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An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity

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Prion (PrP) and amyloid-β (Aβ) peptides are involved in the neuronal loss that occurs in Prion disorders (PrD) and Alzheimer's disease (AD), respectively, partially due to Ca^{2+} dysregulation. Besides, the endoplasmic reticulum (ER) stress has an active role in the neurotoxic mechanisms that lead to these pathologies. Here, we analyzed whether the ER-mediated apoptotic pathway is involved in the toxic effect of synthetic PrP and Aβ peptides. In PrP106–126- and Aβ1–40-treated cortical neurons, the release of Ca^{2+} through ER ryanodine (RyR) and inositol 1,4,5-trisphosphate (IP_3R) receptors induces ER stress and leads to increased cytosolic Ca^{2+} and reactive oxygen species (ROS) levels and subsequently to apoptotic death involving mitochondrial cytochrome *c* release and caspases activation. These results demonstrate that the early PrP- and Aβ-induced perturbation of ER Ca^{2+} homeostasis is a death message that leads to neuronal loss, suggesting that the regulation of ER Ca^{2+} levels may be a potential therapeutical target for PrD and AD.

Keywords: Prion disorders; Alzheimer's disease; Prion peptide; Amyloid-β peptide; Apoptosis; Ca^{2+} homeostasis; Endoplasmic reticulum; Oxidative stress

Introduction

Prion disorders (PrD) and Alzheimer's disease (AD) are progressive neurodegenerative diseases characterized by neuronal loss, cognitive dysfunction and dementia. These diseases are linked by the extracellular deposition of the scrapie isoform of prion protein (PrP^{Sc}) and the amyloid-beta protein (Aβ), respectively (Prusiner, 1996; Wisniewski et al., 1997). In PrD, the PrP^{Sc} results from the conversion of the normal protein PrP^{C}, is insoluble in nondenaturating detergents, aggregates easily and is partially resistant to protease digestion. The PrP106–126, a synthetic peptide homologous to PrP^{Sc}, has been used as a model system to study prion-induced neurodegeneration (Della-Bianca et al., 2001; Pérez et al., 2003). PrP106–126 is rich in β-sheet structure, forms aggregates that are detergent-insoluble and PK-resistant and catalyzes the aggregation of endogenous PrP^{C} to an amyloidogenic form that shares several characteristics with PrP^{Sc} (Ettaiche et al., 2000; Brown, 2000; Singh et al., 2000). In AD, the amyloidogenic material is composed of amyloid-beta peptide (Aβ), derived from β- and γ-secretases cleavage of amyloid precursor protein (APP) in the endoplasmic reticulum (ER), the Golgi apparatus or the endosomal–lysosomal pathway, and most is normally secreted as a 1–40 (Aβ1–40) or 1–42 (Aβ1–42) amino acid peptide (Perez et al., 1999). Synthetic Aβ1–40 and Aβ1–42 are amyloidogenic and neurotoxic peptides that have been widely used to mimic in vitro the degenerative process that occurs in the brain of AD patients (Mattson, 1997; Pereira et al., 1999).

The ER is an essential intracellular organelle involved in intracellular calcium homeostasis, in folding and processing of proteins and in cell death activation (Baumann and Walz, 2001). Ca^{2+} release from the ER is mediated through ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptors (IP_3R) (Berridge et al., 2000). Changes in ER Ca^{2+} homeostasis induce the accumulation of unfolded proteins and activate the ER-stress-induced apoptosis pathway (Kaufman, 1999; Pashen, 2001). In response to ER stress, expression of glucose-regulated protein (GRP) 78, an ER chaperone, is induced (Kaufman, 1999; Rao et al., 2002). Furthermore, caspase-12, that is mainly located on the cytoplasmic side of the ER, is activated (Nakagawa et al., 2000; Nakagawa and Yuan, 2000) and can further activate caspase-9 and -3 (Morishima et al., 2002).

It was recently shown that caspase-12 knock-out mice are resistant to ER stress and to death caused by Aβ protein (Nakagawa et al., 2000) and also that caspase-12 activation and increased expression of GRP58, a chaperone with (PDI)-like activity, occur in cells treated with PrP^{Sc} (Hetz et al., 2003, 2005). These results

Abbreviations: Aβ, amyloid-beta peptide; PrP, prion peptide; AD, Alzheimer's disease; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; Indo-1/AM, Indo-1 acetoxymethyl ester; IP_3R, inositol 1,4,5-triphosphate receptor; PrD, Prion disorders; ROS, reactive oxygen species; RyR, ryanodine receptor.

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strongly support that ER stress is involved in the neuronal death that occurs in PrD and AD. In vitro studies have suggested that ER Ca^{2+} dyshomeostasis and oxidative stress are synergistically related. While ER function is sensitive to the presence of oxidants (Dreher et al., 1995; Racay et al., 1995; Viner et al., 1996; Hayashi et al., 2005), reactive oxygen species (ROS) can also be produced intracellularly from the stress-regulated release of calcium from the ER. This calcium is readily taken up by mitochondria, resulting in the elevation of ROS (Tardif et al., 2005).

The purpose of the present study was to elucidate the role of ER-mediated apoptotic pathway in the neurotoxic effects induced by PrP106–126 and Aβ1–40. Our results suggest that, in ER, the early Ca^{2+} release through the RyR and IP_3R, induced by these peptides, is involved in the perturbation of Ca^{2+} homeostasis and ROS production, leading to cytochrome *c* release and caspase-3 activation and finally to apoptotic cell death. These findings give a contribution to the elucidation of the mechanism of neuronal death that occurs in PrD and AD.

Materials and methods

Materials

Neurobasal medium and B27 supplement were purchased from Gibco BRL, Life Technologies (Scotland, UK). Trypsin, deoxyribonuclease I (DNase I), trypsin inhibitor type II-S-soybean, protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), dantrolene and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SYTO-13, propidium iodide (PI), Indo-1 acetoxymethyl ester (Indo-1/AM), 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), MitoTracker Green, Hoechst 33342 and Alexa Fluor 594 goat anti-mouse IgG conjugate were purchased from Molecular Probes (Leiden, Netherlands). The synthetic Aβ1–40, Aβ40–1, PrP106–126 and PrPscrambled peptides were from Bachem (Bubendorf, Switzerland). Ionomycin and xestospongin C were purchased from Calbiochem (Darmstadt, Germany). The colorimetric substrates for caspase-2 [*N*-acetyl-Val-Asp-Val-Ala-Asp-*P*-nitroanilide (Ac-VDVAD-pNA)], caspase-6 [*N*-acetyl-Val-Glu-Ile-Asp-*P*-nitroanilide (Ac-VEID-pNA)] and caspase-9 [*N*-acetyl-Leu-Glu-His-Asp-*P*-nitroanilide (Ac-LEHD-pNA)] were obtained from BioSource International (Nivelles, Belgium), whereas the substrate for caspase-3 [*N*-acetyl-Asp-Glu-Val-Asp-*P*-nitroanilide (DEVD-pNA)] was from Calbiochem (Darmstadt, Germany) and for caspase-8 [*N*-acetyl-Ile-Glu-Thr-Asp-*P*-nitroanilide (Ac-IETD-pNA)] was from Sigma Chemical Co. (St. Louis, MO, USA). Bio-Rad protein dye assay, reagents and apparatus used in immunoblotting assays were purchased from Bio-Rad (Hercules, CA, USA). The following primary antibodies were from BD Pharmingen (San Diego, CA, USA): monoclonal mouse anti-BiP/Grp78, monoclonal mouse reactive against the native form of cytochrome *c* and polyclonal rabbit anti-caspase-12. The monoclonal mouse anti-microtubule-associated protein 2 (MAP2) and the rabbit anti-glial fibrillary acidic protein (GFAP) antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The goat anti-rabbit IgG and goat anti-mouse IgG antibodies conjugated to alkaline phosphatase, the enhanced chemiFluorescence reagent (ECF) and the polyvinylidene difluoride (PVDF) membrane were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). The DakoCytomation Fluorescent mounting medium was purchased from DakoCytomation (Carpinteria, California, USA). All the other chemicals were obtained from Sigma (St. Louis, MO, USA) or from Merck kgaA (Darmstadt, Germany).

