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Cholesterol esterification reduces the neurotoxicity of prions

Clive Bate*, Mourad Tayebi, Alun Williams

Department of Pathology and Infectious Diseases, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire AL9 7TA, UK

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ABSTRACT

The transmissible spongiform encephalopathies develop following the conversion of a host-encoded protein (PrP^C) into abnormally folded, disease-related isoforms (PrP^{Sc}). Here we report that three acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitors, TMP-153, FR179254 or YIC-C8-434, were more toxic to prion-infected neuronal cell lines (ScGT1 and ScN2a cells) than to their uninfected equivalents (GT1 and N2a cells). The toxicity of ACAT inhibitors for ScGT1 cells was not reversed by the addition of cholesterol esters, rather it was increased by the addition of free cholesterol indicating that the toxicity of ACAT inhibitors was related to the increased free cholesterol content of cells rather than reduced amounts of cholesterol esters. This hypothesis was strengthened by the observation that the addition of free cholesterol killed ScGT1, but not GT1 cells. Treatment with ACAT inhibitors increased caspase-3 activity and prostaglandin E_2 production in ScGT1 cells but not in GT1 cells. The addition of the phospholipase A_2 (PLA_2) inhibitors ($AACOCF_3$ or MAFP) reduced prostaglandin E_2 production and protected ScGT1 cells against the toxicity of ACAT inhibitors. These results indicate that cholesterol esterification is an important cellular response that reduces PrP^{Sc}-induced activation of PLA_2 and protects against cell death in ScGT1 cells.

1. Introduction

The clinical symptoms of the transmissible spongiform encephalopathies (TSEs), otherwise known as prion diseases, arise following the dysfunction and degeneration of neurons. The presence of a host-encoded protein, the cellular prion protein (PrP^C), is essential for the development of prion diseases (Brandner et al., 1996; Bueler et al., 1993; Mallucci et al., 2003). PrP^C is a glycoprotein containing two N-linked glycosylation sites and is attached to cell membranes via a glycosylphosphatidylinositol (GPI) anchor (Stahl et al., 1987). It is thought that prion diseases occur when PrP^C is converted into abnormally folded, disease-related isoforms (PrP^{Sc}), which constitute the main, and possibly the only component of the infectious material (Prusiner, 1998). The conversion of PrP^C to PrP^{Sc} involves a change in secondary and tertiary protein structure; a portion of the α-helix and random coil structure in PrP^C is refolded into a β-pleated sheet (Pan et al., 1993). PrP^{Sc} has increased resistance to proteases and a propensity to self-aggregate into oligomers ranging from dimers to large fibrils (Silveira et al., 2005).

The symptoms of prion infections include the loss of synapses, activation of glial cells and ultimately neuronal apoptosis (Jeffrey et al., 2000; Williams et al., 1997a). However, the mechanisms by which prion infection causes neuronal degeneration remain controversial and poorly understood. Several models of prion-mediated neurodegeneration have been proposed including the production of misfolded, cytoplasmic PrP (Ma et al., 2002) and the identification of disease-specific neurotoxic PrP-derived peptides (Salmona et al., 2003). Since cultured neurons can be killed by preparations of PrP^{Sc} (Bate et al., 2004a; Muller et al., 1993) and prion-infected ScGT1 neuronal cells show signs of apoptosis (Schatzl et al., 1997), we used prion-infected cells to examine the mechanisms of cell death in response to the accumulation of PrP^{Sc}.

There is increasing evidence that cholesterol levels affect the progression of neurodegenerative diseases including Alzheimer's disease (Puglielli et al., 2003; Simons et al., 2001), multiple sclerosis (Neuhaus et al., 2005) and prion diseases (Fai Mok et al., 2006; Kempster et al., 2007). The amounts of free cholesterol within cell membranes is normally tightly controlled via a mixture of biosynthesis, uptake and efflux to and from the extracellular medium and by esterification of free cholesterol in the endoplasmic reticulum (ER) (Simons and Ikonen, 2000). Free cholesterol entering the ER can be esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT) and stored in cytoplasmic droplets. ACAT keeps the levels of free cholesterol within the membrane under tight control (Chang et al., 1997) and the use of ACAT inhibitors has been proposed as treatment for Alzheimer's disease (Hutter-Paier et al., 2004; Puglielli et al., 2001). The main goal of the present study was

* Corresponding author. Tel.: +44 707 666 550.
E-mail address: cbate@rvc.ac.uk (C. Bate).

