ELSEVIER

Brain Research 786 (1998) 270–273

BRAIN RESEARCH

Short communication

Vitamin E protects against Alzheimer's amyloid peptide (25–35)-induced changes in neocortical synaptosomal membrane lipid structure and composition

Tanuja Koppal [a,c], Ram Subramaniam [a,c], Jennifer Drake [a], M. Renuka Prasad [b], Harbhajan Dhillon [b], D. Allan Butterfield [a,c,*]

[a] *Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA*
[b] *Department of Surgery, University of Kentucky, Lexington, KY 40506, USA*
[c] *Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506, USA*

Accepted 25 November 1997

Keywords: Alzheimer's disease; Amyloid; Electron paramagnetic resonance (EPR); Free radicals; Lipid peroxidation; Vitamin E

Alzheimer's disease (AD) is an age-related, neurodegenerative disorder characterized by the presence of brain-resident neurofibrillary tangles, senile plaques (SP), and loss of synapses [9]. The major component of SP is the aggregated form of amyloid β-peptide (Aβ), which is 39–43 amino acids in length [19]. Aβ is associated with free radical oxidative stress as demonstrated in many laboratories, including our own, employing electron paramagnetic resonance (EPR) spin trapping and spin labeling, measuring Aβ-induced protein and lipid oxidation, and investigating effects of Aβ on oxidation of and dysfunctions in proteins of brain membranes and neuronal cultures and their prevention by free radical antioxidants [2,6,7]. We proposed that Aβ-associated free radical lipid peroxidation and the peroxidation product 4-hydroxynonenal (HNE) may be related to the primary mechanism for brain cell death in AD [7,20].

The purpose of the current study was to assess further the damage caused by Aβ (25–35), specifically to the lipid domain of cortical synaptosomal membranes using EPR and the lipid-specific spin label 12-NS (12-nitroxyl stearate) [3] and measuring free fatty acids (FFA), a marker of lipid peroxidation [4]. If Aβ-induced lipid peroxidation is indeed free radical mediated then, a lipid-soluble free radical scavenger like vitamin E is predicted to protect against such damage.

Vitamin E (Sigma, St. Louis) was stored at 0°C as a 1 M stock solution in DMSO, protected from light. Aβ (25–35), lot no. 091396, was obtained from M.D. Enterprises, Manhattan Beach, CA. All FFA standards used for lipid analysis were from Sigma Chemical. Male Mongolian gerbils, 3–5 months of age, were obtained from Tumblebrook Farms (West Brookfield, MA). The animals were decapitated and neocortical synaptosomes were isolated according to methods previously described [8]. Protein concentration of the synaptosomal membranes was measured by the method of Lowry et al. [10].

For the lipid composition studies three gerbils were used. Synaptosomes from each gerbil were divided into four aliquots, each with a protein concentration adjusted to 2 mg/ml. These aliquots were centrifuged in a refrigerated table-top microcentrifuge at 14,000 rpm for 4 min. The supernatant was discarded and 0.5 ml PBS (pH 7.4) added to each pellet following the treatments described below. The first pellet served as a control sample. To the second was added 5 μl of vitamin E stock (final concentration 5 mM), in order to minimize the amount of DMSO in the mixture. To the third pellet was added 0.5 ml of Aβ (25–35), 1 mg/ml, dissolved in PBS (pH 7.4) 1 h prior to this incubation. Finally, the fourth pellet was treated first with vitamin E and the pre-dissolved Aβ (25–35) was added 0.5 h later. All the samples were kept at room temperature for 6 h, with constant shaking. At the end of 6 h, the samples were frozen in liquid nitrogen and stored at −80°C until the lipid extraction was performed. A total of 200 μl of the thawed synaptosomes were used, and the

* Corresponding author. Fax: +1-606-257-5876; E-mail: dabcns@pop.uky.edu

lipid extraction was performed by the method of Bligh and Dyer [1], as modified in Ref. [4]. The lipid extract obtained was then dissolved in 200 μl of chloroform, of which 100 μl was spotted on silica gel G TLC plates for FFA analysis. The respective regions were scrapped into 1.5 ml methanol containing 0.005% BHT (butylated hydroxy toluene). Fatty acids in all the fractions were converted to their methyl esters and then quantitated on a Hewlett-Packard gas chromatograph as previously described [4].

