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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 $A\beta_{42}$ to control cells but did not cause synapse damage in neuronal cultures. These supernatants contained reduced concentrations of $A\beta_{42}$ oligomers and increased concentrations of $A\beta_{42}$ monomers. Treatment of 7PA2 cells with platelet-activating factor (PAF) antagonists had similar effects; it reduced concentrations of $A\beta_{42}$ oligomers and increased concentrations of $A\beta_{42}$ 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 $A\beta_{42}$ oligomers and increased concentrations of $A\beta_{42}$ 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.

Key words - Alzheimer's disease, amyloid-β, cholesterol, platelet-activating factor, synaptophysin, synapses, platelet-activating factor

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 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).

Abbreviations – Alzheimer's Disease (AD), amyloid-β (Aβ), amyloid precursor protein (APP), arachidonyl trifluoromethyl ketone ($AACOCF_3$),

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 $A\beta_{42}$ 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 hours 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 minutes as 16000 x *g*). Both 7PA2-CM and cell extracts were centrifuged at 100,000 x *g* for 4 hours at 4^0C 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 minutes (Disruptor genie, Scientific Instruments) to homogenize tissue. This process was performed 3 times before tubes were centrifuged at 16,000 x *g* for 10 minutes to remove cell debris. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 x *g* for 30 minutes) 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^{o}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 hours. Protein G microbeads were added (10 µl/ml) (Sigma) for 30 minutes and protein G bound-antibody complexes removed by centrifugation (1000 x *g* for 5 minutes).

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 x 10^5 cells/well in 48 well plates in Hams F12 containing 5% foetal calf serum for 2 hours. Cultures were shaken (600 r.p.m

for 5 mins) 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 greater than 90% neurofilament positive. In experiments neurons were subsequently incubated with test compounds/Aβ preparations and synapse damage was assessed after 24 hours. 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 x *g* for 5 mins). To determine cell viability thiazolyl blue tetrazolium bromide was added to cells at a final concentration of 50 μM for 3 hours at 37ºC. 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).

2.6. Isolation of Endoplasmic Reticulum – (ER) membranes – ER membranes were isolated from 7PA2 cells using an ER preparation kit (Sigma) following the manufacturer's instructions. Briefly, homogenised cell extracts were separated on a discontinuous density gradient (Opitiprep) and membranes containing ER components were identified with mAb reactive to the ER marker Grp 78 (Stressgen Biotechnology) (25). ER membranes were pre-treated ± drugs for 30 minutes and incubated with PAF for 1 hour.

2.7. Synaptophysin ELISA - The amount of synaptophysin in neurons were measured by ELISA (26). Maxisorb immunoplates (Nunc) were coated with an anti-synaptophysin mouse mAb (MAB368-Millipore) was used as a capture antibody and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and finally 1 mg/ml 4-nitrophenol phosphate (Sigma). Absorbance was measured on a microplate reader at 405 nm. Samples were expressed as "units synaptophysin" where 100 units was defined as the amount of synaptophysin in control neurons.

2.8. CSP ELISA – Maxisorb immunoplates were coated with a monoclonal antibody (mAb) to CSP ((sc-136468) Santa Cruz) and blocked with 5% milk powder. Samples were added 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 control neurons.

2.9. Cholesterol measurement - The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Life Technologies) (27). Briefly, control and treated cell membranes were washed (16000 × *g*, 10 min) and lipids extracted by suspension in hexane:isopropanol (3:2, v/v) and disruption for 10 min in a cell disruptor ((Disruptor genie, Scientific Instruments). Samples were centrifuged (10,000 × *g*, 1 min), supernatants collected and dried under liquid nitrogen. Lipids were dissolved in 500 µl isopropanol:NP40 (9:1, v/v) and sonicated in a waterbath (30 minutes). Samples were pre-treated with catalase before the enzyme cocktail of the Amplex-red kit was added (0.1 M potassium phosphate buffer, pH 7.4; 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, cholesterol oxidase, (± cholesterol esterase), horse radish peroxidase and 0.4 mM 10-acetyl-3,7-dihydroxyphenoxazine) were added and incubated at 37 °C for 30 minutes. Cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide and ketones. The hydrogen peroxide reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin, which is measured by excitation at 530 nm and emission detection at 590 nm. Each experiment contained cholesterol standards and solvent only controls. Cholesterol concentrations of samples were calculated by reference to the cholesterol standards.

2.10. Sample preparation for ELISA – To detach $A\beta_{42}$ from membrane components that blocked specific epitopes samples (300 µl) were mixed with 700 µl of propan-2-ol and sonicated. Proteins were precipitated by adding 250 µls 100%w/v trichloroacetic acid, incubating on ice for 30 mins and centrifugation (16,000 x *g* for 10 mins at 4^{o}C). The pellet was washed twice with ice-cold acetone, suspended in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS and sonicated.

2.11. $A\beta_{42}$ ELISA – Nunc Maxisorb immunoplates were coated with an anti-Aβ mAb (4G8 - epitope 17-24, Covance) in carbonate buffer overnight. Plates were blocked with 5% milk powder in PBS-tween and samples were applied for 1 hour. The detection antibody was an $A\beta_{42}$ selective rabbit mAb BA3-9 (Covance) followed by biotinylated anti-rabbit IgG and extravidin alkaline phosphatase (Sigma) and 1 mg/ml 4-nitrophenol phosphate solution. Optical density was read in a spectrophotometer at 405 nm.

2.12. $A\beta_{40}$ ELISA - Maxisorb immunoplates were coated with mAb 4G8 (epitope 17-24) and blocked with 5% milk powder in PBS-tween. Samples were applied and $A\beta_{40}$ was detected with rabbit polyclonal PC-149 (Merck) followed by biotinylated anti-rabbit IgG and extravidin alkaline phosphatase. Total Aβ was visualised by addition of 4-nitrophenol phosphate and optical density was read in a spectrophotometer at 405 nm.

2.13. Statistical Analysis - Comparison of treatment effects was carried out using Student's paired t-tests, one-way and two-way ANOVA with Bonferonni's post hoc tests (IBM SPSS statistics 20). Error values are

standard deviation (SD) and significance was determined where $p<0.01$. Bivariate analysis using Pearson's coefficient (IBM SPSS statistics 20) were used to examine correlations between data sets.

3. Results

3.1. CM from 7PA2 cells contains synaptotoxic Aβ - CM from 7PA2 cells caused the loss of synaptic proteins including synapsin-1, vesicle-associated membrane protein-1, CSP and synaptophysin from cultured neurons (26). Here we show that CM from 7PA2 cells, but not CM from CHO cells, reduced amounts of synaptophysin (Figure 1A) and CSP (Figure 1B) in cultured neurons indicative of synapse degeneration. The addition of 7PA2-CM did not reduce neuronal viability as measured by thiazolyl blue tetrazolium ((101% cell survival ± 6 compared with 100% ± 5, n=9, P=0.6) indicating that synapse degeneration occurred in the absence of any significant neuronal death. To demonstrate that it was Aβ within the CM that was responsible for the synaptotoxic effect, Aβ was removed following immunodepletion by mAb 4G8 (reactive with Aβ, epitope 17 to 24) (Figure 1C). Immunodepletion with mAb 4G8 reduced the concentrations of both $A\beta_{40}$ and $A\beta_{42}$ whereas mock-depletion had no significant effect (Table 1). The effects of 7PA2-CM on neuronal synaptophysin levels was removed by immunodepletion with mAb 4G8, but was unaffected by mock-depletion (Figure 1D) indicating that Aβ was the synaptotoxic component. As the 4G8 mAb used in these depletions reacts with an epitope common to several APP breakdown fragments including $A\beta_{40}$ and $A\beta_{42}$ and others (28) the current study does not identify the precise toxic entities. The effects of 7PA2-CM were than compared to that of soluble brain extracts derived from an Alzheimer's patient (15). 7PA2-CM and soluble brain extracts containing similar concentrations of $A\beta_{42}$ caused dose-dependent reductions in synaptophysin (Figure 1E) and CSP (Figure 1F).

