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The Free Radical Antioxidant Vitamin E Protects Cortical Synaptosomal Membranes from Amyloid β-Peptide(25-35) Toxicity But Not from Hydroxynonenal Toxicity: Relevance to the Free Radical Hypothesis of Alzheimer's Disease

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Amyloid β-peptide (Aβ) is a key factor in the neurotoxicity of Alzheimer's disease (AD). Recent research has shown that Aβ-mediated neurotoxicity involves free radicals and that Aβ peptides can initiate multiple membrane alterations, including protein oxidation and lipid peroxidation, eventually leading to neuronal cell death. Research also has emphasized the role of 4-hydroxynonenal (HNE), a downstream product of lipid peroxidation, in being able to mimic some of the effects of Aβ peptides. In the current investigation, electron paramagnetic resonance (EPR) studies of spin labeled cortical synaptosomal membrane proteins has been employed to study conformational changes in proteins, spectrophotometric methods have been used to measure protein carbonyl content, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for mitochondrial function has been used to study the effect of vitamin E on samples that were treated with Aβ or HNE. The free radical dependence of β-amyloid-associated toxicity was confirmed by the ability of the free radical scavenger vitamin E to prevent the toxic effects of Aβ. In contrast, HNE was still toxic in the presence of vitamin E. These results support our Aβ-associated free radical model for neurotoxicity in AD brain and are discussed with reference to potential therapeutic strategies for AD.

KEY WORDS: Amyloid; HNE; Alzheimer's; vitamin E; free radicals; protein oxidation; lipid peroxidation.

INTRODUCTION

Free radical-mediated oxidative stress plays a major role in the pathology of aging in general and several age-related neurological disorders such as Alzheimer's disease (AD), stroke, Parkinson's disease and amyotrophic lateral sclerosis (1–11). Of these disorders AD affects 4–5 million Americans and is thought to be the fourth leading cause of death in the United States (12). Amyloid β-peptide (Aβ), a 39–43 amino acid variable length peptide present in the core of senile plaques (SP), one of the major pathological lesions of AD brain, is thought to be of critical significance in AD pathology [reviewed in (13)]: mutations in the amyloid precursor protein (APP), from which Aβ is derived, are linked to AD; persons with Down's syndrome (trisomy 21) invariably develop AD after sufficient time, and the gene for APP is located on chromosome 21; mutations in the presen-

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ilin-1 and presenilin-2 genes, localized to chromosomes 14 and 1, respectively, and which apparently are associated with APP processing, are linked to early-onset familial AD; and APP-overexpressing transgenic mice develop much of the brain pathology similar to that in AD. The SP density in an AD brain is different in different regions, and it has been shown that plaque-rich regions of an AD brain, such as hippocampus and inferior parietal lobule, are regions high oxidative stress, while the SP-poor region, cerebellum, is not (14).

Electron paramagnetic resonance (EPR) spin trapping studies have shown that Aβ(1–40) and several of its subsets are associated with free radicals in vitro (2,15–19), findings confirmed by other laboratories (20,21).[1] Aβ-associated free radicals induce multiple membrane dysfunctions in neuronal synaptosomes and astrocytes, such as inactivation of key enzymes, protein oxidation, diminution of sodium-dependent glutamate uptake, inhibition of ion-motive ATPases, lipid peroxidation, loss of calcium homeostasis, and cell death (15,16,18,22–25; reviewed in 2).

Recently, the importance of lipid peroxidation products, such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA), have been extensively reviewed relative to free radical-related membrane impairment (26). Aβ is capable of inducing lipid peroxidation (22). Further, addition of Aβ to cultured hippocampal neurons leads to the formation of HNE, a cytotoxic product of lipid peroxidation, and this lipid peroxidation could be prevented by free radial scavengers (24). Based on extensive studies in our laboratory and others, we developed an Aβ-associated free radical oxidative stress model for brain cell death in AD (2,27). In this model, Aβ-associated free radical lipid peroxidation leads to formation of HNE, which then, by rapid Michael addition, covalently forms adducts with and alters the structure and function of transmembrane ion-motive ATPases and key transport proteins, leading to large increases in intracellular calcium that can not be removed, resulting in subsequent cell death.

One prediction of this model is that the initial free radical lipid peroxidation might be prevented by appropriate free radical antioxidants, but that such agents would be relatively ineffectual against HNE, since this alkenal is the product of free radical-induced lipid peroxidation and is downstream from the initial free radical events. In order to test this idea, the effects of the lipid-soluble antioxidant vitamin E on both Aβ-and HNE-mediated toxicity were compared in the present study. We report here results in neuronal systems employing electron paramagnetic resonance in conjunction with a protein-specific spin label, analysis of protein carbonyl levels, which are a biomarker for protein oxidation, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay for mitochondrial function, that show while vitamin E protects biomolecules and brain membrane proteins against Aβ(25-35)-induced damage, this antioxidant is relatively unprotective against the effects of HNE.

[1] R.A. Floyd, Personal communication, 1996

EXPERIMENTAL PROCEDURE

Materials. Pure vitamin E was obtained from Sigma Chemical Co and was stored as a 1M stock solution in DMSO at 0°C in a dark container. Aβ(25–35) (Lot # 091396) was obtained from M.D. Enterprises (Manhattan Beach, CA). HNE was obtained from Caymen Chemicals (Ann Arbor, MI) as a 10 mg/mL ethanol solution. All other chemicals used were of highest grade purity.

