

**$\beta$ -AMYLOID PEPTIDE FREE RADICAL FRAGMENTS INITIATE SYNAPTOSOMAL LIPOPEROXIDATION IN A SEQUENCE-SPECIFIC FASHION: IMPLICATIONS TO ALZHEIMER'S DISEASE**

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We have previously reported (Hensley et al., Proc. Natl. Acad. Sci. USA (1994) in press) that  $\beta$ -amyloid peptide fragments in aqueous media, in a metal-independent reaction, produce reactive peptide free radicals and reactive oxygen species. In contrast to the hours or days necessary to produce neurotoxicity and a detectable free radical for  $\beta$ -amyloid, the extremely neurotoxic A $\beta$ (25-35) fragment of  $\beta$ -amyloid peptide produces a detectable radical in minutes. We now report that A $\beta$ (25-35) is a potent lipoperoxidation initiator, as inferred from peptide-mediated reduction of nitroxyl stearate spin labels bound to rodent neocortical synaptosomal membranes. A $\beta$ (25-35) rapidly quenches the paramagnetism of membrane-bound 12-nitroxyl stearate spin probe deep within the lipid bilayer, but reacts poorly with the 5-nitroxyl isomer whose paramagnetic center is near the lipid/water interface. A $\beta$ (35-25), the non-neurotoxic reverse sequence of A $\beta$ (25-35), shows little proclivity to reduce either spin label. These findings are formulated into a "molecular shrapnel" model of neuronal membrane damage in Alzheimer's disease. © 1994 Academic Press, Inc.

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There has been a growing consensus in recent years that free radical-mediated neuronal damage may be a major contributor to the etiology of age-related degenerative disorders, particularly Alzheimer's disease (AD). Concurrently, much data has been collected to implicate aberrant peptide processing, especially  $\beta$ -amyloid peptide (A $\beta$ ) deposition, in AD. We recently

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**Abbreviations:** AD, Alzheimer's disease; APP, amyloid precursor protein; A $\beta$ ,  $\beta$ -amyloid peptide; CK, creatine kinase; EPR, electron paramagnetic resonance; GS, glutamine synthetase; 5-NS, 5-nitroxyl stearic acid; 12-NS, 12-nitroxyl stearic acid; PBS, phosphate buffered saline, pH 7.4; ROS, reactive oxygen species.

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proposed that these two paradigms are related through the novel radical-generating capacity of A $\beta$  (1). A $\beta$  spontaneously fragments in aqueous solution, producing lower molecular weight, reactive free radical peptides which we detected in electron paramagnetic resonance (EPR) spin trapping experiments and by liquid chromatography-mass spectral analysis (1). Furthermore, reactive oxygen species (ROS) were generated during this reaction, as indicated by salicylate hydroxylation assays, and the reaction components were shown to be potent inactivators of cytosolic glutamine synthetase (GS) and creatine kinase (CK) (1).

We reasoned that reactive A $\beta$ s might compromise neuronal membrane integrity by intercalating themselves into lipid bilayers during the radicalization reaction, thereby initiating lipid peroxidation and damaging multiple membrane components. In order to test this hypothesis, we have formulated an EPR assay based the reduction of synaptosomal membrane-bound, paramagnetic nitroxyl stearate probes. Such assays are becoming popular tools for the investigation of peroxidation processes, including membrane lipoperoxidation (2). Neocortical synaptosomes isolated from gerbils were spin labeled with either 5-nitroxyl stearate (5-NS) or 12-nitroxyl stearate (12-NS). Incubation of labeled synaptosomes with the highly reactive and neurotoxic A $\beta$  fragment consisting of amino acid residues 25-35 [A $\beta$ (25-35)] led to substantial loss of EPR signal from the 12-NS preparation, with relatively little loss of signal intensity from the 5-NS preparation. A $\beta$ (35-25), the non-neurotoxic reverse sequence analogue of A $\beta$ (25-35), had much less effect on 5-NS or 12-NS. We take these results to indicate that A $\beta$  fragments can initiate lipoperoxidation, and that toxicity of A $\beta$  radicals depends on a structural component which varies as a function of peptide primary sequence.

## MATERIALS AND METHODS

A $\beta$ s were prepared by t-BOC solid phase synthesis. Peptides were from batches of verified purity and known neurotoxic function, and known radical producing ability as assayed by EPR spin trapping analysis (1).

