Different Mechanisms of Oxidative Stress and Neurotoxicity for Alzheimer's Aβ(1−42) and Aβ(25−35)

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Abstract: Oxidative stress induced by amyloid β-peptide (Aβ) has been implicated in the neurodegeneration observed in Alzheimer's disease (AD) brain. However, the mechanism by which the predominant form of Aβ found in AD brains, Aβ(1−42), causes oxidative stress and neurotoxicity remains unknown. Numerous laboratories have used the smaller 11-amino acid fragment of the full-length peptide, Aβ(25−35), as a convenient alternative in AD investigations since the smaller peptide mimics several of the toxicological and oxidative stress properties of the native full-length peptide. Our observation that the truncated peptide is more rapidly toxic and causes more oxidative damage than the parent Aβ(1−42) led us to investigate the cause for this enhanced toxicity of Aβ(25−35) in order to gain insight into the mechanism of action of these peptides. These studies reveal that two different mechanisms may be operative in the two peptides; however, the single methionine residue in the peptides appears to play a crucial role in both mechanisms. That methionine is C-terminal in Aβ(25−35) seems to be the cause for its exaggerated effects. When the next amino acid in the sequence of Aβ(1−42) (valine) is appended to Aβ(25−35), the resultant peptide, Aβ(25−36), in which methionine is no longer C-terminal, is neither toxic to cultured neurons nor does it cause oxidative damage. Additionally, oxidizing the sulfur of methionine to a sulfoxide abrogates the damaging effects of both Aβ-(25−35) and Aβ(1−42). The putative mechanistic role of methionine in the observed properties of Aβ peptides is discussed in the context of the obtained results as is the role of Aβ(1−42)-induced oxidative stress in the neurodegeneration found in AD brain.

Introduction

Alzheimer's disease (AD) brain is characterized by extensive oxidative stress, and amyloid β-peptide (Aβ) is thought by many researchers to be central to the pathogenesis of this disorder.[1−3] Oxidative stress induced by Aβ is widely believed to be implicated as one means for the neurodegeneration associated with AD. Aβ, typically a 40- or 42-amino acid peptide (Figure 1a) formed in excess in AD brain by processing of the amyloid precursor protein, is a major insoluble component of senile plaques in AD. Numerous experiments with synthetic Aβ(1−42) have established that this peptide, in ways that are inhibited by free-radical antioxidants, induces protein oxidation, causes lipid peroxidation, exerts oxidative stress in a cellular environment, and is toxic to cultured neurons (reviewed in ref 3).

A smaller 11-amino acid fragment of the Aβ peptide, Aβ-(25−35) (Figure 1a), though not found in the AD brain, has often been studied as the potent component of the full-length peptide following the observation that this peptide produces toxic effects similar to those caused by Aβ(1−42).[4−6] Indeed, Aβ-(25−35) causes neuronal death, protein oxidation, and lipid

(a) H_2N-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]-COOH

(b) H_2N-Gly[25]-Ser[26]-Asn[27]-Lys[28]-Gly[29]-Ala[30]-Ile[31]-Ile[32]-Gly[33]-Leu[34]-Met[35]-**$CONH_2$**

(c) $CH_2CH_2SCH_3$ **(d)** CH_2CH_2**S(O)**CH_3

(e) CH_2CH_2**CH_2**CH_3

Figure 1. Various peptides used in this study. (a) Amino acid sequence of Aβ(1−42) with the single crucial methionine residue 35 underlined. The 11-amino acid fragment Aβ(25−35) widely used in experiments as a substitute for Aβ(1−42) is shown in bold. Note that methionine is C-terminal in this peptide. Shown in italics is the peptide Aβ(25−36) used in our experiments in which the methionine residue is no longer C-terminal. (b) Aβ(25−35)amide in which the C-terminal carboxylate functionality of methionine in Aβ(25−35) is converted into an amide functionality. (c) The side chain of methionine in which the sulfur atom is present as a dialkyl sulfide. (d) The side chain of methionine sulfoxide in which the sulfur is present as a dialkyl sulfoxide as in Aβ(1−42)-Sox and Aβ(25−35)Sox. (e) The side chain of norleucine, in which the S atom of methionine is replaced by a methylene group.

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peroxidation in cell cultures. Similar to the case with the full-length peptide,[7,8] replacement of the methionine residue 35 with structurally similar norleucine (in which the sulfur of methionine is replaced by a methylene group) in Aβ(25−35) abolishes the oxidative stress and neurotoxic properties of the peptide.[9]

Despite the numerous similarities in the properties of Aβ-(1−42) and Aβ(25−35), there are some subtle differences between the two peptides: the shorter peptide is more rapidly toxic than the full-length peptide; in addition, Aβ(25−35) often causes more oxidative damage than Aβ(1−42). Furthermore, Aβ(1−42) has been shown to strongly bind Cu(II) ions and reduce these metal ions to Cu(I), whereas Aβ(25−35) lacks the Cu(II) binding motif that was identified in Aβ(1−42).[10] These observations led us to investigate the possibility that the mechanism of the oxidative stress induced by Aβ(25−35) may be different from that of Aβ(1−42). Since the methionine residue is implicated in damaging effects of both peptides, we wondered whether the environment of the methionine plays a role in the observed differences. The most obvious difference is the fact that the methionine in the shorter peptide is C-terminal. In this report, we describe experiments that elucidate the role of the terminal methionine in the toxic properties of Aβ(25−35) and discuss the possible differences in the mechanism of action of Aβ(1−42) and Aβ(25−35).

