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Review Article

Methionine-35 of Aβ(1–42): Importance for Oxidative Stress in Alzheimer Disease

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1. Introduction

Alzheimer disease (AD) is an age-related progressive neurodegenerative disease. This devastating disease is characterized by the presence of senile plaques (SP), neurofibrillary tangles (NFTs), and loss of synapses [1]. Although AD has been known for over 100 years, the exact mechanism(s) of AD progression or pathogenesis largely remain unknown. Mutations of presenilin-1 (PS-1), presenilin-2 (PS-2), and APP genes cause familial AD (FAD). In addition, other genes, such as apolipoprotein E allele 4 (APOE 4), clusterin (CLU, also called apolipoprotein J (APOJ)), phosphatidylinositol-binding clathrin assembly protein (PICALM), endothelial nitric oxide synthase-3, and alpha-2-macroglobulin, have been suggested as risk factors for AD [2–5]. Studies showed that injection of Aβ cores in rat brain leads to AD-like pathology [6].

2. Oxidative Stress in AD and Mild Cognitive Impairment Brain

Brain of subjects with AD showed increased markers of oxidative stress [7–10]. A number of hypotheses were proposed to explain AD pathogenesis among which oxidative stress hypothesis is well supported by numerous in vitro and in vivo studies [11–15]. Oxidative stress is defined as an imbalance between the amount of reactive oxygen species (ROS)/reactive nitrogen species (RNS) and the antioxidant defense system present (Figure 1). Increased levels of ROS/RNS lead to increased damage of biomolecules, such as proteins, lipids, carbohydrates, and nucleic acid. Studies of postmortem tissue showed that AD brain is under extensive oxidative stress, as supported by the elevated levels of biomarkers of protein oxidation, lipid peroxidation, DNA, and RNA oxidation, and advanced glycation end products.
Further, increased oxidative stress has also been found in MCI [11, 20, 26–30]. The increased markers of oxidative stress in AD and MCI have been shown to be associated with amyloid β-peptide (Aβ), a 40 to 42 amino acid residue neurotoxic peptide, that is derived from the proteolytic cleavage of amyloid precursor protein (APP), by the action of beta- and gamma-secretase [31]. Aβ(1–42) has been shown to aggregate more quickly than Aβ(1–40) and is proposed to play a central role in AD pathogenesis. The evidence of involvement of Aβ(1–42) in AD pathogenesis is largely derived from the observation that FAD cases have increased Aβ load and increased oxidative stress. Additional evidence supporting a role of Aβ(1–42) is provided by Down syndrome, characterized by a trisomy of chromosome 21. The gene for APP also is present on chromosome 21, and in Down syndrome individuals, the increase dosage of APP might be responsible for the observation of AD-like pathology in these individuals, if they live long enough. DS brain also has elevated oxidative stress [32].

3. Oxidative Stress and Aβ(1–42)

Consistent with the role of Aβ in oxidative stress induction and consequently in AD pathogenesis, our laboratory and others showed that Aβ-induced oxidative stress markers can be alleviated by free radical scavengers such as Vitamin E, melatonin, estradiol, EUK-34, and so forth [33–37]. As discussed further below, our laboratory proposed a model for pathogenesis of AD based on the central role of Aβ(1–42) as a mediator of free-radical-induced oxidative stress in AD brain [23]. In this model, Aβ(1–42) as small oligomers can insert into the lipid bilayer and initiate lipid peroxidation, and, consequently, oxidative damage to proteins and other biomolecules [23]. A sequelae of this model is the influx of Ca^{2+} into the neuron, resulting in loss of intracellular Ca^{2+} homeostasis, mitochondrial dysfunction, and ultimately synapse loss and cell death.