For EPR studies, Aβ (25–35) was first dissolved in PBS (pH 7.4), 1 mg/ml, and divided into two aliquots, one aliquot was then treated with vitamin E (final concentration 5 mM). The peptide solutions were then incubated in a water bath at 37°C for 6 h. Protein concentration in the purified synaptosomes was adjusted to 8 mg/ml, and the membranes were spin labeled with 12-NS as described earlier [3,7]. A 0.5 ml of peptide incubate was then added to 0.5 ml of synaptosomes at the time of acquisition of the spectra. The Student's *t*-test was used to determine *P*-values. $P < 0.05$ was considered to be statistically significant for comparison between data sets.

A typical spectrum of 12-NS labeled synaptosomal membrane is shown in Fig. 1A. Due to the partitioning of the spin label between the lipid phase and the aqueous phase, one can observe two environments in the EPR spectrum, the lipid-bound (B) and the aqueous-resident, free (F) component. The amplitude of the mid-field line (B_0) was found to be the most sensitive parameter for lipid peroxidation [3]. Fig. 1B shows the intensity of the mid-field line measured over a period of 30 min and expressed as a percentage of the initial intensity. An earlier study showed that Aβ (25–35) decreased the signal intensity of the 12-NS spectrum in neocortical synaptosomes by about 60% of its initial value within 30 min [3], and the present study confirms this response of the 12-NS spectral parameter to Aβ (25–35) (Fig. 1B). In contrast, when Aβ (25–35), incubated with the free radical scavenger vitamin E, was added to synaptosomes there was almost no loss in the signal intensity of the 12-NS spectrum (Fig. 1B), consistent with an Aβ-associated free radical lipid peroxidation mechanism.

Changes in the amounts of FFA after treating the synaptosomal membranes with vitamin E, Aβ (25–35), and vitamin E followed by Aβ (25–35) are shown in Fig. 2A,B. The level of arachidonic acid (20:4) (Fig. 2A) and the overall change in the amounts of all the FFA examined (palmitic acid, stearic acid, oleic acid, and arachidonic acid) (Fig. 2B), showed the effects of Aβ (25–35) and vitamin E. Treating the membranes with vitamin E alone significantly ($P < 0.03$) lowered the amounts of FFA released, compared to the untreated controls, whereas incubating with Aβ (25–35) caused a significant increase ($P < 0.04$) in all FFA. Pretreatment of the membrane with vitamin E followed by Aβ (25–35) led to levels of FFA lower than those of control samples and statistically comparable to those treated with vitamin E alone. Arachidonic