Animals. Male Mongolian gerbils, 3–4 months of age, were obtained form Tumblebrook Farms (West Brookfield, MA), kept under 12 hour light/dark conditions in the University of Kentucky Central Animal Facility, and fed standard Purina Rodent Laboratory Chow.

Methods.

Peptides. Peptides were dissolved to obtain a final concentration of 1 mg/mL in PBS buffer at pH 7.4. The solubilized peptide was divided into two aliquots. To one aliquot was added vitamin E to obtain a final concentration of 5 mM. The volume of vitamin E added was always less than 0.5% of the total volume, in order to minimize the amount of DMSO in the mixture. The peptide solutions were then incubated in a water bath at 37°C for six hours.

HNE Treatment. Synaptosomes were treated with HNE exactly as described before (27). For the samples that received treatment of both HNE and vitamin E, the synaptosomes were first treated with 5 mM vitamin E for 1 hour, followed by a 3 hour treatment with 50 μM HNE.

Synaptosome Isolation, Spin Labeling and EPR Spectroscopy. Synaptosomes were purified from homogenized neocortices, obtained by dissection from decapitated gerbil brains, ultracentrifugation across discontinuous sucrose gradients, as described previously (28). Crude homogenate was centrifuged at 3,500 rpm for 10 minutes at 4°C in a DuPont Sorvall RC5C refrigerated centrifuge. The pellet was discarded and the supernatant was spun at 13,500 rpm for 10 minutes at 4°C in the same machine. The resulting pellet was resuspended in isolation buffer and layered onto discontinuous sucrose density gradients. Samples were then spun for 2 hours in an SW28 rotor in a Beckman L7-55 refrigerated ultracentrifuge. Removal of the 1.18 M/1.10 M interface yielded the purified synaptosomes used in further protocols. The protein concentration in the purified synaptosomes was adjusted to 8 mg/mL. This was followed by the peptide/HNE incubation as described above. The various aliquots included the following samples: 1. control 2. Aβ(25–35) containing DMSO 3. Aβ(25–35) plus vitamin E in DMSO 4. HNE containing DMSO and 5. HNE plus vitamin E in DMSO.

Synaptosomes were then spin labeled with the protein-specific spin label, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6), and EPR spectra obtained, by methods described previously (5,7–9,14,27–29).

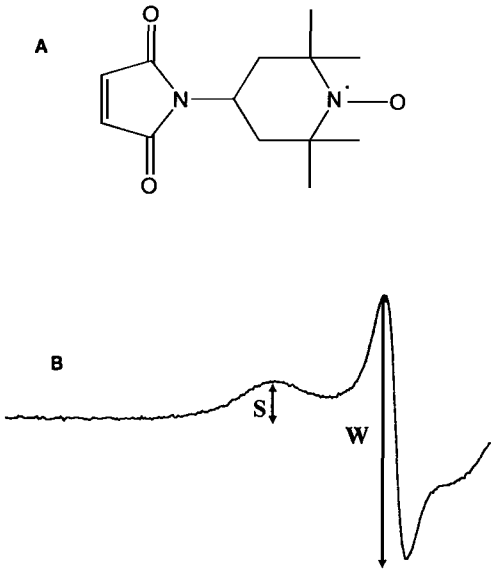

Fig. 1. A. Structure of the protein-specific spin label, MAL-6. **B.** EPR spectrum showing the $M_I = +1$ low-field resonance lines of MAL-6-labeled cortical synaptosomal membranes. The measurements needed to calculate the W/S ratio are shown. See text.

Protein Carbonyl Assay. Measurement of the protein carbonyl content in the synaptosomes was used as a measure of protein oxidation (30,31). The protein carbonyl content of synaptosomes incubated with Aβ(25–35)/HNE/vitamin E (as described above) was measured spectrophotometrically by measuring the absorbance of the dinitrophenylhydrazone at 380 nm using procedures described before (5,27,29–31).

Mitochondrial Function Assay. Mixed cultures of hippocampal neurons and astrocytes were obtained from 18-day old Sprague-Dawley rat fetuses. The cells were treated with solubilized Aβ(25–35) with and without vitamin E and with HNE with and with out vitamin E. The concentrations of the various treatments are exactly as those for the synaptosomal samples. Neuronal mitochondrial function was evaluated by the MTT reduction test by procedures described previously (16,27,32–34).

Statistical Analysis. Statistical significance was determined by the two-tailed Student's *t*-test comparison of means. A p-value of less than 0.05 was considered statistically significant.