Aβ exists in different aggregation states, among which oligomeric Aβ is considered as the highly toxic form [38, 39]. Oligomeric Aβ also has been reported to be localized in mitochondria [40, 41], principal foci for ATP generation and a good source of free radicals. In vitro studies showed that incubation of 9–11-day-old primary hippocampal neuronal cultures with Aβ(1–42) leads to increased oxidative stress markers and neurotoxicity [14, 42]. Consistent with oxidative stress associated with this neurotoxic peptide, the addition of vitamin E, a chain-breaking antioxidant, significantly modulates the oxidative stress and neurotoxic effects of Aβ(1–42) [33, 42, 43], suggesting that the Aβ(1–42)-mediated toxicity is mediated via its ability to lead to induce oxidative stress. Others showed that depletion of vitamin E increases Aβ accumulation by decreasing clearance from brain and blood in a mouse model of AD [44], and we recently showed that the major efflux protein for Aβ(1–42) from brain to blood, LRP-1, was oxidatively modified in AD brain [25]. Studies conducted in our laboratory using Aβ(1–42), Aβ(1–40), Aβ(17–42), and Aβ(25–35) showed that Met-35 of these peptides is critical for Aβ-induced toxicity and oxidative stress [14, 33, 45, 46]. Many researchers have used Aβ(25–35), which mimics several of the oxidative and neurotoxic properties of full-length Aβ(1–42). The shorter peptide is reported to be more toxic than the regular Aβ(1–42) which could be due to the presence of the methionine residue at the peptide C-terminus. Several studies were conducted to explore the role of C-terminal Met in Aβ(25–35). Further, when the Met C-terminal carboxylic acid functionality of Aβ(25–35) was substituted by an amide

Figure 1: Amyloid beta (Aβ) peptide induces oxidative stress and neuronal loss.
oxidative stress in AD. Metal reactivity, and so forth, could play an important role in AD brain. For example, mitochondrial dysfunction, redox and RNS likely contribute to the oxidative stress observed in AD brain, which may lead to increased oxidation of Met [56]. Further, electron paramagnetic resonance (EPR) spin trapping analyses showed that Aβ(1–42M35NLE) did not lead to trapped free radicals, in marked contrast to native Aβ(1–42), suggesting that the sulfur atom of Met is key to the Aβ(1–42)-mediated free radical formation. Three laboratories have confirmed our results that substitution of norleucine for Met in Aβ(1–42) significantly modulated, if not eliminated, oxidative stress and neurotoxicity [59–61]. In contrast, another group reported that while the 33–35 region of Aβ(1–42) was critical for the aggregation and neurotoxic properties of this peptide, substitution of Met by norleucine (NL) was still toxic [47]. This different result from our and others’ studies could be due to different experimental conditions used in these two studies. For example, in our study we have used aged neurons, that is, 9–11 days in culture (by this time transporters and other key cellular components are fully expressed); in contrast, others [47] used 2-day-old neuronal cultures that still lack the complete expression of the cellular proteome.

4. Role of Methionine-35 of Aβ(1–42) in Oxidative Stress

Our laboratory was the first to propose that Aβ(1–42) mediates its toxicity via the single methionine residue of the peptide [46]. As shown in Figure 2, Aβ has Met present at the 35th residue in the peptide. Met is an easily oxidizable amino acid and can undergo 2-electron oxidation to form methionine sulfoxide [48, 49]. Oxidation of Met has been shown to occur in a wide variety of proteins and has been shown to decrease or hinder biological activity [50, 51]. The oxidation of Met to the sulfoxide could serve two purposes biologically: first, as a regulatory function, and secondly, in cellular defense [52]. The notion that Met oxidation to the sulfoxide possibly serves an antioxidant role comes from the fact that cell has an enzyme, methionine sulfoxide reductase (MSR), that catalyzes the conversion of Met-sulfoxide (MetSOx) to Met [53–55]. In AD, MSR activity is diminished compared to control brain [56]. Further, the MetSOx can be further oxidized to form a corresponding sulfone, but this process requires a stronger oxidant; hence, this conversion is not easily accomplished in vivo and is not an easily reversible process.

