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Effects of 4-Hydroxy-Nonenal and Amyloid- β on Expression and Activity of Endothelin Converting Enzyme and Insulin Degrading Enzyme in SH-SY5Y Cells

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Abstract

The cerebral accumulation of amyloid- β ($A\beta$) is a consistent feature of and likely contributor to the development of Alzheimer's disease (AD). In addition to dysregulated production, increasing experimental evidence suggests reduced catabolism plays an important role in $A\beta$ accumulation. Although endothelin converting enzyme (ECE) and insulin degrading enzyme (IDE) degrade and thus contribute to regulating the steady-state levels of $A\beta$, how these enzymes are regulated remain poorly understood. In this study, we investigated the effects of 4-hydroxy-nonenal (HNE) and $A\beta$ on the expression and activity of ECE-1 and IDE in human neuroblastoma SH-SY5Y cells. Treatment with HNE or $A\beta$ upregulated ECE-1 mRNA and protein, while IDE was unchanged. Although both ECE-1 and IDE were oxidized within 24 h of HNE or $A\beta$ treatment, ECE-1 catalytic activity was elevated while IDE specific activity was unchanged. The results demonstrated for the first time that both ECE-1 and IDE are substrates of HNE modification induced by $A\beta$. In addition, the results suggest complex mechanisms underlying the regulation of their enzymatic activity.

Keywords

Alzheimer's disease; amyloid- β ; degradation; endothelin converting enzyme; 4-hydroxy-nonenal (HNE); insulin degrading enzyme; oxidative stress

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that gradually damages the neocortex and hippocampus, reducing memory, learning, and reasoning [1]. The extensive deposition of amyloid- β ($A\beta$) is a pathological hallmark of AD. While still controversial, $A\beta$ deposition and AD development is supported by a variety of *in vivo* and *in vitro* evidence [2–5]. Although the mechanisms that contribute to abnormal $A\beta$ accumulation are not fully understood, both increased production and decreased degradation have been observed [6]. Despite its importance, $A\beta$ catabolism remains understudied [7]. In normal brain, rapid proteolysis attenuates $A\beta$ accumulation. The degradation process occurs primarily through the action of a group of metallopeptidases including neprilysin (NEP), insulysin (insulin degrading enzyme, IDE) and endothelin converting enzyme (ECE). While NEP appears to be the dominant $A\beta$ protease [8–10], other catabolic enzymes such as ECE and IDE likely participate in regulating the steady-state levels of $A\beta$ [10].

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ECEs (including ECE-1 and ECE-2) are homologues belonging to the M13 family of zinc metallopeptidases that also includes NEP [7,11], which degrade A β in acidic intracellular compartments [12]. ECE-1 is widely expressed in human brain [12–14] with the capability to degrade A β [15]. Both A β ₄₀ and A β ₄₂ levels were significantly higher in knockout mice [16] consistent with an *in vitro* role for ECE-1 in amyloid catabolism.

Besides NEP and ECE, IDE also contributes to A β degradation [17]. Purified rat IDE effectively degraded synthetic A β *in vitro* as did IDE from synaptic membrane fractions from postmortem human and fresh rat brain [18,19] or in cultured cells [20,21]. Overexpression of IDE in cells or mice markedly reduced intracellular and extracellular A β ₄₀ and A β ₄₂ [22] while IDE knockout mice demonstrated a clear elevation of brain A β [23]. For unknown reasons, presenilin 1 V97L mutants linked to familial Alzheimer's disease (FAD) related to a functional defect in IDE and demonstrated increased intracellular and extracellular A β ₄₂ [24]. Thus, IDE is likely another important A β -degrading enzyme that may play a role in the pathology of AD.

Oxidative stress has long been recognized as a contributing, early factor in AD [25–30]. A β induces free radical generation [25,31–38] and elevated 4-hydroxy-nonenal (HNE), a marker of lipid peroxidation that is present in plaques [39]. HNE can interact with and inactivate a variety of enzymes including NEP [40]. However HNE adduction and its effects on ECE and IDE activity have not been evaluated. The present study addressed this question by examining the expression and adduction of IDE and ECE-1, in cells treated with A β or HNE. Our results indicate that such treatments rapidly induce IDE and ECE-1 oxidation but paradoxically increase ECE activity.

