

## Review

# The critical role of methionine 35 in Alzheimer's amyloid $\beta$ -peptide (1–42)-induced oxidative stress and neurotoxicity

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## Abstract

Amyloid beta-peptide (1–42) [ $A\beta$ (1–42)] has been proposed to play a central role in the pathogenesis of Alzheimer's disease, a neurodegenerative disorder associated with cognitive decline and aging. AD brain is under extensive oxidative stress, and  $A\beta$ (1–42) has been shown to induce protein oxidation, lipid peroxidation, and reactive oxygen species formation in neurons and synaptosomes, all of which are inhibited by the antioxidant vitamin E. Additional studies have shown that  $A\beta$ (1–42) induces oxidative stress when expressed in vivo in *Caenorhabditis elegans*, but when methionine 35 is replaced by cysteine, the oxidative stress is attenuated. This finding coupled with in vitro studies using mutant peptides have demonstrated a critical role for methionine 35 in the oxidative stress and neurotoxic properties of  $A\beta$ (1–42). In this review, we discuss the role of methionine 35 in the oxidative stress and neurotoxicity induced by  $A\beta$ (1–42) and the implications of these findings in the pathogenesis of AD.

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**Keywords:** Amyloid beta-peptide; Alzheimer's disease; Oxidative stress, including protein oxidation and lipid peroxidation; Methionine

## 1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and affects over 4 million Americans and a growing elderly population worldwide. In the coming decades, with no cure, these numbers are projected to increase to epidemic proportions [1]. Alzheimer's disease is a neurodegenerative disorder associated with cognitive decline and aging and is characterized pathologically by the presence of senile plaques, neurofibrillary tangles (NFT), synapse loss, and neuropil threads. The senile plaque is composed of a core of amyloid beta peptide ( $A\beta$ ) surrounded by dying neurites.  $A\beta$  is produced from the proteolytic cleavage of amyloid precursor protein

(APP), a ubiquitously expressed transmembrane glycoprotein (Fig. 1). The two main forms of  $A\beta$  are  $A\beta$ (1–40) and  $A\beta$ (1–42).  $A\beta$ (1–42) is the primary component of senile plaques.  $A\beta$ (1–40) is the more soluble form of  $A\beta$ , while  $A\beta$ (1–42) forms insoluble fibrillar deposits when injected into rat brain [2] and has been shown to be more neurotoxic than  $A\beta$ (1–40) [3]. Similarly, injection of plaques isolated from AD brain into rat brain leads to neuronal degradation [4]. An increased production of  $A\beta$ (1–42) is associated with familial AD, a form of the disease that constitutes than 5% of all AD cases and results in accelerated onset of disease [5].  $A\beta$ (1–42) has also been implicated in the generation of NFTs, which are composed of hyperphosphorylated tau protein. It has been reported that  $A\beta$ (1–42) caused tau phosphorylation in neuronal cultures [6]. Moreover,  $A\beta$ (1–42) deposition precedes both the hyperphosphorylation of tau in APP/PS1 mutant mice [7] and the formation of NFT in tau transgenic mice and transgenic mice expressing mutant tau and APP [8,9].

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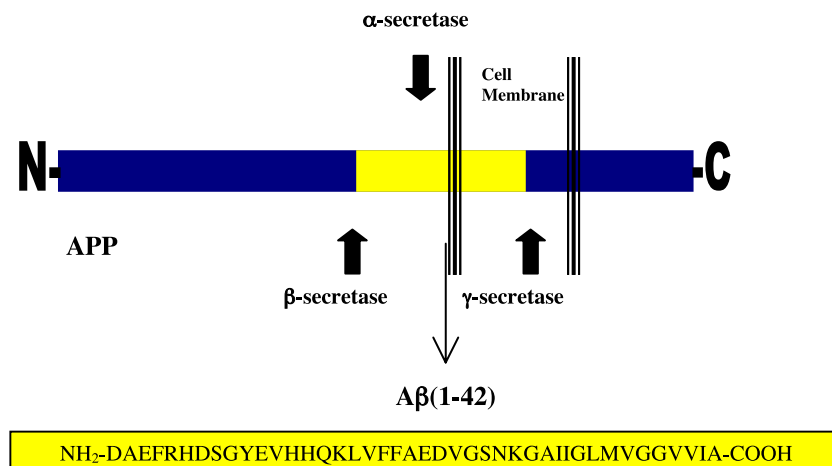


Fig. 1. Schematic of APP processing to generate A $\beta$ (1–42).