3.2. Squalestatin reduced the amounts of synaptotoxic Aβ in CM – The effects of cholesterol depletion on the production of Aβ were examined after collecting CM from 7PA2 cells treated with 500 nM squalestatin, a squalene synthetase inhibitor (29). Squalestatin reduced the concentrations of cholesterol in 7PA2 cells and the concentrations of $A\beta_{42}$ and $A\beta_{40}$ in CM, without affecting cell survival (Table 2). CM from 7PA2 cells treated with 500 nM squalestatin did not cause synapse damage (Figures 2A & B). This effect of squalestatin was dose-dependent (Figure 2C). The effects of squalestatin upon 7PA2 cells were reversed by the addition of 5 μM squalene; it increased cellular cholesterol concentrations and concentrations of $A\beta_{40}$ and $A\beta_{42}$ within CM (Table 2). Furthermore, CM from 7PA2 cells treated with a combination of 500 nM squalestatin and 5 μM squalene caused more synapse damage than CM from 7PA2 cells treated with 500 nM squalestatin alone (Figure 2D). The addition of 5 μM squalene alone did not

affect the concentrations of cholesterol in 7PA2 cells, nor did it affect concentrations of $A\beta_{40}$ and $A\beta_{42}$ found in CM.

3.3. Squalestatin reduced the release of $A\beta_{42}$ oligomers – Although the CM from squalestatin-treated cells contained significant concentrations of $A\beta_{42}$ they did not cause synapse degeneration. To test the hypothesis that the synaptotoxicity of 7PA2-CM was relative to the concentration of Aβ oligomers, rather than the concentration of total Aβ, Aβ oligomers were isolated by filtration. Aβ oligomers were retained (and Aβ monomers removed from 7PA2-CM) by centrifugation with a 10 kDa filter; as shown by electrophoresis (Figure 3A). Treatment with squalestatin reduced the concentrations of $A\beta_{42}$ oligomers in CM in a dose-dependent manner (Figure 3B). In 7PA2 cells treated with squalestatin (0.062 to 0.5 μM) there was a significant correlation between cholesterol concentrations of 7PA2 cells and the concentrations of $A\beta_{42}$ oligomers released into CM, Pearson's coefficient=0.71, $p<0.01$ (Figure 3C). An immunoblot showed that Aβ monomers were isolated from CM passed through a 10 kDa filter (Figure 3D). Treatment with squalestatin caused a dose-dependent increase in the concentrations of $A\beta_{42}$ monomers in CM (Figure 3E). There was a significant inverse correlation between the concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells treated with squalestatin, Pearson's coefficient = -0.79, $p<0.01$ (Figure 3F). Greater than 90% of $A\beta_{40}$ in the CM from control 7PA2 cells was monomeric and there were no major changes in the concentrations of $A\beta_{40}$ monomers/oligomers in CM from squalestatin-treated cells (data not shown). As a consequence we focused upon measuring concentrations of $A\beta_{42}$ monomers and oligomers in the following experiments.

3.4. PLA_2 inhibitors and PAF antagonists controlled the release of Aβ monomers and oligomers – Since PLA_2 affects cholesterol distribution and cell trafficking (30,31) its role in $A\beta_{42}$ production was also examined. CM from 7PA2 cells treated with $cPLA_2$ inhibitors (1 μM $AACOCF_3$ or 1 μM MAFP) did not cause synapse damage to cultured neurons (Figures 4A & B). The concentrations of $A\beta_{42}$ oligomers were significantly reduced in CM from 7PA2 cells treated with 1 μM $AACOCF_3$ or 1 μM MAFP (Table 3) when compared to CM from control cells. As the activation of $cPLA_2$ results in the production of prostaglandins and PAF, specific inhibitors of these were tested. CM from 7PA2 cells treated with PAF antagonists (Hexa-PAF or ginkgolide B) did not cause the loss of synaptophysin (Figure 4C) or CSP (Figure 4D) from neurons. The CM from 7PA2 cells treated with PAF antagonists contained lower concentrations of $A\beta_{42}$ oligomers than CM from control cells (Table 3) and there was significant inverse correlation between the concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells treated with Hexa-PAF, Pearson's coefficient= -0.89, $p<0.01$ (Figure 4E).

3.5. PAF reversed the effects of cPLA$_2$ inhibitors on the release of Aβ monomers and oligomers – It was concluded that PAF, generated following activation of cPLA$_2$, is involved in the production of Aβ. This hypothesis was supported by observations that the addition of 500 nM PAF reversed the effects of the cPLA$_2$ inhibitor AACOCF$_3$. Thus, CM from cells treated with AACOCF$_3$ and PAF caused more synapse damage, as measured by the loss of synaptophysin (Figure 5A) and CSP (Figure 5B) than did CM from cells treated with AACOCF$_3$ alone. Similarly, the addition of 500 nM PAF reversed the effects of AACOCF$_3$ on the forms of Aβ released into CM; it reversed the AACOCF$_3$-induced reduction in Aβ$_{42}$ oligomers (Figure 5C) and increase in Aβ$_{42}$ monomers (Figure 5D).

3.6 Squalene did not reverse the effects of a PAF antagonist - Since PAF antagonists had similar effects upon the release of Aβ as squalestatin, the hypothesis that PAF antagonists affected cholesterol synthesis was explored. However, in contrast to squalestatin, treatment of 7PA2 cells for 24 hours with 1 μM Hexa-PAF did not significantly affect cellular cholesterol concentrations (0.69 μM cholesterol ± 0.09 compared with 0.7 μM ± 0.11, n=6, P=0.71). Furthermore, the addition of 5 μM squalene, which reversed the effects of squalestatin, did not reverse the effects of Hexa-PAF on 7PA2 cells. Thus, CM from cells treated with a combination of 5 μM squalene and 1 μM H-PAF caused similar amounts of synapse damage, as measured by the loss of synaptophysin (Figure 6A) and CSP (Figure 6B), as CM from cells treated with 1 μM Hexa-PAF alone. Similarly, the CM from cells treated with a combination of 5 μM squalene and 1 μM Hexa-PAF contained similar concentration of Aβ$_{42}$ oligomers (Figure 6C) and Aβ$_{42}$ monomers (Figure 6D) as CM taken from cells treated with 1 μM Hexa-PAF alone. Collectively these results indicate that PAF antagonists were not acting like the cholesterol synthesis inhibitor squalestatin.

