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Aβ(31–35) peptide induce apoptosis in PC 12 cells: Contrast with Aβ(25–35) peptide and examination of underlying mechanisms

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Abstract

The toxic behaviour of the two shorter sequences of the native Aβ amyloid peptide required for cytotoxicity i.e., Aβ(31–35) and Aβ(25–35) peptides, was studied. We have shown that Aβ(31–35) peptide induces neurotoxicity in undifferentiated PC 12 cell via an apoptotic cell death pathway, including caspase activation and DNA fragmentation. Aβ(25–35) peptide, like the shorter amyloid peptide has the ability to induce neurotoxicity, as evaluated by the MTS reduction assay and by adherent cell count, but the Aβ(25–35) peptide-induced neurotoxicity is not associated with any biochemical features of apoptosis. The differences observed between the neurotoxic properties of Aβ(31–35) and Aβ(25–35) peptides might result on their different ability to be internalised within the neuronal cells. Furthermore, this study reveals that the redox state of methionine residue, C-terminal in Aβ(31–35) and Aβ(25–35) peptides affect in a different way the toxic behaviour of these two short amyloid fragments. Taken together our results suggest that Aβ(31–35) peptide induces cell death by apoptosis, unlike the Aβ(25–35) peptide and that role played by methionine-35 in Aβ induced neurotoxicity might be related to the Aβ aggregation state.

Keywords: Amyloid β-peptide; AβP(31–35) fragment; AβP(25–35) fragment; Methionine oxidation; Mitochondria; Apoptosis; Neurotoxicity

1. Introduction

Alzheimer's disease (AD) is a neuronal pathology characterised by the presence of senile plaques in several regions of the brain, particularly concentrated in those zones where neurodegeneration occurs. The major protein component of the plaques is the amyloid β-peptide (AβP), which is a 39–43 amino acid peptide that is formed by a much larger transmembrane protein, the amyloid precursor protein (APP) (Selkoe, 1994; Hardy, 1997). It is widely accepted that AD syndrome starts with various gene defects, leading to altered APP expression or proteolytic processing, or to changes in Aβ stability or aggregation. These in turn result in a chronic imbalance between Aβ production and clearance. Aβ is released extra and intracellularly and can also be accumulated extra and intracellularly (Dickson, 2004). It has been reported that toxic conformation of Aβ is a quaternary structure such as an aggregated fiber (Pike et al., 1991) and its gradual accumulation may represent a starting point of a complex, multistep cascade that include inflammatory changes, gliosis and transmitter loss (Selkoe, 2002). However, other reports have indicated that non-aggregated Aβ may intercalate into plasma membrane and directly alter membrane activities (Arispe et al., 1993; Etcheberrigaray et al., 1994; Muller et al., 1998). Recently, we have reported that Aβ(31–35) peptide, although it does not exhibit aggregation phenomena (Yan et al., 1999), induce several toxic and pro-apoptotic effects in isolated brain mitochondria (Misiti et al., 2004).

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This finding supports the hypothesis that Aβ peptide in non-aggregated form might play a key role in neurotoxicity (Pillot et al., 1996, 1999; Schubert et al., 1995; Lambert et al., 1998; Favit et al., 1998; Arispe et al., 1993; Etcheberrigaray et al., 1994). In addition, we have demonstrated that Aβ(31–35) peptide, with the sulfur of methionine oxidised, shows a toxic behaviour different from that displayed by unoxidised Aβ(31–35) (Misiti et al., 2004; Clementi et al., 2004). These and other reports suggest that the active centre of neurotoxicity of AβP may be the 31–35 core sequence and methionine 35 might play a key role in the Aβ dependent mechanism of toxicity (Butterfield and Bush, 2004; Hou et al., 2002; Varadarajan et al., 2001). Because the relation existing between the oxidation of Met-35 and its influence on β-aggregation, neurotoxicity and fibril formation of AβP (Iversen et al., 1995; Varadarajan et al., 1999; Seilheimer et al., 1997), still remain controversial, in the current study, we further investigate the role of methionine 35, comparing the neurotoxic features of Aβ(25–35) peptide, that exhibit significant levels of molecular aggregation, retaining the toxicity of the full-length peptide (Terzi et al., 1995; Shearman et al., 1994) with those evidenced by the soluble AβP(31–35) peptide and by a series of peptides where the methionine at residue 35 is substituted by norleucine or it is oxidised to a sulfoxide.

Our results show that in neuronal PC 12 cells, the soluble Aβ(31–35) peptide induces cell death by apoptosis, unlike the aggregated Aβ(25–35) peptide does. Furthermore, methionine residue, C-terminal in Aβ(25–35) and Aβ(31–35) peptides mediates the degree of neurotoxicity induced by soluble Aβ(31–35) peptide, but not that by the aggregated form of Aβ(25–35) peptide, suggesting that the differential role played by methionine-35 in Aβ induced neurotoxicity is related to the Aβ aggregation form.

