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Role of glycine-33 and methionine-35 in Alzheimer's amyloid β-peptide 1–42-associated oxidative stress and neurotoxicity

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Abstract

Recent theoretical calculations predicted that Gly33 of one molecule of amyloid β-peptide (1–42) (Aβ(1–42)) is attacked by a putative sulfur-based free radical of methionine residue 35 of an adjacent peptide. This would lead to a carbon-centered free radical on Gly33 that would immediately bind oxygen to form a peroxyl free radical. Such peroxyl free radicals could contribute to the reported Aβ(1–42)-induced lipid peroxidation, protein oxidation, and neurotoxicity, all of which are prevented by the chain-breaking antioxidant vitamin E. In the theoretical calculations, it was shown that no other amino acid, only Gly, could undergo such a reaction. To test this prediction we studied the effects of substitution of Gly33 of Aβ(1–42) on protein oxidation and neurotoxicity of hippocampal neurons and free radical formation in synaptosomes and in solution. Gly33 of Aβ(1–42) was substituted by Val (Aβ(1–42G33V)). The substituted peptide showed almost no neuronal toxicity compared to the native Aβ(1–42) as well as significantly lowered levels of oxidized proteins. In addition, synaptosomes subjected to Aβ(1–42G33V) showed considerably lower dichlorofluorescein-dependent fluorescence – a measure of reactive oxygen species (ROS) – in comparison to native Aβ(1–42) treatment. The ability of the peptides to generate ROS was also evaluated by electron paramagnetic resonance (EPR) spin trapping methods using the ultrapure spin trap *N-tert*-butyl-α-phenylnitrone (PBN). While Aβ(1–42) gave a strong mixture of four- and six-line PBN-derived spectra, the intensity of the EPR signal generated by Aβ(1–42G33V) was far less. Finally, the ability of the peptides to form fibrils was evaluated by electron microscopy. Aβ(1–42G33V) does not form fibrils nearly as well as Aβ(1–42) after 48 h of incubation. The results suggest that Gly33 may be a possible site of free radical propagation processes that are initiated on Met35 of Aβ(1–42) and that contribute to the peptide's toxicity in Alzheimer's disease brain.

Keywords: Alzheimer's disease; Reactive oxygen species; Amyloid β-peptide; Methionine free radical; Glycine free radical; Fibrilogenesis

1. Introduction

Amyloid β-peptide (Aβ(1–42)), the chief constituent of senile plaques in Alzheimer's disease (AD) brain, is thought to be central to the pathogenesis of this dementing neurodegenerative disorder [1]. The AD brain is under extensive oxidative stress, manifested by, among other biomarkers, increased protein oxidation [2], lipid peroxidation [3], and

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DNA and RNA oxidation [4–7] as well as elevated levels of redox metals [8]. There is increased interest in the role of Aβ(1–42) in free radical-associated neurotoxicity in AD brain [9]. Although the mechanism of Aβ-associated free radical oxidative stress is still unclear, methionine residue 35 of the peptide (M35) plays a critical role in this process [10–12]. This view is supported by the results of a simple substitution in the approx. 4000 Da peptide, in which the sulfur atom of M35 is replaced by a methylene functionality (–CH_2–) (Aβ(1–42NLE)). This substitution completely abolished oxidative stress and neurotoxic properties caused by the native peptide (i.e. oxidation of neuronal proteins and neuronal death) [12]. Transgenic *Caenorhabditis elegans* worms, in which human Aβ(1–42) is expressed, have oxidized proteins, but substitution of M35 by a different amino acid led to no protein oxidation [12]. Likewise, Aβ(25–35), an oxidative and neurotoxic fragment of Aβ(1–42), loses these properties in Aβ(25–34), i.e. lacking methionine [10,11].

Recently, theoretical calculations based on bond dissociation energies suggested the possibility of formation of an $^{\alpha}$C-centered free radical at glycine in antiparallel β-sheets [13]. Additionally, the glycine at residue 33 (G33) of one Aβ(1–42) peptide molecule was predicted to be susceptible to oxidation by a methionine-based sulfuranyl free radical of a different Aβ(1–42) molecule in a β-sheet aggregate, thus propagating a free radical oxidative process [14]. Only the Gly at position 33 of Aβ(1–42) was shown in these theoretical calculations to have potential for H-atom abstraction by a putative sulfuranyl free radical located at M35. Since the Aβ(1–42) sequence contains a glycine residue in close proximity to methionine (G33 and M35), and the peptide attains a β-sheet secondary structure upon prolonged incubation, i.e. at least two Aβ(1–42) chains interact, we tested the hypothesis that the oxidative stress and neurotoxic properties of Aβ(1–42) involve interaction of M35 and G33.

Using well-established methods published in previous papers from our laboratory, we compared the toxic behavior of native Aβ(1–42) and a mutant peptide in which G33 was substituted with a valine (Aβ(1–42G33V)), thereby precluding H-atom transfer from residue 33. We report for the first time that Aβ(1–42), compared to Aβ(1–42 G33V), displays far greater oxidative stress and toxicity in neuronal cells and synaptosomes and higher levels of free radicals in solution.

