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Alzheimer's amyloid β-peptide (1–42) induces cell death in human neuroblastoma via bax/bcl-2 ratio increase: An intriguing role for methionine 35 ☆

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Abstract

The β amyloid (Aβ), the major protein component of brain senile plaques in Alzheimer's disease, is known to be directly responsible for the production of free radicals toxic to brain tissue and the redox state of Met-35 residue seems to play a particular and critical role in peptide's neurotoxic actions. In this study, we investigated, in human neuroblastoma cells (IMR-32), the relationship between the oxidative state of methionine, and both neurotoxic and pro-apoptotic actions induced by Aβ-peptide, comparing the effects of native peptide, in which the Met-35 is present in the reduced state, with those of a modified peptide with oxidized Met-35 ($A\beta(1–42)^{35Met\text{-}ox}$), as well as an Aβ-derivative with Met-35 substituted with norleucine ($A\beta(1–42)^{35Nle}$). The obtained results show that Aβ induces a time-dependent decrease in cell viability; $A\beta(1–42)^{35Met\text{-}ox}$ was significantly less potent, though inducing a remarkable decrease in cell viability compared to control. On the contrary, no toxic effects were observed after treatment with $A\beta(1–42)^{35Nle}$. Aβ-peptide as well as the amyloid modified peptide with oxidized Met-35 induced the pro-apoptotic gene bax over-expression after 24 h, whereas $A\beta(1–42)^{35Nle}$ had no effect. Conversely, bcl-2, an anti-apoptotic gene, became highly down-regulated by Aβ peptide treatment, in contrast to that evidenced by the $A\beta(1–42)^{35Met\text{-}ox}$ peptide. Finally, Aβ caused an increase in caspase-3 activity to be higher with respect to that shown by $A\beta(1–42)^{35Met\text{-}ox}$ while $A\beta(1–42)^{35Nle}$ had no effect. These results support the hypothesis that Aβ-induced neurotoxicity occurs via bax over-expression, bcl-2 down-regulation, and caspase-3 activation, first indicating that methionine 35 redox state may alter this cell death pathway.

Keywords: Alzheimer's disease; Aβ(1–42) peptide; Methionine; Apoptosis; bax; bcl-2; Caspase

Alzheimer's disease (AD) is a brain pathology characterized by the presence of senile plaques in several regions of the central nervous system (CNS), especially in those areas where neurodegeneration occurs [1]. A major protein component of the plaques is the amyloid peptide (Aβ), a 39–43 amino acid peptide derived from a larger transmembrane protein, amyloid precursor protein (APP). Although controversial, it is established that Aβ-induced neurotoxicity occurs through the induction of apoptotic pathways [2–4].

The prevailing amyloid hypothesis of AD holds that β amyloid becomes toxic when it adopts a fibrillar conformation and that fibrillar Aβ deposition in senile plaques (SP) causes neuronal degeneration. However, recent studies have shown that soluble forms of β amyloid exhibited

☆ *Abbreviations:* Aβ, amyloid β-peptide (Aβ(1–42)); $A\beta(1–42)^{35Nle}$, Aβ(1–42)35Met → Nle; $A\beta(1–42)^{35Met\text{-}ox}$, Aβ(1–42)35Met → Met35 sulfoxide.

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strong neurotoxicity and in monomeric form, Aβ may be responsible for neurodegeneration in AD [5–9].

Apoptosis is a tightly regulated process, which involves changes in the expression of a distinct set of genes [10]. Two of the major genes responsible for regulating apoptotic cell death are bcl-2 and bax. Bcl-2 encodes a 26 kDa protein found in the nuclear envelope, parts of the endoplasmatic reticulum, and the outer mitochondrial membrane. It has been evidenced that Bcl-2 product is able to block apoptotic mechanism [11,12]. In contrast, Bax belongs to the pro-survival subfamily, which promotes apoptosis by translocation into the mitochondrial membrane and facilitating cytochrome c release, downstream apoptotic events [13]. In particular, Bax can homodimerize with itself and heterodimerize with Bcl-2 or Bcl-x_L. It appears that Bax homodimers activate apoptosis while heterodimers inhibit the process [14]. Moreover, an elevated intracellular ratio of bax to bcl-2 occurs during increased apoptotic cell death [15]. Over-expression of bcl-2 reduces apoptosis induced by a wide array of death signals including ROS [16–18]. Recent studies show that Bcl-2 may control the release of mitochondrial proteins into the cytosol, where it takes part in caspase activation in response to various apoptotic stimuli [19]. Since proteins of the Bcl-2 family regulate the activation of apoptosis when translocated to the mitochondrial membrane [20,21], the function of this organelle appears to be critical in apoptosis. The involvement of mitochondria in mediating apoptotic death in Aβ-treated cells is supported by recent studies showing a decrease in mitochondrial functionality following Aβ-peptide exposure [22,23].