2. Materials and methods

2.1. Preparation of peptides

Aβ(25–35), Aβ(25–35)Met-35^{ox}, Aβ(25–35)Met-35^{Nle}, Aβ(31–35), Aβ(31–35)Met-35^{ox} and Aβ(31–35)Met-35^{Nle} were obtained by Peptide Speciality Laboratories GmbH (Heidelberg, Germany). Analysis of the peptide by reverse phase high-performance chromatography (HPLC) and mass spectrometry revealed a purity >98%. Stock solutions of the soluble Aβ peptides were prepared at 8 mM concentration in DMSO, according to Boland et al. (1996) and kept frozen at −20 °C. Stock solutions of the aggregated form of Aβ(25–35) were prepared according to the manufacturer's instructions by leaving a 4 mM Aβ(25–35) solution in ddH_2O at room temperature for 2 h. Thawing and dilutions to the final concentration in the proper medium was performed immediately before use.

2.2. FITC labelling of peptides

Fluorescein isothiocyanate (FITC) was purchased from Sigma. Labelling was conducted essentially according to the manufacturer's recommendations. In brief, FITC was freshly dissolved in Me_2SO to 1 mg/ml, and added to 2 mg/ml of each peptide in 50 mM potassium phosphate buffer (final pH 7.6) to a final concentration of 25 μg/ml. The calculated molar ratio of FITC to peptides was about 1:10. After incubation for 16 h in the dark at 4 °C, 50 mM NH_4Cl was added to inactivate the residual FITC. The solutions were left in the dark for an additional 2 h at 4 °C, and stored in aliquots at 2.5 mM at −20 °C. At the moment of experiments fluorescent compounds, at a final concentration of 40 μM was added to PC 12 cell suspensions kept in ice. Incubation has been performed for 2 h at +4 and +37 °C. Thereafter cells were washed three times with saline solution (NaCl 0.7% v/v) and observed at a fluorescent phase contrast inverted microscope (Olympus IX-70 System). Images were taken by using a 40× objective. Processing images was done on a PC using the software package Photoshop (Adobe Systems Inc., Mountain View, CA, USA).

2.3. Cell culture and Aβ treatment

The PC 12 cell line maintained in 5% CO_2 atmosphere at 37 °C in RPMI with HEPES 10 mM, glucose 1.0 g/l, $NaHCO_3$ 3.7 g/l, penicillin 100 units/ml, streptomycin 100 μg/ml, 10% fetal calf serum and 15% horse serum. The medium was changed every other day and cells were plated at an appropriate density according to each experiment scale. For determination of cytotoxicity of Aβ peptides, PC 12 (rat pheocromocytoma) were initially plated in 96-well plates at a density of 10,000 cells/well and maintained 16 h in complete medium cells were then incubated for 24, 36 and 48 h in the absence (controls) and presence of 40 μM Aβ(31–35) and Aβ(25–35) peptides with methionine in the oxidised and unoxidised form and with Met → Nle substituted Aβ peptides. Staurosporine 10 μM was used as positive control of 100% of cellular death (data do not shown).

2.4. Direct toxicity study

Cell survival was evaluated by two different methods: 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) and the number of adherent cells on collagen type 1 coated plates (SIGMA, St. Louis, USA). The MTS assay is a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflects early cellular redox changes. After incubation, 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-wells plate using the automatic microplate photometer (SpectraCount, Packard Bioscience Co., Groningen, Netherlands) at a wavelength of 490 nm (Cory et al., 1991). The reference wavelength was 690 nm. For adherent cell count, 96-well plates were gently washed twice with phosphate-buffered saline (PBS) and the number of remaining cells was counted, using MTS reduction system above described.

2.5. DNA fragmentation

To prepare cellular DNA, PC 12 cells (6.0×10^5) were centrifuged at 1500 rpm for 10 min and the pellets were washed with PBS. Cellular DNA was isolated according to the method (Jiang et al., 2004). Briefly cells were re-suspended in 200 μl of a Lysis Buffer containing 50 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS, 1% Triton X-100, 0.25 mg/ml RNAse A, 100 μg/ml Proteinase K, and treated for 2 h at 50 °C. After incubation, samples were extracted twice with an equal volume of phenol:-chloroform:isoamyl alcohol (25:24:1) and centrifuged at 10,000 rpm for 10 min. DNA was then precipitated, after adding 0.3 M (f.c.) sodium acetate, with two volume of EtOH at −20 °C overnight. Samples were centrifuged at 13,000 rpm for 10 min, the pellets were then washed in 80% ethanol, centrifuged at 13,000 rpm for 5 min, dried in microvacuum and finally re-suspended in TE 10:1 pH 8.0 (10 mM Tris–HCL pH 8.0 and 1 mM EDTA pH 8.0).