2. Materials and methods

2.1. General

All chemicals used were of the highest purity and were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. *N-tert*-Butyl-α-phenylnitrone (PBN) was synthesized according to well-established methods [15] and subjected to rigorous purification methods (recrystallization in hexane, and triple sublimation). Its purity was verified by EPR, mass spectrometry, and HPLC. Peptides were purchased from US Peptide (Rancho Cucamonga, CA, USA) and AnaSpec (San Jose, CA, USA) and both suppliers provided evidence of purity. The peptides were stored at −20°C in the dry state until use.

2.2. Cell culture experiments

All experiments were conducted in accordance with the University of Kentucky Animal Care and Use Committee. Neuronal cultures were prepared from 18-day-old Sprague–Dawley rat fetuses as described elsewhere [12]. Aβ peptides were dissolved in sterile water that has been stirred over Chelex-100 resin. The peptides were preincubated for 24 h before addition to cultures. The final concentration of the peptides in the cell was 10 μM, and the effects of Aβ on the neurons were measured after 24 h of exposure.

Neuronal survival was evaluated by the Trypan blue exclusion assay. Cells were rinsed three times with 1 ml PBS (pH 7.4) after exposure to the peptides. Trypan blue was added to cells along with 300 μl PBS and incubated for 10 min. Sixteen different microscopic areas were counted for uptake of Trypan blue. Data are given as percentages of corresponding vehicle-treated values [16].

To determine the level of protein oxidation an Oxidized Protein Detection Kit (Intergen, Purchase, NY, USA) was used. This kit is based on immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine (DNPH). The experimental procedure used is described in detail

elsewhere [11,17]. Data are given as percentages of corresponding controls.

2.3. Preparation of synaptosomal membrane systems

The synapse is thought to be the first target of attack in neurons in AD [18], and soluble Aβ(1–42) is reportedly a good predictor of synapse loss in this disorder [19]. Accordingly, we employed synaptosomes as well as neuronal cultures in this study. Three-month-old male Mongolian gerbils were used to obtain synaptosomes. The procedure for isolation of synaptosomes using discontinuous sucrose gradients has been described elsewhere [20]. Briefly, the animals were sacrificed by decapitation, and the brain was isolated immediately. The cortex was placed in 0.32 M sucrose isolation buffer containing 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 2 mM ethylene glycol-bistetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4). Samples were then homogenized by passing in the tube a motor driven pestle 10 times. The homogenate tissue was then centrifuged at $1500\times g$ for 10 min. The supernatant was collected and spun at $20\,000\times g$ for 10 min. The pellet was removed and placed on the top of a discontinuous sucrose gradient (0.85 M, pH 8.0; 1 M, pH 8.0; and 1.18 M, pH 8.5 sucrose solutions each containing 10 mM HEPES, 2 mM EDTA, and 2 mM EGTA) and spun in a Beckman L7-55 ultracentrifuge at $82\,500\times g$ for 2 h at 4°C. The synaptosomal layer was collected at the 1/1.18 molar sucrose interface, and subsequently washed twice with PBS for 10 min at $32\,000\times g$ yielding synaptosomal membranes. Protein concentration was determined by the BCA method on a Bio-tek Instruments PowerWaveX UV-Vis microtiter plate reader.

2.4. Reactive oxygen species (ROS) measurements

For the dichlorofluorescein (DCF) assay for ROS, synaptosomes (1 mg/ml) were dissolved in PBS and incubated with 10 μM of non-fluorescent dichlorofluorescin diacetate (DCFH-DA) for 30 min followed by a cell wash in an Eppendorf centrifuge ($3000\times g$ for 5 min). Dichlorofluorescin (DCFH), formed by esterase cleavage of the diester, is a dianion and cannot exit the synaptosomes. Reaction of this dianion with ROS leads to fluorescent DCF. Synaptosomes were resuspended in 0.5 ml of PBS, and 100 μl aliquots were redistributed to a black, 96-well, microtiter plate (Corning, Acton, MA, USA). Fluorescence was triggered by oxidation of DCFH to DCF following addition of 10 μM of Aβ peptides that were previously preincubated for 8 h at 37°C. The measurements were performed on a Molecular Devices SpectraMax GeminiXS microtiter plate reader with $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm. Data are given as percentage of corresponding controls.

2.5. EPR spin trapping

The fresh peptides (165 μM) were solubilized in PBS buffer containing PBN (50 mM). The buffer was stirred overnight in the presence of Chelex-100 resin, and the chelator deferoxamine mesylate (2 mM) was dissolved in the buffer prior to peptide addition. The peptides, along with a control solution that lacked the peptides, were incubated at 37°C for up to 72 h. The EPR spectra were acquired on a Bruker EMX EPR spectrometer. Instrumental parameters were as follows: microwave power 20 mW, modulation amplitude 1 G, gain 1×10^5, and conversion time 10.28 ms.

