

Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid β -peptide

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The pathogenic event common to all forms of Alzheimer's disease is the abnormal accumulation of the amyloid β -peptide ($A\beta$). Here we provide strong evidence that intracellular cholesterol compartmentation modulates the generation of $A\beta$. Using genetic, biochemical and metabolic approaches, we found that cholesteryl-ester levels are directly correlated with $A\beta$ production. Acyl-coenzyme A:cholesterol acyltransferase (ACAT), the enzyme that catalyses the formation of cholesteryl esters, modulates the generation of $A\beta$ through the tight control of the equilibrium between free cholesterol and cholesteryl esters. We also show that pharmacological inhibitors of ACAT, developed for the treatment of atherosclerosis, are potent modulators of $A\beta$ generation, indicating their potential for use in the treatment of Alzheimer's disease.

Alzheimer's disease is characterized by progressive memory deficits, cognitive impairment and personality changes accompanied by diffuse structural abnormalities in the brain. The pathogenic event that occurs in all forms of Alzheimer's disease is the abnormal accumulation of amyloid β -peptide ($A\beta$). $A\beta$ is a 39–43-amino-acid hydrophobic polypeptide, proteolytically derived from a much larger precursor, the amyloid precursor protein (APP). APP is first cleaved at the amino-terminus of $A\beta$ (β -cleavage), producing a carboxy-terminal fragment (β -APP-CTF), and subsequently in the transmembrane domain (γ -cleavage). The two major sites of γ -cleavage are located at positions 40 and 42 of $A\beta$, generating $A\beta_{40}$ and $A\beta_{42}$, respectively^{1,2}. Although $A\beta_{40}$ is more prevalent (~90% of secreted $A\beta$), $A\beta_{42}$ aggregates far more rapidly into amyloid fibrils and is more toxic^{1–3}. Both genetic and biochemical studies indicate that an increase in the $A\beta_{42}/A\beta_{total}$ ratio accelerates the aggregation and accumulation of $A\beta$ into amyloid fibrils, leading to neurodegeneration and synaptic loss^{1,3}. The molecular mechanisms that regulate APP processing and $A\beta$ generation are still largely unknown.

Epidemiological studies suggest a relationship between serum cholesterol levels and Alzheimer's disease^{4,5}, apparently influenced by the apolipoprotein E- ϵ 4 haplotype⁵. Elevated concentrations of cholesterol in the plasma increase susceptibility to Alzheimer's disease, and patients with Alzheimer's disease have increased concentrations of total serum and low-density lipoprotein (LDL) cholesterol, in comparison with age-matched controls⁵. Furthermore, studies *in vitro* and *in vivo* have identified a direct association of APP and $A\beta$ with cholesterol-rich domains^{6–9} as well as a regulatory effect of cholesterol levels on APP processing and $A\beta$ generation^{10–14}. Specifically, cholesterol depletion decreased^{11,12} and cholesterol overload increased the secretion of both $A\beta_{40}$ and $A\beta_{42}$ (ref. 12). Although these studies clearly indicate a direct relationship between cellular cholesterol and $A\beta$ secretion, they do not discriminate between the roles of free cholesterol (FC) and cholesteryl esters (CEs).

Cellular cholesterol is stored either as FC in the membrane or as CEs in cytoplasmic droplets. The pools of FC and CEs are in a

dynamic equilibrium¹⁵ that is tightly controlled by acyl-coenzyme A:cholesterol acyltransferase (ACAT), an endoplasmic-reticulum (ER)-resident enzyme that catalyses the formation of CEs from cholesterol and long-chain fatty acids¹⁶. Elevated FC concentrations result in the activation of ACAT and the production of CEs. Conversely, when FC levels are lower, they are replenished by CE hydrolysis. A close relationship exists between ACAT, intracellular cholesterol trafficking and the sterol-regulatory-element binding protein (SREBP) pathway, a complex group of membrane proteins that regulate cholesterol homeostasis¹⁷. ACAT keeps the levels of FC in the ER membrane under strict control¹⁶. This pool of FC ultimately regulates the SREBP pathway and cholesterol trafficking, as well as several molecular events in which cholesterol is directly involved, including gene regulation and expression, post-translational modifications and sorting of proteins, signal transduction and membrane trafficking^{18–21}.

Here we show for the first time, through genetic, biochemical and metabolic approaches, that ACAT activity regulates the generation of $A\beta$. We also show that ACAT inhibitors are able to modulate $A\beta$ generation in neuronal and non-neuronal cells. Our results raise the possibility that ACAT might emerge as an important therapeutic target for the treatment of Alzheimer's disease.

Results

Intracellular cholesterol compartmentation regulates $A\beta$ generation.

We initially analysed the role of intracellular cholesterol compartmentation on $A\beta$ generation by using cholesterol-mutant Chinese hamster ovary (CHO) cell lines (M19, 25RA and AC29). M19 mutants are unable to produce the active form of SREBP and therefore cannot activate the expression of the genes involved in cholesterol homeostasis. In contrast, 25RA and AC29 cells produce full-length SREBP and can proteolyse it normally, but they contain a mutation in the sterol-sensing protein SCAP (SREBP cleavage-activating protein), and therefore SREBP is constitutively cleaved. Thus, these cells are unresponsive to sterol overload, overproducing and accumulating cholesterol in a sterol-independent fashion.

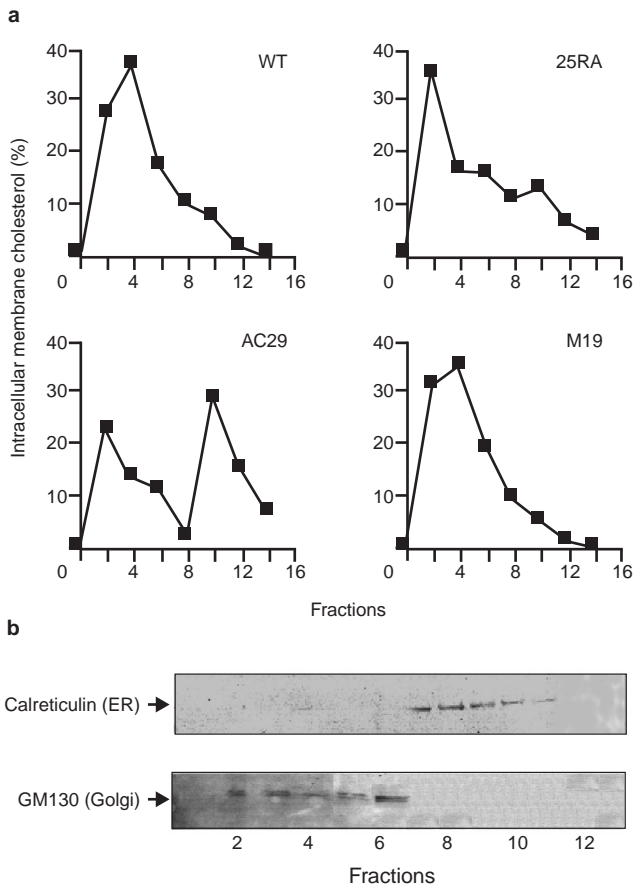


Figure 1 Lack of ACAT activity in AC29 cells elevates FC concentration in the ER membrane. Intracellular membranes from wild-type (WT) and cholesterol-mutant CHO cell lines were separated on a continuous gradient. **a**, In AC29 cells, excess FC distributes equally between Golgi and ER membranes. FC distribution in intracellular membranes is expressed as a percentage of total FC (see also Table 1). Values are means for three different gradients. **b**, Western blot of one representative gradient showing the migration of ER (calreticulin) and Golgi (GM130) markers.