3.7. PAF caused the release of cholesterol from cholesterol esters – A prior study had shown that PAF receptors were concentrated in membranes which also contained Grp78, a marker of the endoplasmic reticulum (ER) (32). The addition of PAF to ER membranes caused dose-dependent increases in cholesterol concentrations (Figure 7A) and reductions in concentrations of cholesterol esters (Figure 7B). There was a significant inverse correlation between concentrations of cholesterol and cholesterol esters in ER membranes incubated with PAF (0.125 to 1μM) (Figure 7C). The PAF-induced increase in cholesterol concentrations in these membranes was not affected by pre-treatment with 500 nM squalestatin indicating that PAF did not stimulate cholesterol synthesis (Figure 7D). The concentrations of cholesterol within cell membranes is partly controlled by the cholesterol ester cycle (33) and membrane cholesterol concentrations can be increased following activation of CEHs, enzymes that release cholesterol from cholesterol esters (34). Pre-treatment of ER membranes with 2 CEH inhibitors (diethylumbelliferyl phosphate (DEUP)) (35) and cholesteryl N-(2-dimethylaminoethyl) carbamate (36) inhibited both the PAF-induced increase in

cholesterol concentrations (Figure 7E) and the reduction in cholesterol ester concentrations (Figure 7F). We concluded that PAF altered cholesterol concentrations via its effects upon CEHs.

3.8. CEH inhibitors reduced the release of synaptotoxic Aβ - Treatment of 7PA2 cells with CEH inhibitors (DEUP or cholesteryl N-(2-dimethylaminoethyl) carbamate) caused a dose-dependent reduction in the concentrations of $A\beta_{42}$ oligomers (Figure 8A) and an increase in $A\beta_{42}$ monomers (Figure 8B) found within CM. There were significant inverse correlations between concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells treated with DEUP, Pearson's coefficient= -0.92, $p<0.01$ or cholesteryl N-(2-dimethylaminoethyl) carbamate, Pearson's coefficient= -0.72, $p<0.01$ (Figure 8C). An immunoblot showed that CM from DEUP-treated cells contained more Aβ monomers and less Aβ oligomers than CM from control cells (Figure 8D). CM from 7PA2 cell treated with either 20 μM DEUP or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate did not cause the loss of synaptophysin (Figure 8E) or CSP (Figure 8F) from neurons. Collectively these results are consistent with the hypothesis that CEHs affect cellular cholesterol concentrations that regulate the release of the Aβ oligomers that cause synapse damage.

3.9. CEH inhibitors did not have a direct effect upon $A\beta_{42}$ oligomers - These results raised the possibility that drugs may interact directly with Aβ oligomers within the CM causing them to dissociate into monomers. This hypothesis was tested by incubating Aβ preparations with 500 nM squalestatin, 1 μM Hexa-PAF or 20 μM DEUP at 37°C for 3 days. An immunoblot showed that there were no significant differences in the amounts of Aβ oligomers and monomers between control and treated preparations (Figure 9). In another experiment Aβ oligomer preparations (containing 10 nM $A\beta_{42}$) were incubated with 500 nM squalestatin, 1 μM Hexa-PAF or 20 μM DEUP at 37°C for 3 days. When these preparations were passed through a 10 kDa filter, monomeric forms of $A\beta_{42}$ were not detected indicating that these drugs had not caused the dissociation of $A\beta_{42}$ oligomers.

3.10. CEH inhibitors reduced cell-associated $A\beta_{42}$ oligomers - The possibility that the drugs tested simply affected the release, rather than the production of Aβ was examined by measuring Aβ in cell extracts from treated 7PA2 cells. The concentrations of $A\beta_{42}$ oligomers in 7PA2 cell extracts were significantly reduced by treatment with 500 nM squalestatin, 1 μM $AACOCF_3$, 1 μM MAFP, 1 μM Hexa-PAF, 1 μM ginkgolide B, 1 μM DEUP or 20 μM cholesteryl N-(2-dimethylaminoethyl) carbamate (Table 4). Treatment with these drugs also increased the amounts of cell-associated $A\beta_{42}$ monomers.

3.11. CM from treated 7PA2 cells protected neurons against Aβ oligomer-induced synapse damage – The CM from 7PA2 cells treated with squalestatin, PAF antagonists or CEH inhibitors contained high concentrations of Aβ monomers which have been reported to have neuroprotective properties (23). Here we

mixed CM from 7PA2 cells treated with either 500 nM squalestatin, 1 μM Hexa-PAF or 20 μM DEUP with either soluble brain extracts or phospholipase A_2-activating peptide (PLAP). Both the soluble brain extracts and PLAP caused the loss of synaptophysin and CSP from neuronal cultures. The presence CM from 7PA2 cells treated with squalestatin, PAF antagonists or CEH inhibitors protected neurons against the brain extract-induced reduction in synaptophysin (Figure 10A). CM from treated CHO cells did not affect the brain extract-induced reduction in synaptophysin (data not shown). The protective effects of CM from treated 7PA2 cells were stimulus specific; they did not affect the loss of synaptophysin caused by PLAP (Figure 10B). CM from DEUP-treated 7PA2 cells was examined in more detail. Immunodepletion with the Aβ-specific mAb 4G8 removed the inhibitory factor from CM (Figure 10C) indicating that the protective component was Aβ. Monomer preparations derived from CM from DEUP-treated 7PA2 cells also protected neurons against the brain extract-induced loss of synaptophysin. Finally, Aβ monomers derived from CM from DEUP-treated 7PA2 cells had a dose-dependent effect against the loss of synaptophysin caused by a brain extract containing 2 nM $Aβ_{42}$ (Figure 10D). We note that in addition to $Aβ_{40}$ and $Aβ_{42}$ there are likely to be other APP fragments in the monomer preparations and that these may contribute to the activity seen here.

4. Discussion

Although many studies have examined the factors that affect Aβ production this is the first study that we are aware of that differentiated between toxic and non-toxic forms of Aβ. The release of biologically active forms of Aβ by 7PA2 cells was examined using synapse damage in cultured neurons as a model. The synapse damage caused by 7PA2-CM was comparable to that caused by soluble brain extracts from AD patients when standardized on their $Aβ_{42}$ content. In addition, the synapse damage caused by both 7PA2-CM and brain extracts (15) were Aβ-dependent, suggesting that 7PA2 cells release synaptotoxic forms of Aβ similar to the Aβ oligomers found in brains of AD patients. The 2 key findings of this study were firstly, that the release of synaptotoxic Aβ oligomers is controlled by pathways sensitive to squalestatin, PLA_2 inhibitors, PAF antagonists and CEH inhibitors. All these compounds affected either cholesterol concentrations or cholesterol distribution within cell membranes. Secondly, that treated cells released increased concentrations of Aβ monomers and that these monomeric forms of Aβ protected neurons against the synaptotoxic Aβ oligomers.

