Vitamin E as an Antioxidant/Free Radical Scavenger Against Amyloid β-Peptide-Induced Oxidative Stress in Neocortical Synaptosomal Membranes and Hippocampal Neurons in Culture: Insights into Alzheimer’s Disease

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SYNOPSIS

Amyloid β-peptide (A\textsubscript{β}), the major constituent in senile plaques in Alzheimer’s disease (AD) brain, is thought by many researchers to be central to neurotoxicity in AD brain. Increasing evidence from many laboratories indicates that AD brain is under oxidative stress, with strong evidence of protein oxidation, lipid peroxidation, and peroxynitrite damage. A link between the central role of A\textsubscript{β} and oxidative stress in AD brain may be A\textsubscript{β}-associated free radical oxidative stress. If so, antioxidants such as vitamin E should modulate A\textsubscript{β}-induced oxidative damage and neurotoxicity in brain cells. This review summarizes studies of A\textsubscript{β}-associated free radical oxidative stress and its inhibition by vitamin E in cortical synaptosomal membranes and hippocampal neuronal cells in culture. Taken together with the recent report that vitamin E slows the progression of AD, this review strongly supports a central role of A\textsubscript{β}-associated free radical oxidative stress in neurotoxicity in AD brain.

KEY WORDS

vitamin E, amyloid β-peptide, free radicals, oxidative stress, protein oxidation, lipid peroxidation, neurotoxicity, Alzheimer’s disease

AMYLOID β-PEPTIDE AND ALZHEIMER’S DISEASE

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder characterized by the progressive loss of memory and cognitive functions /35/. There is yet no cure nor any diagnostic marker for AD. Autopsy studies on AD brain reveal the presence of three pathological hallmarks of the disease - senile plaques (SP), neurofibrillary tangles (NFT) and the loss of synapses. SP are mainly composed of an aggregated core of a protein called amyloid β-peptide (A\textsubscript{β}), surrounded by dystrophic neurons and other moieties. A\textsubscript{β} is a 39-43 amino acid peptide which is deposited extracellularly after cleavage of a larger protein, amyloid precursor protein (APP). A\textsubscript{β} found in SP in AD brain is mostly A\textsubscript{β} (1-42), in a highly aggregated state, resistant to proteolysis, and extremely toxic to neurons in culture. The reasons for the abnormal cleavage of APP leading to excess amounts of extracellular deposition of A\textsubscript{β}, its aggregation, and toxicity in AD are still under study.

Many researchers, though not all, accept the hypothesis that A\textsubscript{β} is key to the pathogenesis of AD (reviewed in /51/). For example, familial AD is associated with mutations in APP and in presenilins, the latter being transmembrane proteins thought to be associated with APP processing. Chromosome 21 is associated with both APP and Down’s syndrome (DS), and DS patients, if they live long enough, invariably develop AD. Finally, APP-overexpressing transgenic mice over time develop many aspects of AD-like pathology /17/.

Oxidative stress is found in AD brain /12,45/. For example, lipid peroxidation /39,40,46,49/ and protein oxidation /29,52/ are found in AD brain regions rich in SP but not in SP-poor cerebellum. It
is conceivable that Aβ-associated free radical oxidative stress is a link between a central role for Aβ in AD and the presence of oxidative stress in AD brain/12/.

Aβ(25-35) is an 11 amino acid subset of Aβ(1-42), located on the C-terminal end in the hydrophobic domain, and is thought to be associated with the toxicological properties of the larger peptide/48/. Both Aβ(25-35) and the larger peptides Aβ(1-40) and Aβ(1-42) are known to be associated with free radicals, cause oxidative stress, and lead to neurotoxicity. Consequently, although Aβ(25-35) is not found in SP in AD brain, in vitro studies of this peptide may provide insights into the role of the larger peptides and may help in investigating the molecular mechanisms of Aβ(1-40) and Aβ(1-42).

