The International Journal of Biochemistry & Cell Biology 79 (2016) 261–270

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

A critical concentration of N-terminal pyroglutamylated amyloid beta drives the misfolding of Ab1-42 into more toxic aggregates

Denise Galante[a,e], Francesco Simone Ruggeri[b,c], Giovanni Dietler[b], Francesca Pellistri[d], Elena Gatta[d], Alessandro Corsaro[e], Tullio Florio[e], Angelo Perico[a], Cristina D'Arrigo[a,*]

[a] Institute for Macromolecular Studies, National Research Council, 16149 Genova, Italy
[b] Ecole Polytechnique Federale de Lausanne (EPLF), 1015 Lausanne, Switzerland
[c] Department of Chemistry, University of Cambridge, CB21EW, United Kingdom
[d] Department of Physics, University of Genova, 16100 Genova, Italy
[e] Section of Pharmacology, Department of Internal Medicine and Centre of Excellence for Biomedical Research (CEBR), University of Genova, 16132 Genova, Italy

ARTICLE INFO

Article history:
Received 23 March 2016
Received in revised form 19 July 2016
Accepted 29 August 2016
Available online 31 August 2016

Keywords:
Alzheimer's disease
β amyloids
Conformational structure
Morphology
Calcium homeostasis
Toxicity

ABSTRACT

A wide consensus based on robust experimental evidence indicates pyroglutamylated amyloid-β isoform (AβpE3-42) as one of the most neurotoxic peptides involved in the onset of Alzheimer's disease. Furthermore, AβpE3-42 co-oligomerized with excess of Aβ1-42, produces oligomers and aggregates that are structurally distinct and far more cytotoxic than those made from Aβ1-42 alone. Here, we investigate quantitatively the influence of AβpE3-42 on biophysical properties and biological activity of Aβ1-42. We tested different ratios of AβpE3-42/Aβ1-42 mixtures finding a correlation between the biological activity and the structural conformation and morphology of the analyzed mixtures. We find that a mixture containing 5% AβpE3-42, induces the highest disruption of intracellular calcium homeostasis and the highest neuronal toxicity. These data correlate to an high content of relaxed antiparallel β-sheet structure and the coexistence of a population of big spheroidal aggregates together with short fibrils. Our experiments provide also evidence that AβpE3-42 causes template-induced misfolding of Aβ1-42 at ratios below 33%. This means that there exists a critical concentration required to have seeding on Aβ1-42 aggregation, above this threshold, the seed effect is not possible anymore and AβpE3-42 controls the total aggregation kinetics.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia with a high incidence in the population over 65, is estimated to globally affect 1 out of 85 people by 2050 (Brookmeyer et al., 1998, 2007). Extracellular plaques of amyloid-β (Aβ) peptides and intraneuronal neurofibrillary tangles, made from tau hyperphosphorylation, are the main histopathological signatures of AD (Tanzi, 2005; Nussbaum et al., 2012). Aβ peptides are the major components of AD plaques and consist of a mixture of peptides having different N and C-termini (Russo et al., 2000). In the last fifteen years, it has been suggested that despite the importance of plaques to AD pathology, soluble Aβ oligomers are the principal toxic forms of Aβ (Kirkitadze et al., 2002; Walsh et al., 2002). The Aβ peptide family is produced by sequential endoproteolytic processing of the amyloid precursor protein (APP) by β-and γ-secretases. Cleavage by γ-secretase at different sites primarily results in Aβ1-40 and Aβ1-42 species that differ at their C-termini. N-terminal heterogeneity is caused by alternative cleavage of APP by β-secretase. Among these, 15–20% are N-terminus pyroglutamylated (pE), catalysed by glutaminyl cyclase (Schilling et al., 2008). The most abundant pyroglutamylated species *in vivo* is AβpE3-42 (Tabaton et al., 1994). N-terminally truncated and *pyro*-modified Aβ peptides show faster aggregation kinetics than full-length β-peptides (D'Arrigo et al., 2009; Schilling et al., 2006). The structural modifications in AβpE3-42 change the attitude of the full-length Aβ1-42 from a slow to a faster aggregation process (Perico et al., 2011). Literature data, about the pattern of fibrillogenesis of AβpE and unmodified peptides are often conflicting,

