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N-Acetylcysteine Prevents 4-Hydroxynonenal- and Amyloid- β -Induced Modification and Inactivation of Neprilysin in SH-SY5Y Cells

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Abstract

As one of the dominant amyloid- β peptide (A β) proteases, neprilysin (NEP) plays a crucial role in maintaining a physiologic balance between A β production and catabolism. We have previously shown that NEP is modified by 4-hydroxynonenal (HNE) adducts, resulting in decreased activity in the brain of AD patients and cultured cells. In order to determine whether antioxidants can rescue NEP, SH-SY5Y cells were treated with HNE or A β , together with N-acetylcysteine for 24 hours, prior to analysis of NEP protein levels, activity, and oxidative modifications. Intracellular NEP developed HNE adducts after 24 hours of HNE or A β treatment as determined by immunoprecipitation, immunoblotting, and double immunofluorescence staining. N-acetylcysteine at 10 to 100 μ M alleviated HNE adduction after HNE or A β treatment. In keeping with previous reports, HNE-modified NEP showed decreased catalytic activity. The present study demonstrates that antioxidants can be used to spare NEP from oxidative modification, suggesting a potential mechanism underlying the neuroprotective effects of antioxidants in aging or Alzheimer's disease.

Keywords

Alzheimer's disease; amyloid- β ; antioxidant; degradation; 4-hydroxynonenal; N-acetylcysteine; neprilysin; oxidative stress

INTRODUCTION

Alzheimer's disease (AD) is an extremely common progressive and irreversible neuropsychiatric disorder characterized by neuronal degeneration and cognitive deterioration [1]. The most prominent neurochemical change in AD brain is a reduced concentration of acetylcholine in the hippocampus and neocortex caused by degeneration of cholinergic neurons [2]. AD is characterized morphologically by extensive amyloid deposition, neurofibrillary tangles, inflammation, and ultimately synaptic and neuronal loss [3, 4]. A large body of evidence suggests that the accumulation of amyloid- β (A β) plays an

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important role in the initiation and maintenance of neuronal degeneration in AD [2, 5–7]. A β neurotoxicity has been shown both *in vitro* [8, 9] and *in vivo* [10–12], but the mechanisms that contribute to abnormal A β accumulation remain unclear. Both increased production and decreased degradation have been observed, although the importance of A β catabolism remains controversial [13].

A β degradation occurs primarily through the action of peptidases, including neprilysin (NEP), insulysin (insulin degrading enzyme), and endothelin converting enzyme. NEP appears to be the predominant A β protease [3, 14–16]. NEP (also known as neutral endopeptidase, EC3.4.24.11, enkephalinase, and CD10), a 97-kDa type II membrane-bound zinc metalloendopeptidase, is ubiquitously expressed by neurons and is capable of degrading both monomeric and oligomeric forms of A β and several other neuropeptides [17]. Animal studies have shown that NEP expression is reduced in the hippocampus and cortex in aged mice [18–20], whereas A β is elevated in NEP knockout mice or those treated with NEP inhibitors [21–23]. Conversely, overexpression of NEP reduced A β levels in a dose-dependent manner [23, 24] and protected neurons from A β toxicity *in vitro* [25]. We previously reported that NEP was selectively decreased in AD brain [26], and there is an inverse correlation between NEP activity and vulnerability to AD pathology. Thus NEP was lower in hippocampus and association cortex, which are vulnerable to senile plaque development, than the cerebellum or peripheral organs, which are relatively resistant [19, 27]. NEP levels were inversely correlated with senile plaque counts and total A β levels in cortical homogenates and positively correlated with clinical cognitive scores [26]. These data support the hypothesis that decreased NEP or its activity contributes to A β deposition and neuronal dysfunction in AD.