AD brain is under extensive oxidative stress, which corresponds regionally to the presence of A $\beta$ (1–42) [i.e., oxidative stress is high in regions rich in A $\beta$ (1–42), such as the hippocampus and cortex, but is negligible in regions that have little A $\beta$ (1–42), such as the cerebellum] [10–13]. A $\beta$ (1–42) has been shown to induce protein oxidative and lipid peroxidation in synaptosomes and neurons and in *C. elegans* expressing human A $\beta$ (1–42) [14–19]. Inheritance of allele 4 of apolipoprotein E is a risk factor for AD [1,3,5]. A $\beta$ (1–42) was added to synaptosomes from mice with human apolipoprotein E allele 2, allele 3, or allele 4 knocked in (the mouse promoter was present; therefore, the correct amount and location of human apolipoprotein E isoform was present) [18]. More oxidative stress induced by A $\beta$ (1–42) added to brain synaptosomes from mice with human apolipoprotein E allele 4 was found compared to that in synaptosomes from human apolipoprotein E alleles 2 or 3 [18]. Along with issues related to clearance of A $\beta$ (1–42), another interpretation of these findings is that the increased risk of AD upon inheritance of allele 4 of apolipoprotein E is the inability of such persons to handle the oxidative stress associated with A $\beta$ (1–42) [18]. A $\beta$ (1–42) has been proposed to play a central role in the pathogenesis of AD [3]. And, taken together, the considerations noted above also have led to the proposal that A $\beta$ (1–42) is central to the oxidative stress and neurotoxicity inherent in AD brain [12,16,20–22].

Our laboratory has proposed a model for the pathogenesis of AD based on the central role of A $\beta$ (1–42) as a mediator of free-radical-induced oxidative stress in AD brain, in a manner that is inhibited by free radical scavengers such as vitamin E [12,15,20–22]. In this model, A $\beta$ (1–42) inserts into the lipid bilayer as a small, soluble aggregate resulting in lipid peroxidation, and, consequently, oxidative damage to proteins [12,17]. Additionally, A $\beta$ (1–42) leads to an influx of Ca<sup>2+</sup> into the neuron, resulting in loss of intracellular Ca<sup>2+</sup> homeostasis, mitochondrial dysfunction, and ultimately cell death [15,23,24].

Recent proteomic studies in our laboratory have identified specific oxidized proteins in AD brain including creatine kinase BB,  $\alpha$ -enolase, triosephosphate isomerase, glutamine synthetase, ubiquitin carboxy-terminal hydrolase L-1, neuropolypeptide h3, and dihydropyrimidinase related protein-2 [25–29]. These studies suggest that there are proteins that are particularly vulnerable to oxidative modification. Moreover, oxidative modification of brain proteins leads to conformational changes and loss of function [12,30,31]. A $\beta$ (1–42) induces the formation of 4-hydroxynonenal (HNE) [17,32]. HNE is a reactive alkenal, formed from lipid peroxidation [33,34], known to be increased in AD brain [35]. HNE can react with protein-bound cystine, lysine, and histidine residues by Michael addition, resulting in the addition of carbonyl functionality [32,34]. HNE, produced by A $\beta$ (1–42), has been shown to bind to the glutamate transporter, EAAT2, in synaptosomes [17]. Additionally, it has been shown that HNE is bound to the EAAT2 in AD brain, which likely accounts for the decrease in activity of this transporter reported in AD brain [17,36]. Similarly, the activities of glutamine synthetase and creatine kinase are reported to be decreased in AD brain and both have been shown to be oxidatively modified in AD brain [10,25,37]. Studies are currently underway in our laboratory to identify specific targets of protein oxidation induced by A $\beta$ (1–42), which will contribute to our understanding of the involvement of A $\beta$ (1–42) in the pathogenesis of AD.