Methionine-35 (Met-35) side chain of Aβ appears to play a critical role in amyloid peptide's neurotoxicity; indeed, this residue is mostly susceptible to oxidation *in vivo* [24,25], and Aβ bearing oxidized Met-35 is found in considerable amounts in post-mortem AD plaques [26,27]. The accumulation of oxidized Met-35 seems to be related to reduced enzymatic reversal of methionine sulfoxide *back* to methionine observed in AD brains [28]. Moreover, the movement of methionine to the N-terminus of the Aβ-peptide and the replacement of Met-35 with another aminoacid, i.e., norleucine, reduces or completely abolishes Aβ(1–40) toxicity and prevents protein oxidation [29–31]. In support of this thesis, several studies have recently evidenced that fragment 31–35 of Aβ, which includes the single methionine-35 residue, represents the shortest sequence of *native* peptide required for cytotoxicity, although it lacks metal binding sites [32–38].

In the present study, with the aim to clarify the putative mechanisms underlying the role played by methionine 35 in the toxic molecular mechanisms induced by Aβ-peptide, we investigated the relationships between the oxidative state of Met-35 and apoptosis related gene expression induced by native Aβ. In consideration of this, the effects of Aβ(1–42), in which the Met-35 is present in the reduced state, were compared to those of a modified peptide with oxidized Met-35 [Aβ(1–42)$^{35Met\text{-}ox}$], as well as those of an Aβ derivative with norleucine substituting Met-35 [Aβ(1–42)^{35Nle}]; knowing that cell death pathway induced by Aβ-peptide involved apoptosis [39–41], we focused on the role of the Bcl-2 family of proteins in the death process. Bcl-2 and bax, two of the main apoptotic-related genes and caspase-3 activity, were investigated in human neuroblastoma (IMR-32) cells after exposure to the above peptides.

Materials and methods

Preparation of Aβ peptides

Aβ(1–42), Aβ(1–42)$^{35Met\text{-}ox}$, and Aβ(1–42)^{35Nle} peptides were obtained by Peptide Speciality Laboratories GmbH (Heidelberg, Germany). Analysis of the peptides by reverse-phase high performance chromatography (HPLC) and mass spectrometry revealed a purity >98%. Stock solutions of Aβ(1–42) peptides, 2.5 mM in DMSO, were prepared according to the manufacturer's instructions and stored at −80 °C. Thawing and dilutions to the final concentration in the proper medium were performed immediately before use. In previous studies [42,43] these conditions have been shown to lead to the predominance of the soluble monomeric form of these peptides. In any case, in order to verify the non-aggregated form of peptides, quantitative measurement of Congo red (from Sigma, St. Louis, MO,USA) binding was carried out as described by Wood [44] (data not shown). In all control experiments, the concentration of DMSO (i.e., <0.5%) was the same as that present in the peptide solutions.

Cell culture and amyloid treatment

Human neuroblastoma IMR-32 cells were grown in minimum essential medium (Biochrom KG, Berlin, Germany) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Grand Island, NY), and cultured at 37 °C in an atmosphere of 5% CO_2 in air. For electrophysiological recordings, cells were plated at a concentration of $10^4/cm^2$ in 35-mm-diameter plastic Petri dishes and cultured at 37 °C in an atmosphere of 5% CO_2 in air. Cell differentiation was induced by 1 mM dibutyryl cAMP and 2.5 μM 5-bromodeoxyuridine (Sigma, St. Louis, MO), which were added to the culture medium three times for week, starting from the day after plating. After a week, the differentiated cells were plated at an appropriate density according to each experimental procedure. For determination of cytotoxicity of peptides, IMR-32 were initially plated in 96-well plates at a density of 10,000 cells/well and maintained 16 h in complete medium cells. Cells were then incubated for 12, 24, 36, and 48 h in the absence (control) and in the presence of examined peptides. The concentration of the peptides was set to 10 μM. The dose-dependent experiment was performed, utilizing Aβ-peptides in a range of concentration between 0 and 20 μM.

