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Review: Alzheimer's Amyloid β-Peptide-Associated Free Radical Oxidative Stress and Neurotoxicity

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Alzheimer's disease, the major dementing disorder of the elderly that affects over 4 million Americans, is related to amyloid β-peptide, the principal component of senile plaques in Alzheimer's disease brain. Oxidative stress, manifested by protein oxidation and lipid peroxidation, among other alterations, is a characteristic of Alzheimer's disease brain. Our laboratory united these two observations in a model to account for neurodegeneration in Alzheimer's disease brain, the amyloid β-peptide-associated oxidative stress model for neurotoxicity in Alzheimer's disease. Under this model, the aggregated peptide, perhaps in concert with bound redox metal ions, initiates free radical processes resulting in protein oxidation, lipid peroxidation, reactive oxygen species formation, cellular dysfunction leading to calcium ion accumulation, and subsequent neuronal death. Free radical antioxidants abrogate these findings. This review outlines the substantial evidence from multiidisciplinary approaches for amyloid β-peptide-associated free radical oxidative stress and neurotoxicity and protection against these oxidative processes and cell death by free radical scavengers. In addition, we review the strong evidence supporting the notion that the single methionine residue of amyloid β-peptide is vital to the oxidative stress and neurotoxicological properties of this peptide. Further, we discuss studies that support the hypothesis that aggregated soluble amyloid β-peptide and not fibrils per se are necessary for oxidative stress and neurotoxicity associated with amyloid β-peptide. © 2000 Academic Press

Key Words: **Alzheimer's disease; electron paramagnetic resonance (EPR); free radicals; fibrils; lipid peroxidation; neurotoxicity; protein oxidation; reactive oxygen species (ROS); spin-trapping.**

1. INTRODUCTION

Amyloid β-peptide (Aβ), in the form of insoluble fibril deposits, is the major component of the senile plaques (SP) that characterize Alzheimer's disease (AD) brain. This observation, in the mid-1980s (Glenner and Wong, 1984; Masters *et al.*, 1985), led to the hypothesis that deposition of amyloid is an early step in the pathogenesis of AD (Masters *et al.*, 1985; Hardy and Higgins, 1992; Masters and Beyreuther, 1993; Selkoe, 1989) and is in some way associated with the neurodegeneration in AD. This hypothesis gained further credence upon the observations that SP are surrounded by degenerated neurons (Katzman and Saitoh, 1991) and that Aβ peptides are toxic to neurons in culture (Yankner *et al.*, 1989; Frautschy *et al.*, 1991; Kowall *et al.*, 1991; Pike *et al.*, 1991; Howlett *et al.*, 1995; Harris *et al.*, 1995a; Aksenov *et al.*, 1995, 1996, 1998a; Yatin *et al.*, 1999a,b,c,d; Varadarajan *et al.*, 1999). Genetic studies of early-onset familial AD (FAD) offer the strongest evidence for a central role of Aβ in the pathogenesis of the disease (Selkoe, 1996). Several FAD mutations have been found in the amyloid precursor protein (APP) and presenilin genes; these mutations invariably lead to increased Aβ deposition (Selkoe, 1996; Scheuner *et al.*, 1996). APP is expressed on chromosome 21 as is Down's trisomy, and persons with Down's syndrome have increased Aβ deposits (Teller *et al.*, 1996) and develop AD eventually. APP-overexpressing mice exhibit some characteristics of AD pathology (Games *et al.*, 1995; Hsiao *et al.*, 1996; Hsiao, 1998; Masliah *et al.*, 1996; Irizarry *et al.*, 1997; Sturchler-Pierrat *et al.*, 1997; Calhoun *et al.*, 1998; Frautschy *et al.*, 1998a; Pappolla *et al.*, 1998; Smith *et al.*, 1998).

The AD brain is subjected to increased oxidative stress resulting from free radical damage (Markesbery, 1997; Markesbery and Carney, 1999; Butter-

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field, 1996, 1997), and the resulting cellular dysfunctions are widely believed to be responsible for neuronal degeneration in this disorder. Considerable evidence supports this view. For example, increased oxidation of proteins (Hensley *et al.*, 1995a; Smith *et al.*, 1991; Lyras *et al.*, 1997) and DNA (Markesbery and Carney, 1999; Mecocci *et al.*, 1993, 1994; Lyras *et al.*, 1997; Gabbita *et al.*, 1998; Hirai *et al.*, 1998) is reported in AD. Decreased levels of polyunsaturated fatty acids (Pettigrew *et al.*, 1988; Nitsch *et al.*, 1992; Svennerholm and Gottfries, 1994; Prasad *et al.*, 1998) coupled with increased lipid peroxidation (Subbarao *et al.*, 1990; Hajimohammadreza and Brammer, 1990; Marcus *et al.*, 1998; McIntosh *et al.*, 1997; Lovell *et al.*, 1995), increased levels of 4-hydroxynonenal (HNE), a toxic product of lipid peroxidation (Lovell *et al.*, 1997; Markesbery and Lovell, 1998), and increased levels of isoprostanes, which are products of free radical-induced oxidation of arachidonic acid (Montine *et al.*, 1998; Roberts *et al.*, 1998), are found in AD brain. Widespread peroxynitrite-induced nitration of tyrosine residues is reported (Smith *et al.*, 1997). There is also evidence for the presence of advanced glycation end-products (AGE), which are formed by oxidation of moieties resulting from the reaction of proteins with carbohydrates, in AD (Smith *et al.*, 1994, 1995; Vitek *et al.*, 1994). Several reviews are available that describe the mounting evidence that establish the elevated oxidative stress levels in AD (Butterfield, 1996, 1997, Butterfield, 1999a, 1999b; Markesbery, 1997; Markesbery and Carney, 1999; Behl, 1999; Retz *et al.*, 1998).

The sites in the AD brain where neurodegeneration occurs and where oxidative stress exists are associated with increased Aβ deposits (Hensley *et al.*, 1995a). Based on these observations and our extensive studies of oxidative stress and neurotoxicity associated with Aβ, we proposed the Aβ-associated oxidative stress model of neurodegeneration in AD (Butterfield *et al.*, 1994; Butterfield, 1997). According to this model, the Aβ peptide, along with other moieties, is directly responsible for free-radical damage to neuronal membrane systems, leading to subsequent neuronal loss in the AD brain. The mechanism by which the amyloid peptides exert toxicity is as yet unknown, but we have suggested, and others have confirmed, that free radicals are associated with Aβ toxicity. Evidence for Aβ-associated free radical formation and Aβ-associated free radical damage to biological membranes is enumerated below. The importance of the single methionine residue of Aβ and the relevance of fibril formation to Aβ toxicity are also discussed in this review.

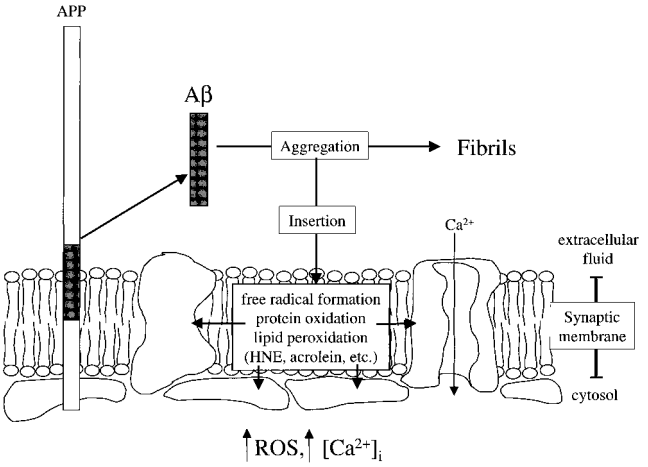


FIG. 1. Model for Aβ-associated free radical oxidative stress in Alzheimer's disease. Aβ-initiated free radicals (ROS) react rapidly with several moieties in the plasma membrane and cause membrane protein oxidation and lipid peroxidation. Toxic products of lipid peroxidation, such as HNE and acrolein, having longer half-lives than free radicals, migrate to different parts of the neuron causing multiple deleterious alterations of cellular function, especially sharp increases in intracellular Ca^{2+}, ultimately leading to neuronal death. This free radical "shrapnel" process could account for multiple alterations of the structure and function enzymatic and transport proteins and lipids in AD, and lipid peroxidation, resulting from a chain reaction process and resulting in reactive aldehydes, provides an "amplification" of an initial free radical event. See text.

2. Aβ-ASSOCIATED FREE RADICAL MODEL FOR OXIDATIVE STRESS IN AD

One of the confounding features of AD is the wide range of modifications in cellular functions that have been observed. Alterations in inflammatory response, membrane enzymes, transport proteins, structural and cytoskeletal proteins, lipids, mitochondrial function, Ca^{2+} homeostasis, etc., have been documented in AD (reviewed in Selkoe, 1991, 1994; Corain *et al.*, 1993; Katzman and Saitoh, 1991; Markesbery, 1997; Markesbery and Carney, 1999; Butterfield, 1997, 1999a, 1999b, 1999c). One way to account for the myriad of changes detected in AD is to invoke a free radical process, in which any protein or lipid moiety attacked by a free radical would have altered structure and function. Considering the centrality of Aβ to AD and the oxidative stress that the AD brain is under, our laboratory developed a model for neuronal death involving Aβ-associated free radical oxidative stress (Fig. 1) (Butterfield *et al.*, 1994; Butterfield, 1997).

According to the model, the APP-derived amyloid β-peptide, probably as a small, soluble aggregate, inserts into the neuronal and glial membrane bilayer and generates oxygen-dependent (and possibly redox metal ion-dependent) free radicals that then

cause lipid peroxidation and protein oxidation. Membrane damage results, either directly due to Aβ-associated free radicals, possibly involving peptide-bound redox metal ions, or indirectly by the action of lipid free radicals or the lipid peroxidation products HNE and acrolein (2-propenal). Loss of membrane integrity leads to cellular dysfunction, such as inhibition of ion-motive ATPases, loss of Ca^{2+} homeostasis, inhibition of glial cell Na^{+}-dependent glutamate uptake system with consequences on neuronal excitatory NMDA receptors, loss of protein transporter function, disruption of signaling pathways, and activation of nuclear transcription factors and apoptotic pathways. Neuronal death is the ultimate consequence of these cellular dysfunctions.

One advantage of this model is that it unifies the seemingly disconnected pathological characteristics of AD into a coherent theoretical framework. The "shower" of amyloid-initiated, highly reactive free radicals can account for the wide range of observed neuronal functional impairment. The secondary toxic products of such free radical attack on membrane systems, such as HNE or acrolein, though less reactive than radicals, with half-lives ranging from minutes to hours and which can therefore diffuse from their site of origin to cause damage at more distant sites, are strongly nucleophilic, reacting easily with cysteine, histidine, or lysine residues on proteins or amino groups on lipids. Further, this model provides an "amplification" process to the original free radical initiation by repeated chain reaction processes in lipids, producing HNE, acrolein, and numerous other reactive aldehyes. This model is consistent with the age dependence of AD. Younger people with greater antioxidant capacity (Smith *et al.*, 1991, 1992; Carney *et al.*, 1991; Starke-Reed and Oliver, 1989) are capable of withstanding the oxidative stress caused by the amyloid-associated free radicals. Since antioxidant mechanisms are compromised with increasing age and other environmental insults (Butterfield *et al.*, 1997b; Butterfield and Stadtman, 1997), the damage caused by free radicals accumulates and could account for, in part, the various membrane and cellular alterations reported in AD. Genetic or environmental factors that contribute to decreased antioxidant status or to altered binding to chaperon proteins, such as apoE4 (Soto *et al.*, 1996; Corder *et al.*, 1993), might also predispose AD patients to these processes.