AC29 mutants are derived from 25RA, but lack a functional ACAT; they therefore still accumulate excess cholesterol but are unable to store it as CEs. A complete review of genetic studies on the above mutant cells is found in refs 17 and 22.

As expected, both 25RA and AC29 mutants contained higher concentrations of total cholesterol than wild-type CHO cells (Table 1). However, in AC29 cells a lack of ACAT activity resulted in a markedly altered intracellular compartmentation of cholesterol. The inability to generate CEs in these cells caused an accumulation of cholesterol solely as FC, resulting in an ~4-fold increase in FC compared with wild-type cells. In contrast, the concentrations of FC in 25RA cells remained unchanged, whereas CE concentrations increased ~6-fold (Table 1). Finally, M19 cells showed an ~90% decrease in FC with comparable concentrations of CEs. The reason for the decrease in FC but not CEs in these cells is not clear. In wild-type cells, ~40% of total FC was found in the plasma membrane, compared with ~60% in 25RA cells and ~70% in AC29 cells (data not shown). FC distribution in intracellular membranes was very similar in wild-type, 25RA, and M19 cells, with most of the intracellular FC residing in the fractions corresponding to the Golgi apparatus (Fig. 1). These results were not unexpected, because mammalian cells normally contain much higher concentrations of FC in the Golgi apparatus than in the ER membrane²³. In contrast,

Table 1 Intracellular cholesterol distribution is altered in cholesterol mutant cell lines

Cell line	Concentration (mg per g protein)		
	Total cholesterol	CEs	FC
Wild type	458 ± 89	206 ± 32	252 ± 32
25RA	1,472 ± 102*	1,310 ± 87*	162 ± 11
AC29	956 ± 78*	7 ± 1*	949 ± 54*
M19	297 ± 59	276 ± 44	21 ± 5*

Values are means ± s.d. for four different determinations
*Significant difference from wild type at $P < 0.05$.

AC29 cells showed two major peaks, indicative of an equal distribution of FC between the Golgi apparatus and the ER. Taken together, these results demonstrate that the absence of functional ACAT in AC29 cells induces a major redistribution of intracellular cholesterol pools, resulting in a lack of CEs and a significant increase of FC in the plasma membrane, ER and Golgi apparatus.

Wild-type and cholesterol-mutant CHO cells were then stably transfected with APP₇₅₁ (751-amino-acid splice form of APP), and several clones were selected on the basis of similar concentrations of APP₇₅₁ expression. Transfection with APP₇₅₁ did not alter intracellular cholesterol distribution (data not shown). In comparison with wild-type cells, AC29 showed a marked decrease (more than 90%; $P < 0.05$ versus wild-type cells) in total Aβ secretion, and undetectable concentrations of Aβ₄₂ (Fig. 2a). Both Aβ secretion and CE generation were recovered after transient transfection with ACAT-1 (Fig. 2b). In contrast, 25RA cells showed a significant increase in the secretion of both Aβ_{total} (~36%; $P < 0.05$ versus wild-type cells) and Aβ₄₂ (~55%; $P < 0.05$ versus wild-type cells), in comparison with wild-type cells (Fig. 2a). The Aβ₄₂/Aβ_{total} ratio also increased by ~20% in 25RA cells. No difference was observed between M19 and wild-type cells, which is consistent with previous reports^{24,25}.

We next asked whether the altered Aβ secretion observed in 25RA and AC29 cells was due to altered secretase activities. We therefore analysed steady-state total and plasma membrane concentrations of APP, Aβ internalization and APP-CTFs in wild-type, 25RA and AC29 cells (Fig. 2c–f). Western blot analyses of cell lysates showed no difference in the steady-state concentrations of APP holoprotein in wild-type and cholesterol-mutant cells (Fig. 2c). Expression of cell surface APP, as determined by a radioimmunoassay, was similar in wild-type, 25RA and AC29 cells (Fig. 2d), indicating that the secretory pathway is not affected in these cell lines. This result is consistent with published reports on normal trafficking of the LDL receptor along the secretory pathway in the same cell lines²². Internalization of secreted Aβ was not altered in AC29 cells (Fig. 2e), excluding the possibility that these cells internalize and degrade Aβ more rapidly than wild-type cells. In contrast, the concentrations of both APP-CTFs, generated by the α- or β-cleavage of APP, as well as secreted APP (sAPP) were increased in 25RA cells and greatly reduced in AC29 cells (Fig. 2f). Taken together, these data clearly indicate that the altered concentrations of secreted Aβ in the cholesterol-mutant cells are a direct result of changes in the rate of Aβ generation.

Stable transfection of wild-type, 25RA, AC29 and M19 cells with APP₇₅₁ containing the EAD V717I mutation (APP_{V717I}; London mutation) yielded similar results with regard to Aβ generation (data not shown). All cell lines transfected with APP_{V717I} showed elevated Aβ₄₂/Aβ_{total} ratios (between 1.5-fold and 2-fold) compared with wild-type APP₇₅₁, the only exception being AC29 cells, in which Aβ₄₂ concentrations remained below detection (data not shown).