The DNA fragments were extracted from PC 12 cells following treatment with Aβ peptides (48 h at 37 °C) in a 5% CO_2 incubator. The typical ladder pattern was observed by agarose gel electrophoresis 1.2% in TBE 1× buffer (Tris 40 mM, EDTA 1 mM, boric acid 44 mM) for 1.30 h at 90 V and visualised after plunging agarose in ethidium bromide 1 μg/ml solution at dark for 20 min. The gel was examined and photographed using an ULTRA Violet Products Gel Documentation System (Cambridge, UK).

2.6. Measurement of caspase activity

Caspase activity was measured by using Sigma (St. Louis, USA) assay kit following manufacture's instructions. DEVD-pNA was used as a colourimetric substrate. PC 12 cells were plated at a density of 1×10^6 cells/culture dish. After treatment with Aβ peptides (40 μM for 48 h at 37 °C in a 5% CO_2 incubator), the PC 12 cells were harvested by centrifugation. The pellets were washed with PBS and lysed in 50 ml of chilled cell lysis buffer and left on ice for 10 min. Lysate was centrifuged at 10,000 rpm for 1 min at 4 °C, and 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*-ntroanilide (pNA) after its cleavage by caspase 3 from the labelled caspase-3-specific substrate (DEVD-pNA). Additional control assays with 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).

2.7. Statistics

Data were analysed for statistical differences by the two-way and one-way analysis of variance as well as by the two-tailed Student's *t*-test; a *p*-value of less than 0.05 was considered significant.

3. Results

We examined the toxicity of Aβ peptides in a cell line of neuronal origin. Toxicity was measured by the MTS reduction assay and by counting the number of adherent cells on collagen type 1 coated plate. First, we examined the effect of Aβ(31–35), Aβ(25–35) and the other amyloid peptides on MTS reduction in PC 12 cells. In each case, the peptides were prepared in ddH_2O, as described in Section 2, in order to optimise formation of aggregates in Aβ(25–35) peptide samples (Buchet et al., 1996). The results depicted in Fig. 1 show that Aβ(25–35)Met-35^{ox} peptide had a similar degree of toxicity on neuronal cell cultures to that of Aβ(25–35) and Aβ(25–35)Met-35^{Nle} peptides. Importantly, neurotoxic effects were already statistically significant after 24 h incubation. In contrast, in the experiments with Aβ(31–35) peptides, MTS assay indicates that a longer incubation period of 24 h results in a significant level of toxicity. This finding suggests the existence of distinct mechanisms of toxicity by Aβ(31–35) and Aβ(25–35) peptides, respectively. Intriguingly, Aβ(31–35) peptide resulted significantly more toxic with respect to that shown by Aβ(31–35)Met-35^{ox} peptide. Under the same conditions, Aβ(31–35)Met-35^{Nle} was not toxic to PC 12 neuronal cells at 24, 36 and 48 h.

Cell viability was further assessed by counting the number of adherent cells on collagen type 1 coated plates after Aβ peptides treatment for 24 and 48 h. As shown in Fig. 2, oxidised Aβ peptides, like the unoxidised Aβ peptides, decreased the number of adherent cells on collagen type-1 coated plates, within 24–48 h of Aβ peptide exposure. Interestingly, cell degeneration count did not correlate with the extent of mitochondrial activity observed by the MTS assay. After 24 h of exposure to Aβ(31–35) and Aβ(31–35)Met-35^{ox} peptides, the number of cells adhering to the collagen-coated plates showed a larger decrease (∼22 and ∼13% reduction relative to control, respectively) compared with the extent of the mitochondrial activity (8 and 7% reduction relative to control, respectively). The observed pattern of Aβ(31–35) peptide-induced toxicity is consistent with an apoptotic pathway, which is characterised by an early cell shrinkage and membrane blebbing, followed