Our observation that squalestatin caused only small differences in the production of $Aβ_{40}$ and $Aβ_{42}$ was in contrast to prior reports of the effects of other cholesterol synthesis inhibitors. This may be due to using

squalestatin, a more specific cholesterol synthesis inhibitor that does not affect isoprenoid function (22), rather than conventional "statins" used in other reports. Furthermore, the reduction in cellular cholesterol concentrations in these studies was mild when compared to other studies (19,37). The key observation, that CM from squalestatin-treated cells contained $A\beta_{42}$ but did not cause synapse damage, showed that measuring concentrations of Aβ alone was a poor indicator of biological activity. That squalestatin selectively affected the production of soluble Aβ oligomers, thought to be major causes of synapse damage in AD (10,16,38), is consistent with a report that reduced cholesterol synthesis did not affect Aβ production but did extend lifespan in a mouse model of AD (39). Cholesterol affects the formation and function of lipid rafts (40,41) in which APP and many of the enzymes involved in the generation of Aβ are found (42-44). Consequently, it seems likely that the squalestatin-induced reduction in cholesterol concentrations would affect the processing of APP to Aβ oligomers.

Cholesterol concentrations within the ER were increased after activation of $cPLA_2$ (25). This enzyme affects tubule formation and consequently intracellular trafficking of proteins (31), specifically within the trans-golgi network (45), a key area of APP metabolism (46). Here we show that inhibition of $cPLA_2$ in 7PA2 cells resulted in CM that did not cause synapse damage. Activation of $cPLA_2$ leads to the production of PAF and PAF antagonists also reduced the release of Aβ oligomers from 7PA2 cells. As the addition of PAF reversed the effects of $cPLA_2$ inhibitors we concluded that it was the key mediator involved in this pathway. 7PA2 cells treated with $cPLA_2$ inhibitors or PAF antagonists, like cells treated with squalestatin, released less $A\beta_{42}$ oligomers and more $A\beta_{42}$ monomers.

In contrast to squalestatin, neither $cPLA_2$ inhibitors, nor PAF antagonists, had a significant effect on 7PA2 cellular cholesterol concentrations, and their effects upon Aβ production were not reversed by squalene indicating that they did not affect cholesterol synthesis. However, it could be argued that Aβ production might be affected by small changes in cholesterol concentrations in specific organelles which would not be seen in total cell membrane extracts. PAF receptors were concentrated in the ER and PAF increased cholesterol concentrations within isolated ER membranes. The cholesterol concentrations in cell membranes are controlled by the cholesterol ester cycle (47) and lipid droplets, containing high concentrations of cholesterol esters are concentrated in the ER (48). The PAF-induced increase in cholesterol in ER membranes was accompanied by a reduction in cholesterol esters; there was an inverse correlation between concentrations of cholesterol and cholesterol esters, leading to the conclusion PAF activates CEHs. The inhibition of PAF-induced changes by CEH inhibitors supported this hypothesis. Notably CM from 7PA2 cells treated with CEH inhibitors were similar to CM from 7PA2 cells treated with

PAF antagonists; they contained less $A\beta_{42}$ oligomers, more $A\beta_{42}$ monomers and did not cause synapse damage.

Each class of drug tested, squalestatin, PAF antagonists or CEH inhibitors demonstrated an increased release of $A\beta_{42}$ monomers that closely correlated with the reduction in $A\beta_{42}$ oligomers. A key finding was that CM from cells treated with either squalestatin, a PAF antagonist or a CEH inhibitor reduced synapse damage caused by soluble Aβ derived from a brain extract. The neuroprotective effect was mediated by Aβ monomer preparations, consistent with reports that Aβ monomers are neuroprotective (23). It is noteworthy that thee Aβ monomer preparations may contain other APP fragments in addition to $A\beta_{40}$ and $A\beta_{42}$ peptides as has been reported in cerebrospinal fluid (49). Critically these results suggest that synapse damage is dependent upon the ratio of Aβ monomers and Aβ oligomers, rather than the total Aβ concentration. We can only speculate how squalestatin, PLA_2 inhibitors, PAF antagonists and CEH inhibitors alter APP metabolism and subsequently the release of Aβ monomers/oligomers. All of the drugs affected cholesterol concentrations which is important for the formation and function of lipid rafts (41). APP and many of the enzymes involved in the generation of Aβ are found in lipid rafts (42,43) and $A\beta_{42}$ is produced within cholesterol-sensitive lipid rafts (50). Protein cargos in lipid rafts traffic via different pathways to those in the normal cell membrane (51) and consequently APP in lipid rafts may be targeted to different cell compartments (and consequently interacts with different enzymes) than APP found in the normal cell membrane. In addition, membrane cholesterol concentrations affect the process of exocytosis (52) and may alter the release of Aβ from cells. The possibility that changes in the concentrations of $A\beta_{42}$ oligomers/monomers within cell supernatants was mediated by cholesterol-sensitive changes in the release of Aβ monomers/oligomers alone appears unlikely as these drugs also reduced concentrations of $A\beta_{42}$ oligomers and increased $A\beta_{42}$ monomers in cell extracts.

5. Conclusions

In conclusion these results indicate that in 7PA2 cells the production of synaptotoxic and neuroprotective forms of Aβ can be modified by pharmacological manipulation. More specifically, the Aβ monomer:oligomer ratio was sensitive to drugs that altered cholesterol concentrations in cell membranes. Furthermore this study demonstrates the need to understand the biological activity of the forms of Aβ measured.

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7. Tables

	$A\beta_{40}$ (nM)	$A\beta_{42}$ (nM)
Control 7PA2-CM	7.42 ± 0.6	2.21 ± 0.3
Aβ-depleted 7PA2-CM	0.67 ± 0.28*	0.04 ± 0.04*
Mock depleted 7PA2-CM	6.95 ± 0.81	2.03 ± 0.21

Table 1. Immunodepletion reduces Aβ concentrations in 7PA2-CM - The concentrations of $A\beta_{40}$ and $A\beta_{42}$ in control 7PA2-CM and the same 7PA2-CM after immunodepletion with the Aβ-reactive mAb 4G8, or after mock depletion (incubation with mAb LN27 reactive with APP but not Aβ). Values are means ± SD from triplicate experiments performed 3 times (n=9). *=concentrations of $A\beta_{40}$/$A\beta_{42}$ significantly less than in untreated 7PA2-CM, $p<0.01$.

Treatment	**Cell viability (%)**	**Cholesterol (μM)**	**$A\beta_{42}$ (nM)**	**$A\beta_{40}$ (nM)**
Control	100 ± 4	0.65 ± 0.05	2.44 ± 0.29	7.12 ± 0.45
Squalestatin	98 ± 6	0.51 ± 0.04*	1.68 ± 0.24*	5.34 ± 0.48*
Squalene	101 ± 5	0.65 ± 0.06*	2.51 ± 0.31	7.28 ± 0.41
Squalestatin + squalene	99 ± 4	0.66 ± 0.05	2.37 ± 0.34	7.06 ± 0.33

Table 2 – Squalene reversed the effects of squalestatin on 7PA2 cells – The survival of 7PA2 cells, the concentrations of cholesterol in 7PA2 cells and the concentrations of $A\beta_{42}$ and $A\beta_{40}$ in CM from 7PA2 cells treated for 3 days with control medium, 500 nM squalestatin, 5 μM squalene or a combination of both. Values are means ± SD from triplicate experiments performed 3 times (n=9). *=concentrations significantly less than in control cells, $p<0.01$.