MATERIALS AND METHODS

Chemicals and reagents

Synthetic human A β peptide 1–42 was purchased from BACHEM. Dithiothreitol (DTT), protease inhibitors cocktail (P8430) were obtained from Sigma-Aldrich. HNE was obtained from A.G. Scientific, Inc. Mca-R-P-P-G-F-S-A-F-K (Dnp)-OH Fluorogenic Peptide Substrate V, RNase-free DNase came from R&D and Promega, respectively. GE nitrocellulose membrane was purchased from ISC BioExpress. An enhanced chemiluminescence (ECL) kit and Seize[®] Classic (G) Immunoprecipitation Kit were obtained from Pierce. Other general chemicals and reagents were from Fisher Scientific.

Antibodies were from the following sources: Rabbit anti-HNE (Chemicon); goat anti-ECE-1 and goat anti-IDE (Santa Cruz); Alexa Fluor[®] 594 donkey anti-goat IgG (H+L), Alexa Fluor[®] 488 chicken anti-rabbit IgG (H+L) were from Invitrogen, goat anti-rabbit and rabbit anti-goat HRP conjugated secondary antibody were purchased from Chemicon. Clean-Blot[™] IP Detection Reagent is the product of Thermo Scientific.

Cell culture

SH-SY5Y neuroblastoma cells, obtained from the American Type Culture Collection (ATCC), were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were seeded into plates or dishes in DMEM/F12 (1:1) medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Cell treatments with HNE and A β peptide

SH-SY5Y cells were seeded into 100 mm dishes at a density of 2×10^4 cells per ml. Experiments were carried out 24–48 h after cells were seeded. Different concentrations of HNE (dissolved in 3% DMSO) or A β (dissolved in 0.4% DMSO) were added to the cultures 24 h

before harvest. Final concentrations of DMSO in medium were < 0.003%. Vehicles (with same concentrations of DMSO) were added to the cultures as controls.

Real time RT-PCR

Total RNA from a well of 6-wells plate was isolated using the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The concentration of nucleic acids was determined spectrophotometrically at 260 nm and 280 nm, taking into account the dilution factor. RQ1 RNase-free DNase (Promega) was used to remove relict DNA which might interfere the output of PCR. The PCR first strand synthesis was performed using the Omiscript® RT Kit (Qiagen Inc.). The resulting cDNA was then assayed by real time RT-PCR.

Real time RT-PCR was performed in 0.2 ml thin wall PCR plates using the iCycler thermal cycler (Bio-rad) and carried out with iQ SYBR Green supermix (Bio-rad) according to the manufacturer's instructions. The standard reaction mix consisted of iQ SYBR Green supermix, forward and reverse primers at a final concentration of 500 nM each, 10 pg DNA template, and DNase free water to give final volume of 20 μ l. The mixture was heated to 95°C for 3 min followed by 35 cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Human S26 (housekeeping gene) [41] was used as reference gene. Primer sequences for real time PCR: S26: 5'-CGC AGC AGT CAG GGA CAT TT-3' (F), 5'-TTC ACA TAC AGC TTG GGA AGC-3' (R); ECE-1: 5'-GAC GCC GAT GAG AAG TTC ATG-3' (F), 5'-GCA AAA CTT CCA GCG AGG AA (R); IDE: 5'-GCC GAA GCC TTG TCT CAA CT (F), 5'-CAA ATA GGC CAT GTT ACA GTG CAA (R). The melting curves were performed to identify the specificity of the primers.

Relative quantification of genes expression was carried out by comparative Ct method according to manufacturer's protocol (User Bulletin #2: ABI PRISM 7700 Sequence Detection System). Briefly, the genes mRNA level was expressed in cycle threshold (Ct) value; the Ct values for each sample were averaged from duplicate. Differences between the mean Ct values of ECE or IDE and reference gene were calculated as $\Delta Ct_{\text{sample}} = Ct_{\text{ECE or IDE}} - Ct_{\text{S26}}$ for different treated groups. The ΔCt for the control groups were set for calibrator ($\Delta Ct_{\text{calibrator}}$). Final results, the sample-calibrator ratio, expressed as N-fold differences of ECE-1 or IDE expression in the HNE or $A\beta_{1-42}$ groups compared with control, were determined as $2^{-(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}})}$.

