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Direct Evidence of Oxidative Injury Produced by the Alzheimer's β-Amyloid Peptide (1–40) in Cultured Hippocampal Neurons

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The β-Amyloid peptide (Aβ) is hypothesized to mediate the neurodegeneration seen in Alzheimer's disease. Recently, we proposed a new hypothesis to explain the toxicity of Aβ based on the free-radical generating capacity of Aβ. We have recently demonstrated using electron paramagnetic resonance (EPR) spectroscopy that Aβ (1–40) generates free radicals in solution. It was therefore suggested that Aβ radicals can attack cell membranes, initiate lipoperoxidation, damage membrane proteins, and compromise ion homeostasis resulting in neurodegeneration. To evaluate this hypothesis, the ability of Aβ to induce neuronal oxidation, changes in calcium levels, enzyme inactivation, and neuronal death were compared with the ability of Aβ to produce free-radicals. Using hippocampal neurons in culture, several methods for detection of oxidation were utilized such as the conversion of 2,7-dichlorofluorescin to 2,7-dichlorofluorescein, and a new fluorescence microscopic method for the detection of carbonyls. The ability of Aβ to produce free-radicals was determined using EPR with the spin-trapping compound *N-tert*-butyl-α-phenylnitrone. Consistent with previous studies, we found that preincubation of Aβ increased the toxicity of the peptide. There is a strong correlation between the intensity of radical generation by Aβ and neurotoxicity. The highest neuronal oxidation and toxicity was seen at a time when Aβ was capable of generating the most intense radical signal. Furthermore, little oxidation and toxicity was seen when cultures were treated with freshly dissolved Aβ, which did not generate a detectable radical signal. These data are consistent with the hypothesis that free-radical-based oxidative damage induced by Aβ contributes to the neurodegeneration of Alzheimer's disease. · 1995 Academic Press, Inc.

INTRODUCTION

Amyloid plaques are the primary pathological feature in the brains of individuals suffering from Alzheimer's disease (AD) (43). β-Amyloid peptide (Aβ), the main constituent in these plaques, is a hydrophobic 39–43 amino acid fragment of a membrane-associated amyloid precursor protein (APP) (29). β-Amyloid peptide spontaneously adopt structures with significant β-pleated sheet content that interact to form large filaments that have low solubility and are resistant to dissociation during urea–SDS–PAGE gel analysis (11).

Several lines of evidence support a causal role of Aβ in AD. Amyloid plaques are surrounded by dystrophic neurites and, in many plaques, the density of astrocytes and microglia is higher than that of the surrounding neuropil (42). Furthermore, following exposure to synthetic Aβ, the neurites of rat hippocampal cultured neurons become dystrophic (37). These degenerative changes are similar to those observed in neurites surrounding plaques. Mattson *et al.* (28) have demonstrated that exposure of neurons in culture to Aβ resulted in an increase in intracellular calcium. Electrophysiological studies of the effects of the smaller neurotoxic fragment Aβ (25–35) in cultured neurons (45) and of Aβ (1–40) in artificial lipid bilayers (2) demonstrate that these peptides can directly increase ionic conductances, including calcium conductances.

Until recently, no unifying hypothesis had been proposed to integrate the pathological features of AD such as inhibited enzyme activities, modified cytoskeletal proteins, and increased lipid peroxidation with a mechanism of Aβ aggregation and neurotoxicity. We have recently presented mass spectroscopic and EPR spin trapping evidence that Aβ, in aqueous solution, fragments and also generates reactive free-radical peptides (19). It was previously shown that Aβ aggregation is promoted by oxidation and prevented by radical scavengers (11). Furthermore, vitamin E has also been shown to protect against Aβ toxicity *in vitro* (4,5). Evidence in support of the hypothesis that there is increased oxidative stress in both aging and in AD has been accumulating for years. Enhanced SOD activity (52), increased brain glucose-6-phosphate dehydrogenase (25), a decrease in the activity of oxidation-vulnerable glutamine synthetase (GS) activity (46), and enhanced lipid peroxidation (48) all support a free radical-based hypothesis for the neurotoxicity of AD.

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In this study, we have examined the relationship between free-radical production by Aβ (1–40) and neuronal oxidation/toxicity. The data presented provide direct evidence that Aβ is capable of oxidizing hippocampal neurons in culture and support the hypothesis that free radical-mediated damage by Aβ contributes to neuronal degeneration.

METHODS

Cell Cultures

The methods for culturing embryonic rat hippocampal cells have been described previously (27). Briefly, rat hippocampi were obtained from 18-day-old Sprague–Dawley fetuses and incubated for 15 min in a solution of 2 mg/ml of trypsin in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS; Gibco) buffered with 10 m*M* Hepes. The tissue was rinsed once in HBSS, exposed for 2 min to soybean trypsin inhibitor (1 mg/ml in HBSS), and rinsed in HBSS. Cells were dissociated by trituration and were distributed to polyethyleneimine-coated glass-bottom dishes (MatTek, Inc.) for confocal laser microscope studies or plastic coated dishes (Corning) for viability and enzyme studies. At the time of plating, the culture dishes contained 1 ml of Eagle's minimum essential medium (Gibco) buffered with 10 m*M* sodium bicarbonate and supplemented with fetal bovine serum (10%, v/v; Sigma), 2 m*M* L-glutamine, 20 mM KCl, 1 mM pyruvate, and 20 mM glucose. After a 3–5 hr period to allow cell attachment, the original medium was removed and replaced with 0.8 ml of medium of the same composition. The cell density was 75–100 cells/mm^2 of culture surface. Cultures were maintained at 37°C in a 5% CO_2/95% room air-humidified incubator. All experiments were performed on 9- to 11-day-old cultures.