Oxidative stress has long been recognized as a contributing, early factor in AD [28–33]. A β induces free radical generation [28, 34–41] culminating in elevated 4-hydroxynonenal (HNE), a marker of lipid peroxidation [42]. HNE can interact with and inactivate a variety of enzymes including NEP [43]. We have previously shown that NEP is modified by HNE adducts in AD brain [43] or in cells treated with HNE and A β protein [44]. HNE-modified NEP showed decreased catalytic activity that was associated with elevations in A β _{1–40} in SH-SY5Y and H4 APP695wt cells [44]. These studies strengthened our hypothesis that NEP can be inactivated by HNE-adduction, contributing to reduced A β cleavage and enhanced accumulation.

Antioxidants have been shown to be a promising therapy in various types of neurodegenerative diseases [45]. Because oxidative modification can inhibit NEP activity, we evaluated if antioxidants could antagonize HNE-adduction. N-acetylcysteine (NAC) is a precursor of glutathione that plays an important role in intracellular redox balance. NAC can replenish intracellular glutathione stores, thus providing protection against oxygen free radicals. NAC also possesses additional antioxidant properties such as directly scavenging hydrogen peroxide [46], inhibiting chemotaxis, inhibiting cytokine-mediated induction of nitric oxide synthase, and protecting against ischemia-reperfusion injury [47, 48], lethal endotoxemia [49], multiple sclerosis [50, 51], and hypoxic ischemic brain injury in neonatal brains [52, 53]. It also prevents apoptosis [54, 55]. In this study, we show NAC antagonized HNE or A β -induced HNE-adduction of NEP and preserved A β protease activity. These

results suggest a novel mechanism for the neuroprotective effects of antioxidants in pathologic aging or AD.

MATERIALS AND METHODS

Chemicals and reagents

Synthetic human A β ₁₋₄₂ was purchased from BACHEM (Torrance, CA). Dithiothreitol (DTT) protease inhibitors cocktail (P8430) was obtained from Sigma-Aldrich (St. Louis, MO). 4-Hydroxy-2-nonenal (HNE) was obtained from A.G. Scientific, Inc. (San Diego, CA). Mac-R-P-P-G-F-S-A-F-K (Dnp)-OH Fluorogenic Peptide Substrate V came from R&D Systems, Inc. (Minneapolis, MN). GE nitrocellulose membrane was purchased from ISC BioExpress (Kaysville, UT). An enhanced emiLuminescence (ECL) kit and Seize® Classic (G) Immunoprecipitation kit were obtained from Pierce (Rockford, IL). N-acetylcysteine (NAC) and other general chemicals and reagents were from Fisher Scientific (Pittsburgh, PA).

Antibodies were obtained from the following sources: rabbit anti-HNE and rabbit anti-NEP antibody (Millipore, Billerica, MA); mouse CD10 ectodomain (R&D Systems, Inc., Minneapolis, MN). Anti-human/mouse Rhodamine conjugated affinity purified secondary antibody, goat anti-rabbit fluorescein conjugated secondary antibody, and horseradish peroxidase (HRP) conjugated secondary antibody were purchased from Millipore.

Cell culture and experimental treatment

SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells were seeded into plates or dishes (BD FALCON, San Jose, CA) in Dulbecco's Modified Eagle Medium/F12 (1:1), supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and 2 mM L-glutamine. Experiments were carried out 24 to 48 hours after cells were seeded. NAC was dissolved and diluted with Dulbecco's phosphate buffered saline (PBS). HNE was freshly prepared in dimethyl sulfoxide (DMSO) and diluted in Dulbecco's PBS prior to each experiment. A β ₁₋₄₂ was dissolved in 0.4% DMSO and incubated in 37°C for 3 days to allow aggregation. The aggregated A β ₁₋₄₂ can be stored at -80°C till usage. To induce oxidative modification, HNE (10–20 µM) or A β ₁₋₄₂ (1 µM) were added to the cultures. Cells were kept in serum-free medium. Final concentrations of DMSO in medium were <0.003%. Vehicles (with the same concentrations of DMSO) were added to the cultures as controls. Preincubation with NAC (10–100 µM) was conducted 2 hours before the HNE or A β addition. Assays for HNE adduction and NEP activity were performed 24 hours after HNE or A β treatment.