Materials and Methods

General Information. Peptides used in these studies were obtained from Bachem Chemical (Torrence, CA) or AnaSpec (San Jose, CA). Purity was certified by HPLC−MS for each of the peptides. The peptides were stored in the dry state, as received, at −20 °C until use. N-*tert*-Butyl-α-phenylnitrone (PBN) was synthesized as described[11] and rigorously purified by repeated recrystallizations and vacuum sublimations. All batches of PBN were verified to be pure by NMR, HPLC, and MS before use in order to eliminate the possibility that impurities may give rise to any EPR spectra. Deferroxamine and Chelex 100 were obtained from Sigma (St. Louis, MO). The protein carbonyl Oxyblot kit was purchased from Intergen, Catalog No. S7150 (Purchase, NY). All other chemicals were of reagent grade or better.

Cell Culture Experiments. All experiments were conducted in accordance with national legislation, with the National Institutes of Health Guide regarding the care and use of animals for experimental procedures, and with the approval of the University of Kentucky Animal Care and Use Committee. Neuronal cultures were prepared from 18-day-old Sprague−Dawley rat fetuses as described elsewhere.[6] Synthetic Aβ peptides were first solubilized in double-distilled sterile water that had been stirred over Chelex 100 resin by indirect sonication in a cold water bath for a few minutes. The full-length peptide solutions were preincubated for 24 h before addition to cultures, while the shorter peptides were added to cultures immediately after dissolution. The final peptide concentration in the cell cultures was 10 μM in all cases. The same volume of sterile deionized water was added to control cultures. The effects of the full-length peptides on neurons was measured after 24 h of exposure, while those of the smaller peptides were measured after 6 h of exposure.

(a) Assessment of Neuronal Survival. Neuronal survival of 9−11-day-old hippocampal neuronal cultures was evaluated by the trypan blue exclusion assay. Cells were rinsed 3 times with 1 mL of phosphate-buffered saline (PBS; pH 7.4) after exposure to the peptides. Trypan blue (0.4%, Sigma) was added to cells along with 300 mL of PBS and the resultant mixture incubated for 10 min. Sixteen different microscopic areas were counted for uptake of trypan blue. Dead neurons (with a damaged cell membrane) internalize the dye while competent neurons do not. Data are given as percentages of corresponding vehicle-treated values.

(b) Assessment of Mitochondrial Function. Mitochondrial function in 9−11-day-old hippocampal neuronal cultures was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) reduction assay.[12] MTT is reduced to blue formazan by dehydrogenases in functional mitochondria.[13,14] Cells were incubated with MTT at 37 °C for 4 h. After incubation, the unreacted dye and the medium were removed by inverting the plate. Into each well, 1 mL of 0.04 M HCl in 2-propanol was added to solubilize MTT formazan. Absorbance was measured by UV−visible spectrophotometry at a wavelength of 570 nm and a reference wavelength of 630 nm. The data are presented as a percentage of corresponding control values.

(c) Protein Carbonyl Assay (Oxyblot Method). To determine the level of protein oxidation an oxidized protein detection kit (Intergen) was used. This kit is based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine (DNPH).[15,16] The experimental procedure used is described elsewhere.[6] Data are given as percentages of corresponding vehicle-treated values.

EPR Spin Trapping. The purity of the PBN used in these studies was extensively verified as described elsewhere.[3] The peptides were solubilized (165 μM for the full-length peptides, 1 mM for the shorter peptides) in PBS buffer (150 mM NaCl, 5 mM sodium phosphate, pH 7.4) containing PBN (50 mM). The buffer was stirred overnight in the presence of Chelex 100 resin, and the divalent metal ion chelator deferroxamine mesylate (2 mM) was dissolved in the buffer prior to peptide addition. The peptide solutions and control solutions lacking peptides were then incubated in a water bath at 37 °C for different periods varying between 0 and 72 h as required. Aliquots of 300 μL of these solutions were placed into an EPR flat cell at appropriate time points for EPR analysis. The EPR spectra were acquired on a Bruker (Billerica, MA) EMX EPR spectrometer. Instrumental parameters were as follows: microwave power 20 mW, modulation amplitude 0.3−1 G, gain 1×10^5, and conversion time 10.28 ms.