EPR Spectroscopy and Spin Trapping

Aβ (1–40) was solubilized to 1.0 mg/ml by addition of buffer [150 m*M* phosphate-buffered saline at pH 7.4 (PBS)] to the lyophilized powder. Buffer used for spin trapping contained 50 m*M* *N-Tert*-butyl-α-phenylnitrone (PBN) (Sigma or Aldrich). Peptide solutions were pipetted into a 300 μl aqueous quartz flat cell that was subsequently sealed at both ends and immersed in a 37°C water bath for 0–48 h and removed periodically for EPR analysis. EPR spectra were acquired on a Bruker (Billerica, MA) 300 EPR spectrometer equipped with computerized data acquisition software. Instrumental parameters were microwave power, 20 mW; modulation amplitude, 0.96 G; gain, 1 × 105; and conversion time, 10.28 ms. Signal intensities were quantitated by conversion of the EPR units to concentration based on comparison with the standard 2,2,6,6-tetramethyl-4-aminopiperdine-1-oxyl (Tempamine). Tempamine is a nitroxide that gives a 3-line spectrum similar to the formed PBN adduct. Serial dilutions of tempamine were prepared and scanned under the same experimental conditions as above to generate a concentration-intensity curve. Intensity was measured using the $M_I(0)$ line.

Enzyme Activity Assays

Following experimental treatment, cells were scraped from the culture plates in the presence of 10 m*M* Hepes buffer, pH 7.4/137 m*M* NaCl/4.6 m*M* KCl/1.1 m*M* KH_2SO_4/0.6 m*M* $MgSO_4$. To prevent artifactual proteolysis of oxidized proteins, the protease inhibitors leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), and aprotinin (0.5 μg/ml) were added to the buffer. The supernatant fraction was prepared by disrupting the cells by sonification followed by centrifugation at 10,000*g* for 10 min. The clear supernatant was recovered and used for the enzyme assay. Protein content was determined by the Pierce BCA method. Glutamine synthetase activity was determined according to the method of Rowe *et al.* (41). Corrections were made for nonspecific glutaminase activity by comparing total activity in the presence and absence of adenosine diphosphate and arsenate. All assays were performed in duplicate and averaged for each sample. Statistical comparisons were made using ANOVA followed by Dunnett's test for multiple comparisons.

Assessment of Neuronal Survival

Neuronal damage was evaluated by morphological criteria that correlate well with vital dye staining methods (27). Cultures were visualized and photographed with a phase-contrast Nikon Diaphot inverted microscope. Viable neurons had neurites that were uniform in diameter and smooth in appearance and somata that were smooth and round to oval in shape. In degenerating nonviable neurons, neurites were fragmented and beaded, and the soma was rough, swollen, vacuolated, and irregular in shape. Subsequent to these morphological changes, the degenerated neurons detached from the culture substrate. Viable neurons in premarked regions (four regions of 1 mm^2/culture) were counted immediately prior to and at the time points following exposure to experimental treatments. Statistical comparisons were made using ANOVA followed by Dunnett's test for multiple comparisons.

Carbonyl Detection by Fluorescence Microscopy

The method of Harris *et al.* (18) and Leedle and Wynia (24) was used to detect oxidation generated carbonyls. The method utilizes biotin-4-amidobenzoic hydrazide to bind carbonyls followed by standard fluorescence visualization techniques. Modifications to this technique were as follows: Cells were fixed in 1,4-phenylene diisothiocya-

nate (5 mg/ml in PEG 200) for 45 min followed by one rinse in PEG 200 and then one rinse in methanol. Cells were covered in 50% PEG 200/50% PBS for 1 h to hydrolyze remaining isothiocyanate groups. Cells were then covered in a 50% acetate/50% PEG 200 buffer (200 m*M* sodium acetate, 0.5% phenol, 5 m*M* EDTA, PEG 200, pH 5.5) for 5 min before covering with the biotin-4-amidobenzoic hydrazide (1.9 mg/ml in acetate/PEG 200 buffer). After 1 h, residual hydrazide was removed and the cells rinsed three times with PBS. This was followed by incubation with DTAF fluorescein-conjugated streptavidin. Fluorescence was amplified by using a biotinylated antistreptavidin antibody. Cultures were then mounted in Vectashield (Vector) mounting medium and examined using a confocal scanning laser microscope (Molecular Dynamics, Sarastro 2000) coupled to an inverted microscope (Nikon). Fluorescence was excited at 488 nm and emission filtered using a 510-nm barrier filter. Cells scanned were chosen on a random basis. Four cells were evaluated in each treatment group. Values for average staining intensity/cell were obtained using the Imagespace (Molecular Dynamics) software. Statistical comparisons were made using ANOVA followed by Student–Newman–Keuls multiple comparisons test.

Dichlorofluorescein Assay

For measurement of intracellular reactive oxygen species, cells were loaded with 2,7-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc.) by incubating for 50 min in the presence of 100 μ*M* of the dye. Cells were washed three times (1 ml/wash) in Hank's balanced saline solution containing 10 m*M* Hepes buffer and 10 m*M* glucose. Imaging utilized a confocal laser scanning microscope as described above. The dye was excited at 488 nm and emission was filtered using a 510-nm barrier filter. The intensity of the laser beam and the photodetector sensitivity were held constant to allow for quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Cells were chosen for analysis on a random basis and scanned only once with the laser since exposure to the laser light itself may induce photo-oxidation. Values for average fluorescence intensity/cell were obtained using the Imagespace software supplied by the manufacturer (Molecular Dynamics). Statistical comparisons were made using ANOVA followed by Student–Newman–Keuls multiple comparisons test.

