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Aβ(25-35) PEPTIDE DISPLAYS H_2O_2-LIKE REACTIVITY TOWARDS AQUEOUS FE^{2+}, NITROXIDE SPIN PROBES, AND SYNAPTOSOMAL MEMBRANE PROTEINS

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Summary

Amyloid beta peptides (Aβs) are found in abnormally high accumulations in brains of persons with Alzheimer's disease, and are believed to contribute to cognitive decline in this disorder. Synthetic Aβ and its peptide fragment 25-35 [Aβ(25-35)] are toxic to cells in culture; however, the exact mechanism of amyloid peptide toxicity is not known. An emerging hypothesis contends that Aβ toxicity results from peptide-mediated free radical reactions and generation of reactive oxygen species [Hensley, et al., Proc. Natl. Acad. Sci. USA 19: 3270-3274 (1994); Harris, et al., Exp. Neurol. 131: 193-202 (1995)]. Recently, we reported that reactivity of Aβ toward the oxidation-sensitive enzyme glutamine synthetase is related to the peptide's reactivity toward the spin trap phenyl-*tert*-butyl nitrone (PBN) [Hensley, et al., Neuroreport 6: 489-492 (1995)]. Neuronal damage may be due, in part, to oxidative processes initiated by amyloid-derived free radicals species. This work presents evidence from electron paramagnetic resonance (EPR) spin labeling techniques and spectrophotometric assays that a portion of synthetic Aβ(25-35) demonstrates hydrogen peroxide-like reactivity toward Fe^{2+}, nitroxide spin probes, and neocortical synaptasomal membrane proteins. These results are discussed with reference to free radical membrane damage and neurotoxicity in Alzheimer's disease.

Key Words: amyloid, protein oxidation, free radicals, spin labels, nitroxides

Synthetic amyloid beta peptides (Aβ) are analogs of a naturally occuring peptide found within the senile plaques of Alzheimer's disease (AD) - affected brains (1). Synthetic amyloid peptide Aβ(1-40) or its fragment Aβ(25-35) both induce Ca^{2+} influx and cytotoxicity

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in a variety of cell culture systems (2,3). Recently, evidence has been proposed that the toxicity of synthetic Aβ peptide may be due to free radical production in peptide incubates (4-7). According to this hypothesis, amyloid peptide-derived oxyradicals act to initiate lipid peroxidation and consequently damage sensitive cell membrane proteins.

Consistent with the free radical theory of synthetic amyloid toxicity are several observations. Amyloid application to hippocampal cell culture causes protein oxidation and oxyradical generation as evidenced by an elevation of protein carbonyl levels and dichlorofluorescein (DCF)-reactive oxidizing equivalents (8,9). Application of synthetic amyloid to rodent hippocampal neurons or human synaptosomes causes rapid inactivation of the plasma membrane-bound Na^+/K^+ ATPase and Ca^{2+} ATPase similar to effects induced by Fe^{2+}/H_2O_2 treatment, i.e. similar to damage produced by hydroxyl radical ($^{\bullet}OH$) (3). Within the timeframe required for amyloid-induced inactivation of ion-motive ATPases, Aβ(25-35) reacts with synaptosomal lipid-bound spin probes in a manner suggestive of a lipoperoxidation initiator (3,7). Amyloid-induced calcium influx, ATPase inactivation, DCF reactivity, and cell death can be effectively inhibited by such antioxidants as α-tocopherol (vitamin E), phenyl-*tert*-butylnitrone (PBN) and propyl gallate, as well as the lipoxygenase inhibitor nordihydroguaiatic acid and the NADPH oxidase inhibitor diphenylene iodonium (8-11).

The complete mechanism by which amyloid induces free radical production in cells is unclear. The initial free radical generation can occur in cell-free peptide solution incubates, as evidenced by spin trapping experiments (4-6). The product of amyloid reaction with the spin trap PBN, for instance, is a stable nitroxide species which has been trapped and partially characterized (4-6). Unfortunately, study of the solution chemistry of synthetic amyloid peptides is inherently limited by a lack of techniques for quantifying peptide-derived free radicals, and by an innate variability in peptide free radical generating capacity which parallels peptide toxicity (5,12).