The mechanism of free radical oxidation of sulfur in Met-containing Aβ peptides has been extensively studied. In AD brain, a significant fraction of SP-resident Aβ peptide has Met in the form of MetSOx [57]. As noted, previous investigations showed that MSR activity is reduced in AD brain, which may lead to increase oxidation of Met [56]. In addition to 2-electron oxidation, the sulfur atom of Met of Aβ(1–42) can undergo a one-electron oxidation leading to the formation of a sulfuranyl radical cation (S⁺) or hydroxysulfuranyl radical cation [58]. Further, it appears that H-atom abstraction, likely from the adjacent methyl group on Met, could lead to the formation of an α(alkylthio)alkyl radical of methionine, which can react with paramagnetic oxygen to form a highly reactive peroxyl radical or other species such as formaldehyde and sulphydryl [58]; H₂N-Asp¹-Ala²-His³-Asp⁴-Ser⁵-Gly⁶-
Tyr⁷-Glu⁸-Val⁹-His¹⁰-Glu¹¹-Val¹²-His¹³-Gln¹⁴-Lys¹⁵-Leu¹⁶-
Val¹⁷-Phe¹⁸-Phe¹⁹-Ala²⁰-Glu²¹-Asp²²-Val²³-Gly²⁴-
Ser²⁵-Asn²⁶-Lys²⁷-Gly²⁸-Ala²⁹-Ile³⁰-Val³¹-Leu³²-Gly³³-
Leu³⁴-Met³⁵-Val³⁶-Gly³⁷-Gly³⁸-Val³⁹-Val⁴⁰-Ile⁴¹-Ala⁴²-COOH

\[ \text{Figure 2: The sequence of amyloid beta (Aβ) peptide. Methione is present in the 35th position of Aβ(1–42).} \]

4.1. In Vitro Evidences. To test the role of Met in Aβ-mediated toxicity, the sulfur atom of methionine in Aβ(1–42) was substituted by a methylene moiety (Aβ(1–42) M35NLE) that has the same side chain length and hydrophobicity as Met [14]. The addition of Aβ(1–42M35NLE) to 9–11-day-old primary hippocampal cultures caused no increase in the markers of oxidative stress and neurotoxicity [14]. Further, electron paramagnetic resonance (EPR) spin trapping analyses showed that Aβ(1–42M35NLE) did not lead to trapped free radicals, in marked contrast to native Aβ(1–42), suggesting that the sulfur atom of Met is key to the Aβ(1–42)-mediated free radical formation. Three laboratories have confirmed our results that substitution of norleucine for Met in Aβ(1–42) significantly modulated, if not eliminated, oxidative stress and neurotoxicity [59–61]. In contrast, another group reported that while the 33–35 region of Aβ(1–42) was critical for the aggregation and neurotoxic properties of this peptide, substitution of Met by norleucine (NL) was still toxic [47]. This different result from our and others’ studies could be due to different experimental conditions used in these two studies. For example, in our study we have used aged neurons, that is, 9–11 days in culture (by this time transporters and other key cellular components are fully expressed); in contrast, others [47] used 2-day-old neuronal cultures that still lack the complete expression of the cellular proteome.

