Nuclear Localization of Endothelin-Converting Enzyme-1 Subisoform Specificity
Farahdiba Jafri, Adviye Ergul

Objective—The biosynthesis of endothelin-1 (ET-1), the most potent vasoconstrictor with mitogenic properties, involves the processing of intermediate protein big ET-1 by a unique metalloprotease, endothelin-converting enzyme-1 (ECE-1). ECE-1 has 4 subisoforms that possess the same catalytic properties but different localization patterns on the plasma membrane and cytosol. We investigated the trafficking of ECE-1 subisoforms using green fluorescent protein–tagged recombinant enzymes in target and nontarget cells.

Methods and Results—ECE-1 localization was studied using confocal microscopy, which provides evidence for the first time that both ET-1 and ECE-1a are also found in the nuclear compartment in transiently transfected cells as well as in native endothelial cells that endogenously possess the ET system. In cells maintained in high-glucose medium, ECE-1a–specific staining shifted from plasma membrane to intracellular compartments. ECE-1b subisoform, however, is mainly in the cytosolic compartment, indicating a subisoform specificity for nuclear localization.

Conclusions—Our findings define a novel localization pattern for the ET system, which may be differentially regulated under pathophysiological conditions. (Arterioscler Thromb Vasc Biol. 2003;23:2192-2196.)

Key Words: endothelin ■ subcellular localization ■ high glucose

The family of endothelins (ETs) consists of 3 related vasoactive peptides, ET-1, ET-2, and ET-3, which play an important role in cardiovascular pathophysiology and embryonic development.1–3 Because ETs mediate a wide spectrum of physiological functions via autocrine and paracrine mechanisms, ET biosynthesis is tightly regulated and involves a 2-step proteolytic maturation process.4–6 Preproendothelins (PPETs) are first processed at conserved multibasic sites by furin or a furin-like enzyme to generate big ET, which is additionally processed by endothelin-converting enzyme (ECE) to release the biologically active peptide.4 During the release of ET-1, unprocessed big ET-1 is also secreted and cleaved to ET-1 in circulation, indicating the presence of the processing enzyme in the extracellular space.

ECE is a type II membrane protein of the neutral endopeptidase family with a single transmembrane domain, a short N-terminal cytosolic tail, and a large extracellular domain that contains the catalytic site.7,8 There are 3 isoforms, and ECE-1 has a broader tissue distribution and higher expression levels than ECE-2. ECE-3, isolated from bovine iris, has specificity for big ET-3.9 The targeted disruption of the ECE-1 gene results in a lethal phenotype, whereas ECE-2 knockout model does not exhibit significant modification of the mouse phenotype.10 ECE-1 is expressed in the endothelium of all organs as well as in nonvascular cells of many tissues, including brain and neuroendocrine tissues.11

There are 4 subisoforms of ECE-1, ECE-1a through d, that are generated by alternative splicing.12–14 These subisoforms share the same catalytic and transmembrane domains and only differ in the cytosolic N terminus, with no difference in their catalytic properties. ECE-1 subisoforms display different subcellular localization patterns. Original localization studies in endothelial cells did not reveal information, because there were no specific antibodies that can distinguish between ECE-1a, 1b, 1c, and 1d. In transfected fibroblasts or epithelial cell models, ECE-1a and ECE-1c were localized to the plasma membrane, whereas ECE-1b was targeted to intracellular organelles and ECE-1d displayed an intermediate distribution. Considering the importance of the intracellular and extracellular processing of big ET to ET, the subcellular distribution of ECE-1 subisoforms may play a central role in the regulation of the ET system and constitutes an important factor for the efficient inhibition of ECE-1 in pathological conditions. However, because of difficulties in distinguishing subisoforms, regulation of cellular trafficking of ECE-1 proteins is not fully understood. Accordingly, in this study we developed an ECE-1/green fluorescent protein (GFP) fusion protein model to investigate the regulation of intracellular localization of ECE-1 sub isoforms. By using scanning confocal microscopy and subcellular protein markers, we identified a novel localization pattern.
Methods

Generation of ECE-1/GFP Fusion Proteins
cDNAs for human ECE1a, ECE1b, and ECE1c, kindly provided by Dr. Olivier Valdenaire (INSERM U 36 College de France, Paris), were subcloned into the expression vector EGFP-N1 in frame with GFP.