Immunoprecipitation, immunoblotting, and protein quantification

After incubation with HNE (5, 10 and 20 μ M) or $A\beta$ (0.5, 1 and 2 μ M) for 24 h, cells were harvested and lysed in Passive Lysis Buffer containing protease inhibitors cocktail (Sigma P8430). Although this cocktail of protease inhibitors with broad specificity for the inhibition of aspartic, cysteine, and serine proteases as well as aminopeptidases, it does not interfere with metallopeptidases such as IDE, ECE, and NEP since it is EDTA free and has been used in the previous study [10,42]. Lysates were incubated for 30 min on ice, and then centrifuged at 10,000 X g for 10 min at 4°C. Protein concentration in the supernatant was determined by Coomassie blue protein binding method using protein quantification Kit-rapid (Sigma/Fluka) with bovine serum albumin as standard. The same amount of protein from HNE-treated, $A\beta_{1-42}$ and vehicle-treated cells were used for western blotting assays and immunoprecipitation.

Immunoprecipitation with anti-ECE or IDE was performed with Seize® Classic (G) Immunoprecipitation Kit according to the manufacturer's protocol. For total cell extract preparation, supernatants (with same amount of protein) were incubated with goat anti-ECE or goat anti-IDE polyclonal antibody (Santa Cruz, 60 μ g/ml) at 4°C overnight. After washing Immobilized Protein G by adding 0.4 ml of BupH™ Modified Dulbecco's PBS twice, the

immune complexes were added to the spin cup containing the equilibrated immobilized protein G and incubated for more than 1 h at 4°C. The samples were centrifuged briefly to remove the flow-through solution. The beads were extensively washed with BupH™ Modified Dulbecco's PBS. The immunoprecipitated proteins were eluted by adding equal volume of ImmunoPure® Elution Buffer and then analyzed by Western blotting with anti-HNE antibody. Briefly, Eluted samples were denatured in protein sample buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 20% H₂O, 200 mM DTT) at 100°C for 10 min and loaded, separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes in a Bio-rad electrophoresis system. After blocking with TBST (Tris-buffered saline with 0.1% Tween) containing 5% non-fat milk, the membranes were kept at 4°C overnight with HNE, ECE, and IDE primary antibody (1:1000, 1:500, 1:500, respectively), followed by Clean-Blot™ IP Reagent (HRP) (1:100) at room temperature (RT) for 2 h. The target protein bands were detected using the ECL Western blotting detection system (Pierce) and autoradiography film (Fisher).

For ECE and IDE protein levels detection, regular Western Blotting assay was performed using ECE-1 and IDE antibodies (1:500), followed by HRP-conjugated second antibodies (1:5000 to 1:10000 dilution) at RT for 2 h. The target protein bands were detected using the ECL Western blotting detection system (Pierce) and autoradiography film (Fisher). β -actin (1:5000) was used as reference.

The density of the ECE-1, IDE, and HNE blotting bands were quantified by ImageJ software (1.37v, Wayne Rasband, National Institutes of Health, USA) as described [43,44].

Double immunofluorescence staining

Cells were fixed with 4% paraformaldehyde at RT for 20 min and permeabilized with 0.2% Triton X-100 in PBS. After blocking for 1 h at RT with 1% goat serum/PBS, cells were incubated overnight at 4°C in a humidity chamber with primary anti-ECE-1 or IDE (1:100 Santa Cruz). At the end of the incubation, the cells were rinsed three times with PBS-Tween-20 (0.05%) and incubated with Alexa Fluor® 594 donkey anti-goat IgG (H+L) (1:5000) for 60 min at RT. Then the cells were fixed with 4% paraformaldehyde at RT for 15 min. After rinsing with PBS for 3 times, second primary rabbit anti-HNE (1:200) were added and incubated for 2 h at RT or overnight at 4°C. Secondary, Alexa Fluor® 488 chicken anti-rabbit IgG (H+L) (1:5000) was incubated for another 60 min at RT. All primary and secondary antibodies were diluted in PBS with 1% normal goat serum. After a rinse with PBS, the nuclei were counterstained with DAPI/Antifade solution (0.4 μ g/mL, Chemicon), and then the cells were photographed and analyzed with a fluorescent microscope (Nikon, E600, Japan). The immunofluorescence staining was done at least three times to assess the reproducibility of the results. In order to confirm the specificity of immunostaining for the second cycle, the primary or secondary antibodies were omitted or replaced with preimmune IgG.

