

# Attenuated amyloid- $\beta$ aggregation and neurotoxicity owing to methionine oxidation

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Aggregation of the amyloid- $\beta$  (A $\beta$ ) peptide into amyloid plaques is a characteristic feature of Alzheimer's disease neuropathogenesis. We and others have previously demonstrated delayed A $\beta$  aggregation as a consequence of oxidizing a single methionine residue at position 35 (Met-35). Here, we examined the consequences of Met-35 oxidation on the extremely aggregation-prone peptides A $\beta$ 1-42 and A $\beta$ 1-40Arctic with respect to protofibril and oligomer formation as well as neurotoxicity. Size exclusion chromatography and mass spectrometry demonstrated that monomer/dimers

prevailed over larger oligomers after oxidizing Met-35, and consequently protofibril formation and aggregation of both A $\beta$ 1-42 and A $\beta$ 1-40Arctic were delayed. The oxidized peptides completely lacked neurotoxic effects in cortical neuronal cultures under these conditions, in contrast to the neurotoxic properties of the unoxidized peptides. We conclude that oxidation of Met-35 significantly attenuates aggregation of A $\beta$ 1-42 and A $\beta$ 1-40Arctic, and thereby reduces neurotoxicity. *NeuroReport* 18:559–563 © 2007 Lippincott Williams & Wilkins.

## Introduction

Soluble amyloid- $\beta$  (A $\beta$ ) oligomers are currently suspected to be the major neuropathogens active in Alzheimer's disease (AD) [1,2]. Cognitive decline has been correlated with cerebral levels of soluble A $\beta$  in AD patients [2] and neurotoxic properties have been attributed to small A $\beta$  oligomers [3] as well as larger oligomers, that is protofibrils [4].

Whether monomeric A $\beta$  induces neurotoxicity is not unambiguous, largely because of the technical difficulties in preparing pure monomeric A $\beta$  solutions at relevant concentrations and maintaining them in a cell culture environment. For instance, the literature is conflicting on whether low molecular weight A $\beta$  (a preparation consisting largely of monomers and possibly smaller oligomers [5]) is nontoxic [6] or toxic [4].

We have demonstrated previously that oxidation of the single methionine residue at position 35 (Met-35) attenuates A $\beta$ 1-40 trimer formation. This finding provides us with a useful tool to evaluate the neurotoxicity of low molecular weight A $\beta$  primarily consisting of monomers and dimers. Therefore, we compared the aggregation and neurotoxic properties of A $\beta$ 1-42 and A $\beta$ 1-40Arctic (E22G) in their reduced and oxidized form. The latter peptide is produced by carriers of the Arctic amyloid precursor protein mutation, and spontaneously generates high levels of protofibrils *in vitro* [1].

## Methods

### $\beta$ -Amyloid peptides and reagents

Synthetic A $\beta$  peptides were purchased from Biosource (Camarillo, California, USA). For the size exclusion chromatography (SEC) experiments, A $\beta$ 1-42 and A $\beta$ 1-40Arctic peptides were Met-35 oxidized according to our previously published protocols using 2.7% H<sub>2</sub>O<sub>2</sub> [7]. For all other experiments, oxidized A $\beta$ Met-35 was purchased from Biosource where oxidation had been carried out using dimethylsulfoxide. All Met-35 oxidized and unoxidized peptides were checked for their correct identity by sequence analysis, and mass determination using mass spectrometry. All reagents and antibodies were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), unless otherwise stated.

### Animals

Timed-pregnant-specific pathogen-free C57Bl/6 mice were purchased from Harlan (Horst, Netherlands). All experiments were performed in accordance with international guidelines to minimize pain and discomfort (NIH-guidelines) and European Community Council Directive 86/609/EEC.

### Kinetics of protofibril formation with size exclusion chromatography (SEC)

A $\beta$ 1-40Arctic was dissolved in cold H<sub>2</sub>O, and diluted with an equal volume 2 × phosphate-buffered saline (final

concentration: 0.05 M phosphate buffer, pH 7.4, 0.10 M NaCl). A $\beta$ 1-42 was dissolved in 1/10 dimethylsulfoxide before ice-cold H<sub>2</sub>O was added. Before SEC analysis peptide samples were incubated for different time intervals using a static kinetic incubation protocol at 30°C. Samples were centrifuged at 17 900g for 5 min at 16°C and the supernatant analyzed on a Merck Hitachi D-7000 HPLC LaChrom system with UV detection using a Superdex 75 PC3.2/30 column (Amersham Biosciences, Uppsala, Sweden) as described earlier [1].