RESULTS

In order to study the effect of Aβ(25–35) and HNE on synaptosomal membrane protein oxidation and conformation, the protein specific spin label MAL-6 was used. The structure of MAL-6 and a typical spectrum of MAL-6 labeled neocortical synaptosomal membranes are shown in Fig. 1A and 1B, respectively. MAL-6 binds specifically to protein thiol groups in the membrane (5,28,29). There are at least two kinds of binding sites for MAL-6 on membrane proteins. Binding of MAL-6 to thiol groups on the surface of the protein molecules enables free motion of the spin label (hence, weakly immobilized), giving rise to relatively sharp lines on the EPR spectrum; binding of MAL-6 to thiol groups buried in a deep pocket in protein molecules restricts the motion of the spin label (hence, strongly immobilized), giving rise to broad lines in the EPR spectrum. The ratio of the EPR spectral amplitude of the low-field line of the weakly-immobilized (W) to strongly-immobilized (S) line is termed as the W/S ratio and has been shown to be a sensitive monitor of protein oxidation (5,7–9,28,29). In-vivo and in-vitro investigations involving oxidative stress associated with ischemia/reperfusion injury, accelerated aging, Fenton chemistry, and hyperoxia showed that the W/S ratio of MAL-6-labeled synaptosomal membrane proteins decreases with protein oxidation, increased protein-protein interactions, and decreased segmental motion (5,7–9,28,29,35). For example, when membrane protein-protein interactions were increased by crosslinking membrane proteins by spermine or hemin, the motion of the MAL-6 spin label slowed and the W/S ratio decreased. In contrast, when membrane protein-protein interactions were decreased by polyphosphate addition or by breaking protein-protein links between cytoskeletal and transmembrane proteins, segmental motion of spin-labeled proteins increased and the W/S ratio of MAL-6 increased [reviewed in (35)].

When Aβ(25–35) was added to spin labeled synaptosomes, the W/S ratio was decreased significantly from the control value ($p < 0.003$, Fig. 2A). A significant decrease in the W/S ratio also was observed in samples treated with 50 μM HNE ($p < 0.003$, Figure 2B). These decreases suggest that both Aβ and HNE can significantly alter the conformation of synaptosomal membrane proteins. However, samples of synaptosomes that were treated with both Aβ and vitamin E did not show any significant loss in the W/S ratio as compared to controls (p was not significant), implying that vitamin E protected against an Aβ-induced alteration in the conformation of synaptosomal membrane proteins (Fig. 2A). In contrast, samples that were treated with both HNE and vitamin E showed a significant decrease in the W/S ratio ($p < 0.001$), similar to samples treated with HNE alone (Fig. 2B). Statistical analysis showed no statistical difference between the mean W/S values of samples treated with HNE alone and HNE plus vitamin E ($p < 0.08$). That is, vitamin E was unable to protect against HNE-induced changes in protein conformation.

Stadtman (30) and Butterfield and Stadtman (31) have shown that protein carbonyl content is an important marker of oxidative stress in proteins. In the case of

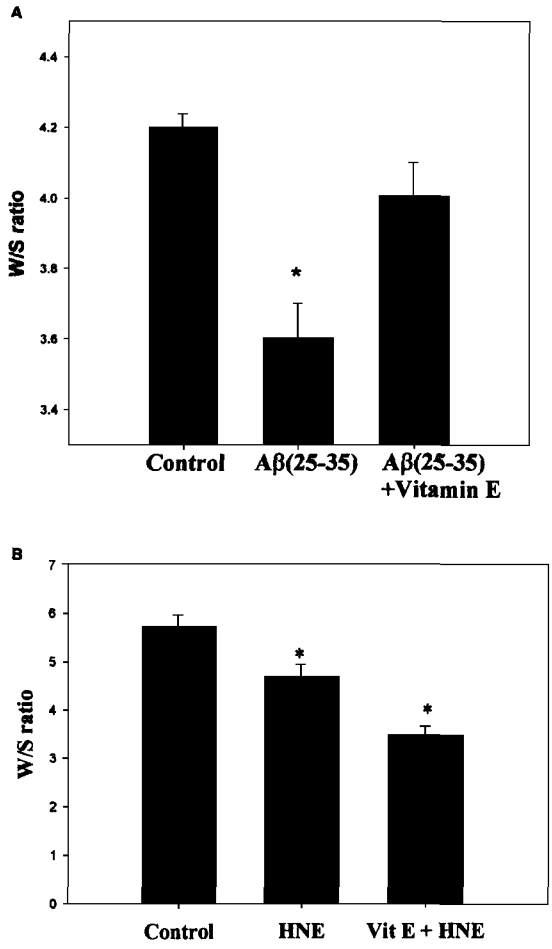

Fig. 2. A. Effect of Aβ(25–35) with and without vitamin E on the W/S ratio of the protein-specific spin label MAL-6 in cortical synaptosomal membranes. Aβ(25–35) significantly reduces the W/S ratio compared to control ($n = 3$, $p < 0.003$). Previous studies showed that protein oxidation leads to a decrease in the W/S ratio of MAL-6 spin labeled membranes. Aβ(25–35) incubated with 5 mM vitamin E prevents a lowered W/S ratio, and this EPR parameter is essentially equal to that in control samples. See text. **B.** Effect of HNE with and without vitamin E on the W/S ratio of the protein-specific spin label MAL-6 in cortical synaptosomal membranes. Samples were pre-incubated at room temperature with 5 mM vitamin E for one hour and then treated with 50 μM HNE for another one hour with constant shaking. HNE significantly reduces the W/S ratio compared to control ($n = 3$, $p < 0.03$), and HNE plus vitamin E has a mean W/S ratio also significantly reduced compared to control ($n = 3$, $p < 0.001$). The mean W/S ratios of HNE-treated and HNE plus vitamin E-treated samples were not statistically different ($n = 3$, $p < 0.08$).

