Susceptibility of Amyloid β Peptide Degrading Enzymes to Oxidative Damage: A Potential Alzheimer's Disease Spiral†

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ABSTRACT: Insulysin (IDE) and neprilysin (NEP) were found to be inactivated by oxidation with hydrogen peroxide, an iron−ascorbate oxidation system, and by treatment with 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). In each case reaction led to the introduction of protein carbonyl groups as judged by reaction with 2,4-dintrophenylhydrazine. IDE was inactivated by reaction with 4-hydroxy-2-nonenal (HNE) with the concomitant formation of protein adducts. NEP was not inactivated to a significant extent by HNE, but some HNE-adduct formation did occur. Prior reaction with hydrogen peroxide or AAPH led to enhanced formation of HNE adducts. Treatment of IDE with AAHP or hydrogen peroxide increased its susceptibility to proteolysis, while treatment of NEP with iron/ascorbate or hydrogen peroxide increased its susceptibility to proteolysis. Since IDE and NEP play a prominent role in the clearance of amyloid β peptides, their oxidative inactivation and enhanced proteolysis can contribute to the onset and/or progression of Alzheimer's disease.

The accumulation and deposition of amyloid plaques is one of the hallmarks of Alzheimer's disease and is thought to result from elevated levels of brain amyloid β peptides (Aβ),[1] in particular Aβ_{1-42}. Although the cause of increased Aβ in Alzheimer's disease is generally unknown, evidence is accumulating that it is not a result of increased synthesis, but rather a decrease in Aβ clearance (*1*). Aβ clearance occurs primarily through the action of a group of peptidases including neprilysin (NEP), insulysin (insulin degrading enzyme, IDE), and endothelin converting enzyme (ECE). The deletion or disruption of any one of these peptidase genes in mice leads to a significant increase in Aβ levels (*2*−*5*). In the case of IDE and NEP the increase in Aβ was proportional to the gene dosage (*2*, *3*).

Based on studies with mouse models it has been suggested that there is a decline in neprilysin activity with aging (*6*−*7*). In particular NEP activity declined in the hippocampus, a region vulnerable to AD pathology, but not in the cerebellum, a region in which Aβ does not accumulate. Yasojima et al. (*8*, *9*) showed that neprilysin mRNA and immunoreactive protein was lowered in high plaque areas of the Alzheimer's disease brain. Recently, it has been shown that in mouse brain the steady-state IDE levels also decrease in the hippocampus as a function of aging, but not in the cerebellum (*7*).

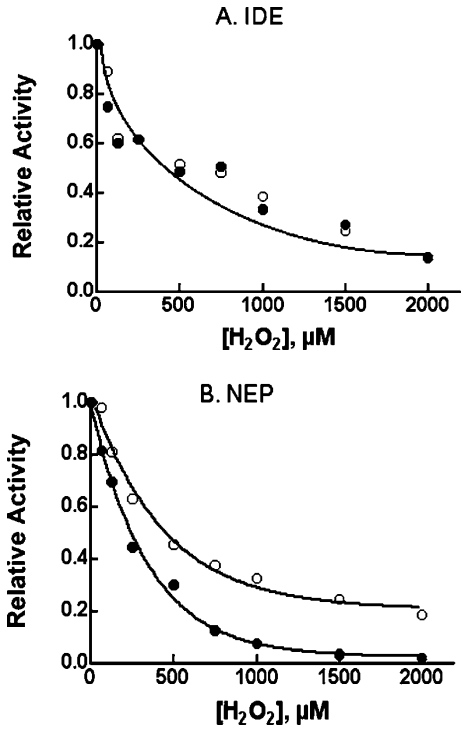

FIGURE 1: Inactivation of IDE and NEP by hydrogen peroxide. Inactivation reactions were conducted in 50 mM potassium phosphate buffer pH, 7.3, containing the indicated concentration of H_2O_2 (closed circles) and when added 100 μM $FeCl_2$ (open circles). The reaction was allowed to proceed for 24 h at room temperature (IDE) or 37 °C (NEP), after which time residual activity was determined. (A) IDE inactivation by hydrogen peroxide. (B) NEP inactivation by hydrogen peroxide.