### Mass spectrometry

All mass spectra were acquired using a Bruker Daltonics (Billerica, Massachusetts, USA) BioAPEX-94e superconducting 9.4 T Fourier transform ion cyclotron resonance electrospray mass spectrometer (FTICR-MS) as earlier described in Palmblad *et al.* [7]. Peptides were dissolved in either double distilled H<sub>2</sub>O or in acetonitrile: H<sub>2</sub>O (50:49) with 1% acetic acid.

### Cell culture and treatment

Murine cortical neurons were isolated from day 14–16 fetal C57Bl/6 mice as described previously [8]. Dissociated neurons were plated on 100  $\mu$ g/ml poly-D-lysine coated dishes at a density of about  $0.25 \times 10^6$  cells/cm<sup>2</sup> and cultured in Neurobasal (Invitrogen, San Diego, California, USA) supplemented with 2% B-27 supplement without antioxidants (Invitrogen), 0.5 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Neurons were fed every third day by replacing half of the medium. Neuronal cultures were exposed to A $\beta$  peptides at 7 days of culture *in vitro* in their own medium. Before addition to the cortical cultures A $\beta$ 1-42 peptides were dissolved in the same manner as for the SEC experiments. Potential aggregates were removed using a 5 min centrifugation at 20 000 g (16°C). The supernatants were used as the A $\beta$  peptide source either directly or once these had been aged at 30°C for 24 h.

### Neuronal toxicity

Plasma membrane integrity and nuclear morphology were assessed by double-staining cortical cultures with the nuclear dyes H-333342 (cell permeant, blue fluorescent) and SYTOX (non cell permeant, green fluorescent) and counting of neuronal condensed nuclei. About 300 cells were counted in three different fields in three different culture wells, and experiments were repeated using at least three different preparations. In addition, the percentage of viable cells was quantified by their capacity to reduce 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrasodium bromide (MTT) after incubation with 0.5 mg/ml MTT for 60 min.

### Immunofluorescence

After desired period of treatment, cells were fixed in 4% paraformaldehyde. To monitor cytoskeletal alterations, we stained neurons with an anti- $\beta$ -III-tubulin monoclonal antibody (1:300, #clone5G8; Promega, Mannheim, Germany), which recognizes only neuronal tubulin. Anti-mouse IgG antibody conjugated with Alexa Fluor 488 (Molecular Probes) was used as the secondary antibody. Nuclei were counterstained with propidium iodide.

## Results

### A $\beta$ oxidation delays oligomer and protofibril formation

Protofibril and monomer content for A $\beta$ 1-40Arctic and A $\beta$ 1-42 as well as their Met-35 oxidized forms was assessed by SEC. Protofibrils are defined as the species eluting in the void volume of the column after sedimentation of fibrils and larger aggregates. The gel included peak is denoted monomer for clarity, but most likely contains some dimers and trimers as well. Monomeric levels of both unoxidized peptides disappeared rapidly while protofibrils were formed in parallel. In contrast to the unoxidized peptides, Met-35 oxidized peptides remained monomeric and produced barely detectable levels of protofibrils (Fig. 1) for up to 66 h of incubation (data not shown). To determine the proportions of monomers and small oligomers, samples of both A $\beta$ 1-40Arctic and A $\beta$ 1-42 as well as their oxidized forms were analyzed at different time points using FTICR-MS. The two unoxidized peptides contained low amounts of monomers, dimers, trimers and tetramers which disappeared after 7–8 h, likely owing to oligomerization (Table 1). The same phenomenon was observed at 100 to 1000-fold lower concentrations (1  $\mu$ M and 100  $\mu$ M), however with much lower signals (data not shown). In contrast, a strong monomer signal was evident for both oxidized A $\beta$  peptides for up to 8 h of incubation (Table 1) and even up to 165 h (data not shown). Some dimers, but no trimers, were detected for both oxidized peptides, indicating delayed trimer formation. Our results by SEC and FTICR-MS demonstrate that Met-35 oxidation of A $\beta$ 1-40Arctic and A $\beta$ 1-42 attenuates A $\beta$  oligomerization and hence protofibril formation.