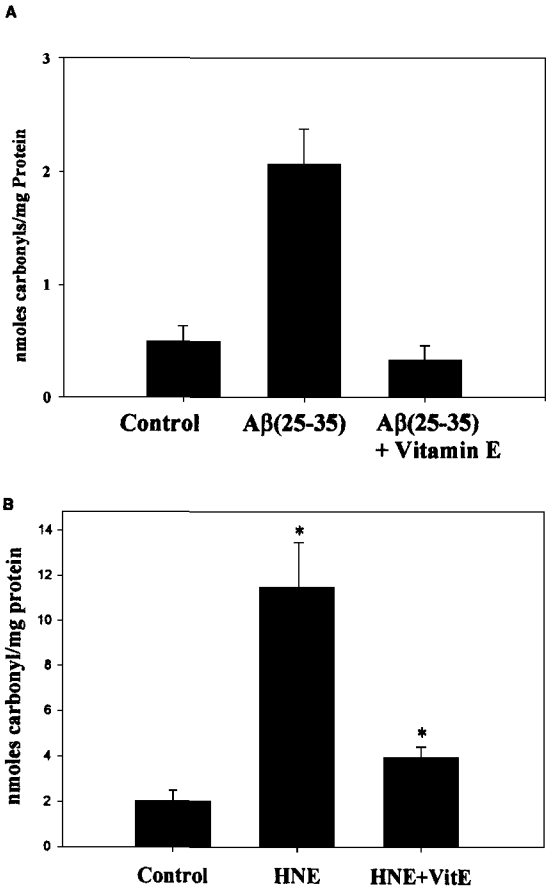

Fig. 3. Protein carbonyl content of synaptosomes measured by the DPNH spectrophotometric assay. **A:** Samples treated with Aβ(25–35) as described in Methods demonstrate a significantly higher level of protein carbonyls than that of untreated controls ($p < 0.01$; $n = 3$), suggesting that Aβ(25–35) caused oxidative modification of proteins. Samples treated with both the peptide and vitamin E as described in Methods were statistically identical to untreated controls. See text. **B:** Samples treated with 50 μM HNE yielded a significantly higher level of protein carbonyls than that of untreated controls ($p < 0.01$; $n = 3$), showing that HNE had bound to proteins. Samples treated with vitamin E and HNE as described in Methods showed a smaller, but significantly increased level of protein carbonyl ($p < 0.04$, $n = 3$). See text.

protein modifications resulting from conjugation of protein cysteine, histidine, or lysine side chains with HNE (26), the increase in carbonyl content in part could be the result of the added carbonyl functionality from the aldehyde group on HNE. Fig. 3A and Fig. 3B show that samples of synaptosomes treated with either Aβ or HNE show a significant increase in the carbonyl content ($p < 0.01$ and $n = 3$ in each case), as measured spectrophotometrically by the DNPH assay. As in the case of conformational changes measured by EPR and the changes in W/S ratio of the protein-specific spin label MAL-6, the increase in carbonyl content was prevented in the Aβ-treated samples that also contained vitamin E (Fig. 3A). In HNE-treated samples containing vitamin E, although the mean value of protein carbonyl content was lower than in HNE-treated samples, a statistically significant increase in carbonyl content over controls was observed ($p < 0.04$).

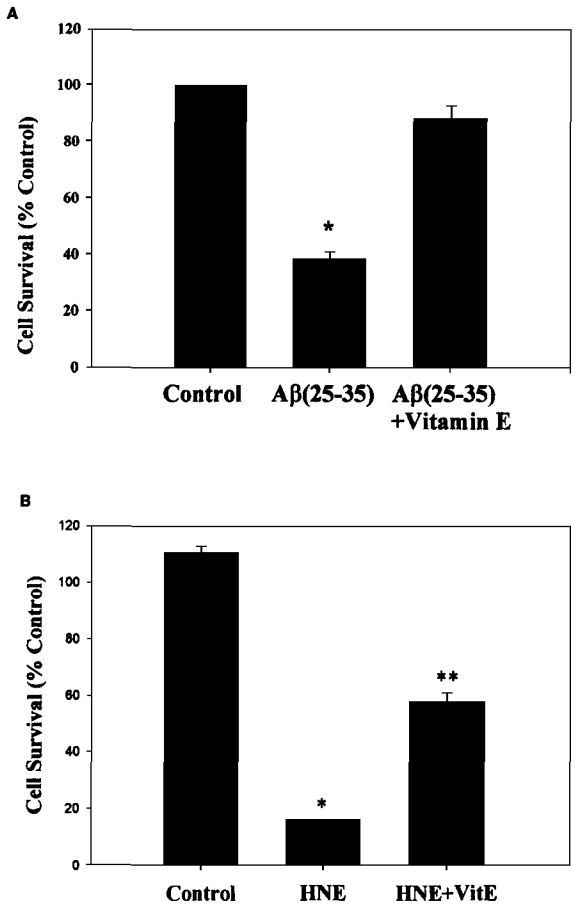

Fig. 4. Hippocampal neuronal mitochondrial function and cell survival assays, as measured by the MTT reduction method. Cell survival was measured six hours after the last treatment addition. **A.** Aβ(25–35) exposure resulted in the death of nearly 60% of the cells ($p < 0.001$, $n = 3$). Vitamin E treatment was able to significantly protect neurons from Aβ(25–35) induced cell death and results in the survival of more than 90% of the cells. See text. **B.** HNE (2 μM) exposure led to nearly 80% cell death ($n = 3$, $p < 0.001$). Toxicity also was significant for cells treated for one hour with 5 mM vitamin E followed by treatment with 2μM HNE for one hour (40% cell death, $n = 3$, $p < 0.02$). See text.

