Neurotoxicity and oxidative stress in D1M-substituted Alzheimer’s Aβ(1-42): relevance to N-terminal methionine chemistry in small model peptides

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Abstract

Small model peptides containing N-terminal methionine are reported to form sulfur-centered-free radicals that are stabilized by the terminal N atom. To test whether a similar chemistry would apply to a disease-relevant longer peptide, Alzheimer’s disease (AD)-associated amyloid beta-peptide 1-42 was employed. Methionine at residue 35 of this 42-mer has been shown to be a key amino acid residue involved in amyloid beta-peptide 1-42 [Aβ/H9252](1-42)-mediated toxicity and therefore, the pathogenesis of AD. Previous studies have shown that mutation of the methionine residue to norleucine abrogates the oxidative stress and neurotoxic properties of Aβ/H9252(1-42). In the current study, we examined if the position of methionine at residue 35 is a criterion for toxicity. In doing so, we tested the effects of moving methionine to the N-terminus of the peptide in a synthetic peptide, Aβ/H9252(1-42)D1M, in which methionine was substituted for aspartic acid at the N-terminus of the peptide and all subsequent residues from D1 to L34 were shifted one position towards the carboxy-terminus. Aβ/H9252(1-42)D1M exhibited oxidative stress and neurotoxicity properties similar to those of the native peptide, Aβ/H9252(1-42), all of which are inhibited by the free radical scavenger Vitamin E, suggesting that reactive oxygen species may play a role in the Aβ-mediated toxicity. Additionally, substitution of methionine at the N-terminus by norleucine, Aβ/H9252(1-42)D1Nle, completely abrogated the oxidative stress and neurotoxicity associated with the Aβ/H9252(1-42)D1M peptide. The results of this study validate the chemistry reported for short peptides with N-terminal methionines in a disease-relevant peptide.

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1. Introduction

Alzheimer’s disease (AD) brain is under extensive oxidative stress [8,17]. Amyloid β-peptide (1-42) [Aβ(1-42)] is abnormally accumulated in the AD brain and is the primary component of extracellular senile plaques—a pathological hallmark of the disease. Aβ(1-42) is produced from the proteolytic processing of amyloid precursor protein, a ubiquitously expressed transmembrane glycoprotein, by β- and γ-secretase [23]. Aβ(1-42) has been shown to cause protein oxidation, lipid peroxidation, and is toxic to neurons in vitro [8], all of which is inhibited by the chain-breaking antioxidant Vitamin E [1,2,28,29]. Consequently, Aβ(1-42) has been implicated as a causative agent in the disease; however, the mechanism of toxicity remains elusive.

Methionine 35 has been shown to play a critical role in the oxidative stress and neurotoxicity properties exhibited by Aβ(1-42) [5–7,13–15,25]. Substitution of methionine 35 by norleucine, which replaces the sulfur atom of methionine by a methylene group, has been shown to abolish the oxidative stress and neurotoxicity induced by Aβ(1-42) [27]. Additionally, in vivo substitution of methionine by cysteine in a mutant Aβ-peptide expressed in C. elegans attenuated the
oxidative stress properties detected in *C. elegans* expressing the native Aβ(1–42) peptide [27]. These effects were found to be independent of the ability of the mutant peptides to form fibrils.

Studies have shown that the α-helical environment of methionine in the C-terminus is important for the oxidative and neurotoxic properties of Aβ(1–42) [14,20]. Additionally, the hydrophobic environment of the lipid bilayer is also important for the methionine 35 mediated oxidative stress and neurotoxicity [13]. Furthermore, in an anti-parallel β-sheet conformation of Aβ(1–42), it has been proposed that methionine may act as an electron donor for reduction of Cu(II) to Cu(I) leading to the formation of a sulfur-centered radical cation which is stabilized by transfer of a hydrogen atom from the α-carbon of glycine 33 to form a carbon centered radical on the α-carbon of glycine 33 [3,22]. These computational studies were supported by experimental results in which mutation of glycine 33 to valine significantly decreased the oxidative stress and neurotoxicity mediated by the peptide [15]. Finally, computational studies have shown that long distance electron transfer from methionine to the Cu(II) binding site in the N-terminus of the peptide is mediated through phenylalanine 20 [21]. Recent studies from our laboratory have suggested that this latter propose mechanism may not be critically important in the oxidative stress and neurotoxic properties of Aβ(1–42) [2].