Calcium Imaging

Following treatment, cultures were incubated for 2 h at 37°C in serum-free Eagle's minimal essential medium with 20 μ*M* of the calcium indicator dye fluo-3/AM, which was added from a 1 m*M* stock solution in dimethyl sulfoxide plus 0.1% Pluronic F-127. Cultures were rinsed by replacing 80% of the medium five times with buffer containing 120 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 15 m*M* glucose, 25 m*M* Tris–HCl, pH 7.4, at 37°C. Cells were imaged and fluorescence quantitated as described above under carbonyl detection. Statistical comparisons were made using ANOVA followed by Dunnett's test for multiple comparisons.

Peptides/Experimental Treatment

Synthetic β-amyloid peptide (1–40) (Lot #ZK600) was obtained from Athena Neurosciences (San Francisco) and (40–1) from Bachem. Aβ stocks of 1 m*M* were prepared in double-distilled water. Preincubation ("aging") of Aβ was performed at 37°C for times ranging from 0 (fresh) to 48 h prior to addition to cell cultures. Peptides were added directly to cultures containing the growth medium at a final concentration of 20 μ*M*.

RESULTS

Detection of Amyloid Radicals and Neurotoxicity

We have previously shown that Aβ (1–40) generates free-radicals in aqueous solution (19). Figure 1 shows the representative spectra and intensity profile of radicalization of Aβ (1–40) in 6-h increments over a 48-h incubation period. Figure 1A demonstrates the characteristic 3-line EPR spectra obtained using the spin-trap PBN with Aβ (spectrum c). There is no signal detectable with PBN alone (spectrum a) or freshly dissolved Aβ (spectrum b). Aβ (40–1) produced a qualitatively different 6-line spectrum (spectrum d). The Aβ (40–1) signal did not appear until after 24 h of incubation. Quantitation of the Aβ (1–40) signal intensity is presented in Fig. 1B. The largest amount of Aβ (1–40) PBN-radical adducts was formed during the first 6 h of incubation (Fig. 1B). The net production of adducts decreased after the first 6 h, most likely due to consumption of the monomer into higher molecular weight aggregates.

To examine the time course of radicalization in relation to the time course of toxicity, we established primary rat hippocampal cultures and treated them with Aβ (1–40). For this experiment, Aβ (1–40) was preincubated in distilled water at a concentration of 1 m*M* for times ranging from 0 to 48 h before addition to cultures for 48 h. Neuronal survival decreased as the preincubation time increased up to 6 h (Fig. 2). After 12 h of preincubation, the peptide was less toxic to hippocampal neurons. The time course of Aβ developing toxicity, with the Aβ becoming increasingly toxic over a 6-h preincubation period, is consistent with the formation of radicals (PBN adducts) over the first 6 h of incubation. Aβ (40–1) was not toxic to cultured hippocampal neurons. Freshly dissolved Aβ (40–1) and 24-h preincubated Aβ (40–1) were both tested for neurotoxicity and the results were 103 ± 5% (n = 3) and 99.7 ± 0.9% (n = 3) of untreated control cultures, respectively.