Direct toxicity study

Cell survival was evaluated by the 3-[(4,5-dimethylthiazol-2-yl)-5,3-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) reduction assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). The MTS assay is a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflect early cellular redox changes. After exposure to the amyloid peptides, cells were treated with the MTS solution (2 mg/ml) and after incubation for 4 h at 37 °C in a 5% CO_2 incubator, the intracellular soluble formazan produced by cellular reduction of the MTS was determined by recording the absorbance of each 96-well plate using the automatic microplate photometer (SpectraCount—Packard Bioscience Company—Groningen— Netherlands) at a wavelength of 490 nm [45]. The reference wavelength was 690 nm.

Analysis of mitochondrial oxygen consumption

Mitochondria were isolated from IMR-32 cells after exposure for 48 h to 10 μM Aβ-peptides, as previously reported [46]. For studying mitochondrial respiration, 1 mg of mitochondrial protein/ml was incubated in 280 mM sucrose, 10 mM Hepes, 1 mM EDTA, and 10 mM KCl, pH 7.4 for 30 min at 37 °C. Respiration rates were measured using substrates that enter the electron transport chain selectively at the following specific complexes: for complex I, glutamate (1.7 mM) and malate (1.7 mM); for complex II, succinate (2.5 mM) with NADH dehydrogenase inhibitor (2 μM rotenone). Oxygen consumption was measured at 37 °C with a Clark-type oxygen electrode (Strathkelvin Instr., Glasgow, UK) under continuous stirring.

Reverse transcription and polymerase chain reaction

Total RNA isolate. Total RNA of the cells is extracted using RNA-Bee™ reagent (Biotech, Italy) according to the supplier's instructions. RNA was quantified by optical density measurements at 260 and 280 nm with a spectrophotometer. Integrity was confirmed by running samples on 1% agarose gel.

Synthesis of cDNA. We have used 4 μg RNA in a 20 μl reaction mixture utilizing M-MLV Reverse Transcriptase Kit (Sigma, St. Louis, MO, USA) according to the supplier's instructions. Resulting reverse transcription products were stored at −20 °C until later use.

Polymerase chain reaction. Human β-actin, bcl-2 and bax primers were synthesized by Invitrogen according to the following sequences [47].

β-actin (606 bp):
forward 5′-GAGACCTTCAACACCCCAGC-3′,
reverse 5′-TCTTCATTGTGCTGGGTGCC-3′;
bcl-2 (370 bp):
forward 5′-TGTGGCCCAGATAGGCACCCAG-3′;
reverse 5′-ACTTCGCCGAGATGTCCAGCCAG-3′;
bax (367 bp):
forward 5′-ACCAAGAAGCTGAGCGAGTGTC-3′,
reverse 5′-ACAAAGATGGTCACGGTCTGCC-3′.

Human β-actin was chosen as internal control. PCR was carried out with Red *Taq* Polymerase (Sigma, St. Louis, MO, USA) according to the supplier's conditions. The PCR conditions were: 94 °C for 2 min, followed by 35 cycles (30 for β-actin) of 1 min denaturation at 94 °C, 1 min annealing at 56 °C, 2 min polymerization at 72 °C, and finally 10 min extension at 72 °C. PCR products were analyzed by electrophoresis in Agarose 1.8% with ethidium bromide (1 μg/ml) in TBE 1× buffer (Tris 40 mM, EDTA 1 mM, boric acid 44 mM) for 2 h at 80 V (constant voltage) with 123 bp ladder as molecular weight marker.

Images of gels were acquired (Bio-Rad Gel Doc 2000, Hercules, CA, USA) and scanned (Bio-Rad GS800, Hercules, CA, USA) using Bio-Rad Quantity One software. The density of the PCR bands were expressed as a ratio of the band density divided by that of the housekeeping gene, β-actin.

Measurement of caspase-3 activity

Caspase-3 activity was measured by using a specific assay kit from Sigma Chemical (St. Louis, MO, USA) following manufacturer's instructions. DEVD-pNA was used as a colorimetric substrate. IMR-32 cells were plated at a density of 2.9×10^6 cells/35 mm dish, coated with 10 μg/ml poly-D-lysine. After treatment with Aβ-peptides, cells were harvested by centrifugation. The pellets were washed with PBS, lysed in 50 ml of chilled cell lysis buffer, and left on ice for 10 min.