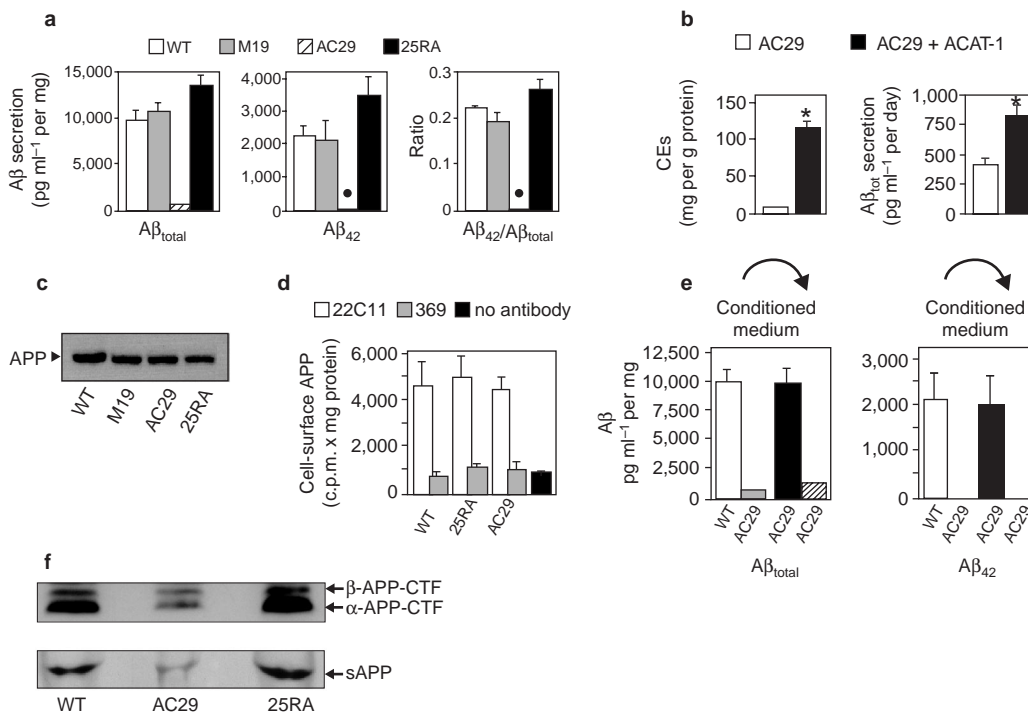


Figure 2 Cholesterol compartmentation regulates both APP processing and Aβ generation. **a**, AC29 and 25RA cells secrete decreased and elevated levels of Aβ, respectively, despite high total cholesterol in both cell lines. Values are the means and s.d. for two separate determinations from three different clones. Filled circles indicate undetectable values. **b**, ACAT activity was reconstituted in AC29 cells by transient transfection with an ACAT-1 cDNA. Aβ generation in AC29 cells was increased by partly reconstituted ACAT activity. Both CE and Aβ levels were lower than expected (we expected ~13% of the levels found in 25RA cells because the transfection efficiency was ~13%). Results are expressed as means and s.d. for at least four different experiments. Asterisk, significant difference from AC29 before ACAT-1 transfection at $P < 0.05$. **c**, Western blot showing similar steady-state levels of APP in wild-type and in cholesterol mutant cell lines stably transfect-

ed with APP₇₅₁. **d**, APP transport to the cell surface is not affected in 25RA and AC29 cells. Cell-surface APP was quantified with 22C11 monoclonal antibody (against the extracellular N-terminal domain of APP; see Methods). **e**, AC29 cells do not degrade secreted Aβ at unusually high rates. White columns, conditioned medium from wild-type (WT) cells; grey columns, conditioned medium from AC29 cells; black columns, conditioned medium from WT cells on AC29 cells for 24 h; hatched columns, conditioned medium from AC29 cells back on AC29 cells for a further 24 h. For details see Methods. **f**, Western blots showing decreased steady-state levels of APP-CTFs and sAPP in AC29 cells and increased levels of APP-CTFs and sAPP in 25RA cells. APP-CTFs were detected with the C7 antibody. sAPP was immunoprecipitated from medium with antibody 22C11 and detected with either 22C11 (shown here) or 6E10 (not shown).

Taken together, these results indicate that increased concentrations of total cholesterol (FC + CE) do not necessarily result in an elevation of Aβ production. Rather, only elevated concentrations of CEs but not of FC increased the generation of Aβ. The drastic decrease in Aβ generation observed in AC29 cells might be explained by either the complete lack of CEs or the 4-fold increase in FC in cellular membranes, perhaps in conjunction with low concentrations of CEs. Because 25RA and AC29 mutants differ only with regard to ACAT activity, these data suggest that ACAT has a role in the regulation of Aβ generation.

ACAT inhibitors downregulate Aβ generation. We next used ACAT inhibitors to test the direct involvement of ACAT in APP processing and Aβ generation. Specifically, we used two compounds: CP113,818 (a fatty-acid anilide derivative designed to mimic acyl-CoA, one of the substrates of ACAT) and Dup128 (a urea derivative). Both of these compounds are potent inhibitors of ACAT activity *in vitro*²⁶.

Wild-type cells were incubated in the presence of different concentrations of either CP113,818 or Dup128. Both compounds inhibited CE biosynthesis in a concentration-dependent manner (Fig. 3a). Complete inhibition was observed at 5 μM for CP113,818 and at 10 μM for Dup128 after 4 days of treatment. Inhibition of CE biosynthesis was accompanied by a parallel increase in the incorporation of [¹⁴C]acetic acid into newly synthesized FC. Total concentrations of cholesterol biosynthesis (FC + CE) were not

affected by either CP113,818 or Dup128, indicating that only ACAT was inhibited, not hydroxymethylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. As expected, inhibition of ACAT induced a progressive shift in cholesterol distribution from the CE pool to the FC pool (Fig. 3b). Treatment with 10 μM CP113,818 for 4 days produced the maximal increase in FC (~42%) and decrease in CEs (~45%).

Both ACAT inhibitors decreased Aβ_{total} and Aβ₄₂ secretion in a concentration-dependent manner (Fig. 3c). A 45% decrease in CEs and a parallel 42% increase in FC engendered by treatment with 10 μM CP113,818 (Fig. 3b) decreased the secretion of Aβ_{total} by ~30% ($P < 0.05$ versus untreated) and Aβ₄₂ by ~50% ($P < 0.05$ versus untreated) (Fig. 3c). The Aβ₄₂/Aβ_{total} ratio also decreased by ~25% (from 0.2 ± 0.02 (mean \pm s.d.) to 0.15 ± 0.02). This effect was accompanied by a decrease in the concentrations of both α- and β-APP-CTFs (Fig. 3d).

Treatment with either CP113,818 or Dup128 for 1 day had no effect on intracellular cholesterol compartmentation; correspondingly, Aβ secretion was also unaffected (data not shown). This result indicates that the decreased Aβ generation observed in the presence of ACAT inhibitors is dependent on changes in the distribution of FC and CEs.