**Aβ IS ASSOCIATED WITH FREE RADICALS**

Free radicals are usually highly reactive and unstable species. In order to detect and analyze free radicals using electron paramagnetic resonance (EPR), the only direct method for doing so, compounds called spin traps are sometimes used /7,33/. Spin traps are usually non-paramagnetic nitrones, and they have the ability to effectively scavenge free radicals to form spin adducts or nitroxides. These adducts are relatively long-lived and stable enough to give a spectrum which is characteristic of the radical forming the adduct. Addition of Aβ(1-42), Aβ(1-40), or Aβ(25-35) to metal-chelated buffers containing the spin trap, N-tert-butyl-α-phenylnitrone (PBN), gave either a 3-line or a 4-line EPR spectrum /25,27,30,31,57,59/., implying that free radicals were present in the solution containing Aβ peptides and PBN. Neither the non-toxic, reverse sequence Aβ(35-25), nor the scrambled sequence of Aβ(25-35) yielded similar signals with PBN, indicating that the presence of free radicals was dependent on the primary sequence of Aβ peptides. Non-toxic Aβ(40-1) gave a 6-line EPR spectrum, completely different from the spectrum shown by either Aβ(1-40) or Aβ(1-42). Further, the trap itself gave no EPR spectrum in the time frame of the Aβ EPR studies /27,30,31,59/. In addition to spin trapping, HPLC analysis of Aβ in the presence of salicylate revealed the presence of 2,3-dihydroxybenzoic acid, a hydroxyl free radical adduct to salicylate /27,59/. Bush and co-workers /6/ reported that Aβ(1-42) could reduce Cu²⁺ to Cu⁺, i.e., confirming by different methods that Aβ donates an electron to a species, forming a free radical. The formation of peroxyl radicals by Aβ(25-35) in solution was also shown using the commercially available PeroOxQuant assay /9/.

EPR was used in a different way to demonstrate Aβ-associated free radicals. Aβ(25-35), added to solutions of nitroxide spin labels, resulted in loss of paramagnetism of the spin label, a process that occurred by a free radical on Aβ reacting with the unpaired electron on the spin label /9/. This technique was used in membrane systems as well. Synaptosomal membranes, isolated from neocortices of gerbil brains, when treated with Aβ(25-35) and labeled with the lipid-specific spin label 12-nitroxide stearate (12-NS), showed decreased EPR signal intensity /8/. Reduction in signal intensity (due to loss of paramagnetism) can occur only when the unpaired electron of the spin label reacts with another free radical existing in the vicinity of the spin label. Also, the signal reduction was found dependent on the primary sequence of the peptide, since the non-toxic, reverse sequence Aβ(35-25) caused a much lower decrease. Aβ(25-35) also significantly reduced the EPR signal intensity of 5-NS in intact PC12 cells and in plasma membranes, mitochondrial membranes, and post-nuclear membranes isolated from PC12 cells /5/. If these cells were transformed to overexpress Bcl-2, the gene product of which is thought to be an antioxidant, no loss of signal occurred /5/. Strengthening the hypothesis of Aβ(25-35)-associated free radical oxidative stress was the finding that addition of vitamin E to synaptosomal membranes prior to addition of Aβ(25-35) completely prevented the loss in signal intensity of the 12-NS spectrum /37/.

**VITAMIN E PROTECTS AGAINST Aβ-INDUCED LIPID PEROXIDATION**

Phospholipids in the membrane bilayer have both saturated and unsaturated fatty acids. The higher the degree of unsaturation the more susceptible the fatty acid is to oxidation. Therefore, polyunsaturated fatty acids (PUFA), such as arach-
idonic acid, are easily oxidizable. The brain has large amounts of PUFA, low antioxidant levels, and a high oxygen consumption. Therefore, the brain is at great risk of undergoing membrane damage due to lipid peroxidation. Oxidation of fatty acids triggers the action of phospholipases which cleave the oxidized fatty acids from the phospholipid backbone. Hence, lipid peroxidation can be followed by an increase in the amounts of free fatty acids (FFA) and a decrease in amounts of fatty acids associated with those phospholipids. Thus, the measurement of amounts of FFA and the composition of phospholipids are considered to be markers of lipid peroxidation /37/.