Metal Reduction Assays. The ability of Aβ peptides to reduce Cu(II) was assessed by an assay based upon modification[17] of established protocols.[18] Briefly, freshly solubilized peptides (10 μM) or vitamin C (10 μM), Cu(II) (25 μM), and the Cu(I) indicator bicinchoninic acid (BCA, 250 μM) were coincubated in Dulbecco's phosphate-buffered saline (PBS: $CaCl_2$ 1.19 mM, $MgCl_2$ 0.6 mM, KCl 2.7 mM, KH_2PO_4 1.4 mM, NaCl 137 mM, Na_2HPO_4 7.68 mM, pH 7.4), in 96-well microtiter plates (Costar Corp., Cambridge MA) at 37 °C, for 1 h. Absorbances were then measured using a plate reader (PowerWaveX, Bio-Tek Instruments, Inc., Winooski, VT). The net absorbances (ΔA) were recorded after subtracting out the appropriate absorbance for controls lacking peptide, Cu(II), and BCA, respectively. Cu(I) concentration (μM) was then calculated as $\Delta A \times 10^6/ML$, where M is the known molar absorptivity for Cu(I)−BC (7700 $M^{-1}cm^{-1}$ at 562 nm) and L is the vertical path length (cm).

Electron Microscopy. The ability of the full-length Aβ peptides to form fibrils upon incubation in solution for 24−48 h was assessed by

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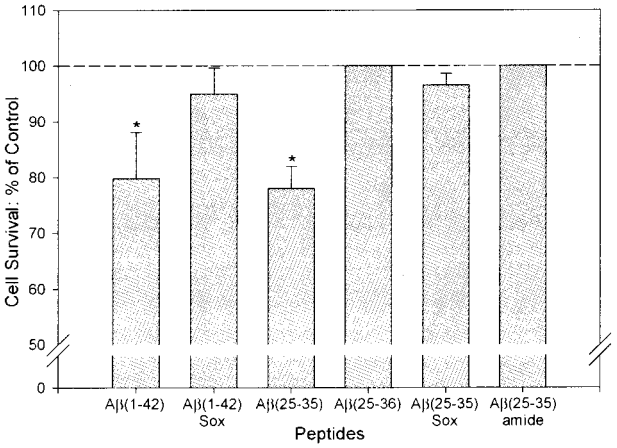

Figure 2. Neurotoxicity in 9−11-day-old hippocampal neurons treated with the various peptides. Neurotoxicity was measured by the trypan blue assay. Dead neurons internalize the dye. The two full-length peptides were preincubated for 24 h before addition to the cell cultures while the smaller peptides were added without any preincubation. Neuronal toxicity was evaluated 24 h after addition of the full-length peptides and 6 h after addition of the smaller peptides. Data shown are the mean of two to four different experiments with each experimental value being the average of three trials. Statistical comparisons were made using the Student's *t*-test. Error bars represent SD values. Only the native Aβ(1−42) and unmodified Aβ(25−35) were significantly toxic with Aβ(25−35) exhibiting levels of toxicity in 6 h that were comparable to those exhibited by the native full-length Aβ(1−42) in 24 h. (*, $p < 0.001$ compared to control values).

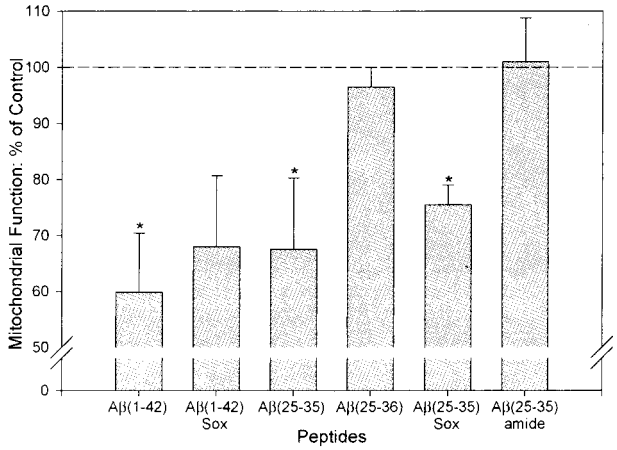

Figure 3. Changes in mitochondrial function, as measured by the MTT reduction assay, in 9−11-day-old cultured hippocampal neurons treated with the various peptides. Neurons with impaired mitochondrial function are unable to reduce the MTT dye effectively relative to healthy cells. The two full-length peptides were preincubated for 24 h before addition to the cell cultures while the smaller peptides were added without any preincubation. Mitochondrial function was evaluated 24 h after addition of the full-length peptides and 6 h after addition of the smaller peptides. Data shown are the mean of two to four different experiments with each experimental value being the average of three trials. Statistical comparisons were made using the Student's *t*-test. Error bars represent SD values. The neurotoxic Aβ(1−42) and Aβ(25−35) significantly inhibited mitochondrial function. The sulfoxides Aβ(1−42)Sox and Aβ(25−35) also lowered mitochondrial function, although to a lesser extent than the corresponding parent peptides. (*, $p < 0.01$ for Aβ-(1−42), Aβ(25−35), and Aβ(25−35)Sox compared to controls; $p < 0.13$ for Aβ(1−42)Sox vs control). Neither Aβ(25−35)amide nor Aβ-(25−36) had any effect on mitochondrial function.

electron microscopy. Aliquots of 5 μL of the peptide solutions that were used for the cell culture experiments were placed on a copper Formvar carbon-coated grid. After 1−1.5 min of incubation at room temperature, excess liquid was drawn off, and samples were counter-stained with 2% uranyl acetate. Air-dried samples were examined in a Hitachi 7000 transmission electron microscope at 75 kV.