We next treated 25RA cells with ACAT inhibitors. The concentration of CE was normalized after 12 days of treatment with 10 μM CP113,818 (Fig. 4a; also compare with wild-type cells in

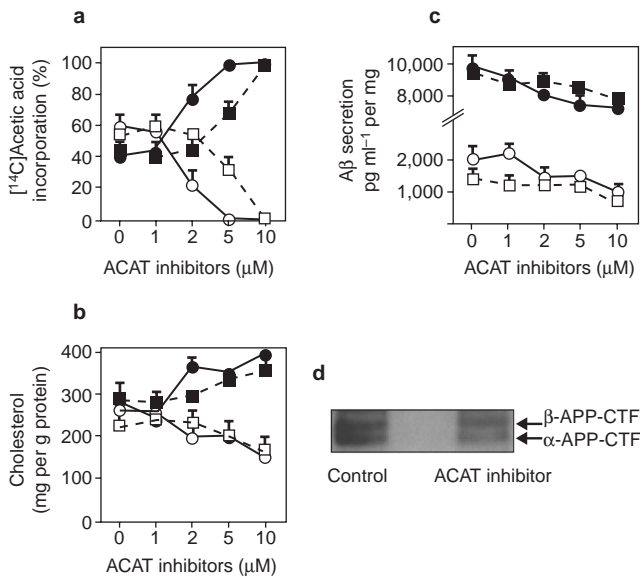


Figure 3 ACAT inhibitors alter cholesterol compartmentation and decrease Aβ generation in wild-type CHO cells. Wild-type CHO cells stably transfected with APP₇₅₁ were grown in the presence of increasing concentrations of either CP113,818 (circles) or Dup128 (squares) for 4 d. **a**, Biosynthesis of FC (filled symbols) and CEs (open symbols). Newly-synthesized FC and CEs are expressed as percentages of total [¹⁴C]cholesterol (FC + CEs). **b**, ACAT inhibitors induced a net shift in cholesterol distribution from the pool of CEs to that of FC. Preformed FC (filled symbols) and CEs (open symbols) were assayed with colorimetric assays. **c**, Aβ_{total} (filled symbols) and Aβ₄₂ (open symbols) decrease in a concentration-dependent manner in the presence of ACAT inhibitors. **d**, Western blot showing a decrease in both α- and β-APP-CTF levels after 4 d of treatment with the ACAT inhibitor CP113,818 (10 μM). APP-CTFs were detected as described for Fig. 2. Values are means and s.d. for two separate determinations from two different APP₇₅₁-transfected wild-type clones.

Table 1). Inhibition of ACAT activity was also accompanied by a net shift of cholesterol from the pool of CEs to that of FC, resulting in an ~6-fold increase in FC compared with wild-type cells (compare Fig. 4a with Table 1). Notably, Aβ secretion correlated with changes in CE concentrations, ultimately normalizing to control wild-type cells as well (compare with wild-type cells in Fig. 2a). The 6-fold increase in FC in these cells is similar to the 4-fold increase in FC in untreated AC29 cells (compare Fig. 4a with Table 1). This indicates that elevated FC alone is not the reason for the ~90% decrease in Aβ generation in AC29 cells. The decrease in Aβ secretion observed after 12 days of ACAT inhibition was accompanied by a parallel decrease in the cellular concentrations of both α- and β-APP-CTFs (Fig. 4b). Treatment with CP113,818 for 4 days decreased but did not normalize CE concentrations, whereas FC increased by ~3-fold (Fig. 4a). Despite this 3-fold increase in FC, Aβ generation remained elevated in these cells, with no significant changes between CP113,818-treated and untreated cells (Fig. 4). In a separate experiment, 25RA cells were grown in log phase, which requires high concentrations of cellular FC. Under these conditions, ACAT inhibitors were able to normalize the pool of CEs in only 3 days. Aβ_{total} and Aβ₄₂ concentrations also decreased to normal (data not shown). These results confirm that the decreased generation of Aβ in the presence of ACAT inhibitors was specific to changes in cholesterol compartmentation.

Taken together, the above results indicate that a selective increase in CE is able to upregulate the generation of Aβ, and that normalization of CE concentrations also normalizes Aβ generation. These data also suggest that changes in the pool of FC are not directly correlated with Aβ production. Aβ generation can either

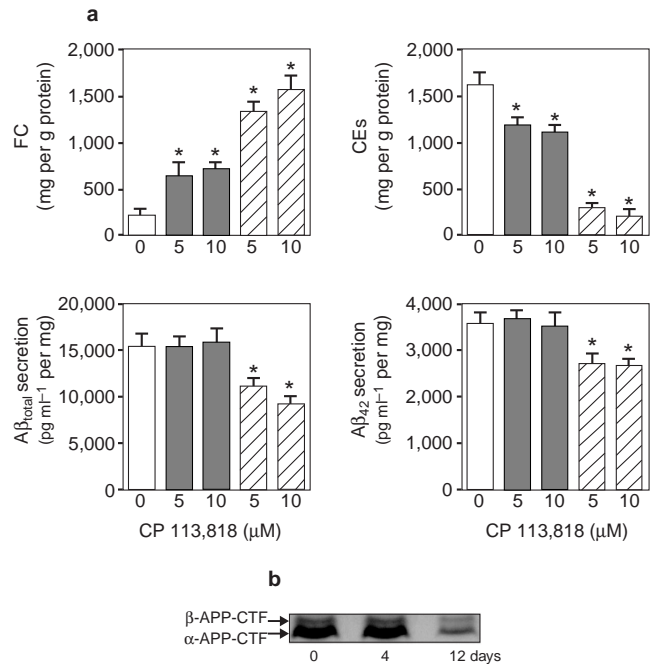


Figure 4 The ACAT inhibitor CP113,818 normalizes CE and Aβ generation in 25RA cells. 25RA mutants stably transfected with APP₇₅₁ were grown in the presence of the ACAT inhibitor CP113,818 (5 and 10 μM) for 0 d (white columns), 4 d (grey columns) and 12 d (hatched columns). **a**, CP113,818 treatment normalized both CE levels and Aβ secretion in a time-dependent manner. Asterisk, significant difference from 0 μM (no treatment) at *P* < 0.05. **b**, Western blot showing a decrease in both α- and β-APP-CTF levels after 12 d of treatment with the ACAT inhibitor CP113,818 (10 μM). No effect was observed after 4 d of treatment. APP-CTFs were detected as described for Fig. 2. Results are means and s.d.

increase or decrease in the presence of high FC concentrations, depending on the concentrations of CEs. Finally, these results indicate that ACAT activity regulates Aβ generation and that ACAT inhibitors are effective in decreasing the production of Aβ.

Sterol depletion normalizes Aβ secretion in 25RA cells. To investigate the effect of CE concentrations on Aβ generation further, we depleted cells of cholesterol. Depletion of the pools of FC and CEs can be achieved by growing cells in the presence of cholesterol-binding molecules, statins or lipoprotein-deficient serum (LDS). In contrast to cholesterol-binding molecules (such as methyl β-cyclodextrin) and statins, cholesterol depletion by LDS-containing medium is more physiological, and it is not associated with cell damage^{27–31}. We therefore grew cholesterol-mutant cell lines in medium in which 10% FBS was replaced by 10% LDS before determining cholesterol compartmentation and Aβ secretion.