	$A\beta_{42}$ oligomers (nM)
Control cells	2.13 ± 0.31
1 μM $AACOCF_3$	0.38 ± 0.28*
1 μM MAFP	0.74 ± 0.23*
1 μM Hexa-PAF	0.28 ± 0.24*
1 μM ginkgolide B	0.36 ± 0.19*

Table 3. Treatment of 7PA2 cells with PLA_2 inhibitors or PAF antagonists reduced concentrations of $A\beta_{42}$ oligomers in CM - The concentrations of $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with squalestatin, $cPLA_2$ inhibitors ($AACOCF_3$ or MAFP) or PAF antagonists (Hexa-PAF or ginkgolide B) or CEH inhibitors (DEUP or cholesteryl N-(2-dimethylaminoethyl) carbamate). Values are means ± SD from three experiments measured in triplicate, n=9. *= concentrations of $A\beta_{42}$ oligomers significantly less than in control cells, $p<0.01$.

	7PA2-cells	
Treatment	**$A\beta_{42}$ oligomers (nM)**	**$A\beta_{42}$ monomers (nM)**
Control	12.47 ± 0.1.2	0.93 ± 0.33
Squalestatin	8.59 ± 0.89*	1.2 ± 0.15*
$AACOCF_3$	9.17 ± 0.71*	1.09 ± 0.1
MAFP	9.13 ± 0.98*	1.11 ± 0.13
Hexa-PAF	9.01 ± 0.99*	1.12 ± 0.12*
Ginkgolide B	8.89 ± 1.04*	1.18 ± 0.19
DEUP	8.51± 0.84*	1.28 ± 0.12*
Cholesteryl-carbamate	8.53 ± 1.1*	1.25 ± 0.1*

Table 4 – Squalestatin, PLA_2 inhibitors, PAF antagonists or CEH inhibitors reduced concentrations of cell-associated $A\beta_{42}$ oligomers – The concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in cell extracts from 7PA2 cells treated with control medium, 500 nM squalestatin, 1μM AACOCF3, 1μM MAFP, 1μM Hexa-PAF, 1μM ginkgolide B, 20 μM DEUP or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate for 3 days. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations significantly different from controls, $p<0.01$.

8. Figures

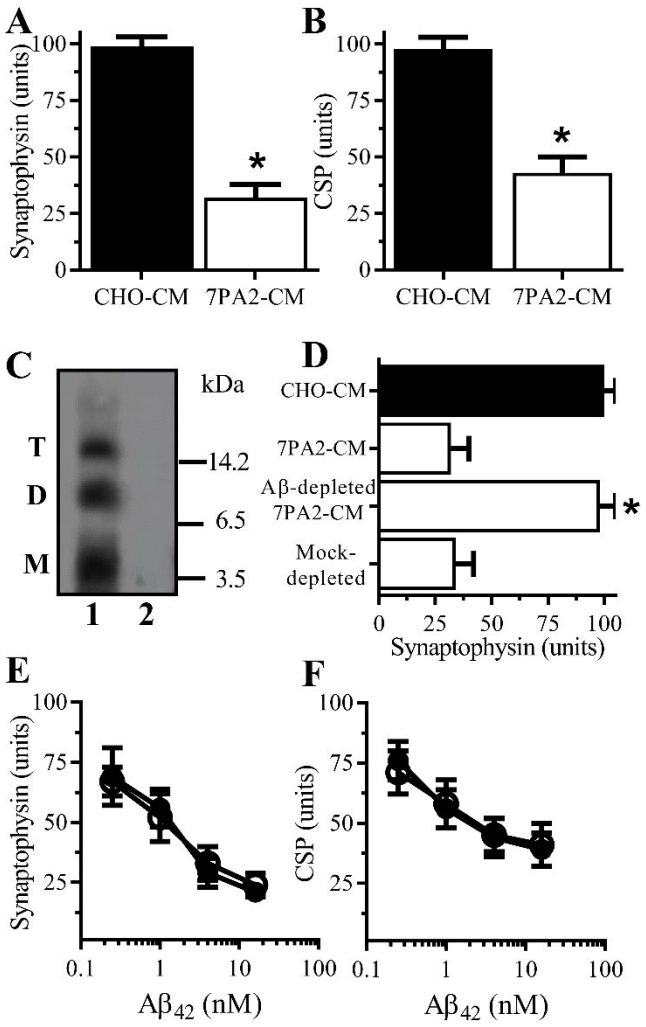

Figure 1. 7PA2 cells release synaptotoxic Aβ - The amounts of synaptophysin (A) and CSP (B) in neurons incubated with CM from 7PA2 cells (■) or CHO cells (□). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=amounts of synaptophysin/CSP significantly less than in neurons incubated with CHO-CM. (C) Immunoblot showing Aβ monomers (M), dimers (D) and trimers (T) in 7PA2-CM (1) and Aβ-depleted 7PA2-CM (2). (D) The amounts of synaptophysin in neurons incubated with CM from CHO cells, 7PA2-CM, Aβ-depleted 7PA2-CM or mock-depleted 7PA2-CM as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=amounts of synaptophysin significantly higher than in neurons incubated with 7PA2-CM. The amounts of synaptophysin (E) and CSP (F) in neurons incubated with 7PA2-CM (●) or soluble brain extract (○) containing $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9.

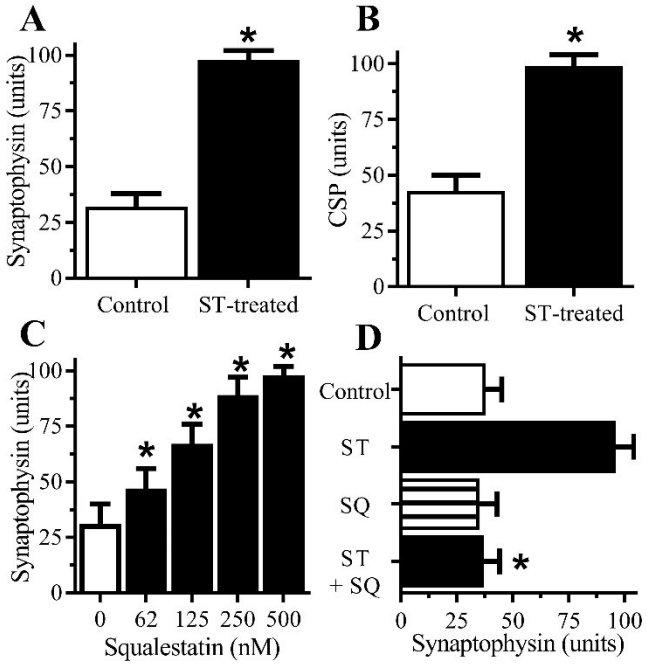

Figure 2. Squalestatin reduced the release of synaptotoxic Aβ - The amounts of synaptophysin (A) and CSP (B) in neurons incubated with CM from 7PA2 cells treated with control medium (□) or 500 nM squalestatin (ST) (■). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=amounts of synaptophysin/CSP significantly higher than in neurons incubated with control 7PA2-CM. (C) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with control medium or squalestatin as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=amounts of synaptophysin significantly higher than in neurons incubated with CM from control cells, $P<0.01$. (D) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with control medium, 500 nM squalestatin (ST), 5 μM squalene (SQ) or a combination of 5 μM squalene and 500 nM squalestatin as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=synaptophysin significantly less than in neurons incubated with CM from 7PA2 cells treated with squalestatin (ST).