This model of Aβ free radical-based neurotoxicity in AD, supported by numerous lines of evidence, also lends itself to a molecular rationale for possible therapeutic strategies in AD that involves the administration of appropriate brain accessible free radical scavengers. This prediction has been borne out by numerous studies in several laboratories. For example, vitamin E (Subramaniam *et al.*, 1998; Yatin *et al.*, 1999a, 2000a; Koppal *et al.*, 1998; Behl and Holsboer, 1998; Harris *et al.*, 1996; Butterfield *et al.*, 1999c), propyl gallate (Harris *et al.*, 1995a), EUK-8 (Bruce *et al.*, 1996), and other antioxidants (Pappolla *et al.*, 1998; Daniels *et al.*, 1998; Gridley *et al.*, 1997) significantly modulate oxidative stress properties and neurotoxicity to brain cells (Markesbery, 1997; Markesbery and Carney, 1999; Butterfield, 1997, 1999a, 1999b, 1999c). Further, high-dose vitamin E treatment is reported to slow the progress of AD (Sano *et al.*, 1997; Grundman, 2000). This model has been rigorously tested in synaptosomal membranes, neuronal and astrocytic cell cultures, control and AD brain, and in *in vivo* studies. These studies and others that support this model are outlined below.

3. ROLE OF AMYLOID FIBRILS IN Aβ TOXICITY

Aβ is a normal product of APP processing (Estus *et al.*, 1992; Golde *et al.*, 1992; Haass *et al.*, 1992) and is a normal soluble component of the plasma and the cerebrospinal fluid (Seubert *et al.*, 1992; Busciglio *et al.*, 1993). The observation of amyloid deposits in the SPs in essentially all cases of AD led to the hypothesis that conversion of soluble Aβ into insoluble fibrils is critical for the onset of the disease. This hypothesis is supported by the fact that fresh Aβ is nontoxic to cultured neurons, while aged Aβ (incubated to form amyloid fibrils) becomes toxic (Yankner *et al.*, 1989; Frautschy *et al.*, 1991; Kowall *et al.*, 1991; Pike *et al.*, 1991; Howlett *et al.*, 1995). Numerous efforts have been made to understand and inhibit fibril formation (Walsh *et al.*, 1999; Tjernberg *et al.*, 1999; Findeis *et al.*, 1999; Ray *et al.*, 1998; Hughes *et al.*, 1998), and prevention of fibril formation has led to the abrogation of toxicity in some cases (Lorenzo and Yankner, 1994).

However, the hypothesis of an *absolute* requirement of fibril formation for toxicity has been challenged (Davis and Chisholm, 1997; Hardy, 1997). Deposition of amyloid does not correlate with dementia (Terry *et al.*, 1991; Arriagada *et al.*, 1992; Roses, 1994; Samuel *et al.*, 1994; Braak and Braak, 1996). Furthermore, amyloid deposits have been found in the brains of nondemented individuals (Davis *et al.*, 1999), and transgenic mice overexpressing Aβ develop amyloid deposits but do not show neuronal loss (Geula *et al.*, 1998). Recently, soluble oligomers of Aβ, termed protofibrils, that are β-sheet intermediates in the development of mature fibrils, have been shown to be toxic to cultured neurons. Additionally, studies have shown that Aβ interacts with proteins such as glutamine synthetase (GS), apolipoprotein J (apoJ, clusterin), α-1-antichy-

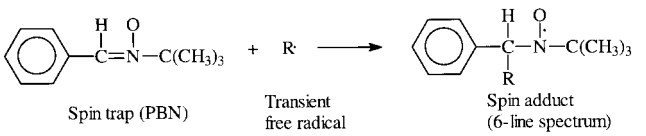
motrypsin, or thrombin, to yield increased Aβ-induced hippocampal neurotoxicity in the absence of fibrils (Aksenov *et al.*, 1995, 1996; Oda *et al.*, 1995; Smith-Swintosky *et al.*, 1995; Lambert *et al.*, 1998). Other proteins can inhibit fibril formation, but not affect the toxicity of Aβ (Aksenova *et al.*, 1996; Yatin *et al.*, 1999c). In contrast, incubation of Aβ(1-42) with vitamin E or replacement of the methionine residue of Aβ(1-42) with norleucine (see below) results in systems that exhibit no neurotoxicity; nevertheless, these systems form fibrils essentially indistinguishable from native Aβ(1-42) (Varadarajan *et al.*, 2000a; Yatin *et al.*, 2000a). These results discussed above, though not inconsistent with the neurotoxic properties of fibrillar Aβ, are inconsistent with the hypothesis of an *absolute* requirement for fibril formation before Aβ toxicity can be displayed.

4. EVIDENCE OF Aβ ASSOCIATION WITH FREE RADICALS

The model for Aβ-associated oxidative stress and neurodegeneration in AD brain (Fig. 1) is based on the generation of free radicals by Aβ, perhaps in concert with redox metal ions. The hypothesis that Aβ could be a source of free radical damage in *in vitro* systems and, by extension, in AD brain was systematically examined. The electron paramagnetic resonance (EPR) technique of spin trapping, among other methods, was used to detect transient Aβ-associated free radicals.

4.1. Spin Trapping

The most direct way of detecting transient, reactive free radicals is by the EPR technique of spin trapping. In EPR spin trapping studies, a nonparamagnetic molecule acting as a trap reacts with a transient free radical (R•) to form a relatively stable paramagnetic adduct (spin adduct) that can be detected by EPR.



This magnetic resonance technique is extremely sensitive, with detection limits close to that of fluorescence, and has the advantage over optical methods in biological systems in that opaque samples can be used (Janzen, 1980; Butterfield, 1982).

In most spin-trapping experiments, *N-tert*-butyl-α-phenylnitrone (PBN) is used as the spin trap. The reaction of an oxygen- or a carbon-centered free radical with PBN normally produces a free radical spin adduct that exhibits a 6-line EPR spectrum (Fig.

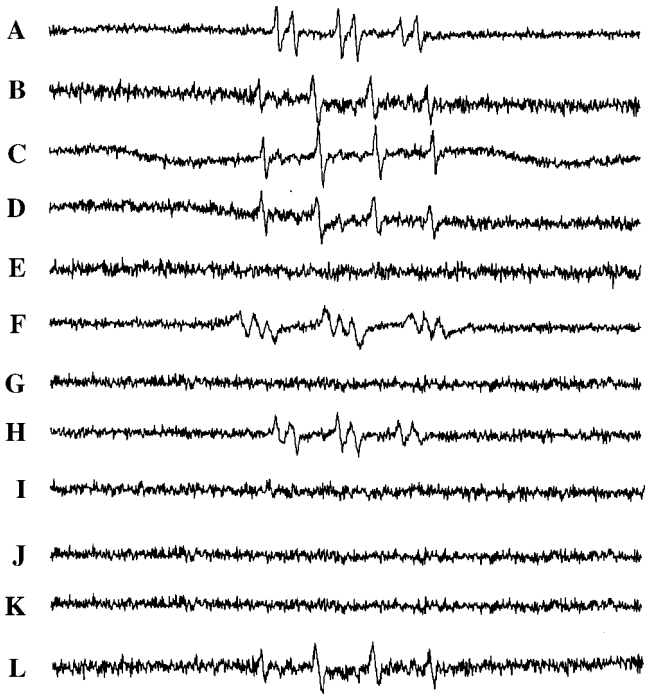


FIG. 2. EPR spectra obtained in various spin trapping experiments with ultrapure PBN (50 mM) upon incubation at 37°C for varying times. (A) Usual 6-line EPR spectrum of a free radical spin adduct of PBN with a C- or an O-centered free radical. Here is shown the $^{\bullet}OH$ adduct of PBN. (B) Aβ(1-42) (165 μM) with PBN (50 mM) after 60 h at 37°C in chelexed PBS containing deferroxamine (2 mM). (C) Aβ(1-40) (250 μM) with PBN (50 mM) after 48 h at 37°C in chelexed PBS containing deferroxamine (2 mM). (D) Aβ(25-35) (1 mM) with PBN (50 mM) after 24 h at 37°C in chelexed PBS containing deferroxamine (2 mM). (E) Control PBN solution (50 mM) plus deferroxamine (2 mM), lacking peptide, after 60 h at 37°C. Note the absence of a spectrum. (F) Aβ(25-35) (1 mM) in deuterated buffer (PBS prepared in D_2O) with PBN (50 mM) and deferroxamine (2 mM) after a 24-h incubation at 37°C. Note the 9-line spectrum. (G) Control PBN solution (50 mM) lacking peptide, in deuterated buffer containing deferroxamine (2 mM) after a 24-h incubation at 37°C. (H) Control PBN solution (50 mM) lacking peptide, in deuterated buffer containing deferroxamine (2 mM) after a 4-day incubation at 37°C. Note that the spectrum has 6 lines, not 9. (I) PBN solution (50 mM) in chelexed PBS containing 1 μM $FeCl_3$ incubated in the absence of peptide at 37°C for 24 h. Note the absence of a spectrum. (J) Aβ(1-42)Met35Nle (165 μM) with PBN (50 mM) after 60 h at 37°C in chelexed PBS containing deferroxamine (2 mM). Note the absence of a spectrum in this peptide in which methionine has been replaced by norleucine. (K) Aβ(1-40)Met35Nle (250 μM) with PBN (50 mM) after 48 h at 37°C in chelexed PBS containing deferroxamine (2 mM). A result similar to that obtained with Aβ(1-42)Met35Nle was found. (L) Aβ(1-42)His6,13,14Tyr (165 μM) with PBN (50 mM) after 60 h at 37°C in chelexed PBS containing deferroxamine (2 mM). Note the spectrum similar to that of native Aβ(1-42) (B). Instrumental parameters were as follows: microwave power 20 mW; modulation amplitude = 0.3 − 1 G; gain = 1×10^5; conversion time = 10.28 ms. Only the neurotoxic peptides Aβ(1-42), Aβ(1-40), Aβ(25-35), and Aβ(1-42)His6,13,14Tyr yield a 4-line spectrum. See text.

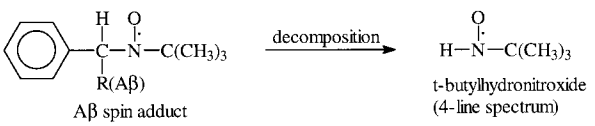
2A), resulting from the hyperfine coupling of the magnetic moment of the unpaired electron of the nitroxide spin adduct with the magnetic moments of the nitrogen nucleus ($I = 1$) and the α-hydrogen atom ($I = 1/2$). The magnitude of the splitting due to the H atom depends on the size and nature of the adduct (Beuttner, 1987; Butterfield, 1982; Janzen, 1980), and therefore, insight into the microenvironment near the paramagnetic center of the free radical can be obtained in favorable cases. If, as in our studies, the highly purified spin trap PBN is itself unable to generate an EPR signal under experimental conditions, then an EPR spectrum in the presence of a radical generator is *prima fascia* evidence for the presence of a free radical.

In our spin-trapping studies, buffers are prepared over Chelex 100, beads that bind redox metal ions, and essentially all our spin-trapping studies employ deferroxamine, a redox metal ion chelator. It has been reported that Fe^{3+} (at concentrations much higher than those found in Chelex- or deferroxamine-treated buffers) can catalyze the decomposition of PBN with subsequent formation of *N-tert*-butyl hydronitroxide (Chamulitrat *et al.*, 1995). This could result in a 3-line EPR spectrum. Additionally, *N-tert*-butylhydroxyl amine, a potential trace contaminant in PBN preparations, can yield a 4-line EPR spectrum upon oxidation by higher valence state redox metal ions like Fe^{3+} (Dikalov *et al.*, 1999). PBN synthesized in our laboratory is rigorously purified by repeated recrystallizations and sublimations to ensure its purity, and PBN purity was verified by NMR, EPR, and HPLC analyses. Further, addition of 1 μM Fe^{3+} to our PBN preparations containing deferroxamine did not lead to an EPR spectrum within the time frame required to generate spectra with Aβ peptides (Varadarajan *et al.*, 1999); i.e., Fe^{3+}, at a concentration that approximates trace amounts of redox metal ions that may be bound to Aβ, did not cause a decomposition of PBN with subsequent formation of EPR spectra in the time frame of the experiments. Additionally, higher concentrations of Fe^{3+} (10 μM) in the presence of 2 mM deferroxamine did not yield any spectrum with PBN. In the absence of deferroxamine, 10 μM Fe^{3+} can cause decomposition of PBN, but all our studies were conducted in the presence of this chelator. This result shows that that in our experiments, deferroxamine is effective in preventing formation of EPR spectra due to Fe^{3+}-induced breakdown of PBN and subsequent oxidation of breakdown products or impurities.

