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Valproic acid and its congener propylisopropylacetic acid reduced the amount of soluble amyloid-β oligomers released from 7PA2 cells

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ABSTRACT

The amyloid hypothesis of Alzheimer's disease suggests that synaptic degeneration and pathology is caused by the accumulation of amyloid-β (Aβ) peptides derived from the amyloid precursor protein (APP). Subsequently, soluble Aβ oligomers cause the loss of synaptic proteins from neurons, a histopathological feature of Alzheimer's disease that correlates with the degree of dementia. In this study, the production of toxic forms of Aβ was examined *in vitro* using 7PA2 cells stably transfected with human APP. We show that conditioned media from 7PA2 cells containing Aβ oligomers caused synapse degeneration as measured by the loss of synaptic proteins, including synaptophysin and cysteine-string protein, from cultured neurons. Critically, conditioned media from 7PA2 cells treated with valproic acid (2-propylpentanoic acid (VPA)) or propylisopropylacetic acid (PIA) did not cause synapse damage. Treatment with VPA or PIA did not significantly affect total $Aβ_{42}$ concentrations; rather these drugs selectively reduced the concentrations of $Aβ_{42}$ oligomers in conditioned media. In contrast, treatment significantly increased the concentrations of $Aβ_{42}$ monomers in conditioned media. VPA or PIA treatment reduced the concentrations of APP within lipid rafts, membrane compartments associated with Aβ production. These effects of VPA and PIA were reversed by the addition of platelet-activating factor, a bioactive phospholipid produced following activation of phospholipase A_2, an enzyme sensitive to VPA and PIA. Collectively these data suggest that VPA and PIA reduce Aβ oligomers through inhibition of phospholipase A_2 and suggest a novel therapeutic approach to Alzheimer's treatment.

1. Introduction

Alzheimer's disease (AD) is a complex neurological disorder characterized by a progressive dementia resulting from synaptic failure (Selkoe, 2002). The amyloid hypothesis maintains that the accumulation of neurotoxic amyloid-β (Aβ) peptides following the proteolytic cleavage of the amyloid precursor protein (APP) (Hardy, 2006) causes neurodegeneration (Lambert et al., 1998). The production of Aβ is a key therapeutic target that can be investigated in cells transfected with the human APP gene; which metabolise APP to toxic forms of Aβ (Walsh et al., 2005). 7PA2 cells are Chinese hamster ovary (CHO) cells transfected with human APP that produce Aβ that are of similar size and potency to soluble Aβ derived from AD patients (Podlisny et al., 1995). Aβ peptides in brains of AD patients are found in a mixture of complex physical forms. Not all forms of Aβ have equal biological significance; toxicity is dependent upon the state of Aβ, whether that is the length of peptide, state of aggregation, homogeneity of aggregates or specific Aβ conformations. Thus, there exist disease-relevant conformations of Aβ, while other conformations are less toxic or biologically inert (Yang et al., 2017). The identity of "toxic Aβ" remains highly controversial; Aβ oligomers of similar size demonstrate dissimilar toxicity (Ladiwala et al., 2012) and consequently it is difficult to ascribe biological function to specific Aβ conformations as identified in biophysical methods. Therefore drug treatments could reduce non-toxic forms of Aβ without affecting the biologically active forms of Aβ. To overcome this problem the effects of Aβ released from treated 7PA2 cells was examined.

Extensive synapse degeneration is observed in Alzheimer's

Abbreviations: Alzheimer's disease, (AD); amyloid-β, (Aβ); amyloid precursor protein, (APP); Chinese hamster ovary, (CHO); conditioned media, (CM); cysteine string protein, (CSP); decanoic acid, (DA); detergent-resistant membrane, (DRM); detergent-soluble membrane, (DSM); enzyme-linked immunoassays, (ELISA); phospholipase A_2, (PLA_2); platelet-activating factor, (PAF); propylisopropylacetic acid, (PIA); prostaglandin, (PG); standard deviations, (SD); valproic acid, (VPA).

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patients (Heinonen et al., 1995) and the reductions in synaptic proteins correlate closely with the degree of dementia in AD (Hamos et al., 1989; Terry et al., 1991). Small, soluble Aβ oligomers are thought to be the main form of Aβ that causes synapse degeneration (Yang et al., 2017). For these reasons the effects of conditioned media (CM) from 7PA2 cells upon synapses in cultured neurons was tested. Synaptic density was determined by quantifying the amounts of synaptophysin and cysteine string protein (CSP) using enzyme-linked immunoassays (ELISA). The amounts of synaptophysin has been used to access synaptic density in the brain (Reddy et al., 2005; Counts et al., 2006) and in cultured neurons (Williams and Bate, 2016). Although the processing of APP is affected by phospholipase A_2 (PLA_2) inhibitors (Emmerling et al., 1993), conventional $cPLA_2$ inhibitors do not readily cross the blood-brain barrier and their use in AD is limited. Several reports demonstrated that valproic acid (2-propylpentanoic acid or VPA), a short branched-chain fatty acid acts like a PLA_2 inhibitor (Williams and Bate, 2016; Bosetti et al., 2003). Furthermore, although VPA has also been reported to reduce Aβ production (Qing et al., 2008a; Su et al., 2004) it was not clear which forms of Aβ were reduced. Here we report that the treatment of 7PA2 cells with VPA and its congener propylisopropylacetic acid (PIA), but not decanoic acid (DA), reduced the production of toxic forms of Aβ. These drugs had only a small effect upon concentrations of Aβ; rather they caused a switching from the production of toxic Aβ oligomers to non-toxic Aβ monomers.