Lysate was centrifuged at 10,000*g* for 1 min at 4 °C, and the supernatant was used for the caspase 3 assay. The protein concentration was confirmed by the BCA assay. The protease activity was determined by the spectrophotometric detection at 405 nm of the chromophore *p*-nitroanilide (pNA) after its cleavage by caspase 3 from the labeled caspase-3-specific substrate (DEVD-pNA). Additional control assays in the presence of specific caspase 3 inhibitor (DEVD-CHO) and in the absence of recombinant human caspase were performed for measuring the non-specific hydrolysis of the substrate (data not shown).

Statistical analysis

The data were analyzed by one-way ANOVA, followed by post hoc Newman–Keul test for multiple comparisons among group means, using a Prism TM computer program (GraphPad, San Diego, CA, USA), and differences were considered statistically significant if $P < 0.05$. All results are presented as means ± SEM of at least three different experiments performed in triplicate, unless otherwise specified.

Results

Studies of cell death in IMR-32 cells treated with Aβ peptides

All Aβ-peptides employed in our experimental procedures were predominantly in non-aggregated form, i.e., monomeric, in accord with previous reports [42,43]. IMR-32 cells were treated with Aβ-peptides and their viability was determined by the reduction of MTS activity. MTS is a tetrazolium salt reduced to formazan by the mitochondrial respiratory chain dehydrogenase enzymes, which are active only in live cells. As shown in Fig. 1A the viability of IMR-32 cells, after 24 h of incubations, was severely affected by Aβ-peptide treatment in a dose-dependent manner. The maximum effect was observed with the concentration fixed at 10 μM when 35% and 19% of IMR-32 cells were killed, respectively, by Aβ(1–42) and Aβ(1–42)$^{35\text{Met-ox}}$ peptides. In contrast, IMR-32 cells treated with Aβ(1–42)$^{35\text{Nle}}$ showed almost no changes in viability at each Aβ concentration tested.

Moreover, as shown in Fig. 1B, fixing the peptide concentration at 10 μM, Aβ(1–42) induced also a time-dependent decrease in cell viability (46.9% of IMR-32 cells after 48 h). Under the same conditions, Aβ(1–42)$^{35\text{Met-ox}}$ was significantly less potent than Aβ(1–42), but was still able to induce a significant decrease in cell viability compared to control (31% of cells after 48 h). No toxic effects were observed after treatment with 10 μM Aβ(1–42)$^{35\text{Nle}}$, and the number of viable cells was broadly similar to control.

Effects of Aβ peptides on mitochondrial functionality

Subsequently, in order to clarify the real intra-cellular target underlying this toxic action, mitochondrial functionality was deeply estimated in intact IMR-32 cells after exposure for 48 h to 10 μM AβP(1–42) and Aβ(1–42)$^{35\text{Met-ox}}$ peptides. Fig. 2A shows that the addition of AβP(1–42) and Aβ(1–42)$^{35\text{Met-ox}}$ to mitochondria suspensions does not significantly modify succinate oxidation when compared to that measured in control cells, whereas the glutamate/malate oxidation (Fig. 2B) was significantly reduced by the presence of Aβ(1–42) and Aβ(1–42)$^{35\text{Met-ox}}$ peptides. In particular, it should be noted that, Aβ(1–42) has a major effect compared to the oxidized form, inducing