In the current study, we evaluated the relationship between the oxidative and neurotoxic properties of Aβ(1–42) and the placement of methionine within the primary sequence of the peptide. Aβ(1–42)D1M, and all subsequent amino acid residues from D1 to L34 were shifted one position towards the carboxy-terminus [Fig. 1]. We report the oxidative stress properties, as measured by protein carbonyl and 4-hydroxynonenal immunoreactivity, of Aβ(1–42) and Aβ(1–42)D1M. Mutation of the methionine residue at the N-terminus of the peptide to norleucine resulted in attenuation of the oxidative stress and neurotoxic properties exhibited by Aβ(1–42)D1M.

### 2. Materials and methods

#### 2.1. Chemicals

All chemicals were of the highest purity and were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted. Peptides were purchased from Anaspec (San Jose, CA, USA) with HPLC and MS verification of purity. The peptides were stored in the dry state at −20 °C until use. The OxyBlot protein oxidation detection kit was purchased from Chemicon International (Temecula, CA, USA). Anti-4-hydroxynonenal was purchased from Alpha Diagnostic International (San Antonio, TX, USA).

#### 2.2. Cell culture experiments

Neuronal cultures were prepared from 18-day-old Sprague–Dawley rat fetuses [27–29]. Aβ peptides were dissolved in sterile water that had been stirred over Chelex:100 resin. The peptides were preincubated for 24 h at 37 °C prior to addition to cultures. The final concentration of the peptides in the cell culture was 10 μM, and the effects of Aβ on the neurons were measured after 24 h of exposure.

Mitochondrial function was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, MTT was added to each well with a final concentration of 1.0 mg/ml, and incubated for 1 h. The dark blue formazan crystals formed in intact cells were extracted with 250 μl of dimethyl sulfoxide (DMSO), and the absorbance was read at 595 nm with a microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA).

#### 2.3. Protein carbonyls

Samples (5 μl) were incubated for 20 min at room temperature with 5 μl of 12% sodium dodecyl sulfate (SDS) and 10 μl of 2,4-dinitrophenylhydrazine (DNPH) that was diluted 10 times with chelexed water from a 200 mM stock.

### Fig. 1. The sequences of Aβ(1–42), Aβ(1–42)D1M and Aβ(1–42)D1Nle.
The samples were neutralized with 7.5 μl of neutralization solution (2 M Tris in 30% glycerol). The resulting sample (250 ng) was loaded per well in the slot blot apparatus. Samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline containing 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 (wash blot) for 1 h and incubated with a 1:100 dilution of anti-DNP polyclonal antibody in wash blot for 1 h. Following completion of the primary antibody incubation, the membranes were washed three times in wash blot for 5 min each. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in wash blot and added to the membrane for 90 min. The membrane was washed in wash blot three times for 5 min and developed using Sigmafast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop (San Jose, CA, USA), and quantitated with Scion Image.

2.4. 4-Hydroxynonenal

Samples (10 μl) were incubated with 10 μl of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol. The resulting sample (250 ng) was loaded per well in the slot blot apparatus. Samples were loaded onto a nitrocellulose membrane under vacuum pressure. The membrane was blocked with 3% (w/v) BSA in wash blot for 1 h and incubated with a 1:5000 dilution of anti-4-hydroxynonenal (HNE) polyclonal antibody in wash blot for 90 min. Following completion of the primary antibody incubation, the membranes were washed three times in wash blot for 5 min each. An anti-rabbit IgG alkaline phosphatase secondary antibody was diluted 1:8000 in wash blot for 1 h. The membrane was washed in wash blot three times for 5 min and developed using Sigmafast Tablets (BCIP/NBT substrate). Blots were dried, scanned with Adobe Photoshop (San Jose, CA, USA), and quantitated with Scion Image.