2.6. Electron microscopy

The ability of the full-length Aβ peptides to form fibrils upon incubation in solution for 48 h was assessed by 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 counterstained with 2% uranyl acetate. Air-dried samples were examined in a Hitachi 7000 transmission electron microscope at 75 kV.

2.7. Thioflavin T binding assay

Thioflavin T (ThT) binding assay was performed according to well-established protocols [21]. Briefly, to a fresh 4.54 μM solution of ThT (dissolved in 50 mM glycine solution, pH 8.5) incubated Aβ peptides

(24 and 48 h incubation) solutions were added to make 10 μM mixture. The resulting fluorescence was measured on a Molecular Devices SpectraMax GeminiXS microtiter plate reader with $\lambda_{ex} = 440$ nm and $\lambda_{em} = 485$ nm. The results are presented as mean and S.D. of three separate measurements.

2.8. Statistics

ANOVA was used to assess the significance; P values less than 0.05 were considered significant.

3. Results and discussion

To investigate the effect of G33 on Aβ(1–42)-induced oxidative stress and neurotoxicity, cultured hippocampal neuronal cells prepared from 18-day-old Sprague–Dawley rat fetuses were treated with 10 μM of Aβ(1–42) or Aβ(1–42G33V). After 24 h of exposure of the respective peptides, oxidative stress and neurotoxicity were assessed by measurement of protein carbonyl levels and the Trypan blue exclusion assay, respectively, using well-established methods [9,12]. Fig. 1A summarizes the results; the values were normalized to the respective controls. While the native peptide displays significantly elevated levels of protein carbonyls – a hallmark of protein oxidation [22] – and considerable cell death ($P < 0.00002$ and $P < 0.00001$ in both cases for Aβ(1–42)), the G33V-substituted peptide only oxidized neuronal proteins to a small extent, and there was no significant cell death caused by the mutant peptide (Fig. 1A). Relative to each other, native Aβ(1–42) causes far more neuronal protein oxidation and neurotoxicity than does Aβ(1–42G33V) ($P < 0.008$ (*) and $P < 0.00027$ (**), respectively).

Often DCFH-DA, a cell permeable dye, is used to assess the levels of ROS in vitro [23]. Synaptosomal membrane systems from Mongolian gerbil cortexes were prepared and preincubated with 10 μM of neutral DCFH-DA for 30 min. As described in Section 2, oxidation of DCFH to DCF leads to fluorescence. Fig. 1B shows the time course of peptide-induced DCF fluorescence in synaptosomes; the results are normalized to the values obtained for the control samples. The levels of ROS generated by the peptides are considerably greater for the native Aβ(1–42) than