Fluorometric assay of ECE activity

ECE activity was determined by Fluorescence Resonance Energy Transfer (FRET). After extraction with a final concentration of 0.1% Triton X-100 in phosphate-buffered saline (PBS; pH 7.2) for 30 min on ice, ECE activity in cell lysates was analyzed using a synthetic ECE fluorogenic peptide substrate (Mca-RPPGFSAFK[Dnp]-OH; R&D Systems, Inc., Minneapolis, MN) [45] at RT in the presence or absence of the ECE inhibitor, Phosphoramidon (1 μ M, Sigma). Phosphoramidon inhibition of ECE-1 is highly pH dependent [46]. The IC₅₀ value of Phosphoramidon is about 50-fold lower at PH 5.8 than at 7.2. In addition, NEP activity is also PH-dependent. There is almost no NEP activity at PH 5.8 [47]. Thus, the inhibition by phosphoramidon at PH 5.8 can be considered to be the inhibition of ECE. Briefly, samples dissolved in 50 mM HEPES buffer (pH 5.8)) were preincubated with 1 μ M Phosphoramidon

or PBS for 10 min prior to adding fluorogenic peptide substrate (dissolved in HEPES). Fluorescence was read after excitation at 320 nm and emission at 405 nm on a fluorescent ELISA plate reader (Spectra Max Gemini; Molecular Devices, Sunnyvale, CA). For kinetic analysis the cell lysates were incubated with increasing concentrations of substrate (4–20 μM) at RT. Fluorescence over the time was measured for 1 h. The specific ECE activity was determined as the fluorescence difference occurring in the presence or absence of 1 μM Phosphoramidon. Kinetic isotherms (V_{max} and K_m values) for ECE activity were determined by means of non-linear least squares fitting to the Michaelis-Menten equation using GraphPad Prism software (version 3.0 for Windows, ISI Software San Diego, CA, USA).

To measure the activity of membrane-bound enzyme, cell suspensions were prepared, and then washed with PBS. 5×10^4 cells were resuspended in 50 μl of PBS contained in each well of black microtiter plates. The 50 μl substrate (10 μM in 50 mM HEPES) was added, and incubated at RT for 60 min. As above, Phosphoramidon was used to determine the specific ECE activity in intact cells.

Immunocaptured specific IDE activity assay

Cells treated by vehicles, HNE, or $\text{A}\beta_{1-42}$ were lysated with Passive Lysis Buffer containing protease inhibitors cocktail (Sigma P8430). After 30 min incubation on ice, the lysates were centrifuged at 1300 rpm for 15 min at 4°C.

IDE specific activity was measured by using immunocapture based fluorometric assay [48]. In brief, Greiner 96-well high binding ELISA plate were coated with capture IDE (Santa Cruz, 50 $\mu\text{g}/\text{ml}$) antibody diluted in carbonate buffer (100 mM, pH 8.6) and left for 18 h at 4°C. The plates were washed with PBS containing 0.5% tween-20 for 3–5 times. After 30 min blocking with PBST containing 2% BSA, the supernatants of cell lysates (50 μl) were added and incubated at 4°C overnight. After 3–5 washes, the fluorogenic peptide (10 μM) diluted in 50 mM HEPES (pH 7.5) was added and incubated at 30°C in dark. Fluorescent readings were taken after 60 min. Control wells included in each plate contained PBS and fluorogenic peptide. Values are the mean $\pm$ SEM, presented in the percentage of control.