NEP and HNE analysis by immunoblotting and immunoprecipitation

After 24 hours incubation of HNE or A β with/without co-incubation of NAC, cells were harvested and lysed in passive lysis buffer containing protease inhibitors cocktail. Lysates were incubated on ice for 30 minutes and then centrifuged at 10,000 X g for 10 minutes at 4°C. Protein concentration in the supernatant was determined by Coomassie blue protein

binding method using the Protein Quantification Kit-Rapid (Sigma/Fluka) with bovine serum albumin as standard. For Western blotting assay, protein from all samples was adjusted to the same concentration.

For NEP protein level detection, samples containing equal amounts of protein 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 minutes and loaded and separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes in a Bio-Rad electrophoresis system (Bio-Rad, provide location). After blocking with TBST containing 5% non-fat milk, the membranes were kept at 4°C overnight with primary antibodies (1:5,000 for β -actin and 1:1,000 for NEP, respectively), followed by HRP-conjugated second antibodies (1:10,000) at room temperature (RT) for 1 hour. The target protein bands were detected using the ECL Western blotting detection system (Pierce) and autoradiography film (Fisher).

Immunoprecipitation with anti-NEP was performed using the Seize® Classic (G) Immunoprecipitation Kit (Pierce) according to the manufacturer's protocol. For total cell extract preparation, SH-SY5Y cells were lysed in passive lysis buffer containing protease inhibitors cocktail. Supernatants, prepared as mentioned above, were incubated with mouse anti-CD10 monoclonal antibody (1 μ g/ml) at 4°C overnight. After washing immobilized protein G with 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 hour 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 ImmunoPure® Elution Buffer and then analyzed by Western blotting with anti-HNE and anti-NEP polyclonal antibodies. The density of the NEP and HNE blotting bands were quantified by ImageJ software (1.37v, Wayne Rasband, National Institutes of Health,) as described [56, 57]. The ratio of HNE/NEP was calculated from the same sample to assess the relative level of HNE-NEP adducts.

Double immunofluorescence staining

Cells with different treatments were fixed with 4% paraformaldehyde at RT for 20 minutes and permeabilized with 0.2% Triton X-100 in PBS. After blocking for 1 hour at RT with 1% goat serum/PBS, cells were incubated overnight at 4°C in a humidity chamber with primary mouse anti-NEP (1:100). At the end of the incubation, the cells were rinsed three times with PBS-Tween-20 (PBST, 0.05%) and incubated with the TRITC conjugated rabbit anti-mouse secondary antibody (1:50) for 60 minutes at RT. After rinsing with PBST 3 times, second primary rabbit anti-HNE (1:200) was added and incubated for 2 hours at RT. FITC conjugated secondary anti-rabbit IgG (1:50) was incubated for another 60 minutes at RT. All primary and secondary antibodies were diluted in PBST with 2% normal goat serum. After rinsing with PBS, 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.

Fluorometric assay of NEP activity in cell lysates and intact cells

NEP activity was determined by fluorescence resonance energy transfer (FRET). After extraction with a final concentration of 0.1% Triton X-100 in PBS (pH 7.4) for 30 minutes on ice NEP activity in cell lysates was analyzed using a synthetic NEP fluorogenic peptide substrate (Mca-RPPGFSAFK[Dnp]-OH(R&D Systems, Inc.) [58] at RT in the presence or absence of the NEP inhibitor thiorphan (1 μ M). Samples dissolved in 50 mM HEPES buffer (pH 7.5) were preincubated with 1 μ M thiorphan or PBS for 10 minutes 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 μ M) at RT. Fluorescence over the time was measured for 1 hour. The specific NEP activity was determined as the fluorescence difference occurring in the presence or absence of 1 μ M thiorphan. Kinetic isotherms (V_0 , V_{max} and K_m values) for NEP activity were determined by means of non-linear least squares fitting to the Michaelis-Menten equation using GraphPad Prim software (version 3.0 for Windows, ISI Software, San Diego, CA).