4.2. Spin Trapping Studies of Aβ Peptides

Incubation of neurotoxic Aβ peptides, viz., Aβ(1-42), Aβ(1-40), and Aβ(25-35), with PBN in metal ion-chelated, oxygenated buffers leads to the formation of EPR-detectable nitroxides (Figs. 2B, 2C, and 2D, respectively) (Butterfield, 1997; Harris *et al.*, 1995b; Hensley *et al.*, 1995b,c; Yatin *et al.*, 1999b; Varadarajan *et al.*, 1999, 2000b). These EPR spectra are not observed in the absence of the peptide (Fig. 2E), suggesting that the peptides are the likely source of the free radicals generated. Our spin-trapping results were recently confirmed in other laboratories that reported a 4-line EPR spectrum with Aβ(1-42) and PBN (Huang *et al.*, 1999b) and a weak 4-line EPR spectrum with Aβ(1-40) or Aβ(25-35) with PBN (Allsop, personal communication, 2000). One report could not confirm an EPR spectrum of the spin adduct of Aβ(25-35) and PBN (Dikalov *et al.*, 1999; see below), and this latter report suggests that redox metal ions are solely responsible for the EPR signal generation with PBN. Our experiments suggest that Aβ, perhaps in concert with redox metal ions, is responsible for the observed neurotoxicity and free radical generation. One possible explanation as to why Dikalov and co-workers did not observe an EPR spectrum with Aβ(25-35) is perhaps the length of incubation of the peptide with PBN (only 6 h). When highly purified PBN is used as the spin trap, our experience is that the generation of EPR spectra requires at least a 12-h incubation for Aβ(25-35), and longer for Aβ(1-40) and Aβ(1-42), with PBN. Tomiyama *et al.* (1996), in their study of the prevention of Aβ(1-40) neurotoxicity and fibril formation by rifampicin, reported a 3-line EPR spectrum of Aβ(1-40) with PBN. We too, in our earlier studies, had observed a 3-line EPR spectrum with Aβ peptides (Hensley *et al.*, 1994b), but have been unable to observe this spectrum in subsequent experiments utilizing ultrapure PBN (Varadarajan *et al.*, 1999).

The spectra observed with the Aβ peptides were not the expected 6-line spectra (Fig. 2A) seen with normal O- or C-centered PBN free radical adducts (Butterfield, 1982; Janzen, 1980). Instead, a 4-line spectrum was observed in most cases (Fig. 2), suggesting an unusual behavior for the Aβ-derived radicals. The highly reactive Aβ-associated free radicals cause the decomposition of the PBN spin trap, leading to the formation of a product that exhibits a 4-line EPR spectrum.



This peptide radical-initiated decomposition of the spin trap was confirmed by spin-trapping experi-

ments with Aβ(25-35) using [^{13}C]PBN with the labeled carbon in the α-position. Hydroxyl radicals, formed by Fe^{3+}/H_2O_2 and trapped by [^{13}C]PBN, yielded the expected 12-line spectrum showing hyperfine coupling of the α-carbon (Hensley *et al.*, 1995c). Aβ(25-35), a truncated form of Aβ(1-42), mimics the neurotoxic and EPR properties of the full-length peptide, albeit in a much shorter time (Hensley *et al.*, 1994b). In contrast to hydroxyl free radical trapped by [^{13}C]PBN, the 4-line EPR spectrum observed using [^{13}C]PBN and Aβ(25-35) was no different from that obtained when unlabeled PBN was used. This result suggests that the peptide-associated radical decomposes the spin trap. Had the spin trap remained intact, further splitting of the 4-line spectra due to the labeled carbon would have been observed.

The 4-line producing species was identified to be *tert*-butylhydronitroxide by comparing the observed EPR spectrum with that of the authentic compound. Further confirmation that the 4-line EPR spectrum producing species is a hydronitroxide was obtained by performing the experiments in D_2O, with the deuterium–hydrogen exchange resulting in the predicted 9-line EPR spectrum (Fig. 2F) (Hensley *et al.*, 1995c; Varadarajan *et al.*, 1999). No signal was observed in the control PBN solution in D_2O (Fig. 2G) within the time frame of the experiment; however, a weak 6-line spectrum was generated after incubation for 4 days at 37°C (Fig. 2H). This result also confirms that the 4-line EPR signal from the peptide/PBN reaction in H_2O is the *tert*-butylhydronitroxide spectrum. This signal is likely associated with the peptide, perhaps in concert with redox metal ions, and likely not with a potential contaminant in the spin trap (Dikalov *et al.*, 1999); had a trace impurity in the PBN led to the *tert*-butyl hydronitroxide formation independent of the peptide, then prolonged incubation of the PBN solution in D_2O would have resulted in a 9-line EPR spectrum and not the observed 6-line spectrum. It is conceivable that some impurity (other than redox metal ions) could be associated with the peptide, and this impurity is responsible for the EPR spectra. Several observations argue against this possibility. Other studies showed that the batch-to-batch variation in properties of synthetic Aβ peptides is not due to impurities in the peptide (Simmons *et al.*, 1994). As shown below, when methionine is replaced by norleucine in Aβ(1-42), Aβ(1-40), or Aβ(25-35), a simple replacement of the S atom in methionine by a CH_2 group, no oxidative stress, no neurotoxicity, and no EPR spectra in the presence of PBN are observed. One might imagine that had an impurity been present in these methionine-substituted peptides, which are prepared by the same peptide supplier as their toxic counterparts, one would have seen an EPR spectrum, yet one does not.

The Aβ-associated EPR spectrum is abolished if solutions of the peptide and spin trap are sparged with nitrogen in order to eliminate the presence of oxygen. Subsequent reoxygenation of these solutions results in the formation of the 4-line spectrum. Superoxide dismutase (SOD) did not affect the Aβ-PBN EPR spectrum, whereas the EPR signal was completely abolished in the presence of catalase (Harris *et al.*, 1995b). A peroxyl free radical was suggested based on the use of a sensitive colorimetric assay specific for peroxyl species (Butterfield *et al.*, 1996b). Other laboratories have subsequently confirmed that catalase will mitigate Aβ-induced free radical damage to cells (Puttfarcken *et al.*, 1996; Manelli and Puttfarcken, 1995).

The mechanism of Aβ-associated free radical formation is not yet clear. The full-length Aβ peptides possess a Cu^{2+}-binding domain (Atwood *et al.*, 1998), and Aβ(1-42) can reduce the bound Cu^{2+} to Cu^{+} (Huang *et al.*, 1999b; Varadarajan *et al.*, 2000b). The resultant Aβ(1-42)-associated Cu^{+} was reported to lead to H_2O_2 production, i.e., oxidative stress (Huang *et al.*, 1999a). This transfer of a single electron from the peptide to the metal would result in the formation of a peptidyl free radical, which is one possible explanation for the formation of Aβ radicals. However, it was reported that the truncated peptide, Aβ(25-35), was incapable of reducing Cu^{2+}, suggesting that a different mechanism is responsible for its toxicity. Mechanistic experiments have established that the methionine at residue 35 is critical to the free radical and neurotoxic properties of the Aβ peptides (see below).

Trace levels of redox metal ion contaminants in the synthetic Aβ peptides were suggested to be responsible for the oxidation of hydroxylamine impurities in PBN to the corresponding nitroxides to account for the signals detected (Fig. 2) (Dikalov *et al.*, 1999). While this is certainly possible (and may explain the formation of the nonreproducible 3-line spectra observed earlier with Aβ peptides and PBN), the spin-trapping results presented in this paper and elsewhere (Varadarajan *et al.*, 1999) strongly suggest that this is not the case in our studies, since the iron chelator deferroxamine was used in all our experiments, and addition of 1–10 μM Fe^{3+} to PBN solutions containing deferroxamine did not produce any EPR signals (Fig. 2I). This result certainly does not rule out the possibility that redox metal ion contamination in the peptide sample may be responsible for the breakdown of the spin trap. However, as noted above, modified Aβ peptides, lacking the methionine residue (see Figs. 2J and 2K) (Varadarajan *et al.*, 1999; Yatin *et al.*, 1999b), presumably synthe-

sized in the same way as the parent peptides that generated EPR signals and therefore containing the same potential trace metal contaminants as native Aβ peptides, in the presence of PBN and putative hydroxylamine impurities, do not produce any EPR signals within the time frame of the experiment. If the EPR signals observed with unmodified Aβ peptides were due to the reaction of redox metal ions with PBN and/or other impurities, then the modified Aβ peptides lacking methionine should also have yielded the same 4-line spectrum, but they do not.

Our spin-trapping studies with Aβ(1-42) are usually conducted for 2–3 days, a time frame in which a 4-line EPR spectrum of Aβ(1-42) appears, but methionine-substituted peptides do not yield EPR spectra with PBN. We have not pursued studies of peptides for longer periods of incubation. In all our experiments, prolonged incubation (4 days or longer) of control PBN solutions (lacking peptide) at 37°C (well beyond the experimental time used for trapping Aβ-associated free radicals) invariably eventually resulted in a 6-line spectrum (similar to Fig. 2H), and not a 4-line spectrum, suggesting that the mechanism of formation of this spectrum is different from the one that is operative in the presence of Aβ peptides. As noted above, this 6-line spectrum was observed even in deuterated water, where no deuterium effect was observed, suggesting that this 6-line spectrum, detected long after Aβ/PBN signals appeared, is possibly due to a breakdown product of PBN, resulting in a C- or O-centered radical being trapped by PBN.

Different batches of Aβ peptides from different sources have been reported to have different toxicity (May *et al.*, 1992; Simmons *et al.*, 1994) and produce EPR detectable signal at different times (Hensley *et al.*, 1995b). The reason for this variation in batch-to-batch properties of Aβ is not clear, but may involve conformational or aggregation differences (May *et al.*, 1992; Simmons *et al.*, 1994). Though trace impurities were shown not to be responsible for this variation (Simmons *et al.*, 1994), it is possible that batch-to-batch variation of redox metal ion impurities in synthetic Aβ peptides may be responsible for the variation in toxicity. As noted, other laboratories have confirmed the 4-line EPR signal of Aβ and PBN (Allsop, personal communication, 2000; Huang *et al.*, 1999b).

4.3. Other Evidence for Aβ Free Radical Generation in Solution

Numerous other experiments have provided direct and indirect evidence for the involvement of free radicals in Aβ-associated oxidative stress. For example, salicylate was used to trap Aβ-derived free radicals in the form of dihydroxybenzoic acids (Hensley *et al.*, 1994b). Creatine kinase (CK) and GS are oxidatively sensitive enzymes (Smith *et al.*, 1992; Oliver *et al.*, 1987) whose activity is decreased in the AD brain (Hensley *et al.*, 1995b). Both these enzymes were deactivated by incubation with Aβ (Harris *et al.*, 1995a; Hensley *et al.*, 1994b; Aksenov *et al.*, 1997; Yatin *et al.*, 1999a). A potential role for redox metal ions associated with Aβ cannot be ruled out in these studies. A number of free radical antioxidants protect against Aβ-induced reactive oxygen species (ROS) formation, protein oxidation, and neurotoxicity (Behl *et al.*, 1992; Behl *et al.*, 1994; Bruce *et al.*, 1996; Daniels *et al.*, 1998; Goodman *et al.*, 1994; Gridley *et al.*, 1997; Harris *et al.*, 1995a, 1996; Koppal *et al.*, 1998; Mark *et al.*, 1997a,b; Yatin *et al.*, 1999a,d). Aβ induces hydrogen peroxide formation in clonal cell lines, and the H_2O_2-degrading enzyme catalase protects cells from Aβ toxicity and free radical production (Behl *et al.*, 1994). Further, in solutions containing both Aβ and nitroxide free radicals, the signal due to the nitroxide species was diminished, indicating that a peptide-associated free radical reacts with the free radical on the nitroxide (Butterfield *et al.*, 1994, 1996b; Koppal *et al.*, 1998; Bruce-Keller *et al.*, 1998a). These observations provide evidence for the involvement of ROS and oxidative damage in the neurotoxic properties of Aβ. Although these findings may indicate an indirect stimulation of ROS formation by Aβ, the observations are consistent with the notion of Aβ being a free radical prooxidant.

