Regulation of Endothelin-Converting Enzyme–1 Expression in Human Neuroblastoma Cells

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In recent years endothelin-converting enzyme (ECE-1) has been suggested to play an important role in amyloid-β peptide metabolism as one of the amyloid-degrading enzymes. In this connection, the analysis of the levels of expression and distribution of ECE-1 in the brain under normal and pathologic conditions could be important in neurodegeneration and pathogenesis of Alzheimer disease. In our previous studies, we have demonstrated that expression of ECE-1 was significantly reduced in the cortex of adult rats after 15 mins of global ischemia. It was also significantly reduced in the striatum of rats subjected to prenatal hypoxia. In the present study, we analyzed effects of hypoxia and oxidative stress on ECE-1 in human neuroblastoma NB7 cells and effects of the cholinergic agonist carbachol and the phorbol ester, phorbol 12-myristate 13-acetate (PMA). We have found that chronic (24 hrs) hypoxia and oxidative stress resulted in 30% and 20% decrease in expression of ECE-1 at the protein level, respectively, although at the level of ECE-1 mRNA there were no statistically significant changes. Serum withdrawal from the incubation medium as well as addition of carbachol or PMA for 24 hrs also led to a significant reduction of the levels of ECE-1 protein in NB7 cells. Further study of the downstream signaling cascades involved in downregulation of ECE expression in NB7 cells and primary neuronal cells might provide us with new insights into possible therapeutic strategies for prevention or treatment of Alzheimer disease in elderly patients and those who suffer from stroke or cerebrovascular disorders. Exp Biol Med 231:1048–1053, 2006

Key words: ECE-1; human neuroblastoma NB7 cells; hypoxia; carbachol; phorbol esters; PMA

Introduction

Endothelin-converting enzyme (ECE) is a zinc metalloprotease that catalyzes the conversion of the 38-residue inactive intermediate molecule of big endothelin (big ET-1) to a very potent vasoconstrictor ET-1 via a specific cleavage (1, 2). It was first purified from rat lung (3), but then was also found in a variety of tissues. It is most abundant in endothelial cells but is also expressed by exocrine cells, smooth muscle cells, neurons, and glia in the brain (4–6). To date, four isoforms of human ECE-1 differing only in a part of their N-terminal cytoplasmic region, but that cleave big ETs with similar efficiencies have been characterized: ECE-1a, ECE-1b, ECE-1c, and ECE-1d (7, 8). Although the relative levels of the isoform mRNA species vary between human tissues, ECE-1c mRNA is generally the predominant isoform message. There are distinct subcellular localizations for the four isoforms: whereas ECE-1a, ECE-1c, and ECE-1d proteins are localized mainly at the cell surface, ECE-1b was found to be intracellular and showed significant colocalization with a marker protein for the trans-Golgi network (7). There are no significant differences in the catalytic properties between them, so it has been suggested that intracellular ECE-1 localized in Golgi and vesicles might be involved in processing of big ET, whereas cell-surface ECE-1 may metabolize other regulatory peptides (9).

Although ECE-1 has been regarded as a highly specific endopeptidase, it was demonstrated to be able to hydrolyze a number of other biologically active peptides, such as bradykinin, substance P, neurotensin, angiotensin I, and insulin B chain, although it is not yet clear whether any of these, or other peptides, are physiologic substrates of ECE-1 (10, 11). Recently, it was demonstrated that ECE-1 can also degrade amyloid beta peptide (Aβ), which made it an important player in the arena of Alzheimer disease (AD) (12).

It is becoming more obvious that the development of AD can be promoted by cardiovascular lesions, ischemia, and stroke (13). Taking into account that ECE-1 can contribute to amyloid metabolism, we have previously