### A $\beta$ oxidation abolishes neurotoxicity

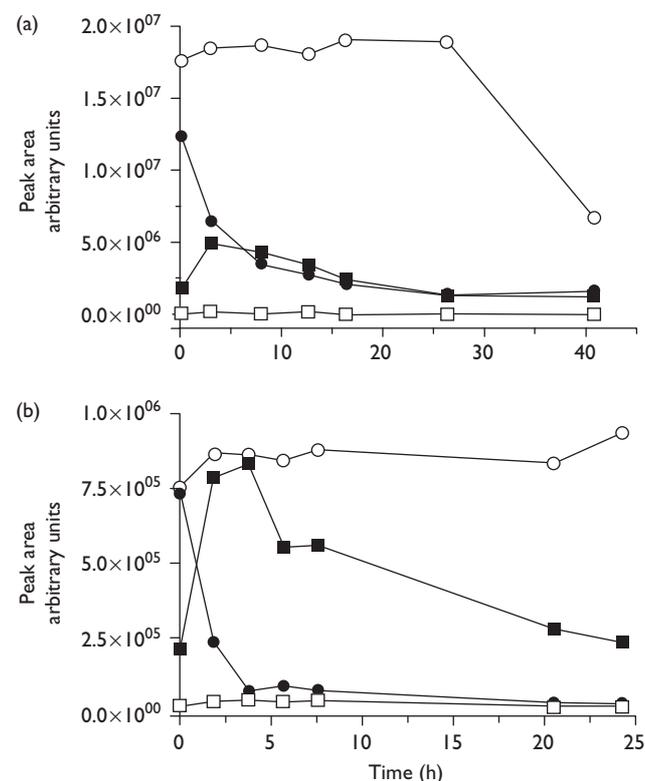
Making use of our finding that Met-35 oxidation attenuated the formation of A $\beta$  oligomers and protofibrils, we evaluated whether Met-35 oxidized peptides still induced neuronal death. Cortical neuronal cultures were exposed to various concentrations of unoxidized and oxidized A $\beta$ 1-40Arctic and A $\beta$ 1-42 either given directly or pre-aged for 24 h. Both peptide preparations (fresh or aged peptide) resulted in similar data. A $\beta$ 1-40Arctic caused cell death in cortical cultures in a concentration range of 10–40  $\mu$ M (Fig. 2). Toxicity was evident after 24 h, and further increased at 48 h as measured by counting of dead neurons (Fig. 2a) and lost capacity to reduce MTT (Fig. 2b). Neurons displayed classical morphological characteristics of apoptosis including shrunken nuclei with chromatin condensation and fragmentation (Fig. 3b, right panel). A $\beta$ 1-42 caused cell death of cortical neurons in a concentration range of 20–40  $\mu$ M (Fig. 2). A $\beta$ 1-42 toxicity was evident after 48 h and increased at 72 h. In contrast to A $\beta$ 1-40Arctic, A $\beta$ 1-42 triggered pyknotic/apoptosis-like morphological characteristics including shrunken nuclei with partially condensed, but not fragmented chromatin clumps (Fig. 3b, left panel). The oxidized peptides did not trigger any signs of cell death in the concentration range and incubation time sufficient for the deleterious effects of the unoxidized analogs (Fig. 2).

A well-known feature of AD is cytoskeletal alterations. We therefore examined whether treatment with unoxidized and oxidized A $\beta$  would lead to a disruption of the neuronal microtubule network (Fig. 3). The first alterations became apparent 24–36 h after exposure to unoxidized A $\beta$  peptides and after 48–72 h the microtubule network was completely

lost, leaving only small dots surrounding the nucleus (Fig. 3a and b). In contrast, oxidized A $\beta$  (Fig. 3a, lower panel) did not compromise the neurite network, which was indistinguishable from that of untreated cultures (Fig. 3b, middle panel).

## Discussion

We have demonstrated that oxidation of a single methionine residue drastically changes the conformational fate of two aggregation-prone A $\beta$  peptides in such a way that aggregation is considerably attenuated. This has previously been observed for the much less aggregation prone wild type A $\beta$ 1-40 peptide [7,9], and recently also for A $\beta$ 1-42 [10].



**Fig. 1** Oxidation of A $\beta$ Met-35 delays protofibril formation. Aggregation kinetics of oxidized and unoxidized A $\beta$ 1-40Arctic and A $\beta$ 1-42 were studied by SEC. A $\beta$  monomer and protofibril peak areas were plotted as a function of A $\beta$  incubation time. (a) 100  $\mu$ M A $\beta$ 1-40Arctic in phosphate-buffered saline (b) 10  $\mu$ M A $\beta$ 1-42 in 10% dimethylsulfoxide (DMSO). Filled circles: unoxidized monomer, open circles: oxidized monomer, filled squares: unoxidized protofibrils, open squares: oxidized protofibrils. One experiment representative of three.