2.5. Thioflavin T binding assay

The thioflavin T (ThT) binding assay was performed according to the method of Levine [16]. A solution of 50 mM glycine, pH 8.5, containing 5 μM ThT was added to Aβi peptides incubated in solution for 24 h to a final peptide concentration of 2.5, 5, 7.5, and 10 μM. The resulting fluorescence was measured with λex = 440 and λem = 485 nm with a SpectraMax GeminiXS microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Copper reduction

The ability of the Aβi peptides to reduce copper was measured by the BCA method described by Huang et al. [12]. Briefly, the peptides (10 μM), copper(II) (25 μM), and the copper(II) indicator BCA (250 μM) were co-incubated in phosphate-buffered saline (PBS) pH 8.0 in a 96-well microtiter plate for 1 h at 37 °C. Controls were conducted with ascorbate (10 μM) in place of the peptide. Absorbance was measured at λ = 562 nm (Bio-Tek Instruments, Winooski, VT, USA). The concentration of Cu(I) was calculated based on the molar absorptivity ε = 7700 M−1 cm−1.

2.7. Electron microscopy

Electron microscopy was used to assess the ability of the Aβi peptides to form fibrils upon incubation in solution for 24 h. Aliquots of 5 μl of the peptide solutions that were used for the cell culture experiments were plated on a copper mesh formvar carbon-coated grid. After 1–1.5 min of incubation at room temperature, excess liquid was drawn off, and samples were counterstained with 2% uranyl acetate. Air dried samples were examined in a Philips Tecnai Biotwin 12 transmission electron microscope (FEI, Eindhoven, Netherlands) at 80 kV. Images were captured with a 2 K × 2 K digital camera (Advanced Microscopy Techniques).

2.8. Analysis of DNA fragmentation

Cultures were rinsed three times in PBS, fixed with 4% paraformaldehyde for 10 min at 37 °C, rinsed, and stained with Hoechst 33258 (1 mg/ml) for 10 min at room temperature. The staining was visualized using a fluorescent microscope with a DAPI filter. The nuclear staining with Hoechst 33258 provided morphological discrimination between normal and apoptotic cells as described by Darzynkiewicz et al. [10].

2.9. Statistics

ANOVA was used to determine statistical significance. P values less than 0.05 were considered significant.

3. Results

3.1. Presence of methionine corresponds with loss of cell viability

As shown in Fig. 2, MTT reduction by Aβi(1-42) was approximately 35% of control, indicating Aβi(1-42)-induced mitochondrial dysfunction. Likewise, Aβi(1-42)D1M demonstrated a similar loss in cell viability. Conversely, treatment of the neuronal cell cultures with Aβi(1-42)D1Nle, which did not contain methionine, did not result in any significant loss of cell viability.

3.2. Vitamin E protects neurons against oxidative stress induced by the methionine-containing Aβi peptides

Oxidative stress was indexed by protein carbonyls and the lipid peroxidation product 4-hydroxynonenal (HNE) [9].
Fig. 2. Neurotoxic properties of Aβ peptides as measured by MTT reduction. Treatment of neurons with 10 μM Aβ(1-42) or Aβ(1-42)D1M showed a significant decrease in MTT reduction. Aβ(1-42)D1Nle, in contrast, showed no such change. (*) P < 0.001, analysis of variance, n = 4. The statistical comparison was performed between control and Aβ treatment data sets.

Oxidative stress can lead to a variety of detrimental effects on neurons including protein crosslinking, decreased protein turnover, loss of protein function, altered redox potential, disruption of Ca2+ homeostasis, and ultimately cell death [9]. Aβ(1-42) and Aβ(1-42)D1M both resulted in a significant increase in protein oxidation and HNE compared to control (Figs. 3 and 4, respectively). Both effects were completely inhibited by pretreatment of the neuronal cell cultures with 50 μM of the chain-breaking antioxidant Vitamin E, consistent with the notion that the protein oxidation and lipid peroxidation were mediated by reactive oxygen species (ROS). In contrast, no change in protein carbonylation or HNE levels were observed in cultures treated with Aβ(1-42)D1Nle, which does not contain methionine.