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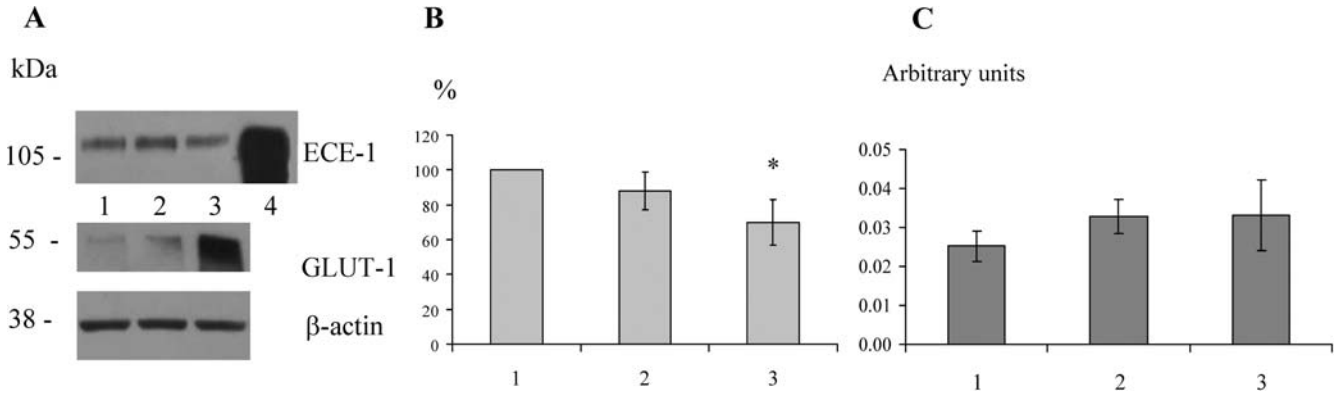

Figure 1. Expression of ECE-1 in control NB7 cells and cells treated with H_2O_2 or under hypoxic conditions. NB7 cell were grown until 60%–70% confluence and treated with 40 μ*M* H_2O_2 or kept in an incubator with reduced O_2 content to 2.5% for 24 hrs. GLUT1 was used as a marker of hypoxia. β-actin was used as a loading control. (A) Representative Western blots showing effects of hypoxia and oxidative stress on expression of ECE-1. (B) Statistical data of blot densitometry. Data are mean ± SEM ($n = 6$). (C) Quantification of the effect of hypoxia and oxidative stress on ECE-1 mRNA levels by real-time PCR. The mean results ± SEM ($n = 10$ for H_2O_2 and $n = 12$ for hypoxia). The data were related to GAPDH mRNA. (1) Control cells, (2) cells treated with 40 μ*M* of H_2O_2, (3) cells grown under 2.5% O_2, and (4) positive control for ECE-1. *$P < 0.05$.

analyzed levels of expression of ECE-1 in the brain cortex of rats after 15 mins of global ischemia and found a significant decrease of ECE-1 protein levels in both hemispheres which returned to normal after 2 hrs of reperfusion (14). We have also demonstrated that ECE-1 levels were lower in the striatum of rats subjected to prenatal hypoxia (15). In the present study, we analyzed the effects of 2.5% hypoxia and oxidative stress on expression of ECE-1 in human neuroblastoma NB7 cells ands the effect on its expression of a cholinergic agonist carbachol and the phorbol ester phorbol 12-myristate 13-acetate (PMA), which were previously shown to upregulate the α-secretase pathway of amyloid precursor protein processing, preventing formation of Aβ peptide (16, 17).

Materials and Methods

Materials. Mini-prep RNeasy purification kit was from Qiagen (Crawley, W. Sussex, UK); the Titanium One-Step RT-polymerase chain reaction (PCR) kit from BD Biosciences (Oxford, UK); ECE-1 mouse monoclonal antibody AEC32–236 was a gift from Dr. K. Tanzawa (Sankyo Research Laboratories, Tokyo, Japan). The affinity purified GLUT1 antibody (0.2 mg/ml) raised against a synthetic peptide corresponding to the C-terminal region of GLUT1 was a gift from Professor S. A. Baldwin (University of Leeds, UK); antibodies for the ECE-1 isoforms were produced as described and characterized previously (18); secondary antibody was either rabbit polyclonal anti-mouse Ig or sheep polyclonal anti-rabbit Ig, both from Amersham Biosciences, Amersham, United Kingdom; anti-actin (20–33) IgG fraction of antiserum developed in rabbit was from Sigma Chemical Company (St. Louis, MO); enhanced chemiluminescent detection system was from Amersham Pharmacia Biotech Ltd., (Bucks, UK); oligonucleotide primers were synthesized by MWG-Biotec (Milton Keynes, UK); recombinant ECE-1 was obtained from Chinese hamster ovary cells expressing ECE-1 plasmid; H_2O_2 from a stable 30% solution (Sigma) was diluted at appropriate concentrations in sterile water; phorbol 12-myristate 13-acetate, carbamylcholine chloride (carbachol) and protein kinase C (PKC) inhibitor GF 109203X (bisindolylmaleimide 1, Bis-I) were from Sigma. All other chemicals used were of reagent grade or higher.