Statistical Analysis. A two-tailed Student's *t*-test was used to compare mean values of the various parameters measured. A *p*-value of <0.05 was considered statistically significant.

Results

Peptide-Induced Neuronal Toxicity. The effect of the Aβ peptides on cell survival was measured by exposing the 9−11-day-old cultured hippocampal neurons to various peptides and measuring the cell viability after a certain time. The full-length peptide Aβ(1−42) and its methionine sulfoxide analogue [Aβ-(1−42)Sox] were preincubated for 24 h prior to addition to cells, and their effect on cell viability was measured 24 h after addition. The shorter peptides Aβ(25−35), Aβ(25−36), the methionine sulfoxide analogue Aβ(25−35)Sox, and Aβ(25−35)amide (where the C-terminal carboxylate is replaced by an amide functionality) were added to cell cultures immediately after dissolution, and their effects were measured 6 h after addition. The values for cell viability were measured by two different methods: the trypan blue exclusion assay, which measures cell membrane integrity, and the MTT assay, which measures mitochondrial function. These results are presented in Figures 2 and 3, respectively. As has been reported before,[7−9] both Aβ(25−35) and Aβ(1−42) were toxic to cells by the trypan blue assay and inhibited mitochondrial function, with Aβ(25−35) exhibiting toxicity levels in 6 h that were comparable to toxicity levels exhibited by Aβ(1−42) in 24 h. Interestingly, while the two sulfoxides Aβ(1−42)Sox and Aβ(25−35)Sox were nontoxic by the trypan blue assay, they both compromised mitochondrial function to some extent. Aβ(25−36), with methionine no longer C-terminal, and Aβ(25−35)amide, in which the C-terminal carboxylic acid is replaced by an amide, were nontoxic to cells by either assay.

Peptide-Induced Neuronal Protein Oxidation. Oxidative stress-induced protein oxidation is usually manifested by an increase in the levels of protein carbonyls.[19] The effect of the various peptides on neuronal protein oxidation is represented as protein carbonyl levels in Figure 4. The two toxic peptides Aβ(1−42) and Aβ(25−35) caused a significant increase in membrane protein oxidation, with the effect of Aβ(25−35) being much more robust (2.5 times control values). None of the other peptides that were examined caused any significant increase in protein oxidation.

Spin Trapping Experiments. The electron paramagnetic resonance (EPR) spectra of the reaction products of Aβ peptides with PBN are shown in Figure 5. Confirming earlier studies, both the toxic Aβ(1−42) and Aβ(25−35) produced a four-line spectrum of *tert*-butylhydronitroxide resulting from the breakdown of the PBN−peptide adduct as described elsewhere.[3,9] Interestingly, Aβ(1−42)Sox and Aβ(25−35)Sox, the two peptides that inhibit mitochondrial function, but exhibit no toxicity indexed by trypan blue, nor protein oxidation, also produced the same four-line EPR spectrum. However, in the case of the sulfoxides, the spectra were generated at a slower rate and were less intense than those generated by the corresponding parent peptides. No spectra were generated with the nontoxic Aβ(25−36) and Aβ(25−35)amide even upon prolonged incubation.

Cu(II) Reduction by the Peptides. The ability of Aβ(1−42) to reduce redox metal ions such as Fe(III) and Cu(II) has been implicated in its toxicity.[20] While one or more of the three

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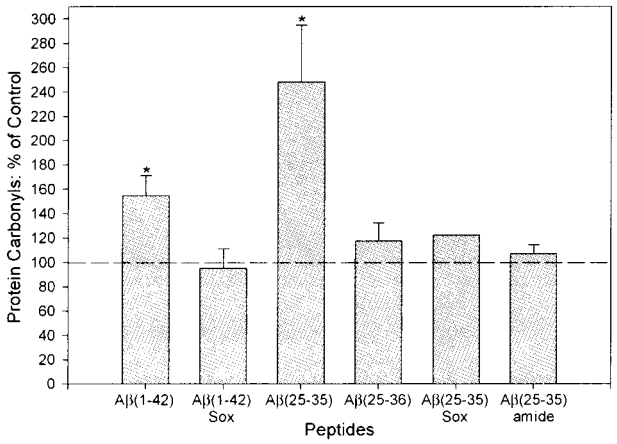

Figure 4. Protein oxidation induced by the peptides as measured by the changes of protein carbonyl content in 9−11-day-old cultured hippocampal neurons treated within the various Aβ peptides. The two full-length peptides were preincubated for 24 h before addition to the cell cultures while the smaller peptides were added without any preincubation. Changes in protein carbonyl levels were evaluated 24 h after addition of the full-length peptides and 6 h after addition of the smaller peptides. Data shown are the mean of two to four different experiments with each experimental value being the average of three trials. Statistical comparisons were made using the Student's *t*-test. Error bars represent SD values. Aβ(1−42) and Aβ(25−35) induced significant protein oxidation, with the smaller peptide being much more damaging. (*, $p < 0.00001$ vs controls). There was no significant increase in protein carbonyl content compared to control values for the other peptides.

