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Cholesterol ester hydrolase inhibitors reduce the production of synaptotoxic amyloid-β oligomers

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ABSTRACT

The production of amyloid-β (Aβ) is the key factor driving pathogenesis in Alzheimer's disease (AD). Increasing concentrations of Aβ within the brain cause synapse degeneration and the dementia that is characteristic of AD. Here the factors that affect the release of disease-relevant forms Aβ were studied in a cell model. 7PA2 cells expressing the human amyloid precursor protein released soluble Aβ oligomers that caused synapse damage in cultured neurons. Supernatants from 7PA2 cells treated with the cholesterol synthesis inhibitor squalestatin contained similar concentrations of $Aβ_{42}$ to control cells but did not cause synapse damage in neuronal cultures. These supernatants contained reduced concentrations of $Aβ_{42}$ oligomers and increased concentrations of $Aβ_{42}$ monomers. Treatment of 7PA2 cells with platelet-activating factor (PAF) antagonists had similar effects; it reduced concentrations of $Aβ_{42}$ oligomers and increased concentrations of $Aβ_{42}$ monomers in cell supernatants. PAF activated cholesterol ester hydrolases (CEH), enzymes that released cholesterol from stores of cholesterol esters. Inhibition of CEH also reduced concentrations of $Aβ_{42}$ oligomers and increased concentrations of $Aβ_{42}$ monomers in cell supernatants. The Aβ monomers produced by treated cells protected neurons against Aβ oligomer-induced synapse damage. These studies indicate that pharmacological manipulation of cells can alter the ratio of Aβ monomer:oligomer released and consequently their effects on synapses.

1. Introduction

Alzheimer's disease (AD) is a complex neurological disorder characterized by a progressive dementia as a consequence of synaptic failure [1,2]. The amyloid hypothesis maintains that the pivotal event in AD is the production of toxic amyloid-β (Aβ) peptides following the proteolytic cleavage of the amyloid precursor protein (APP) [3]. In animal models intracerebral injections of Aβ peptides caused synapse damage and impaired memory formation [4,5]. Neurodegeneration is not directly proportional to concentrations of Aβ; rather that it is dependent upon numerous factors including the state of Aβ aggregation and specific Aβ conformations. Perhaps the key to understanding the amyloid hypothesis is the realization that there exist conformational forms of disease-relevant Aβ, while other conformations are less toxic or even biologically inert. Therefore, we sought to identify factors that specifically affected the release of toxic forms of Aβ. Chinese hamster ovary (CHO) cells stably transfected with cDNA encoding the human $(APP)_{751}$, known as 7PA2 cells, have been extensively used as they release soluble Aβ [6,7] that have similar properties to the soluble Aβ species found within the brains of AD patients [8–11].

The loss of synaptic proteins such as synaptophysin from the brain is indicative of synapse degeneration and provided a good correlate of the degree of dementia in AD [12–14]. Consequently, the loss of synaptic proteins from cultured primary neurons incubated with Aβ provides a useful in vitro model in which to investigate AD-related synapse damage [15]. Synapse density in cultured neurons was measured by quantification of the amounts of synaptophysin and cysteine string protein (CSP) [15]. Picomolar concentrations of soluble Aβ caused the loss of synaptophysin and CSP from cultured neurons [15] and impaired memory formation in animal models [10,16].

Although many studies implicate cholesterol concentrations as a major factor that regulates Aβ production, as reviewed by Chang and colleagues [17], the effects of cholesterol depletion on Aβ production remain controversial. While initial studies demonstrated that cholesterol depletion reduced the production of Aβ [18], another study reported it increased Aβ concentrations [19]. However, the conventional

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; APP, amyloid precursor protein; $AACOCF_3$, arachidonyl trifluoromethyl ketone; CHO, Chinese hamster ovary; CEH, cholesterol ester hydrolase; CM, conditioned media; CSP, cholesteryl *N*-(2-dimethylaminoethyl) carbamate (c-carbamate), cysteine string protein; DEUP, diethylumbelliferyl phosphate; ER, endoplasmic reticulum; Hexa-PAF, 1-O-Hexadecyl-2-acetyl-*sn*-glycerol-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine; MAFP, methyl arachidonyl fluorophosphonate; PLA_2, phospholipase A_2; PAF, platelet-activating factor; SD, standard deviation

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cholesterol synthesis inhibitors (3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) used to deplete cells of cholesterol also block the production of isoprenoids, which also affect Aβ concentrations [20,21]. Consequently it is not clear whether the effects of these drugs on Aβ concentrations are mediated by their effects upon cholesterol, or upon isoprenoid concentrations. In addition, previous studies have not differentiated the effects of cholesterol depletion upon the type of Aβ produced (toxic/non-toxic).