3.3. Aβ-peptide mutants were inefficient at copper reduction

It has been proposed that Aβ(1-42)-induced oxidative stress and neurotoxicity is a result of the ability of the peptide to reduce Cu(II) [12,19]. As a result, the ability of all the peptides tested to reduce copper relative to Vitamin C was evaluated (Fig. 5). Aβ(1-42) was able to reduce 37.2% of the Cu(II) present relative to the Vitamin C control. Placement
of methionine at the N-terminus [Aβ(1-42)D1M] of Aβ(1-42) significantly decreased the ability of the native peptide to reduce Cu(II) (9.6% of Vitamin C control). Additionally, substitution of the methionine residue in Aβ(1-42)D1M by norleucine also resulted in a loss of ability of the peptide to reduce Cu(II) (7.3% of Vitamin C control). Taken together these results indicate that the presence of methionine at position 35 in the native peptide may play a critical role in the ability of the native peptide, Aβ(1-42), to reduce Cu(II) to Cu(I).

3.4. Aβ-peptide fibril formation demonstrates lack of correlation with oxidative stress and neurotoxic properties

Thioflavin T binding is a measure of the β-sheet formation of the peptides and provides insight into their secondary structure and, consequently, the ability of the peptides to form fibrils. All of the peptides produced an increase in β-sheet formation with respect to concentration except...
Fig. 8. Phase contrast microscopy showing morphological changes in the neuronal cultures following 24 h treatment with 10 μM Aβ peptides. Aβ(1-42) or Aβ(1-42)D1M induce loss of neuronal connections and lead to dying cells (Panels B and C, respectively). The effects of Aβ(1-42)D1Nle are represented by Panel D and show cellular morphology similar to that of control, which is represented by Panel A.

Aβ(1-42)D1Nle (Fig. 6). Minimal increase in thioflavin T fluorescence was detected for Aβ(1-42)D1Nle, but, the fluorescence did not change with respect to peptide concentration. These results were confirmed by electron microscopy studies (Fig. 7) showing extensive fibril formation of Aβ(1-42) and Aβ(1-42)D1M. Aβ(1-42)D1Nle showed some fibril formation, but to a much lesser extent than did the native peptide.

3.5. Methionine containing Aβ-peptides induced apoptotic cell death

Morphological changes in the neuronal cell cultures were evaluated by phase contrast microscopy following 24 h treatment with the peptides (Fig. 8). Treatment with Aβ(1-42) and Aβ(1-42)D1M showed extensive loss of neuronal connections as well as the presence of dying cells. Treatment of

Fig. 9. Hoechst staining for DNA fragmentation in the neuronal cultures following 24 h treatment with 10 μM Aβ peptides. DNA fragmentation was detected for neurons treated with Aβ(1-42) and Aβ(1-42)D1M, which correspond to Panel B and C, respectively. Aβ(1-42)D1Nle treatment (Panel D) showed no DNA fragmentation, similar to that of the control, which is represented by Panel A.
neurons with Aβ(1-42)D1Nle resulted in morphology similar to that of control. Hoechst staining was used to detect the presence of DNA fragmentation as an indication of apoptotic cells (Fig. 9). All of the peptides resulted in neuronal DNA fragmentation and apoptotic bodies except for Aβ(1-42)D1Nle, consistent with the lack of oxidative stress and consonant with the morphological studies of this latter peptide in which the S atom of methionine is replaced by a methylene moiety.

4. Discussion

Extensive oxidative stress is prevalent in Alzheimer’s disease (AD) [8,11,17,24]. Aβ(1-42) is toxic to neurons and induces protein oxidation and lipid peroxidation in vitro [8], and in vivo [27], consistent with widely held notion that Aβ(1-42) is central to the pathogenesis of AD [8,17,23]. Methionine 35 is critical for the oxidative stress and neurotoxic properties of Aβ(1-42) [2,3,5–7,13–15,22,26]. These studies include investigation of the α-helical secondary structure of the peptide and the hydrophobic environment of methionine 35 [13,14,20]. Taken together, these studies suggest that the specific placement of methionine at residue 35 and the chemistry of methionine within this environment is critical to the oxidative stress and neurotoxic properties of Aβ(1-42). In the current study, we evaluated the effect of moving the methionine residue within the primary sequence of Aβ(1-42) on the oxidative stress and neurotoxic properties of the peptide. In Aβ(1-42)D1M, the methionine residue was shifted to the N-terminus of the peptide.