Primary cortical neuronal cultures and experimental treatments

Primary cultures of cortical neurons were prepared from 15 to 16 days embryos of Wistar rats according to the method described by Hertz et al. (1989), with some modifications (Agostinho and Oliveira, 2003). Briefly, removed cortices were dissected and placed in Ca^{2+}- and Mg^{2+}-free Krebs buffer (in mM): NaCl 120, KCl 4.8, KH_2PO_4 1.2, glucose 13 and HEPES 10 (pH 7.4) supplemented with BSA (0.3 mg/ml). Minced cortical tissues were washed and incubated in Krebs solution supplemented with BSA, and containing trypsin (0.5 mg/ml) and DNase I (0.04 mg/ml), for 10 min at 37°C. The digestion was stopped with Krebs buffer containing trypsin inhibitor (type II-S) (0.75 mg/ml) and DNase I (0.04 mg/ml) followed by a centrifugation at 140 × *g* for 5 min. After washing the pellet once with Krebs buffer, the cells were resuspended in fresh Neurobasal medium supplemented with 2 mM L-glutamine, 2% B27 supplement, penicillin (100 U/ml) and streptomycin (100 μg/ml) and were dissociated mechanically. Cortical cells were plated on poly-L-lysine (0.1 mg/ml)-coated glass coverslips at a density of 0.10×10^6 cells/cm^2 for immunocytochemistry experiments and nuclear morphology studies or 0.40×10^6 cells/cm^2 for measurement of intracellular Ca^{2+} or ROS levels. For caspase-like activity assays and immunoblotting, neurons were mounted on poly-L-lysine (0.1 mg/ml)-coated dishes at a density of 0.45×10^6 cells/cm^2. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and were used for experiments after 5 to 7 days in vitro. Differentiated cortical neurons were treated with Aβ1–40 or Aβ40–1 (0.5 μM) or with PrP106–126 or PrPscrambled (25 μM) in serum-free Neurobasal medium supplemented with B27. PrP peptides were added from a stock solution prepared in sterile distilled water, at a concentration of 1 mM. Aβ peptides were dissolved in sterile distilled water at a concentration of 6 mg/ml and diluted to 1 mg/ml (231.5 μM) with phosphate saline buffer (PBS) and then incubated for 5–7 days to induce fibril formation. The concentrations of Aβ1−40 and PrP106–126 used were chosen based on previous results demonstrating that, on cortical neurons, 0.5 μM Aβ1–40 and 25 μM PrP106–126 induced a maximal toxic effect (50% decrease in cell viability). Before the addition of Aβ1–40 or PrP106–126, cells were preincubated for 1 h with dantrolene (10 μM) or xestospongin C (1 μM), concentrations that were shown to exert a maximal protective effect, without being toxic by itself (data not shown).

Assessment of neuronal injury

After treatment of cortical cells with Aβ1–40 or PrP106–126, neuronal injury was assessed as described in Ferreiro et al. (2004). Reverse Aβ1–40 (Aβ40–1) or scrambled PrP was also used in order to analyze the specificity of the amino acids sequence of both peptides. Neurons were stained with PI (2.5 μg/ml) and SYTO-13 (3.8 μM), and the nuclear morphology was analyzed by fluorescence microscopy. Cortical cells cultured on glass coverslips were treated with Aβ1–40 or PrP106–126, in the absence or in the presence of dantrolene or xestospongin C. Cells were examined and scored with a Nikon Diaphot TMP microscope, using a triple XF-63 Omega filter. All experiments were performed in duplicate,

and a minimum of 300 cells were scored for each coverslip. The number of viable, necrotic and apoptotic cells was expressed as the percentage (%) of the total number of cells in the microscope field.

Measurement of intracellular Ca^{2+} concentration

Cortical cells cultured on glass coverslips, in the presence or in the absence of peptides, were incubated in the dark with 3 μM Indo-1/AM in a salt solution without phosphate containing (in mM): NaCl 132, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1.4, glucose 6, HEPES–Na 10, pH 7.4 for 45 min at 37°C. The cells were further incubated, for 15 min, in the absence of Indo-1/AM, to ensure a complete hydrolysis of the acetoxymethyl ester of Indo-1. After washing, cells were mounted in a special holder, and the Indo-1 fluorescence was measured with excitation wavelength of 335 nm and 410 nm emission. The free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated as previously described (Bandeira-Duarte et al., 1990).

Caspases activity assay

Untreated or treated cortical cells were lysed with a buffer containing (in mM): HEPES–Na 25, $MgCl_2$ 2, EDTA 1, EGTA 1 supplemented with 100 μM phenylmethylsulphonyl fluoride (PMSF), 2 mM DTT and a protease inhibitor cocktail (containing 1 μg/ml leupeptin, pepstatin A, chymostatin and antipain). The cellular suspension was rapidly frozen/defrosted three times and then centrifuged for 10 min at 20,200 × *g*. The supernatant was collected and assayed for protein content using the Bio-Rad protein dye assay reagent. To measure caspases activity, aliquots of cell extracts containing 25 μg or 40 μg of protein were incubated for 2 h at 37°C, in a reaction buffer containing 25 mM HEPES–Na, 10 mM DTT, 10% sucrose and 0.1% CHAPS (pH 7.4) with 100 μM Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-VEID-pNA, Ac-IETD-pNA or Ac-LEHD-pNA, chromogenic substrates for caspase-2, -3, -6, -8 and -9, respectively (Cregan et al., 1999). Caspases activity was determined by measuring substrate cleavage at 405 nm using a microplater reader, and results were expressed as the increase above control absorbance at 405 nm.