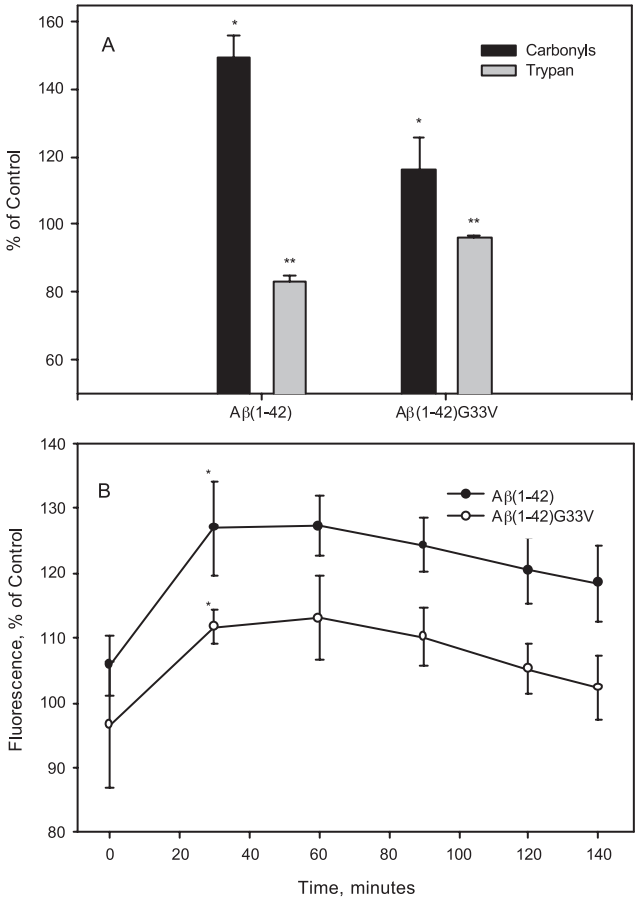

Fig. 1. (A) Levels of oxidation and neurotoxicity in cultured neurons treated with 10 μM of the peptides for 24 h. Protein oxidation (black bars) was indexed by protein carbonyl levels determined immunochemically and whose images were digitized and quantified using Scion Image software (Frederick, MD, USA). Cell death was determined by Trypan blue exclusion (dark gray bars). Dead cells take up the dye, while live cells do not. The numbers for each measure are normalized to respective controls and displayed in groups for Aβ(1–42) and Aβ(1–42G33V), respectively. The results are given as the mean ± S.D. of three different experiments (*$P < 0.008$ and **$P < 0.00027$ for a measure between the two peptides for carbonyls and Trypan blue assay, respectively). (B) ROS induces DCF fluorescence in synaptosomes upon treatment with the peptides. Synaptosomes (1 mg protein/ml in PBS, pH 7.4) were loaded with 10 μM of DCFH-DA and incubated for 30 min at room temperature. After the incubations, the cells were washed and resuspended in PBS. The fluorescence was triggered by addition of 10 μM of the peptide. The results are normalized to the control samples and are shown as mean ± S.D. of three different experiments (*$P < 0.03$ for comparison between two peptide samples). ●, Aβ(1–42); ○, Aβ(1–42G33V).

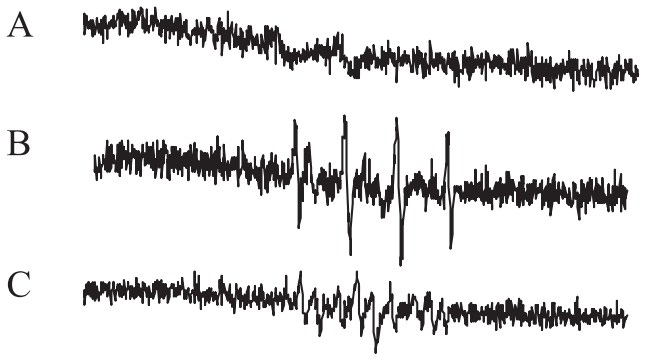

Fig. 2. EPR spectra of peptides (165 μM) individually incubated with 50 mM of ultrapure PBN in the presence of 2 mM deferoxamine mesylate in water stored over Chelex-100. The PBN spin trap used was of the highest purity ensured by repeated recrystallizations and sublimations and verified by HPLC and MS analyses. (A) Control solution, (B) Aβ(1–42), and (C) Aβ(1–42G33V) after 72 h of incubation at 37°C.

that of Aβ(1–42G33V). We previously showed that M35 was critical to the oxidative and neurotoxic properties of Aβ(1–42) [12,24]. The results in Fig. 1A,B are consistent with the notion that the presence of G33 also may be an important factor in these properties of Aβ(1–42), possibly involving propagation events between the peptide chains.

Fig. 2 displays the EPR spectra of the peptides (165 μM) incubated with 50 mM of the ultrapure spin trap PBN for 72 h at 37°C in cell-free solution. The native Aβ(1–42) peptide is responsible for formation of a strong four-line and weaker six-line EPR spectrum as reported previously and confirmed by others [9,25]. In contrast, the EPR intensity of Aβ(1–42G33V) is far less than that of native Aβ(1–42), consistent with the decreased protein oxidation and neurotoxicity of the glycine-substituted peptide. Control experiments with PBN alone show no EPR signal.

We addressed the issue of fibrilogenesis of the substituted peptide. The role of Aβ aggregation and its consequence for pathogenesis of AD is an important issue in AD research. Formation of fibrils was shown to be important in toxicity mechanisms of Aβ [26–28]. However, in the presence of specific proteins, small, soluble aggregated Aβ peptides are formed that are highly toxic species, yet no fibrils appeared [29–32]. In this study, the ability of Aβ(1–42G33V) to form fibrils upon incubation in solution for 24–48 h was investigated by electron microscopy. Fig. 3 shows EM images of Aβ(1–42) and Aβ(1–42G33V)

Table 1
Thioflavin T binding assay for the peptides

Peptide	Aβ(1–42)	Aβ(1–42G33V)
Fluorescence intensity	702 ± 42	178 ± 5

The results are mean ± S.D. of three different measurements.

taken at 75 kV at 30 000× magnification. As reported previously [9,33], Aβ(1–42) clearly formed an extensive network of fibrils (Fig. 3A). The G33V substituted peptide formed only scarce aggregates (Fig. 3B) even viewed at higher magnification (70 000×). We have verified this finding by the ThT binding assay, which indexes the presence of β-sheets. As presented in Table 1, aggregated native Aβ(1–42) binds ThT to a far greater extent than does the G33V mutant, suggesting that upon incubation the Aβ(1–42) secondary structure has the β-sheet conformation necessary for aggregation. In the light of this work and previous reports, the role of fibrilogenesis of Aβ is still unclear. Our previous findings revealed that non-oxidative and non-neurotoxic Aβ(1–42NLE) still generated fibrils [9,33]. In addition, aggregation of Aβ(1–42) cannot be prevented by the antioxidant vitamin E [34], an antioxidant that effectively prevents neuronal death and neuronal protein oxidation caused by Aβ(1–42) [34].