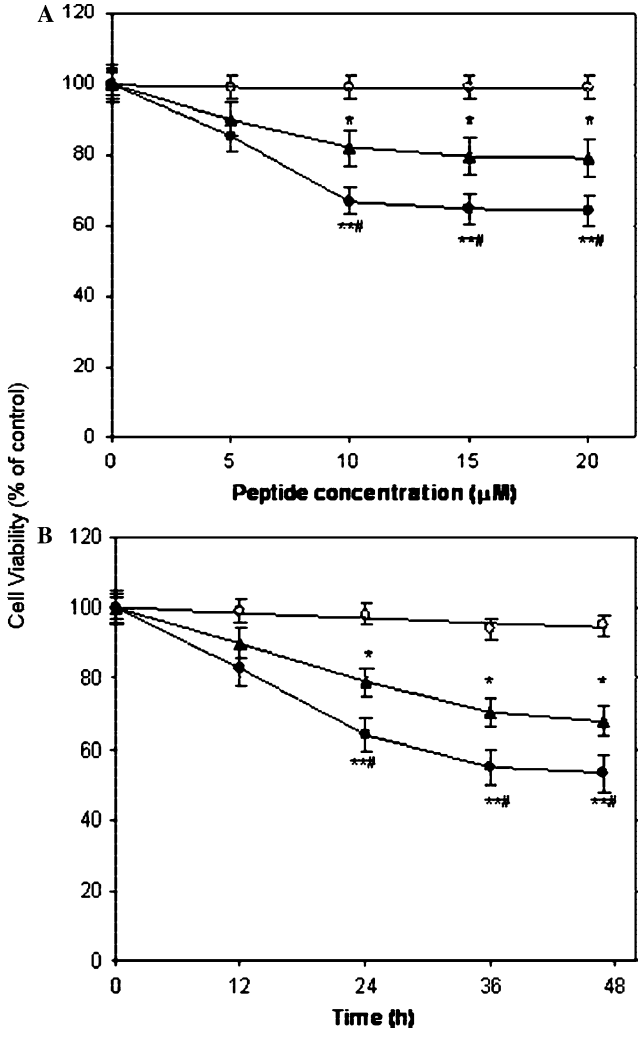

Fig. 1. (A) Dose-dependent effect of Aβ-peptides on IMR-32 cell survival. Cells were incubated with (0–20 μM) Aβ(1–42) (●), Aβ(1–42)$^{Met35\text{-}ox}$ (▲), and Aβ(1–42)^{35Nle} (○). Results are expressed as percent of cells untreated (control line = 100%). Cells (10,000 cells/well) were cultured with peptides under analysis, and the viability of IMR-32 cells was measured by MTS assay after 24 h of incubation. (B) Time course of the effects of Aβ-peptides on IMR-32 cell vitality. Cells (10,000 cells/well) were cultured with 10 μM of peptides under analysis and the viability of IMR-32 cells was assayed after 12, 24, 36, and 48 h of incubation. All values indicate means ± SEM of five independent experiments. Significantly different from controls: $^{*}P < 0.01$; $^{**}P < 0.001$; significantly different from other peptides: $^{\#}P < 0.05$.

a respiration inhibition (with respect to the controls) of ~16% against ~9.5%, respectively, at fourth minute of analysis. Finally, Aβ(1–42)^{35Nle} had no effect whatsoever on mitochondria respiration.

Effects of Aβ peptides on bax and bcl-2 gene expression

Having shown the toxic activity of both reduced and oxidized Aβ(1–42) peptides, we next investigated the molecular mechanisms underlying this action. The gene expression of a number of pro- and anti-apoptotic factors was assessed in neuroblastoma cells exposed to culture medium or medium containing each of the three peptides.

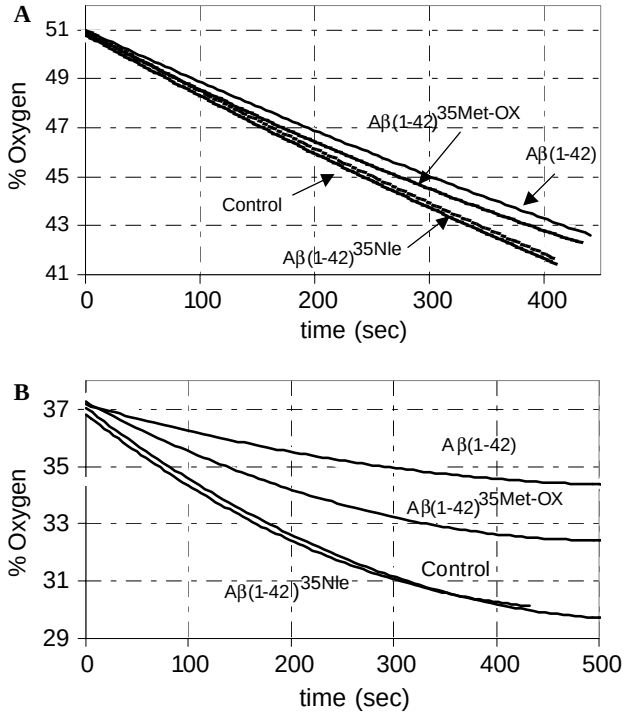

Fig. 2. Effect of 10 μM of Aβ(1–42), Aβ(1–42)$^{Met35\text{-}ox}$, and Aβ(1–42)^{35Nle} peptides on mitochondrial oxygen consumption in the presence of succinate (A) and glutamate/malate (B) as substrates. Experimental conditions are reported in Materials and methods. Absolute values of oxygen consumption (acquired at 4 min of analysis) in the absence of peptides were 54.14 ± 3.0 nmol/min/mg protein in the presence of succinate and 31.6 ± 1.8 nmol/min/mg protein with glutamate/malate. Values presented are means ± SEM obtained for eight independent experiments.

