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Squalestatin protects neurons and reduces the activation of cytoplasmic phospholipase A_2 by $A\beta_{1-42}$

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Abstract

Alzheimer's disease is characterised by the loss of neurons and the production of Aβ peptides. We report that the addition of $A\beta_{1-42}$ to neurons resulted in activation of cytoplasmic phospholipase A_2 ($cPLA_2$), the production of prostaglandin E_2, synapse damage and reduced neuronal survival. Pre-treatment with simvastatin, a clinically relevant statin that penetrates the brain, protected against $A\beta_{1-42}$ induced synapse damage and neuronal death in vitro. The neuroprotective effects of simvastatin were shared by squalestatin, a squalene synthase inhibitor that reduces neuronal cholesterol production and crucially, does not affect isoprenoid formation. The protective effect of both these drugs was reversed by the addition of exogenous cholesterol. These drugs did not alter the amounts of extracellular $A\beta_{1-42}$ ingested by neurons; rather they reduced $A\beta_{1-42}$ induced activation of $cPLA_2$ and prostaglandin E_2 production. Treatment prevented the migration of $A\beta_{1-42}$ and $cPLA_2$ to caveolin-1 containing lipid rafts. We propose that critical concentrations of $A\beta_{1-42}$ trigger the amalgamation of individual micro-domains containing signalling molecules to form lipid raft platforms in which sustained activation of $cPLA_2$ leads to neuronal dysfunction and ultimately neuronal death. This process is dependent on the amounts of cholesterol in neuronal membranes and is susceptible to treatment with squalestatin or simvastatin.

Keywords: Alzheimer's disease; Cholesterol; Phospholipase A_2; Prostaglandins; Caveolae

1. Introduction

Alzheimer's disease (AD) is a complex and genetically heterogeneous disease that is the most common form of dementia, affecting up to 15 million individuals worldwide. This disorder is characterized by the progressive cognitive decline as a consequence of neuronal dysfunction and ultimately neuronal death (Braak and Braak, 1997). The amyloid hypothesis of AD pathogenesis maintains that the primary event is the production of amyloid-β (Aβ) peptides following the abnormal proetolytic cleavage of the amyloid precursor protein (Vassar and Citron, 2000). The accumulation of Aβ peptides leads to the subsequent disruption of neuronal processes, abnormal phosphorylation of tau and ultimately the dysfunction and death of neurons. Initially it was thought that the formation of fibrils consisting of Aβ peptides were effectors of neurodegeneration (Lorenzo and Yankner, 1994). More recently, the importance of smaller soluble oligomers of Aβ or Aβ-derived diffusible ligands in neurotoxicity has been recognised (Lambert et al., 1998).

Biochemical, pharmacological and genetic observations show that cholesterol levels may affect the course of AD (Puglielli et al., 2003). The role of cholesterol in AD was further strengthened by two retrospective reports that showed a strong decrease in the incidence of AD and dementia for patients that were treated with statins (Jick et al., 2000; Wolozin et al., 2000), drugs that are used to treat hypercholesterolemia or ischemic heart disease (Chilton and O'Rourke, 2001). Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyses the production of mevalonate, the rate-limiting step in the synthesis of cholesterol (Alberts et al., 1980). IMR32 neuroblastoma cells treated with the HMG-CoA reductase inhibitor mevastatin, and vascular

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smooth muscle cells with lovastatin, were resistant to the toxicity of $A\beta_{1-40}$ (Paris et al., 2002). However, the HMG-CoA reductase inhibitors are poor research tools, since they block an early step in the metabolic pathway leading to cholesterol production these drugs also affect the production of other molecules including the isoprenoids (Maltese and Sheridan, 1987). Prenylation is a class of lipid modification that is essential for the function of a wide variety of proteins including the Ras-related G proteins (Zhang and Casey, 1996). Recent studies suggest that some of the effects of HMG-CoA reductase inhibitors are independent of their ability to affect cholesterol metabolism (Kalinowski et al., 2002). In addition, BM15.766, an inhibitor of the dehydrocholesterol reductase enzyme that catalyses the last step in cholesterol biosynthesis reduces β-amyloid levels in a transgenic mouse model of Alzheimer's disease (Refolo et al., 2001). Since the neuroprotective effects of HMG-CoA reductase inhibitors have not been proven to be due to cholesterol depletion, we investigated the effects of two cholesterol synthesis inhibitors on neurons. The effects of simvastatin, a conventional HMG-CoA reductase inhibitor that also penetrates the brain (Hess and Fagan, 2001) and squalestatin, a specific inhibitor of squalene synthase that inhibits cholesterol production without affecting the production of non-sterol products (Baxter et al., 1992), on neurons were examined.

