramidon treatment (mean inhibition for fractions 5 through 10 15.9%; range 0% to 28%; n=3) (Figure 4). However, when experiments were repeated at pH 5.4, sensitivity was increased (mean inhibition for fractions 10 through 70.5%; range 60.4% to 81.4%; n=3). No further inhibition in ECE activity was observed when fractions were incubated with 100 μmol/L phosphoramidon at pH 5.4 (mean inhibition 66.6%; range 60.0% to 79.6%; n=3), indicating a maximal or near maximal effect of the inhibitor when used at a concentration of 0.1 μmol/L. The IC50 value for inhibition of ECE activity by phosphoramidon at pH 5.4 was 1.5±0.4 nmol/L (Figure 5).

To determine the localization of ECE-2 in HUVECs, permeabilized cells were labeled with antisera raised against bovine ECE-2 and human von Willebrand factor. Confocal microscopy showed a differential distribution for the two antibodies. A punctate pattern of immunofluorescence staining was observed for ECE-2 within the cell cytosol, whereas von Willebrand factor was detected in discrete, rod-shaped structures resembling endothelial cell storage granules called Weibel-Palade bodies (Figure 6).

**Discussion**

Although the subcellular localization of ECE-1α and ECE-1β has previously been reported in HUVECs, there is less evidence for expression of other ECEs in nonbovine mammalian tissues. Important biochemical information has been obtained from studies using cells transfected with cDNA encoding ECE-2. However, it is not known whether ECE-2 is expressed in native endothelial cells or, indeed, whether the enzyme behaves similarly in native endothelial cells. In the present study, we have identified expression of ECE-2 in human endothelial cells and report on its biochemical characteristics and subcellular localization.

ECEs are characterized by their sensitivity to phosphoramidon, a dual ECE and NEP inhibitor, and can be distinguished from NEP by the NEP selective inhibitor thiorphan. ECE activity was measured in fractions of HUVEC homogenates obtained by sucrose density gradient centrifugation by measuring conversion of exogenously applied big ET-1 to the mature peptide using radioimmunoassay. In all subcellular fractions examined, conversion of big ET-1 was catalyzed by a phosphoramidon-sensitive, thiorphan-insensitive protease consistent with that of an ECE.

The localization of ECE activity was compared with that of 5'-nucleotidase activity, a plasma membrane marker and von Willebrand factor, an endothelial cell storage granule glycoprotein. The two distinct peaks of von Willebrand factor in the subcellular fractions are consistent with the presence of the glycoprotein in the cytosol (buoyant fractions with density <1.06 g/mL) and storage granules (dense fractions ≅1.12 g/mL). It is possible that some of the von Willebrand factor detected in the cytosolic fractions was derived from vesicles or storage granules that degraded during the fractionation procedure. Negligible levels of 5'-nucleotidase activity were detected in fraction 10, which contained both ECE activity and von Willebrand factor, thus providing evidence for localization of ECE to a nonplasmalemmal compartment, possibly endothelial cell–specific storage granules called Weibel-Palade bodies. The colocalization of ECE and 5'-nucleotidase in fractions 6 through 8 suggests conversion of big ET-1 by ECE expressed on the plasma membrane but may also include activity derived from ECE within intracellular compartments located in the plasma membrane–enriched fraction.

The effect of pH on ECE activity has been reported in membranes obtained from Chinese hamster ovary (CHO)/ECE-1 or CHO/ECE-2 cells, with optimal activity determined at pH 6.8 and pH 5.5 for ECE-1 and ECE-2, respectively. In the present study, optimal ECE activity was observed at pH 6.0, a value intermediate between that reported for ECE-1 and ECE-2. This finding may reflect subtle species differences between bovine and human ECEs, or, alternatively, it may indicate expression of both ECE-1 and ECE-2 in HUVECs.