FFA analysis after incubation of synaptosomal membranes with Aβ(25-35) showed a significant increase in the amounts of all FFA, except palmitic acid, 16:0, indicating Aβ-induced lipid peroxidation /37/. The amounts of free stearic acid, 18:0, and arachidonic acid, 20:4, showed greater increase compared to other fatty acids. When vitamin E was added to the membranes 30 minutes prior to the addition of Aβ(25-35), the FFA analysis showed complete protection against Aβ-triggered FFA release, with the amounts of fatty acids being lower than even the control samples and comparable to those treated only with vitamin E /37/. This result is consistent with the notion that vitamin E not only blocked auto-oxidation of membrane lipids but also protected against the oxidative stress induced by agents such as Aβ(25-35). This finding was confirmed by others, using fluorescence and TBARS to measure lipid fluidity and lipid peroxidation, respectively /1/. These researchers found that Aβ(25-35) increased membrane fluidity and caused lipid peroxidation, and that vitamin E prevented both effects, consistent with our results /37/. Aβ-induced lipid peroxidation was confirmed by others, who also showed that the free radical scavengers melatonin or estrogen blocked this effect /14,19/.

**VITAMIN E PROTECTS AGAINST Aβ-INDUCED PROTEIN OXIDATION AND LOSS OF ENZYME ACTIVITY**

Amino acids such as cysteine, methionine, histidine, and others are prone to oxidation /53/. Oxidation of proteins can lead to changes in protein conformation, and changes in chemical composition and structure /13/. Alterations in conformation and structure can cause loss of functionality by inducing changes in active site residues. In particular, the activity of oxidation-sensitive enzymes, such as glutathione synthetase (GS) and creatine kinase (CK), is greatly dependent on oxidation-induced changes /53/.

Changes in protein conformation and in protein-protein interactions can be studied using electron paramagnetic resonance (EPR) in conjunction with a thiol-specific spin label such as 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl (MAL-6) /7/. We have characterized this spin label in several models of oxidative stress, such as ischemia-reperfusion /21,22,23,24/, accelerated aging /10/, hyperoxia /32/, and hydroxyl free radical generation /28/, and found in each case that the low field region of the EPR spectra obtained can be used as a marker of protein conformational changes in brain synaptosomal membranes. On a protein, MAL-6 can react with SH groups to result in weakly (W)- or strongly (S)-immobilized sites, characterized by their motion /7/. These sites are represented in the low-field portion of the EPR spectrum by a sharp peak and a broad peak, respectively. The ratio of the intensities of the W and S peaks (W/S ratio) is a good indicator of protein-conformation /7/. A lower W/S ratio compared to control samples implies increased protein crosslinking, increased protein-protein interactions, or decreased protein segmental motion /7/. In all the in vitro and in vivo models of oxidative stress noted above, the W/S ratio of MAL-6-labeled synaptosomal membranes is decreased relative to controls. Hence, the W/S ratio is indicative of conformational changes and also of protein oxidation causing those changes. The other and more commonly used marker of protein oxidation is the amount of protein carbonyl groups /53/. Increased amounts of carbonyls in samples compared to controls indicates increased oxidation. Loss of enzyme activity by GS and CK by oxidants is also a direct consequence of protein oxidation, and hence measuring their activities can also serve as measure of oxidative stress.