The concentration of Aβ-peptides was fixed to 10 μM and gene expression was estimated after 24 h incubation. Specific bands for bcl-2 and bax (see Fig. 3A) were detected in controls and treated cells. Moreover, we found that Aβ(1–42) and Aβ(1–42)$^{35Met\text{-}ox}$ increased the accumulation of mRNA of the pro-apoptotic gene bax. It is particularly intriguing that mRNA levels of the pro-apoptotic gene bax changed similarly in Aβ(1–42) treated neuroblastoma cells compared with Aβ(1–42)$^{35Met\text{-}ox}$ ones.

On the other hand, Bcl-2 gene expression was largely more down-regulated in the IMR-32 neuroblastoma cells treated with Aβ(1–42) peptide compared with the results shown by Aβ(1–42)$^{35Met\text{-}ox}$ peptide, which was nearly similar to that shown by control cells. Although upregulation of bax and bcl-2 downregulation in these Aβ treated IMR-32 cells demonstrate definitely that Aβ-peptide has a strong effect on genes that mediate apoptosis, Aβ(1–42)$^{35Met\text{-}ox}$-mediated apoptotic action, as evidenced by bax/bcl-2 ratio valuation (Fig. 3B), became much more attenuated. Moreover, as expected, Aβ(1–42)^{35Nle} did not modify the expression of bax and bcl-2 assessed throughout the experiment.

Effects of Aβ peptides on caspase-3 activity

The activation of caspase pathways is a critical event in apoptosis; in particular, caspase-3 is the most prominent

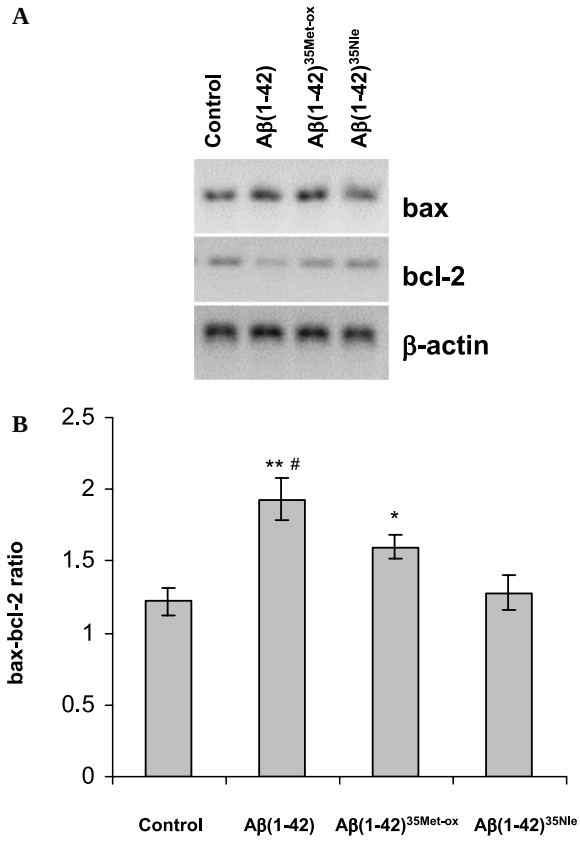

Fig. 3. (A) Effects of Aβ-peptides on bax and bcl-2 expression in IMR-32 cells. Cells were incubated with medium alone (control) or with medium containing Aβ(1–42), $Aβ(1–42)^{Met35\text{-}ox}$ and $Aβ(1–42)^{35Nle}$ (10 μM) for 24 h. β-Actin was used as internal control. (B) Quantification of the intensities of bax and bcl-2 bands determined by densitometric scanning of agarose gel and expressed as bax/bcl-2 ratio. Results are from eight independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$; significantly different from other peptides: $^{\#}P < 0.05$.