Male Mongolian gerbils, 15 months of age, were obtained from Tumblebrook Farms (West Brookfield, MA) and subsequently housed in the University of Kentucky Central Animal Facility. Animals were killed by decapitation and the brain rapidly removed and placed on ice. Unlysed synaptosomes were purified from homogenized cortices via ultracentrifugation across discontinuous sucrose gradients after the method of Ueda et al. (3) as adapted by Barnes (4) and further adapted in our laboratory (5). Isolated synaptosomes were washed 3 times in 150 mM phosphate buffered saline (PBS) pH 7.4 and synaptosomal protein concentration was indexed by the Lowry protein assay (6).

The spin labels, 5-NS or 12-NS, obtained from Sigma or Aldrich Chemical, were dissolved in chloroform to a concentration of 320  $\mu$ g/mL. 32  $\mu$ L of these solutions were placed in 3 ml borosilicate culture tubes and the chloroform evaporated under N<sub>2</sub>. Into these tubes were placed 0.5 mL samples of freshly prepared synaptosomes at a protein concentration of 8 mg/mL. Samples were incubated in the spin-label-coated tubes for 30 minutes at room temperature with gentle agitation. Synaptosomes thus labeled were diluted to a final protein concentration of 4.0 mg/mL by addition of buffer. A 300  $\mu$ L portion of synaptosomal suspension was then added to A $\beta$  powder to give a final A $\beta$  concentration of 0.4 mg/mL (380  $\mu$ M), unless otherwise stated.

This synaptosome / A $\beta$  mixture was immediately placed within a quartz EPR aqueous flat cell. The cell was then placed within the EPR sample cavity and spectral acquisition was executed as described below.

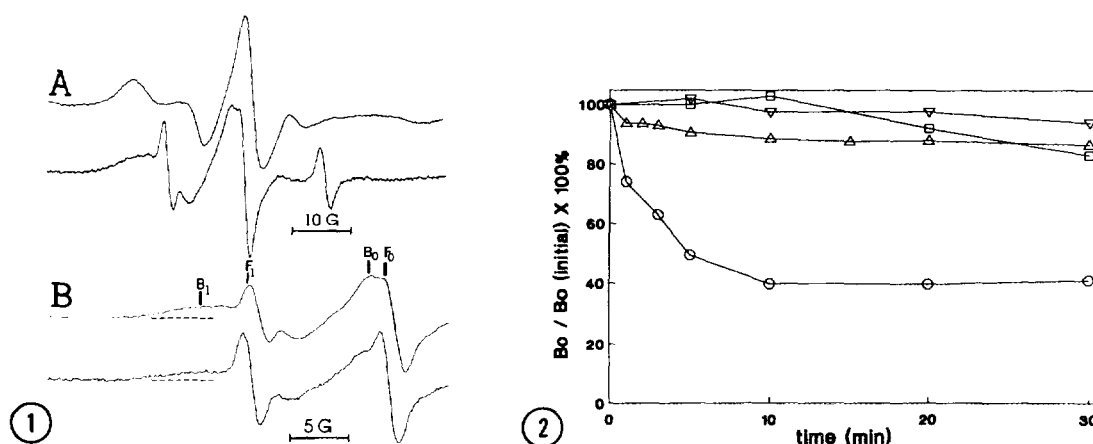
All spectra were acquired on a Bruker model 300 EPR spectrometer equipped with computerized data acquisition and analysis capabilities. Instrumental parameters were: receiver gain =  $1 \times 10^5$ ; conversion time = 10.28 ms; time constant = 1.28 ms; incident microwave power = 19 mW; modulation amplitude = 0.39 G; scan time = 10 seconds. Typically, a spectrum could be obtained within 1 minute of addition of synaptosomes to A $\beta$ . The 5- or 12-NS spectrum was acquired in a single scan after 1, 2, 5, 8, 10, 15, 20, 25, and 30 minutes incubation with A $\beta$ . Spectral line positions and intensities were determined using instrument-resident analysis software. Statistical significance was based on analysis by Student's t-tests.

## RESULTS

The EPR spectrum of an isotropically tumbling nitroxide spin label in dilute solution consists of three lines corresponding to electron spin transitions between  $^{14}\text{N}$  nuclear spin levels  $M_I=+1$ ,  $M_I=0$ , and  $M_I=-1$  (7). In the case of membrane-bound stearic acid nitroxides, however, nitroxide motion is largely restricted to anisotropic rotation about the long axis of the spin label, and this motional restriction causes spectral line broadening, dependent upon the positional isomer employed (7).