In spite of the extensive study of A $\beta$ (1–42), the specific mechanism of A $\beta$ (1–42)-induced oxidative stress and neurotoxicity remains elusive. Among the proposed mechanisms are the role of peptide aggregation [38,39], the role of Cu(II) binding and reduction [37–44], and the role of methionine 35 [16,45–53]. In this review, we will focus on the latter of these hypotheses. The specific chemistry associated with methionine in peptides will be discussed in addition to the specific role of methionine 35 in A $\beta$ (1–42)-induced oxidative stress and neurotoxicity.

## 2. Role of methionine in protein function

Methionine, one of the most easily oxidized amino acids, may have several important cellular functions [54,55]. Among other functions, methionine residues serve as shield of the active site of enzymes against oxidation [54,56,57], as a promoter of alpha-helix secondary structure [55], as oxidation-sensitive sites that may aid in the regulation and maintenance of the redox state of cells [54–59], and in the action of certain repair or chaperone enzymes [56,60–62]. There are two forms of oxidized methionine: methionine sulfoxide and methionine sulfone (Fig. 2). Methionine sulfoxide is the initial oxidation product that must be further oxidized to yield the sulfone product, a biologically unfavorable and irreversible reaction. Methionine sulfoxide leads to a new asymmetric center around the sulfur atom; consequently, two diastereoisomers are formed upon oxidation. These two diastereoisomers are differentiated by their susceptibility to reduction by peptide–methionine sulfoxide reductase [63]. Stadtman and colleagues developed the notion that surface-resident methionine residues may serve as a shield against oxidative insult protecting vulnerable amino acids and possibly the function of proteins [54,56,57]. In AD brain, the activity of peptide–methionine sulfoxide reductase is reportedly decreased [64]. Oxidation of methionine can lead to activation of functional proteins [55,62]. These considerations suggest that the oxidation of methionine may play a role in the regulation of protein activation [54,55].

## 3. Role of methionine in A $\beta$ (1–42)-induced oxidative stress and neurotoxicity

Methionine 35 has been shown to be a critical residue in A $\beta$ (1–42)-mediated oxidative stress and neurotoxicity. Several key findings have led to this conclusion. First, substitution of the sulfur atom of methionine 35 by a methylene group, -CH<sub>2</sub>-, (norleucine) attenuates the oxidative stress and neurotoxicity of A $\beta$ (1–42), but does not affect the morphology of the A $\beta$  fibrils [14]. Second, the

role of methionine 35 in A $\beta$ (1–42)-oxidative stress and neurotoxicity has also been shown in vivo in *C. elegans*-expressing human A $\beta$ (1–42) in which methionine 35 was replaced by cysteine. In contrast to our finding that native human A $\beta$ (1–42) expressed in *C. elegans* leads to oxidative stress, we found that substitution of Cys for Met in human A $\beta$ (1–42) resulted in no protein oxidation in the *C. elegans* even though the deposition of the peptide in both cases was not altered [14]. Additionally, the *C. elegans* model expressing human A $\beta$ (1–42) has been used to show that protein oxidation precedes the deposition of fibrillar aggregates in the muscle tissue of the nematode [19], supporting the role of small soluble aggregates as the toxic species of A $\beta$ (1–42). This finding is consistent with the lack of oxidative stress and neurotoxic properties of the norleucine derivative of A $\beta$ (1–42) that produced fibrils, which were morphologically indistinguishable from the native peptide [14]. Finally, it has been shown that A $\beta$ (1–28) does not induce oxidative stress or neurotoxicity in the absence of methionine [65].