Western blotting analysis

For the preparation of total cell extracts, cells which were either treated or untreated with the peptides were scraped in Neurobasal medium and centrifuged at 140×*g*. The pellet was resuspended in 200 μl of ice-cold lysis buffer containing (in mM): HEPES–Na 25, $MgCl_2$ 2, EDTA 1, EGTA 1, supplemented with 100 μM PMSF, 2 mM DTT and protease inhibitor cocktail (containing 1 μg/ml leupeptin, pepstatin A, chymostatin and antipain). Cell lysates were frozen three times in liquid N_2 and were centrifuged at 140 × *g* to remove nuclei and large debris. Protein concentration in the supernatant was measured using the Bio-Rad protein dye assay reagent. Samples were denaturated at 95°C for 3 min in a 6× concentrated sample buffer (mM): Tris 500, DTT 600, 10.3% SDS, 30% glycerol and 0.012% bromophenol blue. Equal amount of each sample of protein was separated by electrophoresis on a 10% SDS-polyacrylamide gels (SDS-PAGE) and electroblotted onto PVDF membranes. The identification of proteins of interest was facilitated by the usage of a prestained precision protein standard (Bio-Rad) which was run simultaneously. After the proteins were electrophoretically transferred, the membranes were incubated for 1 h at room temperature (RT) in Tris buffer (mM; NaCl 150, Tris–HCl 25 (pH 7.6) with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk) to eliminate nonspecific binding, and were next incubated overnight at 4°C in TBS-T containing 1% nonfat dry milk with a rabbit polyclonal primary antibody against caspase-12 (1:1000 dilution) or a mouse monoclonal primary antibody against GRP78 (1:250 dilution). The membranes were washed several times and then incubated in TBS-T with 1% nonfat dry milk for 2 h at RT, with the appropriate alkaline-phosphatase-conjugated anti-rabbit or anti-mouse secondary antibody at a dilution of 1:25,000 or 1:20,000, respectively. Immunoreactive bands were detected after incubation of membranes with ECF reagent for 5–10 min, on a Bio-Rad Versa Doc 3000 Imaging System.

Immunocytochemistry

Control, Aβ1–40- or PrP106–126-treated cells were washed two times in PBS buffer (pH 7.4) and were fixed with 4% paraformaldehyde for 30 min at RT. For cytochrome *c* release experiments, cells were incubated with 750 nM MitoTracker Green for 45 min at 37°C, in the dark. Then, the cells were permeabilized for 2 min at RT with 0.2% Triton-X100 in PBS buffer (pH 7.4), and the nonspecific binding sites were blocked for 30 min at RT in PBS containing 3% BSA. Cells were incubated for 1 h with a mouse monoclonal anti-cyt *c* antibody that recognizes the native form of the protein (1:100 dilution), or with a mouse monoclonal anti-MAP2 (1:500 dilution) and a rabbit monoclonal anti-GFAP antibody (1:200 dilution), prepared in PBS containing 3% of BSA and incubated with Alexa Fluor 594 goat anti-mouse IgG antibody conjugated (1:100 dilution in 3% BSA/PBS) or with Alexa Fluor 488 goat anti-rabbit IgG antibody conjugated (1:100 dilution in 3% BSA/PBS) for 1 h at RT. Cells labeled with anti-MAP2 and anti-GFAP were also incubated with Hoechst 33342 (15 μg/ml dilution in PBS). Finally, the cells were washed and treated with DakoCytomation Fluorescent mounting solution on a microscope slide, and neurons were visualized in a confocal microscope (Bio-Rad MRC 600, Cambridge, MA, UK) or in an Axiovert 200 fluorescence microscope (Zeiss, Germany).

Measurement of intracellular reactive oxygen species

The levels of ROS, in particular of intracellular hydroperoxides, were assessed using the oxidant-sensitive dye 2′,7′-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA), a cell-permeant nonfluorescent compound that is converted to the acid 2′,7′-dichlorodihydrofluorescin ($DCFH_2$) when the acetate groups are removed by intracellular esterases. This ionized acid is trapped into the cells and can be oxidized to fluorescent 2′,7′-dichlorofluorescin (DCF) (Cathcart et al., 1993). Control cells treated with Aβ1–40 or PrP106–126 were incubated with 5 μM $DCFH_2$-DA in sodium medium for 20 min at 37°C. After $DCFH_2$-DA incubation, cells were washed and further incubated in sodium medium for 10 min to allow its desterification. Then, the glass coverslips containing the loaded cells were mounted in a special holder (Perkin-Elmer L225008) and placed inside a temperature-controlled (37°C) cuvette containing sodium medium, with an alignment of 60° to the excitation beam (to minimize the effect of light reflection). The fluorescence signals, corresponding to intracellular ROS, were monitored for 30 min at 502 nm excitation and 550 nm emission, using a temperature-controlled SPEX Fluorolog spectrometer.

Statistical analysis

Results are expressed as means ± SEM of the number of experiments indicated in the figure captions. Statistical significance was performed using an analysis of variance (ANOVA) followed by Dunnett's post hoc tests for multiple comparisons or by the unpaired two-tailed Student's *t* test. A $P < 0.05$ value was considered statistically significant.

Results

Cortical neurons used throughout this work were cultured in serum-free Neurobasal medium in the presence of B27 supplement, which supports growth of neurons and minimizes glial cell proliferation. The purity of neuronal cultures was confirmed by immunocytochemistry using the neuronal marker microtubule-associated protein 2 (MAP-2) antibody and the anti-glial fibrillary acidic protein (GFAP) antibody, a marker of astrocytic proliferation (Fig. 1A). Quantification of MAP-2- and GFAP-positive cells demonstrated that, under our experimental conditions, ~94% of the cells in culture were neurons (Fig. 1B). Therefore, due to the reduced number of astrocytes in the cultures used in this study (less than 10%), the effects observed could be attributed solely to neurons.

PrP106–126 and Aβ1–40 increase the levels of ER stress markers

To evaluate whether ER stress is induced in cortical neurons by PrP106–126 and Aβ1–40 peptides, the levels of two known ER stress markers, Grp78 and caspase-12 (Rao et al., 2002; Nakagawa and Yuan, 2000), were analyzed by Western blotting. Treatment of cortical neurons with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM) induces a significant increase in Grp78 levels at 6 h which persists at 24 h of incubation, when compared with untreated cells (Fig. 2A). Furthermore, the decrease of the pro-caspase-12 levels in treated cortical neurons suggests that PrP106–126 and Aβ1–40 lead to the activation of caspase-12. After exposure of cells to the synthetic peptides, during 6 or 24 h, the pro-caspase-12 levels decreased significantly (Fig. 2B). These results indicate that PrP106–126 and Aβ1–40 induce ER stress and caspase-12 activation in cortical neurons.