In the current study we utilized electron paramagnetic resonance (EPR) spectroscopy and a simple colorimetric assay to assess and quantify the oxidative reaction of synthetic Aβ(25-35) peptide with nitroxide spin probes and aqueous Fe^{2+}, and with spin-labeled synaptosomal membrane proteins. Solution incubates of synthetic Aβ(25-35) display hydrogen peroxide-like reactivity with an approximate stoichiometry of 10 μM peroxide equivalents per mg/ml peptide incubate. Although this quantity of oxidizing equivalents seems small, biochemical amplification of an initial, localized membrane oxidizing event initiated by amyloid peptide could explain many of the observed cytotoxic effects of Aβ. The results of this work are discussed in reference to potential free-radical reactivity of natural amyloid in the Alzheimer' disease brain.

Materials and Methods

I. Peptide

Amyloid peptide (25-35) was purchased from Bachem Chemical (Torrance, CA) and stored in the lyophilized state at -20°C until use. Three independent samples of Aβ(25-35) were used in this work. Each represents different aliquots of Bachem Chemical Lot ZK650 and are referred to as samples a, b, and c as indicated.

II. Xylenol orange/ferrous ammonium sulfate assay for H_2O_2 equivalents

Hydrogen peroxide equivalents were assayed with the commercially available PeroXOquant™ assay kit (kit No. 23280, Pierce Chemical, Rockford, IL). Reagent A consisted of 25 mM $(NH_4)_2Fe(SO_4)_2$ in 2.5 M H_2SO_4; reagent B consisted of 125 μM xylenol orange (O-cresolsulfonephthalein-3'-3'-bis-[methyliminodiacetic acid sodium salt]) and 100 mM sorbitol in water. Working reagent was prepared by addition of 1 volume reagent A to 99 volumes reagent B. A control working solution was made by combining 1 volume water with 99 volumes reagent B; this solution controlled for Fe^{3+} in the test sample. 1 mM solutions of H_2O_2 (Sigma Chemical, St. Louis, MO) were then prepared by dilution of 30% H_2O_2 stock in deionized water. These peroxide solutions were standardized by measuring H_2O_2 absorbance at 240 nm using a molar absorptivity coefficient of 43.6 M^{-1} cm^{-1}.

Calibration curves were generated by reaction of 900 μL working reagent with 100 μL of H_2O_2/H_2O to give a final H_2O_2 concentration of 0-30 μM in a total volume of 1 mL. Amyloid peptides were assayed for peroxide equivalency in one of two ways. Peptides were either suspended in H_2O to 5 mg/mL, then added to working reagent, or alternatively working reagent was added directly to lyophilized peptide. In either case, samples consisted of 900 μL working reagent plus 100 μL H_2O and appropriate peptide concentrations (0-1 mg/mL peptide, after correction for buffer salt content in peptide lyophilate). Blank solutions consisted of peptide in control reagent (Fe^{2+} excluded) or working reagent plus water (no peptide).

III. Nitroxide reduction assay for reactive radical equivalents

Reagent solutions consisted of 50-100 μM nitroxide in water, plus 100 μM amyloid or 0-20 μM H_2O_2. Nitroxides used as probes (see Figure 1) were tempol (4-hydroxy tempo), tempamine (4-amino tempo), and MAL-6 (4-maleimido tempo). Nitroxide reaction with amyloid was initiated by addition of the nitroxide solution to lyophilized peptide. Nitroxide reaction with H_2O_2 was initiated by addition of small volumes (0-10 μL) of 1 mM peroxide to a 300 μL volume of the nitroxide. Solutions were immediately pipetted into an aqueous quartz EPR flat cell and placed within the EPR resonant cavity; spectral acquisition began 30 seconds to 1 minute after mixing of reagents. Spectra were acquired on a Bruker 300 EPR using the following parameters: sweep width = 80 G; receiver gain = 5.00×10^3; modulation frequency = 100 kHz; modulation amplitude = 0.962 G; conversion time = 10.24 ms; time constant = 1.28 ms. The resulting acquisition time was 10.5 seconds. Changes in nitroxide concentration were determined from measurement of the $M_I = 0$ resonance line.