To measure the activity of membrane-bound enzyme, cells treated by 24 hour-incubation of HNE (10 μ M) or A β (1 μ M) with/without NAC were harvested and washed with PBS. 5×10^4 cells were resuspended in 50 μ l PBS contained in each well of black microtiter plates. The 50 μ l substrate (10 μ M in 50 mM HEPES) was added and incubated for 60 minutes at RT. As mentioned above, thiorphan was used to determine the specific NEP activity in intact cells.

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

N-acetylcysteine prevents NEP from HNE adduction

HNE-adducted and unmodified NEP levels were assessed in SH-SY5Y cells treated with vehicle, HNE or A β_{1-42} , and HNE or A β_{1-42} co-incubated with NAC were analyzed by immunoblotting for NEP levels and immunoprecipitation followed by Western blot, the results were expressed as HNE/NEP ratios. Cells exposed to HNE for 24 hours with or without co-treatment of NAC did not display an obvious alteration in NEP levels (Fig. 1A).

In order to determine if NEP was modified by oxidative damage, we first pulled down NEP protein with mouse anti-NEP (CD10) antibody, followed by Western blot with anti-HNE and rabbit anti-NEP antibodies. Consistent with our previous study [44], treatment with HNE markedly increased HNE-NEP levels. 10 μ M to 100 μ M of NAC administration decreased HNE adduction in NEP. The ratio of HNE to NEP (Fig. 1B) was 60% lower in control than in HNE-treated cells (Fig. 1B), $p < 0.05$. All concentrations of NAC tested in this study decreased the HNE/NEP ratio to control levels.

In A β -treated cells, NEP protein level as determined by western blotting showed no statistically significant change, though it does show a trend to increase in A β treatment. NAC at 10, 100 μ M had little to no effect on total NEP levels (Fig. 2A). Similar to HNE treatment, A β increased HNE-adduction of NEP (approximately 65% higher than control, $p < 0.01$, Figure 2B). However, only 100 μ M NAC reduced A β -induced HNE adduction ($p < 0.05$, Fig. 2B). Thus, NAC showed differential effects on HNE versus A β mediated adduction, suggesting that A β may induce continuous and high level production of endogenous HNE that requires more antioxidant to take effect, although it cannot be excluded that there may be more than one mechanisms involved in these two pro-oxidants induced NEP modification.

To further determine the subcellular location of modified protein, we performed double fluorescence staining followed by confocal analysis of oxidative stressed SH-SY5Y cells (Figs. 3, 4). HNE was undetectable in controls (Fig. 3b, Fig. 4b) but positive in both HNE- and A β -treated cells (Fig. 3f, Fig. 4f). Merged images revealed NEP and HNE the colocalized diffusely in the cytoplasm in cells treated with HNE or A β (Fig. 3h, Fig. 4h) consistent with the results observed after immunoprecipitation and western blotting. Therefore, NEP was modified by exogenously added (Fig. 3) or endogenously produced HNE induced by A β (Fig. 4). NAC reduced HNE-positive immunosignals both in HNE- and A β -treated cells (Fig. 3f and Fig. 4f).

These results suggest that both HNE and A β can induce NEP oxidative modification by HNE adduction, which has been widely reported as a crucial factor in inactivating NEP activity. The antioxidant, NAC, can resist variable HNE- and A β -induced NEP modification.