Treatment of synaptosomal membranes with pre-incubated Aβ(25-35) followed by incubation
with MAL-6 showed significantly lower W/S ratios compared to control values /55/. The same membranes also showed increased protein carbonyl levels /55/, showing that Aβ peptides induced protein oxidation in synaptosomal membranes. In contrast, incubation of membranes with Aβ(25-35) that had been pre-incubated with vitamin E showed no significant lowering of the W/S ratio compared to control samples, nor did they show any increase in protein carbonyl amounts /55/. Thus, vitamin E effectively blocked protein conformational changes and protein oxidation induced by Aβ(25-35). Similarly, hippocampal neurons and astrocytes, in culture, when treated with Aβ(25-35), Aβ(1-40) or Aβ(1-42), showed increased protein carbonyl content /25,55,60/, and this increase was prevented when the cells were pre-treated with vitamin E /55,60/ or propyl gallate /25/. In vivo protein oxidation was found in transgenic C. elegans expressing Aβ(1-42) /63/, consistent with our model of Aβ-associated free radical oxidative stress in AD brain /12/.

Measurement of GS and CK activity in the cytosolic fraction of rodent brain homogenates treated with Aβ(1-40) or Aβ(25-35) showed a significant loss in activity of both enzymes /11,27, 55,60/. The reverse sequences of the peptides Aβ(40-1) or Aβ(35-25) did not cause any significant loss in enzyme activity. Pre-treatment of Aβ(25-35) with vitamin E before addition to GS significantly reduced the loss in enzyme activity /55/. Aβ(1-40) also decreased GS activity in hippocampal neurons in culture /25/. Semi-quantitative determination of brain CK activity from the crude neuronal cell extract, using gel electrophoresis, showed significant loss in enzyme activity in neurons treated with Aβ(25-35) /60/. Neurons treated with both vitamin E and Aβ(25-35) showed almost no loss of CK activity compared to control untreated neurons.

VITAMIN E PREVENTS CELL TOXICITY INDUCED BY Aβ

Oxidative stress in neurons can trigger cell death either by apoptosis or necrosis. As noted above, the brain in particular is not well equipped to deal with oxidative stress because it has low antioxidant levels, high amounts of redox metal ions which can produce reactive oxygen species through Fenton reactions, and also because neurons are non-mitotic. In addition, consumption of high amounts of oxygen by the brain and the presence of oxidizable components such as PUFA make it more prone to oxidation. The trypan blue exclusion assay to determine the integrity of cellular membranes, which is lost after cell death, is a good assay for studying cell survival following oxidative stress.

One of the first studies showing involvement of free radicals in Aβ toxicity was in PC 12 cells. The antioxidants, vitamin E and propyl gallate, were found to protect PC 12 cells against Aβ(25-35)- and Aβ(1-40)-induced toxicity /2/. Later, incubation of hippocampal neurons with Aβ(25-35) and Aβ(1-40) was shown to reduce cell survival /18,55, 60,62/. The reverse sequence Aβ(40-1) or scrambled Aβ(25-35) had no effect on neuronal survival /25/. Pre-incubation of cells with vitamin E prior to treatment with Aβ(1-40) or Aβ(25-35) was shown by many laboratories to prevent the drastic Aβ-induced cell loss /4,12,16,18,42,47,55,56,61, 62/. Furthermore, vitamin E protected against Aβ-induced damage to non-neuronal cells as well, e.g., lens /34/, smooth muscle /20/, and vascular endothelial tissue /56/. Micrographs of neurons treated with Aβ(25-35) and Aβ(1-40) showed shrunken cell bodies and broken dendrites and axons /60/. However, neurons pre-treated with vitamin E prior to Aβ(25-35) addition showed little change in morphology compared to the untreated neurons. In contrast to these results from many different laboratories, others reported that vitamin E was ineffective against Aβ-induced neurotoxicity /38/. These researchers also suggested that catalase was ineffective as well, in marked contrast to other researchers /26,41/. The exact mechanism of Aβ toxicity to neurons is unknown but that the overwhelming majority of laboratories report that vitamin E protects against Aβ-induced cell toxicity suggests that cells die of oxidative stress and free radical damage, consistent with an Aβ-associated free radical oxidative stress model for neurotoxicity in AD brain /12/.
VITAMIN E BLOCKS ROS PRODUCTION INDUCED BY Aβ

Accumulation of intracellular reactive oxygen species (ROS) can be monitored by the addition of 2,7-dichlorofluorescin diacetate to cells. This dye is converted to 2,7-dichlorofluorescin anion by the action of esterases, and to 2,7-dichlorofluorescin, which fluoresces in the presence of ROS, in particular, peroxides. Color images of the cells can then be obtained using a fluorescence confocal microscope and digitized for partial quantification.