IV. EPR spectroscopy and synaptosomal membrane protein spin labeling

Synaptosomes were isolated from Mongolian gerbil neocortex. Animals were obtained from Tumblebrook Farms (West Brookfield, MA) and housed in the University of Kentucky Animal Care Facility and were fed ad libitum in the home cage. Gerbil neocortex was removed as previously described (13-18) and suspended in 20 mL of ice-cold isolation buffer, which was 0.32 M sucrose with the protease inhibitors: 4 μg/mL leupeptin, 4 μg/mL pepstatin, 5 μg/mL aprotinin, 20 μg/mL trypsin inhibitor, and the chelators: 0.2 mM phenylmethylsulfonyl fluoride, 2 mM ethyleneglycolbistetracetic acid, 2 mM ethylenediaminetetracetic acid, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and homogenized with a Wheaton 10 mL homogenizer with a teflon pestle.

The mixture was then centrifuged for ten minutes at 1500 x g at 4°C in a Sorvall RC-5B centrifuge. The supernatant was removed and centrifuged again for ten minutes at 20,000 x g at 4°C. The supernatant was discarded and the resulting pellet suspended in 10 mL of the isolation buffer. The suspended pellet was layered on top of sucrose gradients consisting of 10 mL of 1.18 M sucrose (pH 8.5), 1.0 M sucrose (pH 8.0), 0.85 M sucrose (pH 8.0). The gradients were centrifuged for two hours at 85,000 x g at 4°C in a SW28 rotor Beckman L2-65B ultracentrifuge. The synaptosomes were then isolated from the 1.18 M/1.0 M sucrose interface.

The synaptosomes were then washed three times in lysing buffer (10 mM HEPES, 2 mM EDTA, 2 mM EGTA at pH 7.4), centrifuging each time for 10 minutes at 20,000 x g at 4°C. The protein concentration was determined by the Lowry method (19) and adjusted to 4.0 mg/mL and spin labeled with MAL-6 (4-maleimido-tempo, Sigma) at 20 μg/mg protein in lysing buffer (15-17). The solutions were incubated for 18 hours. The samples were then washed six times in lysing buffer to remove the excess spin label, centrifuging each time for 5-10 minutes at 14000 rpm at 4°C in a tabletop microcentrifuge. The protein concentration was again determined by the Lowry method and adjusted to 4.0 mg/mL. Varying concentrations of H_2O_2, 2,2'-azobis(2-methylpropionitrile) (AIBN), or 1 μg/μL Aβ(25-35) were added to the synaptosomes in order to induce oxidative stress on the synaptosomes. The synaptosomes were incubated for one hour at room temperature following these additions at which time the EPR spectra were obtained.

Results

I. Assay for peroxide equivalents

The peroXOquant™ assay for peroxide equivalents was performed according to the manufacturer's recomendations. The basis for the peroXOquant™ assay is the oxidation of Fe^{2+} by a peroxide to form Fe^{3+} (20,21). Subsequently, Fe^{3+} reacts with xylenol orange to form a purple complex which is detectable by visible absorption spectrometry at λ_{max} = 560 nm. Specifically,

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

$$Fe^{3+} + \text{Xylenol Orange} \longrightarrow \text{Purple Complex} \quad (\lambda_{max} = 560 \text{ nm})$$