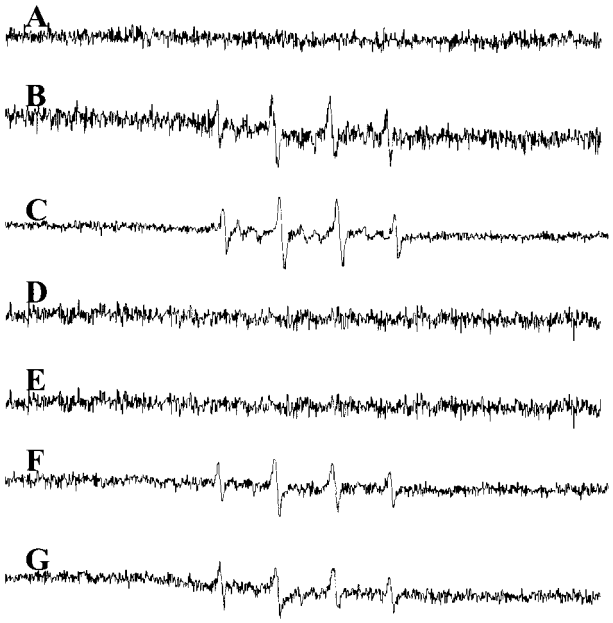

Figure 5. EPR spectra obtained in various spin-trapping experiments with PBN (50 mM) upon incubation at 37 °C in chelexed PBS for varying times. All solutions contained deferroxamine (2 mM). (A) Control PBN solution, lacking peptide, after 72 h. (B) Aβ(1−42) (165 μM) after 60 h. (C) Aβ(25−35) (1 mM) after 24 h. (D) Aβ(25−36) (1 mM) after 48 h. (E) Aβ(25−35)amide (1 mM) after 48 h. (F) Aβ(1−42)Sox (165 μM) after 72 h. (G) Aβ(25−35)Sox (1 mM) after 48 h. Instrumental parameters were as follows: microwave power 20 mW; modulation amplitude 0.3−1 G; gain 1×10^5; conversion time 10.28 ms. All the peptides that inhibited mitochondrial function [Aβ(1−42), Aβ(25−35), Aβ(1−42)Sox, and Aβ(25−35)Sox] yielded a four-line EPR spectrum.

histidine residues (Figure 1a) is believed to be involved in the strong Cu(II) binding by the peptide,[21] the source of the electron transferred to Cu(II) was not identified. Only a few amino acids, viz. tyrosine, tryptophan, methionine, and cysteine, are oxidized at modest potentials. Of these, only the methionine and tyrosine residues are present in Aβ(1−42) and are likely candidates for the electron-transfer reaction. We measured the ability of various peptides to reduce Cu(II), relative to vitamin C (as a positive control). The results of these experiments are shown in Figure 6. Confirming previous findings of others,[17] freshly solubilized Aβ(1−42) reduced Cu(II) to Cu(I) with about 65% efficiency within 1 h. Only freshly solubilized Aβ(1−42) was able to reduce Cu(II); peptide solutions that had been incubated for over 24 h did not reduce any Cu(II). The freshly solubilized Aβ(1−42)Sox, containing oxidized methionine, did not reduce any Cu(II). All the smaller peptides, Aβ(25−36), Aβ(25−35)amide, and the toxic Aβ(25−35), that contain methionine but lack the tyrosine and the Cu(II) binding histidines did not reduce any Cu(II). As reported,[20] Aβ(1−28), which contains the histidine residues of Aβ(1−42) and the tyrosine, but no methionine, did not reduce Cu(II) (data not shown).

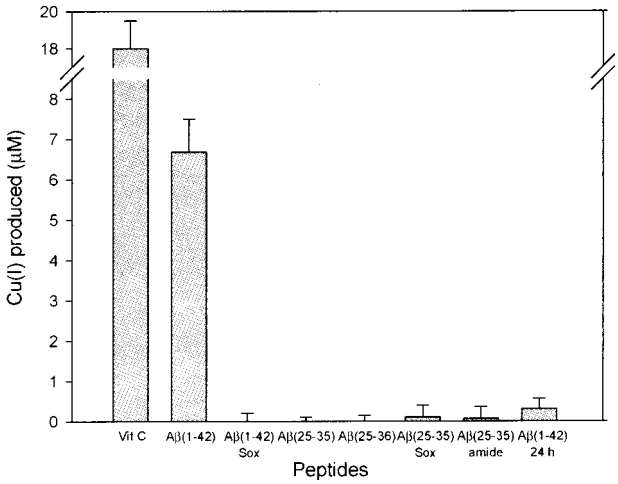

Figure 6. Reduction of Cu(II) by peptides. Freshly solubilized peptides (10 μM) or vitamin C (10 μM), Cu(II) (25 μM), and the Cu(I) indicator BCA (250 μM) were coincubated in Dulbecco's PBS (pH 7.4), at 37 °C, for 1 h, and the absorbance of the BCA−Cu(I) complex was measured. The net absorbances (ΔA) were recorded after subtracting out the appropriate absorbances for controls lacking peptide, Cu(II), and BCA, respectively. Cu(I) concentration (μM) was then calculated as $\Delta A \times 10^6/LM$, where L is the vertical path length (cm) and M is the known molar absorptivity for Cu(I)−BCA (7700 $M^{-1}cm^{-1}$ at 562 nM). Freshly solubilized Aβ(1−42) reduced Cu(II) with about 65% efficiency within 1 h, while Aβ(1−42) that had been preincubated for 24 h did not reduce any significant amount of Cu(II). None of the other Aβ peptides reduced Cu(II). Data are expressed as the average of three replicate wells from a single microplate. Error bars represent SD values.