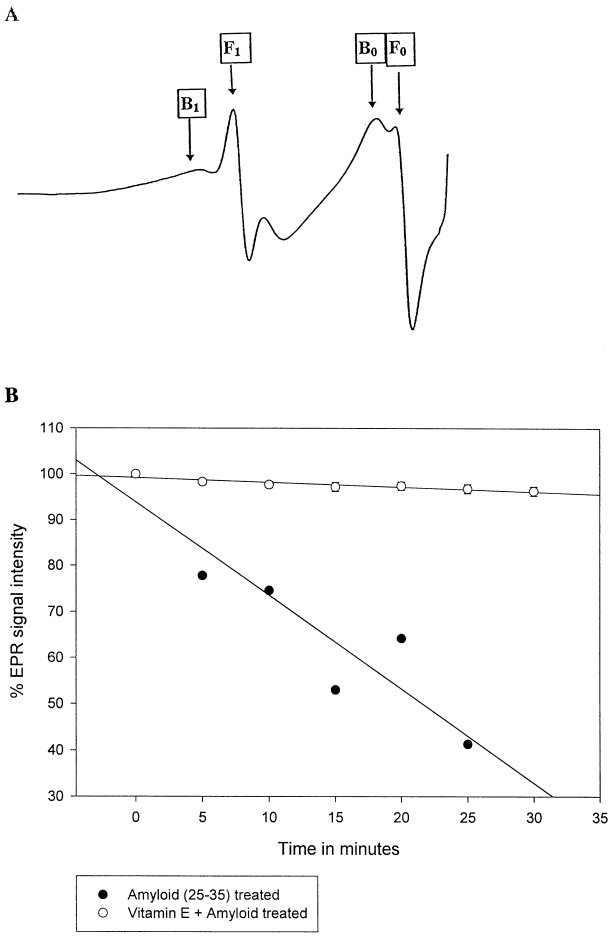

Fig. 1. (A) A typical EPR spectrum of the spin label 12-NS when it is intercalated in the membrane lipid bilayer. B_1 and F_1, and, B_0 and F_0, represent the bound and free components of 12-NS, due to partitioning between the aqueous and lipid phase, on the low field and mid field lines of the EPR spectrum respectively. (B) Percent change in the intensity of the EPR signal of the 12-NS spectrum as observed over time.

acid, containing free radical prone unsaturated sites, was the fatty acid showing maximum release, ~20% over control values ($P < 0.04$), after Aβ (25–35) treatment of synaptosomal membrane samples (Fig. 2A). On pretreatment with vitamin E, the levels of arachidonic acid dropped to ~80% of control values, i.e., FFA release was prevented by this free radical scavenger. Free oleic and stearic acids also were increased after Aβ (25–35) treatment but to a lesser extent than 20:4, and like the latter, pretreatment with vitamin E prevented this increase in oleic and stearic acids (data not shown).

Loss in the EPR signal intensity of the 12-NS spectrum in synaptosomes labeled with 12-NS and treated with Aβ (25–35) was due to the loss of paramagnetism of the spin label. Aβ is known to insert into lipid bilayers [3,13], and if an Aβ (25–35)-associated free radical interacted with the unpaired electron of 12-NS, also present deep in the bilayer, the signal intensity would decrease, as observed. Hence, these results are consistent with the notion that the

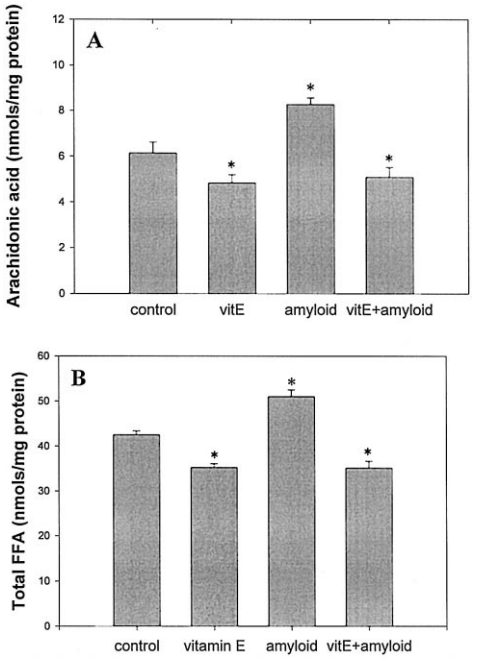

Fig. 2. (A) Effects of vitamin E, Aβ (25–35) and vitamin E + Aβ (25–35) on arachidonic acid (20:4) liberation. Treatment of synaptosomal membranes with Aβ (25–35) did cause a significant increase in the amount of 20:4 as compared to the control ($P < 0.04$). Preincubating both control and Aβ (25–35) treated membranes with vitamin E lead to a significant reduction in the amounts of 20:4 released ($P < 0.04$). (B) Effects of vitamin E, Aβ (25–35) and vitamin E + Aβ (25–35) on the total free fatty acid release observed. Treatment of synaptosomal membranes with Aβ (25–35) did cause an overall significant increase in the amounts of FFA as compared to the control ($P < 0.04$). Preincubation of controls and Aβ (25–35) treated samples with vitamin E showed a statistically significant reduction in the amounts of FFA released ($P < 0.04$). $N = 3$ was required for all FFA measurements.