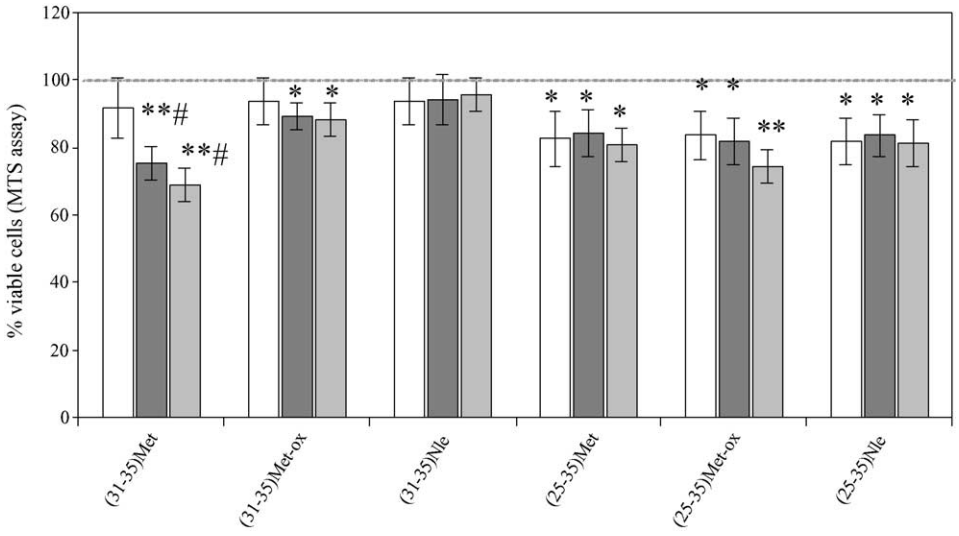

Fig. 1. Effect of Aβ(31–35), Aβ(31–35)Met-35^{ox}, Aβ(31–35)Met-35^{Nle}, Aβ(25–35), Aβ(25–35)Met-35^{ox} and Aβ(25–35)Met-35^{Nle} peptides on PC 12 cell survival. PC 12 cells were treated with 40 μM Aβ(31–35), Aβ(31–35)Met-35^{ox}, Aβ(31–35)Met-35^{Nle}, Aβ(25–35), Aβ(25–35)Met-35^{ox} and Aβ(25–35)Met-35^{Nle} peptides for 24 h (white bars), 36 h (grey bars) and 48 h (dotted bars) and viable cells were evaluated as MTS transformation as described in Section 2. Results are expressed as the percentages of non-treated cells. Bars are means of five separate experiments ± S.D. (*) Indicates a significant difference from control at $P < 0.05$; (**) indicate a significant difference from control at $P < 0.001$; ($^{\#}$) indicates a significant difference from oxidised Aβ(31–35) at $P < 0.05$ (control at 24, 36 and 48 h were not significantly different).

by impairment of mitochondria and late membrane lysis. In Alzheimer's disease, mechanisms of programmed cell death and especially apoptosis have been suggested (Kienlen-Campard et al., 2000). To further investigate this hypothesis, an analysis of DNA fragmentation was carried out on Aβ peptides treated neurones. PC 12 cells treated with Aβ(31–35) and Aβ(31–35)Met-35^{ox} peptides, unlike cells treated with Aβ(25–35) and Aβ(25–35)Met-35^{OX}, underwent