Aggregation of Aβ(1–42) conceivably could be a consequence of the oxidation of M35. Methionine can be easily oxidized under relatively mild conditions leading to formation of methionine sulfoxide

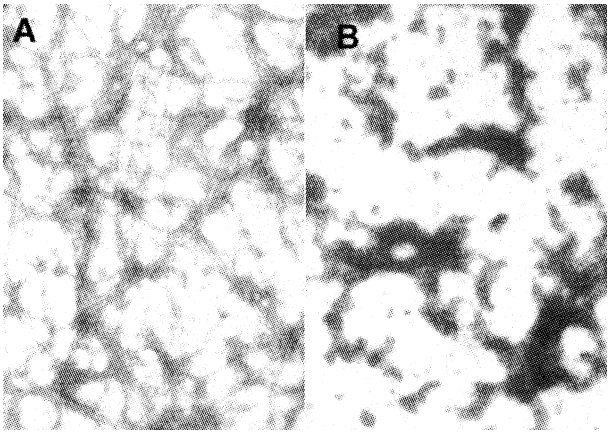

Fig. 3. Electron microscopy of (A) Aβ(1–42) and (B) Aβ(1–42G33V) incubated for 48 h. Aβ(1–42G33V) solutions show lack of extensive fibrils compared to Aβ(1–42) solutions in which fibrils are present. Magnification is 30 000× in A and 70 000× in B.

via several possible pathways [35,36]. As predicted from theoretical calculations [14], a radical formed on methionine can possibly abstract a H-atom from the neighboring G33 amino acid of an adjacent peptide, leading to formation of an $^{\alpha}$C-centered radical on the peptide backbone (structure **1** in Fig. 4). Several possible pathways are possible at this stage of reaction. Two G33 radicals could combine to form a covalent dimer of Aβ(1–42). This is highly unlikely, because of steric issues, plus oxygen likely will get to the radical site quicker, resulting in the diffusion-controlled reaction of a C$^{\bullet}$ radical with molecular oxygen to form a peroxyl radical on the peptide. This latter possibility is consistent with the experimental observation that the toxicity of Aβ(25–35) was abolished in the presence of catalase [37]. Moreover, a radical propagation step is possible, in which there is radical transfer between the G33 residues of two antiparallel β-strands of Aβ(1–42) [13] or between Aβ(1–42) and a lipid chain or another peptide or protein [9,10,38]. The latter would form a lipid radical or protein radical that would immediately bind paramagnetic oxygen, forming a peroxyl lipid or protein radical. Additionally, such peroxyl radicals can, in certain cases, form protein carbonyls or the reactive lipid reaction products, hydroxynonenal or acrolein, each of which are elevated in AD brain [2,3,39,40].

The possibility of an alternate peroxyl free radical pathway exists, one that does not involve Gly33, but rather involves H-atom abstraction from the methyl group bonded to the sulfuramyl radical forming an α-(alkylthio)alkyl radical (structure **2** in Fig. 4) [41]. The latter, C-center radical would undergo a diffu-