peptide initiates lipid peroxidation. In this experiment vitamin E was highly successful in ameliorating the Aβ (25–35)-induced lipid damage primarily because it is a lipid soluble, chain breaking, free radical scavenger [16].

In this study, the levels of free arachidonic acid show the maximum increase under oxidative stress associated with the amyloid peptide. Inositol lipids, enriched in arachidonic acid, can undergo phosphodiesteratic cleavage, mediated by PLC (phospholipase C), and the products of this PLC action are IP_3 (inositol 1,4,5 triphosphate) and DAG (diacyl glycerol) [4]. Hydrolysis of DAG by diacylglycerol lipase and monoacylglycerol lipase can further lead to the accumulation of arachidonic acid. FFA release of both saturated and unsaturated fatty acids can also be caused by the action of PLA_2 (phospholipase A_2). Both these phospholipases are sensitive to calcium, and IP_3 can act as a cellular second messenger and mobilize the release of intracellular Ca^{+2}. Intracellular calcium levels are known to be increased in AD and in response to Aβ [5,14], and hence, the activities of these enzymes may be aberrant in AD brains. Vitamin E has been shown to inhibit PLA_2 [15], consistent with the present data and the notion that FFA release following Aβ treatment is associated with free radical oxidative stress.

Lipid peroxidation, assessed here by FFA release and EPR, can lead to alterations in ion homeostasis and cellular signal transductions, resulting in cellular dysfunctions [12,14]. Also, arachidonic acid can act as a precursor for the synthesis of inflammatory agents like leukotrienes and prostaglandins, through the lipoxygenase and cyclooxygenase pathways, and cause impairment of various cellular functions [21]. In addition, arachidonic acid is hypothesized to be a precursor for HNE [17], formed in neuronal systems exposed to Aβ [12]. HNE alters brain membrane protein conformation [20] and causes the same dysfunction of transmembrane ion-motive ATPases as Aβ itself [11,12].

This study showed the damaging effects of Aβ (25–35) on the structure and composition of lipids isolated from synaptosomal membranes. The exact mechanism of Aβ (25–35) toxicity to cells is not known, but that it is free radical-mediated is strengthened by the observation that vitamin E, a free radical scavenger, could mitigate the damage to a considerable extent. A recent report demonstrated beneficial effects in AD patients, of a high-dose vitamin E treatment [18], consistent with our hypothesis that AD is a disease associated with oxidative stress [2,7].

Acknowledgements

This work was supported by NIH (AG-05119).

References

[1] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[2] D.A. Butterfield, β-amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease, Chem. Res. Toxicol. 10 (1997) 518–526.
[3] D.A. Butterfield, K. Hensley, M. Harris, M.P. Mattson, J.M. Carney, β-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease, Biochem. Biophys. Res. Commun. 200 (1994) 710–715.
[4] H.S. Dhillon, D. Donaldson, R.J. Dempsey, M.R. Prasad, Regional levels of free fatty acids and Evans Blue extravasation after experimental brain injury, J. Neurotrauma 11 (1994) 405–415.
[5] Y. Goodman, M.R. Steiner, S.M. Steiner, M.P. Mattson, Nordihydroguaiaretic acid protects hippocampal neurons against amyloid β-peptide toxicity, and attenuates free radical and calcium accumulation, Brain Res. 654 (1994) 171–176.
[6] M.E. Harris, K. Hensley, D.A. Butterfield, R.A. Leedle, J.M. Carney, Direct evidence of oxidative injury produced by the Alzheimer's amyloid beta protein (1–40) in cultured hippocampal neurons, Exp. Neurol. 132 (1995) 1–10.
[7] K. Hensley, J.M. Carney, M.P. Mattson, M. Aksenova, M. Harris, J.F. Wu, R.A. Floyd, D.A. Butterfield, A model for β-amyloid aggregation and neurotoxicity based on free radical generated by the peptide: relevance to Alzheimer's disease, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 3270–3274.