The neurotoxicity of Aβ and HNE were also investigated using the MTT reduction assay in which the percentage of hippocampal neuronal cells surviving the treatment of Aβ or HNE was measured. These results are represented graphically in Fig. 4A and B. Samples treated with Aβ or HNE resulted in a significant loss in mitochondrial function compared to untreated controls ($p < 0.001$ in each case). Once again, vitamin E was able to prevent this loss in mitochondrial function in samples treated with Aβ, but a significant, 40 % cell death in neurons treated with HNE and vitamin E was observed ($p < 0.02$).

DISCUSSION

This study reconfirms that both Aβ(25–35) and HNE can significantly alter the conformation of cortical synaptosomal membrane proteins, as measured by the decrease in W/S ratio of MAL-6 labeled proteins using EPR spectroscopy (2,27). Previous studies have shown that Aβ is associated with free radicals (2–4,6,14–25,34).[1] Based on analysis of EPR spin trapping spectra, inhibition of the spectra by catalase but not superoxide dismutase, and colorimetric assays using peroxyl sensitive reagents, these Aβ-associated free radicals may be peroxyl in nature [reviewed in (2)]. Multiple membrane dysfunctions caused by Aβ, including protein oxidation (16), lipid peroxidation (22), inhibition of ion-motive ATPases (23) [providing a possible mechanism by which increased Ca^{2+} accumulation could arise], inhibition of glutamate uptake (18,34) and glutamine synthetase activity (25,36) [which conceivably in part could explain the reported synergy between Aβ toxicity and excitotoxicity (37)], were attributed to its association with free radicals [reviewed in (2)]. Changes in membrane protein conformation caused by Aβ are therefore likely due to oxidation of the membrane proteins by Aβ-associated free radical oxidative stress. The changes in membrane protein conformation resulting from exposure to HNE has been suggested to being due to the ability of HNE to react with side chains of protein amino acids, such as lysines, histidines and cysteines by Michael addition (26,27,30,31). In addition, HNE can react with the ε-amino group of the lysine residue of proteins, leading to the formation of a Schiff base, and causing even greater protein crosslinking. Aβ significantly inhibits Na^+/K-ATPase and Ca^{2+} in neuronal cultures (23). Such an inhibition could lead to alteration of the cell potential, with consequent opening of voltage-gated Ca^{2+} channels followed by influx of Ca^{2+}. Because the Ca^{2+} pump, too, is inhibited by Aβ (23), this excess intracellular Ca^{2+} is unable to be extruded form the neuron. Mattson and coworkers (24,38–41) showed that similar inhibition of these ion-motive ATPases and the glutamate transporter, as well as alterations of glucose transport system, transmembrane signaling mechanisms, Ca^{2+} homeostasis, and enhancement of apoptosis could be induced by HNE treatment of hippocampal neurons. Our laboratory has recently shown that HNE, at concentrations formed following exposure of neurons to Aβ, leads to alterations in the protein conformation in cortical synaptosomal membranes (27), which likely accounts for the diminished activity and inhibited function of neuronal membrane proteins following HNE treatment (24,38–41).

Studies from our laboratory and others have reported the protective effects of several antioxidants on Aβ-treated systems (2,3,6,16–18,20,23,24,34,42–50). In addition, increased antioxidant activity is reported for Aβ-resistant PC-12 cells (51) and the decreased antioxidant activity of apoE 4 relative to the other isoforms of this lipoprotein was suggested as being involved with the former's association with AD (52). Aβ-associated free radicals are capable of inducing lipid peroxidation (22), and HNE is known to be a major product of lipid peroxidation resulting from the breakdown of arachidonic acids due to free radical attack (26). As noted, synaptosomes treated with Aβ lead to the formation of HNE (24). Based on these findings we hypothesized (2) that in AD brain free radicals associated with Aβ initiate lipid peroxidation, which is then followed by the production of HNE that could then conjugate with the membrane proteins to cause structure/function alterations in membrane proteins (27,38–41). Our model states that these alterations lead to irreversible Ca^{2+} accumulation and eventual neuronal death. Consistent with our model, evidence exists for lipid peroxidation in AD brain, including elevated levels of HNE (53,54). A corollary of this model is that appropriate free radical scavengers should be able to prevent Aβ-induced effects, but, because this alkenal is formed downstream from the initial free radical events, these free radical scavengers are predicted to be relatively ineffective toward HNE-induced effects, entirely consistent with the current findings. A variety of well established and new potential antioxidant compounds are under investigation using the paradigm of this paper and other measures of oxidative stress.