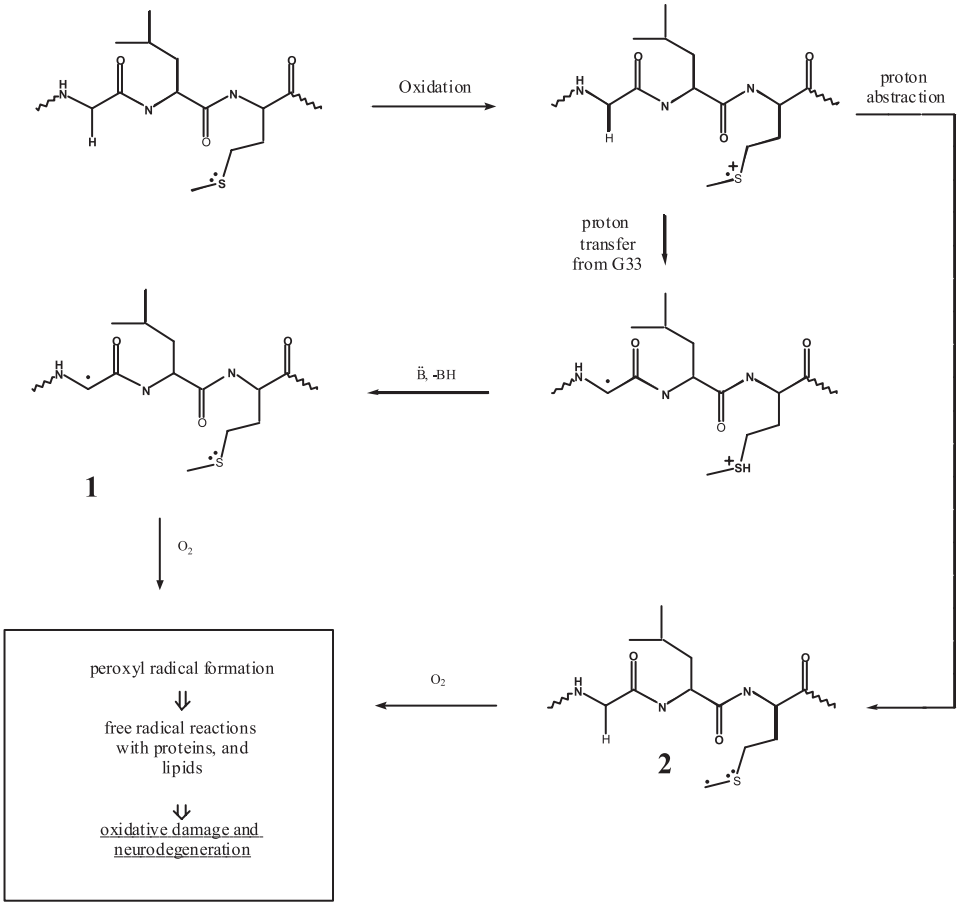

Fig. 4. Possible pathways for methionine-initiated free radical pathways that involve G33 (**1**) or the terminal methyl group of the α-(alkylthio)alkyl radical (**2**), both leading to peroxyl free radical formation. Subsequent free radical reactions with proteins or lipids could lead to the reported vitamin E-inhibited protein or lipid oxidation and neurotoxicity induced by Aβ(1–42).

sion-controlled reaction with molecular oxygen leading to the formation of peroxyl radicals that can undergo similar reactions as in the case of the glycine-derived $^{\alpha}$C-centered radical on the peptide backbone discussed above. In either case, Aβ(1–42)-associated free radical-induced oxidative stress and neurotoxicity, in processes that critically depend on the methionine residue and that are inhibited by vitamin E [9,11,12,34,42], lead to lipid peroxidation, protein oxidation, ROS formation, and neuronal death.

Others have suggested that Aβ(1–42) is a redox metal ion carrier that facilitates transfer of the redox metal ion to the membrane where the oxidative processes associated with Fenton-type chemistry can occur [43]. This was supported by the cell survival studies in which cell cultures treated with Aβ and Fe(III/II) showed greatly decreased survival, an effect that was inhibited by the chelator deferoxamine. Our results are not inconsistent with this finding, only we believe it is the methionine residue 35 that facilitates the oxidative process. This hypothesis is strengthened by the recent results of Curtain et al. [44], who showed that Aβ(1–28) could not reduce Cu(II) nor was it toxic. Yet, all the principal metal binding sites are present, e.g. His6, 13, 14 as well as Glu22. As noted above, we showed earlier that if Met were replaced by norleucine (a simple substitution of a CH_2 for the S-atom in Met, the only change in the entire 4000 Da peptide), and additional Cu(II) were added, there was still not toxicity nor oxidative stress to neurons, in marked contrast to native Aβ(1–42) [9]. Curtain et al. [44] showed that if exogenous Met were added to the non-active Aβ(1–28), then Cu(II) reduction took place.

Given the suggested centrality of Aβ(1–42) to the pathogenesis of AD, such processes may be important for neurodegeneration in AD brain. Additional experiments are underway to elucidate the chemical mechanisms underlying the role of G33 in Aβ(1–42) free radical oxidative stress processes.