We have proposed that, due to the hydrophobic nature of the carboxy terminus of A $\beta$ (1–42), the peptide inserts itself as a small aggregate into the lipid bilayer in an  $\alpha$ -helical conformation [45]. A methionine sulfuranyl radical (MetS<sup>•</sup>) is formed by a one-electron oxidation of methionine in A $\beta$ (1–42). This radical, in turn, can abstract a hydrogen atom from a neighboring unsaturated lipid resulting in the formation of a carbon-centered lipid radical (L<sup>•</sup>). The carbon-centered radical on the lipid can readily react with molecular oxygen to form a peroxy radical (LOO<sup>•</sup>). Hydrogen abstraction from a neighboring lipid results in the formation of a lipid hydroperoxide (LOOH) and another carbon-centered lipid radical (L<sup>•</sup>), thus propagating the free radical chain reaction [45,46,52,53,66]. This model of A $\beta$ (1–42)-induced lipid peroxidation and subsequent protein oxidation is consistent with the results of theoretical and experimental studies in which it was shown that the  $\alpha$ -helical secondary structure of the peptide provides stabilization of the sulfuranyl radical formed by a one-electron oxidation of methionine [46,48]. These studies have shown that mutation in A $\beta$ (1–42) of isoleucine 31 to proline, an  $\alpha$ -helix breaker, attenuated the oxidative stress and neurotoxic properties of the native peptide suggesting that the amide oxygen of isoleucine 31 in the  $\alpha$ -helix conformation (i+4) interacts with a lone pair of electrons on the sulfur atom of methionine 35, priming this atom for a one-electron oxidation. Subsequently, the sulfuranyl radical of methionine can react with other moieties of methionine to form an  $\alpha$ -(alkylthio)alkyl radical of methionine (-CH<sub>2</sub>-CH<sub>2</sub>-S-CH<sub>2</sub> or -CH<sub>2</sub>-CH-S-CH<sub>3</sub>) [46,48]. Such carbon-centered radicals provide potential substrates for reaction with molecular oxygen leading to the formation of peroxy radicals, and consequently, potentiation of free radical generation [67].

Alternatively, computational studies have shown that in an antiparallel  $\beta$ -sheet conformation of A $\beta$ (1–42), the sulfur-centered radical cation of methionine 35 can lead to

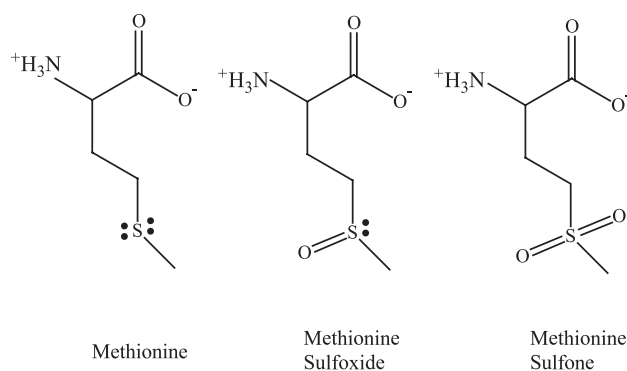


Fig. 2. Structures of methionine, methionine sulfoxide, and methionine sulfone.

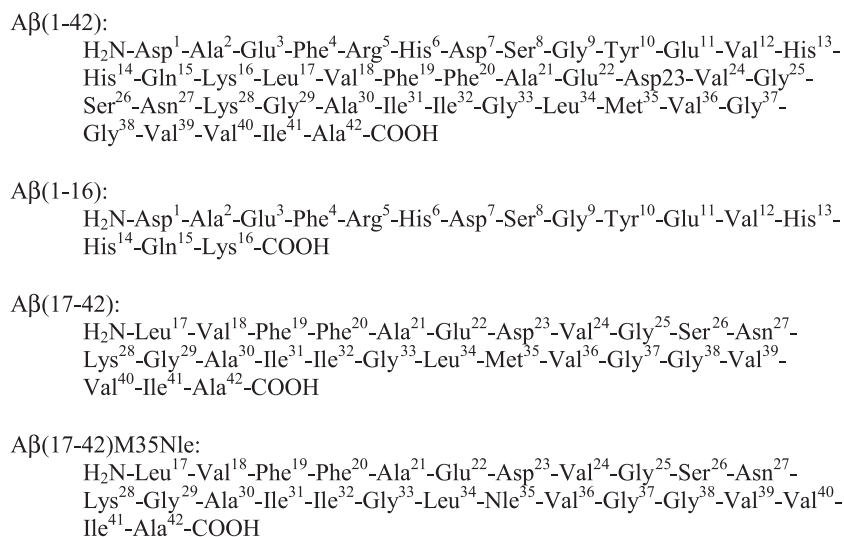


Fig. 3. Primary sequence of A $\beta$ (1–42), A $\beta$ (1–16), A $\beta$ (17–42), and A $\beta$ (17–42)M35Nle.

the generation of an  $\alpha$ -carbon-centered radical on glycine 33, thus producing a long-lived hydrophobic species that can initiate lipid peroxidation in the lipid bilayer [68,69]. Experimental studies in which glycine 33 was mutated to valine showed significantly lower levels of protein oxidation and almost no neuronal toxicity compared to the native A $\beta$ (1–42) peptide [47].

