Phosphorylation of Endothelin Converting Enzyme-1 Isoforms: Relevance to Subcellular Localization

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Endothelin-converting enzyme (ECE)-1 is a metalloenzyme with four subisoforms, which differ only in their amino-terminal domain. ECE-1a and c are the most common isoforms and are found at the plasma membrane and in the Golgi complex, whereas ECE-1b displays lysosomal localization. We have recently shown that ECE-1a but not ECE-1b also colocalizes with nuclear membrane markers, and that maintenance of cells in high glucose (25 m*M*) promotes relocalization of ECE-1a from the membrane to the intracellular compartment. To investigate the mechanisms involved in this process, we conducted a search for potential phosphorylation sites, which yielded a different number of putative sites for protein kinase (PK)-C and PKA in the amino-terminal region. Stimulation of Chinese hamster ovary (CHO) cells expressing a green fluorescent protein (GFP)-tagged human ECE-1a or ECE-1b with 100 n*M* phorbol myristate acetate (PMA) resulted in phosphorylation of ECE-1a, as determined by immunoprecipitation with an antibody to GFP followed by immunoblotting with an antibody to phosphoserine. Stimulation of cells with PMA also promoted intracellular relocalization, as seen in cells grown under high-glucose conditions. Incubation of cells grown in 25 m*M* glucose with the PKC inhibitor, calphostin C (100 n*M*), partially prevented the relocalization of ECE-1a from the plasma membrane to intracellular compartments. Stimulation of cells with 100 n*M* forskolin caused phosphorylation of ECE-1b and not ECE-1a, which is consistent with the lack of a putative PKA site in the ECE-1a amino-terminal sequence. Although phosphorylation is not required for ECE-1 enzymatic activity, these results suggest that ECE-1 isoforms are phosphorylated and that phosphorylation might play an important role in the regulation of intracellular trafficking of ECE-1 subisoforms. Exp Biol Med 231:713–717, 2006

Key words: ECE; phosphorylation; localization

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Introduction

Endothelin (ET)-1 is derived from posttranslational processing of a precursor (preproendothelin-1 [PPET-1]) in multiple enzymatic steps. This precursor protein is cleaved by dibasic-specific endopeptidases and a carboxypeptidase to yield big ET-1, which is then processed to ET-1 by ET-converting enzyme (ECE)-1 (1–5). The complementary DNAs (cDNAs) for two ECE isoforms, ECE-1 (1–3) and ECE-2 (6), were cloned. Based on their pH properties, ECE-1 has been proposed to be the physiologically significant enzyme (6). There are at least four subisoforms of ECE-1 generated by alternative splicing (7–10), and a comparison of the predicted protein sequences of bovine ECE-1 cDNAs obtained by several groups revealed differences only in the amino-terminal region (1, 2). These subisoforms (ECE-1a–d) display different subcellular localizations. For example, human ECE-1a is strongly expressed at the cell surface, whereas ECE-1b is located intracellularly and ECE-1c displays an intermediate distribution (10). ECE-1d is also found at the cell surface, but in much less quantity than ECE-1a.

ECE-1 undergoes several posttranslational modifications. First, ECE-1 is heavily glycosylated, and deglycosylation abolishes ECE-1 activity (1). Second, Schweizer *et al.* demonstrated that all ECE-1 subisoforms are palmitoylated; however, this modification has no effect on enzyme activity or localization (11). MacLeod and colleagues reported that ECE is constitutively phosphorylated and that casein kinase I phosphorylates ECE isoforms (12). Using mass spectrophotometry, investigators identified Ser 18 and 20 (numbering based on the ECE-1c subisoform) as the phosphorylated residues. However, the regulation and functional consequence of ECE phosphorylation remains unknown. We have recently shown that ECE-1a but not ECE-1b also colocalizes with nuclear membrane markers, and that maintenance of cells in high glucose (25 m*M*) promotes relocalization of ECE-1a from the membrane to the intracellular compartment (13). Based on these observations, we hypothesized that phosphorylation of ECE-1a and ECE-1b by protein kinase (PK)-C, which is activated under high-glucose conditions, may play a role in cellular ECE-1 dynamics.