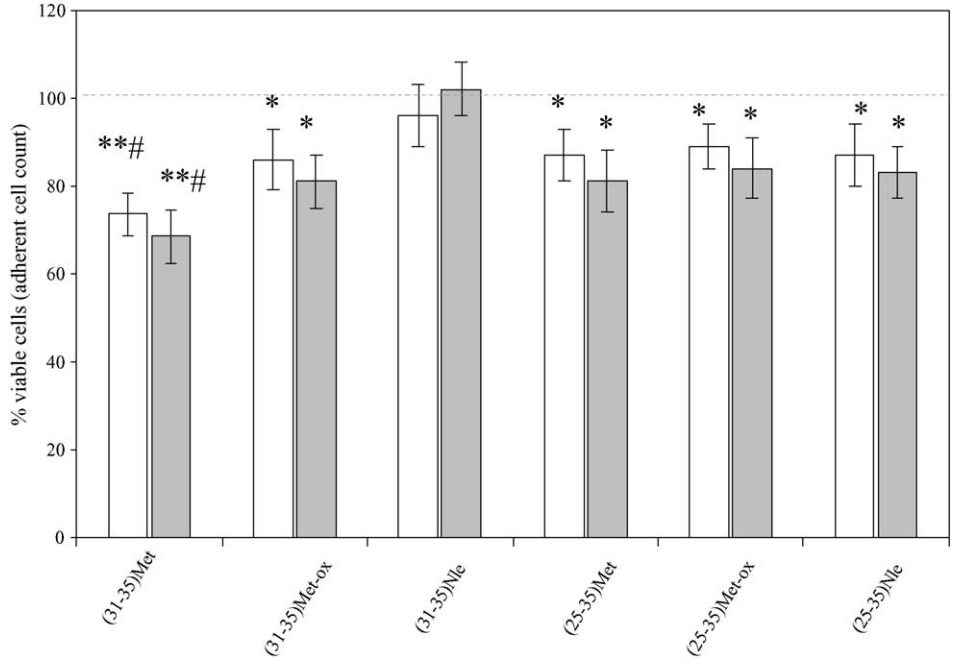

Fig. 2. Effect of Aβ(31–35), Aβ(31–35)Met-35^{ox}, Aβ(31–35)Met-35^{Nle}, Aβ(25–35), Aβ(25–35)Met-35^{ox} and Aβ(25–35)Met-35^{Nle} peptides on PC 12 cell survival. PC 12 cells were treated with 40 μM Aβ(31–35), Aβ(31–35)Met-35^{ox}, Aβ(31–35)Met-35^{Nle}, Aβ(25–35), Aβ(25–35)Met-35^{ox} and Aβ(25–35)Met-35^{Nle} peptides for 24 h (white bars) and 48 h (grey bars) and viable cells were evaluated by adherent cell count as described in Section 2. Results are expressed, as the percentages of non-treated cells Bars are means of six separate experiments ± S.D. (*) Indicates a significant difference from control at $P < 0.05$; (**) indicate a significant difference from control at $P < 0.001$; ($^{\#}$) indicates a significant difference from oxidised Aβ(31–35) at $P < 0.05$ (control at 24 and 48 h were not significantly different).

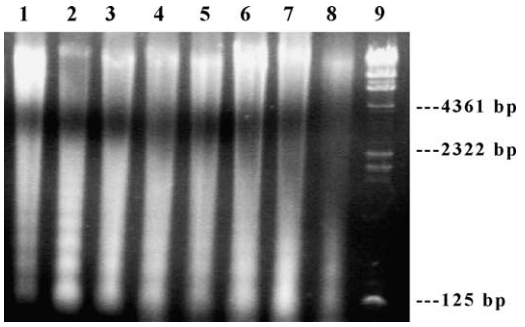

Fig. 3. The detection of apoptosis by analysis of DNA fragmentation in PC 12 neuronal cells after 48 h treatment with 40 μM Aβ(31–35), Aβ(31–35)Met-35^{ox}, Aβ(31–35)Met-35^{Nle}, Aβ(25–35), Aβ(25–35)Met-35^{ox} and Aβ(25–35) Met-35^{Nle} peptides. Cells were harvested after being incubated with each peptide for 48 h and DNA was isolated. When apoptosis occurs, a ladder pattern shows on agarose gels after electrophoresis. Lane 1, control; lane 2, 450 μM of H_2O_2 for 24 h; lane 3, Aβ(31–35) peptide; lane 4, Aβ(31–35)Met-35^{ox} peptide; lane 5, Aβ(31–35)Met-35^{Nle} peptide; lane 6, Aβ(25–35) peptide; lane 7, Aβ(25–35)Met-35^{ox} peptide; lane 8, Aβ(25–35) Met-35^{Nle}; lane 9, standard molecular weights.

apoptosis as determined by analysis of DNA fragmentation. As shown in Fig. 3, DNA extracted from PC 12 cells after 48 h of exposure to Aβ(31–35) and Aβ(31–35)Met-35^{ox} peptides resulted clearly fragmented. DNA fragmentation was slightly present in treated PC 12 neuronal cells with Aβ(31–35)Met-35^{Nle} and with all the variant of Aβ(25–35) peptides. Because, in addition to DNA nuclear fragmentation, caspase activation is recognised as a critical event in apoptosis, it was of interest to determine whether caspase 3 activation occur in PC 12 cells following to Aβ peptides exposure. Fig. 4, shows that a 7- and 4-fold stimulation induction of caspase 3 activity occur in PC 12 cells treated

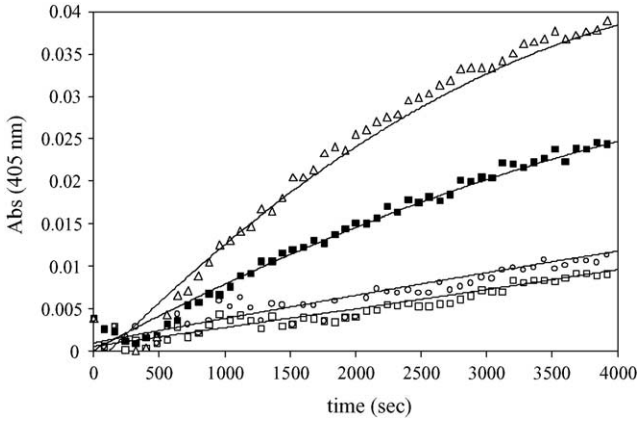

Fig. 4. The effect of Aβ(31–35), Aβ(31–35)Met-35^{ox}, Aβ(31–35)Met-35^{Nle} on Caspase 3 activity. PC 12 cells were plated at a density of 1×10^6 cells/culture dish. Aβ peptides were added to each dish at a final concentration set at 40 μM for 48 h. Activity of caspase 3 was investigated at 37 °C (followed as DEVD-pNA cleavage and increase in optical absorbance at 405 nm) in cell lysates obtained by PC 12 cells at the end of treatment time. Aβ(31–35) (△), Aβ(31–35)Met-35^{ox} (■), Aβ(31–35)Met-35^{Nle} (○) peptides. Not treated cells (□). The experiment shown is typical for five separate assays.