Six passages in LDS-containing medium decreased the total cholesterol concentration in 25RA cells by ~67% (from 1472 ± 102 to 488 ± 34 mg per g protein). Depletion of cholesterol in 25RA cells did not alter the concentration of FC but resulted in an ~75% decrease in the pool of CEs, thus normalizing CE concentrations to those of wild-type cells (Fig. 5). Normalization of the CE concentration in 25RA cells was paralleled by normalized secretion of both Aβ_{total} (~30% decrease) and Aβ₄₂ (~37% decrease) (Fig. 5). These results indicate that normalization of the concentration of CEs, without any change in the pool of FC, is sufficient to normalize the levels of Aβ generation in 25RA mutants. Given that LDS conditions do not directly alter ACAT activity (as opposed to ACAT inhibitors), these data also suggest that Aβ generation is not altered by structural changes of ACAT, but rather by changes in cholesterol compartmentation.

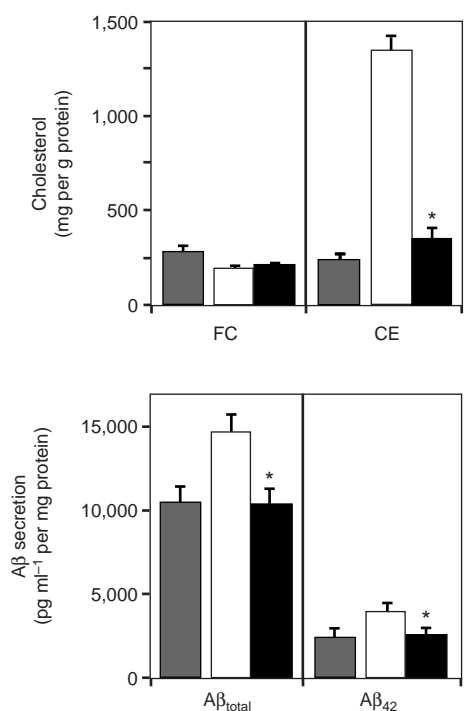


Figure 5 Sterol deprivation normalizes both CE levels and Aβ secretion in 25RA cells. 25RA cells stably transfected with APP₇₅₁ were grown in FBS- or LDS-containing medium for six passages and then analysed for cholesterol compartmentation and Aβ secretion. Grey columns, wild-type (FBS); white columns, 25RA (FBS); black columns, 25RA (LDS). In these experiments, six passages corresponded to ~5 weeks of LDS treatment. Normalization of CE levels in 25RA cells also normalized Aβ generation. FC, CEs and Aβ secretion were determined as in Fig. 2. Asterisk, significant difference from FBS at $P < 0.05$. Results are means and s.d.

We then grew AC29 cells in LDS-containing medium to exclude the possibility that abnormally high concentrations of FC in the ER membrane of these cells are responsible for the downregulation of Aβ generation. After six passages under these conditions, the FC concentration decreased by ~50% (from 949 ± 54 to 482 ± 83), still remaining higher (~2-fold) than those of wild-type cells grown in FBS-containing medium (see also Table 1). However, intracellular FC was redistributed from the ER membrane to the Golgi membrane (data not shown). Although this new FC distribution was similar to that found in the other cell lines (see Fig. 1), it was not sufficient to raise Aβ_{total} or Aβ₄₂ in AC29 cells (data not shown). Therefore, an abnormal accumulation of FC in the ER membrane of AC29 is not responsible for the decreased Aβ generation observed in these cells.

ACAT activity regulates APP processing in neuronal cell lines and primary neurons. To show that the effect of ACAT inhibition on APP processing was not limited to CHO cells, we treated neuronal cell lines and primary neurons with the ACAT inhibitor CP113,818. In H4 (human neuroglioma) cells stably transfected with APP₇₅₁, ACAT inhibition decreased the CE concentration by ~50%, inducing a shift in cholesterol distribution from CEs to FC and a decrease in the steady-state concentrations of both α- and β-APP-CTFs (Fig. 6a). Very similar results were obtained with untransfected SY5Y (human neuroblastoma) cells (Fig. 6b) and primary neurons from Tg2576 (APP_{695/swe}) transgenic mice (Fig. 6c). In contrast to H4 and SY5Y cells, primary neurons secrete quantifiable amounts of Aβ into the medium. We therefore analysed the concentration of Aβ in the conditioned medium of primary neurons after treatment with CP113,818. The decrease in α- and β-APP-CTF engendered by 4

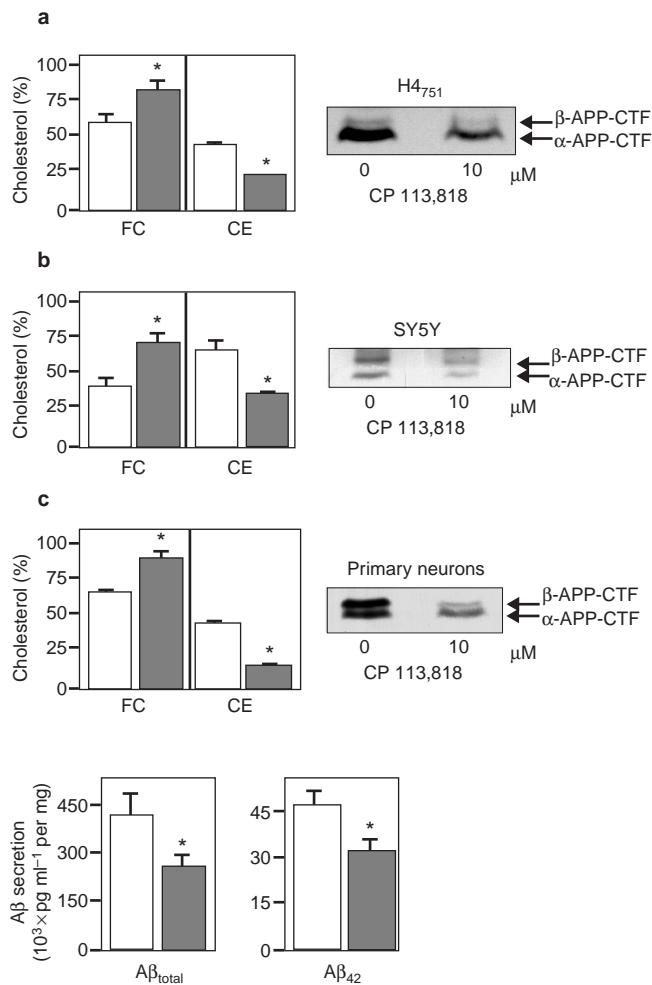


Figure 6 The ACAT inhibitor CP113,818 regulates APP processing and Aβ generation in neuronal cell lines and primary neurons. **a**, H4 (human neuroglioma) cells stably transfected with wild-type APP₇₅₁ were grown in the presence (grey columns) or absence (white columns) of 10 μM CP113,818 for 4 d. ACAT inhibition induced a net shift in cholesterol distribution from the CE to the FC pool and decreased the steady-state levels of both α- and β-APP-CTFs. **b**, Untransfected SY5Y (human neuroblastoma) cells were grown in the presence (grey columns) or absence (white columns) of 10 μM CP113,818 for 4 d. Again, ACAT inhibition resulted in decreased CE levels and increased FC levels, whereas steady-state levels of both α- and β-APP-CTFs decreased. **c**, Primary neurons from Tg2576 (APP_{695/swe}) transgenic mice were grown in the presence (grey columns) or absence (white columns) of 10 μM CP113,818 for 4 d. A net shift in cholesterol distribution from the pool of CEs to that of FC induced a decrease in the steady-state levels of α- and β-APP-CTF and in the secretion of both Aβ_{total} and Aβ₄₂. Asterisk, significant difference from 0 μM (no treatment) at $P < 0.05$. Results are means and s.d.