4.2. In Vivo Evidences. Most of the studies performed to better understand the role of Met of Aβ(1–42) in oxidative stress and neurotoxicity involved in vivo studies. However, recently our laboratory performed the first in vivo study of a mammalian system to address this issue [62]. The PDAPP mouse is a transgenic mouse in which the human APP gene having mutations corresponding to the Swedish and Indiana familial forms of AD are expressed (APP_{Sw,In}) behind a platelet-derived growth factor (PDGF) β-chain promoter, resulting in Aβ accumulation, plaque formation, and memory deficits [62]. In our study, a third mutation in APP was introduced, namely, M631, corresponding to Met35 of Aβ(1–42), was substituted by leucine (M35L) [62]. Leucine was chosen since it is a naturally occurring amino acid with an extant codon, unlike norleucine, which does not have a codon in the cell. Moreover, Leu, like NLE, also exhibits similar length and hydrophobicity as Met.
The PDAPPM631L mice showed similar levels of Aβ(1–42) at 9 months of age, the age at which the biochemical and pathological aspects of this study were conducted, as the PDAPP_Sw,In mice. However, in contrast to the brain of PDAPP_Sw,In human double mutant Tg mice, which contained Aβ-rich plaques, the PDAPPM631L mouse brain presented punctate deposits of Aβ(1–42), suggesting that in addition to affecting oxidative stress, the single Met residue of Aβ(1–42) is also important in plaque formation. Evidence of microglial activation was found in the PDAPPM631L mice. Importantly, the brain of 9-month-old PDAPPM631L mice showed no elevated oxidative stress compared to PDAPP_Sw,In mice and compared to WT littermate mice, demonstrating in vivo in mammalian brain that the Met-35 residue of Aβ(1–42) is essential for oxidative stress associated with this peptide. Surprisingly, this substitution did not rescue the learning and memory deficits assessed by the Morris water maze at 6 months of age of the PDAPP_Sw,In mice. However, to determine if at a later age the deficits in learning and memory observed in the PDAPP_Sw,In mice would be modulated in the PDAPPM631L mouse, a longitudinal study would be required. Indeed, as noted above, while indices of oxidative stress and pathology in brain were determined at 9 months of age, behavior necessarily had to be determined at an earlier age, and in our study mice were tested for behavior at 6 months of age. So, it is possible that we missed behavioral changes that would have been manifested later. This notion is consistent with studies of mice with other APP mutations in which behavioral alterations observed later in life (13 months) were not manifested earlier in life [63]. Also, that microglial activation occurred in brain of PDAPPM631L mice may have contributed to the loss of rescue of behavior compared to the PDAPP_Sw,In mice. Finally, Aβ(1–42) is but one neurotoxic peptide produced from APP proteolytic processing: other, non-Aβ peptides are produced, that is, sAPPα, Jcasp, and C31, each of which has been implicated in neurotoxicity or other components of the AD phenotype [64–67]. Hence, while the oxidative stress in brain of PDAPP_Sw,In mice is clearly related to Aβ(1–42), the behavioral aspects inherent in this mouse and the PDAPPM631L mouse conceivably involve one or more of these other APP proteolytic processing products. More research on this topic is necessary. Our laboratory is currently employing proteomics to explore the proteome to better understand the role of Met-35 of Aβ(1–42) in oxidative stress in brain in vivo.

The earliest study demonstrating in vivo the importance of methionine in Aβ(1–42)-associated oxidative stress involved transgenic Caenorhabditis elegans (C. elegans). C. elegans were genetically modified using an unc-54 promoter (a muscle promoter) to express human Aβ(1–42) which led to the deposition of Aβ(1–42) in the muscle wall of the worm. Increased protein oxidation was found correlated with the phenotypic expression (e.g., paralysis) of the worm [14, 38]. Our result was recently confirmed by others [68]. If in the C. elegans that express human Aβ(1–42), Met-35 were substituted by cysteine, no oxidative stress was found, but the deposition of modified Aβ(1–42) was not altered [14]. One caveat to this study is that it is possible that the ability of Cys residues to form disulfide linkages contributed to some degree to the lack of oxidative stress in C. elegans that expressed human Aβ(1–42)M35C.

5. Methionine and Aβ(1–42) Oligomer

A number of studies also suggest that aggregation of Aβ peptides is essential for determining the toxicity of oxidative stress and neurotoxicity [69, 70]. In vitro studies that compared the toxic effect of Aβ(1–42)M35SOx and native Aβ(1–42) showed that the when Met-35 is oxidized to the sulfoxide in Aβ(1–42), the resulting peptide has decreased toxicity assessed 24 h after incubation [45]. A report suggests that oxidation of Met-35 to the sulfoxide makes this Aβ less neurotoxic by altering production of toxic Aβ oligomers that are reported to be the toxic species Aβ [71, 72]. However, others showed that at long incubation times (96 h), Aβ(1–42)M35SOx is still toxic [73]. Likely, mechanisms involved are different than those in which shorter incubation periods (24 h) showed no toxicity of Aβ(1–42)M35Ox and may involve aggregated extracellular peptide activating RAGE receptors or other processes.