Statistical analysis

All results are given as mean $\pm$ SEM unless otherwise stated. Statistical analyses were performed with One-way ANOVA followed by least significant difference post hoc analysis (multiple comparisons) with threshold of $P < 0.05$.

RESULTS

HNE and $\text{A}\beta$ treatment induce ECE and IDE modification

First we evaluated the effects of HNE and $\text{A}\beta$ on intracellular ECE-1 and IDE mRNA and protein expression. Vehicle-, HNE-, or $\text{A}\beta$ -treated SH-SY5Y cells were analyzed by real-time RT-PCR for ECE-1 and IDE mRNA. Low dose HNE (5 μM) for 12 h upregulated ECE expression, which was suppressed at higher doses (10 μM) (Fig. 1A). Similar treatments with $\text{A}\beta_{1-42}$ upregulated ECE-1 mRNA, with the greatest change at 2 μM (Fig. 1B). IDE mRNA showed no change after HNE treatment (Fig. 1A), but was upregulated by $\text{A}\beta_{1-42}$ at 1 μM , and returned to initial levels after 2 μM treatment (Fig. 1B).

ECE-1 protein levels as determined by western blotting displayed dramatic increases both in HNE and $\text{A}\beta_{1-42}$ -treated cells in a concentration-dependent manner (Fig. 2). The results suggest that ECE-1 has a prolonged *in vitro* half-life (> 12 h) and that ECE-1 gene expression is sensitive to both HNE and $\text{A}\beta$. Compared to ECE-1, IDE protein changes were insignificant.

Only slight decreases of IDE in HNE-treated cells (Fig. 2A, C), while no obvious changes in A β -treated cells (Fig. 2B, D) were observed.

In order to determine if HNE-modification occurred in the cultured SH-SY5Y cells treated with HNE or A β _{1–42}, ECE-1 and IDE were immunoprecipitated followed by western blotting. As shown in Fig. 3, ECE-1 or IDE were variably upregulated and modified after treatment with HNE or A β . While both proteins showed adduction, only IDE was significantly more modified after HNE than control (Fig. 3A–D).

To determine the subcellular location of modified proteins, we performed double fluorescence staining followed by confocal analysis of oxidatively stressed SH-SY5Y cells (Fig. 4). ECE-1 was detected both in cytosol and membrane, which is consistent with previous reports [49–53]. HNE was almost undetectable in controls (Fig. 4b) but positive in a punctal pattern both in HNE- and A β -treated cells (Fig. 4f, j). Merged images revealed ECE-1 and HNE partly colocalized in cells treated with HNE or A β (Fig. 4h, l) consistent with the results observed after western blotting and immunoprecipitation. However, ECE-1, which did not colocalize with HNE, was mainly located on the cell surface. Therefore, ECE-1 is partially modified by exogenously added or endogenously produced HNE induced by A β .

As previous reports [52,54], IDE staining was mostly cytosolic (Fig. 5a, e, i). As for ECE-1, (Fig. 5f, j), HNE and IDE were both colocalized and independent after HNE or A β treatment (Fig. 5h, l). These data suggested that HNE adduction develops both after exogenous HNE and endogenous HNE induced by A β protein.

ECE, but not IDE, activity is altered after HNE and A β treatment

ECE activity in cell lysates or intact SH-SY5Y cells treated by HNE or A β for 24 h was determined with a commercially available fluorogenic substrate. The activity assay was highly sensitive, linear over-time (up to 60 min examined, data not shown), and sensitive to the ECE inhibitor, phosphoramidon (data not shown). Treatment of cells with HNE led to a concentration dependent increase of ECE activity in cell lysates (Fig. 6a). In intact cells, HNE at low concentration (5 μ M) significantly increased ECE activity ($p < 0.01$), which returned to normal at higher concentrations (10 and 20 μ M) (Fig. 6b). Specific ECE activity in cell lysates was increased ~300% ($p < 0.05$) while intact cells showed up to a 70% increase. Despite adduction, samples treated with A β showed increased ECE activity with similar trends in cell lysates and intact cells (Fig. 6d and e). Specific IDE activity in SH-SY5Y cells treated by HNE or A β for 24 h was determined by immunocapture based activity assay with a commercially available fluorogenic substrate. Consistent with the protein level, IDE activity treated by HNE or A β did not show significant differences compared to control (Fig. 7).

