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Knockdown of ACAT-1 reduces amyloidogenic processing of APP

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Abstract Previous studies have shown that acyl-coenzyme A:cholesterol acyl transferase (ACAT), an enzyme that controls cellular equilibrium between free cholesterol and cholesteryl esters, modulates proteolytic processing of APP in cell-based and animal models of Alzheimer's disease. Here we report that ACAT-1 RNAi reduced cellular ACAT-1 protein by ~50% and cholesteryl ester levels by 22% while causing a slight increase in the free cholesterol content of ER membranes. This correlated with reduced proteolytic processing of APP and 40% decrease in Aβ secretion. These data show that even a modest decrease in ACAT activity can have robust suppressive effects on Aβ generation.

Keywords: RNAi; Cholesterol; Cholesteryl esters; Amyloid-β; Alzheimer's disease; Amyloid precursor protein; ACAT

1. Introduction

Progressive accumulation of amyloid β-peptide (Aβ) in senile plaques in brain regions responsible for memory and cognitive functions is a major pathological hallmark of Alzheimer's disease [1]. Aβ is a 39–43-amino acid peptide generated from β-amyloid precursor protein (APP) by sequential proteolytic cleavages mediated by β- and γ-secretases. Alternatively, APP can be processed by the non-amyloidogenic α-secretase pathway, which cuts APP in the middle of the Aβ region [1]. Genetic, epidemiological and biochemical studies have suggested that cholesterol is an important risk factor for AD [2,3]. Statins, a highly successful class of drugs that inhibit HMG CoA reductase, have been shown to attenuate Aβ production in cell-based and animal models of AD and in humans [4]. Although the beneficial effects of statins for AD may be at least partially due to their pleiotropic actions, other cholesterol-modifying strategies for suppression of Aβ production in Alzheimer's disease have recently gained considerable interest.

ACAT is an endoplasmic reticulum (ER)-resident enzyme responsible for conversion of excess free cholesterol to cholesteryl esters [5–7]. Of the two human ACAT isoforms (two different genes in most mammals), ACAT-1 is ubiquitously expressed whereas ACAT-2 expression is restricted to the liver and the intestine [6]. Inhibition of ACAT function in cells by pharmacological means has been shown to efficiently suppress Aβ generation in vitro [8]. Importantly, a two-month treatment with ACAT inhibitor CP-113,818 remarkably reduced amyloid pathology and correlated with improved spatial learning in transgenic mice expressing human APP_{751} containing the London (V717I) and Swedish (K670M/N671L) mutations [9]. To provide an important cell biological proof of principle for ACAT-1 as a therapeutic target and as a modulator of APP metabolism, we set up and characterized a cell-based RNAi model for ACAT-1. Here, we show that reduction of cellular ACAT-1 protein level to half by a single transfection of ACAT-1 siRNA oligonucleotides reduces cellular cholesteryl ester levels while significantly suppressing amyloidogenic processing of APP and Aβ production.

2. Materials and methods

2.1. Cell culture and RNA interference

Human H4 neuroglioma cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS (Atlanta Biologicals), 1% (v/v) L-glutamine–penicillin–streptomycin solution (Sigma) at 37 °C in a water–saturated air/5% CO_2 atmosphere. H4 cells were transfected with pcDNA3.1-APP_{751}, selected and maintained with G418 sulphate (Calbiochem). A clone ($H4_{APP751}$) with ~5-fold overexpression of APP was used in this study. For silencing ACAT-1 expression, cells were transfected with 1 or 3 μg of a manufacturer-optimized mixture of human-specific ACAT-1 siRNAs (Santa Cruz Biotechnology) using Nucleofector™ technology according to the manufacturer's instructions (Amaxa). Control cells were transfected with 3 μg of a mixture of mouse-specific ACAT-1 siRNAs (Santa Cruz Biotechnology). Culture media was changed once at 72 h post-transfection, and 24-h conditioned media was collected when the cells were harvested at 96 h post-transfection.