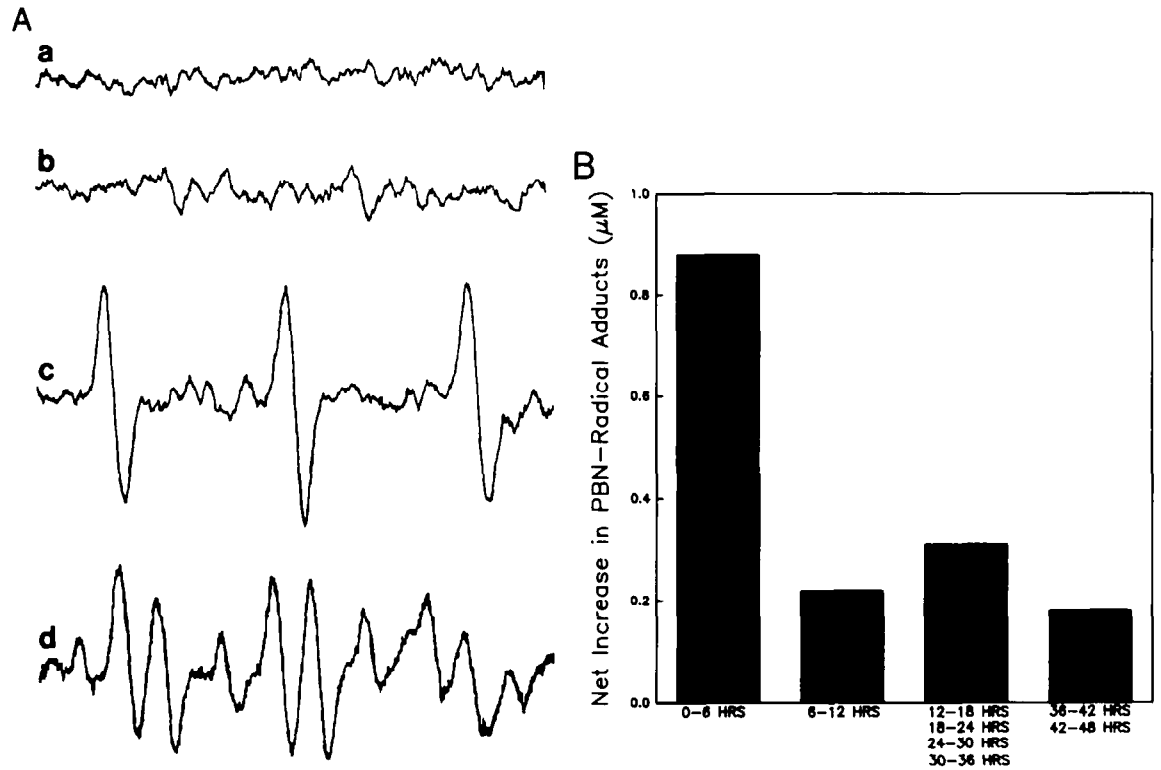

FIG. 1. (A) EPR spectra. (a) background signal with PBN alone. (b) Freshly dissolved Aβ (1–40) and PBN. (c) Aβ (1–40) and PBN after 6 h incubation. (d) Aβ (40–1) and PBN after 24 h of incubation. (B) Quantitation of EPR signal generated when Aβ (1–40) is incubated in t presence of PBN over a 48-h time period. Arbitrary units output by the EPR spectrometer are converted to molar concentration based comparison with the standard 2,2,6,6-tetramethyl-4-amino-piperdine-1-oxyl (Tempamine).

Enzyme Toxicity

Glutamine synthetase, a key metabolic enzyme found in astrocytes, is necessary for conversion of glutamate to glutamine and prevention of excitotoxicity. GS is known to be vulnerable to oxidation (14) and we have previously reported that Aβ (25–35) dose-dependently inactivates GS in cell-free extracts (19). For this experiment, Aβ (1–40) was preincubated for 0–48 h before addition to cultures for 24 h. Figure 3 demonstrates that GS activity decreases as the preincubation time of Aβ (1–40) increased up to 6 h. Consistent with the radical formation and viability data, 6-h preincubation of the peptide produced the greatest loss in GS activity. After 12 h of preincubation, the peptide's ability to decrease GS activity declined.

Based on the EPR, neurotoxicity, and GS results, we chose specific times of peptide preincubation to study neuronal oxidation and injury induced by Aβ (1–40). Freshly dissolved peptide was not neurotoxic whereas 6 h of preincubation was the most toxic. We chose these two times points to examine the temporal changes in oxidative stress in neurons induced by the peptide. We examined early (2 h) and late (24 h) effects of the peptide.

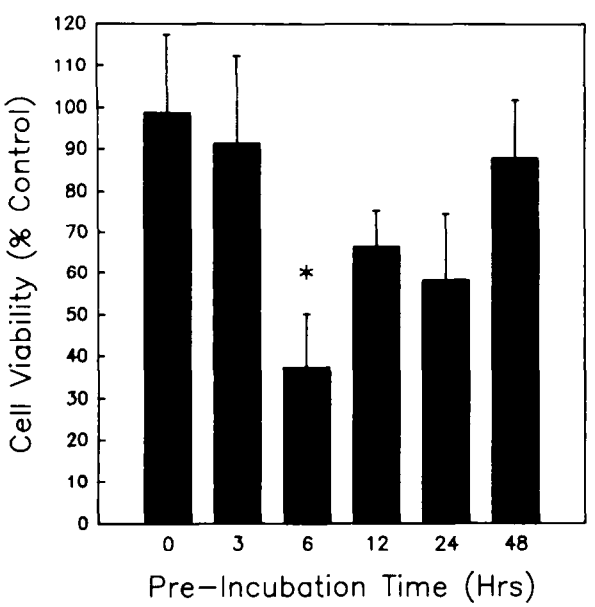

FIG. 2. Viability following treatment with Aβ (1–40) in hippocar pal neurons in culture. Peptide was preincubated for times indicat on the x-axis before addition to cultures for 48 h. Bars represent me percentage cell loss (±SEM) of four separate cultures. Significant c loss was observed with treatment of 6-h preincubated Aβ (1–4 Statistical comparisons were made using ANOVA followed by Du nett's test. $^*P < 0.05$, relative to untreated control groups.

Intracellular Calcium

Consistent with previous findings (27, 29), we have found that hippocampal neurons exposed to Aβ (1–40) have an increased intracellular calcium level. Fresh Aβ (1–40) increased intracellular calcium although this increase was not significantly different from untreated control cultures (Fig. 4). Six-hour preincubated Aβ (1–40) did produce a significant increase in intracellular calcium after 24 h of exposure.

Reactive Oxygen Species (ROS)

Intracellular ROS were increased following Aβ (1–40) treatment (Fig. 5). Fresh Aβ (1–40) added to cultures for 24 h did not produce a significant increase in ROS as detected by the conversion of 2,7-dichlorofluorescin to 2,7-dichlorofluorescein (DCF). However, Aβ (1–40) preincubated for 6 h before addition to cultures produced a large increase in DCF fluorescence over that of nontreated controls ($P < 0.05$) and cultures treated with fresh Aβ ($P < 0.05$).

Detection of Oxidation by Fluorescence Microscopy

We have recently developed a technique that allows sensitive detection of carbonyl groups by fluorescence microscopy (18, 24). The method utilizes a biotin-conjugated hydrazide to bind carbonyl groups followed by standard fluorescein-labeled streptavidin visualization techniques. Treatment groups for this set of experiments were chosen based on the toxicity of the peptide as shown in Figs. 1–3. Six hours of preincubation was the most toxic to cells and freshly dissolved Aβ (1–40) was the least toxic. Figure 6 illustrates cultured neurons treated with Aβ (1–40), stained for carbonyl content, and imaged using a confocal scanning laser microscope. Figure 6A shows the background fluorescence of a nontreated neuron. Following treatment with freshly dissolved Aβ (1–40), the fluorescence intensity increased slightly indicating a low level of oxidation (Fig. 6B). This finding of a low level of oxidation agrees with the low toxicity of the freshly dissolved peptide. A dramatic increase in fluorescence (oxidation) was seen when neurons were treated with Aβ (1–40) that had been preincubated for 6 h prior to culture treatment (Fig. 6C). This oxidation is again consistent with our findings of high neuronal toxicity, GS inactivation, and radical production at that time of preincubation. Table I presents the quantitation of the carbonyl fluorescence for the different treatment groups. Cells treated with 6-h preincubated Aβ show significantly more carbonyl fluorescence than nontreated controls ($P < 0.05$) and cells treated with fresh Aβ ($P < 0.05$). Several controls were performed to ensure that these findings were not artifactual. Salicylic hydrazide was used in place of biotin-4-aminobenzoic hydrazide to test for specificity of the