Aβ(1-40) was shown to induce ROS production in neuronal cells /18,25,60/. The reverse sequence Aβ(40-1) or scrambled Aβ(35-25) did not induce any ROS accumulation in cells /3,25,42/. Pre-treatment of neurons with vitamin E and astrocytes with trolox, a water soluble analogue of vitamin E, prior to Aβ addition, considerably reduced the amount of ROS formed /18,25,60/.

Aβ-MEDIATED OXIDATIVE STRESS AND Ca²⁺ ACCUMULATION

As described above, Aβ, when added to neurons, triggers ROS formation and induces membrane damage by oxidizing proteins and lipids, and these alterations can increase intracellular Ca²⁺. Proteolytic cleavage, phospholipase- and endonuclease-based hydrolysis, and other destructive processes are stimulated by excess Ca²⁺. Membrane damage by ROS, proteases, and lipases can also affect organelles such as mitochondria. These latter alterations can cause electron leak through the electron transport system and lead to release of superoxide, which can then react with nitric oxide to form peroxynitrite. Ca²⁺ can also activate enzymes such as nitric oxide synthase, which can trigger nitric oxide production. Peroxynitrite can cause further membrane damage and oxidize DNA and amino acids, and affect key cellular enzymes. Increase in intracellular Ca²⁺ can also trigger apoptosis, leading to cell death.

There are several lines of evidence showing increased intracellular Ca²⁺, and decrease in activity of Na⁺/K⁺-ATPase and Ca²⁺-ATPase induced by Aβ(1-40) and Aβ(25-35) in neuronal cell cultures /18,42/. Inhibition of Na⁺/K⁺-ATPase affects the cell potential, opening voltage-gated Ca²⁺ channels, thereby permitting extracellular Ca²⁺ to rush down its concentration gradient to the interior of the neuron. Inhibition of Ca²⁺-ATPase slows the calcium pump, thereby keeping intracellular Ca²⁺ levels high. Release of Ca²⁺ from intracellular stores under a calcium ion stress, including that from opening the mitochondrial membrane permeability transition pore, further exacerbates this toxic condition. Aβ has also been shown to induce impairments in glucose and glutamate transporters /36,43,44/. Supporting the Aβ-associated free radical hypothesis, vitamin E has been shown to block Aβ-induced increased intracellular Ca²⁺ /18, 42/, decreased Na⁺/K⁺-ATPase activity /42/, and impairment of glucose transporter /43/. Others reported that Aβ peptides alter Ca²⁺ homeostasis through L-type calcium ion channels and confirmed that vitamin E and other antioxidants blocked this effect /38,64/. Trolox also prevented the loss in activity of the Na⁺-dependent glutamate transporter mediated by Aβ(25-35) in astrocyte cultures /26/.

MECHANISMS UNDERLYING THE ACTION OF VITAMIN E

Aβ-induced lipid peroxidation leads to increase in FFA, such as arachidonic acid, as noted above. Arachidonic acid is the precursor for cytotoxic species such as 4-hydroxynonenal and malondialdehyde, which in turn can attach to key membrane proteins through reactions with the amino acids lysine, cysteine and histidine, and alter their structure and functionality. Arachidonic acid is also the precursor for the biosynthesis of prostaglandins and leukotrienes which stimulate cellular inflammatory responses. Inflammation can activate microglia to produce nitric oxide. Nitric oxide can lead to peroxynitrite formation which can rekindle the whole cascade.