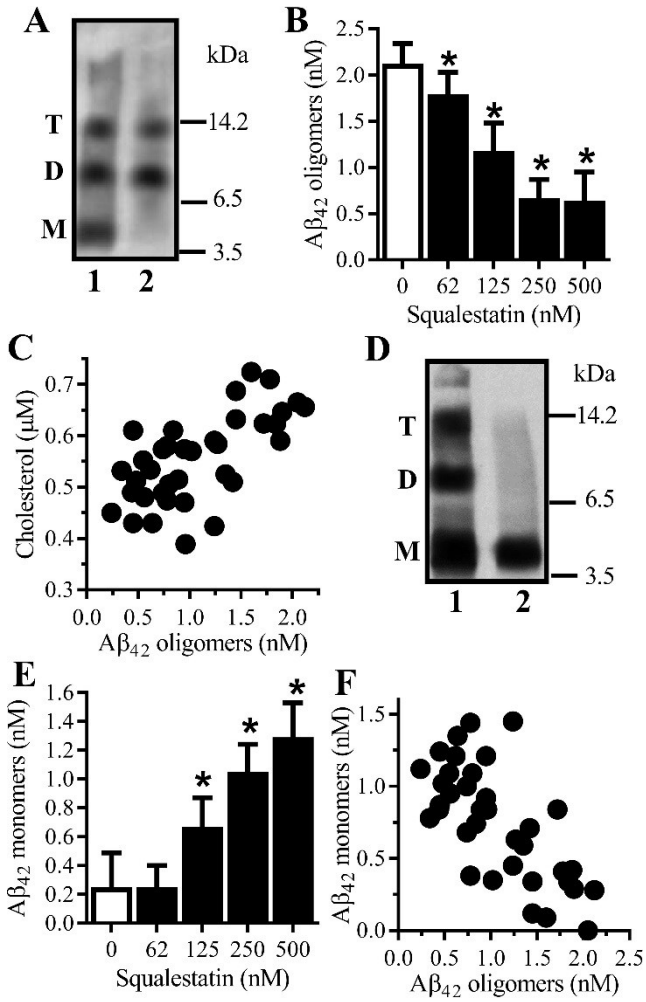

Figure 3. Squalestatin altered the release of Aβ42 oligomers and Aβ42 monomers (A) Immunoblot showing forms of Aβ monomers (M), dimers (D) and trimers (T)) in 7PA2-CM (1) and 7PA2-CM oligomer preparations (>10 kDa) (2). (B) The concentrations of $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with control medium or squalestatin as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of $A\beta_{42}$ oligomers significantly less than in control 7PA2-CM, $p<0.01$. (C) There was a significant correlation between concentrations of cholesterol in 7PA2 cells treated with squalestatin (0.062 to 0.5 μM) and concentrations of $A\beta_{42}$ oligomers in CM, Pearson's coefficient=0.71, $p<0.01$. (D) Immunoblot showing Aβ monomers, dimers and trimers in 7PA2-CM (1) and in monomer preparations (7PA2-CM passed through a 10 kDa filter) (2). (E) The concentrations of $A\beta_{42}$ monomers in CM from 7PA2 cells treated with control medium or squalestatin as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of $A\beta_{42}$ monomers significantly higher than in control CM. (F) There was a significant inverse correlation between concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells treated with squalestatin (0.062 to 0.5 μM), Pearson's coefficient = -0.79, $p<0.01$.

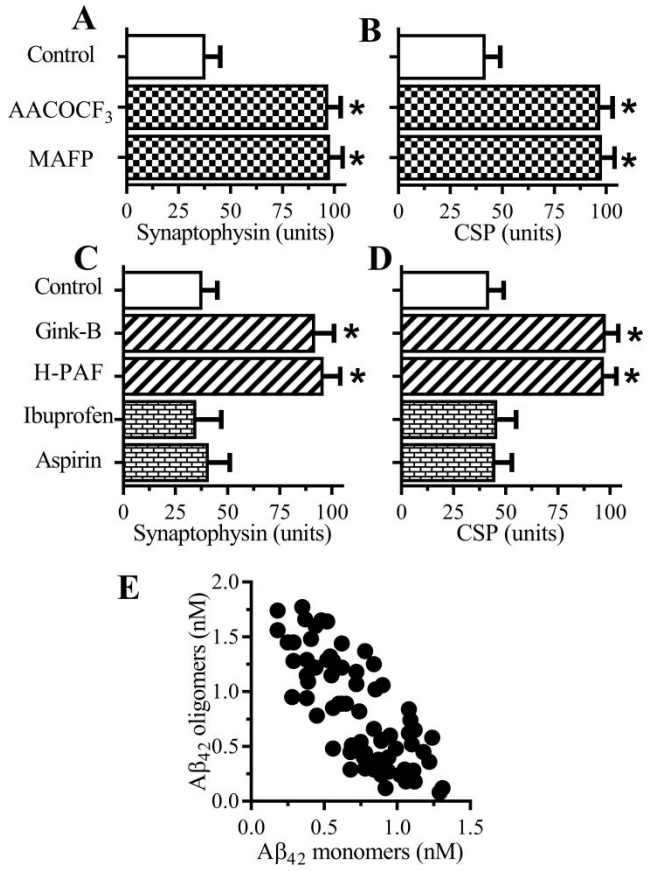

Figure 4. PLA_2 inhibitors and PAF antagonists reduced the release of synaptotoxic Aβ - The amounts of synaptophysin (A) and CSP (B) in neurons incubated with CM from 7PA2 cells treated with control medium or $cPLA_2$ inhibitors (1 μM MAFP or 1 μM $AACOCF_3$). Values are means ± SD from triplicate experiments performed 3 times, n=9. The amounts of synaptophysin (C) and CSP (D) in neurons incubated with CM from 7PA2 cells treated with control medium, PAF antagonists (1 μM Hexa-PAF or 1 μM ginkgolide B) or cyclooxygenase inhibitors (2 μM aspirin or 5 μM ibuprofen) as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=synaptophysin/CSP significantly higher than in neurons incubated with CM from control cells. (E) There was a significant inverse correlation between the concentrations of $Aβ_{42}$ oligomers and $Aβ_{42}$ monomers in CM from 7PA2 cells treated with Hexa-PAF (.125 to 1 μM), Pearson's coefficient= -0.89, p <0.01.