Whereas toxicity induced by Aβ is preventable by free radical scavengers as indicated above, toxicity of HNE has been reported to be preventable by the endogenous antioxidant glutathione (24,26,27). Likely, the thiol functionality of glutathione covalently reacts with HNE by Michael addition, presumably making the HNE unavailable for further deleterious reactions with membrane proteins. In this case, Michael addition involves a nucleophilic attack of a lone pair of electrons on the sulfur atom of cysteine on the partially-positive carbon number 3 of HNE, typical of α,β-unsaturated aldehydes (31). The hydroxyl group on the fourth carbon of HNE, by withdrawing electron density towards itself, further contributes to making carbon number 3 of HNE partially positive, which augments the potential of carbon number 3 being the site of attack of a lone pair of electrons on sulfur, i.e., Michael addition.

With respect to potential therapeutic strategies in AD, the research discussed here suggests that it is imperative to intercept the Aβ-associated free radical oxidative stress *prior* to lipid peroxidation; otherwise, HNE, with its subsequent significant deleterious effects on membrane protein function and cell death, would be formed. Alternatively, means of increasing endogenous glutathione levels could be investigated as a potentially useful therapeutic modality in AD. Initial clinical studies of high-dose vitamin E in moderate to severe AD patients are promising (55), and the significant beneficial outcomes of therapy with this lipid-soluble free radical antioxidant support the concept that AD is associated with free radical oxidative stress. Given the increasingly strong evidence of the role of Aβ and HNE in oxidative stress mechanisms for neuronal toxicity in AD (2,24,27,38–41,56,57), additional studies with even better brain-accessible antioxidants are warranted. Studies with these compounds in oxidative stress conditions are in progress in our laboratory.

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REFERENCES

1. Halliwell, B., and Gutteridge, J. M. C. 1989. Free Radicals in Biology and Medicine, Clarendon Press, Oxford.
2. Butterfield, D. A. 1997. β-Amyloid-associated free radical oxidative stress and neurotoxicity: relevance to Alzheimer's disease. Chem. Res. Toxicol. 10:495–506.
3. Butterfield, D. A., Hensley, K., Hall, N., Subramaniam, R., Howard, B. J., Cole, P., Yatin, S., Lafontaine, M., Harris, M. E., Aksenova, M., Aksenov, M., and Carney, J. 1996. β-Amyloid derived free radical oxidation: a fundamental process in Alzheimer's disease. In Molecular Models of Dementia Wasco, W., and Tanzi, R. E. (eds.) Humana Press, Totawa, New Jersey. 145–167.
4. Markesbery, W. R. 1997. Oxidative stress hypothesis in Alzheimer's disease. Free Rad. Biol. Med. 23:134–147.
5. Butterfield, D. A., Howard, B. J., Yatin, S., Allen, K. L., and Carney, J. M. 1997. Free radical oxidation of brain proteins in accelerated senescence and its modulation by N-tert-butyl-α-phenylnitrone. Proc. Nat. Acad. Sci. USA 94:674–678.
6. Hensley, K., Butterfield, D. A., Hall, N., Cole, P., Subramaniam, R., Mark, R., Mattson, M. P., Markesbery, W. R., Harris, M. E., Aksenov, M., Aksenova, M., Wu, J. F., and Carney, J. M. 1996. Reactive oxygen species as causal agents in the neurotoxicity of the Alzheimer's disease-associated amyloid beta peptide. Ann. N.Y. Acad. Sci. 786:120–134.
7. Hall, N. C., Carney, J. M., Cheng, M. S., and Butterfield, D. A. 1995. Ischemia/reperfusion induced changes in membrane proteins and lipids of gerbil cortical synaptosomes. Neuroscience 64:81–89.
8. Hall, N. C., Carney, J. M., Cheng, M., and Butterfield, D. A. 1995. Prevention of ischemia/reperfusion-induced alterations in synaptosomal membrane-associated proteins and lipids by N-tert-butyl-α-phenylnitrone and difluoromethylornithine. Neuroscience 69: 591–600.
9. Hall, N. C., Carney, J. M., Plante, O. J., Cheng, M., and Butterfield, D. A. 1997. Effect of 2-cyclohexene-1-one-induced glutathione diminution on ischemia/reperfusion-induced alterations in