Alternatively, species other than hydrogen peroxide may give a positive test for peroxide equivalents due to radical-mediated iron oxidation. For example, a peroxyl-type free radical (e.g., ROO•) might react by the following scheme to yield the characteristic purple complex:

$$ROO^{\bullet} + Fe^{2+} + H^+ \longrightarrow Fe^{3+} + ROOH$$

$$ROOH + Fe^{2+} \longrightarrow Fe^{3+} + \text{Products}$$

$$Fe^{3+} + \text{Xylenol Orange} \longrightarrow \text{Complex}$$

Figure 1A shows the effect of varying concentrations of hydrogen peroxide on the peroXOquant™ assay. The 560 nm absorbance of hydrogen peroxide solutions yielded a straight line with a positive slope, as expected. The assay was sensitive to micromolar concentrations of H_2O_2. The Aβ(25-35) response is depicted in Figure 1B. Each of the

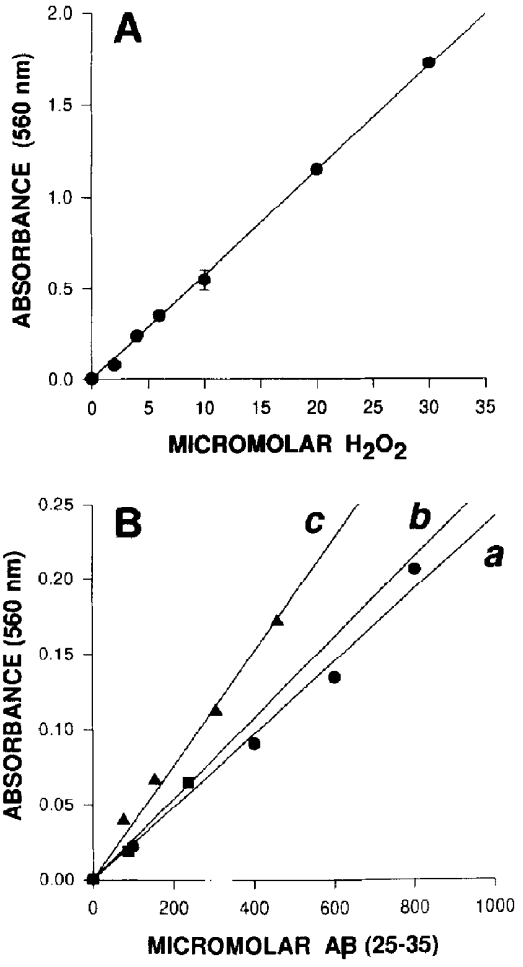

Fig. 1

A: Response of H_2O_2 to the PeroXOquant™ assay for hydrogen peroxide equivalents. Error bar represents standard error about the mean. B: Response of three samples ("a", "b", and "c", as discussed in text) of Aβ(25-35) to the PeroXOquant™ assay.

three Aβ(25-35) aliquots also showed linearity when absorbance was plotted against peptide concentration. The slope of the peptide response curve varied among the three Aβ(25-35) samples tested, which is to be expected given the reported variability in peptide spin trap reactivity and peptide-mediated toxicity (5,6,12). The peroxide equivalency of synthetic Aβ(25-35) was found to be 5-10 μM H_2O_2 equivalents / mg peptide by the Fe^{2+} / xylenol orange assay method.

II. Nitroxide reduction assay for reactive oxygen species

The spin probes used for nitroxide reduction assays are shown in Figure 2. Reaction of the nitroxide with paramagnetic species (e.g., hydroxyl radicals generated by Fenton chemistry or Aβ(25-35)-derived free radicals) in water converts the nitroxide to an EPR-silent hydroxylamine (22). Although the steady-state level of oxyradical may be too low to detect directly by EPR spectroscopy or other methods, the cumulative effect of ROS on the nitroxide solution is readily discernible by monitoring the intensity of the nitroxide EPR spectrum.