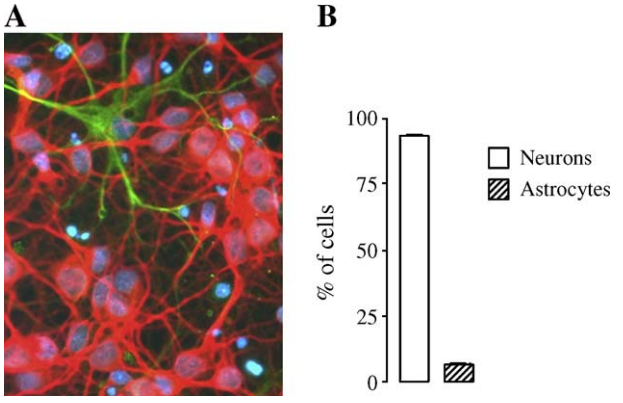

Fig. 1. Relative proportion of neurons and astrocytes in cortical cultures. (A) Representative image of cortical cells labeled with the neuronal marker microtubule-associated protein 2 (MAP-2) antibody (red) and the anti-glial fibrillary acidic protein (GFAP) antibody, a marker of astrocytic proliferation (green). Nucleus was stained with Hoechst 33342 (blue) (magnification 400×). (B) Quantification of the relative proportion of neurons and astrocytes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

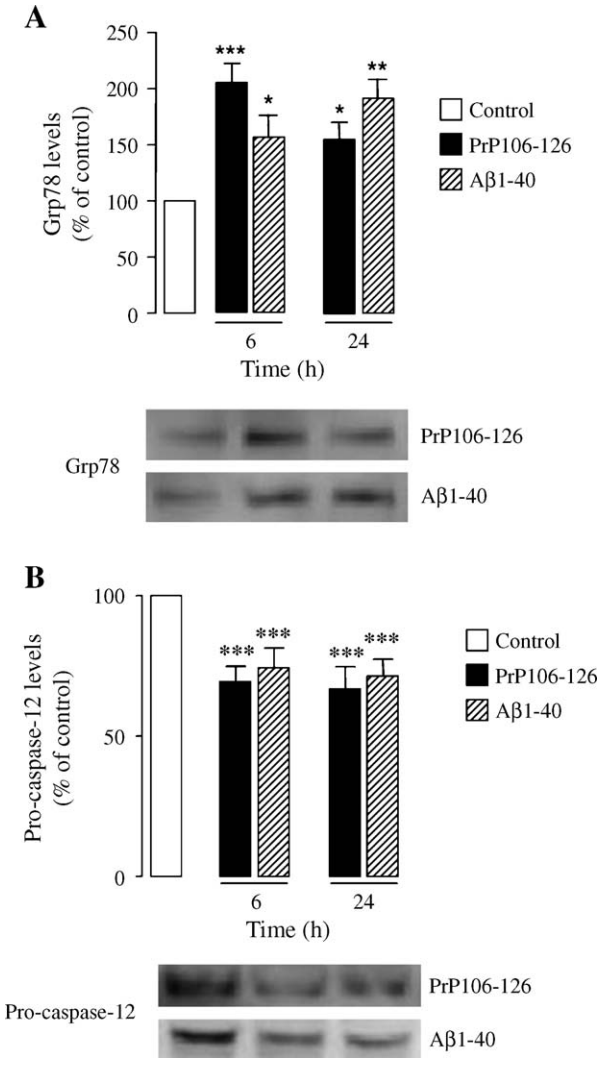

Fig. 2. PrP106–126 and Aβ1–40 induce the increase of Grp78 levels and caspase-12 activation. Total cell extracts were obtained from cortical neurons treated with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM), for 6 h or 24 h, and the levels of Grp78 (A) and pro-caspase-12 (B) were determined by Western blotting. To determine the significance of the differences in expression level, data from at least three different experiments were analyzed by densitometry (Graphs). Data were expressed as the mean ± SEM. $*P < 0.5$; $**P < 0.01$; $***P < 0.001$ with respect to control values.

Cortical neurons treated with PrP106–126 and Aβ1–40 have high intracellular Ca^{2+} levels. ER Ca^{2+} release contributes to perturbed Ca^{2+} homeostasis

ER stress and caspase-12 activation can be mediated by Ca^{2+} signaling (Ferri and Kroemer, 2000), and therefore we analyzed if under our conditions Ca^{2+} homeostasis was perturbed. We also analyzed the contribution of ER Ca^{2+} release by the determination of intracellular Ca^{2+} levels in the presence or absence of dantrolene and xestospongin C, inhibitors of RyR

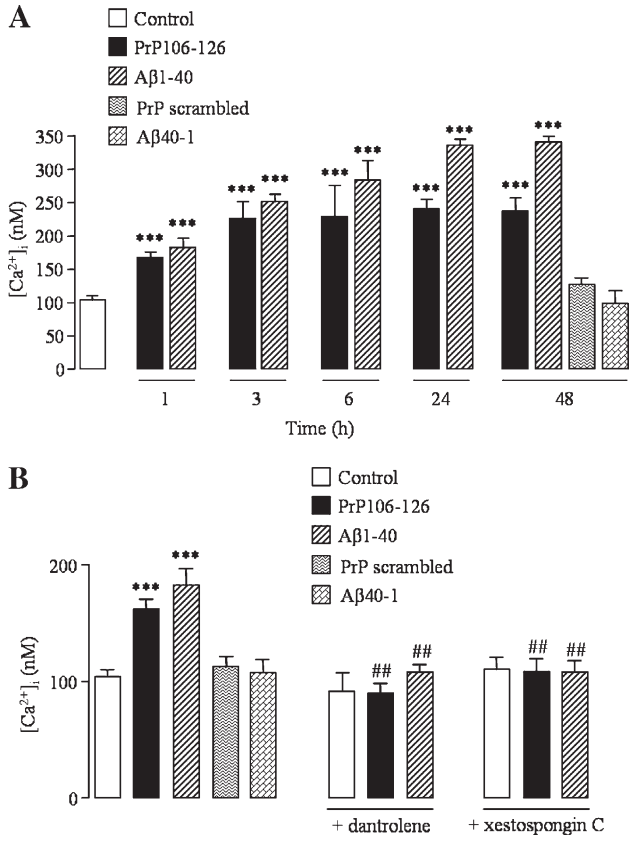

Fig. 3. Effect of PrP106–126 and Aβ1–40 on basal intracellular Ca^{2+} levels in cortical neurons. (A) Cells were treated with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM) for 1 to 48 h. (B) Cortical neurons were incubated with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM) for 1 h, in the absence or in the presence of dantrolene (10 μM) or xestospongin C (1 μM). The fluorescence of Indo-1 loaded cells was measured, and the $[Ca^{2+}]_i$ was calculated as described in the Materials and methods section. The results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. ***$P < 0.001$ with respect to control values. ##$P < 0.01$ with respect to PrP and Aβ addition.