days of ACAT inhibition was accompanied by an ~40% decrease in the secretion of both Aβ_{total} and Aβ₄₂ (Fig. 6c). No significant change was observed in the Aβ₄₂/Aβ_{total} ratio.

The data presented so far show that decreased ACAT activity modulates both α- and β-cleavage of APP, resulting in decreased Aβ generation and lower concentrations of APP-CTFs (Figs 2–4 and Fig. 6). However, they do not indicate whether the lower rates of Aβ generation are also the result of decreased γ-cleavage of APP. To address this question, we stably transfected H4 neuroglioma cells with a deletion construct of APP harbouring the C-terminal 105 amino acids of the protein. This short protein

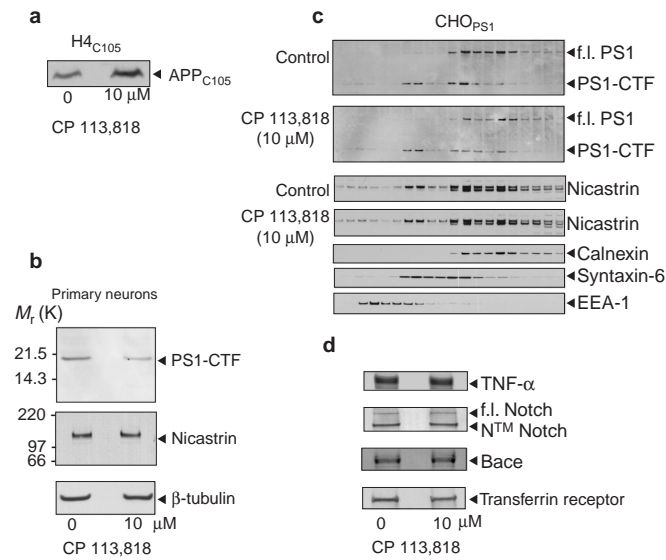


Figure 7 The ACAT inhibitor CP113,818 regulates the γ -secretase cleavage of APP and the steady-state levels of PS1 holoprotein and PS1 fragments. **a**, H4 cells were stably transfected with the C-terminal 105 amino acids of APP, which mimics β -APP-CTF. ACAT inhibition for 4 d increased the steady-state levels of APP_{C105}. **b**, Primary neurons from Tg2576 (APP_{695/swe}) transgenic mice were grown in the presence or absence of 10 μ M CP113,818 for 4 d (see also Fig. 6c). Inhibition by ACAT decreased endogenous PS1-CTF and PS1-NTF by ~50% (PS1-NTF levels are not shown). No effect was observed on the steady-state levels of nicastrin. Polyclonal antibody against nicastrin identified a single band at M_r ~110K that was not detected with the preimmune serum. **c**, Subcellular fractionation of membranes from CHO cells stably transfected with PS1 and treated with 10 μ M CP113,818 for 4 d. ACAT inhibition did not change the subcellular distribution of PS1 holoprotein, PS1 terminal fragments or nicastrin. However, CP113,818 treatment decreased the steady-state levels of both full-length (f.l.) PS1 and PS1-CTF. The migration of ER (calnexin), Golgi (syntaxin-6), and early endosome (EEA-1) markers is also shown. **d**, H4 cells were grown in the presence or absence of CP113,818 for 4 d. ACAT inhibition did not affect 'alpha-like' cleavage of TNF- α by TACE, or f.l. Notch cleavage by furin. NTM Notch indicates the mature transmembrane domain of Notch produced by furin-cleavage of f.l. Notch⁴⁹. CHO cells stably transfected with BACE and primary neurons from Tg2576 (APP_{695/swe}) transgenic mice were also grown in the presence or absence of 10 μ M CP113,818 for 4 d. Inhibition by ACAT did not affect the steady-state levels of either BACE in CHO cells, or transferrin receptor in primary neurons.

mimics the β -APP-CTF. Treatment with CP113,818 for 4 days increased the steady-state concentration of APP_{C105}, showing that γ -cleavage activity is decreased by ACAT inhibition (Fig. 7a). These results contrast with those obtained with cells expressing full-length APP (see Figs 3, 4 and 6), in which ACAT inhibition decreased the steady-state concentrations of both α - and β -APP-CTFs. Therefore, our data indicate that all three major processing events of APP (α -, β - and γ -cleavages) are affected by ACAT activity, suggesting that the activities of all three secretases (α , β and γ) and/or the availability of APP for cleavage are affected by intracellular cholesterol distribution.

To investigate the effect of ACAT inhibition on γ -secretase activity further, we analysed the steady-state concentrations and subcellular localization of both presenilin-1 (PS1) and nicastrin³². CP113,818 treatment of primary neurons from Tg2576 transgenic mice decreased the steady-state concentrations of endogenous PS1-CTF but not those of nicastrin or β -tubulin (Fig. 7b). Similar results were obtained in SY5Y cells (data not shown) and CHO cells (Fig. 7c) stably transfected with PS1. ACAT inhibition did not affect the subcellular localization of either PS1 or nicastrin (Fig. 7c).

Table 2 Brief summary of the effects of cholesterol distribution on A β production

FC	CEs	A β	Model	Cell type
=	=	=	LDS medium	25RA
	↑	↑	Untreated	25RA
↑	↓	↓	Untreated	AC29
			LDS medium	AC29
			ACAT inhibitors, 4 days	Wild type, H4, SY5Y, primary neurons
	=	=	ACAT inhibitors, 12 days	25RA
	↑	↑	ACAT inhibitors, 4 days	25RA

In the presence of normal or increased levels of FC, A β production correlated only with CE levels.