To further analyze enzymatic kinetics, ECE activity was compared over a range of substrate concentrations (4–20 μ M) in SH-SY5Y cells. Activity was saturable, and followed Michaelis-Menten kinetics in all cell lysates treated with vehicle, HNE and A β peptide. Lineweaver-Burk double-reciprocal plots of reaction velocities and substrate concentrations allowed calculation of the apparent Michaelis Constant (K_m) for the enzyme in all samples (Fig. 6c and f, Table 1). After HNE treatment, the K_m was slightly decreased compared to control (with no statistic significance) which implies that the enzyme affinity for substrate tended to be increased (Table 1, Fig. 6c). Similar results were observed in the A β treatment. There was no significant effect on the K_m at any of the three concentrations of A β (Table 1, Fig. 6f). The change of v_{max} was not significant after either HNE or A β treatment (Table 1). These kinetics features are consistent with the alteration of ECE activity. Together with the Western blotting data and immunofluorescence staining, these results suggest that HNE adduction induced either by HNE or A β treatment does not play a substantial role in suppressing ECE or IDE activity.

DISCUSSION

Although many factors such as abnormal protein cross-linking [55–57], trauma [58], abnormal phosphorylation of multiple proteins [59], synaptic loss [60], white matter changes [61], and some imbalanced metabolism [62–64] have been implicated in the AD pathogenesis, excessive A β deposition is still considered to play a major role in AD. ECE expression and activity correlates well with A β deposition and the onset of AD [16,65–68], and data from cultured cells and animal model indicated that A β is a physiologically relevant substrate of ECE [16]. Animals lacking ECE-2 are deficient in learning and memory [69]. Homozygous carriers of the A allele, which is associated with increased ECE-1 mRNA expression in human neocortex, had a reduced risk of AD [13] while the upregulation of ECE and IDE in mice was associated with decreased A β [68]. However, there are little data from studies on human tissue and the responses of ECE to HNE or A β . As AD typically develops over decades, the data from postmortem samples provides limited mechanistic insights into how AD-related factors such as A β peptides and oxidative stress alter ECE. In our study, ECE-1 mRNA was upregulated after the treatment of HNE and A β . Consistent with mRNA level, the changes of ECE-1 were more obvious in protein level after 24 h. ECE-1 protein was dramatically increased both in HNE- and A β -treated cells in a dose-dependent manner. These results agree with our study in human brain [70]. Moreover, we showed previously that HNE could enhance A β accumulation in culture media [70]. Thus increased ECE-1 levels, either mRNA or protein, may be the compensatory response to the increased endogenous or exogenous A β . In our previous study, cell viability after the treatments with increasing concentrations of HNE or A β showed a concentration dependent decrease (~25% with 20 μ M HNE and ~15% in 2 μ M A β) [70]. The evidence also indicated the cell death induced by HNE and A β was not dominant on mRNA depression.

Considerable evidence exists that A β can increase the production of free radicals by neurons [6,71–73]. The formation of HNE is initiated by oxidative stress and culminates in lipid, DNA, and protein adduction. The HNE-macromolecular adducts are abundant in the brain of patients with AD suggesting a role for oxidative damage in disease pathogenesis [36,39,74]. Consistent with previous reports [75], we also show A β -induced HNE-adductions determined by western blotting and immunofluorescence staining. Among A β degrading enzymes, NEP is clearly one of the targets of HNE-mediated modification in AD brain and cultured neurons [76]. HNE adduction could inactivate endogenous and recombinant NEP [70,77], which likely contributed to A β accumulation [76]. Since ECE and IDE are also capable of degrading A β , we explored whether HNE adduction on ECE-1 or IDE occurred in HNE- or A β -treated cells and whether these changes altered their activities in cultured cells. The data in this study show that both exogenous HNE and endogenous HNE induced by A β mediate ECE-1 and IDE modification. However, the extent of modification was significantly different. The ratio of HNE to ECE-1 was moderately higher to control both in HNE- and A β -treated cells. The ratio of HNE to IDE was higher after HNE than A β treatments although both treatments induced adduction. These results, along with prior analysis of human brain tissue and culture cells, suggest that although multiple A β degrading enzymes are oxidatively modified, the biological consequences from the oxidization of different enzymes may vary.