Materials and Methods

Generation of ECE-1/Green Fluorescent Protein (GFP) Fusion Proteins. cDNAs for human ECE1a and ECE-1b, kindly provided by Dr. Olivier Valdenaire, 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 and 6 were used for transfection. Cells were cultured in Ham's F12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, and antifungal and antibiotic agents at 37°C in humidified air. CHO cells were transiently transfected with three constructs *via* a lipofectamine-DNA mixture for 5 hrs at 37°C in a 75-cm^2 tissue culture flask; transfection efficiency was determined by monitoring for GFP protein and was approximately 70%. To determine *in vivo* phosphorylation of ECE-1a by PKC and PKA, 48-hr posttransfection cells were incubated with 100 n*M* phorbol myristate acetate (PMA) or forskolin for 30 mins. At the end of the stimulation, cell extracts were prepared, immunoprecipitated with an antibody to GFP, and probed with antibodies to phosphoserine or phosphothreonine after separation on a sodium dodecyl sulfate (SDS) gel. Untreated cells were included to determine basal ECE-1 phosphorylation levels. In addition, mock-transfected CHO cells that do not express endogenous ECE were included as negative controls.

Preparation of Cell Extracts and Immunoprecipitation. After stimulation with PMA or forskolin, cells were rinsed with PBS, and pellets were resuspended in 20 ml of Buffer A (20 m*M* Tris-HCl, pH 7.4; 20 μ*M* pepstatin A; 1 m*M* phenylmethylsulfonyl fluoride; and 250 m*M* sucrose), were homogenized at 400 rpm with a Tekmar tissumizer. The homogenate was centrifuged at 1000 *g* for 10 mins, and the resulting supernatant was further centrifuged at 100,000 *g* for 60 mins. The crude membrane fraction was solubilized in buffer A containing 2.5% polyoxyethylene-10-lauryl ether for 30 mins. The mixture was then centrifuged at 50,000 *g* for 60 mins, and the supernatant was incubated with a monoclonal antibody to GFP (Invitrogen, Carlsbad, CA) overnight at 4°C. Protein G beads provided with the Protein G Immunoprecipitation Kit (Sigma, St Louis, MO) were added and the incubation was continued for 2 hrs at 4°C. After washing, the ECE-1a/GFP fusion protein was eluted with sample buffer and 50 μg protein was separated by SDS-polyacrylamide gel electrophoresis under reducing conditions (5% mercaptoethanol) and transferred to nitrocellulose membranes. Blots were incubated with antibodies to phosphoserine or phosphothreonine (Santa Cruz Technologies, Santa Cruz, CA) and visualized by horseradish peroxidase–conjugated rabbit anti-mouse IgG (1:1000; ECL kit, Amersham Pharmacia Life Science Products, Arlington Heights, IL). Bands corresponding to the ECE-1/GFP fusion protein were quantified by scanning densitometry.

Immunohistochemistry. CHO cells transfected with ECE-1a cDNA were maintained in normal (5 m*M*) or high (25 m*M*) glucose medium. Cells were fixed in 2% formaldehyde 48 hrs after transfection. 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 not treated. After blocking, slides were incubated with an anti–ECE-1 antibody overnight, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Sections were mounted with ProLong Antifade Kit (Molecular Probes, Eugene, OR). The slides were viewed with a Zeiss Axiovert microscope interfaced with a digital Spot camera. In a subset of experiments, cells were grown in 5 m*M* glucose, stimulated with 100 n*M* PMA for 30 mins, and then fixed. To determine the role of PKC in high glucose–mediated translocation of ECE-1a, 24 hrs after transfection, the PKC inhibitor calphostin C (100 n*M*) was added to the medium of cells grown in high glucose; these cells were fixed at 48 hrs. An antibody to ECE-1 that recognizes the carboxy-terminus was kindly provided by Dr. Emoto from Kobe, Japan.