5. Aβ-ASSOCIATED OXIDATIVE STRESS

Oxidative stress reflects a situation wherein reactive oxygen species, such as free radicals and their products, are in excess of the antioxidant defense systems. The link between the amyloid deposits and oxidative stress in AD brain is not readily apparent due to the fact that studies of postmortem tissue cannot reveal whether these deposits are the by-products of neurodegeneration or precede the degenerative process. However, there is increasing evidence that shows that Aβ itself is associated with oxidative stress. Several markers of excess oxidative stress, such as an increase in ROS, accumulation of oxidized products such as protein carbonyls from protein oxidation and aldehydes and isoprostanes from lipid peroxidation, serve to establish the direct role of Aβ in the oxidative damage associated with AD. This section of the review describes the oxidative stress caused by Aβ in biological systems.

5.1. Oxidation of Membrane Proteins

One of the predictions of the model for Aβ-associated free radical oxidative stress-induced neuronal

death in AD brain (Fig. 1) is that Aβ will cause membrane protein oxidation. The resulting protein damage may be manifested in the form of physical, chemical, or functional changes.

Changes in the physical or functional state of proteins can be detected by the sensitive EPR technique of spin labeling utilizing protein-specific spin labels, e.g., 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl (MAL-6) (Butterfield, 1982). The motion of the spin label, covalently bound to sulfhydryl sites on the protein, is either relatively free or highly restricted, depending on the site of protein attachment. This is reflected in the EPR spectrum as weakly immobilized (W) or strongly immobilized (S) components. The ratio of the EPR signal amplitude of the low-field resonance line in the two populations of immobilized spin label, the W/S ratio, is an indirect measure of protein oxidation. The W/S ratio is highly sensitive to oxidative modifications of proteins and has been shown to decrease relative to controls in several models of oxidative stress ranging from hydroxyl free radical generation, to models of stroke, hyperoxia, ischemia, aging, sepsis, Huntington's disease, and peroxynitrite damage (Howard *et al.*, 1996; Hensley *et al.*, 1994a; Hall *et al.*, 1995a,b,c, 1997; Butterfield *et al.*, 1997b; Bellary *et al.*, 1995; Koppal *et al.*, 1999a; La Fontaine *et al.*, 2000).

Chemical changes in membrane proteins, manifested in the form of increased protein carbonyls, an index of protein oxidation (Butterfield and Stadtman, 1997; Stadtman, 1992), are detected using UV–Vis spectroscopy, immunochemistry, and histofluorescence methods. Kinetic studies measure oxidatively induced changes in enzyme function (Smith *et al.*, 1991; Hensley *et al.*, 1994b; Butterfield *et al.*, 1997a). Each of these methods was used to demonstrate protein oxidation in Aβ-treated brain samples.

Synaptosomal membranes obtained from Aβ-rich hippocampus and inferior parietal lobule regions of AD brains following the University of Kentucky rapid autopsy protocol (2–4 h postmortem interval) showed decreased W/S ratios of MAL-6 relative to Aβ-poor cerebellum and relative to all areas of similarly obtained control brains (Hensley *et al.*, 1995a). Synaptosomal membranes isolated from rodent brain and treated with Aβ resulted in a decreased W/S ratio of MAL-6, and, consistent with Aβ-induced protein oxidation, the antioxidant vitamin E protected rodent synaptosomal membranes treated with Aβ against the decrease in the W/S ratio of MAL-6 (Subramaniam *et al.*, 1998; Butterfield, 1997; Butterfield *et al.*, 1999c).

Cortical or hippocampal synaptosomes isolated from AD brain or from rodent brain or rodent cultured hippocampal neurons incubated with Aβ(1-42), Aβ(1-40), or Aβ(25-35) have increased protein oxidation (Fig. 3) (Aksenov *et al.*, 1998a; Aksenova *et al.*, 1999; Butterfield, 1997; Hensley *et al.*, 1995a; Smith *et al.*, 1991, 1996; Subramaniam *et al.*, 1998; Harris *et al.*, 1995a; Yatin *et al.*, 1999a,b,d,e), and this oxidation is significantly reduced by the antioxidants propylgallate or vitamin E (Butterfield, 1997; Butterfield *et al.*, 1999c; Subramaniam *et al.*, 1998; Harris *et al.*, 1995a, 1996; Yatin *et al.*, 1999a,b,d, 2000a). Nontoxic reverse peptides did not cause protein oxidation. Maximum protein oxidation occurred at the time when, in separate spin-trapping experiments, Aβ gave the most intense EPR spectrum (Harris *et al.*, 1995a; Yatin *et al.*, 1999e). Other studies have confirmed protein oxidation assessed by protein carbonyl formation following Aβ treatment (Frautschy *et al.*, 1998b).

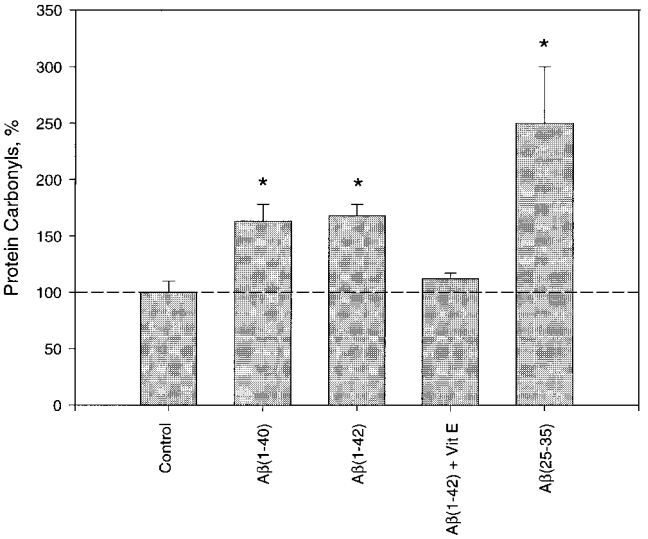


FIG. 3. Protein oxidation induced by Aβ peptides as assessed by measurement of protein carbonyls. The figure represents results from several studies using immunochemical methods. Results are presented as an average of two to three different trials. $*P < 0.006$. Error bars represent SD values. The neurotoxic Aβ(1-40), Aβ(1-42), and Aβ(25-35) all significantly increased protein carbonyl levels over control values. See text.

Free radical oxidation of proteins may damage enzymes critical to neuronal function (Stadtman, 1992). GS and CK are two enzymes that are particularly sensitive to oxidative modification. The function of GS is to convert glutamate to glutamine, thereby lessening the opportunity for glutamate-activated NMDA receptor-mediated excitotoxicity. CK plays a central role in energy transfer in cells with high energy requirements. A significant decline in GS and CK activity has been observed in AD brain (Hensley *et al.*, 1995a; Smith *et al.*, 1991; Burbaeva *et al.*, 1992). Aβ(1-42), Aβ(1-40), or Aβ(25-35) signif-

icantly decreased GS activity in cytosolic factions of mammalian brain homogenates (Aksenov *et al.*, 1995, 1996, 1997; Butterfield *et al.*, 1997a; Hensley *et al.*, 1994b; Varadarajan *et al.*, 2000b) and in cultured hippocampal neurons and astrocytes (Harris *et al.*, 1995a, 1996). Using purified GS, in the absence of any cellular components, GS was oxidatively inhibited by Aβ and protein carbonyls were incorporated in the enzyme (Aksenov *et al.*, 1997), suggesting that Aβ-associated oxidative stress occurs independent of cellular processes. As expected, the free radical spin trap, sulfonated PBN, blocked the effects of Aβ on GS (Aksenov *et al.*, 1997). Also, in sheep brain GS oxidized by Aβ, the rate of uptake of the protein-specific spin label 1-oxyl-2,2,5,5,-tetramethyl-delta^3-pyrroline-3-methyl)methanethiosulfonate (MTS) into GS sulfhydryl groups was markedly reduced compared to control GS (Butterfield *et al.*, 1997a); similar results were found in purified GS isolated from AD and control brain (Butterfield *et al.*, 1997a). CK activity is also inhibited by Aβ (Hensley *et al.*, 1994b; Yatin *et al.*, 1999a), and this loss of activity is blocked by vitamin E (Yatin *et al.*, 1999a), further supporting the concept of Aβ-associated free radical oxidative stress.

The decline in CK activity in AD brain may be related to altered endogenous antioxidant levels (Maret, 1995; Maret *et al.*, 1999). Under oxidative stress the level of intracellular glutathione decreases, and in AD brain the activity of glutathione *S*-transferase is diminished (Lovell *et al.*, 1998). This latter enzyme also protects neurons from damaging effects of the lipid peroxidation product, HNE (Xie *et al.*, 1998). Lymphoblasts from familial AD that carry PS-1 or APP mutations are reported to have decreased glutathione levels (Cecchi *et al.*, 1999). Decreased glutathione makes neurons more prone to damage in ischemia reperfusion (Hall *et al.*, 1997) and in models of Down's syndrome (Schuchmann and Heinemann, 2000). In contrast, elevated glutathione protects synaptosomal membranes from oxidative damage due to peroxynitrite (Koppal *et al.*, 1999a,b) and hydroxyl free radicals (Pocernich *et al.*, 2000). Under oxidative stress conditions, a shift of glutathione redox balance causes release of Zn^{2+} from metallothionein (MT) (Maret, 1995). Zn^{2+} is reported to accelerate Aβ aggregation (Huang *et al.*, 1997); however, the apo MT (thionein) is able to activate certain enzymes that contain Zn^{2+} at an inhibitory site. It is possible that thionein could also bind Zn^{2+} from other sources, offering protection against Aβ aggregation. Thus, a decline in cellular CK, especially near the synapse that is under extensive oxidative damage in AD brain and thus in need of energy, would lead to depletion of ATP levels, which could affect the levels of GSH. This, in turn, could cause the release of Zn^{2+} from MT, which could affect Aβ aggregation. This conceivably could be an area of fruitful study in AD research.

Peroxynitrite, a powerful oxidant that is formed by the reaction of relatively less potent ROS nitric oxide and superoxide, can oxidize membrane lipids, proteins, and DNA and, in addition, can generate the highly reactive hydroxyl radical. Peroxynitrite causes the nitration of tyrosine residues yielding nitrotyrosine, which is used as an indicator of peroxynitrite damage (Butterfield and Stadtman, 1997). Nitrotyrosine has been found in NFT in the hippocampus in AD (Good *et al.*, 1996; Smith *et al.*, 1997). Nitrotyrosine immunoreactivity has also been found in non-NFT-bearing neurons and in nuclei of glia in AD (Smith *et al.*, 1997). Peroxynitrite also led to protein oxidation in cortical synaptosomal membranes, an effect that was blocked by the pretreatment of the synaptosomal membranes with glutathione (Koppal *et al.*, 1999a). *In vivo* reduction of glutathione levels in rodent brain by the intraperitoneal injection of cyclohexen-1-one led to significantly greater peroxynitrite-induced protein oxidation, whereas *in vivo* elevation of glutathione levels by *N*-acetylcysteine provided partial protection of synaptosomal membranes from peroxynitrite-induced protein oxidation (Koppal *et al.*, 1999b). Peroxynitrite is formed by the reaction of NO with superoxide radical anion (Estevez *et al.*, 1995). Aβ is reported to stimulate i-NOS, from which NO is derived, and mitochondrial dysfunction is reported in AD (Beal, 1998), which could increase superoxide levels. The Aβ-associated oxidative stress model for neurodegeneration in AD brain is consistent with these observations (Fig. 1) (Butterfield, 1997).