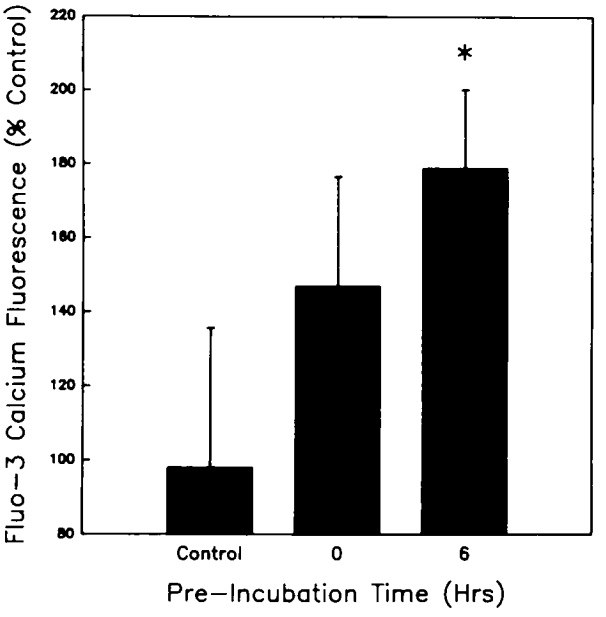

FIG. 4. Aβ (1–40) induced changes in intracellular free calcium in hippocampal neurons. Calcium fluorescence was quantitated using the calcium indicator dye Fluo-3. Cultures were treated with either fresh Aβ (1–40) which produces little cellular toxicity or 6-h preincubated Aβ (1–40) which was the most toxic in this study. A significant increase in intracellular calcium was observed in cultures treated with the 6-h preincubated peptide for 24 h. Bars represent mean percentage increase over untreated control cultures (±SEM) of 16–24 neurons in four separate cultures. Statistical comparisons were made using ANOVA followed by Dunnett's test. *$P < 0.05$, relative to untreated control group.

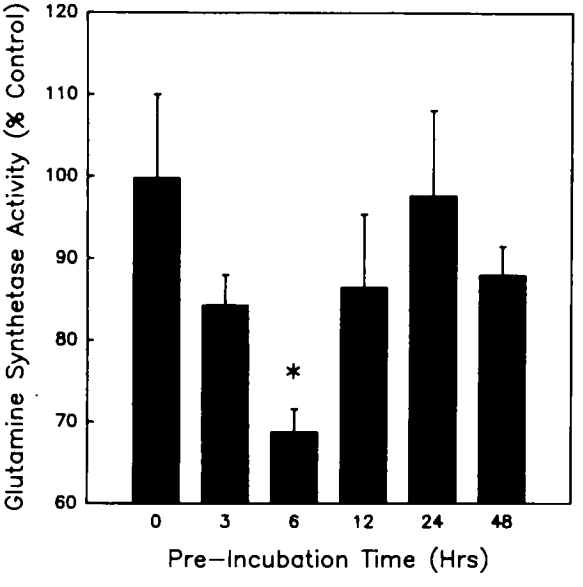

FIG. 3. Aβ (1–40) is capable of inactivating GS in hippocampal cultures. Peptide was preincubated for times indicated on the x-axis before addition to cultures for 24 h. Bars represent mean percentage GS activity (±SEM) of four separate cultures. Statistical comparisons were performed using ANOVA followed by Dunnett's test. *$P < 0.05$, relative to untreated control.

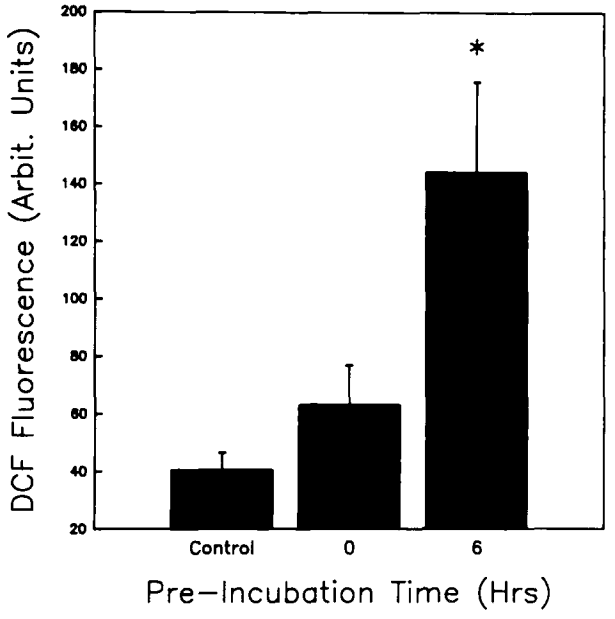

FIG. 5. Increase in intracellular reactive oxygen species as detected by the conversion of 2,3-dichlorofluorescin to 2,3-dichlorofluorescein (DCF). Preincubated Aβ treatment for 24 h produced a significant increase in DCF fluorescence. Bars represent mean percentage increase over untreated control cultures (±SEM) of 16–24 neurons in four separate cultures. Statistical comparisons were made using ANOVA followed by Dunnett's test. $*P < 0.05$, relative to untreated control group and 0 h preincubated Aβ (1–40) treated group.