Recent studies from our laboratory have provided supporting evidence for the role of methionine in A $\beta$ (1–42)-mediated oxidative stress and neurotoxicity.<sup>1</sup>

In particular, one study utilized two physiological peptides: A $\beta$ (1–16) and A $\beta$ (17–42) (Fig. 3). A $\beta$ (1–16) is produced from the proteolytic processing of APP by  $\beta$ - and  $\alpha$ -secretase and contains only the amino acids proposed to play a role in the binding and reduction of Cu(II). On the other hand, A $\beta$ (17–42) is produced by the proteolytic cleavage of APP by  $\alpha$ - and  $\gamma$ -secretase and contains only methionine at residue 35. A $\beta$ (17–42) was shown to induced mitochondrial dysfunction, protein oxidation, and lipid peroxidation similar to A $\beta$ (1–42) in neuronal cell cultures (Fig. 4). Consistent with previous findings using A $\beta$ (1–42) [15], vitamin E inhibited the oxidative stress induced by A $\beta$ (17–42), suggesting the involvement of reactive oxygen species. Conversely, A $\beta$ (1–16) did not induce any oxidative or neurotoxic effects, consistent with the reported lack of toxicity of A $\beta$ (1–28) in the absence of methionine [65]. Moreover, substitution of methionine in A $\beta$ (17–42) by norleucine [A $\beta$ (17–42)M35Nle], which replaces the sulfur atom of methionine by a methylene group, abrogated the oxidative stress and neurotoxicity induced by A $\beta$ (17–42). This finding is consistent with previous reports that substitution of methionine 35 by norleucine in A $\beta$ (1–42)

abolishes the oxidative and neurotoxic properties of A $\beta$ (1–42) [14]. Taken together, these results suggest that methionine plays a critical role in the oxidative stress and neurotoxicity induced by A $\beta$ (1–42), and if Cu(II) is involved, such involvement is a consequence of the presence and properties of methionine.

Recent studies provided some experimental indication that a sulfuranyl free radical, as proposed initially by us [12,14,16,46,52,66] and others [48,51,67], was obtained with A $\beta$ (1–40) [70]. In contrast, the nontoxic reverse peptide A $\beta$ (40–1) had a Tyr radical on residue 10 [70]. The hypothesis for Cu(II) binding and reduction being the basis of the oxidative stress and neurotoxicity of A $\beta$ (1–42) requires that an electron emanate from Tyr at residue 10, to reduce Cu(II) to Cu(I), leaving a tyrosyl free radical [40,41,44]. Thus, a principal tenant of the Cu(II) binding

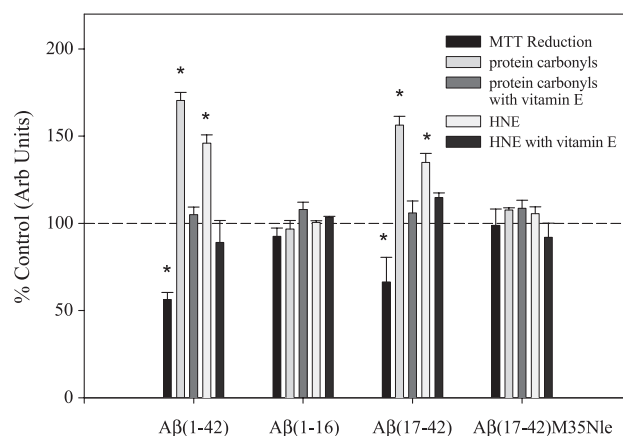


Fig. 4. Relative changes in MTT reduction, protein carbonyls, and HNE. MTT is an assessment of mitochondrial function, while protein carbonyls index protein oxidation, and HNE is a index of lipid peroxidation. Only A $\beta$ (1–42) and A $\beta$ (17–42) induced a significant decrease in mitochondrial function. Additionally, only A $\beta$ (1–42) and A $\beta$ (17–42) induced significant increases in protein oxidation and lipid peroxidation, both of which were inhibited by vitamin E. \* $P$ <0.05.