Protein oxidation affects the activity of ion motive ATPases, which can lead to disruption of ion homeostasis and increase intracellular calcium. Loss of activity of enzymes such as CK, which plays a key role in ATP synthesis, can drastically affect cellular energy status /60/. The inability to meet the increasing demands of the cell to deal with oxidative stress at a point in the neuron’s life when
energy demand is critical, e.g., under oxidative stress due to Aβ, could be detrimental to cell survival. Loss of activity of GS can increase the levels of glutamate leading to glutamate excitotoxicity through over-stimulation of NMDA receptors. Thus, the mechanisms of Aβ-mediated toxicity in cells are multiple, and the results discussed above point towards a free radical role in this cascade.

Vitamin E was highly successful in ameliorating Aβ-induced lipid damage, primarily because it is a lipid soluble, chain breaking, free radical scavenger. Vitamin E has a preference for associating with the fluid domains in the membrane, which usually contain the most unsaturated fatty acyl chains. While the aromatic head group of vitamin E lies close to the aqueous interface, its side chain lies embedded in the lipid phase and so the phenolic head group can scavenge radicals that are generated both in the aqueous medium as well as those present in the lipid bilayer. The vitamin E molecule reacts with a free radical to form the tocopheroxy radical, which is unreactive, thus inhibiting the propagation of a chain reaction. The molecule, because of its structure, has a dual mode of protection, its phenolic head group scavenging free radicals, while its long carbon side chain aligns with the fatty acyl chains of the lipid bilayer, thereby stabilizing the membrane and restoring its structure. Also, vitamin E in biological systems can be recycled by both vitamin C and glutathione, and, hence, the levels of the reduced form of the molecule can be maintained. Finally, NMR studies have shown that vitamin E can form a complex with either FFA or lysophospholipids, thereby diminishing the disorder in the lipid environment caused by these molecules.

The hypothesis that the antioxidant properties of vitamin E are primarily due to its free radical scavenging ability is further strengthened by studies which showed that vitamin E showed only partial protection against the action of HNE on synaptosomal membranes /54/. HNE is not a free radical but is formed after free radical action on membranes causes lipid peroxidation /15/. The toxicity of HNE is due to its reaction with lysines, histidines and cysteines, forming Michael adducts and causing alterations in protein structure and functionality /15/.

Synaptosomal membranes incubated with HNE and labeled with MA1-6 showed lower W/S ratios compared to untreated samples, and pre-incubation of the membrane with vitamin E prior to HNE addition did not alter the lowering of the W/S ratios observed, in contrast to the effect of vitamin E with Aβ(25-35)-treated synaptosomal membranes /54, 55/. Similarly, vitamin E was unable to protect the membranes against HNE-induced protein oxidation, measured by increased protein carbonyls. HNE added to hippocampal neurons in culture dropped cell survival values to less than 20% of controls, as measured using the MTT assay for mitochondrial dysfunction. Vitamin E added to neurons prior to HNE addition was only partially protective, in contradistinction to its protective effect against Aβ /55/. It is reasonable to suggest that the free radical scavenging ability of vitamin E, that protected membranes from Aβ(25-35) action, was ineffective against HNE, because HNE is formed as a consequence of free radical effects. The efficacy of vitamin E lies in its ability to intercept free radicals at their inception. However, it is ineffective in preventing the deleterious effects of agents formed downstream from the initial free radical reactions.

The therapeutic benefit of vitamin E in AD has already been demonstrated through clinical trials in which AD patients given 2000 units of vitamin E per day showed delayed progression of the disease /50/. Thus, the use of free radical scavengers such as vitamin E against Aβ toxicity and in AD, has strengthened the hypothesis implicating free radicals in the pathogenesis of AD /12,45/ and has provided impetus for the development of more efficient brain-accessible antioxidants.

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