To exclude the possibility that ACAT inhibition also affects proteolytic cleavage of other membrane proteins in a nonspecific manner, we analysed the processing and/or steady-state concentrations of four different membrane proteins. Treatment with CP113,818 did not affect another ' α -secretase-like' processing pathway, namely the cleavage of tumour necrosis factor- α (TNF- α) by TNF- α -converting enzyme (TACE) in H4 cells (Fig. 7d). Similarly, furin-dependent cleavage of full-length Notch in H4 cells was also unaffected by ACAT inhibition (Fig. 7d). The steady-state concentration of β -site APP-cleaving enzyme (BACE) (β -secretase) was normal in stably transfected CHO cells treated with CP113,818 for 4 days (Fig. 7d), although the CE concentration in these cells was lower (data not shown). Finally, the steady-state concentration of the transferrin receptor in primary neurons from Tg2576 (APP_{695/swe}) transgenic mice was unaffected by CP113,818 (Fig. 7d).

Taken together, these results show clearly that ACAT-mediated regulation of APP processing is not restricted to CHO cells but is extended to both neuronal cell lines and primary neurons. Moreover, α -, β - and γ -secretase cleavages of APP are all affected by ACAT inhibition. The lack of any effect on the processing and/or steady-state concentrations of other membrane proteins indicates that ACAT-mediated regulation of APP processing is not likely to be a generalized phenomenon that could potentially interfere with other cellular events.

Discussion

We investigated the effect of intracellular cholesterol compartmentation on A β generation with cholesterol-mutant cell lines, ACAT inhibitors and cholesterol depletion. We found that a delicate balance between membrane FC and CE levels, regulated by ACAT, modulates total A β and A β ₄₂ production. Specifically, our results suggest that CE levels are correlated with A β levels and that ACAT activity directly modulates A β generation through the tight control of CEs generation. As ACAT inhibitors have been developed and

widely studied for the treatment of atherosclerosis, these compounds are available and, on the basis of our results, carry strong potential for the treatment and prevention of Alzheimer's disease.

The mechanism by which CE levels modulate the generation of A β is not clear. APP processing occurs along the secretory pathway via the α/γ and β/γ secretases^{1,2}. APP must therefore be accessible to the different secretases (α , β and γ) or to cofactors of these enzymes. In our study, ACAT activity was correlated not only with changes in A β generation but also with changes in α -, β - and γ -secretase-cleaved APP-CTFs, indicating a direct effect on APP processing. We have not analysed a possible effect on the amyloid precursor-like proteins (APLPs), which are homologous with APP but do not contain the A β sequence, or on the cleavage of membrane-bound Notch, which has been linked to PS1 activity³³. Nevertheless, the normal processing of TNF- α and full-length Notch (by furin), and the unchanged steady-state levels of BACE, nicastrin and transferrin receptor, indicate that this effect is specific to APP. Even if the most plausible explanation for our results is that changes in cholesterol distribution and/or compartmentation mainly regulate the availability of APP for cleavage, we cannot rule out a direct effect on the activity of the secretases (α , β or γ). The fact that ACAT activity also regulates the steady-state levels of both PS1 holoprotein and PS1 fragments suggests that this might indeed be so. Interestingly, time-course degradation experiments revealed an increased half-life of full-length PS1 in 25RA cells (D.M.K., unpublished observations). A similar phenomenon has already been described for perilipins and apolipoprotein B, both of which are post-translationally stabilized by neutral lipids and CEs stored in cytoplasmic droplets^{34,35}.

Although our results point to the pool of CEs as a regulator of A β generation (Table 2), we cannot totally rule out the involvement of FC. Ultimately, the ratio of FC to CEs, in addition to the levels of CEs, might modulate APP (and PS1) metabolism. Interestingly, the correlation between A β generation and CE levels was observed only in the presence of physiological or above-normal FC levels. At low FC concentration (wild-type cells grown in LDS), decreased CEs did not alter A β levels. Thus, although CE levels are closely correlated with those of A β , a minimal amount of FC might be necessary for the effect to be observed. Modulation of the generation of A β by ACAT inhibitors was dependent on the depletion of CEs, because no effect was observed when ACAT inhibitors were used under conditions that did not induce changes in cholesterol distribution (that is, short periods and highly confluent cells). ACAT inhibitors seemed to be less effective in 'fast-growing' cell lines such as HEK-293 (data not shown) than in 'slow-growing' cell lines (H4, SY5Y), probably owing to differences in the half-life of the CE cycle³⁶. CHO cells were less sensitive to ACAT inhibition than neuronal cell lines and primary neurons. Ultimately, primary neurons were so sensitive to ACAT inhibition (Fig. 6c) that treatment with CP113,818 for as little as 2 days was enough to affect both cholesterol distribution and APP processing (data not shown).

Mammals, including humans, express two different ACAT forms, called ACAT-1 and ACAT-2 (refs 37–41). Whereas ACAT-1 is almost uniformly distributed between several tissues, including the brain, ACAT-2 is selectively expressed in the liver and intestine^{37,40,41}. It is worth remembering that CHO cells express only ACAT-1 (ref. 37) and that AC29 cells have a selective disruption of the gene encoding ACAT-1 (ref. 22). Moreover, disruption of the gene encoding ACAT-1 in mice caused an abnormal and extensive deposition of cholesterol as FC in the brain⁴², indicating that ACAT activity has a pivotal role in maintaining cholesterol homeostasis in the brain. The cholesterol esterification activities of ACAT-1 and ACAT-2 exhibit different IC₅₀ values when assayed in the presence of different ACAT inhibitors, suggesting that ACAT inhibitors can selectively target the specific forms of ACAT^{26,40}. ACAT inhibitors have already been the object of extensive studies as potential anti-atherosclerotic and hypocholesterolaemic agents in both animals^{43–46} and humans^{47,48}. Because levels of cholesterol in the plasma also seem to be associated with an increased risk for Alzheimer's disease⁴, the use of ACAT inhibitors that cross the blood–brain barrier

could provide the double effect of lowering levels of cholesterol in the plasma and inducing a shift in cholesterol distribution from CEs to FC in neurons of individuals at high risk for Alzheimer's disease. Studies in transgenic animals and patients are needed to test ACAT-1 inhibitors as possible therapeutic agents for the treatment and prevention of Alzheimer's disease. □

Methods

Cell culture and antibodies.

Wild-type and cholesterol-mutant CHO cell lines were grown in DMEM and nutrient mixture F-12 Ham (Sigma Chemicals, St Louis, Missouri), respectively. Media were supplemented with 10% (v/v) FBS (Atlanta Biologicals, Norcross, Georgia), 1% (v/v) L-glutamine/penicillin/streptomycin solution (Sigma), and 0.4% (v/v) G418 sulphate (Calbiochem, La Jolla, California) as a selection marker for stably transfected cells. Cells were cultured at 37 °C in a water-saturated air/CO₂ atmosphere.