6. Mechanism of Methionine-35 of Aβ(1–42) Toxicity

The proposed Aβ-induced oxidative stress and neurotoxicity is based on the one-electron oxidation of Met-35 to form the sulfuranyl radical cation (Figure 3). The nature of the oxidant is not known for certain, but two candidates are possible among others. Molecular oxygen (zero dipole moment; therefore, soluble in lipid bilayers), would form superoxide radical anion as it oxidized the S atom in Met to the sulfuranyl radical. Superoxide, in turn, could reduce Cu²⁺ to Cu⁺ that is able to catalyze Fenton chemistry to produce reactive and damaging hydroxyl free radicals. Moreover, there is a report that Aβ(1–42) has Cu/Zn SOD-like properties [74], which, if sustained by further studies, would imply that there must be a nearby source of superoxide radicals needed to be scavenged to prevent further damage. The second possible oxidant to convert Met in Aβ(1–42) to a sulfuranyl free radical is Cu²⁺. Copper is shown to bind to Aβ with a high affinity via His present at 6, 13, 14, of Aβ(1–42) [75]. In amyloid plaques, high levels of copper were reported and have been shown to form complex with Aβ [76]. In vitro Aβ(1–42) and Cu(II) interaction promotes the reduction of peptide-bound Cu²⁺ to Cu⁺ and form H₂O₂·Cu⁺, in turn, can react with the H₂O₂ to form hydroxyl free radicals [77, 78]. Moreover, it has been shown that when methionine was added exogenously to a fragment of Aβ that lacks Met (Aβ(1–28)), an increased rate of Cu(II) reduction occurred, supporting the importance of methionine in Aβ-induced toxicity [77, 79]. In vitro studies have shown that chelation of copper by a suitable ligand inhibited the formation of ROS. An effective chelator clioquinol (CQ, 5-chloro-7-idoquinolin-8-ol), hydroxyquinoline antibiotic was found to be an effective high-affinity chelator in blocking the formation of hydrogen peroxide by Aβ [75, 80] and resulted
Figure 3: Methionine-35 residues in Aβ can undergo one electron oxidation to form a sulfuranyl radical, which can then initiate the process of lipid peroxidation. For details of this proposed mechanism, please refer to the text.
isoprostanes, each of which is found in AD and MCI brain [87]. However, if a chain-breaking antioxidant like vitamin E is present, the chain reaction will be halted and reactive and damaging lipid peroxidation products will no longer be made. This is likely the mode by which vitamin E is able to block ROS formation and neurotoxicity in neuronal systems [33].

Referring to Figure 3, when the allylic H-atom is abstracted by the sulfuranyl free radical, an acid of pKa-5 is formed. This acid can react with any base, for example, water, to lose the H⁺ to form reduced Met again. That is, the whole reaction is catalytic. This model of Aβ(1–42)-induced lipid peroxidation and subsequent oxidative damage to proteins is consistent with the results of theoretical and experimental studies which showed that the α-helical secondary structure of the peptide provides stabilization of the sulfuranyl radical formed by a one-electron oxidation of methionine. Further, in vitro studies showed that substitution of Ile-31, that interacts with the sulfur atom of Met-35 by the i+4 rule of α-helices, by proline, an alpha-helix breaker, abrogates the oxidative stress and neurotoxicity associated with Aβ(1–42) [85]. That is, the secondary structure of Aβ(1–42) contributes to reactivity of the neurotoxic peptide.

That lipid peroxidation requires the radical source to be within a van der Waals distance from the allylic H-atoms with which they react, that lipid peroxidation is well documented in AD and MCI [11, 88], and that Aβ(1–42) directly leads to lipid peroxidation [24], indicates that the radical source to initiate the free radical must be soluble in the bilayer, since free radicals are too reactive to be formed outside the bilayer and diffuse into the bilayer to abstract an allylic H-atom. That is, oligomeric Aβ(1–42) must be soluble within the bilayer to initiate lipid peroxidation. Another sequelae of lipid soluble Aβ(1–42) that adopts an α-helix secondary structure conceivably may be related to the greater neurotoxicity of Aβ(1–42) compared to Aβ(1–40), namely, all α-helices have an intrinsic dipole moment, which has the effect of stabilizing a radical intermediate, that is, sulfuranyl free radical. Since Aβ(1–42) has two additional amino acids in length than Aβ(1–40), the dipole moment of the former is larger than the latter, and this increased dipole moment conceivable could stabilize the sulfuranyl free radical on Met-35 longer, thereby providing greater opportunity for allylic H-atom abstraction to occur, that is, for lipid peroxidation to be initiated.