Cell Culture and Expression of ECE-1 Subisoforms
Chinese hamster ovary (CHO) cells between passage numbers 3 to 6 were used for transfection. Cells were cultured in Ham F-12 medium supplemented with 10% (vol/vol) heat-inactivated FBS, antimycotic, and antibiotic at 37°C in a humidified air. CHO cells were transiently transfected with 3 constructs by using lipofectamine-DNA mixture for 5 hours at 37°C for a 75-cm² tissue culture flask. Twenty-four hours after transfection, cells were seeded on chamber slides for confocal microscopy studies. Human microvascular endothelial cells (HMECs) were obtained from the Center for Disease Control and maintained as recommended.

Imaging Studies
Forty-eight hours after transfection, cells grown on chamber slides were fixed in 2% formaldehyde and 0.5% Triton X-100 followed by an overnight incubation in blocking solution. For visualization of cells expressing ECE-1/GFP fusion proteins, cells were mounted in the medium containing glycerol, sodium azide, and PBS. For colocalization, studies cells were incubated with the primary marker antibodies for plasma membrane (CD59), lysosome (Lamp-1), and Golgi complex (58K protein) for 1 hour followed by incubation with secondary antibody (Texas red–conjugated goat anti-mouse IgG). Next, slides were stained with nuclear dye DAPI and visualized using a Molecular Dynamics confocal microscope with an Argon-Krypton laser.

In separate experiments, CHO cells transfected with ECE-1a cDNA were maintained in normal-glucose (5 mmol/L) and high-glucose (25 mmol/L) medium. Forty-eight hours after transfection, cells were fixed in 2% formaldehyde. To study plasma membrane and intracellular localization of ECE-1a, one set of slides prepared from cells maintained in normal- or high-glucose media was permeabilized with 0.5% Triton X-100 and another set was untreated. After blocking, slides were incubated with an anti–ECE-1 antibody overnight followed by incubation with FITC-conjugated secondary antibody. Sections were mounted with ProLong Antifade Kit (Molecular Probes). The slides were viewed with a Zeiss Axiosvert microscope interfaced with a digital spot camera.

Preparation of Cell Extracts
Forty-eight hours after transfection, nuclear and cytoplasmic extracts were prepared using a sucrose gradient method as previously described. Supernatants were aliquoted and stored at −80°C until the day of experiment. The purity of extracts was confirmed by investigating the presence of nuclear (Mab 414, nuclear pore protein) and cytoplasmic (Lamp-1) proteins in nuclear and cytoplasmic fractions.

Immunoblotting
Nuclear and cytoplasmic fractions (30 μg) were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions (5% mercaptoethanol) and transferred to nitrocellulose membranes. Blots were incubated with a mouse monoclonal antibody against GFP and visualized by ECC kit (Amersham Pharmacia Life Science Products).

ELISA of ET-1 and Big ET-1
The concentration of ET-1 in nuclear and cytoplasmic fractions from HMEC and CHO cells was measured using an ELISA kit specific for ET-1. The sensitivity of ET-1 assay is 0.1 to 50 fmol/mL, and the cross reactivity of the antibodies used are reported to be 100% with ET-1. The sensitivity of ET-1 assay is 0.1 to 50 fmol/mL, and the cross reactivity of the assay with both ET-1 and big ET-1 (1-38) is 0.1 to 70 fmol/mL. The cross reactivity of the assay with both ET-1 and big ET-1 is <5% with big ET-3, and <1% with big ET-1 (1-38) and the C-terminal fragment (22-38). Sensitivity of big ET-1 assay is 0.1 to 70 fmol/mL. The cross reactivity of the assay with both ET-1 and C-terminal fragment is <1%.