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to determine the role of cholesterol metabolism in the death of prion-infected cells. We report that compounds which inhibited ACAT were significantly more toxic to prion-infected neuronal cell lines than to their uninfected cell line equivalent. These drugs reduced amounts of esterified cholesterol in cells but also increased the amounts of free cholesterol within cell membranes suggesting that esterification of free cholesterol is an important protective mechanism in ScGT1 cells. Treatment with ACAT inhibitors altered cell signalling in ScGT1 cells, it increased activation of phospholipase A_2 (PLA_2) and prostaglandin E_2 (PGE_2) production, consistent with reports that inhibition of the PLA_2 protects neurons against PrP^{Sc} (Bate et al., 2004b).

2. Methods

2.1. Cell lines

The prion-infected neuronal cell lines (ScGT1 and ScN2a) and their non-infected controls (GT1 and N2a) were grown in Hams F12 medium supplemented with 2 mM glutamine, standard antibiotics (100 U/ml Penicillin, 100 μg/ml Streptomycin) and 5% foetal calf serum. Cells were seeded (2×10^5cells/well) in 48-well plates and allowed to adhere overnight. The following day, varying concentrations and/or combinations of drugs were added; after a further 72 h cell viability was measured by the addition of 25 μM thiazolyl blue tetrazolium for 3 h. The dye produced was solubilised in propan-2-ol and read in a spectrophotometer at 595 nm. Neuronal survival was reported as a percentage of control cultures (untreated neurons). In other cultures cell extracts were made after 72 h treatment.

2.2. Cell extracts

At the end of the treatment, cells were washed twice in phosphate buffered saline (PBS) and homogenised in an extraction buffer containing 10 mM Tris–HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS at 10^6 cells/ml. Mixed protease inhibitors (AEBSF, Aprotinin, Leupeptin, Bestain, Pepstatin A and E-46; Sigma, Dorset, UK) were added to some cell extracts. Membranes were prepared by repeated passage with a Wheaton homogeniser; nuclei and large fragments were removed by centrifugation ($300 \times g$ for 5 min).

2.3. Cholesterol and protein content

Cellular cholesterol and protein content were determined in cell extracts. Protein concentrations were measured using a micro-BCA protein assay kit (Pierce, Cramlington, UK). The amounts of free cholesterol were measured using the Amplex Red cholesterol assay kit (Invitrogen), according to the manufacturer's instructions. Briefly, free 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 550 nm and emission detection at 590 nm. The "total cholesterol" content of samples was determined by measuring the cholesterol concentration following digestion with cholesterol esterase (50 units/ml for 1 h at 37 °C). The amounts of cholesterol esters were calculated by subtracting the amounts of free cholesterol in each sample from those of the total cholesterol.

2.4. Caspase-3 activity

Caspase-3 activity was measured in cell extracts using a fluorometric immunosorbent enzyme assay (FIENA) kit as per the manufacturer's instructions (Roche Diagnostics, Lewes, UK).

2.5. PGE_2 assay

The amounts of PGE_2 produced by cells were determined by using an enzyme-immunoassay kit (Amersham Biotech, Amersham, UK) according to the manufacturer's instructions. This assay is based on competition between unlabeled PGE_2 in the sample and a fixed amount of labelled PGE_2 for a PGE_2 specific antibody. The detection limit of this assay is 20 pg/ml.