Here we show that treatment of 7PA2 cells with squalestatin, a squalene synthetase inhibitor that inhibits cholesterol synthesis without affecting the production of isoprenoids [22], reduced the release of synaptotoxic Aβ; conditioned media (CM) from these cells did not cause synapse degeneration when added to cultured neurons. Surprisingly, squalestatin had only a small affect upon the total concentrations of $Aβ_{42}$ in CM, rather it changed the ratio of Aβ oligomers to Aβ monomers. Treatment reduced concentrations of the synaptotoxic Aβ oligomers and increased concentrations of neuroprotective Aβ monomers [23]. This study also identified other drugs that affected the type of Aβ released; the release of soluble Aβ oligomers was reduced by treating 7PA2 cells with either phospholipase A_2 (PLA_2) inhibitors, platelet-activating factor (PAF) antagonists or cholesterol ester hydrolase (CEH) inhibitors. The observation that PAF activated CEHs, resulting in increased cholesterol concentrations within the endoplasmic reticulum (ER), led to the hypothesis that PAF-induced activation of CEH releases cholesterol that consequently affected the production of Aβ oligomers.

2. Methods

2.1. Culture of 7PA2 cells

Chinese hamster ovary (CHO) cells stably transfected with cDNA encoding APP_{751} (referred to as 7PA2 cells) originated from Professor E Koo's laboratory (National University of Singapore) were grown in Dulbecco's minimum essential medium supplemented with 10% foetal calf serum as described [6]. For experiments 7PA2 cells were grown in 6 well plates until 80% confluent. Culture media was replaced with neurobasal medium containing B27 components (Invitrogen) ± test compounds including squalestatin (a gift from GlaxoSmithKline), squalene, arachidonyl trifluoromethyl ketone ($AACOCF_3$), platelet-activating factor (PAF), 1-O-Hexadecyl-2-acetyl-*sn*-glycerol-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine (Hexa-PAF), methyl arachidonyl fluorophosphonate (MAFP), ginkgolide B, diethylumbelliferyl phosphate (DEUP), cholesteryl *N*-(2-dimethylaminoethyl) carbamate (Sigma) and the cells cultured for a further 3 days. Conditioned medium (CM) from these cells (7PA2-CM) was collected. To determine cell viability thiazolyl blue tetrazolium bromide was added to cells at a final concentration of 50 μM 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). Cells were washed 3 times with ice cold PBS and then homogenised in an extraction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS) containing mixed protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) and a phosphatase inhibitor cocktail (PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma)) at 10^6 cells/ml. Cellular debris was removed by centrifugation (20 min as 16,000 × *g*). Both 7PA2-CM and cell extracts were centrifuged at 100,000 × *g* for 4 h at 4 °C and passed through a 50 kDa filter (Sartorius). The CM from treated 7PA2 cells was then desalted (3 kDa filter, Sartorius to remove any residual drugs) and the retained material (> 3 kDa) diluted to its original volume. CM and cell extracts from non-transfected CHO cells (CHO-CM) were used as controls. 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).

2.2. Soluble brain extracts

Were prepared from the temporal lobes of patients with a clinical, and pathologically-confirmed, diagnosis of Alzheimer's disease using methods as described [10]. Briefly, brain tissue was cut into pieces of approximately 100 mg and added to 2 ml tubes containing lysing matrix D beads (Q-Bio). PBS was added so that there was the equivalent of 100 mg brain tissue/ml. The tubes were shaken for 10 min (Disruptor genie, Scientific Instruments) to homogenize tissue. This process was performed 3 times before tubes were centrifuged at 16,000 × *g* for 10 min to remove cell debris. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 × *g* for 30 min) followed by desalting, retention by a 3 kDa filter (Sartorius) to eliminate bioactive small molecules and drugs. The retained material was collected (preparation contains peptides with molecular weights between 3 and 50 kDa) and stored at − 80 °C.

2.3. Western blotting

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.

2.4. Aβ 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).

2.5. Primary neuronal cultures

Cortical neurons were isolated from the brains of mouse embryos (day 15.5) after mechanical dissociation and cell sieving as described [24]. Cells were plated at 2 × 10^5 cells/well in 48 well plates in Hams F12 containing 5% foetal calf serum for 2 h. Cultures were shaken (600 r.p.m. for 5 min) and non-adherent cells removed by 2 washes in PBS. Neurons were subsequently grown in neurobasal medium containing B27 components and nerve growth factor (5 ng/ml) (Sigma) for 10 days. Immunohistochemistry showed that the cells were > 90% neurofilament positive. In experiments neurons were subsequently incubated with test compounds/Aβ preparations and synapse damage was assessed after 24 h. 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. Neurons were washed 3 times in PBS and homogenised in an extraction buffer containing mixed protease and phosphatase inhibitors (as above) at 10^6 cells/ml. Nuclei and cell debris were removed by centrifugation (300 × *g* for 5 min). To determine cell viability thiazolyl blue tetrazolium bromide was added to cells at a final concentration of 50 μM 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. Neuronal survival was calculated with reference to untreated cells (100% survival).