As Fig. 1 shows, the spectrum of 5-NS incorporated into synaptosomal membranes consists purely of an anisotropic component; the  $M_I=+1$ , 0, and -1 lines have no overlapping peaks or shoulders since the spin label is completely transferred to the membrane during the labeling procedure. However, as previously documented in other membrane systems (7), 12-NS consistently partitions between the lipid (membrane) and aqueous (extrasynaptosomal) phases, and one can resolve in the 12-NS spectrum both a "bound" and a "free" population (Fig. 1A, bottom). The bound population predominates in untreated 12-NS labeled synaptosomes, with the three sharp lines of the free population providing only a minor contribution to the total integrated spectral intensity. In order to quantify the data collected in this experiment, we designated the bound and free components of the  $M_I(0)$  mid-field 12-NS line as  $B_0$  and  $F_0$ , respectively (Fig. 1B).

We found that the amplitude of the mid-field  $M_I(0)$  line(s) was the most sensitive indicator of lipid peroxidation. Treatment of 12-NS labeled synaptosomes with A $\beta$ (25-35) induced a 60 % decrease of the intensity of the  $B_0$  component within 30 minutes ( $p < 0.002$ , Fig. 2). The  $F_0$  component of the 12-NS spectrum was less affected by A $\beta$ (25-35) than was the  $B_0$  component, and in fact the relative line heights of these two components became reversed after A $\beta$ (25-35) treatment (Fig. 1B). Incubation of 5-NS labeled synaptosomes with A $\beta$ (25-35) induced a small decrease in the intensity of the  $M_I(0)$  5-NS line, but this effect was much less dramatic than that observed in the case of the 12-NS label (Fig. 2). The spectra of both 5-NS and 12-NS labeled synaptosomes remained unchanged throughout the time course of the



**Figure 1.** A: Spectrum of 5-NS (*top*) and 12-NS (*bottom*) incorporated into synaptosomal membranes. B: Scale expanded to show  $M_1(+1)$  and  $M_1(0)$  components of 12-NS spectra before (*top*) and after 30 minutes incubation (*bottom*) with Aβ(25-35), as described in the text.

**Figure 2.** Time course of membrane-bound spin label reduction induced by incubation with β-amyloid peptides, as described in the text. Treatments were: 5-NS + Aβ(25-35) (Δ, N=2); 5-NS + Aβ(35-25) (▽, N=2); 12-NS + Aβ(25-35) (○, N=3-4); 12-NS + Aβ(35-25) (□, N=2).

experiment in samples incubated without peptide; observed line heights fluctuated less than  $\pm 3\%$  over 30 minutes.

As a control to investigate the relationship between peptide radical structure and reactivity, spin labeled synaptosomes were treated with Aβ(35-25) peptide, the non-neurotoxic reverse sequence analogue of Aβ(25-35). Aβ(35-25) was much less reactive than Aβ(25-35) with regard to both spin labels (Fig. 2). Aβ(35-25) induced only a 10-15% decrease in 12-NS  $B_0$  signal intensity relative to untreated controls.

We compared the above-described reactivity of Aβ(25-35) with the pattern of spin label reduction exhibited by hydrogen peroxide, a commonly studied lipoperoxidation initiator. In samples treated with 3 mM  $H_2O_2$  [8-fold higher concentration than Aβ(25-35) used], the free 12-NS population, rather than the bound population, was reduced preferentially. Consistent with this observation, we found that membrane-bound 5-NS was much more susceptible to peroxide than was the 12-NS isomer, and in the 5-NS case  $H_2O_2$  was more destructive to the spin probe than was Aβ(25-35) (data not shown). Our  $H_2O_2$  data corroborate previous reports that hydroxyl radicals generated external to the membrane bilayer cannot effectively permeate the lipid leaflet, and therefore preferentially reduce nitroxides localized near the lipid/aqueous interface (2).