Cell Culture. The NB7 human neuroblastoma cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 5% penicillin/streptomycin, and 5% L-glutamine. Cells were grown routinely in 175-cm^2 tissue culture flasks containing 30 ml medium at 37°C in 5% (v/v) carbon dioxide. In some experiments, the cells, after reaching the confluent stage, were kept in serum-free media with or without addition of carbachol, the phorbol ester PMA, or Bis-I at the concentrations described in the Results section. To induce oxidative stress in the NB7 cells, H_2O_2, to a final concentration of 40 μ*M*, was added to the culture media, which were kept under normoxic conditions for 24 hrs. Cell viability was estimated by a routine trypan blue uptake test.

Hypoxia in NB7 Cells. The NB7 cells at 70% confluence stage were incubated in an O_2/CO_2 incubator (MC0-175M, Sanyo) under 2.5% of O_2 for 24–48 hrs. Oxygen content in the incubator in control experiments was close to its content in atmospheric air.

Cell Lysis and Membrane Preparation. Pellets of the cultured cells were resuspended in 2 ml of MES buffer (25 m*M* MES, 0.15 *M* NaCl, pH 6.5) with a standard cocktail of protease inhibitors (without EDTA) and 1% Triton X-100. Cells were lysed for 30 mins and the lysates were centrifuged at 100,000 *g* for 1 hr at 4°C in Optima TL Ultracentrifuge (Beckman, High Wycombe, UK). The supernatant was discarded and the membrane pellet resuspended in 50 μl MES buffer plus 1% (v/v) Triton X-100. The amount of protein in the fractions obtained was analyzed by the routine bicinchoninic acid protein assay

using the bicinchoninic acid kit (Sigma) and bovine serum albumin 1 mg/ml as a standard.

Western Blotting and Immunodetection of Proteins. Proteins from membrane preparations were denatured for 5 mins at 100°C with reducing agent and the sample buffer (10% v/v of 1 *M* Tris pH 6.8, 40% v/v of 10% sodium dodecyl sulfate (SDS), bromophenol blue, 20% v/v glycerol, 20% H_2O, 10% β-mercaptoethanol) and loaded (20–40 μg) onto 8% (w/v) acrylamide-SDS gels in a BioRad electrophoresis system (Hemel Hempstead, UK). The electrophoresis, transfer of proteins onto polyvinylidene fluoride (PVDF) membranes, and incubation with appropriate primary and secondary (rabbit anti-mouse or sheep anti-rabbit, dilution 1:2000) antibodies were performed by routine protocols. Sample loading was monitored either by β-actin or by staining the PVDF membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid (v/v). Blots were visualized by an enhanced chemiluminescent detection system and subjected to densitometry using software for densitometric analysis of immunoblotting data (Bio-Rad Multi-Analist/PC, Version 1.1.1). Data of densitometry were calculated as percent of changes in the density of bands compared with control samples. The results were presented as means ± SEM. Statistical significance was determined by *t* test, and *P* values less than 0.05 were considered as significant.

Real-Time PCR. Total RNA was isolated from the cells using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Real-time PCR was performed using iQ SYBR Green supermix (BioRad) according to the manufacturer's protocols. The standard reaction mix consisted of iQ SYBR Green supermix, forward and reverse primers at final concentration of 500 n*M* each, 10 pg DNA template, DNAse free water to give final volume of 20 μl. A series of dilutions of ECE-1 plasmid from 0.01 pg to 100 pg was used as standards.

Primer Sequences.

ECE1-F 5′-GGA CTT CTT CAG CTA CGC CTG T-3′
ECE1-R 5′-CTA GTT TCG TTC ATA CAC GCA CG-3′
GAPDH-F 5′-AGC TGA ACG GGA AGC TCA CT-3′
GAPDH-R 5′-AGG TCC ACC ACT GAC ACG TTG-3′

Results

Effect of Hypoxia and Oxidative Stress on Expression of ECE-1. The data on ECE-1 expression under chronic hypoxic conditions (24 hrs, 2.5% oxygen content in the incubator) or under conditions inducing oxidative stress (24 hrs, 40 μ*M* H_2O_2 under normal atmospheric oxygen content) both at mRNA and protein levels are given in Figure 1. As a marker of hypoxia, we used expression of the glucose transporter GLUT1, which is known to be upregulated under hypoxic conditions (19). We also observed a dramatic increase in GLUT1 expression in NB7 cells under hypoxic conditions in our experiments (Fig. 1A). Cell viability after hypoxia, serum withdrawal, or drug treatment did not differ by more than 5% from the control cells under the conditions used in the experiments. ECE-1 expression at the protein level was found to be decreased by 30% compared with control values under hypoxic conditions and on average by 20% under the conditions of oxidative stress (Fig. 1A and B). However, under both conditions, there were no significant changes in ECE-1 expression at mRNA levels when it was assessed by real-time PCR against GAPDH as a housekeeping gene (Fig. 1C).