Fibril Formation. The ability of Aβ(1−42) to form fibrils in solution has often been linked to its toxicity.[5,22] Therefore, we investigated the ability of the nontoxic Aβ(1−42)Sox to form fibrils in solution. Upon incubation for 24−48 h (the time frame in which cell toxicity is measured in our experiments), Aβ(1−42)Sox formed an extensive network of fibrils similar to native Aβ(1−42) as shown in Figure 7.

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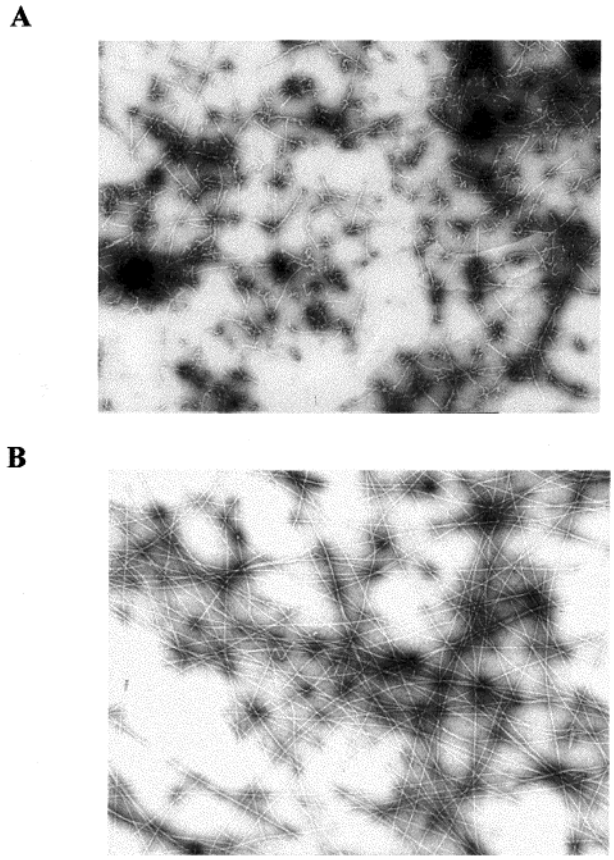


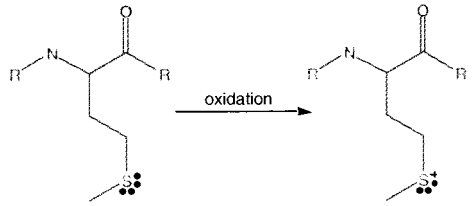
Figure 7. Electron microscopy of (A) Aβ(1−42) and (B) Aβ(1−42)-Sox incubated at 37 °C for 48 h. Both peptides form an extensive network of fibrils.

Discussion

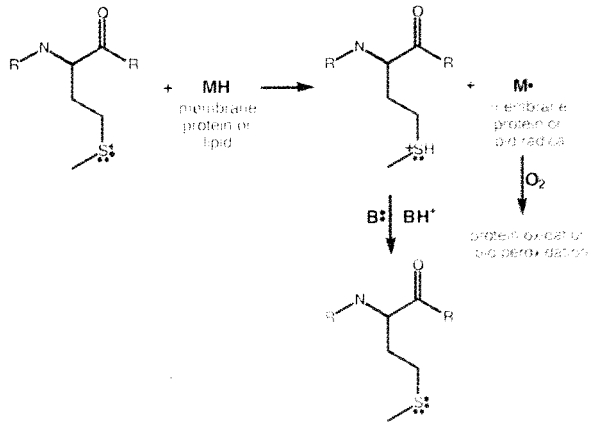
Aβ(25−35) has been frequently used in investigations of Aβ properties since it is a less expensive and more easily manipulated substitute for the native full-length peptide, Aβ(1−42), the latter being implicated in the pathogenesis of AD. Implicit in these experiments is the assumption that the mechanism of action of Aβ(25−35) is similar to that of Aβ(1−42). Indeed, Aβ(25−35) mimics the toxicological and aggregation properties of the full-length peptide, though these characteristics are exaggerated; i.e., the shorter peptide is more toxic to cultured neurons, exhibits toxicity earlier, causes more membrane protein oxidation, and aggregates faster than the native Aβ(1−42).