and IP_3R, respectively. PrP106–126 and Aβ1–40 induce a sustained increase of intracellular Ca^{2+} levels, starting at 1 h until 48 h of incubation, when compared with the control (Fig. 3A). Since PrP106–126 and Aβ1–40 induce a significant increase of intracellular Ca^{2+} levels at 1 h, we analyzed the contribution of ER Ca^{2+} release through RyR and IP_3R at this incubation time. When co-incubated with PrP106–126 and Aβ1–40, dantrolene (10 μM) or xestospongin C (1 μM) is protective as demonstrated by the decrease in intracellular Ca^{2+} concentration to values similar to the control (Fig. 3B). Dantrolene and xestospongin C alone have no effect in intracellular Ca^{2+} levels of cortical neurons. These results show that PrP106–126 and Aβ1–40 induce an early increase of intracellular Ca^{2+} levels due to the release of Ca^{2+} from ER through RyR and IP_3R, leading to the consequent perturbation of Ca^{2+} homeostasis. The reverse Aβ40–1 and the scrambled PrP peptides did not lead to any significant increase in the intracellular Ca^{2+} levels (at 1 and 48 h), demonstrating that the specific sequence of amino acids of Aβ and PrP peptides is needed for the perturbation of Ca^{2+} homeostasis (Figs. 3A and B).

PrP106–126 and Aβ1–40 induce the increase of reactive oxygen species mediated by ER Ca^{2+} release

Several studies suggest that, altogether with the changes in calcium dynamics, abnormalities in oxidative stress are central to the toxic effects of PrP106–126 and Aβ1–40 (Gibson, 2002; O'Donavan et al., 2003; Agostinho and Oliveira, 2003). In order to analyze the role of ER Ca^{2+} release in oxidative stress induced by these amyloidogenic peptides, the production of reactive oxygen species (ROS) was determined. PrP106–126 induces a time-dependent increase in intracellular ROS levels that goes from 3 h to 24 h of incubation, decreasing at 48 h (Fig. 4A). At 1 h, PrP106–126 has no significant effect on intracellular ROS levels. On the

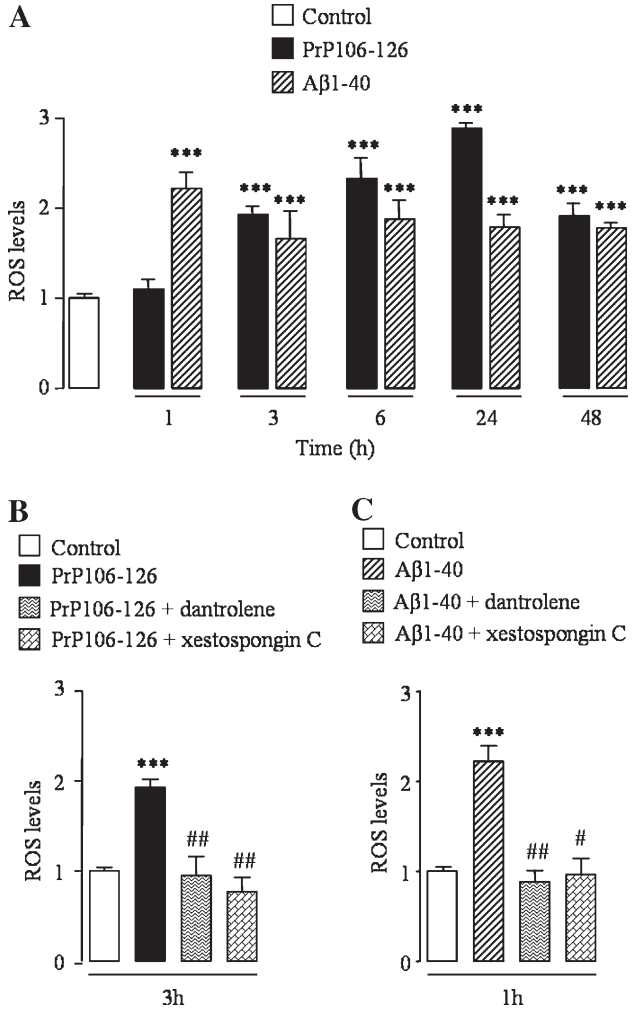

Fig. 4. PrP106–126 and Aβ1–40 induce the increase in intracellular reactive oxygen species (ROS) levels. Dantrolene and xestospongin C protected against intracellular ROS increase induced by PrP106–126 and Aβ1–40. (A) Cortical neurons were treated with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM) for 1 to 48 h. (B) Cortical neurons were incubated with PrP106–126 (25 μM) for 3 h, in the absence or in the presence of dantrolene (10 μM) or xestospongin C (1 μM). (C) Cortical neurons were incubated with Aβ1–40 (0.5 μM) for 1 h, in the absence or in the presence of dantrolene (10 μM) or xestospongin C (1 μM). Intracelullar ROS levels were determined by DCF fluorescence. The results are the means ± SEM of values corresponding at least 3 experiments, each value being the mean of duplicate assays. ***$P < 0.001$ with respect to control values. #$P < 0.05$; ##$P < 0.01$ with respect to PrP and Aβ addition.

other hand, Aβ1–40 induces a significant increase of ROS levels at 1 h, maintaining these levels until 48 h of incubation (Fig. 4A). When cortical cells were treated for 3 h with PrP106–126 or for 1 h with Aβ1–40, in the presence of dantrolene or xestospongin C, a significant decrease in ROS levels is observed, demonstrating that RyR- and IP_3R-mediated ER Ca^{2+} release is involved in oxidative stress triggered by PrP and Aβ peptides in cortical neurons (Figs. 4B and C). Dantrolene or xestospongin C per se did not affect intracellular ROS levels (data not shown).

PrP106–126 and Aβ1–40 induce cytochrome c release and caspase-2, -3, -6, -8 and -9 activation upon ER Ca^{2+} release

The release of cytochrome *c* from mitochondria in cortical neurons treated with PrP106–126 or Aβ1–40 was analyzed by confocal microscopy determining the co-localization of the mitochondrial fluorescent marker MitoTracker Green and an anti-cytochrome *c* antibody. The involvement of ER Ca^{2+} release was evaluated co-treating cells with PrP or Aβ peptides and the ER Ca^{2+} channels inhibitors dantrolene and xestospongin C. After 6 h of treatment with PrP106–126 and Aβ1–40, a reduction in the overlay of MitoTracker Green and anti-cytochrome *c* fluorescence is observed when compared with the control (Fig. 5). When cells treated with PrP and Aβ were preincubated with dantrolene or xestospongin C, the co-localization of cytochrome *c* with the mitochondria was observed, as in the control cells, suggesting the involvement of ER Ca^{2+} in cytochrome *c* release from mitochondria induced by both peptides. Similar results were obtained in cells treated with PrP106–126 and Aβ1–40 for 24 h (data not shown).

Cytochrome *c* release from the intermembrane space of mitochondria is one of the triggers of the activation of several caspases and apoptosis (Desagher and Martinou, 2000). Therefore, we determined the activity of caspase-2, -3, -6, -8 and -9 upon PrP106–126 or Aβ1–40 treatment. In cortical cells incubated with PrP106–126 and Aβ1–40 for 48 h, the activation of caspase-2, -3, -6, -8 and -9 was demonstrated, the most significant increase being observed for caspase-6 (Fig. 6A).