2. Materials and methods

2.1. 7PA2 cells

CHO cells stably transfected with cDNA encoding human APP_{751} (referred to as 7PA2 cells) (Podlisny et al., 1995) were provided by Professor E Koo (University of California). These were cultured in neurobasal medium supplemented with B27 components (Invitrogen) in the presence of compounds for 72 h. CM was collected, filtered, desalted and passed through a 50 kDa filter (Vivaspin-Sartorious). CM from CHO cells (CHO-CM) were used as controls. To determine cell viability thiazolyl blue tetrazolium bromide (Sigma) was added to cells at a final concentration of 50 μM for 3 h at 37 °C. The medium 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). Cell extracts were collected from treated cells washed 3 times with ice cold PBS and homogenised in extraction buffer (containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS and mixed protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) (Sigma)) at 10^6 cells/ml. Cellular debris was removed by centrifugation (20 min as 16000 × g) and the supernatant collected. 7PA2-CM containing Aβ monomers were prepared by filtration of 2 mls 7PA2-CM through a 10 kDa filter (Sartorius). Oligomer preparations were the 7PA2-CM retained by the 10 kDa filter diluted back to the original volume (2 mls). For immunoblot analysis, 7PA2-CM/monomer or oligomer preparations were concentrated from 2000 to 100 μls using a 3 kDa filter (Sartorius). 10 μl of sample were mixed with an equal volume of in 0.5% NP-40, 5 mM CHAPS, 50 mM Tris, pH 7.4 and separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidene fluoride membrane by semi-dry blotting and blocked using 10% milk powder. Aβ was detected by incubation with mAb 6E10 (Covance), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence. Soluble brain extracts (containing peptides between 3 and 50 kDa) were prepared from brain tissue derived from Alzheimer's patients as described (Williams and Bate, 2016).

2.2. Brain extracts

Samples of temporal lobes from 3 patients with a pathologically-confirmed, clinical diagnosis of Alzheimer's diseasewas supplied by Asterand. Soluble extracts were prepared using methodology as described (Shankar et al., 2008). Briefly, pieces of brain tissue of approximately 100 mg were added to tubes containing lysing matrix D beads (Q-Bio). Ice cold 20 mM Tris, pH 7.4 containing 150 mM NaCl was added to an equivalent of 100 mg brain tissue/ml, tubes were shaken for 10 min (Disruptor genie, Scientific Instruments). This process was performed 3 times before tubes were centrifuged at 16,000 × *g* for 10 min to remove particulate matter. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 × *g* for 30 min). The remaining material was desalted (3 kDa filter (Sartorius)) the retained material collected (preparation contains molecules with molecular weights between 3 and 50 kDa). Monomers were prepared by passage through a 10 kDa filter (Sartorius) and oligomers were collected from the material that was retained (10–50 kDa). Preparations were stored at −80 °C. For cell experiments preparations were diluted in neurobasal medium containing B27 components. For immunoblots, preparations were separated by electrophoresis and visualised as outlined above.

2.3. Immunodepletions

To deplete preparations of Aβ they were incubated with 1 μg/ml mAb 4G8 (reactive with amino acids 17–24 of Aβ, Covance) or 1 μg/ml mAb LN27 (reactive with amino acids 45 to 53 of APP, (mock-depletion)) and incubated on rollers for 2 h. Protein G microbeads were added (10 μl/ml) (Sigma) for 30 min and protein G bound-antibody complexes removed by centrifugation (1000 × *g* for 5 min) and passed through a 0.2 μm filter.

2.4. Isolation of detergent-resistant membranes (DRMs (lipid rafts))

These membrane domains were isolated by their insolubility in non-ionic detergents as described (London and Brown, 2000). Briefly, cells were homogenised in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM EDTA and mixed protease inhibitors. Nuclei and large fragments were removed by centrifugation (300 × g for 5 min at 4 °C). The post nuclear supernatant was incubated on ice (4 °C) for 1 h (shaken at 10 min intervals) and centrifuged (16,000 × g for 20 min at 4 °C). The supernatant was reserved as the detergent-soluble membrane (DSM) while the insoluble pellet was homogenised in an extraction buffer containing 10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS and mixed protease inhibitors at 10^6 cells/ml, centrifuged (10 min at 16,000 × g) and the soluble material was reserved as the DRM fraction.

2.5. 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 polyvinylidene fluoride membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; APP was detected with rabbit polyclonal anti-APP (Sigma), caveolin with rabbit polyclonal anti-caveolin (Upstate) and platelet-activating factor (PAF) receptor with rabbit polyclonal anti-