for 48 h with Aβ(31–35) and Aβ(31–35)Met-35^{ox} peptides, respectively. Importantly, caspase activation resulted almost completely absent in Aβ(31–35)Met-35^{Nle} treated cells. Under the same conditions, Aβ(25–35) and Aβ(25–35)Met-35^{ox} did not induce any effect on cytosolic caspase 3 in neuronal PC 12 cells (data not shown), ruling out the possibility that, under our experimental conditions, Aβ(25–35) and Aβ(25–35)Met-35^{ox} could induce apoptosis in PC 12 cells.

With the goal of identifying the real mechanism that could account for the distinct mechanisms of Aβ(31–35) and Aβ(25–35) peptides induced neurotoxicity, we examined the interaction of different Aβ peptides with PC 12 neuronal cells. We initially characterised the amount of FITC-derivative soluble forms of Aβ(31–35) and Aβ(25–35) peptides internalised by PC 12 cells during 2 h incubation at 4 and 37 °C. A significant larger amount of Aβ(31–35) peptide is internalised over 2 h incubation period at 37 °C in comparison to the soluble forms of Aβ(25–35) peptide (Fig. 5 panel A–C, respectively). A behaviour similar to the parental peptide was observed by the soluble forms of the oxidised and substituted Aβ(31–35) and Aβ(25–35) peptides, respectively (data not shown).

Temperature may affect the binding of the ligand (rate) as well as the lateral mobility of the ligand-receptor complex. Some ligands will not bind well at low temperatures. However, others will bind, but not be taken in. Our result shows that internalization of soluble forms of Aβ peptides do not occur at 4 °C (data not shown), suggesting the involvement of a patching or capping mechanism in the entire process of internalization of Aβ peptides.

The ability to be internalised within the PC 12 cells was restricted to the FITC-derivative soluble form of the Aβ(25–35) peptide, whereas the aggregated form of the Aβ(25–35) peptide, at the same concentration was totally ineffective (Fig. 5 panel B). The oxidised and the Met-35 → Nle aggregate form of Aβ(25–35) peptides were again unable to be internalised (data not shown).

4. Discussion

Whereas a correlation between the neurotoxicity of Aβ and its aggregated form has been proposed, previous data suggests that neurodegeneration could occur independently of the presence of amyloid aggregates. (Etcheberrigaray et al., 1994; Pillot et al., 1996, 1999; Schubert et al., 1995; Lambert et al., 1998; Favit et al., 1998). Recently, it has been reported that soluble forms of Aβ might contribute to neurodegeneration mechanisms by altering neuronal functions by means other than molecular aggregate formation (Barnham et al., 2003). With the aim to distinguish between the neurotoxic mechanisms of soluble and aggregated forms of Aβ peptides, we have used as peptide model, the two shorter sequences of the native Aβ amyloid peptide required for cytotoxicity (Yan et al., 1999; Misiti et al., 2004; Milton,