Because caspase-3 is a key executioner of apoptosis (Kilic et al., 2002), the protective effect of dantrolene and xestospongin C against PrP- and Aβ-induced caspase-3 activation was analyzed. As shown in Fig. 6B, when cortical cells are preincubated with the Ca^{2+} channel inhibitors dantrolene or xestospongin C, before PrP106–126 treatment, the caspase-3 activation induced by the PrP peptide is significantly reduced. Similar results were obtained in cells treated

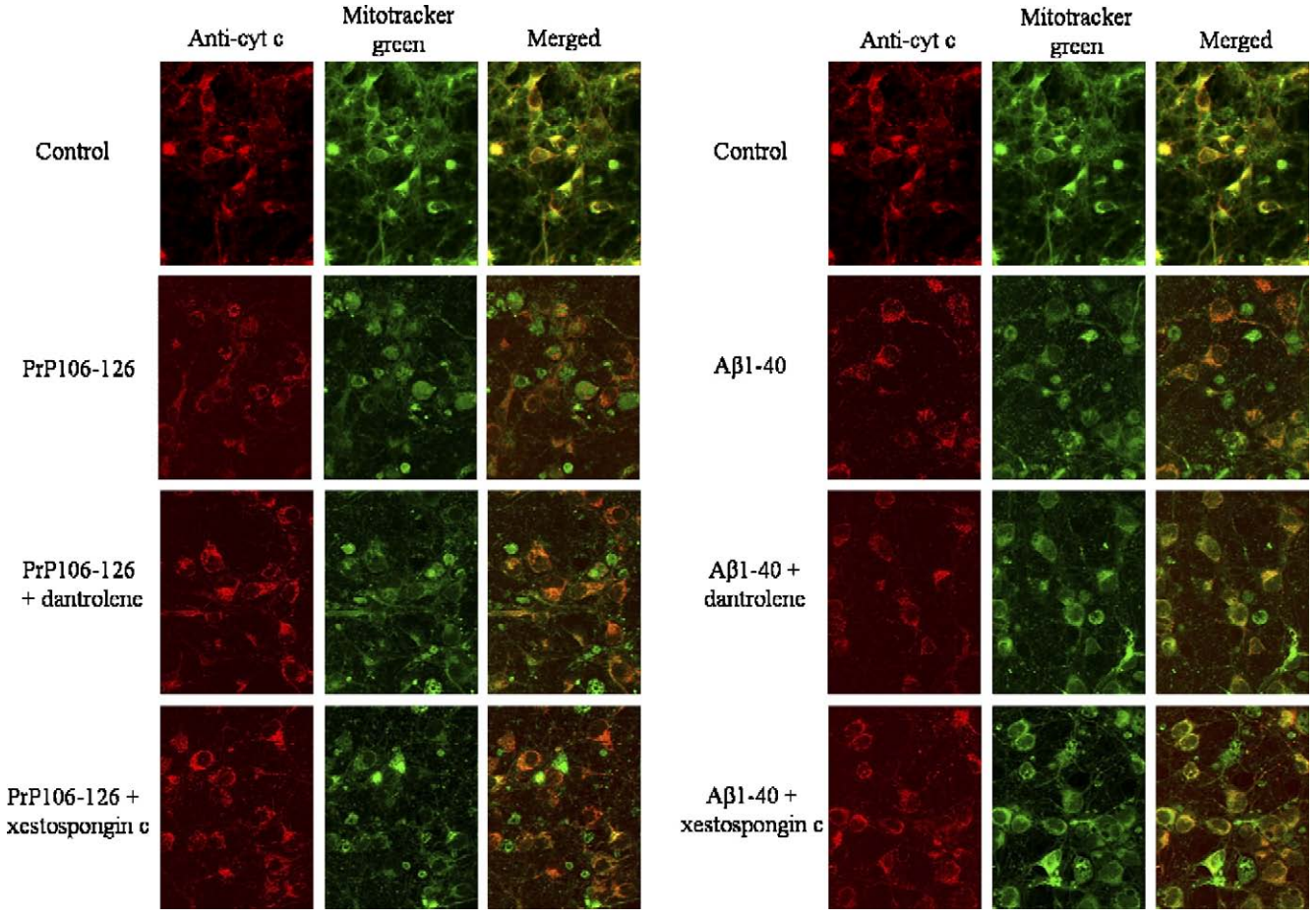

Fig. 5. PrP106–126 and Aβ1–40 induce cytochrome *c* release from mitochondria. Ca^{2+} release through RyR and IP3R is involved in cytochrome *c* release induce by PrP and Aβ peptides. Cortical neurons were treated for 6 h with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM), in the absence or the presence of dantrolene (10 μM) or xestospongin C (1 μM). Cytochrome *c* localization was evaluated by immunocytochemistry, using a confocal microscope. Cells were co-labeled with anti-cyt *c* and MitoTracker green. Merged images provide evidence about the co-localization of cyt *c* immunoreactivity with mitochondria. Overlay of fluorescence (yellow-orange) indicates retention of cyt *c* in mitochondria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with Aβ1–40 and preincubated with dantrolene or xestospongin C. Treatment of cortical cells with dantrolene or xestospongin C for 48 h did not significantly affect the caspase-3-like activity (Fig. 6B).

Blockage of ER Ca^{2+} release protects against PrP106–126- and Aβ1–40-induced increase in the number of apoptotic cells

In cortical neurons, apoptotic cell death upon exposure to PrP and Aβ peptides was analyzed by fluorescence microscopy after SYTO-13/PI labeling. Fig. 7A depicts that PrP106–126 and Aβ1–40 significantly increase the number of apoptotic cells after 24 and 48 h of incubation. The most prominent effect on apoptotic death, observed 48 h after addition of PrP and Aβ to cortical neurons, was prevented by preincubation with dantrolene and xestospongin C (Fig. 7B). Dantrolene and xestospongin C alone did not affect the number of apoptotic cells. Results show that the appearance of neurons with apoptotic morphology, upon exposure to PrP and Aβ peptides, can be prevented by the inhibition of Ca^{2+} release through ER channels. The reverse Aβ40–1 and the scrambled PrP peptides were not toxic to cortical neurons (Fig. 7B).