* Corresponding author at: Institute for Macromolecular Studies, National Research Council, Via De Marini 6, 16149 Genova, Italy.
E-mail address: cristina.darrigo@ge.ismac.cnr.it (C. D'Arrigo).

http://dx.doi.org/10.1016/j.biocel.2016.08.037
1357-2725/

because of the inherent polymorphism of the peptides and the experimental conditions adopted (Petkova et al., 2005; Jeong et al., 2013). A detailed characterization of the oligomeric and prefibrillar states, allowed the discrimination of the intermediate states mainly responsible of oligomer toxicity, confirming the peculiar role of AβpE3-42 in the pathogenesis of AD (Galante et al., 2012). In 1995, by examination of cortical sections from AD patients, it was found that AβpE3-x peptides were present in equivalent or greater densities than full length peptides (Saido et al., 1995). Subsequently, the neurotoxic effect of oligomers was related to the predominance of AβpE3-42 (Russo et al., 2002). However, the relative abundances of AβpE and unmodified peptides remain controversial. While several reports indicate that AβpE3-x is more abundant than Aβ1-x in AD (Frost et al., 2013), others reported that the two species were in similar concentrations (Moore et al., 2012) and in advance stage AD (Wu et al., 2014). By the contrast, Pivtoraiko et al. (2015) found in posterior cingulated cortex same concentrations for AβpE3-x and Aβ1-x in insoluble pool but only for Aβ1-42 in soluble pool. Low concentrations of soluble AβpE3-x reflect its rapid aggregation into plaques. Upadhaya et al. (2014) demonstrated that AβpE are not only detectable in the plaques but also in soluble and dispersible Aβ aggregates outside of the plaques. Biophysical studies showed that mixtures of the two peptides (*pyro*-modified/truncated and unmodified Aβ) cause an initial delay in β-sheet formation from both equimolar and no-equimolar samples, concluding that AβpE3-42 affects early aggregation process of unmodified peptides (Sanders et al., 2009). A tight interaction between AβpE3-42 and the unmodified peptide was showed by Fourier Transform Infrared Spectroscopy (FTIR) experiments, demonstrating that AβpE3-42, in 10% and 50% mixtures, transmits specific structural features to Aβ1–42 by a prion-like mechanism (Matos et al., 2014). These data leave the open question of which are the amounts of AβpE3-42 that have a sensitive influence on the biological activity, aggregation kinetics, secondary structure, and morphology of Aβ1-42. Is there a correlation between biological activity and structure or morphology? How does the structure of the mixed aggregates change, compared to the structure of the unmodified, Aβ1-42, aggregates?

In this manuscript we compare the biological activity, aggregation kinetics, conformational behavior, structure and morphology of mixtures of the Aβ1-42 and AβpE3-42 peptides, from 2.5% to 50% of AβpE3-42, to those of the two peptides alone. The biological activity is evaluated by measuring the variations of intracellular calcium concentrations, and by *in vitro* neurotoxicity, evaluating cell viability. The secondary structure is analysed by circular dichroism (CD) and by infrared nanospectroscopy (NanoIR). The aggregation is followed by turbidity experiments. Finally, the morphology of the aggregates is obtained by Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM).