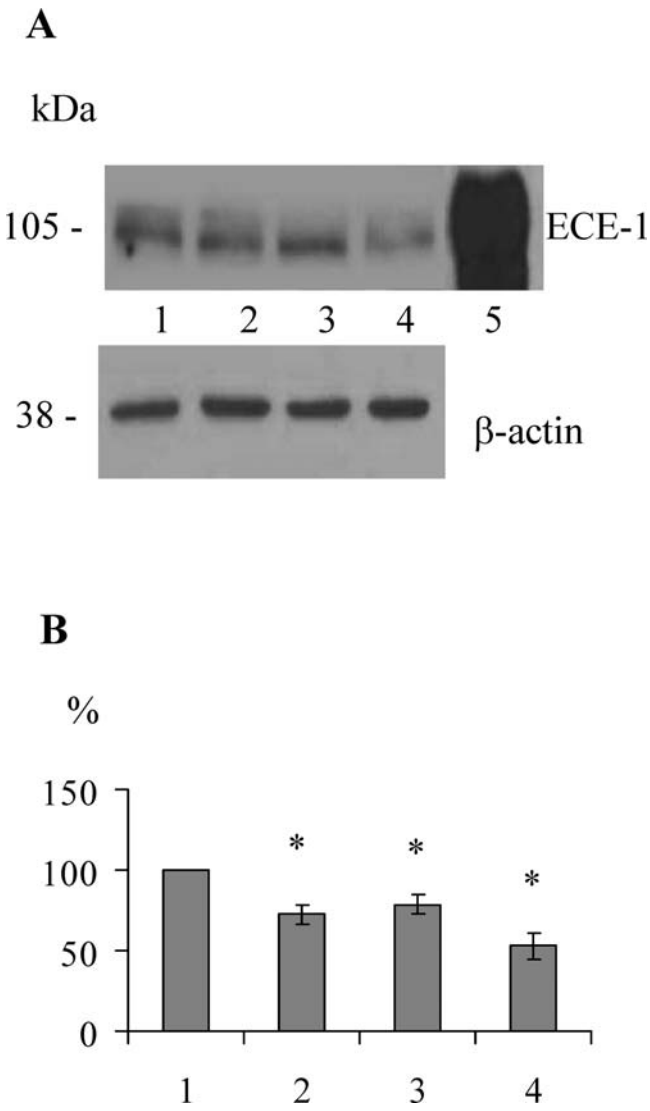

Figure 2. Effect of withdrawal of serum and addition of carbachol on expression of ECE-1 in NB7 cells. (A) NB7 cells were grown until 60%–70% confluence and incubated in serum-free medium with or without 20 μ*M* carbachol. Cells were collected 24 hrs after treatment. β-actin was used as a loading control. (B) Statistical data of blot densitometry. Data are mean ± SEM ($n = 3$). (1) Control cells, (2) cells treated with 20 μ*M* of carbachol, (3) cells grown in serum-free medium; (4) cells grown in serum-free medium with 20 μ*M* of carbachol, and (5) positive control for ECE-1. *$P < 0.05$.

Effect of Carbachol or Serum Deprivation on Expression of ECE-1. Because human neuroblastoma