Figure 3A illustrates the reduction in the EPR signal for the spin probe, 4-hydroxy-tempo (tempol) by Aβ(25-35)-derived free radicals as a function of time and for varying concentrations of H_2O_2. Figure 3B shows a similar response from the spin probes 4-maleimido-tempo (MAL-6) and 4-amino-tempo (tempamine) upon the addition of Aβ(25-35). Like H_2O_2, Aβ(25-35) induces time-dependent decay of the nitroxide EPR signals such that a concentration of 5-10 μM H_2O_2-like reducing equivalents were generated per mg peptide during the 20 minutes of spectroscopic measurement. This figure agrees quantitatively with the results from the PeroXOquant™ analysis (above). As in the case of PeroXOquant™-analyzed peroxide equivalency, synthetic Aβ(25-35) demonstrated sample variability with respect to reaction with nitroxides. Sample "c", which reacted most avidly with the PeroXOquant™ reagent, reacted more vigorously with spin probes than did sample "a", yielding approximately twice the maximum level of nitroxide consumption.

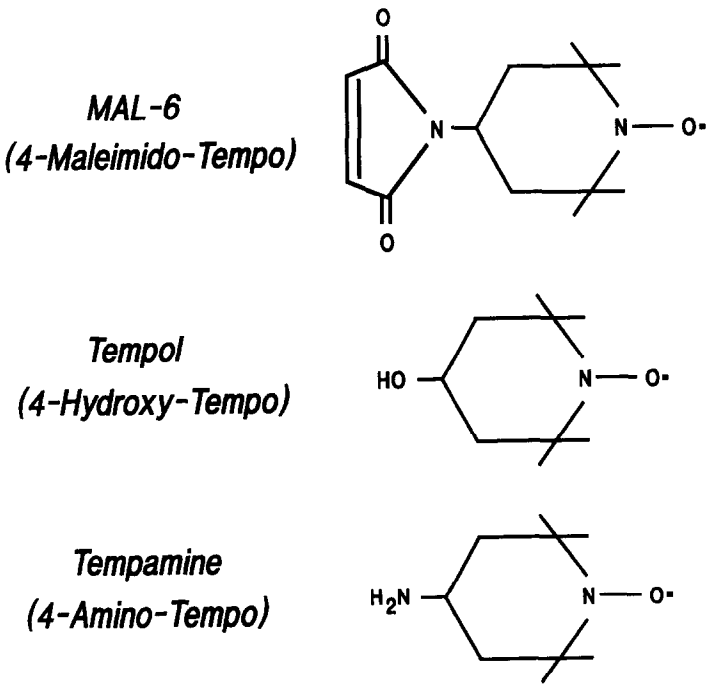

Fig. 2

Nitroxide spin labels and probes used in this study.

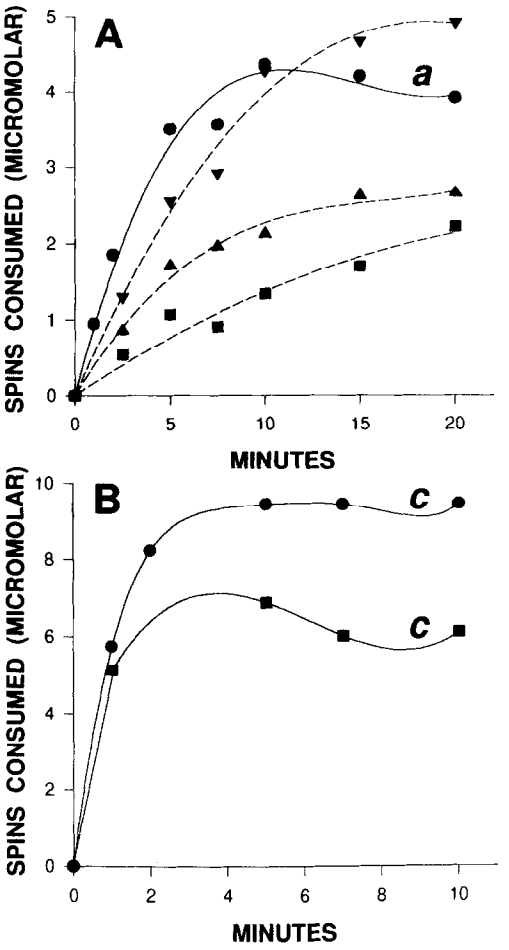

Fig. 3

A: H_2O_2 and Aβ(25-35)-mediated reduction of the EPR signal of tempol spin probe. Symbols: ■ = 5 μM H_2O_2; ▲ = 10 μM H_2O_2; ▼ = 15 μM H_2O_2; ● = 100 μM Aβ(25-35) sample "a" as described in text. B: Aβ(25-35)-stimulated reduction of the EPR signal of tempamine (●) and MAL-6 (■) spin probes; sample "c" as described in text.