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[1] Abbreviations: Aβ, amyloid β peptide; IDE, insulysin (insulin degrading enzyme); NEP, neprilysin; AD, Alzheimer's disease; HNE, 4-hydroxy-2-nonenal; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride.

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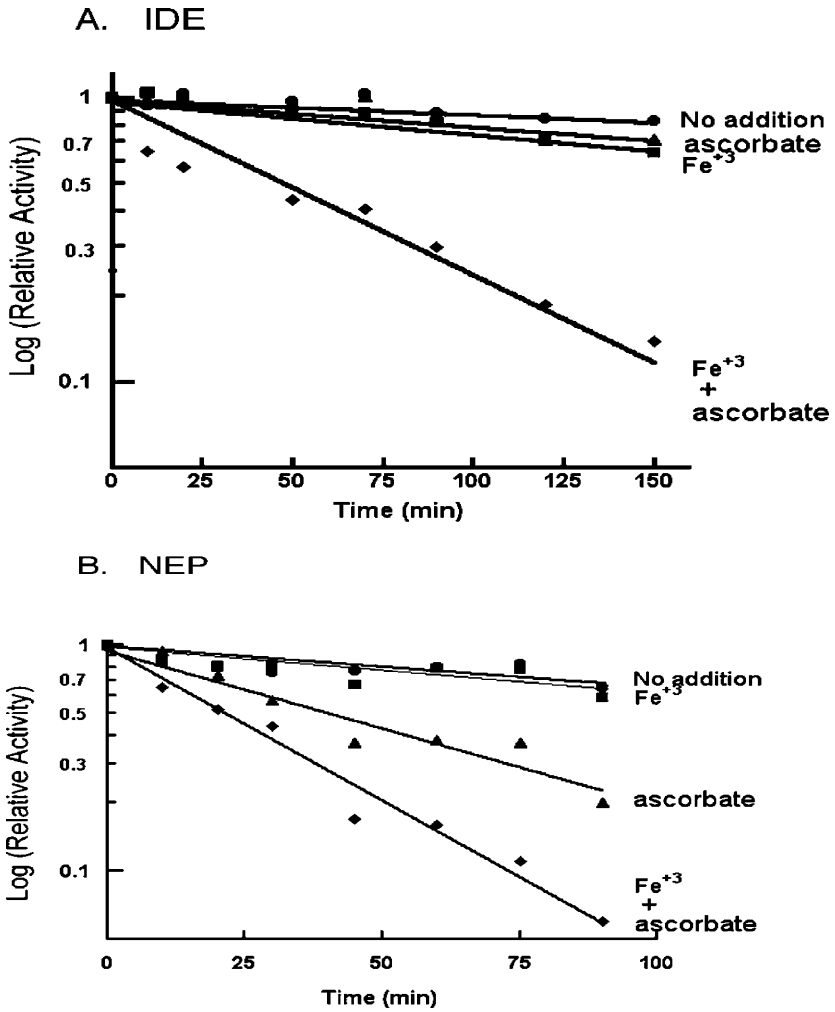

FIGURE 2: Inactivation of IDE and NEP by metal catalyzed oxidation. (A) IDE was incubated in 25 mM HEPES, pH 7.2, and when added 10 mM ascorbate, 100 μM $FeCl_3$, or a combination of ascorbate and iron. The reaction was allowed to proceed for the indicated time at 37 °C, after which the residual activity was determined. Data are plotted as a pseudo-first-order inactivation curve. (B) NEP was incubated in 25 mM HEPES, pH 7.2, and when added 25 mM ascorbate, 25 μM $FeCl_3$, or a combination of ascorbate and iron. The reaction was allowed to proceed for the indicated time at 37 °C, after which time the residual activity was determined. Data are graphed as in panel A above.