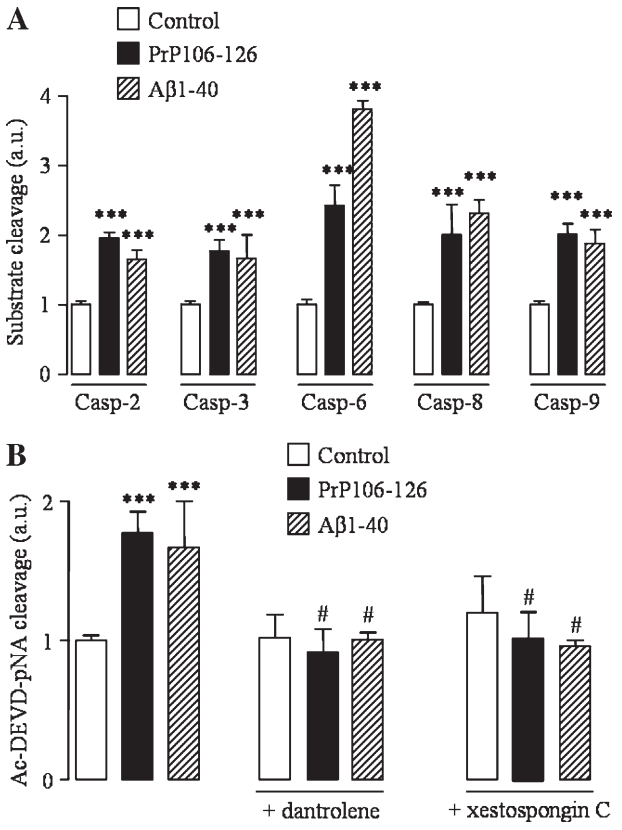

Fig. 6. Caspases activation in cortical neurons induced by PrP106–126 and Aβ1–40. Effect of dantrolene and xestospongin C on the increase of caspase-3-like activity induced by PrP and Aβ peptides. (A) Cells were incubated for 48 h with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM), and caspase-2, -3, -6, -8 and -9 activity was determined. (B) Cells were incubated for 48 h with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM), in the absence or the presence of dantrolene (10 μM) or xestospongin C (1 μM), and caspase-3 activity was determined. Caspase activity was determined using protein extracts from cortical cells, and the activity measured by the quantity of chromogenic substrates cleavage at 405 nm, as described in the Materials and methods section. The results, expressed as the increase above control values, are the means ± SEM of values corresponding at least to 3 experiments, each value being the mean of duplicate assays. ***$P < 0.001$ with respect to control values. ###$P < 0.05$ with respect to PrP or Aβ addition.

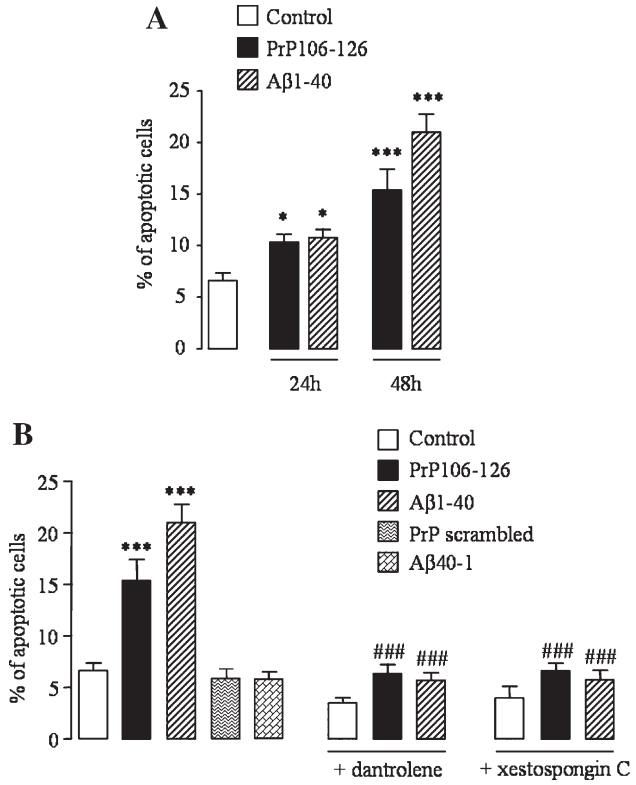

Fig. 7. DNA fragmentation induced by PrP106–126 and Aβ1–40. (A) The number of apoptotic cells induced by 25 μM PrP106–126 or 0.5 μM Aβ1–40 for 24 and 48 h was determined by SYTO-13 and PI staining, as described in the Materials and methods section. (B) Neurons were incubated with PrP106–126 (25 μM) or Aβ1–40 (0.5 μM) for 48 h in the absence or the presence of dantrolene (10 μM) or xestospongin C (1 μM). The results, expressed as the percentage of the total number of cells, are the means ± SEM of values corresponding at least to 3 experiments, each value being the mean of duplicate assays.*$P < 0.05$; ***$P < 0.001$ with respect to control values. ###$P < 0.001$ with respect to PrP or Aβ addition.

Discussion

In the present study, we show that treatment of cortical neurons with PrP106–126 or Aβ1–40 induces ER stress. This is accompanied by a significant increase in the $[Ca^{2+}]_i$ and in the levels of ROS, cytochrome *c* release from mitochondria and activation of several caspases including caspase-2, -3, -6, -8 and -9, which culminates in apoptotic cell death. These parameters were shown to be prevented by dantrolene and xestospongin C, inhibitors of the ER Ca^{2+} release through RyR and IP_3R, demonstrating that the ER Ca^{2+} release through RyR and IP_3R is involved in PrP- and Aβ-induced apoptotic death.

Several studies have shown the involvement of ER stress in neuronal death that occurs in PrD and AD and suggest that PrP and Aβ peptides play a role in ER-mediated cell injury. In fact, Hetz et al. (2003) refer that caspase-12 activation is detected in cells treated with PrP^{Sc} and that GRP58, a chaperone with protein disulfide isomerase (PDI)-like activity, protects cells against PrP^{Sc} toxicity and decreases the rate of caspase-12 activation (Hetz et al.,

2005). In addition, Nakagawa et al. (2000) point out that cortical neurons deficient in caspase-12 are more resistant to Aβ protein neurotoxicity. Furthermore, cells that express presenilin 1 mutants, linked to familial AD, have increased susceptibility to ER stress (Katayama et al., 2004; Imaizumi et al., 2001).

Our results show that PrP106–126 and Aβ1–40 induce the increase of GRP78 levels and caspase-12 activation in cortical neurons, indicating that these peptides induce ER stress, which can be activated by the unfolded protein response (UPR) and by disturbance of Ca^{2+} homeostasis (Kaufman, 1999). The balance between intracellular Ca^{2+} levels and the ER Ca^{2+} content is guaranteed by two different processes: the pumping in the ER by SERCA ATPases and the release by the opening of IP_3R or RyR Ca^{2+} release channels (Berridge et al., 2000). These Ca^{2+} release channels of the ER participate in the signal transduction pathway of apoptosis (Guo et al., 1997; Jayaraman and Marks, 1997; Pan et al., 2000). In agreement with results obtained by other authors using PC12 cells (Guo et al., 1996, 1997), we have recently demonstrated that Ca^{2+} release from RyR but also from IP_3R is involved in Aβ25–35 toxicity in cortical neurons (Ferreiro et al., 2004). IP_3-mediated Ca^{2+} release from the ER was previously demonstrated to trigger apoptosis in response to diverse signals (Distelhorst and Roderick, 2003). It has been shown in our laboratory that Aβ25–35/1–40 and PrP106–126 can induce the increase of cytosolic Ca^{2+} levels in cortical neurons following 24 h of incubation (Ferreiro et al., 2004; Agostinho and Oliveira, 2003). In the present study, we show that in cortical neurons treated with PrP106–126 and Aβ1–40 intracellular Ca^{2+} levels increase before 24 h of incubation. In fact, at 1 h of incubation, a significant rise in cytosolic Ca^{2+} concentration was measured. The Ca^{2+} release through the RyR and IP_3R contributes to this early $[Ca^{2+}]_i$ increase.