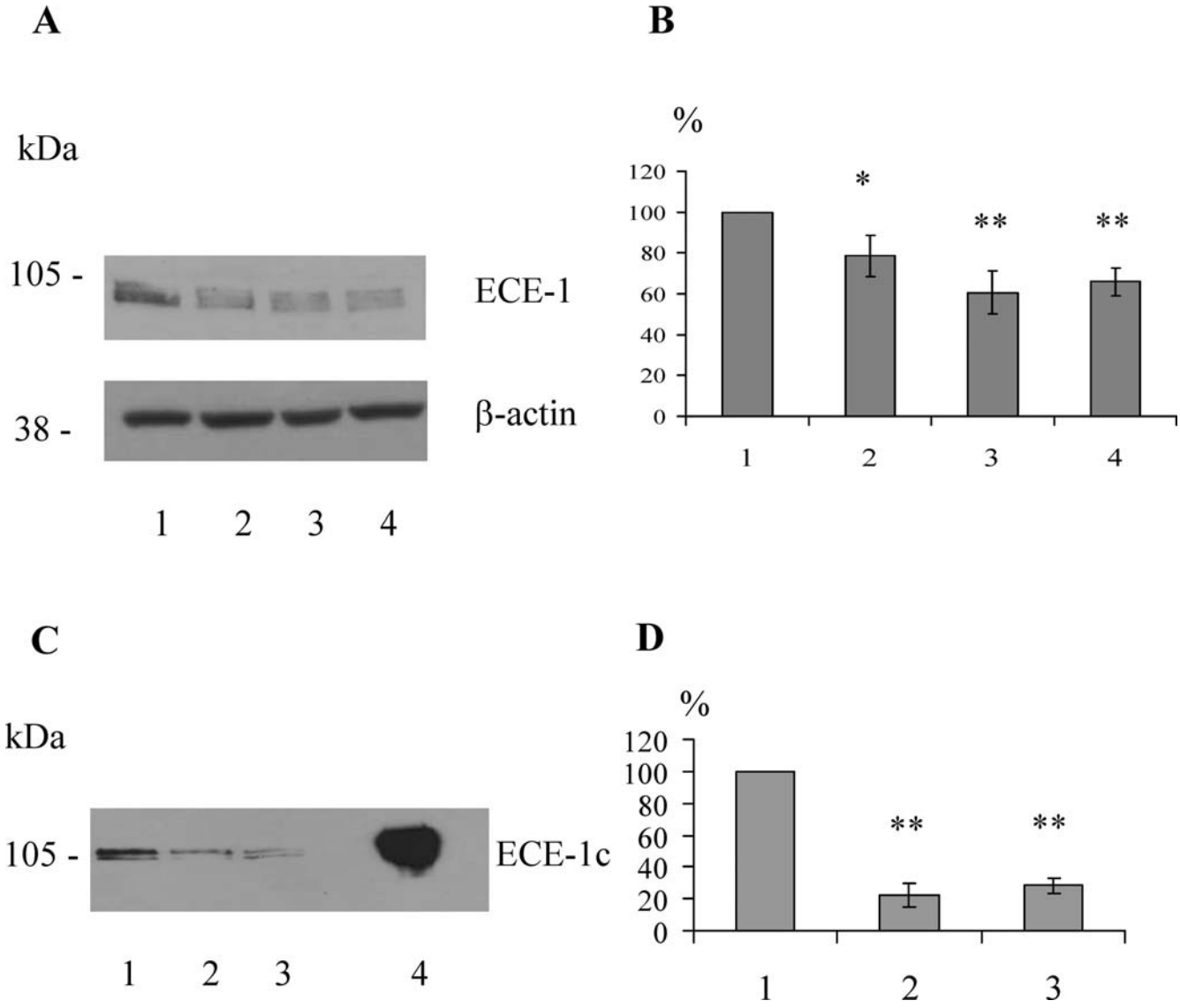

Figure 3. Expression of ECE-1 and ECE-1c in NB7 cells after treatment for 24 hrs with phorbol ester (PMA) at various concentrations. NB7 cells were grown until 60%–70% confluence and treated with various concentrations of PMA. Cells were collected 24 hrs after treatment. (A) A representative blot for ECE-1. (B) Densitometric analysis of the blots with the mean results ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$; (1) control, (2) 10 μ*M*, (3) 50 μ*M*, (4) 100 μ*M* PMA. (C) A representative blot of the effect of 100 μ*M* PMA and PKC inhibitor Bis-I on ECE-1c. (D) Data of densitometry ($n = 3$). (1) Control, (2) 100 μ*M* PMA, (3) 100 μ*M* PMa + 10 μ*M* Bis-I, (4) positive control for ECE-1c. *$P < 0.05$; **$P < 0.01$.