5.2. Oxidation of Membrane Lipids

A key prediction of the Aβ-associated free radical model for neurotoxicity in AD brain (Fig. 1) is that Aβ induces lipid peroxidation. Membrane bilayer resident phospholipid unsaturated fatty acids (PUFAs) are especially vulnerable to free radical attack. Free radical H atom abstraction from unsaturated fatty acid chains and the subsequent immediate reaction of the C-centered radicals with molecular oxygen result in the formation of lipid peroxyl radicals or hydroperoxides (Halliwell and Gutteridge, 1989). Lipid peroxidation can lead to changes in the membrane fluidity, formation of conjugated dienes, HNE, acrolein, and isoprostanes, the release of free fatty acids, and a consequent decrease in levels of PUFA, etc. Several such markers are commonly used to index free radical attack on phospholipids (Esterbauer *et al.*, 1991; Mathews *et al.*, 1997; Morrow and Roberts, 1997; Tsai *et al.*, 1998; Borchman *et al.*, 1996; Akiba *et al.*, 1997). Increased

free fatty acid release and elevated levels of HNE, acrolein, isoprostanes and conjugated dienes are reported in AD (Prasad *et al.*, 1998; Lovell *et al.*, 1995; Lovell and Markesbery, 1998; Markesbery and Lovell, 1998; Montine *et al.*, 1998; Marcus *et al.*, 1998).

EPR in conjunction with lipid-specific nitroxyl stearate spin labels, such as 5-NS and 12-NS, were used to examine changes in lipid bilayer order and motion (fluidity) and to monitor free radical-induced loss of spin label paramagnetism. Cortical synaptosomal membranes treated with Aβ(25-35) and incubated with the NS spin labels exhibited a large reduction in signal intensity of the EPR spectrum, indicating loss of paramagnetism of the spin label (Butterfield *et al.*, 1994, 1996a; Koppal *et al.*, 1998; Bruce-Keller *et al.*, 1998a). The paramagnetic nitroxide moiety of the 12-NS is located deep in the lipid bilayer, near the most common sites of unsaturation, i.e., near the sites of lipid radical formation. In addition, nonpolar oxygen, which is required for Aβ free radical generation (Hensley *et al.*, 1994b) and for the formation of lipid peroxides, is highly soluble deep in the hydrophobic portion of biological membranes. Small-angle X-ray studies showing the insertion of Aβ into the lipid domain of membranes (Mason *et al.*, 1996), and electron microscopic immunolocalization of Aβ to the neuronal plasma membrane of cultured cells (Mattson *et al.*, 1993), confirm membranes as the target for Aβ damage. No loss of the intensities of 12-NS in synaptosomal membranes was observed with the nontoxic reverse peptide, Aβ(35-25) (Butterfield *et al.*, 1994). The antioxidant vitamin E inhibited the Aβ-induced loss of paramagnetism in cortical synaptosomal membranes (Koppal *et al.*, 1998). Also, PC-12 cells overexperessing Bcl-2, the gene product of which is thought to be an antioxidant (Hockenbery *et al.*, 1993), did not show Aβ-induced lipid peroxidation in contrast to nearly 50% loss in signal following Aβ addition to PC-12 control cells (Bruce-Keller *et al.*, 1998a).

Oxidation of PUFAs marks them for breakdown by phospholipases. Lipid peroxidation is increased in AD, and, as expected, PUFA levels, especially arachidonic acid and docosahexenoic acid which are more vulnerable to attack by ROS, are diminished (Prasad *et al.*, 1998). The decreases are significant in the case of the phospholipids that are rich in oxidizable arachidonic and docosahexenoic acids (phosphatidylethanolamine and phosphatidylinositol) and not so in the case of phosphatidylcholine, which contains lesser amounts of these fatty acids, suggesting that free radicals are responsible for the alterations in membrane phospholipids. Aβ(25-35) incubated with synaptosomal membranes stimulated the release of phospholipid resident fatty acids (Koppal *et al.*, 1998). The greatest release was for arachidonic acid, and this release was inhibited by pretreatment of the synaptosomal membranes with the free radical scavenger vitamin E. Conjugated dienes, a marker for lipid peroxidation, were significantly elevated in brain membranes following Aβ addition (Butterfield *et al.*, 1996a).

Free radical-induced oxidation of arachidonic acid results in the formation of isoprostanes, whose concentration has been shown to be a reproducible quantitative marker of lipid peroxidation *in vivo* (Morrow and Roberts, 1997). Oxidation of docosahexenoic acid leads to the formation of compounds called neuroprostanes (Roberts *et al.*, 1998). Concentrations of isoprostanes and neuroprostanes in the CSF of AD patients are significantly elevated compared to controls (Montine *et al.*, 1998; Roberts *et al.*, 1998). Aβ addition to rat hippocampal cultures leads to increased isoprostane levels (Mark *et al.*, 1999), confirming Aβ-induced lipid peroxidation (Butterfield *et al.*, 1994).

Oxidation of PUFAs also results in the formation of multiple aldehydes, of which HNE is one of the more reactive and more prevalent ones. HNE is a highly reactive α,β unsaturated aldehyde, capable of inhibiting DNA, RNA, and protein synthesis, inhibiting glycolysis, and modifying proteins (Esterbauer *et al.*, 1991). This alkenal forms covalent adducts with proteins through Micheal addition or by Schiff base reactions (Esterbauer *et al.*, 1991; Uchida and Stadtman, 1992). HNE levels are significantly elevated in multiple brain regions (Markesbery and Lovell, 1998) and in ventricular cerebrospinal fluid (Lovell *et al.*, 1997) in AD. The activity of glutathione *S*-transferase (GST), an enzyme that detoxifies HNE, is significantly lowered in AD (Lovell and Markesbery, 1998). This diminished activity of GST suggests a loss of protection against HNE in AD and could lead to increased deleterious consequences of oxidative stress. Exposure to Aβ causes a significant increase in free and protein-bound HNE in cultured rat hippocampal neurons when exposed to Aβ (Mark *et al.*, 1997a). HNE has been shown to induce apoptosis in PC12 cells and cultured rat hippocampal neurons, suggesting that this alkenal is a mediator of oxidative stress-induced apoptosis (Kruman *et al.*, 1997). The membrane-damaging effects of Aβ are also produced by HNE (Mark *et al.*, 1995, 1997a) and are discussed below.

Acrolein, the most reactive among the α,β-unsaturated aldehyde products of lipid peroxidation (Esterbauer *et al.*, 1991), can be rapidly incorporated into proteins (Uchida *et al.*, 1998a,b). Acrolein modification of proteins has been shown to occur in the neurofibrillary tangles in AD brains and not in con-

trol brains (Calingasan *et al.*, 1999). *In vitro,* acrolein modifies axonal cytoskeletal proteins (He *et al.*, 1995) and reacts rapidly with and depletes the antioxidant glutathione (Horton *et al.*, 1997). Both HNE (Subramaniam *et al.*, 1997) and acrolein (Butterfield, *vide infra*) significantly alter the conformation of synaptosomal membrane proteins as measured by EPR spin labeling methods.

Numerous other aldehydic products of lipid peroxidation, with different carbon chain lengths, such as malondialdehyde, propanal, butanal, pentanal, and hexanal, have been identified (Esterbauer *et al.*, 1991). These reactive aldehydes, including HNE and acrolein, with half-lives ranging from minutes to hours, can diffuse to sites distant from that of their origin and alter neuronal function. In addition to the direct effect of Aβ on membrane proteins and phospholipids leading to neuron death, secondary indirect mechanisms induced by Aβ, involving HNE, acrolein, and other products of lipid peroxidation, may play an important role in neuronal toxicity.

5.3. Carbohydrate Oxidation

Nonenzymatic glycation of proteins through the Maillard reaction, followed by subsequent Amadori chemistry results in the formation of AGE involving free radical intermediates (Munch *et al.*, 1997). Recent studies indicate a role for AGE in AD. AGE have been found in diffuse and neuritic SP in AD (Smith *et al.*, 1994; Vitek *et al.*, 1994). Aggregation of soluble nonfibrillar Aβ *in vitro* is accelerated by AGE-modified Aβ, suggesting that AGE may enhance SP formation *in vivo* (Vitek *et al.*, 1994). Fibrillar Aβ binds to RAGE, which is one of the cell receptors for AGE, and generates oxidative stress, activating NF-κB (Yan *et al.*, 1997). This study suggests that a free radical-dependent inflammatory pathway, triggered by interaction of Aβ on RAGE, may be present in AD. Modification of proteins by oxidation and glycooxidation and products of lipid peroxidation can occur in an additive and synergistic manner (Smith *et al.*, 1995).

5.4. DNA Oxidation

Oxidation of DNA can result in numerous modifications including strand breaks and base modifications (Davies, 1995). Oxidized DNA bases, particularly the adduct 8-hydroxy-2′-deoxyguanosine (8-OHdG), serve as markers of DNA oxidation (Gabbita *et al.*, 1998; Lovell *et al.*, 1999). A twofold increase in DNA strand breaks has been described in the brain in AD (Mullaart *et al.*, 1990). 8-OHdG levels are increased in AD (Gabbita *et al.*, 1998; Lovell *et al.*, 1999; Lyras *et al.*, 1997), and there is a threefold increase in mitochondrial DNA oxidation in the parietal lobe in AD subjects compared with normal controls (Mecocci *et al.*, 1994). The 5-kb deletion, the most common DNA alteration in human mitochondria, was prominent in large hippocampal pyramidal neurons in AD (Hirai *et al.*, 1998) and is believed to potentiate oxidative damage in vulnerable neurons. Oxidative DNA alterations in AD may be related to altered message for antioxidant enzymes (Aksenov *et al.*, 1998a, Aksenov *et al.*, 1998b, 1999; Davis *et al.*, 1997; Chandrasekaran *et al.*, 1997). Aβ can induce, directly or indirectly, the production of $O_2^{\bullet -}$ (Beal, 1998), peroxynitrite (Estevez *et al.*, 1995), H_2O_2 (Huang *et al.*, 1999a), and $^{\bullet}OH$ (via Fenton chemistry), all of which can cause DNA damage.

5.5. Production of Reactive Oxygen Species

Aβ has been shown to directly produce hydrogen peroxide through metal ion reduction (Huang *et al.*, 1999a). Further, Aβ induces protein oxidation and lipid peroxidation as discussed above. We reasoned that ROS should, therefore, be detectable using fluorescence methods (Harris *et al.*, 1995a, 1996; Yatin *et al.*, 1999a). The redox-sensitive neutral dye, 2′,7′-dichlorofluorescin diacetate, once transported into hippocampal neuronal or astrocytic cultures, is converted by esterases to anionic 2′,7′-dichlorofluorescin (DCF), which, following reaction with peroxyl radicals or hydrogen peroxide, is converted to fluorescent 2′,7′-dichlorofluorescein. Aβ led to fluorescence in neuronal (Harris *et al.*, 1995a; Yatin *et al.*, 1999a) and astrocytic (Harris *et al.*, 1996) cell cultures, indicating that Aβ-induced ROS production had occurred. In both cell types, fluorescence was inhibited by free radical scavengers (Harris *et al.*, 1995a, 1996; Yatin *et al.*, 1999a). Figure 4 shows that vitamin E blocks Aβ(1-42)-induced ROS formation in hippocampal neurons. Similar results, using immortalized PC12 cells, and the redox-sensitive dye MTT, which is converted by mitochondrial reductive processes to colored formazan, have been reported (Behl *et al.*, 1994). Treatment of cells with H_2O_2 or Aβ reduced this conversion, consistent with a more oxidizing intracellular environment. These results are consistent with the model of Aβ-associated free radical oxidative stress and neurotoxicity (Figs. 1 and 8), wherein Aβ triggers the formation of ROS.