NAC protects NEP from HNE adduction induced inactivation after HNE and A β treatment

Because HNE adduction can inactivate NEP, we hypothesized that NAC would stabilize NEP activity after HNE and/or A β treatment. NEP activity in lysates or intact SH-SY5Y cells treated for 24 hours was determined with a commercially available fluorogenic substrate. The activity assay was highly sensitive, linear over-time (up to 60 minutes examined, data not shown) and sensitive to the NEP inhibitor thiorphan (data not shown). Treatment of cells with HNE led to a significant loss of NEP activity, particularly in intact cells (Fig. 5). Specific NEP activity in cell lysates was decreased ~50% ($p < 0.05$) (Fig. 5A) whereas intact cells showed up to a 60% reduction (Fig. 5B). Similar to HNE, samples treated with A β showed decreased NEP activity (Fig. 6A–B). NAC co-incubation at two concentrations all significantly increased NEP activity compared to HNE- or A β -treated cells (Figs. 5, 6).

To further analyze NEP activity biochemically, we assessed a range of substrate concentrations (4–20 μ M) in SH-SY5Y cells. Activity was saturable, and followed Michealis-Menten kinetics in all cells (Fig. 5C and Fig. 6C). V_0 and V_{max} decreased after HNE or A β treatment. NAC at 10 to 100 μ M partially normalized the V_0 and V_{max} , but did not reach statistical significance due to the small sample size (Table 1, Fig. 5C and Fig. 6C). 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.

The K_m values showed no obvious differences in the different treated groups (data not shown).

DISCUSSION

Proteins become oxidatively modified during the aging process and in various neurodegenerative diseases including AD. An imbalance of prooxidants and antioxidants leads to oxidative stress. Excess HNE produced by oxidative injury culminates in membrane lipid, DNA, and protein peroxidation. HNE-macromolecular adducts are abundant in the brain of patients with AD, consistent with a role for oxidative damage in disease pathogenesis [36, 42, 59]. Increasing evidence, including ours, has demonstrated that NEP is clearly one of the targets of HNE-mediated modification in AD brain, and likely contributes to accelerated NEP catabolism and reduced levels compared to controls [43]. Our previous data demonstrated that NEP is modified by HNE adducts in the brain of AD patients and *in vitro* treatment of either exogenous HNE or endogenous HNE induced by A β in SH-SY5Y neuroblastoma cells. HNE-modified NEP showed decreased catalytic activity that was associated with elevations in A β ₁₋₄₀ in SH-SY5Y and H4 APP695wt cells [44]. The results demonstrate that NEP can be inactivated by HNE-adduction, which is associated with reduced A β cleavage and enhanced A β accumulation. In the present study, NEP protein levels in cells treated with HNE did not show obvious changes, while they were slightly increased in A β -treated cells, which is in accordance with previous studies [44]. NAC did not affect NEP levels in HNE-treated cells but decreased A β -induced NEP enhancement. Previous studies indicated that A β increased the production of free radicals by neurons [60–63]. To measure HNE-modified NEP, we used immunoprecipitation followed by western blotting approaches. Consistent with previous reports [64], in addition to the increased HNE-NEP adducts in HNE treatment, we also showed that A β -induced HNE production within 24 hours. As seen with exogenous HNE treatment, A β increased HNE-NEP conjugates as measured by immunoprecipitation, western blotting, and double immunofluorescence staining. 10 μ M and 100 μ M of NAC decreased HNE adduction in NEP both in HNE- and A β -treated cells. Immunoprecipitation data indicated the ratio of HNE to NEP was 60% and 65% higher than controls in HNE- and A β -treated cells, respectively. When cells treated with HNE or A β were co-incubated with NAC at concentrations >10 μ M, the HNE/NEP ratios were significantly decreased. However, NAC was only partially effective in antagonizing A β induced HNE-NEP adducts, suggesting that different mechanism might be involved in A β -induced HNE-NEP adduct development. These data strongly suggest that NAC is capable of preventing NEP from HNE adduction induced by either exogenous or endogenous HNE treatments.