Results and Discussion

Localization of ECE-1/GFP Fusion Proteins in Transfected CHO Cells
To develop a cell culture model to study the regulation of the intracellular localization of ECE-1 subisoforms individually, we first generated subisoform-specific constructs encoding human ECE-1a/GFP, ECE-1b/GFP, and ECE-1c/GFP fusion proteins and expressed in CHO cells that do not possess endogenous ECE activity. Confocal images taken 48 hours after transfection demonstrated that in addition to plasma membrane localization of ECE-1a, as previously reported, there was a strong nuclear localization signal (Figure 1C). Colocalization with nuclear dye DAPI showed significant staining along the nuclear envelope (for colocalization with other subcellular markers, please see online Figures IA through IN, available at http://atvb.ahajournals.org). hECE-1c/GFP also displayed similar localization pattern (data not shown). In contrast, in similar experiments using hECE-1b/
in nuclear and cytoplasmic extracts prepared from CHO cells expressing ECE-1a/GFP and ECE-1b/GFP fusion proteins. The purity of nuclear (N) fractions prepared from cells transfected with ECE-1a/GFP, whereas ECE-1b/GFP was present only in the cytoplasmic fractions. Whole-cell extracts (W) from mock-transfected CHO cells, there was no immunoreactivity. In native HMEC cells that endogenously express ET-1, using an antibody that recognizes all isoforms, an ~120-kDa band corresponding to the size of ECE-1 was detected in both nuclear and cytoplasmic fractions. A representative of 3 independent experiments is shown.

GFP construct, there was only cytoplasmic expression, which was mainly localized to lysosomal compartment and Golgi complex, as indicated by colocalization with lysosomal marker protein Lamp-1 and Golgi marker 58K protein (online Figures II and IIM) but not nuclear localization (Figure 1F).

Because nuclear localization of ECE-1 has never been reported before, to provide additional support for subisoform-specific localization pattern of ECE-1/GFP subisoforms, we next examined ECE-1a/GFP and ECE-1b/GFP protein levels in nuclear and cytoplasmic extracts prepared from CHO cells transfected with each subisoform. The purity of nuclear fractions was investigated by immunoblotting and confirmed by the lack of any immunoreactivity to cytoplasmic proteins CD59 and Lamp-1 in the nuclear fraction (data not shown). As shown in Figure 2A, an ~150-kDa band corresponding to the ECE-1a/GFP fusion protein was detected in both the cytoplasmic and nuclear fractions (lanes 3 and 4). Consistent with the confocal data, hECE-1b/GFP was present only in the cytoplasmic extracts (lane 1). Cells transfected with GFP alone were included as control, and GFP protein was detected in both fractions at ~30 kDa (data not shown), as previously reported. The lack of nuclear localization for ECE-1b/GFP provided support that ECE-1a/GFP nuclear localization is not directed by signals in the GFP protein but specific for ECE-1a protein.

To gain an understanding of the physiological significance of this localization pattern and determine whether ECE-1 localization is subject to regulation under different growth conditions, CHO cells expressing ECE-1a were maintained in normal- and high-glucose conditions after transfection.

ECE-1 localization was then assessed by immunohistochemistry using an antibody against ECE-1. To separate plasma membrane and intracellular staining, in one set of slides, cells were not permeabilized to determine only the plasma membrane localization. In another set, cells were solubilized and both plasma membrane and intracellular staining pattern were assessed. In cells grown under high-glucose conditions, plasma membrane staining seems to be less than in cells maintained in normal glucose (Figures 3A and 3B). In contrast, when permeabilized, intracellular staining intensity was higher in high-glucose conditions (Figures 3C and 3D), suggesting that there is a shift from plasma membrane to intracellular compartments when cells are cultured in high-glucose medium. There was no significant nonspecific staining, as determined in the absence of primary antibody (not shown).

**ECE-1 Expression Pattern in Target Endothelial Cells**

CHO cells do not endogenously express ECE-1 or ET-1. To investigate whether this nuclear localization pattern is a result of using transfected cells that are normally devoid of ECE-1 activity or is attributable to overexpression of ECE-1/GFP, additional immunoblotting experiments with nuclear and cytoplasmic extracts from HMECs were performed. An approximately ~120-kDa band corresponding to the molecular weight of native ECE-1 protein was detected in both fractions (Figure 2B). The low-molecular-weight band represents a degradation product of ECE-1, which we have previously shown using purified ECE-1. Because the antibody used cannot differentiate between ECE-1 subisoforms, we cannot conclude that nuclear immunoreactivity is attributable to ECE-1a but provides evidence that in native cells that endogenously synthesize ET-1, ECE-1 is found in both nuclear and cytoplasmic compartments and supports our findings obtained with transfected CHO cells.
Evidence for Nuclear ET-1