5.6. Cellular Dysfunction

Aβ oxidatively modifies membrane protein and lipids as discussed above, and therefore, a prediction of the Aβ-associated free radical oxidative stress model for neurotoxicity (Fig. 1) in AD brain is that Aβ, either directly or through various ROS and lipid

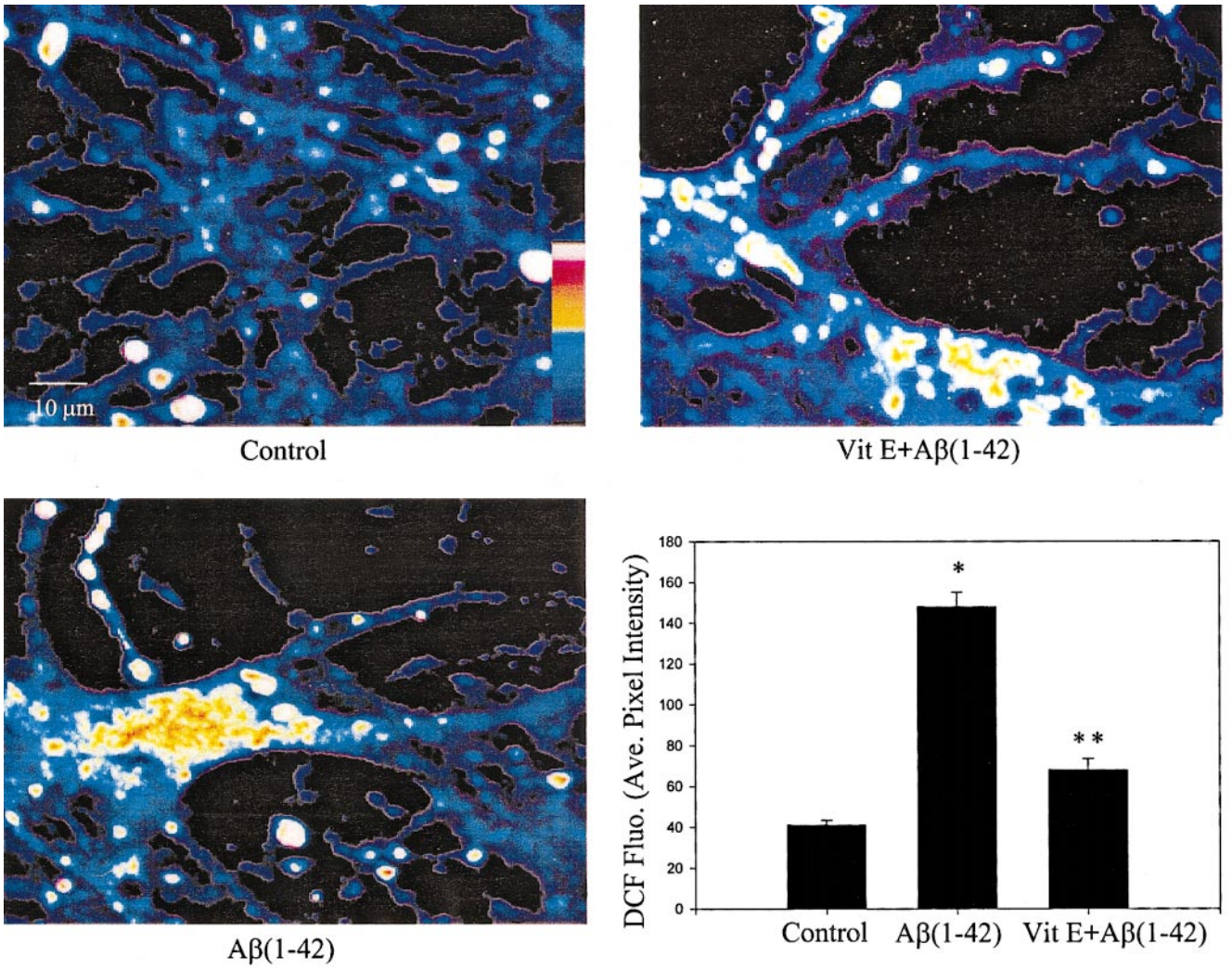


FIG. 4. Laser confocal fluorescence micrographs showing Aβ(1-42)-induced generation of ROS (free radicals) in cultured hippocampal neurons and their modulation by the free radical scavenger vitamin E. Increased yellow color represents more ROS. (Top left) Control hippocampal neurons with all reagents except Aβ(1-42) are added. (Bottom left) Aβ(1-42) added to hippocampal neurons containing the dye 2,7-dichlorofluorocin that is converted to fluorescent 2,7-dicholorfluoroscein by reaction with ROS results in a significant increase in fluorescence over control values. (Top right) Pretreatment of hippocampal neurons with vitamin E significantly modulates Aβ-induced ROS formation detected by the dye. (Bottom right) Digitization of the fluorescence (average pixel intensity) showing the results. Error bars represent SEM values. *$P < 0.001$ vs control, **$P < 0.005$ vs Aβ(1-42) ($n = 3$; each n is the average of 8–11 neurons).

peroxidation products produced, alters cellular function, leading to neurotoxicity. One locus where Aβ-initiated oxidative processes lead to cellular dysfunction is probably the plasma membrane.

Oxidative damage to the plasma membrane by Aβ appears to play a key role in the disruption of ion homeostasis. Aβ administration to rat hippocampal neurons causes impairment of Na^+/K^+-ATPase activity followed by elevation of intracellular Ca^{2+} levels and ultimately cell degeneration (Harris *et al.*, 1995a, 1996; Mattson *et al.*, 1993; Mark *et al.*, 1995). That the increase in levels of intracellular Ca^{2+} is secondary to loss of Na^+/K^+-ATPase activity was shown by the observation that manipulations that reduce Na^+ influx protected neurons (Mark *et al.*, 1995). Exposure of synaptosome preparations from neurologically normal human brains to Aβ caused a highly significant decrease in both Na^+/K^+-ATPase and Ca^{2+}-ATPase activities (Mark *et al.*, 1995), suggesting that this mechanism of Aβ toxicity is likely to occur in the human brain. The loss of Mg^{2+}-ATPase required longer times, while the Na^+/Ca^{2+}-exchanger was unaffected by Aβ peptides. HNE, too, is toxic to neurons and astrocytes in cultures (Subramaniam *et al.*, 1997, 1998; Mark *et al.*, 1997a; Montine *et al.*, 1996) and caused crosslinking of tau

into high-molecular-weight species. HNE, similar to Aβ, impairs Na^+/K^+-ATPase activity and disrupts calcium homeostasis in rat hippocampal neurons, which finally leads to neuron death (Mark *et al.*, 1997a). Alterations in ion-motive ATPases could affect the cell potential, thereby leading to the opening of voltage-gated Ca^{2+} channels with subsequent Ca^{2+} accumulation. Alterations in ion homeostasis, particularly Ca^{2+}, following Aβ free radical oxidative damage could have serious consequences on cell function, ranging from disruption of various signaling pathways and second messenger levels, to alterations in membrane cytoskeletal proteins following Ca^{2+}-activated proteolysis, to compromised mitochondrial function and loss of ATP, and/or to activation of endonucleases. Nuclear transcription factor activation and apoptotic processes are also Ca^{2+} sensitive. The Aβ-induced impairment of the ion-motive ATPase activities was blocked by antioxidants, suggesting that free radicals mediated the inhibition process (Mark *et al.*, 1995).

Aβ and HNE cause impairment of glucose and glutamate transport and mitochondrial function in rat neocortical synaptosomes and cultured neurons (Mark *et al.*, 1997b; Keller *et al.*, 1997; Harris *et al.*, 1995b, 1996). Both species also induced accumulation of mitochondrial reactive oxygen species and reduced cellular ATP levels significantly. Aβ-induced impairment of glucose and glutamate transport was inhibited by antioxidants, suggesting that free radicals are causally linked to this adverse action of Aβ (Keller *et al.*, 1997).

Normally glutamate is sequestered from neurons by the astrocyte-resident, Na^+-dependent glutamate transport system. Once transported to the astrocyte interior, glutamate is converted to glutamine by the oxidation-prone enzyme GS. Aβ-induced oxidative inhibition of glutamate transporters would result in excessive accumulation of extracellular glutamate and consequent sustained activation of excitotoxic glutamate receptors. As noted above, Aβ peptides also inhibit the activity of GS (Harris *et al.*, 1995a; Hensley *et al.*, 1994b; Butterfield *et al.*, 1997a; Aksenov *et al.*, 1997). Hydroxyl radicals inhibit both GS and the glutamate transporter (Volterra *et al.*, 1994; Stadtman, 1992). Others, using EPR, showed that glutamate stimulation of NMDA receptors led to intracellular free radicals (Lafon-Cazal *et al.*, 1993).

Aβ- or HNE-associated impairment of glucose transport would increase neuronal vulnerability to excitotoxicity by depleting ATP levels and compromising function of ion-motive ATPases. Energy deprivation causes mitochondrial dysfunction and depletion of ATP levels in rat hippocampal neurons (Mattson *et al.*, 1993). Impairment of glucose transport precedes ATP depletion in cultured rat cortical neurons exposed to Aβ (Mark *et al.*, 1997b), suggesting that depletion of ATP levels does not contribute to impairment of glucose transport induced by Aβ and HNE. These results suggest that the cumulative effects of Aβ and HNE on membrane transport systems and mitochondria in synaptosomes may play an important role in the energy failure in AD (Ogawa *et al.*, 1996; Swaab *et al.*, 1998). Since creatine phosphate is involved in cellular energy metabolism, inhibition of CK, known to occur in AD brain (Hensley *et al.*, 1995b) and by Aβ (Hensley *et al.*, 1994b; Yatin *et al.*, 1999a), may augment the effects of inhibition of the glucose transporter by Aβ or HNE on the decreased energy utilization in AD brain (Swaab *et al.*, 1998; Ogawa *et al.*, 1996; Munch *et al.*, 1998; Meier-Ruge *et al.*, 1997).

Aβ disrupts carbachol-stimulated, G-protein-facilitated signal transduction in cultured rat cortical neurons (Kelly *et al.*, 1996). This effect probably involves the "uncoupling" of the muscarinic receptors from the G-protein because ligand-binding studies suggested that Aβ did not interfere with the binding of carbachol to receptors (Kelly *et al.*, 1996). Experiments on postmortem brain tissue obtained from AD patients showed evidence of impaired coupling of muscarinic receptors to G-proteins (Pearce and Potter, 1991). Disruption of this cholinergic signaling pathway is probably free radical mediated since the antioxidant vitamin E attenuated this effect of Aβ (Kelly *et al.*, 1996). HNE added to rat forebrain damages cholinergic neurons and impairs visuospatial memory (Bruce-Keller *et al.*, 1998b). This finding may have relevance to loss of cholinergic neurons and memory dysfunction in AD. These results suggest that this defect may partially explain the relative ineffectiveness of cholinergic agents in ameliorating cognitive symptoms of AD.

Alterations in brain polyamine metabolism may be critical for neuron survival after free radical-initiated neurodegenerative processes (Bernstein and Muller, 1995). Treatment of rat embryonic hippocampal neuronal cultures with Aβ peptides increased ornithine decarboxylase activity and spermidine uptake, suggesting that oxidative stress upregulates the polyamine mechanism for the repair of free radical damage (Yatin *et al.*, 1999d). Pretreatment of the cells with vitamin E prior to Aβ exposure decreased ODC activity and spermidine uptake to control levels. Subsequent studies showed that spermine, in concert with Aβ(1-42), is especially toxic to neurons (Yatin *et al.*, 2000b). These polyamine results provide further evidence for the involvement of free radicals in Aβ-induced oxidative stress.