2.6. Drugs

FR179254 and lovastatin were obtained from Calbiochem (Nottingham UK). $AACOCF_3$, methyl arachidonyl fluorophosphonate (MAFP), YIC-C8-434, cholesterol, cholesterol myristate and cholesterol arachidonate were obtained from Sigma. TMP-153 was obtained from Biomol, Exeter, UK. Squalestatin was a gift from GlaxoSmithKline, Stevenage, UK.

2.7. Statistical analysis

Comparison of treatment effects was carried out using one and two way analysis of variance techniques as appropriate.

3. Results

3.1. Prion infection reduced the survival of neuronal cells exposed to ACAT inhibitors

The current study examined whether prion infection affected the sensitivity of neuronal cell lines to drugs that affect cholesterol metabolism. There were no significant differences in the sensitivity of GT1 and ScGT1 cells in response to the cholesterol synthesis inhibitors, lovastatin or squalestatin. In contrast, the survival of ScGT1 cells incubated with the ACAT inhibitor TMP-153 for 72 h was significantly less than that of GT1 cells (Fig. 1). The effects of TMP-153 were time-dependent; there were no significant differences in the survival of GT1 and ScGT1 cells incubated with TMP-153 after only 24 h (data not shown). Similar results were obtained following titrations of two other ACAT inhibitors, FR179254 and YIC-C8-434, both of which were significantly more toxic for ScGT1 cells than for GT1 cells after 72 h. The survival of ScGT1 or GT1 cells was not affected by the addition of 100 μM diethylumbelliferyl phosphate (DEUP), which inhibits the hydrolysis of cholesterol esters. To confirm that the effects of ACAT inhibitors were not cell-type specific they were titrated on the ScN2a prion-infected cell line. All three ACAT inhibitors tested were more toxic for ScN2a than for N2a cells (Table 1).

To confirm the effects of these drugs on cholesterol metabolism the amounts of total, free and esterified cholesterol were measured in ScGT1 and GT1 cells. Untreated ScGT1 cells contained significantly more free cholesterol than GT1 cells (517 ng cholesterol/ml ± 54 vs 440 ± 52, $n = 8$, $P < 0.05$). In GT1 cells, treatment with 20 nM TMP-153, FR179254 or YIC-C8-434 for 72 h did not significantly affect the amounts of total, free or esterified cholesterol (Table 2). In contrast, treatment of ScGT1 cells with TMP-153 for 72 h

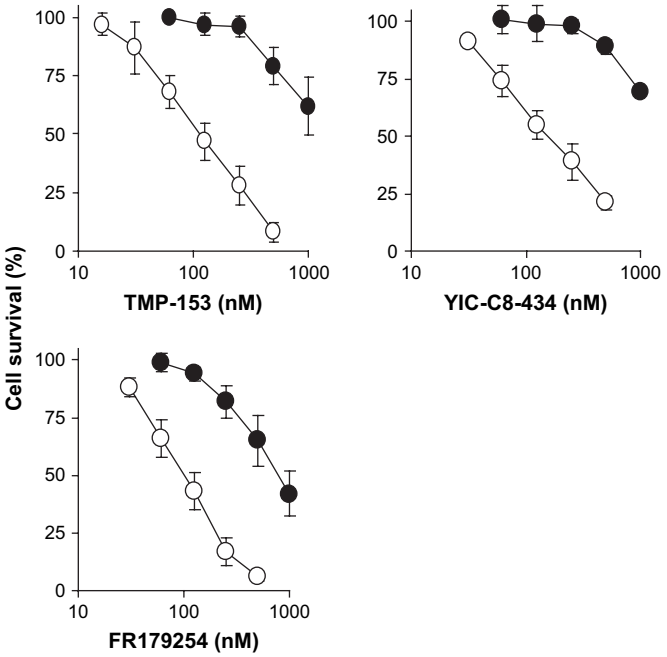

Fig. 1. Prion infection increased the toxicity of the ACAT inhibitors for ScGT1 cells: the survival of GT1 cells (●) or ScGT1 cell (○) incubated with varying concentrations of the ACAT inhibitors TMP-153, YIC-C8-434 or FR179254 for 72 h. Values shown are the mean average cell survival ± SD, $n = 12$.