The precise mechanisms by which Aβ peptides lead to neuronal damage remain to be fully determined. Some of the events that lead to neuronal dysfunction and death in AD can be examined in vitro by incubating neurons with peptides derived from the Aβ protein (Yankner et al., 1989). The earliest clinical phase of AD is characterised by memory impairment which is thought to be due to synaptic failure (Selkoe, 2002). Synapse damage occurs during the early stages of AD (Heinonen et al., 1995) where the loss of synapses and the reduction in synaptophysin levels are features of AD that strongly correlate with cognitive decline (DeKosky and Scheff, 1990). In the present study we examined mechanisms by which $A\beta_{1-42}$ caused synapse damage and neuronal death. Firstly, the effect of $A\beta_{1-42}$ on the amounts of synaptophysin (a presynaptic membrane protein essential for the generation of synaptic vesicles and neurotransmission (Elferink and Scheller, 1993)) in neuronal cultures was measured as a surrogate marker of synapse integrity. In addition we examined the effects of higher concentrations of $A\beta_{1-42}$ on neuronal survival and on activation of the phospholipase A_2 (PLA_2) pathway. PLA_2 immunoreactivity is raised in the AD brain (Stephenson et al., 1996), and Aβ peptides stimulate PLA_2 (Lehtonen et al., 1996). PLA_2 inhibitors prevented $A\beta_{1-42}$-induced neurotoxicity (Bate et al., 2004b). Since antisense oligonucleotide studies showed that cytoplasmic PLA_2 ($cPLA_2$) was responsible for neuronal death induced by oligomers of Aβ (Kriem et al., 2004; Malaplate-Armand et al., 2006) we measured amounts of activated $cPLA_2$ in neurons incubated with $A\beta_{1-42}$, Finally we measured the production of prostaglandin E_2 (PGE_2) in $A\beta_{1-42}$ treated neurons as PGE_2 is a marker of neuronal death in vitro (Bate et al., 2003) and PGE_2 levels are raised in the CSF of patients with probable AD (Montine et al., 1999).

2. Methods

2.1. Cell lines

The human neuroblastoma SH-SY5Y and the murine neuroblastoma (NB4-1A3) cell lines (European Collection of Cell Cultures) were grown in Hams F12 media supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% foetal calf serum (FCS). For survival assays neurons were plated into 48 well plates at 5×10^4 cells/well, and allowed to adhere overnight before use. Cells were treated for 24 h before the addition of Aβ peptides, and cell survival was determined 48 h later.

2.2. Primary neuronal cultures

Primary cortical or cerebellar neurons were prepared from the brains of mouse embryos (day 15.5). The cortex or cerebellum was dissected from embryonic mice and a single cell separation was produced following mechanical dissociation (repeated passage through a 1 ml pipette tip), cell aggregates were removed after passage through a 100 μM cell strainer (Becton Dickinson) cells were isolated by flotation on histopaque ($500 \times g$ for 10 min) (Sigma, Poole, UK). Neuronal precursors were washed twice with phosphate buffered saline (PBS) and plated at 2×10^5 cells/well in 48 well plates in Hams F12 containing 5% FCS for 2 h. Cultures were shaken (600 rpm for 5 min) and non-adherent cells removed by 2 washes in PBS. Neurons were subsequently grown in neurobasal medium (NBM) containing B27 components (Invitrogen, Paisley, UK) for 7 days before use. Neurons were pre-treated with drugs for 24 h, cells were then washed twice in PBS and Aβ peptides in fresh medium were added. For some experiments drugs and peptides were given simultaneously or drugs were added 30 min after the addition of peptides. The amounts of synaptophysin in treated cultures were measured 24 h after the peptides were added and cell survival was determined after 4 days.

2.3. Cell extracts and isolation of detergent-resistant membranes

Treated/untreated neurons were washed twice with PBS before cell extracts were obtained. For whole cell extracts neurons were solubilised in a buffer containing 10 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulphate (SDS) and mixed protease inhibitors (AEBSF, Aprotinin, Leupeptin, Bestain, Pepstatin A and E-46) (Sigma) at 1×10^6 cells/ml. Membranes were prepared by repeated passage with a Wheaton homogeniser followed by mechanical agitation on a cell disruptor (10 min full power); nuclei and large fragments were removed by centrifugation ($300 \times g$ for 5 min). For some experiments cell extracts were mixed 1:1 with Laemmli buffer (Bio-Rad) and boiled for 5 min. A total of 20 μl of each sample was subjected to electrophoresis on a 15% polyacrylamide gel and proteins were transferred onto a Hybond-P PVDF membrane (Amersham Biotech, UK) by semi-dry blotting. Membranes were blocked using 10% milk powder in PBS containing 0.2% Tween 20. Synaptophysin was detected by incubation with mouse monoclonal antibody (mab) anti-synaptophysin MAB368 (Chemicon, UK) and β-actin was detected with a mouse mab (Sigma). Bound antibodies were detected with a secondary anti-mouse IgG conjugated to biotin followed by extravidin alkaline phosphatise (Sigma). Detection of bound antibody was by 5-bromo-4-chloro-3-inodyl-phosphate and nitroblue tetrazolium substrate (Pierce, Cramlington, UK). To differentiate between the normal bulk membrane and the detergent-resistant lipid raft micro-domains, neurons were solubilised in a buffer containing 1% Triton × 100, 10 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA and mixed protease inhibitors at 1×10^6 cells/ml at 4 °C. Membranes were prepared by repeated passage with a Wheaton homogeniser followed by mechanical agitation on a cell disruptor (10 min full power); nuclei and large fragments were removed by centrifugation ($300 \times g$ for 5 min). The subsequent post nuclear supernatant was incubated on ice (4 °C) for 1 h and centrifuged ($16{,}000 \times g$ for 30 min at 4 °C). The supernatant was reserved as the normal bulk membrane while the insoluble pellet was homogenised in an extraction buffer containing 10 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS and mixed protease inhibitors,