6. MODULATION OF Aβ-INDUCED OXIDATIVE STRESS BY FREE RADICAL SCAVENGERS

If an Aβ-associated free radical oxidative mechanism is operative in AD neurotoxicity, as predicted by the shrapnel model (Fig. 1), then free radical scavengers may provide protection against Aβ-derived membrane damage (Butterfield *et al.*, 1999c). Throughout this article, numerous references have been cited that provide evidence for such protection. Additionally rifampicin was effective in preventing the formation of the PBN-Aβ spin adduct (Tomiyama *et al.*, 1996). The semiquinone component of the drug was thought to trap the free radicals produced. Cocktails containing catalase are reported to protect neurons from Aβ damage (Puttfarcken *et al.*, 1996; Manelli and Puttfarcken, 1995). Other antioxidants ranging from new experimental antioxidants such as EUK-8 (Bruce *et al.*, 1996), U-83836E (Zhou *et al.*, 1996), and U-78517F (Kumar *et al.*, 1994) to the spin-trapping antioxidant compound PBN (Behl *et al.*, 1994) are reported to prevent Aβ-induced neurotoxicity. Other compounds with antioxidant capability, such as nordihydroguaiaretic acid (Goodman *et al.*, 1994) and estrogens (Goodman *et al.*, 1996), are reported to offer protection to neurons from Aβ toxicity. The antioxidants melatonin and vitamin E protected against Aβ-induced lipid peroxidation (Daniels *et al.*, 1998; Koppal *et al.*, 1998), but not against HNE-induced alterations (Subramaniam *et al.*, 1998; Mark *et al.*, 1997a; Keller *et al.*, 1997) as expected, since HNE is a product of free radical-induced lipid peroxidation and not a free radical itself. Lipid peroxidation stimulated by Aβ (Butterfield *et al.*, 1994) and blocked by antioxidants (Koppal *et al.*, 1998) has also been reported by others (Gridley *et al.*, 1997; Daniels *et al.*, 1998; Avdulov *et al.*, 1997; Mark *et al.*, 1997; Behl *et al.*, 1994).

A few papers have appeared that suggest that although Aβ is confirmed to cause lipid peroxidation that is blocked by free radical scavengers, antioxidants did not rescue cells from apoptosis and cell death (Yao *et al.*, 1999; Lockhart *et al.*, 1994; Pike *et al.*, 1997). However, methodological differences, such as the use of 1- to 3-day-old, still-developing cells, which may not have fully expressed receptors, such as the NMDA receptor and transport proteins, may account for the lack of antioxidant protection against neurotoxicity seen in these studies. While one must remain open-minded about Aβ and its effects on cell death, the numerous references cited above together with a large number of papers showing that antioxidants do protect neurons from Aβ-induced apoptosis and prevention of cell death (Butterfield, 1999a,b; Mook-Jung *et al.*, 1999; Chyan *et al.*, 1999; Stephenson *et al.*, 1999; Mark *et al.*, 1999; Calingasan *et al.*, 1999) put the great preponderance of evidence as supporting the notion that Aβ-induced lipid peroxidation and cell death are related.

~Asp[1]-Ala[2]-Glu[3]-Phe[4]-Arg[5]-His[6]-Asp[7]-Ser[8]-Gly[9]-Tyr[10]-Glu[11]-Val[12]-His[13]-His[14]-Gln[15]-Lys[16]-Leu[17]-Val[18]-Phe[19]-Phe[20]-Ala[21]-Glu[22]-Asp[23]-Val[24]-Gly[25]-Ser[26]-Asn[27]-Lys[28]-Gly[29]-Ala[30]-Ile[31]-Ile[32]-Gly[33]-Leu[34]-Met[35]-Val[36]-Gly[37]-Gly[38]-Val[39]-Val[40]-Ile[41]-Ala[42]

FIG. 5. Amino acid sequence of Aβ(1-42). The peptide contains a single methionine, residue 35, which is susceptible to oxidation.

7. IMPORTANCE OF METHIONINE TO Aβ-ASSOCIATED OXIDATIVE STRESS

The precise chemical mechanisms involved in Aβ-associated free radical ROS production and observed neurotoxicity are not yet known. A number of studies have focused on the role of Met35 in AD since it is the residue in Aβ most susceptible to oxidation *in vivo*, especially under conditions of oxidative stress (Vogt, 1995) (Fig. 5). Indeed, examination of senile plaque-resident Aβ(1-40) showed a high proportion of methionine sulfoxide present (Naslund *et al.*, 1994). Methionine, like other dialkyl sulfides, is known to participate in unusual free radical reaction chemistry (Schoneich *et al.*, 1994). In addition, oxidation of methionine residues in model peptides is known to significantly alter secondary structure (Dado and Gellman, 1994); namely, methionine oxidation to the sulfoxide leads to predominantly β-sheet conformation, which is the conformation adopted by toxic Aβ (Selkoe, 1994). This section describes our experiments that establish the critical role that methionine plays in Aβ-induced free radical oxidative stress.

7.1. Free Radical Production

As noted above and displayed in Fig. 2, the full-length amyloid peptides Aβ(1-40) and Aβ(1-42) produced a 4-line EPR spectrum with purified PBN, and in both cases the EPR spectrum was abolished upon replacement of the methionine with norleucine (Figs. 2J and 2K) (Yatin *et al.*, 1999b; Varadarajan *et al.*, 1999). This replacement is a simple substitution of the sulfur atom in the thioether of methionine by a CH_2 group. One or more of the three histidines of Aβ have been suggested to be part of a copper-binding domain, and the bound Cu^{2+} is suggested as being responsible for abstracting an electron from the amyloid peptide and generating a peptidyl radical (Huang *et al.*, 1999a,b). We observed that Aβ(1-42) with all three histidines replaced by tyrosine, which has a Cu^{2+}-binding affinity at least two or-

ders of magnitude lower (Martell and Smith, 1974), still produced a 4-line EPR spectrum with PBN (Fig. 2L) (Butterfield *et al.*, 1999b; Varadarajan *et al.*, 2000b). Cu^{2+} binding cannot be ruled out in the tyrosine substituted peptide. However, addition of Cu^{2+} to Aβ(1-42)Met35Nle-PBN mixtures did not yield a 4-line EPR signal; instead, a 6-line spectrum was observed (Butterfield *et al.*, 1999b; Varadarajan *et al.*, 2000b). Control PBN solutions, lacking peptide, also produced a 6-line spectrum with added Cu^{2+}, probably due to a Cu^{2+}-mediated breakdown of PBN and subsequent trapping of a C- or an O-centered radical such as a *tert*-butyl radical by the excess (50 mM) PBN present. The results suggest that the methionine residue of Aβ(1-42) and Aβ(1-40) is critical to the free radical generation. This suggestion was strengthened by studies with the truncated peptide, Aβ(25-35) (Varadarajan *et al.*, 1999). Incubation of the 11-amino-acid amyloid fragment Aβ(25-35), containing the methionine as the terminal residue, with PBN produces a strong 4-line EPR spectrum. As noted above, the 4-line-producing species has been identified to be *tert*-butyl hydronitroxide. There was no spectrum observed in the case of the truncated peptide Aβ(25-34) lacking the methionine residue (Varadarajan *et al.*, 1999). Similarly, Aβ(25-35) with the methionine residue replaced by either valine or structurally similar norleucine, also produced no EPR-detectable signal upon incubation with PBN (Varadarajan *et al.*, 1999). Similar to the cases of Aβ(1-42) and Aβ(1-40) (Yatin *et al.*, 1999b; Varadarajan *et al.*, 1999), Aβ(25-35) with methionine replaced by norleucine no longer caused protein oxidation in or toxicity to hippocampal neurons, in marked contrast to the unmodified peptides in each case (Varadarajan *et al.*, 1999; Yatin *et al.*, 1999b).

7.2. Neuronal Toxicity

Aβ(1-42), Aβ(1-40), and Aβ(25-35) are all toxic to cultured hippocampal neurons, as mentioned above (Yatin *et al.*, 1999a,b,c,d; Varadarajan *et al.*, 1999; Butterfield, 1997; Mattson *et al.*, 1997). Replacement of methionine by norleucine in all three peptides completely abrogates neuronal toxicity (Fig. 6) (Varadarajan *et al.*, 1999; Yatin *et al.*, 1999b). Replacement of the methionine with valine or removal of the methionine in Aβ(25-35) also abolished the peptide's toxicity to cultured neurons (Varadarajan *et al.*, 1999). Replacement of the three histidine residues by tyrosine did not affect the toxicity of the parent Aβ(1-42). These results suggest that if Cu^{2+} is important in Aβ(1-42)-induced neurotoxicity, it is so only because of the methionine. These toxicity results are consistent with the EPR results listed above and with the notion of the methionine residue being critical to a free radical process of Aβ toxicity to neurons.

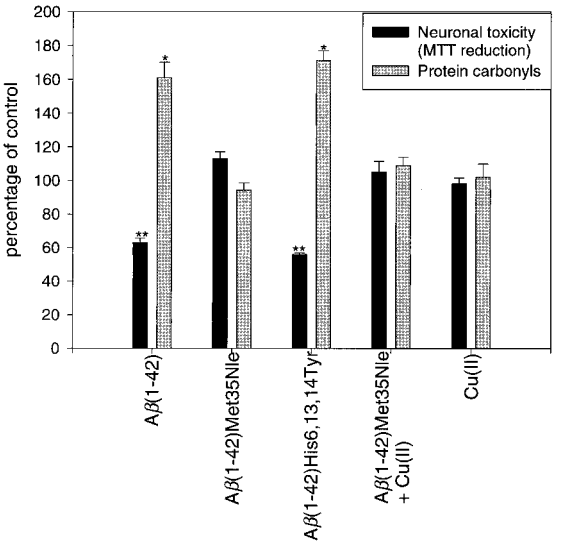


FIG. 6. Neurotoxicity and changes of protein carbonyl content in hippocampal neurons treated within the various Aβ peptides. Neurotoxicity was measured by the MTT assay. Neurons with impaired mitochondrial function are unable to reduce the MTT dye effectively relative to healthy cells. Neuronal toxicity was evaluated 24 h after addition of the peptides. Statistical comparisons were made using the Student's *t* test. Error bars represent SD values. $^{**}P < 0.002$ for Aβ(1-42) and Aβ(1-42)His6,13,14Tyr vs control ($n = 3$). None of the other peptides were significantly toxic to the neurons. Protein carbonyl levels are presented as an average of two to three different trials. For each trial, anti-DNP staining was performed three times and averaged. Statistical comparisons were made using the Student's *t* test. Error bars represent SD values. $^{*}P < 0.006$ for Aβ(1-42) and Aβ(1-42)His6,13,14Tyr vs controls. There was no significant increase in protein carbonyl content compared to control values for the other peptides. See text.

7.3. Membrane Protein Oxidation

Concomitant with the spin-trapping and neuronal toxicity studies cited above, addition of Aβ(1-42), Aβ(1-40), and Aβ(25-35) to neurons caused a significant increase in protein oxidation manifested by increased levels of protein carbonyls, whereas the carbonyl levels in hippocampal neurons treated with peptides having methionine substituted by norleucine, even with added Cu^{2+} (in the case of Aβ(1-42)), were no different from control values (Fig. 6) Varadarajan *et al.*, 1999; Yatin *et al.*, 1999b). Consistent with the EPR and neurotoxicity studies cited above, the extent of protein oxidation by the tyrosine substituted Aβ(1-42) was similar to that of the parent peptide, suggesting the importance of methionine in Aβ-associated free radical oxidative stress and neurotoxicity. These findings also suggest an essential requirement for methionine in any role of metal ion-mediated oxidative stress and cell death.