Real-time RT-PCR indicated that IDE mRNA was upregulated after cell treatment with 1 μ M A β , but western blotting did not show statistically significant changes in IDE protein levels. These data imply that IDE mRNA expression is more sensitive than protein and may reflect compensation to reduced IDE (protein levels and catalytic activity) induced by A β modifications.

We also evaluated if HNE adduction altered ECE activity. The specific activity of ECE increased significantly in cell lysates and intact cells after HNE or A β treatment. $V_{\max}$, and

K_m revealed that ECE activity in SH-SY5Y cells followed Michealis-Menten kinetics, and demonstrated a hyperbolic dependence of v (velocity) on substrate concentration. The ECE kinetic features are similar in HNE- and $A\beta$ -treated cells. The K_m of ECE after HNE or $A\beta$ treatment was slightly decreased when compared to control (Table 1), which implies that substrate affinity was increased but not significant. The V_{max} was unchanged in HNE and $A\beta$ incubated cells compared to controls. Overall, these kinetic parameters suggest some changes in affinity for substrate after HNE and $A\beta$ treatment. However, together with the result of co-localization exam, HNE adduction induced either by HNE or $A\beta$ treatment is not to be crucial to ECE activity.

Unlike ECE, the alteration of IDE specific activity was not significant after HNE or $A\beta$ treatments although a downward trend was observed in $A\beta$ -treated cells. Shinall and colleagues reported that IDE was inactivated by reaction with HNE with the concomitant formation of protein adducts in a cell-free system [77]. In this study, there was marked adduction without significant decrease of activity after HNE treatment, while $A\beta$ treatment showed more inactivation of IDE activity but less adduction. These results suggest that the HNE adduction may not be the predominant means of inactivating IDE and imply that the mechanism of inactivation is more complicated. Alternatively, $A\beta$ -induced HNE modifies IDE at a different intracellular location than exogenous HNE, accounting for dissimilar changes in enzymatic activity.

The experiments on cell viability after the treatments with increasing concentrations of HNE or $A\beta$ performed along with other experiments reported in this manuscripts showed that there was a concentration dependent decrease (~25% with 20 μ M HNE and ~15% in 2 μ M $A\beta$) in cell viability as determined by MTT reduction (data not shown). Although both HNE and $A\beta$ are known to be toxic to cultured neuronal cells, it is possible that other mechanisms also involved in neuronal cell death induced by $A\beta$ in addition to the induction of HNE.

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Fig. 1.

ECE-1 and IDE mRNA are altered in SH-SY5Y cells after HNE or A β ₁₋₄₂ treatment. SH-SY5Y cells were untreated or treated with HNE (5, 10 μ M) (Panel A) or A β ₁₋₄₂ (1, 2 μ M) (Panel B) for 12 h, respectively. Total RNA were analyzed by real-time RT-PCR and normalized to S26 mRNA levels. Shown are means $\pm$ SEM % of untreated control. * represents $P < 0.05$ (ECE-1), ## represents $P < 0.01$ (IDE) versus control.

Fig. 2.

ECE-1 and IDE protein changes in HNE or $A\beta_{1-42}$ treated SH-SY5Y cells. After incubation with vehicle, HNE (5, 10 and 20 μ M) or $A\beta$ (0.5, 1 and 2 μ M) for 24 h, SH-SY5Y cells were harvested and equal amounts of protein western blotted. (A) and (B) are representative western blots. (C) and (D) are quantification of (A) and (B) of 3 independent experiments. * $p < 0.05$ compared to vehicle-treated control.

Fig. 3.