For neuronal cultures, hippocampi and frontal cortices were dissected from embryonic-day 16–18 (E16–18) mice and placed in Eagle's modified medium with 10% fetal calf serum. The tissue was mechanically dissociated by pipetting. Neurons were plated overnight on poly-(L-lysine)-coated glass chamber slides (0.2 mg ml⁻¹) and on culture dishes for western blot and cholesterol compartmentation analyses. Neurons were then maintained in Neurobasal medium with 2% B27 supplement (Gibco) at 37 °C with 5% CO₂ in air. The individual embryo tails were subjected to assays based on the polymerase chain reaction to establish the genotype of each culture (transgenic or non-transgenic).

The following antibodies against APP were used throughout this study: 22C11 (monoclonal, against N terminus; Chemicon International, Temecula, California); 369 (monoclonal, against C terminus; gift from Dr Sam Gandy, Cornell University School of Medicine, New York, New York); C7 (against C terminus; gift from Dr Dennis J. Selkoe, Harvard Medical School, Boston, Massachusetts); 4G8 (monoclonal, against residues 18–25 of A β ; Endogen, Woburn, Massachusetts); 6E10 (monoclonal, against residues 1–17 of A β ; Senetek, St Louis, Missouri). Polyclonal antibodies against BACE and nicastrin were obtained from Affinity BioReagents (Golden, Colorado). Antibodies against calreticulin (ER marker), GM130 (Golgi marker) and EEA-1 (early endosomes) were obtained from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada) and Transduction Laboratories (Lexington, Kentucky). Monoclonal antibody (MSXPS1) against CTF-PS1 was obtained from Chemicon International (Temecula, California). Monoclonal antibody (M32255a) against TNF- α was obtained from Fitzgerald Industries International (Concord, Massachusetts). The hybridoma antibody developed by S. Artavanis-Tsakonas⁴⁹ was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa (Iowa City, Iowa).

Cholesterol determinations.

For the determinations of intracellular pools of FC and CE, cells were first incubated to equilibrium (for 3 days) in the presence of [1-¹⁴C]acetic acid (57.0 mCi mmol⁻¹) (Amersham Life Sciences, Elk Grove, Illinois), then washed twice in PBS and extracted in chloroform:methanol (2:1, v/v). The chloroform phase was dried, resuspended again in chloroform and applied, together with standards, to a Silica Gel-G (EM Science, Gibbstown, New Jersey) thin layer chromatography (TLC) plate. Plates were developed in hexane:ethyl ether:acetic acid (87:20:1, v/v) and revealed with I₂ vapour. Spots were then scraped off and counted in a liquid-scintillation counter. The values, expressed as percentages of total radiolabelled cholesterol, were used to extrapolate the concentration (in mg per g of protein) from the total amount of cholesterol obtained with the enzymatic assay (Sigma; catalogue no. 402-20). As control, FC was also determined with the enzymatic assay from Boehringer Mannheim (R-Biopharm GmbH, Darmstadt, Germany; catalogue no. E0139050). CEs were determined by subtracting FC from total cholesterol.

For the determination of cholesterol distribution in the plasma membrane, cells were subjected to the cholesterol oxidase assay⁵⁰ before extraction with chloroform:methanol.

Subcellular fractionation.

Cells were first radiolabelled with [1-¹⁴C]acetic acid to equilibrium, then scraped and homogenized through a ball-bearing homogenizer. Homogenates were centrifuged at 1,000g for 20 min at 4–8 °C. Postnuclear supernatants (PNSs) were collected, applied to an 8–34% Nycomed OptiPrep (Gibco BRL, Grand Island, New York) continuous gradient and centrifuged at 100,000g in an SW41 rotor for ~18 h at 4 °C. Fractions (0.8 ml) were collected, extracted and resolved on a Silica Gel-G TLC plate. Pretreatment of intact cells with cholesterol oxidase was used to determine the degree of contamination of PNSs with sterols contained in the plasma membrane. Only gradients containing less than 5% oxidized cholesterol (from the plasma membrane) were processed.

Modified radioimmunoassay.

Wild-type and cholesterol-mutant cell lines stably transfected with APP₂₅₁ were incubated overnight at 4 °C in PBS containing 22C11 (against the N terminus) or 369 (against the C terminus) anti-APP antibodies. Plates were then washed twice in PBS, and antibody binding was detected with 0.5 nM [¹²⁵I]-labelled Protein A (NEN Life Sciences Products, Boston, Massachusetts; specific radioactivity more than 80 mCi per mg of protein). Cells were then washed in PBS, scraped and counted on a γ -counter. As an additional control, the first antibody was omitted from some experiments. As expected, only the 22C11 antibody, against the N-terminal (extracellular) domain of APP, was able to detect cell-surface APP.

Clearance of secreted A β .

To assess the degradation of secreted A β by AC29 cells, both wild-type and AC29 cells were first grown in fresh medium for 24 h. Wild-type cells secreted elevated levels of A β _{total} and A β ₄₂ into the medium, whereas AC29 cells did not (see 'conditioned medium' in Fig. 2e). AC29 cells were then incubated with this 'conditioned medium' from either wild-type or AC29 cells for a further 24 h. A β _{total} and A β ₄₂ were then quantified as described below.

Aβ determinations and ACAT inhibitor treatment.

For Aβ determination, cells stably transfected with APP₇₅₁ were grown in six-well plates (Becton Dickinson Labware, Franklin Lakes, New Jersey). When ~80–90% confluent, cells were washed in PBS and incubated in 1 ml of fresh medium for 24 h. Secreted Aβ₁₋₄₀ and Aβ₄₂ were quantified by standard sandwich ELISA (Aβ ELISA Core Facility, Center for Neurological Diseases, Harvard Institutes of Medicine, Harvard Medical School, Boston, Massachusetts).

For the experiments with ACAT inhibitors, cells were plated at low confluence (as single cells carefully dissociated from each other) in six-well plates 12–24 h before treatment. Cells were then treated for 72 h, washed in PBS containing the inhibitor and incubated in 1 ml fresh medium containing ACAT inhibitors for additional 24 h. If splitting of cells was needed during 12 days of treatment, the inhibitor was added to both PBS and trypsin-EDTA.

Cholesterol depletion.

For cholesterol depletion, cells were incubated in the presence of medium containing LDS and always maintained below ~70% confluence. LDS was obtained after the elimination of total lipoproteins from FBS, as described previously⁵⁰. In brief, FBS was brought to a density of 1.25 g ml⁻¹ with KBr and centrifuged to separate total lipoproteins (very-low-density lipoprotein, intermediate-density lipoprotein, low-density lipoprotein and high-density lipoprotein). LDS was then dialysed against 15 litres of 0.15 M NaCl, 0.3 mM EDTA, pH 7.4, for 36 h with five changes at 4 °C. In our hands this strategy decreased the concentration of serum cholesterol by more than 97%.

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