Oxidative stress has long been implicated as a contributing factor to Alzheimer's disease (*10*−*16*). In fact as noted by Ghanbari et al. (*17*) oxidative damage and the cellular response to oxidative damage is an early event in Alzheimer's disease and as proposed by Nunormura et al. (*18*, *19*) appears to precede the clinical and pathological manifestations of the disease. It has been reported that in the human AD brain NEP is more oxidatively modified by 4-hydroxy-2-nonenal compared to control brain (*20*). IDE was found to also be oxidatively modified by 4-hydroxy-2-nonenal in the AD brain, although a comparison was not made with control brain (*7*). Furthermore the level of oxidatively modified IDE was greater in the hippocampus than in the cerebellum. In neither of these studies was the effect of oxidation on activity determined. We have studied the susceptibility of NEP and IDE to oxidative stress and find that such oxidation leads to inactivation and increased susceptibility to proteolysis. Inactivation of these peptidases in brain could initiate a cascade effect in which oxidative stress leads to decreased neprilysin and insulysin activity resulting in a concomitant increase in Aβ due to impaired clearance.

METHODS

Hydrogen peroxide, trypsin, chymotrypsin, and proteinase K were obtained from Sigma. The concentration of hydrogen peroxide stock solutions was determined by measuring the absorbance at 240 nm ($\epsilon = 43.6\ M^{-1}\ cm^{-1}$) and/or by the PeroXOquant Quantitative Peroxide Assay Kit obtained from Pierce. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAHP) was a product of Wako Chemicals, while 4-hydroxy-2-nonenal (HNE) was purchased from Cayman Chemical. Rabbit anti-HNE antisera and the Oxyblot kit for the detection of protein carbonyl groups were purchased from Chemicon International.

Recombinant insulin degrading enzyme (EC 3.4.24.56; IDE, insulysin) was expressed in Sf9 cells and purified on a His-Select HC nickel affinity gel column (Sigma) as previously described (*21*). Recombinant neprilysin (EC 3.4.24.11; NEP) was expressed as its secreted extracellular domain in HEK293 cells using a lentivirus construct. The media from these cells were collected after 7 days, concentrated, dialyzed against 20 mM Tris-HCl, pH 7.9, and chromatographed on a 1 mL column of Source^T 15Q quaternary ammonium strong anion exchanger resin (Amersham Biosciences) in 20 mM

Tris-HCl, pH 7.9. The enzyme was eluted by a linear salt gradient from 0 to 0.5 M NaCl. This procedure generally produced enzyme of greater than 90% purity as judged by Coomassie stained gels of the enzyme subjected to SDS−PAGE. Occasionally a second anion exchange column (Pharmacia mono Q column) was used for the final purification step.

IDE activity was measured fluorometrically (excitation at 318 nm and emission at 419 nm) with Abz-GGFLRKHGQ-EDDnp as substrate as previously described (*21*). Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, and 2 μM substrate. NEP activity was measured with glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide as substrate in a modification of the assay described by Li and Hersh (*22*). NEP releases Phe-4-methoxy-2-naphthylamide, which is then converted to the fluorescent 4-methoxy-2-naphthylamine by inclusion of recombinant puromycin sensitive aminopeptidase (*23*) in the assay. Reaction mixtures (200 μL) contained 50 μM glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide in 20 mM MES buffer, pH 7.4, and ~1 μg of purified recombinant human puromycin sensitive aminopeptidase. The 4-methoxy-2-naphthylamine was measured at an excitation wavelength of 340 nm and an emission wavelength of 425 nm.

Both IDE and NEP assays were conducted in a 96-well ELISA fluorescence plate reader (SpectraMAX GeniniXS, Molecular Devices).

Protein was quantified by the Bradford method using the Coomassie Plus Protein Assay Reagent from Pierce.

For reaction with hydrogen peroxide, IDE or NEP was incubated in 50 mM potassium phosphate buffer, pH 7.3, at room temperature (IDE) or 37 °C (NEP) with varying concentrations of hydrogen peroxide. The AAHP reaction was conducted at room temperature for both IDE and NEP. For reaction with iron ($FeCl_3$)/ascorbate, IDE or NEP was incubated in 25 mM HEPES buffer, pH 7.2 with ascorbate alone, $FeCl_3$ alone, or ascorbate plus $FeCl_3$. Aliquots were removed and assayed for residual activity.

The incorporation of HNE into IDE was determined by Western blot analysis. Following reaction of the enzyme with HNE, the sample was reduced with 10 mM sodium borohydride in 10 mM sodium hydroxide, and then subjected to SDS−PAGE and Western blot analysis using anti-HNE antisera.