ECE-2 mRNA has been detected in cultured HUVECs, and so in this study, we investigated the possibility that...
ECE-2 contributes to the converting enzyme activity in these cells. Although both ECE-1 and ECE-2 are phosphoramidon-sensitive, thiorphan-insensitive proteases, the two enzymes can be differentiated by the aforementioned pH dependence for optimal activity\textsuperscript{10,19} and by differential sensitivity to phosphoramidon, which has IC\textsubscript{50} values for inhibition of ECE-1 and ECE-2 of 1 \textmu mol/L and 4 nmol/L, respectively.\textsuperscript{10} With the use of the mass action equation, 0.1 \textmu mol/L phosphoramidon would be expected to inhibit only 10% of ECE-1 but >95% of ECE-2. A novel series of quinazoline inhibitors of ECE can also be used to differentiate the two enzymes.\textsuperscript{14} PD069185 inhibits ECE-1 with an IC\textsubscript{50} value of 1.1 \textmu mol/L with no effect on ECE-2 at 100 \textmu mol/L. PD159790, which was used in the present study, has slightly lower potency at ECE-1 than PD069185 but has no cellular toxicity at concentrations up to 100 \textmu mol/L.\textsuperscript{14} The higher sensitivity of big ET-1 conversion by PD159790 in fractions 5 through 10 at pH 6.9 compared with pH 5.4 is indicative of expression of ECE-2 in human endothelial cells. This hypothesis is supported by the higher sensitivity of big ET-1 conversion by phosphoramidon, which was observed at pH 5.4 compared with pH 6.9. The IC\textsubscript{50} value determined for phosphoramidon at pH 5.4 (IC\textsubscript{50} 1.5 nmol/L) is similar to that previously reported for ECE-2–transfected CHO cells (IC\textsubscript{50} 4 nmol/L).\textsuperscript{10}

Several findings have recently indicated the possible existence of other converting enzymes in mammalian tissues. Neuronal cells, which lack detectable levels of ECE-1 mRNA,\textsuperscript{19} produce ET-3,\textsuperscript{20,21} suggesting the existence of a big ET-3–specific converting enzyme. Interestingly, a novel ECE (ECE-3) was purified by SDS-PAGE from bovine iris microsomes that has specificity for big ET-3.\textsuperscript{22} Although we cannot exclude the possibility that ECE-3 is expressed in human endothelial cells, this is probably unlikely. Whereas ECE-3 has specificity for big ET-3, ET-1 is the predominant mature peptide isoform in human endothelial cells with no detectable production of ET-3 reported.\textsuperscript{23,24} In addition, optimal ECE-3 activity was observed at pH 6.6,\textsuperscript{25} which is higher than the pH determined for optimal ECE activity in HUVECs, suggesting the presence of an ECE that does not have the characteristics of either ECE-1 or ECE-3.

Findings from experiments carried out using CHO/ECE-2 cells transfected with a prepro–ET-1 construct suggest that ECE-2 may convert endogenous big ET-1 to the biologically active peptide in acidic intracellular vesicles of the secretory pathway and not on the cell surface.\textsuperscript{10} The punctate pattern of ECE-2–like immunoreactive staining in the HUVEC cytosol would be consistent with expression of ECE-2 in secretory vesicles and suggests the involvement of ECE-2 in the basal release of ET via the constitutive secretory pathway. The immunofluorescence staining did not colocalize with von Willebrand factor in storage granules previously shown to express ECE-1a and ECE-1b.\textsuperscript{15} This suggests a distinct role for ECE-2 in conversion of big ET-1 to ET-1 in human endothelial cells within the constitutive secretory pathway.

It remains to be determined whether ECE-2, which has acidic pH optimum, has a pathogenic role in diseases in which cellular pH is reduced, eg, ischemic heart disease. Lactate accumulation and a concomitant intracellular acidosis (pH 5.8) has been detected in hearts subjected to global ischemia,\textsuperscript{25} and a correlation between myocardial ischemia and increased plasma levels of ET is now well established.\textsuperscript{1,26} In conclusion, the findings of the present study indicate expression of ECE-2 in human endothelial cells. It is suggested that an inhibitor that is nonselective for ECE-1 and ECE-2 may be beneficial in reducing ET production in diseases in which a pathogenic role of the peptide has been implicated. The findings also suggest that an effective converting enzyme inhibitor will be one that can access intracellular vesicles and is therefore permeable to the plasma membrane.

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