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Monomeric amyloid-β reduced amyloid-β oligomer-induced synapse damage in neuronal cultures

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disease characterized by the accumulation of amyloid-β (Aβ) in the brain. Aβ oligomers are believed to cause synapse damage resulting in the memory deficits that are characteristic of this disease. Since the loss of synaptic proteins in the brain correlates closely with the degree of dementia in Alzheimer's disease, the process of Aβ-induced synapse damage was investigated in cultured neurons by measuring the loss of synaptic proteins. Soluble Aβ oligomers, derived from Alzheimer's-affected brains, caused the loss of cysteine string protein and synaptophysin from neurons. When applied to synaptosomes Aβ oligomers increased cholesterol concentrations and caused aberrant activation of cytoplasmic phospholipase A_2 ($cPLA_2$). In contrast, Aβ monomer preparations did not affect cholesterol concentrations or activate synaptic $cPLA_2$, nor did they damage synapses. The Aβ oligomer-induced aggregation of cellular prion proteins (PrP^C) at synapses triggered the activation of $cPLA_2$ that leads to synapse degeneration. Critically, Aβ monomer preparations did not cause the aggregation of PrP^C; rather they reduced the Aβ oligomer-induced aggregation of PrP^C. The presence of Aβ monomer preparations also inhibited the Aβ oligomer-induced increase in cholesterol concentrations and activation of $cPLA_2$ in synaptosomes and protected neurons against the Aβ oligomer-induced synapse damage. These results support the hypothesis that Aβ monomers are neuroprotective. We hypothesise that synapse damage may result from a pathological Aβ monomer:oligomer ratio rather than the total concentrations of Aβ within the brain.

1. Introduction

Alzheimer's disease (AD) is a complex neurological disorder characterized by a progressive dementia resulting from synapse failure (Selkoe, 2002; Tanzi, 2005). The amyloid hypothesis maintains that the pivotal event in AD is the production of amyloid-β (Aβ) peptides following the proteolytic cleavage of the amyloid precursor protein (APP) (De Strooper et al., 2010; Hardy and Selkoe, 2002). The accumulation of C-terminal fragments (Aβ) within the brain is thought to cause the synapse dysfunction and the memory loss that is characteristic of AD. Aβ has the capacity to self-aggregate, and consequently is found in different forms ranging from monomers and small soluble oligomers to much larger fibrils and plaques. The soluble Aβ oligomers are currently considered to be the principal mediators of synapse damage (Yang et al., 2017).

The mechanisms of Alzheimer's-related synapse damage can be examined by incubation of cultured neurons with Aβ. Since the loss of synapses and synaptic proteins is a feature of AD that strongly correlates with cognitive decline in AD (Counts et al., 2006; Masliah et al., 1991; Reddy et al., 2005) synapse density was measured by determining the amounts of synaptophysin and cysteine string protein (CSP) in cultured neurons (Lipton et al., 2001). Soluble forms of Aβ derived from the brains Alzheimer's patients caused synapse degeneration in neurons (Yang et al., 2017). The biological effects of these Aβ oligomers occurred at picomolar concentrations, similar concentrations to those found in the cerebrospinal fluid of Alzheimer's patients (Bibl et al., 2007; McLean et al., 1999; Mehta et al., 2000). The role of Aβ monomers that are found in high concentrations in the brain are poorly understood; while some studies report that Aβ monomers are not toxic (Shankar et al., 2007; Walsh et al., 2002) another study suggested that they were neuroprotective (Giuffrida et al., 2009). Confusion may arise due to the heterogeneity of Aβ in monomer preparations, in addition to

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β; PrP^C, cellular prion protein; $cPLA_2$, cytoplasmic phospholipase A_2; CSP, cysteine-string protein; ELISA, enzyme-linked immunoassay; Fab, fragment antigen-binding; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; PLAP, phospholipase A_2-activating peptide; PG, prostaglandin; SDS, sodium dodecyl sulphate; SD, standard deviation; VAMP, vesicle-associated membrane protein

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$A\beta_{40}$ and $A\beta_{42}$ peptides there are other APP fragments in cerebrospinal fluid (Brinkmalm et al., 2012) and that some of these carboxy-terminal fragments of APP have biological activity (Willem et al., 2015). In the present study Aβ monomer preparations and Aβ oligomers were isolated from soluble brain extracts and their biological activity studied.

Increasing evidence suggests that aberrant activation of cytoplasmic phospholipase A_2 ($cPLA_2$) plays an important role in Aβ oligomer-induced synapse damage. For example, Aβ activates $cPLA_2$ (Anfuso et al., 2004; Shelat et al., 2008) and pharmacological inhibition of $cPLA_2$ protects cultured neurons against Aβ-induced synapse damage (Bate et al., 2010). In addition, $cPLA_2$ inhibitors ameliorated cognitive decline in a mouse model of AD (Sanchez-Mejia et al., 2008). Here we demonstrate that whereas Aβ oligomers increased synaptic cholesterol concentrations, activated $cPLA_2$ and caused synapse damage in cultured neurons, Aβ monomers had none of those effects. The cellular prion protein (PrP^C) mediates Aβ-induced memory defects (Lauren et al., 2009) and aggregation of PrP^C by Aβ oligomers caused the activation of $cPLA_2$ and synapse damage (Bate and Williams, 2011). Here we report that Aβ monomers did not cause aggregation of PrP^C; rather that they reduced the aggregation of PrP^C by Aβ oligomers. The presence of Aβ monomers significantly reduced the Aβ oligomer-induced activation of $cPLA_2$ and synapse damage.