Anti-IDE monoclonal antibody 9B12 was a generous gift of Dr. Richard Roth, Stanford University, anti-NEP monoclonal antibody (anti-CD10) was purchased from Novo Castra Labs., and rabbit anti-HNE antisera was from Calbiochem, while rabbit anti-DNP antisera was obtained from Chemicon as a part of the Oxyblot kit.

RESULTS

Treatment of IDE (Figure 1A) or NEP (Figure 1B) with hydrogen peroxide led to a concentration dependent loss of enzyme activity. The lack of an effect of ferrous iron suggests that hydrogen peroxide may react directly with these enzymes rather than go through a Fenton type reaction involving metal catalyzed radical formation. It is also possible that sufficient metal is present to negate the effect of added ferrous iron. In the case of IDE there appeared to be ~20% residual activity, whereas NEP inactivation appeared complete.

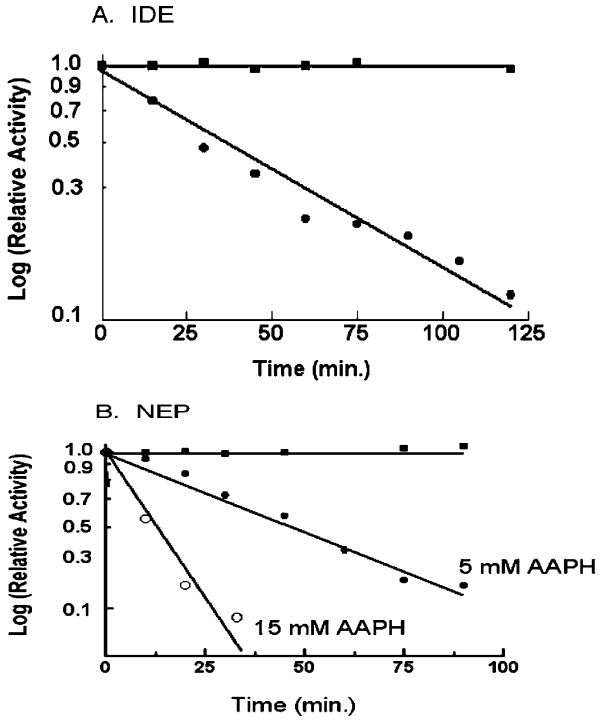

FIGURE 3: Inactivation of IDE and NEP by AAHP. Inactivation reactions were conducted in 50 mM potassium phosphate buffer, pH 7.3, at room temperature. At the time points indicated an aliquot of the reaction was removed and residual activity was determined. (A) IDE incubated in the presence (filled circles) or absence (filled squares) of 25 mM AAHP. (B) NEP incubated in the presence of 5 mM AAHP (filled circles), 15 mM AAHP (open circles), or with no addition (filled squares).

Since metal-catalyzed oxidation systems have been implicated in aging (*24*), we tested the ability of an ascorbate, oxygen, ferric iron system (*25*, *26*) to modify NEP and IDE. This system is reported to mimic the action of various mixed function oxidase systems (*26*). As shown in Figure 2A and Figure 2B, both IDE and NEP are rather sensitive to inactivation by this oxidation system.

Ma et al. (*27*) established the use of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) to produce alkylperoxy radicals and hydroperoxides that serve as reactive oxygen species to inactivate glutamine synthase. We used this system to study the effect of these reactive oxygen species on IDE and NEP. As shown in Figure 3, AAPH was rather effective in inactivating both of these enzymes. IDE is rather susceptible to oxidative inactivation by AAHP as 50% inactivation is seen in less than 10 min at 25 mM AAHP, Figure 3A. For comparison, glutamine synthase is ~50% inactivated after ~2 h with 15 mM AAHP (*27*). As seen in Figure 3B, NEP is even more sensitive, exhibiting 50% inactivation in less than 10 min at 15 mM AAHP, and 50% inactivation in ~30 min with 5 mM AAHP.