NB7 cells do not represent morphologically typically differentiated neuronal cells and lack developed neurites, we have induced their differentiation either by serum withdrawal or addition of the cholinergic agonist carbachol, which were previously shown to induce differentiation and neuritogenesis in human neuroblastoma SH-SY5Y cells (20, 21). In parallel, we have also assessed levels of expression of ECE-1 in these cells. Serum deprivation and addition of 20 μ*M* carbachol both to full or serum-free media induced formation of short neurites; however, it also resulted in a decrease of expression of ECE-1 in these cells (Fig. 2) down to 60%–70% compared with the control levels.

Effect of the Phorbol Ester PMA on Expression of ECE-1 in Neuroblastoma NB7 Cells. It has been reported that the phorbol ester PMA upregulates expression of the ECE-1a isoform in human endothelial cells (22) at the mRNA level. Therefore, we have compared the effect of PMA on ECE-1 expression at the protein level in NB7 cells. In contrast to the reported ECE-1 upregulation in human endothelial cells, PMA at various concentrations down-regulated the expression of ECE-1 protein down to 60% of control values (Fig. 3A and B). The analysis of ECE-1 isoforms by specific antibodies for 1a, 1b, and 1c isoforms (18) demonstrated that ECE-1c was the only isoform expressed at detectable levels in NB7 cells (Fig. 3C). Addition of the protein kinase C–specific inhibitor Bis-I (GF

109203X) at a concentration of 10 μ*M* did not diminish the effect of PMA on ECE-1c expression (Fig. 3C and D).

Discussion

Although ECE-1 is a rather well-characterized enzyme, its expression and functioning in the nervous system and neuronal cells have been little explored. However, in recent years, interest in the role and regulation of ECE-1 in the brain and neuronal cells has increased dramatically since it was suggested to be one of the Aβ-degrading enzymes. In this context, a study of expression and regulation of ECE-1 in various types of neuronal cells is important and timely.

In this study, we have found that, in addition to human neuroblastoma SH-SY5Y cells, which express ECE-1 (6), NB7 cells and SK-N-SH (data not shown) also express ECE-1. ECE-1c isoform was found to be the dominant ECE-1 isoform in human neuroblastoma NB7, whereas ECE-1a and ECE-1b isoforms were not detectable. These data for the first time demonstrate the ECE-1c isoform as specific for neuronal cells. Our data also for the first time demonstrate that ECE-1 decreases in neuroblastoma cells after chronic 24 hrs of hypoxia and to a lesser extent after oxidative stress. The data on a similar level of decrease in the activity of the ECE-1 homologue neprilysin detected by a high-performance liquid chromatography assay method have recently been reported in another neuroblastoma cell line SH-SY5Y (23). The data of this study and our previous data obtained in animal models suggest that hypoxia leading to a decrease in expression of ECE-1 and neprilysin might result in a deficit of amyloid-degrading enzymes in the brain, allowing its accumulation.

After differentiation of NB7 cells either by serum withdrawal or addition of the cholinergic agonist carbachol, expression of ECE-1 was found to decrease by 40%, which might reflect a changed physiologic role of this enzyme in differentiated cells. As shown in glioblastoma cells, inhibition of ECE-1 had an antiproliferative effect (24); therefore, decreased ECE-1 activity in NB7 cells can reflect the decreased rate of proliferation of these cells in serum-deficient medium or under cholinergic activation.

Because upregulation of Aβ-degrading enzymes is considered a viable strategy in AD therapy, it is important to undertake a search for compounds and signaling pathways involved in ECE-1 regulation in neuronal cells. Although treatment with cholinergic (muscarinic) agonists and, in particular, carbachol is well known to stimulate the nonamyloidogenic (α-secretase) pathway of amyloid precursor protein processing (16), this study demonstrates that it downregulated expression of ECE-1 in neuroblastoma NB7 cells. The phorbol ester PMA was also shown to enhance nonamyloidogenic processing of amyloid precursor protein via a PKC-dependent mechanism (25). Although PMA was also found to upregulate ECE-1 in human endothelial cells (22), our data demonstrate that ECE-1 is downregulated by PMA but after a more prolonged treatment (24 hrs). Because the inhibitor of PKC Bis-I (GF 109203X) did not restore the PMA-induced decrease in ECE-1 (ECE-1c) expression, this effect is not coupled to the PKC-signaling cascade (26). In view of these data, downregulation of ECE-1 by agents activating α-secretase processing of amyloid precursor protein suggests that selection of compounds for AD therapeutic purposes has to take into account the possibility of downregulation of ECE-1 and subsequent reduction in the rate of Aβ cleavage.

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