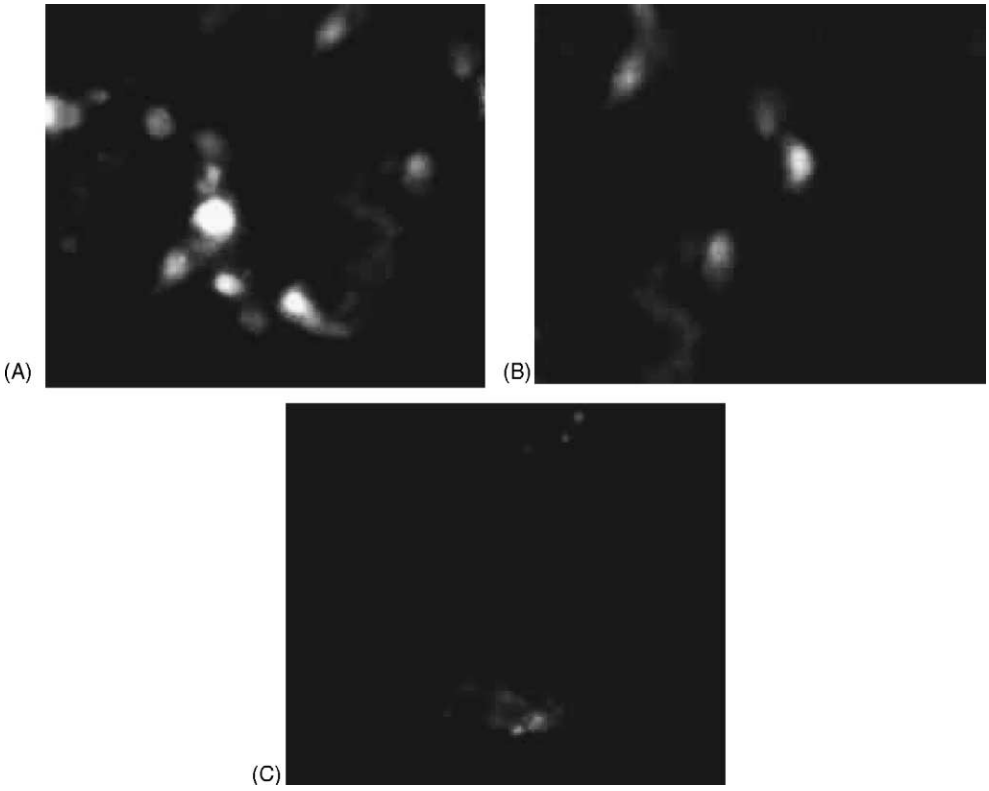

Fig. 5. Analysis of Aβ peptides cellular uptake. Confocal fluorescence microscopy images of PC 12 neuronal cells after 2 h treatment at 37 °C with 40 μM FITC labelled Aβ peptides solubilised under different experimental conditions: Aβ(31–35) solubilised in ddH_2O (panel A), Aβ(25–35) solubilised in ddH_2O (panel B), Aβ(25–35) solubilised in DMSO (panel C). The presence of FITC-linked fluorescence signal in cells shown in panels A and C confirms the Aβ peptide localization within the PC 12 cell.

2001). The data presented here show that both the soluble Aβ(31–35) peptide and the aggregated Aβ(25–35) peptide induce neurotoxicity in PC 12 neuronal cell, but neurotoxicity by Aβ(31–35) peptide, unlike that by Aβ(25–35) peptide, is mediated by an apoptotic mechanism. The shorter fragment of Aβ peptide has been previously shown to induce apoptosis in cultured cortical neurons (Yan et al., 1999). Apoptosis describes a type of cell death exhibiting a distinct set of morphological and biochemical features such as cell shrinkage, caspase activation, chromatin condensation, break up of nucleus followed by DNA fragmentation (Kerr et al., 1972). In this study, some of these changes, such as caspase activation and DNA fragmentation are clearly observed in PC 12 cells after 48 h treatment of cells with Aβ(31–35) peptide, unlike in Aβ(25–35) treated cells. Cytochrome *c* release and mitochondrial membrane potential ($\Delta\Psi$) collapse, two critical events in the mechanisms promoting apoptosis in response to death inducing signals from mitochondria, occur in brain mitochondria, following exposure to Aβ(31–35) peptide (Misiti et al., 2004).

On the basis of these findings, we suggest that Aβ(31–35) peptide might induce toxicity in PC 12 cells, through mitochondria damage, release of cytochrome *c* from inner mitochondrial membrane and activation of caspase activities, both events associated with the observed nuclear DNA cleavage into ladders.

With the goal of identifying the real mechanism that could account for the distinct mechanisms of Aβ(31–35) and Aβ(25–35) neurotoxicity, we examined the interaction of these Aβ peptides with PC 12 neuronal cells. There are two prominent differences in the manner in which Aβ(31–35) and Aβ(25–35) interacts under physiological conditions with PC 12 cells: the first preferentially adsorb to the cell surface and as a consequence is preferentially internalised, the second has not the ability to enter within the cell. The most obvious explanation for this distinct behaviour is that if Aβ(31–35) has not the ability to form aggregates while Aβ(25–35) peptide shows significant levels of molecular aggregation (Iversen et al., 1995; Terzi et al., 1995; Shearman et al., 1994), this alone could account for why Aβ(31–35) is preferentially internalised within the neuronal cells. This suggestion is supported by a recent report, that indicate that the serpin enzyme complex receptor (SEC-R) may bind the FVFLM pentapeptide similar to the sequence of Aβ(31–35), but it does not recognise the aggregated form of Aβ(25–35) (Boland et al., 1996). Further, Matter et al. (1998) demonstrated that α5β1 integrin receptor binds solely soluble Aβ peptides. On the basis of these findings, we suggest that soluble Aβ(31–35) peptide could initiate a cascade of events leading to neurotoxicity and cell death solely after its internalisation within the cells by means of a receptor mediated mechanism (Skovronsky et al., 1998).