Statistics. All experiments were repeated in four independent experiments and results are given as mean ± SEM. The density of phosphorylated ECE-1/GFP isoforms with and without stimulation was compared by Student's *t* test.

Results

Phosphorylation of ECE-1 Subisoforms. Using the NetPhos 2 program (http://www.cbs.dtu.dk/databases/PhosphoBase/predict; Ref. 14), we conducted a sequence analysis to identify potential phosphorylation sites in ECE-1, which yielded consensus sequences for PKC and PKA (Table 1). To determine whether phosphorylation occurs in intact cells, CHO cells expressing ECE-1/GFP isoforms were stimulated with 100 n*M* PMA or forskolin for 30 mins. Untreated cells were included to determine basal ECE-1 phosphorylation levels. In addition, mock-transfected CHO cells that do not express endogenous ECE were included as negative controls. As shown in Figure 1, in untreated control cells transfected with ECE-1/GFP isoforms, a band corresponding to ECE-1/GFP isoforms (~150 kDa) was detected, indicating a basal level of ECE-1 phosphorylation that is higher in ECE-1b. After treatment with the PKC activator, PMA, for 30 mins, the band intensity of all phosphorylated ECE-1 isoforms, especially that of ECE-1a, was increased. Similarly, when probed with an antibody to phosphothreonine, after treatment with the PKA activator, forskolin, there was a basal level of phosphorylation for both subisoforms, but PKA activation caused an increase in phosphorylated ECE-1b and not in ECE-1a.

Role of PKC in Intracellular Dynamics of ECE-1a. To gain an understanding of the physiologic significance and mechanism of ECE-1a phosphorylation, CHO cells expressing ECE-1a were maintained in normal and

Table 1. Putative Phosphorylation Sites in ECE1a Subisoforms[a]

	ECE-1a	ECE-1b
PKC	S39 NFH**S**PRS	S51 NFH**S**PRS
	—	T20 GMS**T**YKR
PKA	—	T25 KRA**T**LDE

[a] NetPhos 2 program (http://www.cbs.dtu.dk/databases/PhosphoBase/predict).

high-glucose conditions after transfection. ECE-1 localization was assessed by immunohistochemistry using an antibody against ECE-1. In cells grown under high-glucose conditions, plasma membrane immunoreactivity is less than in cells maintained in normal glucose (Fig. 2A and B). In contrast, when permeabilized, intracellular immunoreactivity was higher in high-glucose conditions (Fig. 2C and D), suggesting that 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).

In cells maintained in normal glucose and challenged with the PKC activator, PMA (100 n*M*), for 30 mins before fixation of the cells, there was a similar shift of plasma membrane immunoreactivity toward the intracellular compartments (Fig. 3A and C). Incubation of cells grown in high-glucose conditions with the PKC inhibitor, calphostin C, partially prevented the relocation of membrane ECE-1a into intracellular compartments, indicating that PKC is involved in this process (Fig. 3B and D).

Discussion

This study tested the hypothesis that ECE-1a and ECE-1b subisoforms are phosphorylated by PKC and PKA and that phosphorylation of ECE-1 subisoforms under normal and/or pathophysiologic conditions, such as hyperglycemia, contribute to the regulation of intracellular localization of