Previous studies have shown that HNE adduction could inactivate endogenous and recombinant NEP [44, 65], which contributes to A β accumulation [43, 44]. Since NAC markedly attenuated HNE-adducted NEP levels, we explored whether NAC can prevent NEP inactivation due to HNE adduction after HNE or A β treatment in SH-SY5Y cells. The data in this study show that exogenous HNE or endogenous HNE induced by A β decreased NEP activity both in cell lysates and intact cells, in keeping with our previous results [44]. V_0 , V_{max} , and K_m revealed that NEP activity in SH-SY5Y cells followed Michealis-Menten kinetics, and demonstrated a hyperbolic dependence of v (velocity) on substrate

concentration. After 24-hour treatment of HNE or A β , V_0 and V_{max} trended to decrease. These results, together with the data from immunoprecipitation and western blotting, suggested that NEP is inhibited by HNE adduction, while was sensitive to NAC despite HNE or A β treatment (Table 1, Fig. 5C and Fig. 6C). Overall, these data suggest that HNE adduction reduces NEP activity. Whether NAC prevents or reverses NEP-HNE adduction remains to be determined.

Our previous study indicated that A β treated cells showed increased NEP mRNA and protein expression. However, despite elevations in NEP protein, the activity was significantly lower compared to the NEP protein level [44]. In order to better observe the effect of NAC, in the present study, we optimized the experimental condition via serum deprivation during HNE and A β treatments to induce more active HNE adduction. NEP levels did not change obviously in HNE treatment, while they significantly decreased in A β treatment, suggesting serum may interfere with biological effects of A β in these experimental systems.

Based on *in vitro* and *in vivo* studies, antioxidants and antioxidant drugs have been proposed as an alternative AD therapy. An increasing number of studies have demonstrated that direct antioxidants such as flavonoids, indirect antioxidants such as nitric oxide synthase inhibitors, and metabolic antioxidants such as NAC [66] are able to reduce or block neurodegenerative characteristic of AD [67]. Results from this study showed that specific NEP activities were lower in both HNE- and A β -treated cells due to oxidative modifications demonstrated by the increased ratio of HNE/NEP. Decreased A β -catabolic activity of NEP might contribute, at least partly, to the reduced A β cleavage and enhanced accumulation during the AD pathogenesis. NAC, a metabolic antioxidant, can protect the enzyme activity by preventing NEP modification by HNE. These results indicate that it may be worth the further study on the biological effects of antioxidants in addition to their general neuroprotective effects.

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Figure 1.

NAC prevents HNE-adduction of NEP. After incubation with vehicle, HNE (20 μ M) with/without NAC (10, 100 μ M) for 24 hours, SH-SY5Y cells were harvested and equal amounts of protein were analyzed by western blot to determine NEP protein levels. HNE-NEP adducts were detected by anti-NEP IP followed by western blot. (A) Representative NEP western blot with quantification. (B) Representative anti-NEP immunoprecipitation followed by HNE and NEP western blotting. Data was quantified and expressed as HNE/NEP ratio. The data are expressed as mean $\pm$ SEM from three independent experiments. * p < 0.05

compared to vehicle-treated control. # $p < 0.05$, ## $p < 0.01$ compared to HNE treated group.

Figure 2.

NAC partially blocks NEP and HNE-NEP adducts after A β ₁₋₄₂ peptide treatment. After incubation with vehicle, A β ₁₋₄₂ (1 μ M) with/without NAC (10, 100 μ M) for 24 hours, SH-SY5Y cells were harvested and equal amounts of protein were analyzed by western blot to determine NEP protein levels. HNE-NEP adducts were detected by anti-NEP IP followed by western blot as shown. (A) Representative NEP western blot and quantification. (B) Representative blot of NEP immunoprecipitation followed by HNE and NEP western blotting. Quantified data was presented as HNE/NEP ratio. The data are expressed as mean $\pm$ SEM from 3 independent experiments. ** p < 0.01 compared to vehicle-treated control. # p < 0.05 compared to A β ₁₋₄₂ treated group.

Figure 3.

NEP and HNE colocalize in the cytoplasm of HNE-treated SH-SY5Y cells. Cells were untreated (a–d), treated with 10 μ M HNE (e–h) or 100 μ M NAC (i–l) for 24 hours prior to fixation and staining with anti-NEP (red) (a, e, i) or anti-HNE (green) (b, f, j). The nuclei were counterstained with DAPI (c, g, k). Colocalization was determined by confocal imaging (d, h, l) and is represented by yellow.