At this regard, mitochondria–Aβ peptide interaction seems to represent one of the initial events in the mechanisms promoting toxicity (Yan et al., 1997). The exact mechanism responsible for Aβ-associated activation of cell death pathways still remains unclear, but there is convincing evidence supporting the involvement of Met-35 side chain. Methionine-35 is the residue in Aβ most susceptible to oxidation in vivo and necessary for the generation of ROS (Butterfield, 1997; Yatin et al., 1999; Vogt, 1995). We provide evidence that although oxidation of Met-35 to Met-35^{ox} does not affect the internalization process of the Aβ(31–35) peptide, it significantly affect the amplitude of the AβP(31–35) induced toxicity within the cell. Among the different Aβ peptides used in this study, the Aβ(31–35) peptide with the methionine 35 in the reduced form resulted more active in the induction of caspase activation, in contrast to the behaviour showed by variant peptides of Aβ(31–35) with methionine 35 oxidised or substituted by another aminoacid i.e., norleucine. These findings outline the importance of the redox state of Met-35 in the modulation of Aβ-mediated event (Butterfield and Bush, 2004). Because Aβ(31–35) peptide does not exhibit aggregation phenomena (Yan et al., 1999), a different fibril assembly can not explain the different toxic behaviour shown by Aβ(31–35) and Aβ(31–35)Met-35^{ox} peptides. The differences observed between the neurotoxic properties of the Aβ(31–35) and Aβ(31–35)Met-35^{ox} peptides might result from differences in the ability to interact with lipid bilayer of the membranes. Indeed it is known that the oxidation of methionine 35 in the Aβ(31–35) peptide reduces the hydrophobicity of the amyloid peptide, limiting probably its ability to intercalate into mitochondrial membranes (Misiti et al., 2004; Watson et al., 1998).

Although, the aggregated form of Aβ(25–35) peptide has not the ability to be internalised within the PC 12 cells, unlike its correspondent soluble form, probably it interacts with plasma membrane, inducing cytotoxic effects independently and/or dependently of a receptor mediated pathway. At this regard, an increased cytosolic calcium concentration linked to a Ca^{2+} channel protein overexpression and a marked decrease in some key enzyme activities have been evidenced in Aβ(25–35) treated cells (Ba et al., 2004; Bielarczyk et al., 2003). Further, Verdier et al. (2004) showed that several membrane proteins might mediate the interaction between the aggregated forms of Aβ peptides and the plasma membrane.

A recent research reported that Aβ(25–35) activates early neurotoxic events, but these events do not result in the activation of apoptotic pathways (Saito et al., 2001). These report supports our results, because in this study we evidenced neurotoxicity in PC 12 following to aggregated Aβ(25–35) exposure, as evaluated by the MTS assay, but we did not evidence neither caspase activation and DNA fragmentation, differently with that observed in Aβ(31–35) treated cells. It may be that the toxic effects of Aβ(25–35) are weak and that aggregated Aβ(25–35) in itself does not induce activation of cell death pathways, as previously reported (Behl et al., 1994; Suzuki, 1997).

Previous studies have yet suggested the possibility that Aβ with different length may have distinct mechanisms of toxicity (Woods et al., 1995; Mattson et al., 1997). With the aim to characterise the factors that are responsible for the Aβ(25–35) induced mechanism of toxicity, we evaluated the link existing between the redox state of the C-terminal residue and neurotoxicity in the Aβ(25–35) peptide. Although, it has been reported that oxidation of methionine 35 may attenuate and slow the fibrillisation process in Aβ peptides (Hou et al., 2002; Palmblad et al., 2002), our results evidence that oxidation of Met-35 or the substitution with another aminoacid (i.e., norleucine) in Aβ(25–35) peptide did not affect significantly the amplitude of the Aβ(25–35) induced neurotoxicity. At this regard it has been reported that the oxidation of the sulfur of methionine 35 does not affect the metal binding properties of Aβ peptide, suggesting that oxidised and the unoxidised aggregated Aβ peptides may share a common mechanism of toxicity via a metal-dependent H_2O_2 formation (Kienlen-Campard et al., 2000). All these findings indicate that factors that govern the toxicity of aggregated Aβ(25–35) peptides may be deeply distinct with respect to that observed for the soluble Aβ(31–35) peptide. This different behaviour may be likely to arise because in the Aβ soluble forms, methionine 35 residues is more accessible than in the aggregated forms.

In summary, we have reported an analysis of the neurotoxic mechanisms of Aβ(25–35) and Aβ(31–35) peptides. The results of these studies confirm and extend previous findings (Pillot et al., 1996, 1999; Schubert et al., 1995; Lambert et al., 1998; Favit et al., 1998) that at least in some cases soluble Aβ peptides, that precede formation of mature amyloid aggregates, may be the primary species responsible for neurotoxicity. Such early toxicity seems to be mediated by an apoptotic mechanism.

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