To rule out the possibility that the oxidative inactivation of IDE and NEP was not simply due to protein denaturation, we looked for carbonyl group formation which is characteristic of protein oxidation. We thus measured the generation of carbonyl groups by reaction of the oxidized protein with 2,4-dintrophenylhydrazine, followed by SDS−PAGE and Western blot analysis with an anti-2,4-dinitrophenylhydrazine specific antisera (Oxyblot system). As shown in Figure 4, reaction with hydrogen peroxide, Fe^{3+}/ascorbate, and AAHP

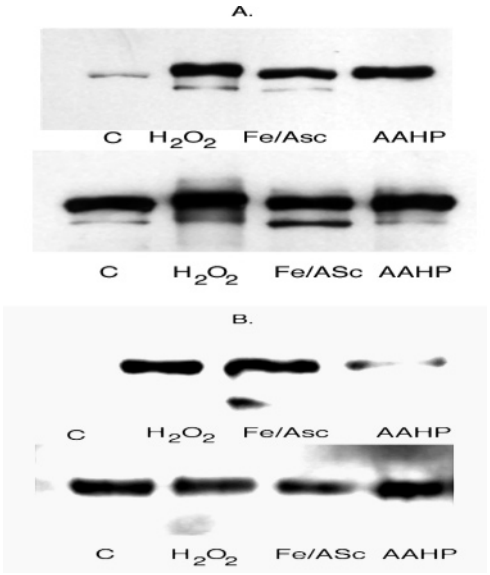

FIGURE 4: Generation of protein carbonyls by reaction of IDE and NEP with hydrogen peroxide, Fe/ascorbate, and AAPH. IDE (A) in 50 mM potassium buffer, pH 7.3, was treated with 1 mM H_2O_2 (H_2O_2) or 10 mM $FeCl_3$ + 100 μM ascorbate (Fe/Asc) for 16 h at 37 °C, or with 15 mM AAHP (AAHP) for 3 h at room temperature. NEP (B) was treated in the same manner except that 2.5 mM $FeCl_3$ + 25 μM ascorbate and 5 mM AAHP were used. Control was untreated enzyme. Carbonyl groups generated were then derivatized with 2,4-dinitrophenylhydrazine using the Oxyblot kit from Chemicon, and aliquots run in duplicate on an 8% polyacrylamide gel. Samples were subjected to Western blot analysis with anti-DNP antisera (top part of A or B) or anti-IDE or anti-NEP monoclonal antibodies (bottom part of A or B).

all resulted in the generation of protein carbonyl groups in NEP and IDE.

The formation of the aldehyde 4-hydroxy-2-nonenal (HNE) and its subsequent reaction with DNA and proteins occurs as a result of oxidative stress through membrane lipid peroxidation. HNE can form adducts with protein amine and thiol functions. We thus studied the reaction of HNE with IDE and NEP. The NEP reaction with HNE resulted in either no change in activity or up to a ~20% loss in activity following an overnight incubation with up to 100 μM HNE. However, HNE adducts were detected by Western blot analysis with anti-HNE antisera. In the case of IDE we observed a concentration and time dependent inactivation by HNE, Figure 5. Western blot analysis with anti-HNE antisera showed incorporation of HNE into the enzyme, Figure 5. The rather weak reaction of NEP with HNE appears contradictory to the report of HNE modified NEP in brain (*20*). However, as shown in Figure 6 prior oxidation of NEP by hydrogen peroxide or AAHP increased its reactivity toward HNE.

Protein oxidation can lead to increased degradation by cellular proteases. We thus looked for increased protease susceptibility of oxidized IDE and NEP. As shown in Figure 7, treatment with AAHP or H_2O_2 increased the susceptibility of IDE to proteolysis by trypsin and chymotrypsin. Similarly treatment of NEP with iron/ascorbate and H_2O_2 increased its susceptibility to proteolysis by trypsin, chymotrypsin, and proteinase K, Figure 8. However, neither reaction with AAHP nor reaction with HNE increased proteolysis with these enzymes.