2.2. Protein extraction and Western blotting

Cells were washed twice, scraped in ice-cold PBS and extracted on ice for 30 min in a buffer containing 10 mM Tris–HCl, pH 6.8, 1 mM EDTA, 150 mM NaCl, 0.25% Nonidet P-40, 1% Triton X-100 and a protease inhibitor mixture (Roche Molecular Biochemicals). Cell debris was removed by a spin at $16000 \times g$. The protein concentrations were determined using the BCA protein assay kit (Pierce). For Western blot analysis, 30 μg of total protein per lane was resolved in a 4–12% gradient Bis–Tris gels (Novex) under reducing conditions. The filters were probed with a C-terminal APP antibody (A8717; Sigma), ACAT-1 (Santa Cruz) and GAPDH (Chemicon) antibodies. Calreticulin (Calbiochem) and GM130 (BD Biosciences) antibodies were used to identify ER and Golgi fractions, respectively. After incubation with horseradish-conjugated secondary antibodies the signal was developed using ECL Western Blotting detection reagent (Amersham). Western blot images were quantitated using Quantity One software package (Bio-Rad).

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2.3. Lipid extraction and cholesterol assay

For the determination of cellular pools of free cholesterol (FC) and cholesteryl esters (CE), cells were extracted in chloroform/methanol/ddH_2O (4:2:1; v/v/v). Chloroform phase was separated, mixed with 1:100 volume of polyoxyethylene 9-lauryl ether ('polidocanol'; Sigma), dried and resuspended in assay reaction buffer (100 mM potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM cholic acid, 0.1% Triton X-100). Free cholesterol was measured enzymatically using Amplex Red Cholesterol Assay kit (Molecular Probes/Invitrogen). To directly measure cholesteryl esters in samples, free cholesterol was first converted to cholest-4-ene-3-one by cholesterol oxidase and the resulting hydrogen peroxide decomposed by catalase after which the enzymatic cholesterol assay was performed in the presence of cholesterol esterase [10].

2.4. Lipid droplet staining

Four days after transfection with 3.0 μg of ACAT-1 siRNA and 0.5 μg of pEGFP plasmid (Amaxa), cells were washed once with PBS and fixed with 3% paraformaldehyde in PBS for 20 min. Cells were stained with HCS LipidTOX™ red neutral lipid stain and Hoechst 33342 (Molecular Probes/Invitrogen) for 20 min according to manufacturers instructions. Images were taken with Olympus DSU/IX70 spinning disc confocal microscope. Control cells were treated for 4 days with 10 μM CP-113,818 or vehicle (DMSO).

2.5. Aβ ELISA

For Aβ determination, the conditioned media was cleared from debris and secreted $A\beta_{40}$ and $A\beta_{42}$ were quantitated by standard sandwich ELISA (Aβ ELISA Core Facility, Center for Neurological Diseases, Harvard Institutes of Medicine, Harvard Medical School).

2.6. Subcellular fractionation

ER membranes from transfected $H4_{APP751}$ cells were prepared as described previously [11].

3. Results

3.1. Knockdown of ACAT-1 reduces cholesteryl ester levels in cells

Human H4 neuroglioma cells overexpressing human APP_{751} ($H4_{APP751}$) were transfected with an increasing dose of chemically synthesized ACAT-1 siRNA oligonucleotides (specific for human ACAT-1). As a control, the cells were transfected with siRNA oligonucleotides specific for mouse ACAT-1 which had no detectable effect on endogenous ACAT-1 protein levels in $H4_{APP751}$ cells. In previous studies, we have noted that for ACAT inhibitors the maximal efficacy in reducing cholesteryl ester levels in cultured cells requires prolonged (up to 4 days) incubation times [8]. Thus, cells were harvested 96 h after transfection with ACAT-1 siRNA oligonucleotides for analysis of ACAT-1 expression and cholesterol levels. At this point, ACAT-1 protein levels were down by 42.7 ± 7.6% ($P = 0.0052$) for 1.0 μg siRNA dose and 54.4 ± 11.0% ($P = 0.0067$) for 3.0 μg of siRNA dose as compared to the control siRNA-transfected cells (Fig. 1A and B).