Several studies indicate that, in addition to perturbed Ca^{2+} homeostasis, oxidative stress also occurs in PrD and AD (reviewed in Brown et al., 1996; Agostinho and Oliveira, 2003; Fernaeus and Land, 2005; Sheehan et al., 1997; Zhu et al., 2004). In fact, the ER is especially vulnerable to oxidative stress since it is one of the cell organelles that produce ROS (Hayashi et al., 2005). Furthermore, oxidative stress may lead to perturbations of the ER Ca^{2+} homeostasis because many of the regulatory proteins of ER Ca^{2+}, such as IP_3R and RyR, the Ca^{2+} ATPases and the ER-resident proteins, are sensitive to oxidants (Huang et al., 2004). In addition, Ca^{2+} that results from the depletion of ER Ca^{2+} stores can be taken up by juxtaposed mitochondria, inducing ROS formation (Tardif et al., 2005). Our study demonstrates that PrP106–126 and Aβ1–40 induce ROS formation in cortical neurons, and this effect was prevented by dantrolene and xestospongin C, indicating that Ca^{2+} release through RyR and IP_3R is involved in PrP- and Aβ-induced ROS formation. In the case of PrP106–126, the intracellular Ca^{2+} increase precedes the ROS formation, indicating that intracellular Ca^{2+} rise is an early event in PrP peptide toxicity.

ER Ca^{2+} release has been shown to have immediate effects on mitochondrial function, leading to rapid Ca^{2+} accumulation in mitochondria which promotes cytochrome *c* release and activation of downstream caspase pathways in cells exposed to proapoptotic agents (Nutt et al., 2002). In the cytosol, cytochrome *c* binds Apaf-1 and dATP to form a complex that activates caspase-9, which can activate the executioner caspase-3 (Desagher and Martinou, 2000). Cytochrome *c* has been shown to be involved in the neurotoxicity of Aβ25–35 and PrP106–126 (Cardoso et al., 2002; Agostinho and Oliveira, 2003). Our results show that, beyond the fact that Aβ and PrP peptides induce cytochrome *c* release from mitochondria, ER Ca^{2+} release through RyR and IP_3R is involved in the process, demonstrating that mitochondria and the ER cooperate in the neuronal death induced by Aβ and PrP peptides. These results are in agreement with data obtained in cells treated with ER stressors in which ER/mitochondria crosstalk was demonstrated (Häcki et al., 2000; Boya et al., 2002). Although our results demonstrate that ER stress induced by PrP and Aβ peptides occurs upstream of mitochondrial dysfunction, since cytochrome *c* release is prevented by dantrolene and xestospongin C, we cannot rule out the possibility that mitochondrial dysfunction could also trigger ER stress, propagating the initial injury. Recently, it was demonstrated that both full-length APP and Aβ accumulate in the mitochondria compartment causing mitochondrial dysfunction (Anandatheerthavarada et al., 2003; Lustbader et al., 2004), which may in turn deregulate ER Ca^{2+} homeostasis. In fact, inhibition of mitochondrial respiratory chain with rotenone was shown to induce ER stress through the phosphorylation of the key ER stress kinases IRE1α and PKR-like ER kinase (PERK) (Ryu et al., 2002).

Several caspases have been shown to be involved in ER stress. Hitomi et al. (2003) have shown that activation of caspase-12 indirectly activates cytoplasmic caspase-3 and activation of this caspase is completely suppressed in cells overexpressing Bcl-2 targeted specifically to the ER (Häcki et al., 2000). Dahmer (2005) has shown that thapsigargin, an activator of ER stress, induces activation of the caspase-2, -3 and -7. He et al. (2002) had already shown that thapsigargin-mediated perturbation in Ca^{2+} homeostasis upregulates DR5 (death receptor 5) and TRAIL (tumor necrosis factor-related apoptosis inducing ligand), and this is coupled with caspase-8 activation and Bid cleavage. In the present study, it was demonstrated that caspase-2, -3, -6, -8 and -9 activities are increased upon exposure to PrP106–126 and Aβ1–40. Also under our experimental conditions, the caspase-3 activity increased upon treatment with PrP 106–126 and Aβ1–40 was prevented by the ER Ca^{2+} release inhibitors dantrolene and xestospongin C, suggesting that perturbation of ER Ca^{2+} homeostasis mediated by Ca^{2+} release from RyR and IP_3R is involved in the activation of caspase-3 induced by the peptides.

One possible site of initiation of apoptosis is the synapse. Perturbed synaptic ER Ca^{2+} homeostasis promotes activation of apoptotic cascades (Mattson, 2000). Therefore, since PrP and Aβ peptides are known to be produced and to accumulate in high amounts at the synapses (Gong et al., 2003; Sales et al., 1998; Herms et al., 1999), our results support the view that loss of ER Ca^{2+} homeostasis induced by the peptides is involved in synaptic dysfunction and loss of dendritic spines, which are early events that occur in PrD and AD (Belichenko et al., 2000; Selkoe, 2002). Furthermore, perturbation of Ca^{2+} homeostasis induced by these peptides can lead to the activation of several kinases, such as GSK-3 and Cdk5, and to the consequent phosphorylation of the microtubule-associated protein tau, leading to the formation of neurofibrillary tangles (Pérez et al., 2003; Lee et al., 2000; reviewed in Fuentalba et al., 2004). Therefore, the ER stress induced by PrP and Aβ in the early stages of the diseases, in which they are implicated, may be associated with synaptic loss and with the presence of neurofibrillary tangles, central hallmarks of AD that may be also related to PrD. We then suggest that drugs that stabilize neuronal Ca^{2+} homeostasis, including dantrolene and xestospongin C to suppress Ca^{2+} release from ER, are potential therapeutical targets for PrD and AD.

Altogether, our in vitro results show that PrP106–126 and Aβ1–40 induce Ca^{2+} homeostasis deregulation, oxidative stress and

apoptotic cell death by a mechanism that involves the early release of Ca^{2+} from ER through RyR and IP_3R. Furthermore, the results presented here demonstrate that PrP- and Aβ-induced Ca^{2+} release from ER leads to mitochondrion-mediated cell death, suggesting that both organelles cooperate in PrP- and Aβ-induced apoptosis.

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