7.4. In Vivo Studies

If the results of *in vitro* studies of Aβ in neuronal and synaptosomal membranes suggesting that Aβ is associated with free radical oxidative stress and that the methionine residue of Aβ is important in this process are applicable for neurotoxicity in AD brain, then *in vivo* models in which Aβ(1-42) are expressed should show protein oxidation. Consistent with this prediction, *Caenorhabditis elegans* transgenic animals expressing human, full-length Aβ(1-42) showed strong evidence of protein oxidation (Yatin *et al.,* 1999b), a key marker of free radical oxidative stress. Mutation of Met-35 to Cys-35 resulted in healthy animals that showed no evidence of protein oxidation, consistent with the hypothesis that Met-35 is important in Aβ-associated free radical oxidative stress (Yatin *et al.,* 1999b). The totality and self-consistent nature of the findings suggest that methionine is critical to Aβ-associated neuronal protein oxidation and neurotoxicity and to the Aβ-associated EPR spectra observed. The results also suggest that redox metal ions, potentially involved in Aβ-associated oxidative stress properties, are important only because of the methionine residue. In the absence of methionine both *in vitro* and *in vivo* studies suggest that no oxidative stress occurs.

The results suggest that the methionine residue and Aβ-associated free radical oxidative stress are intimately linked. Numerous other reports confirm this view. Pike *et al.* (1995) reported that the C-terminal region of Aβ(25-35) was critical in its neurotoxicological properties and that modifications of the 33-35 region of the amyloid peptide led to a loss of peptide aggregation. Also in agreement with the potential importance of methionine in Aβ chemistry and pathology, Snyder *et al.* (1994) reported that synthetic Aβ(1-40) containing methionine sulfoxide in residue 35 formed fibrils at twice the rate of unmodified Aβ(1-40), and Naslund *et al.* (1994) found that SP resident Aβ(1-40) in AD brain is rich in methionine sulfoxide. Non-active-site-resident methionine residues in several enzymes protect the enzyme from oxidative insults and become converted to methionine sulfoxide in the process (Levine *et al.,* 1999). The enzyme methionine sulfoxide reductase in brain then reduces the oxidized methionine residues back to methionine (Levine *et al.,* 1999), acting as a neuroprotective enzyme. The activity of this reducing enzyme is lowered in AD (Gabbita *et al.,* 1999), consistent with an oxidative environment in AD brain.

Consistent with the notion that Aβ induces oxidative stress *in vivo,* injection of Aβ directly into rodent brain together with protease inhibitors produced protein oxidation and lipid peroxidation (Frautschy *et al.,* 1998b).

8. FIBRILS AND Aβ-ASSOCIATED OXIDATIVE STRESS

An important question concerning the protein chemistry of Aβ toxicity is whether it is the three-dimensional conformation of Aβ fibrils that is necessary for inducing neuron death or whether it is oxidative stress associated with the formation of fibrils (i.e., aggregation) that damages neurons. Soluble Aβ monomers in the AD brain probably aggregate to form oligomers or fibrils (Podlisny *et al.,* 1998), and soluble Aβ oligomers have been isolated from normal and AD brains (Kuo *et al.,* 1996). The levels of soluble Aβ were found to be greater in AD brain than in controls, and the proportion of soluble Aβ(1-42) was significantly increased over soluble Aβ(1-40) species in AD patients. Similar results have been obtained by Funato *et al.* (1998). Several recent studies have shown that low-molecular-weight oligomers of Aβ are neurotoxins. Walsh *et al.* (1999) reported that Aβ monomers and dimers are nontoxic, while low-molecular-weight oligomers, termed *protofibrils,* are toxic. One study has suggested that water-soluble dimeric species are also neurotoxic (Roher *et al.,* 1996). Lambert *et al.* (1998) found that small, low-molecular-weight oligomers of Aβ(1-42) are several orders of magnitude more potent neurotoxins than high-molecular-weight fibrillar species of Aβ(1-40). Interaction of Aβ(1-42) or Aβ(1-40) with different brain-resident proteins can lead to different results, but of importance to this discussion, GS or apoJ interaction with Aβ leads to soluble, aggregated, nonfibrillar peptides that are more toxic than Aβ in fibrillar form (Oda *et al.,* 1995; Aksenov *et al.,* 1996).

All the neurotoxic Aβ peptides that we investigated, viz. Aβ(1-42), Aβ(1-42)His6,13,14Tyr (Fig. 7), Aβ(1-40), and Aβ(25-35), form fibrillar structures upon incubation. The reverse sequence, Aβ(42-1), does not form fibrils under identical conditions, nor does the scrambled Aβ(25-35). That fibrils per se were not required for toxicity (Oda *et al.,* 1995; Aksenov *et al.,* 1996; Walsh *et al.,* 1999; Lambert *et al.,* 1998) was confirmed by studies with the nonneurotoxic, non-protein-oxidizing, and non-free-radical-forming Aβ(1-42)Met35Nle, which also forms fibrils (Fig. 7). It is therefore evident that the methionine residue is critical to the observed neurotoxicity, while fibril formation itself, per se, is not required for neurotoxicity. Consistent with this notion, vitamin E incubation with Aβ(1-42) blocks protein oxidation in and toxicity of hippocampal neurons (Yatin *et al.,* 2000a), but does not inhibit fibril formation (Varadarajan *et al.,* 2000a). Rather, small aggre-

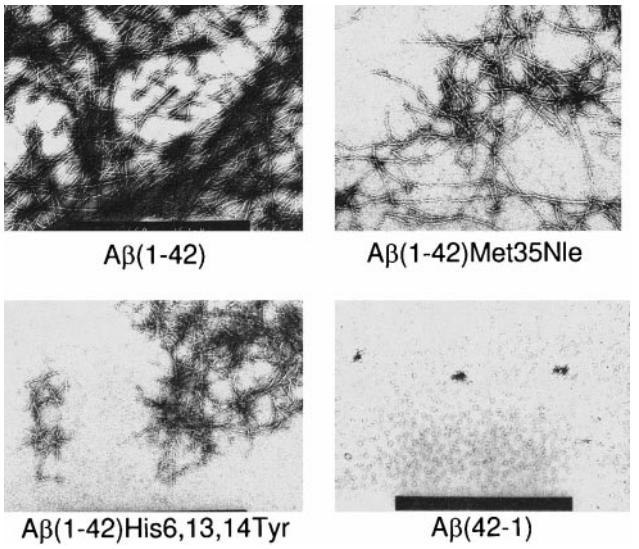


FIG. 7. Electron microscopy of Aβ peptides incubated at 37°C for 48 h. Neurotoxic and oxidative stress-producing Aβ(1-42) and Aβ(1-42)His 6,13,14Tyr and the nonneurotoxic and the nonoxidative stress-producing Aβ(1-42)Met35Nle form fibrils. The nonneurotoxic reverse sequence Aβ(42-1) does not form fibrils. See text.

gates are likely the toxic species of Aβ, and the inhibitory role of vitamin E likely stems from its antioxidant properties rather than blockage of Aβ fibril formation.

Numerous studies indicate a central role for Aβ in AD pathogenesis. None of these studies, however, definitively indicate the form or site of action of Aβ neurotoxicity. Until the mechanism of Aβ neurotoxicity is understood, it will be difficult to explain the topography of neurodegeneration (Small, 1998). Strong circumstantial evidence supports the notion that low-molecular-weight diffusible forms of Aβ may be important for neurotoxicity (Walsh *et al.*, 1999; Lambert *et al.*, 1998; Aksenov *et al.*, 1996; Oda *et al.*, 1995), and our results, confirmed in many laboratories as discussed above, suggest that oxidative stress, perhaps emanating from the methionine residue of Aβ, plays an important role in neurotoxicity in AD brain.

9. CONCLUSIONS

Given the centrality of Aβ to the pathogenesis of AD, and the significant oxidative stress present in AD brain, an Aβ-associated oxidative stress model for neurodegeneration in AD provides a framework that unites these observations. The aggregate of all the studies presented in this review overwhelmingly support, in our opinion, the notion of a pivotal role for Aβ-induced oxidative stress in AD (Fig. 8). In addition to its direct neurotoxic oxidative effects, perhaps with the involvement of redox metal ions, Aβ can potentiate the toxic effects of a variety of different neuronal insults including excitatory amino acids, glucose deprivation, energy depletion, and protein and lipid oxidation. Addition of Aβ to primary neuronal cultures results in inhibition of ion-motive ATPases, alteration of cell potential, and consequent influx of Ca^{2+}. Since Aβ also inhibits the Ca^{2+} pump, intracellular levels of Ca^{2+} attain deleterious levels, resulting in many destructive processes, such as proteolysis, breakdown of nuclear and mitochondrial DNA, and induction of apoptotic processes. Aβ can impair mitochondrial redox activity and increase the generation of free radicals such as superoxide. Aβ stimulates i-NOS, resulting in an increase in levels of nitric oxide. Fibrillar Aβ can trigger an inflammatory response, most likely by binding to RAGE receptors or by activation of microglia; the latter causes a respiratory burst resulting in the generation of nitric oxide and superoxide.

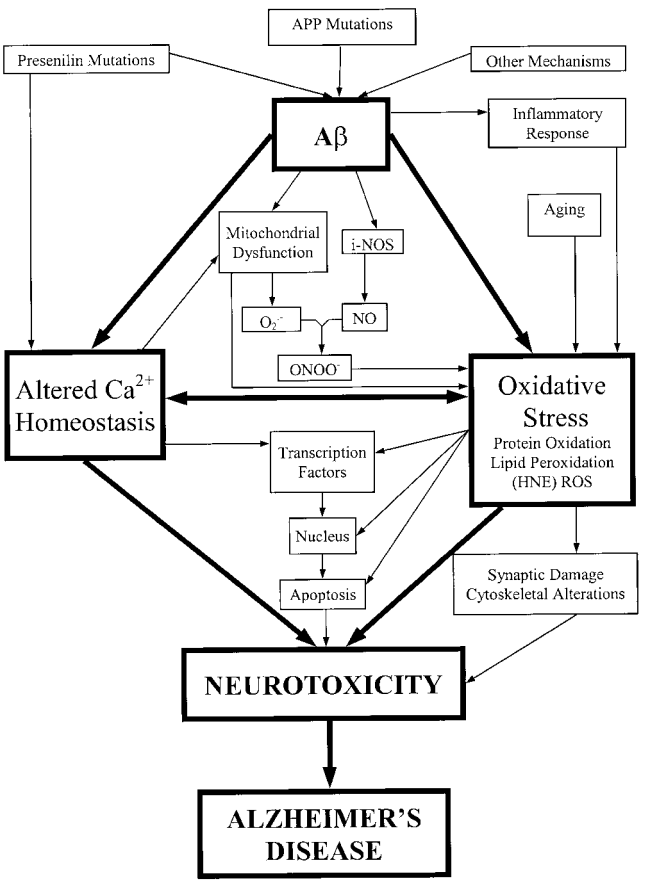


FIG. 8. Schematic diagram of Aβ-associated oxidative stress and neurotoxicity in Alzheimer's disease brain. This model is consistent with a central role of Aβ in AD pathogenesis and the extensive oxidative stress under which AD brain exists. The model unites much of the literature on Aβ-related research into a single theoretical framework. See text.

Nitric oxide combines with superoxide to form the highly toxic peroxynitrite. Aβ has also been shown to cause H_2O_2 accumulation, which could lead to the toxic hydroxyl radical formation via Fenton chemistry.

It is likely that AD is associated with multiple etiologies and pathogenic mechanisms. This review demonstrates that Aβ-associated free radicals and the resultant oxidative stress are part of the mechanism that is involved in the pathogenic cascade that leads to neurodegeneration in AD brain. The prevention of several of the Aβ-associated deleterious effects by free radical antioxidants strengthens the notion of free radical involvement in Aβ toxicity and suggests the potential usefulness of brain-accessible free radical antioxidants or elevating levels of endogenous antioxidants as therapeutic strategies for AD.

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