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References

[1] D.J. Selkoe, Amyloid β-protein and the genetics of Alzheimer's disease, J. Biol. Chem. 271 (1996) 18295–18298.
[2] K. Hensley, N. Hall, R. Subramaniam, P. Cole, M. Harris, M. Aksenov, M. Aksenova, S.P. Gabbita, J.F. Wu, J.M. Carney, D.A. Butterfield, Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation, J. Neurochem. 65 (1995) 2146–2156.
[3] W.R. Markesbery, M.A. Lovell, Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease, Neurobiol. Aging 19 (1998) 33–36.
[4] P. Mecocci, U. MacGarvey, M.F. Beal, Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease, Ann. Neurol. 36 (1994) 747–751.
[5] S.P. Gabbita, M.A. Lovell, W.R. Markesbery, Increased nuclear DNA oxidation in the brain in Alzheimer's disease, J. Neurochem. 71 (1998) 2034–2040.
[6] M.A. Lovell, S.P. Gabbita, W.R. Markesbery, Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF, J. Neurochem. 72 (1999) 771–776.
[7] A. Nunomura, G. Perry, M.A. Pappolla, R. Wade, K. Hirai, S. Chiba, M.A. Smith, RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease, J. Neurosci. 19 (1999) 1959–1964.
[8] M.A. Lovell, J.D. Robertson, W.J. Teesdale, J.L. Campbell, W.R. Markesbery, Copper, iron and zinc in Alzheimer's disease senile plaques, J. Neurol. Sci. 158 (1998) 47–52.
[9] S. Varadarajan, S. Yatin, M. Aksenova, D.A. Butterfield, Review: Alzheimer's amyloid β-peptide-associated free radical oxidative stress and neurotoxicity, J. Struct. Biol. 130 (2000) 184–208.
[10] S. Varadarajan, J. Kanski, M. Aksenova, C.M. Lauderback, D. A Butterfield, Different mechanisms of oxidative stress and neurotoxicity for Alzheimer's Aβ(1–42) and Aβ(25–35), J. Am. Chem. Soc. 123 (2001) 5625–5631.
[11] S. Varadarajan, S. Yatin, J. Kanski, F. Jahanshahi, D.A. Butterfield, Methionine residue 35 is important in amyloid-beta-peptide-associated free radical oxidative stress, Brain Res. Bull. 50 (1999) 133–141.
[12] S.M. Yatin, S. Varadarajan, C. Link, D.A. Butterfield, In vitro and in vivo oxidative stress associated with Alzheimer's amyloid β-peptide (1–42), Neurobiol. Aging 20 (1999) 325–330.
[13] A. Rauk, D.A. Armstrong, Influence of β-sheet structure on the susceptibility of proteins to backbone oxidative damage: preference for $^{\alpha}$C-centered radical formation at glycine residues of antiparallel β-sheets, J. Am. Chem. Soc. 122 (2000) 4185–4192.
[14] A. Rauk, D.A. Armstrong, D.P. Fairlie, Is oxidative damage by β-amyloid and prion peptides mediated by hydrogen atom transfer from glycine α-carbon to methionine sulfur within β-sheets, J. Am. Chem. Soc. 122 (2000) 9761–9767.
[15] R. Huie, W.R. Cherry, Facile one-step synthesis of phenyl-