biotin-4-amidobenzoic hydrazide. Sets of cultures were taken through the entire procedure with the omission of the hydrazide to check for nonspecific fluorescence. We also determined the fluorescence of the Aβ (1–40) peptide itself before the staining procedure and at each step throughout the staining procedure to control for nonspecific binding of the fixative hydrazide or streptavidin to Aβ (1–40). In each of these control evaluations of the staining procedure, no background or nonspecific fluorescence was observed. Aβ (40–1) treatment produced a slight but insignificant increase in carbonyl fluorescence. This was partially attributable to nonspecific DTAF–streptavidin binding to particles of Aβ (40–1) on the surface of the culture dish. Furthermore, Aβ (40–1) does produce a detectable radical signal; therefore, a low level of oxidation may be occurring. Pretreatment with the antioxidant propyl gallate (5 μM) provided protection against the carbonyl increase induced by 6-h preincubated Aβ (1–40).

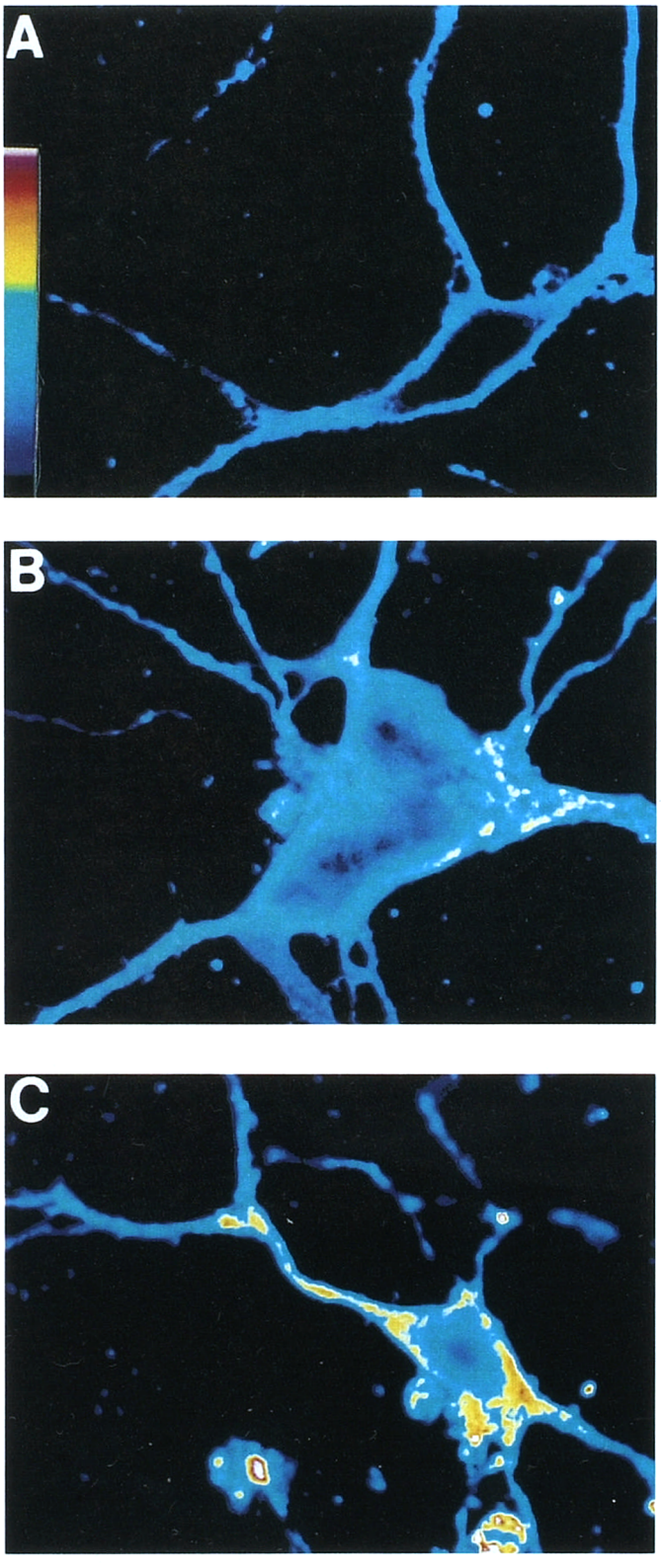

FIG. 6. Increase in protein carbonyl content produced by exposure of neurons to Aβ (1–40) in culture. False-color images taken using a confocal laser microscope to detect carbonyl content following Aβ treatment. (A) Untreated control neuron displaying low levels of background fluorescence. (B) Fresh Aβ (1–40) added to cultures for 2 h. Carbonyl content was slightly increased over control. (C) Six-hour preincubated Aβ (1–40) treatment for 2 h demonstrating a large increase in carbonyl content of the neuron. Quantitation of fluorescence is displayed in Table I.

TABLE 1

Quantitation of Carbonyl Fluorescence in Rat Hippocampal Neurons

Treatment group	Fluorescence (% control)	Statistics
Control	100 ± 8.8	
AB (1–40), 0 h preincubation	109.7 ± 11.4	
AB (1–40), 6 h preincubation	324.8 ± 15	$P < 0.05$ vs control and 0 h preincubated
AB (40–1)	133.3 ± 8.5	
AB (1–40), 6 h preincubation plus 5 μ*M* propyl galiate	73.2 ± 9.7	

Note. Culture treatments were 2 h in duration. Fluorescence units are expressed as the mean ± SEM of 16–24 neurons. Units were obtained using the Imagespace software as outlined in the methods section. Statistical analysis was performed using ANOVA followed by Student–Newman–Keuls test for multiple comparisons.