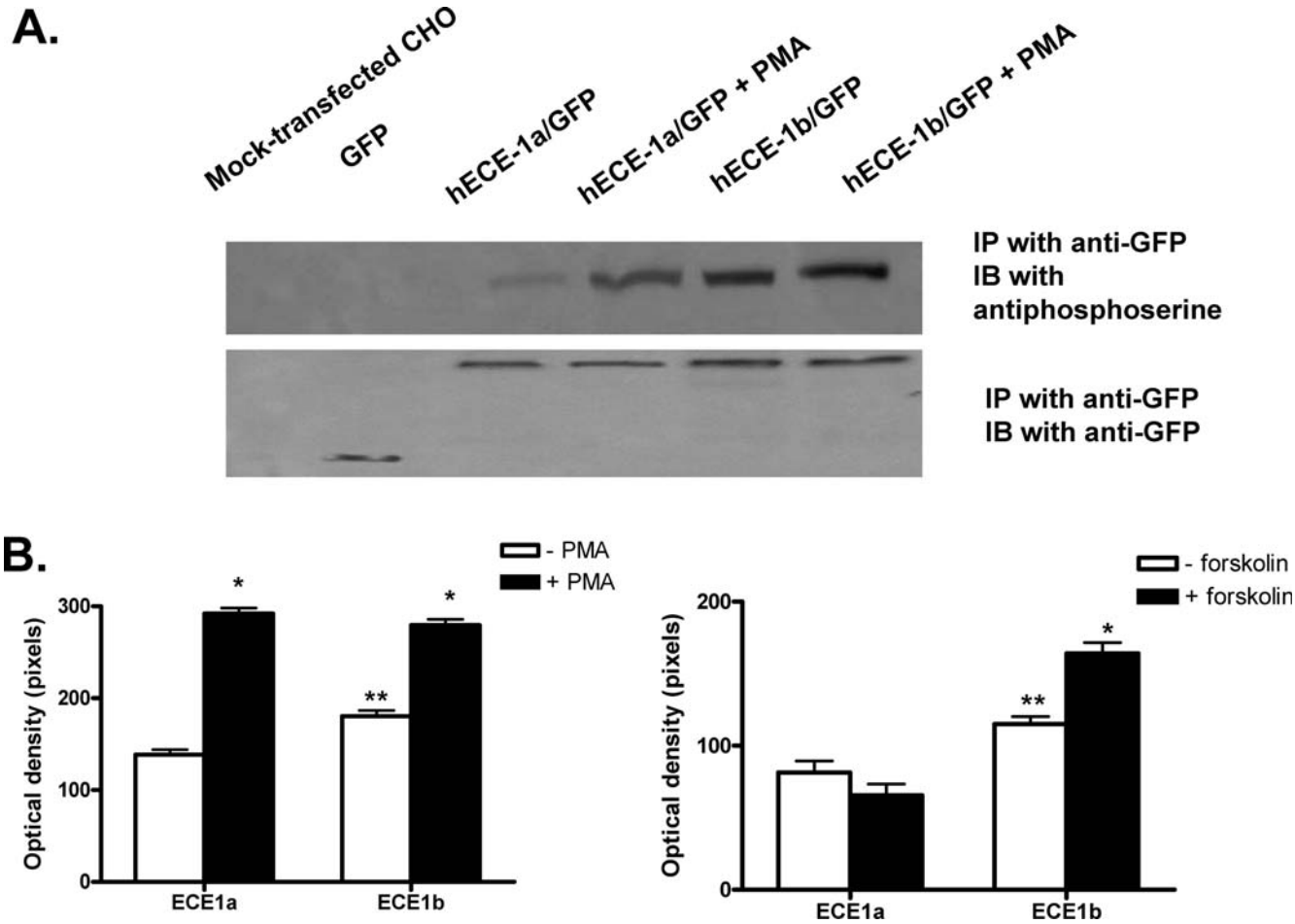

Figure 1. (A) A representative immunoblot for phosphorylation of ECE-1 isoforms in CHO cells transfected with ECE-1/GFP cDNAs. Cells were left untreated or stimulated with PMA or forskolin. After immunoprecipitation with an antibody to GFP, 50 μg of membrane protein prepared from CHO cells expressing ECE-1/GFP fusion proteins was separated on an SDS gel and immunoblotted using an antibody against phosphoserine or phosphothreonine for PMA and forskolin stimulations, respectively. The membranes were stripped and reprobed with an antibody against GFP to ensure equal protein loading. CHO cells expressing the GFP protein alone, and mock-transfected cells were included as controls. Densitometric analysis of bands in four individual experiments are summarized in Panel B. Results are presented as mean ± SEM. $^{*}P < 0.005$ vs. respective unstimulated ECE-1a or ECE-1b; $^{**}P < 0.05$ vs. unstimulated ECE-1a.