Also notable in these data is the observation that Aβ(25-35) induced approximately twice the nitroxide reduction of the tempamine probe as the MAL-6 probe, all other experimental factors being constant. This effect may be due to side reactions occuring at the double bond of the maleimide group, for instance free radical addition to the double bond, which could compete with reaction at the nitroxide center. At prolonged incubation times, both amyloid and H_2O_2-generated nitroxide reduction profiles demonstrated a slight rebound effect, possibly due to reoxidation of hydroxylamine to regenerate the nitroxide

group.

III. H_2O_2-, AIBN-, and Aβ(25-35)- induced perturbations to membrane proteins

Membrane proteins spin labeled with the protein-thiol specific MAL-6 compound yield characteristic EPR spectra which are exquisitely sensitive to perturbations in the physical state of membrane proteins (15-18,23,24). Conformational changes which alter protein structure about the spin label binding site, by increasing steric hindrance to spin label motion, broaden EPR resonance lines. Conversely, membrane protein disruption or protein unfolding alleviate steric hindrance to spin label motion and the EPR lines become more narrow. The EPR spectrum of MAL-6 covalently bound to neocortical synaptosomal membrane proteins shows at least two classes of binding sites, discernable by their motion: those bound to thiol groups on hidden clefts (S-sites), which prevent rapid motion of the spin label, and those bound in more solvent-exposed sites (W-sites) on the surface of membrane proteins (Fig. 4). The EPR spectral amplitude ratio (W/S ratio) of the M_I = +1

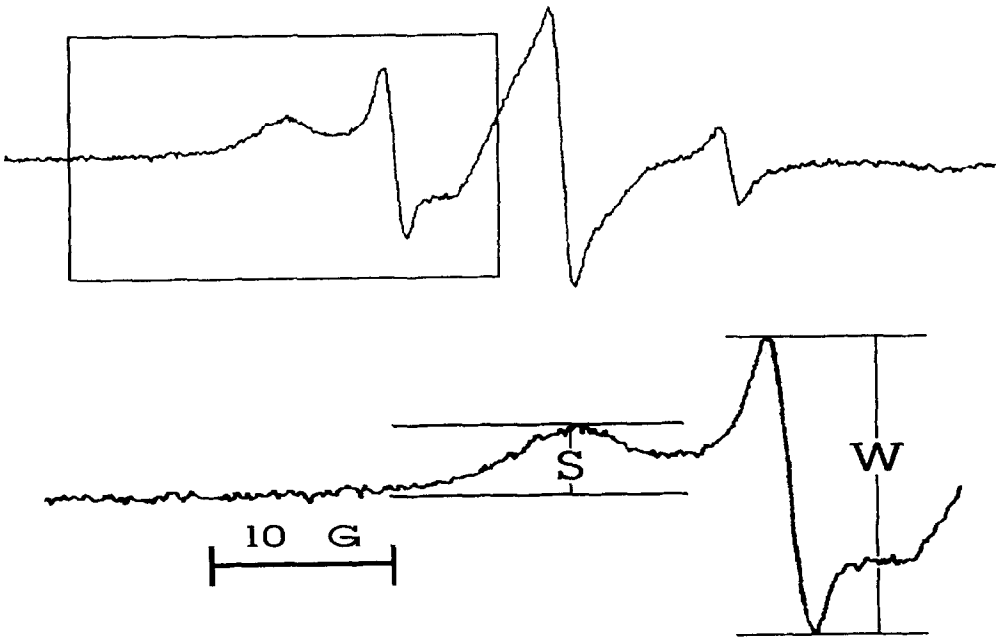

Fig. 4

EPR spectrum of MAL-6 labeled rodent synaptosomes; the M_I = +1 low-field resonance component of the MAL-6 EPR spectrum is boxed and expanded to illustrate the W and S components used in determining spectral W/S ratio.