<sup>1</sup> Boyd-Kimball D, R. Sultana, H. Mhammad-Abdul, D.A. Butterfield, Role of methionine 35 vs. Cu(II) in Alzheimer's A $\beta$ (1–42)-mediated oxidative stress and neurotoxicity in primary neuronal cell cultures: a critical comparison, submitted for publication, 2004.



and reduction hypothesis for A $\beta$ (1–42)-mediated oxidative stress and neurotoxicity appears to be associated with a nontoxic peptide [70].

We suggest that the methionine residue 35 plays a critical role in the oxidative stress and neurotoxic properties of A $\beta$ (1–42). This suggestion was strengthened by recent studies of rodent A $\beta$ (1–42) [71]. A $\beta$ (1–42) from rat has only one His and Tyr replaced by Phe. Rodent A $\beta$ (1–42) is unable to reduce Cu(II) to Cu(I), unlike human A $\beta$ (1–42) [41]. Thus, the assumption has been that this rodent A $\beta$ (1–42) is not toxic nor oxidative. However, if rodent A $\beta$ (1–42) is incubated with neurons sufficiently long (3–4 days), oxidative stress and neurotoxicity occur [71]. Therefore, the methionine residue 35, even in rodent A $\beta$ (1–42), appears paramount in the oxidative stress and neurotoxic characteristics of this peptide. This suggestion was bolstered by recent studies with brain from the senescence accelerated prone (SAMP8) mouse [72]. This mouse deposits A $\beta$  in the brain and shows learning and behavioral deficits as a function of age. Use of an antisense oligonucleotide directed against the A $\beta$  region of APP of 12-month-old SAMP8 mice led to about a 40% reduction in brain A $\beta$ (1–42) levels, a diminution of brain oxidative stress to that of 4 month-old mice, and a significant improvement in learning and behavior [72]. Thus, rodent A $\beta$ (1–42) can be oxidative, even though the rodent peptide does not reduce Cu(II).

Recent theoretical studies suggested that Met-mediated reduction of Cu(II) found on His residues 6,13,14 of A $\beta$ (1–42) would be facilitated by the aromatic pi orbitals of Phe20, and that substitution of this residue by aliphatic Ala would abrogate this role of Phe [50]. We performed experiments to test these predictions and showed that there is little contribution of Phe20 to the reduction of Cu(II) by Met of A $\beta$ (1–42) [73]. Rather, it is likely that the Cu(II) ions being reduced in A $\beta$ (1–42) are not those bound to His. Pertinent to this notion, a proposed therapy of AD centers around the use of clioquinol to remove Cu(II) bound to A $\beta$ (1–42) [74]. This agent has a  $K_D$  for Cu binding of approximately nanomolar. However, these same researchers showed that Cu(II) was bound to A $\beta$ (1–42) with a  $K_D$  of attomolar [41]. It seems highly unlikely that, contrary to the initial claims, an agent with 8–10 orders of magnitude difference in affinity for Cu(II) could displace Cu(II) from the peptide. Rather, we hypothesize that the loss of plaques in transgenic animal models of AD following clioquinol treatment may be due to sequestration of loosely bound Cu(II). In particular, since Cu(II) is reduced by A $\beta$ (1–42) to Cu(I) and a sulfuranyl radical is found, we suggest that a possible low-affinity Cu(II)-binding site that is of importance in redox chemistry associated with the peptide may be found on Met-35. Further studies will be necessary to confirm this hypothesis.

A sequence within A $\beta$ (1–42) is A $\beta$ (25–35). This peptide, which has a C-terminal methionine residue, is not found in AD brain. However, this peptide is oxidative and neurotoxic [16,66], yet this peptide is unable to reduce

Cu(II) [41]. We have shown that the mechanism of oxidative stress associated with this peptide is different than full-length A $\beta$ (1–42), due to the different chemistry associated with C-terminal methionine vs. intrachain methionine [66]. Thus, this peptide, which kills neurons with equal efficacy as native A $\beta$ (1–42), is associated with oxidative stress independent of Cu(II) reduction, and, since A $\beta$ (25–34), which lacks terminal methionine, is not toxic, the obvious residue leading to oxidative stress and neurotoxicity in this 11-mer is methionine.