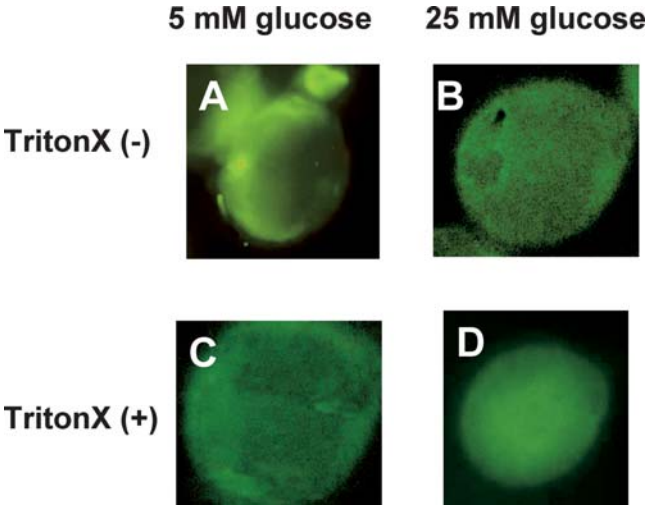

Figure 2. ECE-1a localization pattern under high-glucose conditions. Slides were prepared from CHO cells expressing ECE-1a and grown in normal (A and C) and high (B and D) glucose conditions. Cells were incubated with an antibody to ECE-1 followed by a FITC-conjugated secondary antibody in untreated (A and B) or permeabilized (C and D) cells. Under high-glucose conditions, the diffuse immunoreactivity pattern for plasma membrane decreased, whereas intracellular immunoreactivity increased. (Color figures are available in the on-line version.)

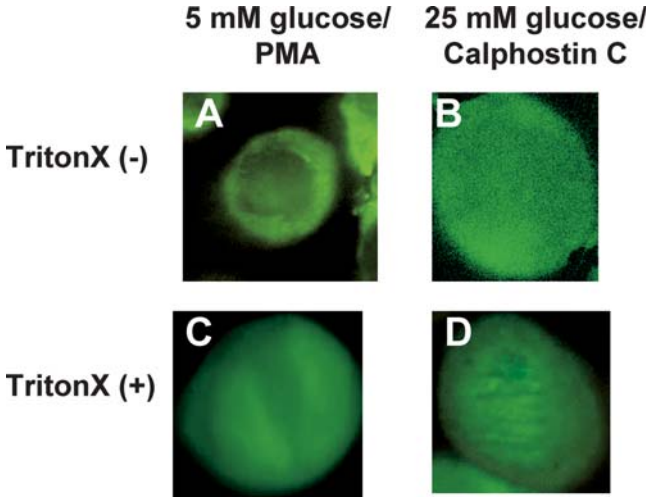

Figure 3. Role of PKC in ECE-1a localization. (A and C) Cells were grown in normal glucose–containing medium and stimulated with PMA before fixation. (B and D) Cells were incubated with calphostin C for 24 hrs. Slides were incubated with an antibody against ECE-1 followed by a FITC-conjugated secondary antibody in untreated (A and B) or permeabilized (C and D) cells. PMA treatment promoted intracellular relocalization of ECE-1a, whereas calphostin C treatment partially prevented the intracellular relocalization of ECE-1a in cells grown in 25 m*M* glucose. (Color figures are available in the on-line version.)

these enzymes. Our findings provide evidence that the PKC activator, PMA, stimulates serine phosphorylation of ECE-1a and ECE-1b. ECE-1, mainly localized to the plasma membrane, is shifted to intracellular compartments under high-glucose conditions and this is partly mediated by PKC. Direct activation of PKC by PMA also promotes relocalization of ECE-1a. Taken together, these results suggest that phosphorylation of ECE-1a by PKC may affect the intracellular localization of ECE-1a, and this may be a mechanism for the regulation of ECE-1a dynamics under conditions, such as diabetes, associated with increased PKC activity.