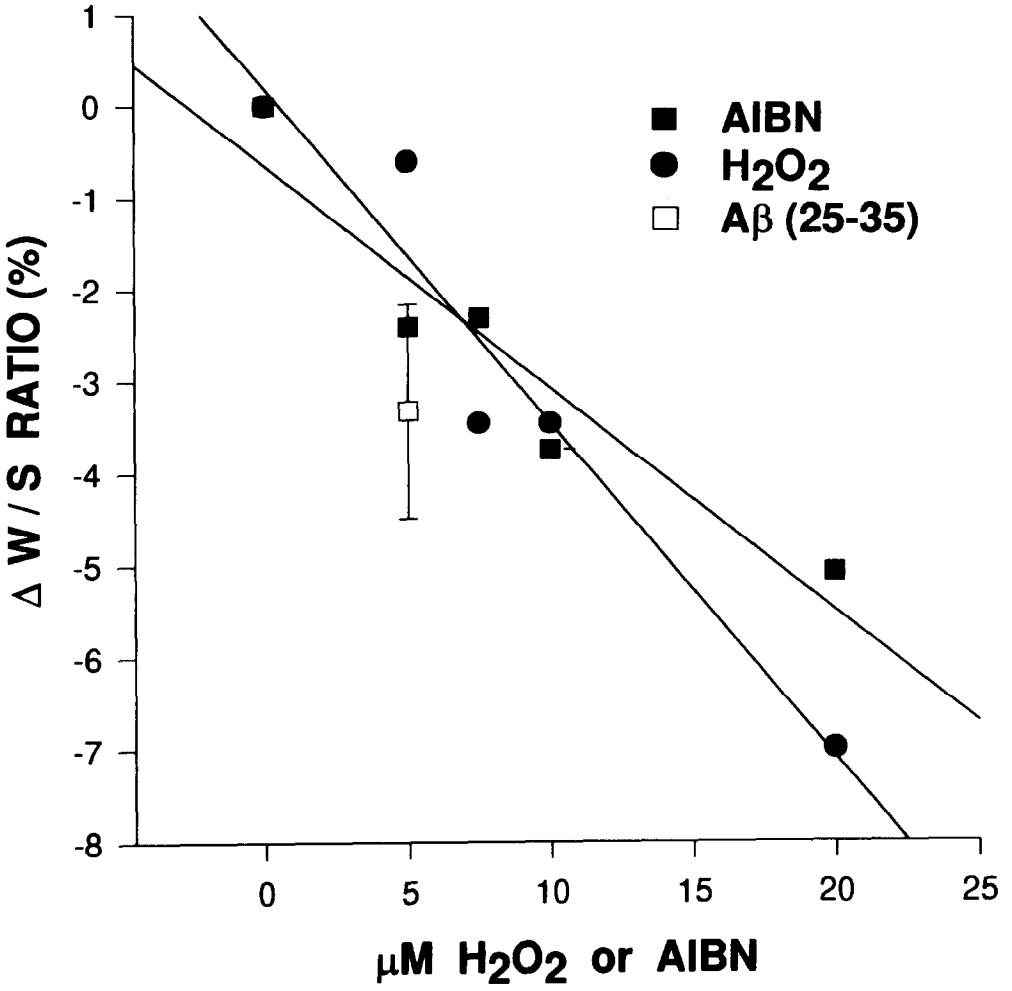

Fig. 5

Response of the W/S ratio of MAL-6 labeled synaptosomes to low levels of H_2O_2 or AIBN, and to treatment with synthetic Aβ(25-35); n = 12, $p < 0.008$. Data represent pooled results of experiments conducted with peptide samples "a", "b", and "c"; error bar indicates standard error about the mean.

low-field EPR line is the most sensitive indicator of MAL-6 tumbling rate and protein segmental conformation (Fig. 4).