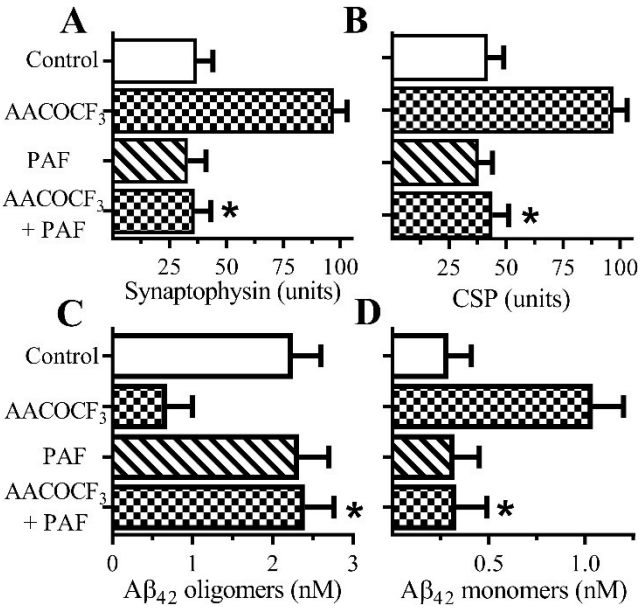

Figure 5. PAF reversed the effects of a PLA_2 inhibitor on the release of synaptotoxic Aβ – The amounts of synaptophysin (A) and CSP (B) in neurons incubated with CM from 7PA2 cells treated with control medium, 1 μM $AACOCF_3$, 500 nM PAF or a combination of 1 μM $AACOCF_3$ and 500 nM PAF as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of synaptophysin/CSP significantly less than those of neurons incubated with CM from $AACOCF_3$-treated cells. The concentrations of $A\beta_{42}$ oligomers (C) and $A\beta_{42}$ monomers (D) in CM from 7PA2 cells treated with control medium,1 μM $AACOCF_3$, 500 nM PAF or a combination of 1 μM $AACOCF_3$ and 500 nM PAF as shown. Values are mean $A\beta_{42}$ ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of $A\beta_{42}$ oligomers/monomers significantly different to those in CM from $AACOCF_3$-treated cells.

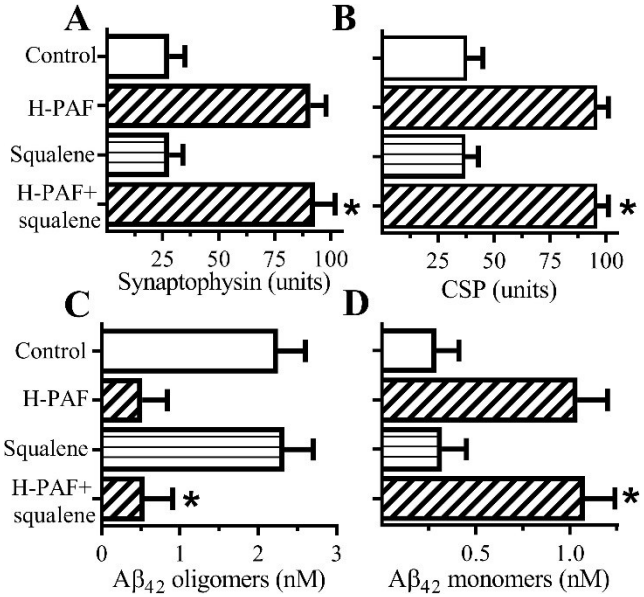

Figure 6. Squalene did not reverse the effects of a PAF antagonist on the release of synaptotoxic Aβ – The amounts of synaptophysin (A) and CSP (B) in neurons incubated with CM from 7PA2 cells treated with either control medium, 1 μM Hexa-PAF, 5 μM squalene or a combination of 1 μM Hexa-PAF and 5 μM squalene as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of synaptophysin/CSP not significantly different from those of neurons incubated with CM from H-PAF-treated cells. The amounts of $A\beta_{42}$ oligomers (C) and $A\beta_{42}$ monomers (D) in CM from 7PA2 cells treated with either control medium, 1 μM Hexa-PAF, 5 μM squalene or a combination of 1 μM Hexa-PAF and 5 μM squalene as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of $A\beta_{42}$ oligomers/monomers not significantly different from those in CM from H-PAF-treated cells.

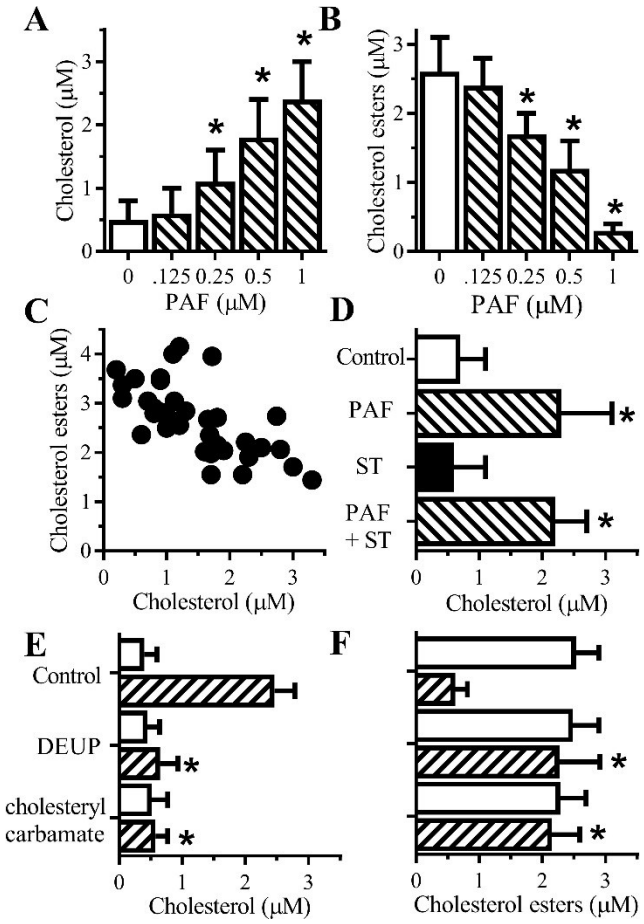

Figure 7. PAF increased cholesterol concentrations in ER membranes – The concentrations of cholesterol (A) and cholesterol esters (B) in ER membranes treated with control medium or PAF as shown. Values are means ± SD from triplicate experiments performed twice, n=6.*=concentrations of cholesterol/cholesterol esters significantly different to controls. (C) There was a significant inverse correlation between the concentrations of cholesterol and cholesterol esters in ER membranes incubated with PAF (0.125 to 1 μM), Pearson's coefficient = -0.78, p <0.01. (D) The concentrations of cholesterol in ER membranes incubated with either control medium, 1 μM PAF, 500 nM squalestatin (ST) or pre-treated with 500 nM ST and incubated with 1 μM PAF as shown. Values are mean cholesterol concentrations ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of cholesterol significantly higher than those in control ER membranes. The concentrations of cholesterol (E) and cholesterol esters (F) in ER membranes pre-treated with control medium, 20 μM DEUP or 5 μM cholesteryl carbamate as shown and then incubated with control medium (□) or 1 μM PAF (striped bars). Values are means ± SD from triplicate experiments performed 3 times, n=9. *=concentrations of cholesterol/cholesterol esters significantly different to those in ER membranes incubated with PAF alone.