Figure 4.

NEP and HNE colocalize in the cytoplasm of A β -treated SH-SY5Y cells. Cells were untreated (a–c), treated with 10 μ M HNE (d–f) or 1 μ M A β _{1–42} (g–i) for 24 hours prior to fixation and staining with anti-NEP (green) (a, d, g) or anti-HNE (red) (b, e, h). Colocalization was determined by confocal imaging (c, f, i) and is represented by yellow.

Figure 5.

NAC prevents HNE-induced reduction of NEP activity in SH-SY5Y cells. After 24-hour incubation with vehicle, HNE (10 μ M), or together with NAC (10 and 100 μ M), cells were harvested. NEP activity in the cell lysates (A) or whole cells (B) was analyzed by fluorescence resonance energy transfer (FRET). Average NEP activity represented by fluorescence intensity was measured from 0 to 60 minutes with a reading interval of 5 minutes. Specific NEP activity was calculated by subtracting residual fluorescent intensity after incubation with the NEP inhibitor thiorphan. (A) Specific NEP activity of cell lysates

after HNE with/without NAC treatments. (B) Whole cell specific NEP activity after HNE with/without NAC treatments. (C) Dependence of the mean NEP activity on increasing substrate concentration in SH-SY5Y lysates. Values are the mean $\pm$ SEM expressed as the percentage of control value (A, B). Three independent experiments were performed in triplicate. ** $p < 0.01$, vs control. # $p < 0.05$, ## $p < 0.01$, vs HNE treatment.

Figure 6.

NAC alleviates Aβ₁₋₄₂-induced reductions in NEP specific activity in SH-SY5Y cells. After 24-hour incubation with vehicle, Aβ₁₋₄₂ (1 μM) or together with NAC (10 and 100 μM), cells were harvested, NEP activity in the cell lysates (A) or whole cells (B) was analyzed by fluorescence resonance energy transfer (FRET). Average NEP activity represented by fluorescence intensity was measured from 0 to 60 minutes with a reading interval of 5 minutes. Specific NEP activity was calculated by subtracting residual fluorescent intensity after incubation with the NEP inhibitor thiorphan. (A) Specific NEP activity of cell lysates after Aβ with/without NAC treatments. (B) Whole cell specific NEP activity after Aβ with/without NAC treatments. (C) Dependence of the mean NEP activity on increasing substrate concentration in SH-SY5Y lysates. Values are the mean ± SEM expressed as the percentage

of control value (A, B). Three independent experiments were performed in triplicate. ** $p < 0.01$, vs control. # $p < 0.05$, ## $p < 0.01$, vs A β treatment.

Table 1

NAC enhances V_0 and $V_{\max}$ values after treated by HNE and $A\beta_{1-42}$ peptide in SH-SY5Y cells.

Treatment	V_0 (nmol/min)	$V_{\max}$ (nmol/min)
Control	101.20±4.93	149.30±23.72
HNE 10 μ M	77.24±2.88	98.29±11.63
HNE +NAC 10 μ M	79.15±25.61	103.00±51.26
HNE +NAC 100 μ M	112.32±28.43	176.70±90.60
Control	61.30±8.59	76.67±10.75
$A\beta_{1-42}$ 1 μ M	58.16±5.75	64.39±6.00
$A\beta$ +NAC 10 μ M	56.61±3.73	61.58±4.18
$A\beta$ +NAC 100 μ M	64.43±1.72	77.01±3.82

Data represent mean $\pm$ SEM. All assays were performed in duplicate for three to five independent experiments. Cells were pre-treated with NAC (10–100 μ M) 2 hours prior to the addition of vehicle, HNE (10 μ M) or $A\beta_{1-42}$ (1 μ M) and continue cultured for 24 hours.