the physical state of brain synaptosomal membrane proteins and lipids. Neuroscience 77:283–290.
10. Jenner, P., and Olanow, C. W. 1996. Oxidative stress and the pathogenesis of Parkinson's disease. Neurology 47:S161–S170.
11. Beal, M. F., Howell, N., and Bodis-Wollner, I. 1997. Mitochondria and Free Radicals in Neurodegenerative Diseases. Wiley-Liss, Inc. New York.
12. Katzman, R., and Saitoh, T. 1991. Advances in Alzheimer's disease. FASEB J. 4:278–286.
13. Selkoe, D. J. 1996. Amyloid β-protein and the genetics of Alzheimer's disease. J. Biol. Chem. 271:18295–18298.
14. Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenov, M., Aksenova, M., Gabbita, P., Wu, J. F., Carney, J. M., Lovell, M., Markesbery, W. R., and Butterfield, D. A. 1995. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. J. Neurochem. 65: 2146–2156.
15. Hensley, K., Carney, J. M., Mattson, M. P., Aksenova, M., Harris, M., Wu, J. F., Floyd, R. A., and Butterfield, D. A. 1994. A model for β-Amyloid aggregation and neurotoxicity based on free radical generating capacity by the peptide: relevance to Alzheimer's disease. Proc. Nat. Acad. Sci. USA 91:3270–3274.
16. Harris, M., Hensley, K., Butterfield, D. A., Leedle, R. A., and Carney, J. M. 1995. Direct Evidence of Oxidative Injury Produced by the Alzheimer's β-Amyloid Peptide (1-40) in Cultured Hippocampal Neurons. Exp. Neurol. 131:193–202.
17. Subramaniam, R., Howard, B. J., Hensley, K., Aksenova, M., and Carney, J. M. 1995. β-Amyloid (32–35) toxicity to biomolecules: Implications to Alzheimer's disease. Alzheimer's Res. 1:141–144.
18. Harris, M. E., Carney, J. M., Cole, P., Hensley, K., Howard, B. J., Martin, L., Bummer, P., Wang, Y., Pedigo, N., and Butterfield, D. A. 1995. β-Amyloid peptide-derived, oxygen-dependent free radicals inhibit glutamate uptake in cultured astrocytes: implications to Alzheimer's disease. NeuroReport 6:1875–1879.
19. Hensley, K., Aksenova, M., Carney, J. M., Harris, M., and Butterfield, D. A. 1995. Amyloid β-peptide spin trapping I: peptide enzyme toxicity is related to free radical spin trap reactivity. NeuroReport 6:489–492.
20. Tomiyama, T., Shoji, A., Kataoka, K. L., Suwa, Y., Asano, S., Kaneko, K., and Endo, N. 1996. Inhibition of amyloid β protein aggregation and neurotoxicity by rifampicin. J. Biol. Chem. 271: 6839–6844.
21. Manelli, A. M., and Puttfarcken, P. S. 1995. β-Amyloid-induced toxicity in rat hippocampal cells: in-vitro evidence for the involvement of free radicals. Brain Res. 38:569–578.
22. Butterfield, D. A., Hensley, K., Harris, M., Mattson, M. P., and Carney, J. M. 1994. β-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: Implications to Alzheimer's disease. Biochem. Biophys. Res. Comm. 200:710–715.
23. Mark, R. J., Hensley, K., Butterfield, D. A., and Mattson, M. P. 1995. Amyloid β-peptide impairs ion-motive ATPase activities: Evidence for a role in loss neuronal Ca^{2+} homeostasis and cell death. J. Neurosci. 15:6239–6249.
24. Mark, R. J., Lovell, M. A., Markesbery, W. R., Uchida, K., and Mattson, M. P. 1997. Evidence that 4-hydroxynonenal mediates disruption of ion homeostasis and neuronal death by amyloid-β peptide. J. Neurochem. 68:255–264.
25. Butterfield, D. A., Hensley, K., Cole, P., Subramaniam, R., Aksenov, M., Aksenova, M., Bummer, P., Haley, B. E., and Carney, J. M. 1997. Oxidatively induced structural alteration of glutamine synthetase assessed by spin label incorporation kinetics: relevance to Alzheimer's disease. J. Neurochem. 68:2451–2457.
26. Esterbauer, H., Schaur, R. J., and Zollner, H. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde, and related aldehydes. Free Rad. Biol. Med. 11:81–128.
27. Subramaniam, R., Roediger, F., Jordan, B., Mattson, M. P., Keller, J. N., Waeg, G., and Butterfield, D. A. 1997. The lipid peroxidation product, 4-hydroxy-2-transnonenal, alters the conformation of cortical synaptosomal membrane proteins. J. Neurochem. 69: 1161–1169.
28. Hensley, K., Hall, N. C., Shaw, W., Carney, J. M., and Butterfield, D. A. 1994. Electron paramagnetic resonance investigation of free radical induced alterations in neocortical synaptosomal membrane protein infrastructure. Free Rad. Biol. Med. 17:321–331.
29. Howard, B. J., Yatin, S., Hensley, K., Allen, K. L., Kelly, J. P., Carney, J. M., and Butterfield, D. A. 1996. Prevention of hyperoxia-induced alterations in synaptosomal membrane-associated proteins by N-tert-butyl-α-phenynitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol). J. Neurochem. 67: 2045–2050.
30. Stadtman, E. R. 1992. Protein oxidation in aging. Science 257: 1120–1124.
31. Butterfield, D. A., and Stadtman, E. R. 1997. Protein oxidation processes in aging brain. In The Aging Brain (Mattson, M. P. and Geddes, J. W., eds.), JAI Press, Greenwich, CT, pp. 161–191.
32. Mossman, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity. J. Immunol. Methods 65:55–63.
33. Shearman, M. S., Hawtin, S. R., and Tailor, V. J. 1995. The intracellular component of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction is specifically inhibited by β-amyloid peptides J. Neurochem. 65:218–227.
34. Harris, M., Wang, Y., Pedigo, N. W., Hensley, K., Butterfield, D. A., and Carney, J. M. 1996. Amyloid β Peptide (25–35) Inhibits Na^+-dependent Glutamate Uptake in Rat Hippocampal Astrocyte Cultures. J. Neurochem. 67:277–286.
35. Butterfield, D. A. 1990. Principles of biological membrane structure: alteration in the physical state of one side of the membrane by modulation of the physical state of the other side. J. Membrane Sci. 53:3–17.
36. Aksenov, M. Y., Aksenova, M. V., Butterfield, D. A., Hensley, K., Vigo-Pelfrey, C., and Carney, J. M. 1996. Glutamine synthetase-induced neurotoxicity accompanied by abrogation of fibril formation and amyloid β-peptide fragmentation. J. Neurochem. 66:2050–2056.
37. Mattson, M. P., Burger, S. W., Cheng, B., Lieberburg, I., Smith-Swintosky, V. L., and Rydel, R. E. 1993. β-Amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. Trends Neurosci. 16:409–414.
38. Mark, R. J., Pang, Z., Geddes, J. W., Uchida, K., and Mattson, M. P. 1997. Amyloid beta-peptide impairs glucose transport in hippocampal and cortical neurons: involvement of membrane lipid peroxidation. J. Neurosci. 17:1046–1054.
39. Blanc, E. M., Kelly, J. F., Mark, R. J., Waeg, G., and Mattson, M. P. 1997. 4-Hydroxynonenal, an aldehydic product of lipid peroxidation, impairs signal transduction associated with muscarinic acetylcholine and metabotropic glutamate receptors: possible action on G"qll. J. Neurochem. 69:570–580.
40. Keller, J. N., Pang, Z., Geddes, J. W., Begley, J. G., Germeyer, A., Waeg, G., and Mattson, M. P. 1997. Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid β-peptide: role of the lipid peroxidation product, 4-hydroxynonenal. J. Neurochem. 69:273–284.
41. Kruman, I., Bruce-Keller, A. J., Bredesen, D., Waeg, G., and Mattson, M. P. 1997. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. J. Neurosci. 17:5089–5100.
42. Bruce, A. J., Malfroy, B., and Baudry, M. 1996. β-Amyloid Toxicity in Organotypic Hippocampal Cultures: Protection by EUK-8, a Synthetic Catalytic Free Radical Scavenger. Proc. Nat. Acad. Sci. USA 93:2312–2316.
43. Kumar, U., Dunlop, D. M., and Richardson, J. S. 1994. The Acute Neurotoxic Effect of β-Amyloid on Mature Cultures of Rat Hippocampal Neurons is Attenuated by the Antioxidant U-78517F. Internat. J. Neurosci. 79:185–190.
44. Behl, C., Davis, J. B., Lesley, R., and Shubert, D. 1994. Hydrogen peroxide Mediates Amyloid β Protein Toxicity. Cell 77:817–827.