We reasoned that studying the factors that cause the smaller peptide to be much more potent than the full-length peptide may help us understand the mechanism of toxicity. Earlier studies from our laboratory established the link between the methionine residue of Aβ(25−35), Aβ(1−40), and Aβ(1−42) and the oxidative stress and neurotoxicity caused by these peptides.[3,7−9] It followed that factors that enhance the deleterious effects in the smaller Aβ(25−35) may involve the environment of the methionine residue. The main difference in the environment of methionine between Aβ(1−42) and Aβ(25−35) is that methionine is a C-terminal residue in the smaller peptide (Figure 1a). To investigate whether the terminal nature of the methionine plays a role in its enhanced toxicity, we tested Aβ(25−36) (Figure 1a), in which the next amino acid in the series (valine) is added to the C-terminus of methionine, and Aβ(25−35)amide (Figure 1b), in which the terminal carboxylate functionality is converted into an amide functionality. Both these modifications completely blocked the oxidative stress and neurotoxicity properties of the peptide, confirming that the terminal nature of methionine is indeed important to these properties of the smaller peptide. In contrast, such a simple increase in the length of the native Aβ(1−40) to Aβ(1−42) does not significantly affect its toxicological properties.[9,23]

Scheme 1



Scheme 2



The role of methionine in the toxicological properties of Aβ most likely involves an oxidative event at the sulfur atom. We therefore investigated the effect of oxidizing the sulfide of methionine to a sulfoxide (Figure 1c,d) in both Aβ(1−42) and Aβ(25−35) on their behavior. The result of this modification was similar in both cases. While cellular toxicity and membrane protein oxidation were blocked, mitochondrial function was still affected, albeit to a lesser extent than the corresponding parent peptides. Interestingly, all the peptides that inhibit mitochondrial function also generate an EPR spectrum upon incubation with PBN in cell free solutions.

The event that may initiate the oxidation of methionine is not yet clear. One or more of several reactive oxygen species present in biological systems may play a role. Also, Cu(II), postulated to bind to histidines of Aβ(1−42), may be involved. The single electron oxidation of a sulfide to a sulfuranyl radical cation may be represented as in Scheme 1. Usually such S-oxidized radical cation species form reversible complexes via three-electron, two-center bonding to water, sulfides, or other Lewis bases and tend to be very short-lived.[24] If this radical cation is sufficiently long-lived, it can cause oxidative damage by several mechanisms that have been theoretically examined in the literature.[25] For example, such sulfuranyl radical cations can abstract a H atom from other proteins (leading to protein oxidation) or lipids (leading to lipid peroxidation) in the membrane bilayer as shown in Scheme 2. The resultant sulfonium ion is highly acidic (p$K_a \cong -5$)[26] and can be easily deprotonated even by weak bases to restore the methionine. Such a catalytic role for methionine, coupled with compromised free-

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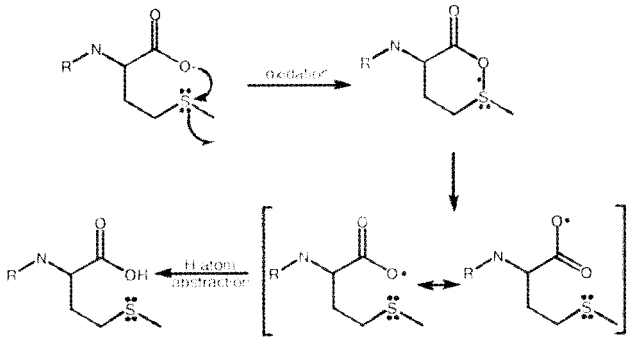
Scheme 3



radical antioxidant mechanisms in AD victims, could explain the vast amount of neuronal damage observed around amyloid deposits in the AD brain.

The ease of oxidation of the methionine sulfur and the stability of the resultant radical cation can be expected to affect the ability of the peptide to mediate oxidative damage. In the case of Aβ(25−35), the methionine is a terminal residue. At neutral pH, the C-terminus of the peptide is a carboxylate anion. The negatively charged oxygen can aid in the oxidation of the sulfur and in the subsequent stabilization of the resultant radical cation as shown in Scheme 3. Six-membered sulfuranyl radical cation intermediates have been reported.[27] Such *strong* neighboring group assistance to oxidation would not be possible in Aβ(25−36), where methionine is not C-terminal, or in Aβ(25−35)amide, which contains the neutral amide terminus. Consistent with this notion, Figures 2−5 show that neither of these modified Aβ(25−35) peptides is toxic to cells, causes mitochondrial dysfunction, induces neuronal protein oxidation, or leads to an EPR spectrum in the presence of the spin trap PBN. Also, in the case of Aβ(25−35)Sox, since the methionine is already in the oxidized form, further oxidation, though possible, would be difficult. While insight into the mechanism of oxidative damage and neurotoxicity of the shorter Aβ(25−35) has been gained, the exact identity of the oxidizing species of this non-AD peptide remains unknown.