[8] K. Hensley, N.C. Hall, W. Shaw, J.M. Carney, D.A. Butterfield, Electron paramagnetic resonance investigation of free radical induced alterations in neocortical synaptosomal membrane protein infrastructure, Free Radic. Biol. Med. 17 (1994) 321–331.
[9] R. Katzman, T. Saitoh, Advances in Alzheimer's disease, FASEB J. 4 (1991) 278–286.
[10] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[11] R.J. Mark, K. Hensley, D.A. Butterfield, M.P. Mattson, Amyloid β-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca^{2+} homeostasis and cell death, J. Neurosci. 15 (1995) 6239–6249.
[12] R.J. Mark, M.A. Lovell, W.R. Markesbery, K. Uchida, M.P. Mattson, Evidence that 4-hydroxynonenal mediates disruption of ion homeostasis and neuronal death by amyloid-β peptide, J. Neurochem. 68 (1997) 255–264.
[13] R.P. Mason, J.D. Estermyer, J.F. Kelly, P.E. Mason, Alzheimer's disease amyloid β peptide 25–35 is localized in the membrane hydrocarbon core: X-ray diffraction analysis, Biochem. Biophys. Res. Commun. 222 (1996) 78–82.
[14] M.P. Mattson, S.W. Barger, B. Cheng, L. Lieberburg, V.L. Smith-Swintosky, R.E. Rydel, β-Amyloid precursor protein metabolites and loss of neuronal Ca^{+2} homeostasis Alzheimer's disease, Trends Neurosci. 16 (1993) 409–414.
[15] A.P. Pentland, A.R. Morrison, S.C. Jacobs, L.L. Hruza, J.S. Herbert, L. Packer, Tocopherol analogs suppress arachidonic acid metabolism via phospholipase inhibition, J. Biol. Chem. 267 (1992) 15578–15584.
[16] B. Perly, I.C.P. Smith, L. Hughes, G.W. Burton, K.U. Ingold, Estimation of the location of natural α-tocopherol in lipid bilayers by ^{13}C-NMR spectroscopy, Biochim. Biophys. Acta 819 (1985) 131–135.
[17] W. Pyror, N. Porter, Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autooxidation of polyunsaturated fatty acids, Free Radic. Biol. Med. 8 (1990) 541–543.
[18] M. Sano, C. Ernesto, R.G. Thomas, M.R. Klauber, K. Schafer, M. Grundman, P. Woodbury, J. Growdon, C.W. Cotman, E. Pfeiffer, L.S. Schneider, L.J. Thal, A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's disease cooperative study, New Engl. J. Med. 336 (17) (1997) 1216–1222.
[19] D.J. Selkoe, Amyloid β-protein and the genetics of Alzheimer's disease, J. Biol. Chem. 271 (1996) 18295–18298.
[20] R. Subramaniam, F. Roediger, B. Jordan, M.P. Mattson, J.N. Keller, G. Waeg, D.A. Butterfield, The lipid peroxidation product, 4-hydroxy-2-*trans*-nonenal, alters conformation of cortical synaptosomal membrane proteins, J. Neurochem. 69 (3) (1997) 1161–1169.
[21] R.P. White, Cerebrospinal fluid eicosanoids as an index of cerebrovascular status, Anal. N.Y. Acad. Sci. 570 (1989) 131–145.