2. Materials and methods

2.1. Primary neuronal cultures

Cortical neurons were prepared from the brains of day 15.5 mouse embryos as described (Bate et al., 2010). Cells were suspended in Ham's F12 medium containing 5% foetal calf serum and seeded at 2×10^5 cells/well in 48 well plates that had been coated with poly-L-lysine. After 2 h, cultures were shaken and washed to remove non-adherent cells. Neurons were grown in neurobasal medium containing B27 components and 5 ng/ml nerve growth factor (Sigma) for 10 days. Immunohistochemistry revealed that approximately 95% of cells were neurofilament positive. All experiments were performed in accordance with European regulations (European Community Council Directive, 1986, 56/609/EEC) and approved by the local authority veterinary service/ethical committee. To determine cell viability they were incubated with 50 μM thiazolyl blue tetrazolium bromide for 3 h at 37 °C. The supernatant was removed, the formazan product solubilized in 200 μl of dimethyl sulfoxide, transferred to an immunoassay plate and absorbance read at 595 nm. Cell survival was calculated with reference to untreated cells (100% survival). Neurons were incubated with Aβ preparations for 24 h. For other studies, neurons were pre-treated with Aβ monomers or control medium for 1 h and then incubated with Aβ oligomers or a phospholipase A_2-activating protein (PLAP) (Bachem) for 24 h. Treated neurons were washed 3 times with PBS and homogenized in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS at 10^6 cells/ml. Mixed protease inhibitors (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma) were added and nuclei and large fragments were removed by centrifugation (1000 × *g* for 5 min).

2.2. Western blotting

Samples were mixed with Laemmli buffer containing β-mercaptoethanol, heated to 95 °C for 5 min and proteins were separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidiene fluoride membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; synapsin-1 was detected with goat polyclonal (Santa Crux Biotech), synaptophysin with MAB368 (Abcam), cysteine-string protein (CSP), with rabbit polyclonal anti-CSP ((sc-33154) Santa Cruz), vesicle-associated membrane protein (VAMP)-1 with monoclonal antibody (mAb) 4H302 (Abcam), caveolin with rabbit polyclonal antibodies to caveolin (Upstate). These were visualised using a combination of biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma), extravidin-peroxidase and enhanced chemiluminescence.

2.3. Synaptophysin ELISA

The amount of synaptophysin in samples was determined by ELISA as described (Bate et al., 2010). Maxisorb immunoplates (Nunc) were coated with an anti-synaptophysin mAb (MAB368-Chemicon) and blocked with 5% milk powder. Samples were added for 1 h and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam) followed by a biotinylated anti-rabbit IgG (Sigma), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution (Sigma). Absorbance was measured at 405 nm. Samples were expressed as "units synaptophysin" where 100 units was the amount of synaptophysin in 10^6 untreated cells.

2.4. CSP ELISA

Maxisorb immunoplates were coated with a mouse mAb to CSP ((sc-136468) Santa Cruz) and blocked with 5% milk powder. Samples were added for 1 h and bound CSP was detected using rabbit polyclonal anti-CSP ((sc-33154) Santa Cruz) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. Samples were expressed as "units CSP" where 100 units was the amount of CSP in 10^6 untreated cells.

2.5. Isolation of synaptosomes

Synaptosomes were prepared from mouse cortical neuronal cultures, as described above, on a discontinuous Percoll gradient as described (Dunkley et al., 2008). Neurons were homogenized at 4 °C in 1 ml of SED solution (0.32 M sucrose, 5 mM Tris-HCl pH 7.2, 1 mM EDTA, and 0.25 mM dithiothreitol) and centrifuged at 1000 × *g* at 4 °C. The supernatant was transferred to a 4-step gradient of 3, 7, 15, and 23% Percoll in SED solution and centrifuged at 16,000 × *g* for 30 min at 4 °C. The synaptosomes were collected from the interface between the 15% and 23% Percoll and washed (16,000 × *g* for 10 min at 4 °C) and suspended in neurobasal medium containing B27 components at a concentration equivalent to 5×10^6 neurons per ml. All synaptosomes were used on the same day of preparation. Synaptosomes were incubated with Aβ monomers or Aβ oligomers for 1 h at 37 °C. In one experiment synaptosomes were pre-treated with Aβ monomers for 30 min and then incubated with Aβ oligomers for 1 h at 37 °C. Treated synaptosomes were washed with ice cold PBS and suspended in ice cold extraction buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.2% SDS and mixed protease/phosphatase inhibitors (as above)).

2.6. Synaptic vesicle recycling

The fluorescent dye FM1-43 that is taken up into synaptic recycling vesicles was used as an indicator of synaptic activity (Parodi et al., 2010). Treated synaptosomes were pulsed with 5 μM FM1-43 and 1 μM ionomycin, a calcium ionophore used to stimulate neurotransmitter release, for 5 min, washed 5 times in ice cold PBS and homogenized in methanol. Soluble extracts were transferred into Sterilin 96 well black microplates and fluorescence was measured using excitation at 480 nm and emission at 625 nm. Samples were expressed as "% fluorescence" where 100% fluorescence was the fluorescence of 10^6 control synaptosomes pulsed with FM1-43 and ionomycin.