With respect to the full-length Aβ(1−42), since the methionine is not terminal and therefore lacks the nearby carboxylate functionality, there is less neighboring group assistance for oxidation of the sulfur, and consequently, the methionine is likely to be more difficult to oxidize. As noted above, perhaps redox metal ions such as Cu(II) play a role in the oxidation of methionine in this case. There is no evidence as yet to indicate that methionine is the source (direct or indirect) of the electron transferred to Cu(II) during the reduction of the metal ion by freshly solubilized Aβ(1−42). However, we previously showed that the single atom substitution of S in methionine residue 35 to CH_2, i.e., norleucine in place of methionine in Aβ(1−42), resulted in a peptide that is nonoxidative and nonneurotoxic[7] even in the presence of added Cu(II).[3] This norleucine-substituted Aβ(1−42) still contains the purported Cu(II) binding histidines and the easily oxidizable tyrosine. Thus, in the absence of methionine, Cu(II) appears to be a bystander.[3] Hence, if redox metal ions such as Cu(II) do play a role in Aβ(1−42) toxicity, then the mechanism of metal ion-mediated toxicity has to involve methionine. However, Aβ(1−42) incubated in solution for more than 24 h no longer reduces metal ion, while it has been well documented that incubation of Aβ(1−42) for long periods (up to 7 days) prior to exposure to cells enhances its toxicity.[28] Therefore, although the oxidation of Aβ(1−42) by a redox metal ion may explain the origin of the free-radical process that we believe to be involved in the oxidative stress exerted by the peptide, the role of metal ion reduction in Aβ(1−42) toxicity is not clear. Other amino acids, such as tyrosine (residue 10), conceivably could be involved in Cu(II) reduction. However, other factors must be involved in the metal ion reduction since Aβ(1−28), which does not contain methionine, but does have tyrosine and the Cu(II) binding histidines, does not reduce Cu(II) nor does Aβ(1−42)Sox. More research is necessary to address this issue. But reduction of transition metal ions clearly plays no role in the toxicity of Aβ(25−35), which does not contain the strong Cu(II) binding sites identified in Aβ(1−42), since, unlike Aβ(1−42), this shorter peptide does not reduce Cu(II) to Cu(I).

Fibril formation by the Aβ peptides has often been postulated to be an essential prerequisite for their toxic effects on neurons. As a consequence, much research has been devoted toward inhibiting fibril formation as a tactic to prevent Aβ toxicity. Indeed, several such approaches have succeeded in inhibiting the lethal effects of these peptides. However, we earlier reported that replacing the methionine in Aβ(1−42) with structurally similar norleucine (S replaced by CH_2) had no effect on fibril formation but completely abrogated the damage to cells.[29] Similarly, we now show that Aβ(1−42)Sox (in which the sulfur of methionine is oxidized to sulfoxide) forms an extensive network of fibrils (Figure 7) similar to the native Aβ(1−42), but the former peptide is not lethal to cells, nor does it cause increased protein oxidation, unlike Aβ(1−42). Furthermore, minor modifications around the methionine residue in Aβ(25−35), such as increasing its length by one amino acid (valine) in Aβ(25−36) or converting the carboxylate terminal to an amide as in Aβ(25−35)amide, completely block the poisonous effects of the peptide. Our results seem to suggest that the factors that govern the toxicity of these peptides may be molecular in nature. Prevention of toxicity of Aβ peptides in other studies by modifying their aggregation properties conceivably could be due to the change in the electronic nature of the environment surrounding methionine caused by these modifications.

Since not all methionine-containing peptides are lethal, it follows that additional contributing factors must be present in Aβ peptides that render the methionine harmful. These supplementary factors in Aβ could be structural, due to the unique sequence of amino acids relative to methionine. It has been suggested that perhaps the proximity of this methionine in Aβ peptides to a glycine (residue 33) on an adjacent peptide chain may be the critical factor.[25] The hypothesis was that if the lifetime of the highly reactive sulfuranyl radical cation generated upon oxidation of the methionine sulfur was directly proportional to the damage caused by the peptide, then perhaps H atom abstraction by the sulfuranyl radical from the α-carbon of glycine, resulting in the formation of a glycyl radical, would prolong the life of a radical species and thus increase the damage. Such an α-H atom abstraction, which in the presence of oxygen would form a peroxyl free radical, would not be possible from other amino acids in a hydrophobic β-sheet environment since the formation of a radical species at the α-carbon requires that the geometry around this α-carbon be planar. All other amino acids with bulky side chains would be unable to adopt this planar geometry around the α-carbon in a β-sheet environment. Studies to test this notion are in progress.

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Conclusion

Aβ(1−42)-associated oxidative stress and neurotoxicity may unite the known oxidative stress under which the AD brain exists and the centrality of Aβ(1−42) to the pathogenesis of this neurodegenerative disorder.[1−3] Although the toxicological and oxidative stress effects of Aβ(1−42) and Aβ(25−35) are quite similar, our experiments reveal that there may be two different mechanisms that govern their toxicological properties. Only one of these mechanisms is probably operative in the truncated peptide containing C-terminal methionine. However, both mechanisms may be operative in the full-length peptide. Both mechanisms seem to be intimately associated with the environment around the methionine residue. Studies conducted on Aβ-(25−35) may be invaluable in obtaining some molecular insights into the mechanism of toxicity. However, given the distinctly different molecular mechanism involved in the oxidative and neurotoxic properties of this shorter peptide, it may be misleading and nonproductive to explore therapeutic strategies for the treatment of AD utilizing the smaller, non-native peptide.

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