2. Materials and methods

2.1. Aβ sample preparation

One milliliter of dimethyl sulfoxide (DMSO; Sigma) was added to 1 mg of lyophilized synthetic peptide (Aβ1-42 or AβpE3-42; AnaSpec), reaching a final concentration of 1 mg/ml. Aliquots of 75 μl were lyophilized and stored at −20 °C until used. For all experiments, stock peptides were reconstituted as reported Galante et al. (2012). The concentration of the peptide was estimated using a molar extinction coefficient at 214 nm, by Shimadzu UV 2700 Spectrophotometer (Hung et al., 2008). For the preparation of the mixtures, Aβ1-42 and AβpE3-42 were first mixed in microfuge tubes at different concentration ratios and diluted at 10 μM of total peptides concentration in Phosphate Buffered Saline (PBS, 150 mM, pH 7.4). pH of mixtures was corrected at 7.4 with few μl of 1 M HCl. Using these peptide solutions, we prepared mixtures at 2.5%, 5%, 10%, 20%, 33%, 50%, in which the percentage indicates the amount of AβpE3-42 over the total concentration of peptides.

2.2. Neurons preparation and Aβ treatments

Cerebellar granule cells were prepared from Sprague–Dawley rats (Harlan, Bresso, Italy). Experimental procedures and animal care complied with the EU Parliament and Governing Council of 22 September 2010 (2010/63/EU) and were approved by the Italian Ministry of Health (protocol number 2207) in accordance with D.M. 116/1992. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable results. Granule cells were prepared from cerebella of 7–8 day old rats following the procedure of Levi et al. (1984) as previously described (Robello et al., 1993). Cells were plated at 1×10^6 per dish on 20 mm poly-L-lysine-coated glass coverslips and maintained in Basal Eagle's culture medium, containing 10% fetal calf serum, 100 μg/ml gentamicin and 25 mM KCl, at 37 °C in a humidified 95% air, 5% CO_2 atmosphere. Cultures were treated with 10 μM cytosine arabinoside since the first day in order to minimize proliferation of non-neuronal cells. In a range of time between 6 and 12 days after plating, cells were treated for 20 h with Aβ peptides alone and their mixtures (1 μM), which were oligomerized for 24 h at 37 °C at the concentration of 10 μM before dilution into culture media. Then, the intracellular free Ca^{2+} concentrations increase, was measured in the cells by means of the fluorescent probe Oregon Green.

2.3. Intracellular Ca^{2+} concentration measurements by oregon green fluorescence

Following Aβ treatments, neurons were incubated at 37 °C for 40 min in a 6.0 μM solution of the cell-permeant AM ester of Oregon Green (Molecular Probes, Eugene, OR) and then washed several times with washing solution (135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM Hepes, 10 mM glucose, pH 7.4). Then cultures were transferred to a recording chamber mounted onto a Nikon Eclipse TE300 inverted microscope. Cells were visualized using a ×100 objective in oil (N.A. 1.3) as previously described (Pellistri et al., 2008). The fluorescence signal was detected using a Hamamatsu digital CCD camera with a 450–490 nm excitation filter, a 505 nm dichroic mirror, and a 520 nm emission filter (Nikon Italia, Florence, Italy). Images were acquired with the Simple PCI software (Hamamatsu, Sewickley, PA). Fluorescence intensity was calculated in arbitrary units by building a scale of the pixel intensity located in the region of interest. Fluorescence intensity changes were calculated as $(F - F_0) \times 100/F_0$, where F was the fluorescence intensity measured after Aβ treatment and F_0 the basal fluorescence level. Three independent experiments in duplicate were done. For each replicate the fluorescence of at least 40 cells was analyzed to obtain an average of the signal. Statistical analysis was performed by means of one-way ANOVA using "Newman-Keuls multiple comparison post-hoc test".

2.4. A1 neuron cultures and Aβ treatment

Neuronal Mes-c-myc A1 (hereafter A1) cell line was generated by immortalization of primary cultures of mouse embryonic mesencephalic neurons. Phenotypical characterization and neuronal features of these cells have been described elsewhere (Gentile et al., 2012). A1 was plated in MEM/F12 (Gibco-BRL, Milan, Italy), supplemented with 10% FBS (Invitrogen, USA) medium. When cells reached 70% of confluence, were treated with Aβ1-42, AβpE3-42, or the peptides mixtures (5%, 33%, 50%) at a final concentration of 1 μM. The peptides and the mixtures were aggregated for 24 h