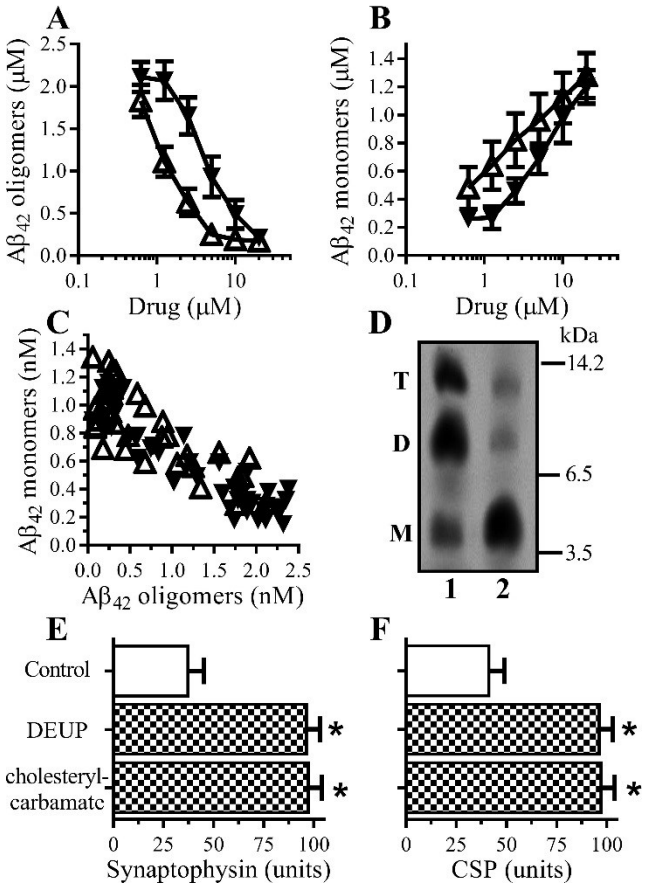

Figure 8. CEH inhibitors reduced the release of synaptotoxic Aβ– The concentrations of $A\beta_{42}$ oligomers (A) and $A\beta_{42}$ monomers (B) in CM from 7PA2 cells treated with either DEUP (△) or cholesteryl N-(2-dimethylaminoethyl) carbamate (▼) as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. (C) There was a significant inverse correlation between the concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells incubated with DEUP (△), Pearson's coefficient = -0.92, $p<0.01$ or cholesteryl N-(2-dimethylaminoethyl) carbamate (▼), Pearson's coefficient = -0.71, $p<0.01$. (D) Immunoblot showing forms of Aβ in CM from (1) control 7PA2 cells and (2) DEUP-treated 7PA2-cells.The amounts of synaptophysin (E) and CSP (F) in neurons incubated with CM from 7PA2 cells treated with control medium or with CEH inhibitors (5 μM DEUP or 20 μM cholesteryl N-(2-dimethylaminoethyl) carbamate as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=amounts of synaptophysin/CSP significantly higher than in neurons incubated with control 7PA2-CM.

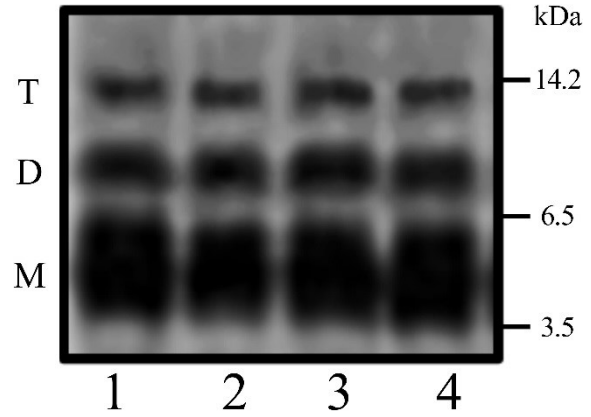

Figure 9 – Drugs do not cause the dissociation of Aβ oligomers - Immunoblot showing forms of Aβ in CM from 7PA2 cells incubated for 3 days at 37ºC with (1) control medium, (2) 500 nM squalestatin, (3) 1 μM Hexa-PAF or (4) 20 μM DEUP.

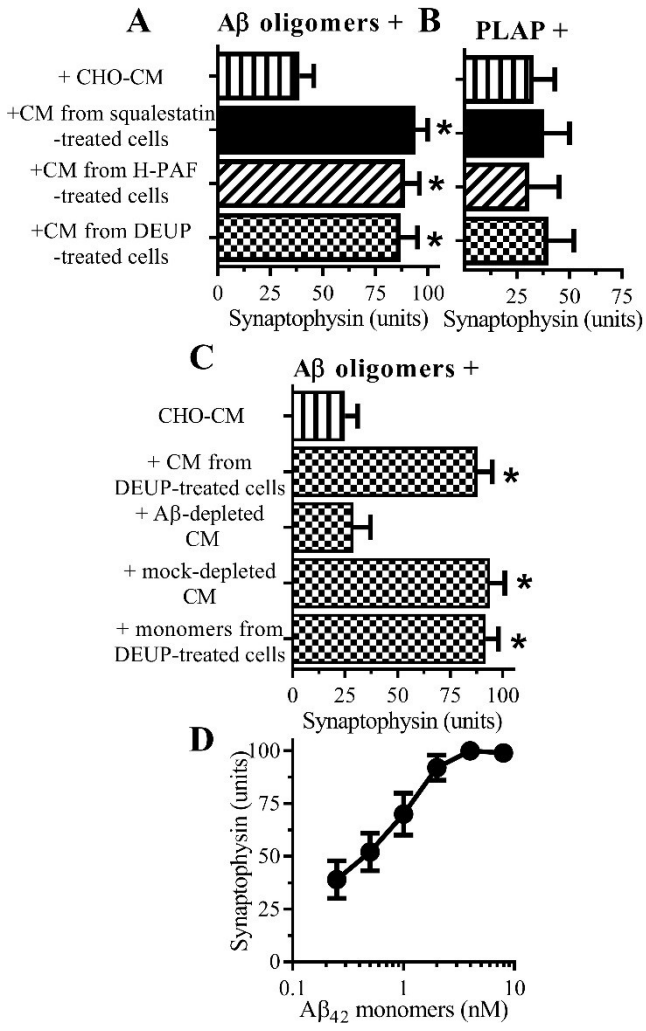

Figure 10. CM from DEUP-treated 7PA2 cells protected neurons against Aβ-induced synapse damage – The amounts of synaptophysin in neurons incubated with 2 nM $A\beta_{42}$ (A) or 250 nM PLAP (B) mixed with CM from CHO cells or CM from 7PA2 cells treated with 1 μM squalestatin, 1 μM Hexa-PAF or 20 μM DEUP as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=amounts of synaptophysin significantly greater than in neurons incubated with Aβ oligomers and CHO-CM. (C) The amounts of synaptophysin in neurons incubated with 2 nM $A\beta_{42}$ mixed with CM from CHO cells, CM from DEUP-treated 7PA2 cells, or CM from DEUP-treated 7PA2 cells depleted of Aβ or mock-depleted or monomer preparations derived from CM from DEUP-treated 7PA2 cells as shown. Values are means ± SD from triplicate experiments performed 3 times (n=9). *=amounts of synaptophysin significantly greater than in neurons incubated with Aβ oligomers and CHO-CM. (D) The amounts of synaptophysin in neurons pre-treated with Aβ monomers derived from DEUP-treated 7PA2 cells (containing $A\beta_{42}$ monomers as shown) and incubated with soluble brain extract containing 2 nM $A\beta_{42}$. Values are means ± SD from triplicate experiments performed 3 times, n=9.