ECE-1 is a type II integral membrane protein with no apparent signal sequence. Its structural features include a short cytoplasmic amino-terminal domain followed by a hydrophobic membrane segment (signal anchor domain) and a large COOH-terminal ectodomain containing the catalytic site. Several groups have reported that this cytoplasmic tail is important for the intracellular localization of the enzyme (7, 8, 10, 15, 16). For example, human and rat ECE-1a and ECE-1c isoforms are located at the plasma membrane, whereas ECE-1b exhibits an intracellular localization (7, 8, 10, 15, 16). Emoto and colleagues demonstrated that novel proline-containing signals in the cytoplasmic domain of bovine ECE-1a target the enzyme to the lysosomal compartments in a constitutive fashion (17). These studies provided evidence that the amino-terminal cytoplasmic domain may be involved in the sorting of ECE-1 subisoforms. We have previously shown that deletion of the cytoplasmic amino-terminal tail (residues 1–55) of ECE-1a results in the cleavage of a potential signal peptide located in the signal anchor domain, leading to the partial secretion of the recombinant enzyme into the media. However, the truncation of the amino-terminal and/or the signal anchor domain does not affect the activity of ECE-1a, providing evidence that the hydrophobic signal anchor domain alone is not sufficient for the membrane anchoring of ECE-1a and that the amino-terminal domain of ECE-1a is important for membrane targeting as well as for the intracellular localization of the enzyme (4, 5). The intriguing findings of the current study, that constitutive phosphorylation of ECE-1b, which is mainly found intracellularly in the lysosomal compartment, is higher than that of ECE-1a, and that phosphorylation of ECE-1a coincides with relocalization of the enzyme to intracellular compartments after exposure to PKC or high glucose, indicate a role for phosphorylation in the regulation of ECE-1a localization.

ECE-1 is a zinc-dependent metalloenzyme and, to date, glycosylation is the only posttranslational modification known that is required for enzyme activity (1). Based on our findings that high glucose or PKC activation promotes relocalization of the enzyme, it is not unreasonable to speculate that in pathologic conditions, such as diabetes, which is associated with hyperglycemia, increased PKC activity, and elevated ET-1 levels (18–21), regulation of ECE-1a localization by phosphorylation may be a regulatory step in ET-1 biosynthesis. However, further experiments regarding ECE-1 isoform localization using intracellular markers and confocal microscopy are needed to determine the trafficking of ECE-1 isoforms. Another

point of interest is that our results differ from those reported by MacLeod *et al*. (12). They reported that casein kinase I (CK I) phosphorylates ECE-1b and ECE-1c isoforms in the amino-terminal domain in human umbilical vein endothelial cells or CHO cells expressing FLAG-tagged ECE-1 isoforms by using an *in vivo* labeling method. The authors discuss the idea that addition of the FLAG tag may have affected the phosphorylation under those conditions. In our study, ECE-1 isoforms were tagged with GFP. Although expression of GFP alone was not associated with phosphorylation, it is possible that addition of GFP influences the overall structure and make it more accessible to phosphorylation by PKC. Future experiments in which putative sites are mutated are warranted to provide direct evidence. Nevertheless, the results of the current study are important to demonstrate that ECE-1 is phosphorylated and that phosphorylation of ECE-1 subisoforms may play an important role in the intracellular dynamics.

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