Oxidative modification of methionine 35 to methionine sulfoxide has been shown constitute a major component of the various amyloid beta peptides isolated from AD brain [75–77], consistent with the role of methionine in the oxidative properties of A $\beta$ (1–42). In vitro oxidation of methionine to methionine sulfoxide has been shown to abolish the oxidative stress and neurotoxic properties of A $\beta$ (1–42) following a 24-h incubation with neurons [66]. However, at 24-h incubation, mitochondrial dysfunction as measured by MTT reduction was altered by the methionine sulfoxide-modified A $\beta$ (1–42) [66]. This finding was confirmed in recent study [78], in which the methionine sulfoxide of A $\beta$ (1–42) was shown to induce mitochondrial dysfunction following a 24-h treatment. Additionally, it was noted that following a 96-h treatment the methionine sulfoxide of A $\beta$ (1–42) resulted in neuronal death as observed by phase contrast microscopy. In contrast, others confirmed our finding of lack of neurotoxicity of the methionine sulfoxide derivative of A $\beta$ (1–42) and suggested that oxidation of A $\beta$ (1–42) at the methionine residue may be a mechanism to make the native A $\beta$ (1–42) no longer neurotoxic [79,80]. The methionine sulfoxide derivative of A $\beta$ (1–42) is not associated with the lipid bilayer after 96 h of incubation with neurons due to the hydrophilic oxidized sulfur atom [78]. Initial studies of fibril morphology described fibrils formed by A $\beta$ (1–42)-containing methionine sulfoxide as similar to the fibrils formed by the native peptide [81]. However, further studies have shown that A $\beta$ (1–42)-containing methionine sulfoxide may not form fibrils rapidly, but only slowly [82], and NMR studies of the monomeric peptides showed that the methionine sulfoxide A $\beta$ (1–42) maintained a random coil structure rather than the  $\beta$ -sheet structures formed by the native peptide [83]. These findings are consistent with the lack of correlation between fibrillar aggregates and oxidative stress and neurotoxicity [14,19]. Due to the differences between that native peptide and the methionine sulfoxide derivative, including incubation time to induce toxicity, rate of fibril formation, and lack of integration of the methionine sulfoxide derivative into the lipid bilayer, it is possible that if toxicity is induced by methionine-sulfoxide-modified A $\beta$ (1–42), such toxicity may occur by a different mechanism compared to the native A $\beta$ (1–42). For example, delayed formation of fibrils by the methionine sulfoxide derivative of A $\beta$ (1–42) may take longer to activate the RAGE receptor on the outside of the neuron. Such activation is known to lead to oxidative stress

[84]. A $\beta$ (1–42) inserts into the lipid bilayer, as a small aggregate, where it causes lipid peroxidation [12]. In contrast, A $\beta$ (1–42) with methionine sulfoxide is found outside the neuron, as noted above [78], which would place the resulting fibrils in a good position to interact with the RAGE receptor. Thus, this scenario would reconcile the findings of Varadarajan et al. [66] with respect to A $\beta$ (1–42), its sulfoxide derivative, and the findings of Barnham et al. [78]. Similar considerations may explain the recent observation that substitution of methionine by valine, which had slower Cu(II) reduction kinetics than native A $\beta$ (1–42), increased the toxicity of the resulting peptide: such a substitution would increase the hydrophobic nature of the peptide, making fibril formation, and therefore RAGE activation, more plausible [85].

This review has summarized the critical involvement of methionine in A $\beta$ (1–42)-induced oxidative stress and neurotoxicity. Given the extensive oxidative damage in AD brain, A $\beta$ (1–42) is thought to be central to the pathogenesis of AD as a mediator of oxidative stress. Additionally, the mechanism of A $\beta$ (1–42)-induced oxidative stress and neurotoxicity involves methionine-associated formation of reactive oxygen species. These findings provide important additional information regarding the potential pathogenesis of AD, and suggest that endogenous antioxidants and brain-accessible exogenous antioxidants may be a promising therapeutic strategy for this devastating dementing disorder.

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