ECE-1 and IDE show HNE adduction after HNE or A β ₁₋₄₂ treatment. (A) SH-SY5Y cells were incubated with HNE and A β ₁₋₄₂ for 24 h. Cell lysates were prepared and immunoprecipitated with monoclonal anti-ECE-1 and anti-IDE antibodies, respectively. Immunoprecipitates were analyzed by immunoblotting with the polyclonal HNE, ECE-1, and IDE antibodies. CON, vehicle control; HNE (10 μ M); A β ₁₋₄₂ (1 μ M). The results are representative of three independent replicates. Values are the mean $\pm$ SEM expressed as the percentage of control value. C, vehicle control; H, HNE (10 μ M) treated; A, A β ₁₋₄₂ (1 μ M) treated. (B) and (D), Quantification of the immunoblots by phosphorimaging of three independent experiments. * $p < 0.05$ compared to vehicle-treated control.

Fig. 4. ECE-1 and HNE colocalize in oxidatively stressed SH-SY5Y cells. Cells were untreated (a–d), treated with HNE (e–h) or $A\beta_{1-42}$ (i–l) for 24 h, prior to fixation and staining with anti-ECE-1 (red) (a, e, i) or anti-HNE (green) (b, f, j). The nuclei (c, g, k) were counterstained with DAPI. Colocalization was determined by confocal imaging (d, h, l) and is represented by yellow.

Fig. 5. IDE and HNE colocalize in oxidatively stressed SH-SY5Y cells. Cells were untreated (a–d), treated with HNE (e–h) or $A\beta_{1-42}$ (i–l) for 24 h, prior to fixation and staining with anti-IDE (red) (a, e, i) or anti-HNE (green) (b, f, i). The nuclei (c, g, k) were counterstained with DAPI. Colocalization was determined by confocal imaging (d, h, l) and is represented by yellow.

Fig. 6.

HNE or A β increases ECE specific activity in SH-SY5Y cells. After 24 h incubation with vehicle, HNE (5, 10 and 20 μ M) (a, b and c) or A β (0.5, 1, and 2 μ M) (d, e and f), cells were harvested, ECE activity in the cell lysates (a, c, d, f) or whole cells (b and e) was analyzed by Fluorescence Resonance Energy Transfer (FRET). Average ECE activity (FL intensity) was measured from 0 to 60 min with a reading interval of 5 min. Specific ECE activity was calculated by subtracting residual fluorescent intensity after incubation with the ECE inhibitor phosphoramidon. a and d, the specific ECE activity of cell lysates after HNE or A β treatments. b and e, whole cell ECE activity after HNE or A β treatments. c and f, dependence of the mean ECE activity on increasing substrate concentration in SH-SY5Y. Values are the mean $\pm$ SEM expressed as the percentage of control value (a, b and d, e). Three independent experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$, vs control.

Fig. 7. Effect of HNE or Aβ on IDE specific activity. After 24 h incubation with vehicle, HNE (10 μM) or Aβ₁₋₄₂ (1 μM), cells were harvested, specific IDE activity in the cell lysates was analyzed by the assay of immunocapture based Fluorescence Resonance Energy Transfer (FRET). Fluorescent readings were taken after 60 min. Values are the mean ± SEM, presented by the percentage of control.

Table 1ECE K_m and $V_{\max}$ values alteration after treated by HNE and $A\beta$ peptide in SH-SY5Y cells

Treatments	$V_{\max}$ ($\mu\text{M}/\text{min}$)	K_m (μM)
HNE (μM)		
0	7.19 ± 4.20	5.08 ± 3.87
5	6.36 ± 2.24	3.35 ± 1.70
10	5.17 ± 4.13	3.11 ± 3.67
20	5.70 ± 3.03	2.67 ± 2.20
$A\beta_{1-42}$ (μM)		
0	15.38 ± 4.82	7.41 ± 2.0
0.5	16.42 ± 3.60	5.40 ± 2.07
1	15.66 ± 2.71	5.32 ± 1.67
2	17.38 ± 3.38	4.71 ± 1.88

Data represent mean $\pm$ SEM. All assays were performed in duplicate for three to five independent experiments. Cells were treated with HNE (5, 10 and 20 μM) and $A\beta_{1-42}$ (0.5, 1 and 2 μM) for 24 h. Kinetics isotherms ($V_{\max}$ and K_m values) for ECE activity were determined by means of non-linear least squares fitting to the Michaelis-Menten equation using GraphPad Prism software.