Confirming previous findings [2,8,13,14,25–29], Aβ(1-42) induced a significant increase in both protein oxidation and lipid peroxidation coupled with a significant loss in cell viability. Aβ(1-42)D1M exhibited a significant loss in cell viability and a significant increase in both protein carbonyl and HNE immunoreactivity, similar to that induced by Aβ(1-42). The oxidative effects of both peptides were inhibited by pretreatment of neuronal cell cultures with the free radical chain-breaking antioxidant Vitamin E, suggesting that reactive oxygen species play a role in the oxidative stress and neuronal toxicity induced by both peptides. Additionally, substitution of the methionine residue at the N-terminus of the peptide by norleucine, Aβ(1-42)D1Nle, abrogated the oxidative stress and neurotoxic properties of the native peptide, suggesting that methionine is involved in the oxidative stress and neuronal toxicity induced by Aβ(1-42)D1M.

This finding is further supported by the copper reduction results of our study. It has been proposed that Aβ(1-42) induced oxidative stress and neurotoxicity are due to the ability of the peptide to reduce Cu(II) to Cu(I) [10,17]. In this study, we have shown that movement of methionine 35 to the N-terminus of Aβ(1-42) results in a decrease in the ability of the peptide to reduce Cu(II) relative to the native peptide. However, Aβ(1-42)D1M induces oxidative stress and neurotoxic effects similar to those induced by Aβ(1-42). These results support the previous findings of our laboratory for the critical role of methionine in Aβ(1-42)-mediated oxidative stress and neurotoxicity, suggesting that, while Cu(II) may be involved, these effects are consequence of methionine rather than the ability of the peptide to reduce Cu(I) [4–7].

In small model peptides containing N-terminal methionine a sulfur radical cation of methionine (sulfuranyl free radical) can be stabilized by the N-terminal nitrogen to yield a nitrogen-sulfur radical cation [18]. This could result in an intramolecular Aβ(1-42) proton abstraction from the neighboring methyl or methylene group on methionine to form an α(alkylthio)alkyl radical of methionine (–CH2–CH2–S–CH2 or –CH2–CH–S–CH3, respectively) (Fig. 10),

Fig. 10. Schematic representation for the one-electron reduction of an N-terminal methionine by Cu(II) to form a sulfuranyl radical that is stabilized by formation of a sulfur-nitrogen radical cation, leading subsequently to the formation of α(alkylthio)alkyl radicals. See text.
which could react with molecular oxygen leading to the formation of a highly reactive peroxyl radical [18]. Consequently, the chemical interaction of the methionine residue with the free amino terminus of the peptide can result in the formation of peroxyl radicals leading to the oxidative stress and neuronal toxicity exhibited by Aβ(1-42)D1M. This is supported by the lack of neurotoxicity and oxidative stress associated with Aβ(1-42)D1Nle, reported here, in which the sulfur atom of methionine is replaced by a methylene group. Thus, the current study shows that the chemistry of N-terminal methionine in small peptides is replicated in the 42-mer Aβ(1-42)D1M.

In summary, movement of the methionine residue within the primary amino acid sequence of Aβ(1-42) to the N-terminus did not significantly alter the oxidative stress and neurotoxicity mediated by the native peptide. This finding is a result of the chemistry of methionine associated with the environment in which the residue is located. In this case, the influence of N-terminal methionine within the peptide is likely to account for these observations. Taken together, these results validate in AD-relevant peptide the reported influence of N-terminal methionine within short peptides, and further suggest that the secondary structure of Aβ(1-42) plays a critical role in Aβ(1-42)-mediated oxidative stress and neurotoxicity.

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References

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