DISCUSSION

The present data provide strong support for the hypothesis that oxidation is involved in the mechanism of neurotoxicity of Aβ. We have previously shown that Aβ is capable of generating free radicals in solution that are detectable by EPR (19). In the current study, we have examined the temporal pattern of neuronal oxidation induced by Aβ (1–40) by systematic evaluation of the EPR signal generated by Aβ (1–40) and early and late signs of oxidative damage induced by the peptide and the resulting neurotoxicity. We demonstrate here that the capacity of Aβ (1–40) to produce radicals is consistent with its ability to promote neuronal oxidation and death.

It has been previously reported that the activity of Aβ changes from a trophic or nontoxic soluble peptide to a toxic aggregate following incubation (35). Consistent with published findings, we found that freshly dissolved Aβ is not neurotoxic. According to our previous studies, freshly dissolved Aβ has not yet begun to generate a radical signal detectable by EPR spectroscopy (19). Incubation of the peptide in buffer or distilled water results in the generation of a highly neurotoxic peptide which is consistent with the peptide's ability to generate free radicals as seen in this study and as previously reported (19). There is a strong relationship between aggregation and neurotoxicity (8, 30, 38) and secondary structure and neurotoxicity (44). It is therefore important to determine the relationship between radical formation, aggregation, and Aβ-induced neurotoxicity. We have demonstrated that this lot of Aβ (1–40) displays an increase in radical formation during the period from 0 to 6 h of preincubation. This net increase in radical signal diminishes thereafter. We speculate that the decrease in signal intensity over time may either be the result of consumption of the peptide monomer into higher molecular weight aggregates or the breakdown of the peptide into fragments (19). Tomski and Murphy (49) in their studies on the kinetics of Aβ (1–40) aggregation demonstrated a rapid initial aggregation rate constant that decreased over time as the aggregates grew.

Based on the free-radical generating capability of the peptide, it should therefore be possible to make predictions of the neurotoxic potency of the peptide from the EPR signal/intensity. Peptides with an intense triplet EPR signal, like that presented in this study, should produce high neurotoxicity. Consistent with this hypothesis, we have shown that toxicity correlates with the EPR signal. At times longer than 6 h of preaggregation, the EPR signal intensity diminishes as does the toxicity. Thus, we have effectively "overaged" the peptide. Consistent with these results, Simmons *et al.* (44) have noted that neurotoxic potencies can reach a maximum and then decrease with longer aging protocols. Loss of the peptide monomer by peptide breakdown or consumption of the peptide monomer by free radical-mediated processes to form higher molecular weight aggregates may lead to the reduction in toxicity over time. Furthermore, Aβ (40–1), which produced a qualitatively different six-line spectrum was not toxic in our cultures. Aβ (40–1), which does not form fibrillar structures but only amorphous particles, has been reported to be neurotoxic over extended time periods in culture (16). The significance of the differences in spectra in relation to toxicity and secondary structure has yet to be determined.

We have previously demonstrated using HPLC-mass spectral analysis that Aβ (1–40) fragments during incubation generating lower molecular weight breakdown products (19). These fragments may be responsible for the persistent low intensity EPR signal seen out to 48 h of incubation. We hypothesize based on these data that radicalization leads to a toxic form of the peptide that can interact with neurons, oxidizing them and resulting in death. Reactive oxygen species and smaller fragments of the peptide generated over time may also contribute to this toxicity.

Our free radical-based hypothesis of Aβ neurotoxicity is consistent with previously reported hypotheses of Aβ aggregation. The work of Jarrett and Lansbury (21) discusses nucleation-dependent polymerization or "seeding" of Aβ that then leads to the continued aggregation of Aβ. The current study and our previous work (19) suggests that this seeding mechanism is mediated by free radical formation by the Aβ (1–40) peptide alone. Consistent with previous reports (35, 36, 28, 22, 31), we have observed that freshly dissolved Aβ (1–40) is not toxic to neurons compared to neurons exposed to preincubated peptide. The kinetics of aggregation of amyloid peptides have been shown to be concentration dependent (7, 44). Radicalization and aggregation of the peptide would be unfavorable at the low concentrations in the culture dish (20 μ*M* final concentration compared to 1 m*M* for preaggregation), which may explain the

subsequent lack of toxicity of freshly dissolved peptide even over extended time periods (48 h). Fresh Aβ (1–40) did produce some changes in the hippocampal neurons such as an increase in intracellular calcium and ROS as well as a slight increase in carbonyl content (not significantly different from controls). There may be a threshold level of oxidative insult that neurons are capable of withstanding. Although there were indications of slight oxidative stress in cells treated with fresh Aβ, the longer term viability of these cells was not compromised. The chronicity of Alzheimer's disease and its progression may therefore be a result of the time needed to develop a critical mass of reactive amyloid species that are capable of developing toxicity and leading to an overwhelming level of oxidative stress.

Further evidence for radical involvement comes from the work of Dyrks *et al.* (11) who have reported that radical treatment transforms nonaggregated Aβ into stable aggregates. Although we have previously shown that radical formation by synthetic Aβ is not metal-dependent but instead requires only molecular oxygen (19), amino acid oxidation and protein cross-linking induced by radical generation may be key to Aβ aggregation and toxicity.