## DISCUSSION

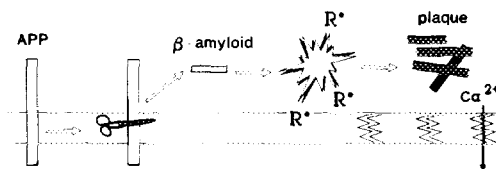
We have previously reported that Aβs spontaneously fragment in aqueous solution in a metal-independent reaction characterized by the production of reactive peptide free radicals and

ROS (1). A $\beta$ (25-35) is particularly reactive and may be a major breakdown product of A $\beta$ (1-40). A $\beta$ (25-35) is extremely toxic to certain brain enzymes; however, the reverse [A $\beta$ (35-25)] and scrambled [A $\beta$ (25-35)scram] sequences, which also generate EPR-detectable radicals, are much less cytotoxic (1).

The observations reported in the current study indicate that A $\beta$ -derived species can initiate neuronal lipoperoxidation in a sequence-specific manner reminiscent of the previously reported enzyme inactivation data (1). A $\beta$ (25-35), known to be highly toxic to hippocampal cultures and cytosolic enzyme extracts, appears most capable of reducing membrane-bound nitroxides. 400  $\mu$ M A $\beta$ (25-35) produces a deep, intramembranous lipid effect not observed near the lipid/water interface. The non-neurotoxic A $\beta$ (35-25) isomer, however, is much less competent as a lipoperoxidation initiator. Therefore, we conclude that the presence of a detectable peptide radical may be a necessary but not sufficient criterion for A $\beta$  toxicity. Our results suggest that the sequence-dependent structure of the peptide is also a crucial determinant of the peptide radical reactivity. The importance of a putative structure-activity relationship has been noted by other researchers who have reported a correlation between  $\beta$ -sheet content and peptide neurotoxicity (8). We propose that peptide structure could influence toxicity either directly, by affecting the kinetic reactivity of the peptide radical center(s), or indirectly, by targeting the insertion of peptide radical(s) into cell membranes. These two possibilities are not mutually exclusive.

A $\beta$ (25-35) preferentially induces reduction of 12-NS over the 5-NS isomer. This result may be due to the abundance of lipid double bonds deep within the membrane, which favors propagation of a lipoperoxidation chain. Alternatively, it could reflect the location of the peptide radical center. We have suggested methionine-35-sulfoperoxyl radical as a likely candidate for this center (1). Insertion of A $\beta$ (25-35) into the lipid leaflet parallel to the lipid chains, with the amino terminus facing the extrasynaptosomal medium and the carboxyl terminus deep within the bilayer [as previously modeled by Kang et al. (9)], could conceivably position the putative methionine sulfoperoxyl radical in favorable proximity to react with the 12-NS nitroxide, but not with the 5-NS probe. The capacity of A $\beta$ (25-35) to selectively initiate deep intramembranous peroxidation distinguishes this peptide as unique among commonly studied radical generators (e.g., H<sub>2</sub>O<sub>2</sub>).

Based on our observations (1, and this work), and on previously published data, we hypothesize the following "molecular shrapnel" model for A $\beta$  toxicity (Fig. 3), with obvious implications for the etiology of AD. Initial perturbation of proteolytic or other pathways encourages cleavage of amyloid precursor protein and release of A $\beta$  peptides [particularly A $\beta$ (1-40)] into the extracellular space of neuronal tissue. We propose that A $\beta$ s then fragment to form smaller, toxic oligopeptide radicals, which act as molecular "shrapnel". These radicals can attack



**Figure 3.** "Molecular shrapnel" model of  $\beta$ -amyloid neurotoxicity.

nearby cell membranes, initiating lipoperoxidation and damaging sensitive membrane proteins. The membrane barrier function is compromised, precipitating loss of ion homeostasis and resulting  $\text{Ca}^{2+}$  influx. Neurodegeneration follows, possibly expedited by stimulation of  $\text{Ca}^{2+}$ -dependent pathways. Also, radical-induced damage to critical glutamate transporters could explain the  $\text{A}\beta$  potentiation of glutamate excitotoxicity observed by some researchers (10). Explicit tests of this model, with potential applications to AD, are currently underway in our laboratory.

The results presented in this paper suggest that  $\text{A}\beta$  aggregation and neurotoxicity depend upon the presence of a radical center as well as a structural component. Therefore, compounds which either trap  $\text{A}\beta$  radicals, modulate  $\text{A}\beta$  structure, or inhibit lipoperoxidation deep within the lipid bilayer, might be promising candidates for the therapeutic interdiction of AD. Studies to address these issues are currently in progress.

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