45. Behl, C., Widmann, M., Trapp, T., and Holsboer, F. 1995. 17-β estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem. Biophys. Res. Commun. 216:473–482.
46. Goodman, Y., Steiner, M. R., Steiner, S. M., and Mattson, M. P. 1994. Nordihydroguaiaretic acid protects hippocampal neurons against amyloid β-peptide toxicity, and attenuates free radical and calcium accumulation. Brain Res. 654:171–176.
47. Goodman, Y., Bruce, A. J., Cheng, B., Mattson, M. P. 1996. Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid-β toxicity n hippocampal neurons. J. Neurochem. 66:1836–1844.
48. Puttfarcken, P. S., Manelli, A. M., Neilly, J., and Frail, D. E. 1996. Inhibition of age-induced β-amyloid neurotoxicity in rat hippocampal cell. Exp. Neurol. 138:73–81.
49. Prehn, J. H., Bindokas, V. P., Jordan, J., Galindo, M. F., Ghadge, G. D., Roos, R. P., Boise, L. H., Thompson, C. B., Krajewski, S., and Reed, J. C. 1996. Protective effect of transforming growth factor-beta 1 on beta-amyloid neurotoxicity in rat hippocampal neurons. Mol. Pharmacol. 49:319–328.
50. Aksenov, M. Y., Aksenova, M., Carney, J. M., and Butterfield, D. A. 1997. Oxidative modification of glutamine synthetase by amyloid beta peptide. Free Rad. Res. 27:267–281.
51. Sagara, Y., Dargusch, R., Klier, F. G., Schubert, D., and Behl, C. 1996. Increased antioxidant enzyme activity in amyloid beta protein-resistant cells. J. Neurosci. 16:497–505.
52. Miyata, M., and Smith, J. D. 1996. Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. Nature Genetics 14:55–61.
53. Lovell, M. A., Ehmann, W. D., Butler, S. M., and Markesbery, W. R. 1995. Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. Neurology 45:1594–1601.
54. Lovell, M. A., Ehmann, W. D., Mattson, M. P., and Markesbery, W. R. 1997. 4-Hydroxynonenal in ventricular fluid in Alzheimer's disease. Neurobiol. Aging 18:457–461.
55. Sano, M., Ernesto, C., Thomas, R. G., Klauber, M. R., Schafer, K., Grundman, M., Woodbury, P., Growdon, J., Cotman, C. W., Pfeiffer, E., Schneider, L. S., and Thal, L. J. 1997. A controlled trail of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's disease cooperative study. N. Eng. J. Med. 336:1216–1222.
56. Koppal, T., Subramanian, R., Drake, J., Prasad, R., Dhillon, H., and Butterfield, D. A. 1998. Vitamin E protects against Alzheimer's amyloid peptide (25–35)-induced changes in neocortical synaptosomal membrane lipid structure and composition. Brain Res. 786:270–273.
57. Bruce-Keller, A. J., Begley, J. G., Fu, W., Butterfield, D. A., Bredesen, D. E., Hutchins, J. B., Hensley, K., and Mattson, M. P. 1998. Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid β-peptide. J. Neurochem. 70:31–39.