Oxidative damage to proteins produces an increase in the carbonyl content of the protein due to oxidation of sensitive amino acids such as histidine, proline, arginine, and lysine (47). Oxidative modification of individual amino acids in proteins can result in inactivation of key metabolic enzymes such as GS and creatine kinase (32). For example, the oxidation of a single histidine residue in GS will inactivate this enzyme. Altered forms of enzymes and levels of protein carbonyl have been shown to increase with age (46). Smith *et al.* (46) reported both an increase in carbonyl content and decrease in the oxidation sensitive GS and CK enzymes in AD. Oxidation products accumulate in the brains of both neurologically normal and AD patients due to a progressive decrease with age in the defense mechanisms that protect against oxidation in combination with an increase in levels of free radicals (49). We have demonstrated an increase in cellular carbonyl content following Aβ (1–40) treatment. This increase in carbonyl was rapid following treatment with Aβ and was prevented by the antioxidant propyl gallate. Propyl gallate, at the same concentration used in this study, has been shown to protect against loss of viability and increased peroxide production due to Aβ (1–40) treatment (5).

Consistent with this hypothesis, free radicals produced by Aβ (1–40) may be capable of damaging cells by attacking lipid membranes, initiating lipoperoxidation, and damaging vital membrane proteins. In support of this hypothesis, there have been reports of abnormal membrane lipid status/composition in AD (15) and increased sensitivity of AD lipid membranes to peroxidation (48). Behl *et al.* have recently demonstrated lipid peroxidation and increased intracellular hydrogen peroxide following exposure of B12 cells to Aβ (5). We recently reported that the smaller neurotoxic fragment Aβ (25–35) is a potent initiator of lipid peroxidation (9). Damage to the lipid membrane can result in loss of ion homeostasis and membrane potential. Indeed, it has been demonstrated that aggregation of Aβ on or near the plasma membrane of neurons was correlated with membrane depolarization and the increase in intracellular calcium (30).

Loss of calcium homeostasis contributes to Aβ-mediated neuronal death. Arispe *et al.* (2) have provided evidence that Aβ (1–40) can form cation channels in lipid bilayers that conduct calcium current. Several studies have demonstrated that Aβs destabilize intracellular calcium homeostasis (28, 30) and that calcium channel blockers attenuate Aβ toxicity (50). Increases in free intracellular calcium could result in a further increase in oxidative stress. For example, a calcium-mediated increase in phospholipase activities could result in increased arachidonic acid levels and, as a metabolic consequence, the generation of oxygen radicals associated with fatty acid metabolism (23, 33). Excessive calcium loading of the mitochondria will result in aberrant electron transport function and an increase in superoxide anion concentrations in the cytosol due to leakage of electrons from the mitochondria (3). Our finding of increased neuronal calcium levels following Aβ exposure is consistent with previous findings (28, 30).

There is evidence for an increase in intracellular ROS production induced by Aβ. Our data, and that of Goodman and Mattson (17), demonstrate that Aβs can induce ROS production in hippocampal neuronal cultures. Based upon previous studies (23, 33), it is reasonable to hypothesize that the Aβ-mediated increase in intracellular ROS results from an increase in intracellular calcium. Excitotoxicity has also been implicated in the neurodegeneration of Aβ. Previous studies have demonstrated that Aβ enhances the vulnerability of neurons in culture to excitotoxic-mediated death (22, 28). Stimulation of glutamate receptors, particularly *N*-methyl-D-aspartate (NMDA) receptors results in increased calcium and sodium influx. Bondy and Lee (6) have reported increased ROS generation induced by ionotropic glutamate agonists. NMDA receptor activation can lead to increased arachidonic acid levels (34) and subsequent increased ROS formation by lipoxygenases. It is also possible that lipid membrane damage by Aβ radicals can result in changes in membrane potential and removal of the Mg^{2+} blockade of NMDA receptors.

It has been postulated that astrocytes are involved in the neuropathology of AD (13, 39). Reactive astrocytes surrounding senile plaques have been described as hallmarks of the pathology of AD (10). In addition, aberrant

processing of the APP in astrocytes has been implicated in the formation of plaques (20, 40). We have demonstrated in this study the inhibition of glutamine synthetase, an astrocytic enzyme, by Aβ under incubation conditions that produce significant oxidative stress. Astrocytes play an important role both in the maintenance of neuronal function/synapse development and in the prevention of neurotoxic effects due to excitotoxicity. Inhibition of GS may exacerbate Aβ-excitotoxic cell injury by accumulation of glutamate. In addition, astrocytes are important in that they secrete many growth factors such as basic fibroblast growth factor, which has been shown to protect hippocampal neurons in culture against oxidative stress induced by ferrous sulfate (53) and the disturbance in calcium homeostasis and neuron death induced by Aβ (30). Free radicals generated by Aβ may also damage lipids and critical membrane proteins of astrocytes.

In conclusion, the demonstration of free radical production by Aβ (1–40) and direct evidence of oxidative damage in neurons by Aβ (1–40) extends the hypothesis of free radical-mediated neurotoxicity of Aβ (19). Aβ (1–40) produces a 3-line spectrum, whose intensity over time parallels neurotoxicity. In addition, Aβ (1–40) produces an increase in cellular carbonyl content. This increase is preventable when the cells are pretreated with the antioxidant propyl gallate. We speculate that free radical generation by Aβ (1–40) leads to a reactive and toxic form of the peptide that has a relatively short half-life but can interact with cells and lead to oxidative stress. Subsequently there is a continued cascade of cellular events that ultimately leads to neuronal death. The precise relationship between peptide secondary structure, radical formation, aggregation, and toxicity are still unknown and further work is needed to understand the complex chemistry of Aβ and other beta-pleated sheet proteins.

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