These previous observations are here extended by demonstrating both delayed oligomer and protofibril formation for Met-35 oxidized A $\beta$ 1-42 as well as A $\beta$ 1-40Arctic. Trimer formation seemed to be attenuated also for these highly aggregating A $\beta$  peptides, similarly to what has previously been shown for A $\beta$ 1-40 [7]. Moreover, we have demonstrated that these oxidized A $\beta$  peptides completely lost their neurotoxic capacity, supposedly because of the decreased production of A $\beta$  oligomers.

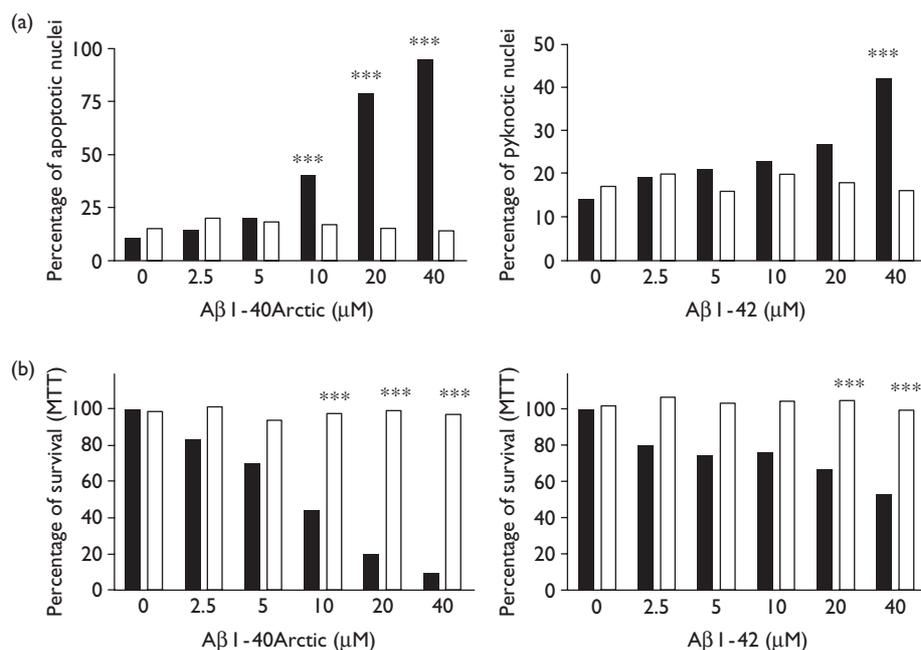
It has previously been proposed that A $\beta$  Met-35 plays a critical role in neurotoxicity. Substitution of Met-35 in A $\beta$  [11–13] and studies of A $\beta$  fragments lacking Met-35 [14,15] have demonstrated an attenuated ability for A $\beta$  to exert oxidative stress and cytotoxicity. Recent studies comparing the toxic actions of Met-35 oxidized A $\beta$  and unoxidized A $\beta$  on isolated rat brain mitochondria [16] and human neuroblastoma cells [13] showed attenuated toxicity for the oxidized form. One suggestion for the mechanism behind these observations is that the Met-35 residue plays a critical role as a mediator of oxidative stress [14], as substitution of Met-35 [11] or studies of A $\beta$  fragments lacking Met-35 [14,15] have shown attenuated ability to exert oxidative stress as well as neurotoxicity. Another possibility, although not thoroughly investigated, is that Met-35 oxidation prevents apoptosis, as Met-35 oxidation has been described to prevent the A $\beta$ 1-42-mediated down regulation of the antiapoptotic gene *bcl-2* [13]. Our findings support the view that oxidation of Met-35 renders A $\beta$  less neurotoxic by altering production of toxic A $\beta$  oligomers, oligomers that are claimed to have a range of unwanted, deleterious effects on neurons, including oxidative stress [17] and apoptosis [18]. Thus, attenuated ability of Met-35 oxidized A $\beta$  to exert oxidative stress and apoptotic events might be secondary effects to the altered aggregation behavior of this peptide.