Another study showed that substitution of Met35 to Val leads to enhanced toxicity and cellular binding of amyloid peptide [89]. In this study, the authors suggested that substitution of M35 by Val might lead to a change in the conformation of Aβ, that is, change the structure from alpha-helix to a mixture of alpha-helical and beta-sheet conformations, and thereby increasing the binding of Cu²⁺ to this peptide and making it more toxic [89]. Studies from our laboratory conducted by substituting His 6, 13, 14 in Aβ(1–42) by Tyr, which binds Cu²⁺ at least 100-fold less well than does His, showed that this peptide still retains its ability to induce oxidative stress and neurotoxicity emphasizing the importance of Met-35 in the Aβ-induced toxicity and oxidative stress [42, 45]. Also, theoretical studies suggested that Met-mediated reduction of Cu²⁺ found on His residues 6, 13, 14 of Aβ(1–42) would be facilitated by the aromatic pi orbitals of Phe20 [90]. To test this notion, our laboratory substituted aromatic Phe by aliphatic alanine (Ala) and showed that while there was a slight reduction in the oxidative stress and neurotoxicity associated with Aβ(1–42), basically this peptide had quite similar properties as native Aβ(1–42) [42].

A computational study from Brunelle et al. reported that the sulfuranyl free radical on Met of one molecule of Aβ(1–42) could abstract an H-atom from the α-carbon of Gly-33 of an adjacent peptide in a β-sheet conformation. The His-mechanism suggests that the H-atom abstraction would lead to generation of C-centered free radicals which should be able to interact with molecular oxygen leading to the formation of a peroxyl radical and consequently to the protein and other biomolecules damage. Basically this mechanism suggests that no redox metals are necessary to trigger the oxidative stress process [91]. To test this idea, we substituted Gly-33 in Aβ(1–42) by Val (Aβ(1–42G33V)), to prevent the H-atom transfer to Met-35 and observed that this substitution leads to reduced levels of oxidative stress markers and does not cause cell death [92].

7. Methionine-35 of Aβ(1–42) Localization in Membrane is Key to Oxidative Stress

Noting that lipid peroxidation by Aβ(1–42) oligomers is critical in our model of Aβ-induced oxidative stress and neurotoxicity, we tested the soundness of this hypothesis. By substitution of negatively charged aspartic acid for glycine residue 37, which is predicted to remove the Met35 residue out of the hydrophobic lipid bilayer, we hypothesized that the sulfuranyl free radical would no longer be resident in the bilayer, and that oxidative stress and neurotoxicity in cells would be prevented [85]. This study supported our hypothesis, and is a direct test of our model (Figure 3), in which lipid solubility of Aβ(1–42) as small oligomers is essential for subsequent oxidative stress and neurotoxicity [7, 87, 93, 94]. Therefore, providing evidence that lipid peroxidation is the initial event in the Aβ-induced toxicity, as demonstrated by us and others [7, 93, 94], would be a major advance in understanding potential roles of this neurotoxic peptide.

In contrast to the previous studies on M35Nle and M35Val substitutions studies discussed above, a recent study suggested that while M35 is important for Aβ assembly, this residue was not important for Aβ-induced toxicity [95]. However, there are a number of differences in the methodologies of this study and those normally employed, one of which is use of a thin film of peptide onto which buffer is added. Oligomerization and deposition of Aβ(1–42) show some dependence on Met-35 of this toxic peptide [62]. Consequently, in addition to the role of Met-35 described in the review, differences in aggregation, location (i.e., mem-
brane bilayer), and role of H-transfer from Gly-33 could play roles in the oxidative stress observed in AD brain. Taken together, most laboratories that have examined the issue have provided evidence for the involvement of Aβ(1–42) in AD oxidative stress (reviewed in [11, 23, 88] and indicated that Met-35-associated formation of free radicals is critical to Aβ(1–42)-induced oxidative stress and neurotoxicity [46, 59–61]. Consistent with this notion, we suggest that either augmenting brain-accessible exogenous antioxidants or upregulating endogenous antioxidants may be promising therapeutic strategies to prevent or delay this devastating disorder. More research is needed to delineate the exact mechanism of Aβ-induced toxicity, and our laboratory is using redox proteomics approaches to identify oxidatively modified proteins and altered pathways involved in brain as a result of Aβ(1–42)-associated, Met-35-mediated oxidative stress that may play a role in pathogenesis and progression of AD.

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