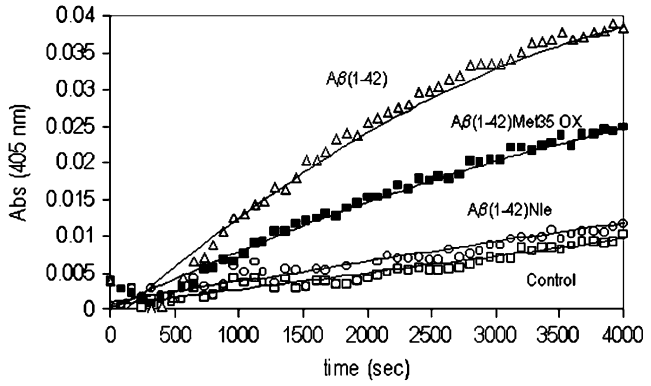

Fig. 4. Effects of Aβ-peptides on Caspase-3 activity levels in IMR-32 cells. Cells were incubated with medium alone (□) or with medium containing Aβ(1–42) (△), $Aβ(1–42)^{Met35\text{-}ox}$ (■) and $Aβ(1–42)^{35Nle}$ (○) peptides (10 μM each) for 24 h. Data are representative of four independent experiments.

effector involved in apoptosis in neural tissues [48]; therefore, in consideration of the results obtained on cell availability, it was of interest to determine whether caspase-3 activation occurs in IMR-32 cells following Aβ(1–42) peptide exposure.

As shown in Fig. 4, Aβ(1–42) is able to increase (+68% with respect to control) caspase-3 activity: the effect was observed after 48 h of incubation with a peptide concentration of 10 μM. Under the same experimental conditions, $Aβ(1–42)^{35Met\text{-}ox}$ caused a smaller effect (+52%). Finally, $Aβ(1–42)^{35Nle}$ had no effect whatsoever on caspase-3 activation.

Discussion

The precise molecular mechanisms responsible for AD-associated neuro-degeneration are not fully understood. However, it has been proposed that Aβ-peptide plays a crucial role in the pathogenesis of the disease. Aβ induced toxicity is a multi-factorial process that is thought to involve generation of reactive oxygen species, alteration of intracellular calcium homeostasis, mitochondrial function alteration, and caspase activation.

We have previously reported that oxidation of methionine at position 35 of the shortest fragments of the native Aβ-peptide reduces the toxic effects induced by Aβ(31–35) and (25–35) peptides [37,38].

This study provides further evidences regarding the role of methionine 35 in the mechanisms underlying the Aβ-dependent toxicity, showing that Aβ(1–42) peptide containing methionine 35 oxidized to sulfoxide attenuates the Aβ(1–42)-induced cell death pathways.

To investigate the putative relationship between the oxidative state of Met-35 and Aβ(1–42) toxicity, we compared the toxic profile of Aβ(1–42) in which the Met-35 is present in the reduced state to that of a modified peptide with oxidized Met-35 ($Aβ(1–42)^{35Met\text{-}ox}$), as well as to that of an Aβ-derivative in which Met-35 is substituted with norleucine ($Aβ(1–42)^{35Nle}$). We show that either Aβ(1–42), as recently reported [41], and $Aβ(1–42)^{35Met\text{-}ox}$ are able to induce IMR-32 cells degeneration; however, Aβ(1–42) appears to be significantly more potent with respect to the oxidized derivative, a new finding consistent with previous studies, performed with some short fragments of the amyloid β-peptide and with the native Aβ [25,35,37].

Distinct mechanisms of action for the *reduced* and for the methionine sulfoxide derivative Aβ-peptides have been suggested [49,50]. To verify this hypothesis, we investigated the expression of bax and bcl-2 genes, two of the main important apoptosis-related genes [51]. Both Aβ(1–42) and $Aβ(1–42)^{35Met\text{-}ox}$ increased bax expression in IMR-32 cells, in line with other reports [52,53]. Although controversial, Bax protein levels have also been reported to increase in AD brain [54,55]. On the other hand, our results show that the analysis of the expression of the anti-apoptotic gene bcl-2 showed a different expression for both peptides; in particular in IMR-32 cells treated with $Aβ(1–42)^{35Met\text{-}ox}$ peptide, bcl-2 down-regulation became attenuated with respect to that shown by Aβ(1–42) peptide-treated cells. Previous reports showed that another anti-apoptotic gene, as bcl-xl, is down-regulated in Aβ(25–35) treated cells [56].