A $\beta$ Met-35 may be oxidized in AD brains through exposure to free radicals produced and released by microglia, such as superoxide, which quickly forms reactive hydrogen peroxide [19]. In concert, the association of microglia with A $\beta$  plaques is one hallmark of AD neuropathology [20]. In addition, the proportion of methionine sulfoxide could increase through decreased activity of methionine sulfoxide reductase, which reduces methionine sulfoxide back to methionine. Interestingly, Gabbita *et al.* (1999) have reported a decline in methionine sulfoxide reductase in brains of AD patients [21]. We propose that A $\beta$  Met-35 oxidation may constitute a means by which A $\beta$  is rendered less toxic in the brain, if oxidation of Met-35 occurs *in situ* to a significant extent. One may speculate how increased levels of Met-35 oxidized A $\beta$  could be beneficial in AD: (i) build-up of toxic oligomeric species may be delayed;

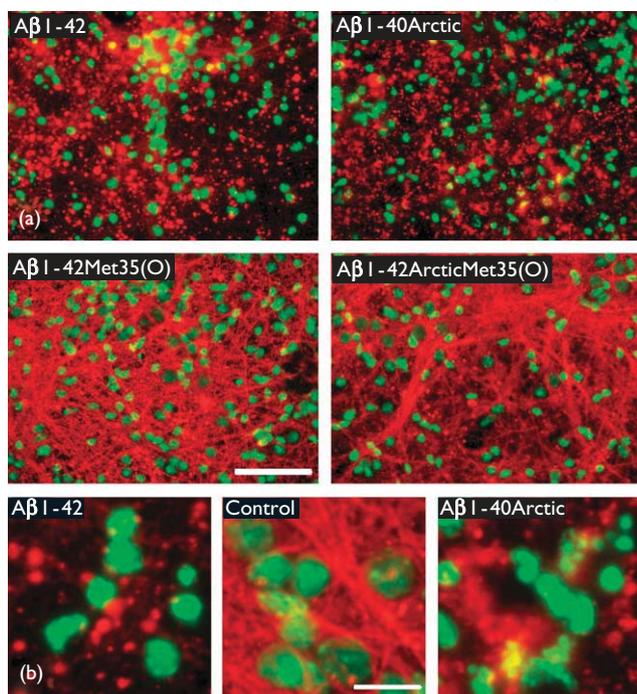
**Table 1** Observed oligomeric A $\beta$  species for oxidized [Met35(O)] and unoxidized A $\beta$ 1-40Arctic and A $\beta$ 1-42

Observed form	A $\beta$ 1-42		A $\beta$ 1-42Met35(O)		A $\beta$ 1-40Arctic		A $\beta$ 1-40Arctic Met35(O)	
	t = 0	t = 8	t = 0	t = 8	t = 0	t = 8	t = 0	t = 8
Monomer	+	(+)	+++	+++	+++	(+)	+++	+++
Dimer	(+)	–	+	++	+	(+)	(+)	+
Trimer	(+)	–	–	–	+	–	–	–
Tetramer	(+)	–	–	–	+	–	–	–

Oligomeric species measured by electrospray mass spectrometry after incubation of 100  $\mu$ M A $\beta$  for 0 and 8 h at room temperature. The number of + signs reflects the intensity of the signal.



**Fig. 2** Attenuated neurotoxicity following oxidation of Aβ Met-35. Cortical neurons were incubated with oxidized or unoxidized Aβ1-40Arctic and Aβ1-42 for 48 and 72 h, respectively. (a) Neurons were stained with SYTOX and H-33342, and the percentage of condensed (apoptotic or pyknotic) nuclei was counted. (b) Cell survival was assessed using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay. Filled bars: unoxidized Aβ, open bars: oxidized Aβ, data are means ± SD of triplicate determinations from three experiments. Significance was determined using one-way analysis of variance followed by the Tukey's test; \*\*\**P* < 0.001.



**Fig. 3** Oxidation of AβMet-35 prevents microtubule disruption in neurons. Cortical cultures were either treated with 20 μM Aβ1-40Arctic for 48 h or 40 μM Aβ1-42 for 72 h or untreated (control). Cells were fixed and stained for β-III-tubulin and chromatin (propidium iodide). Green channel: nuclei. Red channel: axodendritic network. (a) Low magnification images of unoxidized and oxidized Aβ1-40Arctic and Aβ1-42. Scale bar = 80 μm (b) Note different nuclear morphology of Aβ1-40Arctic and Aβ1-42 treated neurons: condensed and fragmented nuclei (apoptotic) versus shrunken nuclei (pyknotic). Scale bar = 15 μm.

(ii) delaying oligomerization may allow for more substantial proteolytic processing of Aβ, perhaps also by the 20S proteasome [22]; (iii) the potentially neurotoxic interaction of oxidized Aβ with cellular membranes could be compromised owing to the decreased hydrophobicity of methionine sulfoxide.

### Conclusion

Posttranslational modification of Met-35 to a methionine-sulfoxide in aggressively aggregating Aβ peptides attenuates oligomerization and reduces neurotoxicity. We propose that Aβ Met-35 oxidation could be an example of an oxidative process that works in an advantageous direction to delay on-set and/or slow down progression of AD.

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