A variety of oxidation paradigms have been shown to decrease the W/S ratio including hydroxyl radical generation, menadione treatment, hyperoxia, ischemia / reperfusion trauma, and cortical X-irradiation (15-18,23,24). Figure 5 illustrates the similar effects of Aβ(25-35), H_2O_2, and AIBN on the W/S ratio of MAL-6 labeled gerbil synaptosomal

membrane proteins. Hydrogen peroxide generates •OH radical upon reaction with transition metals resident in the synaptosomal preparation, while AIBN generates a tertiary carbon-centered radical [NC-C$(CH_3)_2$•] which subsequently reacts with O_2 to form a hydroperoxyl species (other reactions of the AIBN radical are possible). Data were expressed as percent change in the W/S ratio between paired treated and control samples. The W/S ratio decreased linearly with increasing H_2O_2 or AIBN concentration, and a spectroscopically measurable effect could be observed at micromolar prooxidant levels (Fig. 5A). The quantitative effects of AIBN and H_2O_2 were virtually identical. The change observed in the W/S ratio as a result of membrane oxidation is not the same effect observed in solution nitroxide reduction. Analysis of line amplitudes and line widths and comparison with double integrated total spectral intensity argues against any measurable reduction of protein-bound MAL-6 as a result of •OH generation or amyloid treatments employed in this study (16).

Similar to the case of H_2O_2 and AIBN treatments, incubation of synaptosomal membranes with synthetic Aβ(25-35) resulted in a significant decrease in the W/S ratio equivalent to approximately 10 μM H_2O_2 (N=12, P<0.008; Fig. 5B). Quantitatively, the peroxide equivalency of amyloid as inferred from synaptosomal membrane protein perturbation agrees with that inferred from both the PeroXOquant™ assay and from nitroxide reduction assays (above).

Discussion

A better understanding of amyloid peptide-mediated cellular oxidation may help clarify mechanisms of neuronal death pertinent to Alzheimer's Disease pathology and may lead to potential therapeutic intervention of Alzheimer's disease.

In this study, three different experimental approaches yielded data consistent with the hypothesis that synthetic Aβ(25-35) peptide demonstrates hydrogen peroxide-like reactivity, perhaps due to conversion of some fraction of the lyophilized peptide to a metastable peroxyl species.

The H_2O_2- like response of Aβ(25-35) to the PeroXOquant™ assay suggests that in solution, the synthetic oligomer may form a peroxyl-type free radical. Theoretically, such a moiety would form rapidly upon reaction of a putative tertiary carbon radical, for instance a leucine centered radical, with molecular oxygen. The existence of a carbon or sulfur-centered free radical at low levels within some amyloid peptide lyophilates would be difficult to detect directly, but would explain much of the reported free radical generating capacity of synthetic Aβ upon solubilization. The ability of Aβ(25-35) to reduce nitroxide spin probes' EPR signals, likewise, is consistent with oxyradical combination in peptide incubates, reaffirming our previous spin trap studies (4-7).

Finally, the amyloid peptide-mediated depression of the W/S ratio of synaptosomally bound MAL-6 is consistent with multiple models of membrane protein oxidation (15-18,23,24). It should be remembered that in a biological tissue preparation, the role of enzymatic processes cannot be ruled out. Much of the effect of synthetic amyloid on these *in vitro* systems may stem from interaction of the peptide with membrane bound flavin oxidases or lipoxygenases, as has been suggested in several recent studies (8-11)

We and others have shown that amyloid peptide-mediated ROS generation can be

affected by known or potential antioxidant compounds in solution and in cell culture (9-11). The data from the current studies suggests that intervention by free radical scavengers may modulate the neurotoxic properties of Aβ. Such studies are currently in progress in our laboratory.

Acknowledgements

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