Thus, we demonstrate that the large shift of bax/bcl-2 *ratio* in favour of bax in Aβ(1–42) treated IMR-32 cells may explain the larger amount of cell death found in Aβ(1–42) treated IMR-32 cells compared with Aβ(1–42)$^{35Met\text{-}ox}$ treated ones [57].

Therefore, in our study, caspase-3 the most prominent executor involved in apoptosis in neural tissues [48] was increased when IMR-32 cells were treated with both reduced and oxidized Aβ(1–42) peptides, but as expected, the reduced peptide was far more effective than the oxidized derivative in activating the enzyme, confirming its overall higher toxic potential. These results support the importance of the Bcl-2 family proteins in mediating cell survival through regulating caspase activity, as suggested by previous studies [16,58].

Bcl-2 has been linked to mitochondrial function during inhibition of apoptosis. Many scenarios causing apoptosis have been reported to reduce mitochondrial functions [59], suggesting that the alteration of the mitochondrial functions, i.e., oxygen consumption, occurs during apoptosis [60]. Moreover, drugs that inhibit the opening of the permeability transition pore prevent apoptosis induced by various stimuli [61]. Thus, loss of mitochondrial functionality appears to play a key role in mediating apoptosis. Our results showing decreased mitochondrial oxygen consumption following exposure to Aβ(1–42) peptides are consistent with previous data showing a loss of mitochondrial functionality when cells were exposed to Aβ-peptides [22].

The role of Bcl-2 in regulating mitochondrial functions has been observed in isolated mitochondria following exposure to pharmacological agents (e.g., hydrogen peroxide) [62]. Our results are in line with this finding because we evidenced that altered amplitude of bcl-2 expression is directly linked with different mitochondrial functionality.

Bcl-2 family of proteins has been suggested to be directly dependent on the elevation of Bax and its translocation to the mitochondrial membrane [63,64]. When translocated to the mitochondrial membrane, Bax can homodimerize and trigger the activation of terminal caspases by alteration of mitochondrial functions, which results in the release of apoptosis-promoting factors into the cytoplasm [14,65]. Conversely, Bcl-2/Bax heterodimer formation may prevent or reduce some of these downstream events [15], as evidenced in Aβ(1–42)$^{35Met\text{-}ox}$-mediated effects.

Although still controversial, it is known that there are multiple factors that act upstream of bax and bcl-2 expression [11,66,67]. In particular, JNKs are a family of serine/threonine kinases involved in a variety of cellular responses, including cell proliferation and death [68]. Activation of JNK signaling has been closely linked to a variety of apoptotic stimuli, and inhibition or loss of the JNK pathway provides protection against neuronal apoptosis in multiple paradigms, including Aβ neurotoxicity [69–71]. In line with this finding, it is reasonable to hypothesize that the presence of methionine 35 in the oxidized form renders the Aβ(1–42) peptide in some way unable to affect the expression or the function of some of the key factors involved in the Aβ-mediated bcl-2 down-regulation.

In conclusion, our results indicate that Aβ(1–42)-mediated degeneration in IMR-32 cells is due, at least in part to bax over-expression and to a contemporaneous down-regulation of bcl-2 gene expression, that leads to a large shift of bax/bcl-2 *ratio* in favour of the pro-apoptotic bax. The single Met-35 residue seems to play a crucial role, since its substitution with norleucine causes the loss of Aβ(1–42) neurotoxicity. In addition, oxidation of Met-35 to Aβ(1–42)$^{35Met\text{-}ox}$ significantly ameliorates the toxicity of Aβ(1–42) peptide, probably mediated by the reduction of the pro-apoptotic Bax/Bax homodimers through the formation of Bcl-2/Bax heterodimers. From a structural point of view, we previously suggested that the differential toxic behavior elicited by Aβ fragments containing methionine-35 in the reduced or in the oxidized form might be related to the Aβ aggregation level, i.e., monomeric or aggregated form [38].

It has been pointed out that the oxidation of Met-35 in Aβ(1–42) peptide may represent a mechanism affording protection against the neurotoxic actions of Aβ(1–42) [38,72,73]. Thus, this paper may provide a new opportunity for the development of an effective AD therapy as well as elucidating the pathological mechanism of AD.

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