To determine how this reduction in ACAT-1 protein levels affect cellular cholesterol levels, both free cholesterol and cholesteryl esters were determined from chloroform:methanol-extracted total lipids by enzymatic assay. We noted a moderate, statistically insignificant rise [5.9% for 1.0 μg siRNA (from 376.4 ± 18.5 to 398.4 ± 14.2 mg/g protein) and 6.2% for 3.0 μg siRNA (from 376.4 ± 18.5 to 399.7 ± 12.2 mg/g protein)] in free cholesterol level and highly significant 14.6 ± 2.9% (from 289.8 ± 16.9 to 247.2 ± 10.8 mg/g protein; $P = 0.00013$) and 21.6 ± 4.4% (from 289.8 ± 16.9 to 227.2 ± 17.0 mg/g protein; $P = 0.00010$) decreases in cholesteryl ester

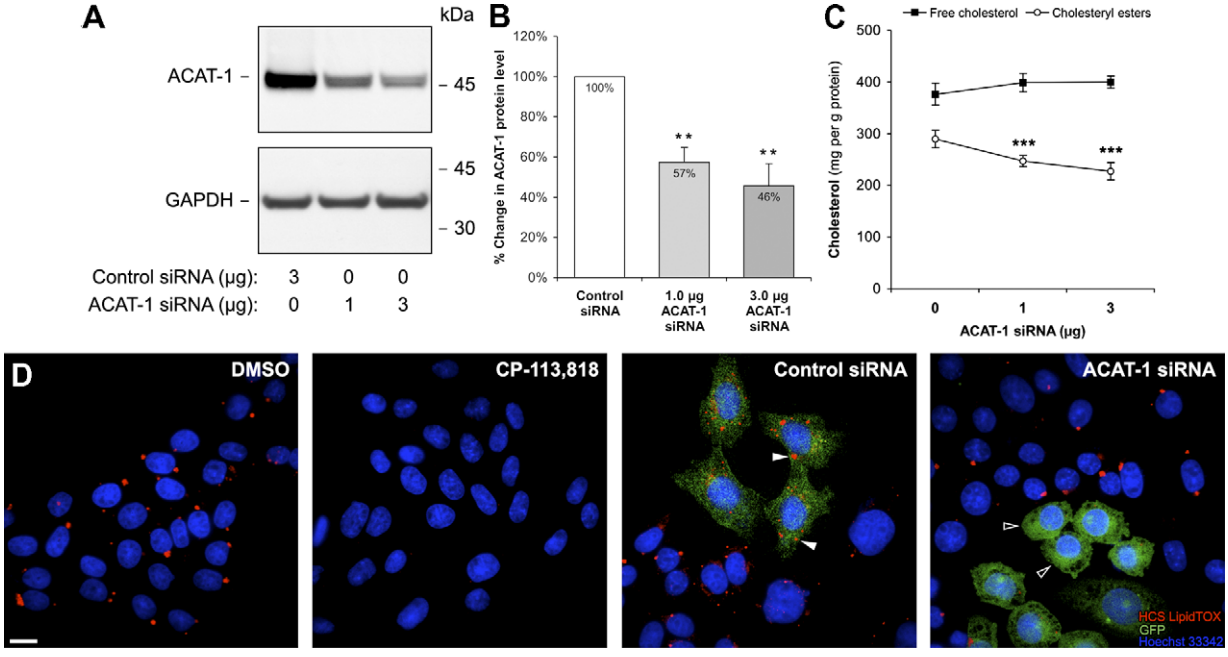

Fig. 1. Knockdown of ACAT-1 reduces cholesteryl ester levels. $H4_{APP751}$ cells transfected with either mouse ACAT-1-specific (control siRNA) or an increasing dose of human ACAT-1-specific siRNA oligonucleotides were analyzed 96 h after transfection for ACAT-1 expression and cholesterol levels. ACAT-1 expression determined by Western blot analysis (A) was quantitated and normalized to GAPDH levels (B). (C) Cellular free cholesterol and cholesteryl ester levels are shown as the means ± S.D. of three independent experiments. (D) $H4_{APP751}$ cells were treated with DMSO or 10 μM CP-113,818 or transfected with 0.5 μg GFP plasmid together with 3.0 μg of either mouse ACAT-1 (control) or human ACAT-1 siRNA oligonucleotides. After 96 h, cells were stained with HCS LipidTOX™ red neutral lipid stain (lipid droplets) and Hoechst 33342 (nuclei). $^{**}P < 0.01$, $^{***}P < 0.001$. Bar = 10 μm.