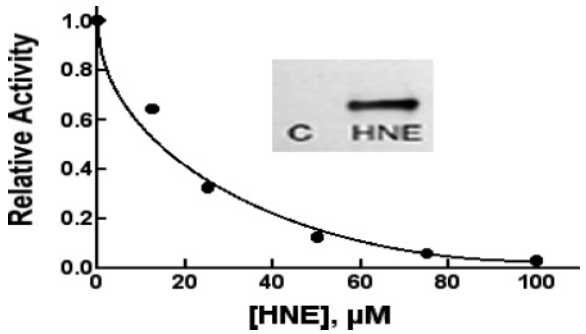

FIGURE 5: Reaction of IDE with 4-hydroxy-2-nonenal (HNE). IDE was incubated in 50 mM potassium phosphate buffer, pH 7.3, with the indicated concentration of HNE. The reaction was allowed to proceed for 24 h at 37 °C, after which time residual activity was determined. The inset shows the results of Western blot analysis with anti-HNE antisera of aliquots of enzyme incubated for 24 h in the absence of HNE (C) or incubated with 50 μM HNE (HNE).

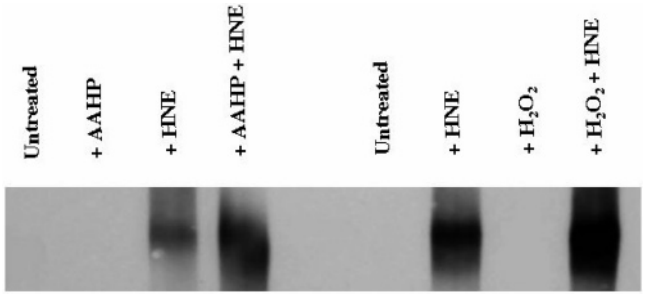

FIGURE 6: Oxidation increases NEP reactivity toward HNE. NEP was treated first with 25 mM AAHP (+AAHP) for 4 h at 22 °C in 50 mM potassium phosphate, pH 7.3, or 5 mM H_2O_2 (+H_2O_2) for 16 h at 37 °C in the same buffer, and then reacted with 150 μM HNE (+AAHP+HNE) or (+H_2O_2+HNE) for 16 h at 37 °C. Samples were then subjected to SDS−PAGE followed by Western blot analysis with anti-HNE antisera. Also shown are untreated NEP (untreated) and NEP reacted with HNE only (+HNE).

DISCUSSION

It is generally accepted that an accumulation of amyloid β peptides (Aβ) in the brain is associated with the pathogenesis of Alzheimer's disease. Thus the presence of mutant APP genes as well as presenilin-1 and presenilin-2 genes is linked to AD, and each leads to enhanced Aβ formation as a likely causative agent of the disease. The increase in Aβ seen in late onset Alzheimer's disease is due to an imbalance between Aβ synthesis and clearance. As noted by Wang et al. (*20*) there is little experimental evidence that Aβ synthesis is increased in sporadic AD. On the other hand it is now appreciated that a decrease in Aβ peptide clearance could represent a significant risk factor in AD (*1*). Three peptidases have been implicated in the clearance of Aβ, NEP, IDE, and ECE, (*28*, *29*). Of these neprilysin and insulysin have been the most widely studied.

That a decrease in the level of NEP mRNA and immunoreactive protein occurs in the AD brain was reported by Yasojima et al. (*8*, *9*) and by Carpentier et al. (*30*). Similarly Cook et al. (*31*) reported lower IDE levels in the hippocampus of AD patients that expressed the apolipoprotein e4 allele. These enzyme changes could be a consequence of cell death or may be due to removal of inactivated enzyme. As noted by Carpentier et al. (*30*), the observed decrease in neprilysin protein did not correlate with the smaller changes in NEP mRNA, suggesting a mechanism other than cell loss.