tert-butylnitrone (PBN) and its derivatives, J. Org. Chem. 50 (1985) 1531–1532.
[16] D.A. Butterfield, S.M. Yatin, S. Varadarajan, T. Koppal, Amyloid β-peptide-associated free radical oxidative stress, and Alzheimer's disease, Methods Enzymol. 309 (1999) 746–768.
[17] C.M. Lauderback, J.M. Hackett, J.N. Keller, S. Varadarajan, L. Szweda, K. Kindy, W.R. Markesbery, D.A. Butterfield, Vulnerability of synaptosomes from apoE knock-out mice to structural and oxidative modifications induced by Aβ(1–40): implications for Alzheimer's disease, Biochemistry 40 (2001) 2548–2554.
[18] M.P. Mattson, J.N. Keller, J.G. Begley, Evidence for synaptic apoptosis, Exp. Neurol. 153 (1998) 35–48.
[19] L.F. Lue, Y.M. Kuo, A.E. Roher, L. Brachova, Y. Shen, L. Sue, T. Beach, J.H. Kurth, R.E. Rydel, J. Rogers, Soluble amyloid beta peptide concentration as a predictor of synaptic changes in Alzheimer's disease, Am. J. Pathol. 155 (1999) 853–862.
[20] V.P. Whittaker, Thirty years of synaptosome research, J. Neurocytol. 22 (1993) 735–742.
[21] H. LeVine III, Quantification of β-sheet amyloid fibril structures with thioflavin T, Methods Enzymol. 309 (1999) 274–284.
[22] D.A. Butterfield, E.R. Stadtman, Protein oxidation processes in aging brain, Adv. Cell Aging Gerontol. 2 (1997) 161–191.
[23] H. Wang, J.A. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radic. Biol. Med. 27 (1999) 612–616.
[24] D.A. Butterfield, J. Kanski, Review: methionine residue 35 is critical for the oxidative stress and neurotoxic properties of Alzheimer's amyloid β-peptide 1–42, Peptides (2002) in press.
[25] X. Huang, C.S. Atwood, M.P. Cuajungco, M.A. Hartshorn, T. Tyndall, G.R. Hanson, K.C. Stokes, M. Leopold, G. Multhaup, L.E. Goldstein, R.C. Scarpa, A.J. Saunders, J. Lim, R.D. Moir, C. Glabe, E.F. Bowden, C.L. Masters, D.P. Fairlie, R.E. Tanzi, A.I. Bush, Cu(II) potentiation of Alzheimer Aβ neurotoxicity: correlation with cell-free hydrogen peroxide production and metal reduction, J. Biol. Chem. 274 (1999) 37111–37116.
[26] A. Lorenzo, B.A. Yanker, β-Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red, Proc. Natl. Acad. Sci. USA 91 (1994) 12243–12247.
[27] C.J. Pike, A.J. Walencewicz-Wasserman, D. Kosmoski, H. Cribbs, C.G. Glabe, C.W. Cotman, Structure-activity analyses of β-amyloid peptides: contributions of the β25–35 region to aggregation and neurotoxicity, J. Neurochem. 64 (1995) 253–265.
[28] B. Seilhneimer, B. Bohrmann, L. Bondolfi, F. Müller, D. Stüber, H. Döbeli, The toxicity of the Alzheimer's β-amyloid peptide correlates with a distinct fiber morphology, J. Struct. Biol. 119 (1997) 59–71.
[29] T. Oda, P. Wals, H. Osterbung, S. Johnson, G. Pasinetti, T. Morgan, I. Rozovosky, W.B. Stein, S. Synder, T. Holzman, G. Kraft, C. Finch, Clusterin (apo J) alters the aggregation of amyloid β peptide (1–42) and forms slowly sedimenting Aβ complexes that cause oxidative stress, Exp. Neurol. 136 (1995) 22–31.
[30] M.Y. Aksenov, M.V. Aksenova, D.A. Butterfield, K. Hensley, C. Vigo-Pelfrey, J.M. Carney, Glutamine synthetase-induced neurotoxicity accompanied by abrogation of fibril formation and amyloid β-peptide fragmentation, J. Neurochem. 66 (1996) 2050–2056.
[31] D.M. Walsh, D.M. Hartley, Y. Kusumoto, Y. Fezoui, M.M. Condron, A. Lomakin, G.B. Benedek, D.J. Selkoe, D.B. Teplow, Amyloid β-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates, J. Biol. Chem. 274 (1999) 25945–25952.
[32] M.P. Lambert, A.K. Barlow, B.A. Chromy, C. Edwards, R. Freed, M. Liosatos, T.E. Morgan, I. Rozovsky, B. Trommer, K.L. Viola, P. Wals, C. Zhang, C.E. Finch, G.A. Krafft, W.L. Klein, Diffusible, nonfibrillar ligands derived from Abeta1–42 are potent central nervous system neurotoxins, Proc. Natl. Acad. Sci. USA 95 (1998) 6448–6453.
[33] S. Varadarajan, S. Yatin, D.A. Butterfield, Alzheimer's amyloid β peptide (1–42) fibrils are not always neurotoxic, Alzheimer Rep. 3 (2000) 71–76.
[34] S.M. Yatin, S. Varadarajan, D.A. Butterfield, Vitamin E prevents Alzheimer's amyloid β-peptide (1–42)-induced protein oxidation and reactive oxygen species formation, J. Alzheimer Dis. 2 (2000) 123–131.
[35] B.L. Miller, T. Williams, C. Schöneich, Mechanism of sulfoxide formation through reaction of sulfur radical cation complexes with superoxide or hydroxide ion in oxygenated aqueous solution, J. Am. Chem. Soc. 118 (1996) 11014–11025.
[36] B.L. Miller, K. Kuczera, C. Schöneich, One-electron photooxidation of N-methionyl peptides. Mechanism of sulfoxide and azasulfonium diastereomer formation through reaction of sulfide radical cation complexes with oxygen or superoxide, J. Am. Chem. Soc. 120 (1998) 3345–3356.
[37] M.E. Harris, J.M. Carney, P. Cole, K. Hensley, B.J. Howard, L. Martin, P. Bummer, Y. Wang, N. Pedigo, D.A. Butterfield, β-Amyloid peptide-derived, oxygen-dependent free radicals inhibit glutamate uptake in cultured astrocytes: implications to Alzheimer's disease, NeuroReport 6 (1995) 1875–1879.
[38] D.A. Butterfield, β-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer's disease, Chem. Res. Toxicol. 10 (1997) 495–506.
[39] M.A. Lovell, C. Xie, W.R. Markesbery, Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal neurons, Neurobiol. Aging 22 (2001) 187–194.
[40] D.A. Butterfield, J. Drake, C. Pocernich, A. Castegna, Evidence of oxidative damage in Alzheimer's disease brain: central role of amyloid β-peptide, Trends Mol. Med. 7 (2001) 548–554.
[41] C. Schöneich, F. Zhao, K.P. Madden, K. Bobrowski, Side chain fragmentation of N-terminal threonine or serine resi-

due induced through intramolecular proton transfer to hydroxy sulfuranyl radical formed at neighboring methionine in dipeptides, J. Am. Chem. Soc. 116 (1994) 4641–4652.

[42] T. Koppal, R. Subramaniam, J. Drake, M.R. Prasad, D.A. Butterfield, Vitamin E protects against amyloid β-peptide (25–35)-induced changes in neocortical synaptosomal membrane lipid structure and composition, Brain Res. 786 (1998) 270–273.

[43] C.A. Rothkamp, A.K. Raina, X. Zhu, E. Gaier, A.I. Bush, C.S. Atwood, M. Chevion, G. Perry, M.A. Smith, Redox-active iron mediated amyloid β-toxicity, Free Radic. Biol. Med. 30 (2001) 447–450.

[44] C.C. Curtain, F. Ali, R.A. Cherny, R.S. Norton, K. Beyreuther, C.J. Barrow, C.L. Masters, A.I. Bush, K.J. Barnham, Alzheimer's disease amyloid-beta binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits, J. Biol. Chem. 276 (2001) 20466–20473.