Because big ETs are the only substrates known for ECE-1 subisoforms, we wanted to determine whether ET-1 is present in the perinuclear compartment close to ECE-1. Confocal analysis of CHO cells transfected with PPET-1 and hECE-1 as well as native HMEC cells that constitutively express ET-1 demonstrated diffuse cytoplasmic staining for ET-1 that also colocalized to the nucleus (Figures 1G through 1L, online Figure III). Next, we measured ET-1 and big ET-1 levels in the cytoplasmic and nuclear fractions of both CHO and HMEC cells using specific ELISA kits. There was no measurable ET-1 in mock-transfected CHO cells. ET-1/big ET-1 levels (femtomole per milliliter) in nuclear fractions from transfected CHO cells and HMECs were 26±9/3±1 and 16±7/2±1, respectively. Corresponding values in the cytoplasmic fractions were 53±19/9±2 and 29±13/6±2. These results confirmed the confocal microscopy findings. Low levels of big ET-1 provided additional support for ECE-1 activity.

Perspectives

This study focused on ECE-1 localization for several reasons. Unlike ECE-1 knockout mice, ECE-2 knockout model does not exhibit any abnormal phenotypes, indicating that ECE-1 is the physiologically significant enzyme. ECE-3 has been recently cloned and preferentially cleaves big ET-3. Although there is no information available on ECE-3 knockout mice, because of substrate selectivity, ECE-3 is not believed to play an important role for ET-1 processing. Another issue that cannot be addressed by the present study is the role of ECE-1a subisoforms in ET-1 processing. Technically, it is extremely difficult to fractionate subcellular compartments and obtain sufficient protein to conduct enzyme kinetics experiments under conditions that mimic the milieu of that particular compartment. Therefore, in this study we cannot determine whether plasma membrane ECE-1 behaves differently than the subisoforms detected in Golgi complex or nucleus. Kinetic studies with purified enzymes do not reveal a difference in catalytic properties of ECE-1a subisoforms. Thus, we do not anticipate a difference in the processing of big ET-1 to ET-1 by different ECE-1a subisoforms. However, as determined in this study, under high-glucose conditions, plasma membrane ECE-1a seems to be shifted to intracellular compartments and provides evidence that regulation of ECE-1 localization might play an important role in ET-1 production. Lastly, markers for nucleus, lysosomes, and plasma membrane were used in colocalization studies. With this present experimental design, we cannot visualize the transport vesicles to assess the localization of other proteases such as furin involved in the processing of PPET-1 to big ET-1.

In conclusion, using several approaches, we have shown that in addition to the conventional localization of ECE-1a and ECE-1b on the plasma membrane and cytosolic compartment, respectively, ECE-1a is also targeted to nuclear membrane. Moreover, ET-1, the major product of this enzyme, can also be found in the nuclear fractions. To the best of our knowledge, this study is the first to report nuclear localization of the ET system. Previous studies have investigated ECE-1 localization using direct immunofluorescent techniques and provided evidence for plasma membrane localization for ECE-1a and ECE-1c, whereas ECE-1b was mainly found in the lysosomes. The present study using endogenously fluorescent ECE-1/GFP fusion proteins and visualizing cells at multiple sections with confocal microscopy was able to detect nuclear ECE-1a localization. Our findings with ECE-1a then prompted us to study nuclear ET-1. ET-1 is believed to exert its potent contractile and mitogenic effects via stimulation of cell-surface receptors. A recent study demonstrated that basic fibroblast growth factor was targeted to both nuclear and cytosolic compartments in pituitary adenomas and nuclear localization correlated significantly with the maximum tumor diameter and invasiveness without its receptor. Although we cannot exclude the possibility that ECE-1a and ET-1 are first targeted to plasma membrane and then taken in to nuclear compartment, our intriguing results suggest that some of the secretory vesicles carrying ET-1 and ECE-1a may be directly sorted to the nucleus and regulated differently under pathological conditions.

Acknowledgments

This work was supported by an AHA Scientist Development Grant (to A.E.).

References


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Arterioscler Thromb Vasc Biol. 2003;23:2192-2196; originally published online October 9, 2003;
doi: 10.1161/01.ATV.000099787.21778.55
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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