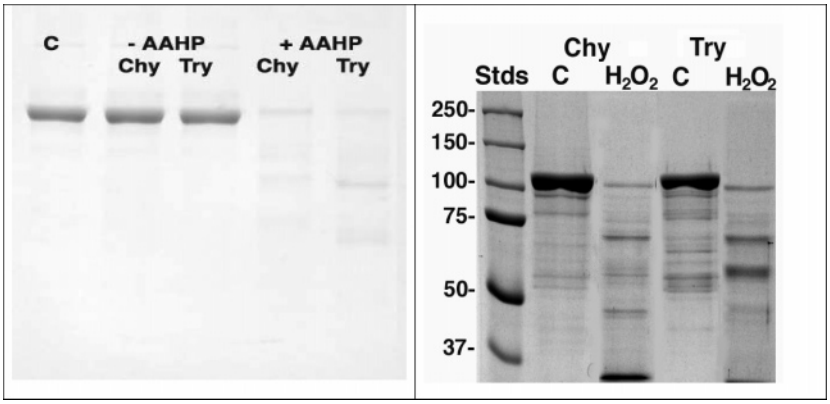

FIGURE 7: Increased susceptibility of oxidized IDE to proteolysis. Left: IDE was treated with 15 mM AAHP for 2 h at room temperature. The reaction mixture was then divided into aliquots, which were then treated with chymotrypsin (Chy) or trypsin (Try) for 1 h at 37 °C at a 1/50 molar ratio of protease to IDE. The reaction was stopped by 50 μM TLCK plus 500 μM PMSF (for chymotrypsin), and a 1.1 to 1 molar equivalent of soybean trypsin inhibitor (for trypsin). Samples were then subjected to SDS−PAGE, followed by Coomassie staining. Although not shown, addition of the stopping reagent at zero time completely prevented proteolysis. Right: IDE was treated with 5 mM H_2O_2 overnight at room temperature. The reaction mixture was treated as above using chymotrypsin (Chy) or trypsin (Try) for 1 h at 37 °C at a 1/50 molar ratio of protease to IDE.

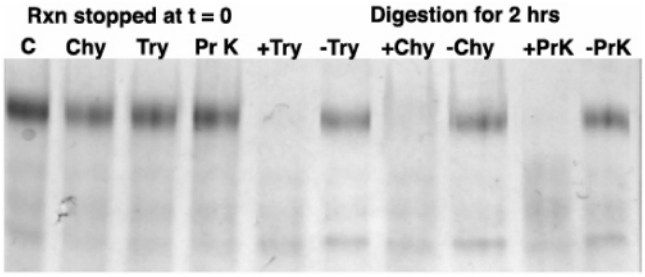

FIGURE 8: Increased susceptibility of oxidized NEP to proteolysis. NEP was treated with 25 μM $FeCl_3$/25 mM ascorbate for 16 h at 37 °C. The reaction mixture was then divided into aliquots, which were then treated with chymotrypsin (Chy), trypsin (Try), or proteinase K (PrK) for 2 h at 37 °C at a 1/100 molar ratio of protease to NEP. The reactions with chymotrypsin and trypsin were stopped as in Figure 7, while 500 μM PMSF was added to stop the reaction with proteinase K. Samples were then subjected to SDS−PAGE, followed by Coomassie staining. Although not shown, addition of the proteases to native NEP did not result in a significant amount of proteolysis.

Additionally an age dependent decrease in NEP activity and immunoreactive protein has been observed in the mouse hippocampus (*6*).

Wang et al. (*20*) showed that NEP is modified by HNE in both normal aged brain and the AD brain, but that the extent of HNE modification is greater in the AD brain. However, in the study of Wang et al. (*20*) it was not determined what if any consequence on enzyme activity or turnover was produced by HNE modification of NEP.

In this study we show that both NEP and IDE are susceptible to oxidative inactivation, and with the exception of AAHP and HNE modified NEP, oxidized enzyme exhibits enhanced susceptibility to proteolytic degradation. Interestingly, NEP reaction with HNE did not significantly affect its activity nor did it enhance its degradation by three proteases; trypsin, chymotrypsin, and proteinase K. This appears to be in contradiction to the finding of HNE modified NEP in normal and AD brain (*20*). This apparent paradox can be explained by the observation that NEP oxidized by hydrogen peroxide or AAHP shows enhanced reactivity toward HNE. Thus the reaction of NEP with HNE appears to be a secondary reaction of the oxidized enzyme. The oxidative inactivation of NEP and the subsequent proteolysis of the oxidized enzyme can explain the finding that NEP levels are reduced in the AD brain.

It is believed that the accumulation of Aβ leads to oxidative stress and the formation of reactive oxygen species. The results of this study suggest that reactive oxygen species so formed would lead to inactivation of IDE and NEP. This decrease in IDE and NEP would in turn lead to an increase in Aβ due to a decrease in its clearance. The resulting increase in Aβ would lead to an enhanced formation of reactive oxygen species through Aβ induced cell damage, increased NEP and IDE activation, and a further increase in Aβ. This vicious cycle would lead to a spiraling effect in the development and progression of AD.

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