Secretory processing of amyloid precursor protein is inhibited by increase in cellular cholesterol content

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Plasma-membrane composition plays a crucial role in most of the cellular functions that depend on membrane processes. In virtually all cell types the proteolytic processing of Alzheimer amyloid precursor protein (APP) to generate soluble APP (sAPP) is believed to occur at the plasma membrane or in its immediate proximity. Alteration of this metabolic pathway has been linked to the pathogenesis of Alzheimer's disease. We analysed the effect of membrane cholesterol enrichment on APP metabolism. Incubation of COS cells with increasing concentrations of non-esterified cholesterol carried by rabbit β -very low-density lipoprotein caused a dose-dependent inhibition of sAPP release: 70% inhibition with 10 μ g/ml non-esterified cholesterol. A less pronounced inhibitory effect was observed on treatment with

human low-density lipoprotein. Inhibition of sAPP release was independent of receptor-mediated lipoprotein metabolism since simultaneous treatment with chloroquine did not modify the effect of lipoprotein treatment. In addition, treatment with cholesterol dissolved in either ethanol or methyl- β -cyclodextrin elicited the same effect. Excess non-esterified cholesterol did not cause cell toxicity. Cell cholesterol mass inversely correlated with sAPP release. Progesterone, which inhibits shuttling of non-esterified cholesterol between the plasma membrane and intracellular pools, had no effect on the inhibition of sAPP release from cholesterol-loaded cells, providing indirect evidence that cholesterol may act at the plasma membrane.

INTRODUCTION

Cholesterol has a wide variety of effects on the physical properties of membranes, such as membrane ordering, membrane permeability and lateral diffusion [1]. Overall, cholesterol exerts a homogenizing effect in both natural membranes and lipid bilayers. All membrane-associated proteins are likely to be affected by the lipid composition of their environment [2]. Cholesterol has been reported to interact directly with some membrane proteins [3]. Specific modulation of membrane protein activity by cholesterol has been observed [4–7]. Membrane-associated proteolytic cleavage and subsequent translocation of the perinuclear sterol-regulatory-element-binding protein (SREBP-1) has recently been shown to be strikingly dependent on sterol membrane content [8].

Many proteins exist as both membrane-bound and soluble isoforms. The two isoforms can be generated by alternative RNA splicing of a common transcript or by post-translational proteolytic cleavage of their membrane anchor [9]. The latter mechanism has been described for a number of integral membrane proteins such as angiotensin-converting enzyme [10], transforming growth factor α [11,12], L-selectin [13] and the amyloid precursor protein (APP) [14,15]. APP is an integral glycosylated protein with a single membrane-spanning region, a short intracellular C-terminal segment and a long extracellular N-terminal domain [16–19]. The protein follows the normal constitutive secretory pathway and is cleaved by a so-called ' α -secretase' immediately before or after reaching the cell surface

[20] to release the N-terminal ectodomain into the extracellular space. APP is the source of the β -amyloid protein (β A4), a highly aggregating peptide that is the major constituent of senile plaques of Alzheimer's disease [21]. Normally, proteolytic cleavage occurs within the β A4 sequence and therefore formation of amyloidogenic fragments does not occur [14,15]. APP molecules that escape α -secretase cleavage can be internalized and degraded, possibly in a lysosomal compartment, to generate fragments containing the entire amyloidogenic β A4 sequence [22]. The observation that β A4 is physiologically produced and secreted by cells [23] suggests that the secretory metabolism of APP and the production of amyloidogenic fragments (including β A4) are reciprocal mechanisms, the latter being predominant during α -secretase APP processing [20]. This hypothesis is supported by observations on transfected cells [20] as well as peripheral cells from patients with Alzheimer's disease [24].

Recently a significant inhibition of soluble (s)APP secretion by very high cholesterol concentrations in the culture medium (0.4–2.4 mg/ml) was observed in HEK 293 cells overexpressing APP [25]. Maintenance of cellular non-esterified cholesterol concentrations within small ranges appears to be critical for preservation of cellular function and cell viability. In the present study, we demonstrate that secretion of endogenously expressed APP may be strongly and rapidly inhibited at much lower cholesterol concentrations; the increase in the cellular non-esterified cholesterol content did not induce any cell toxicity; the inhibition of APP secretion inversely correlates with cell membrane cholesterol content.

Abbreviations used: SREBP-1, sterol-regulatory-element-binding protein; APP, amyloid precursor protein; sAPP, soluble secreted APP; β A4, β -amyloid protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; FCS, fetal calf serum; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; MEM, minimal essential medium; ACAT, acyl-CoA-cholesterol acyltransferase.

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These findings may have direct correlations with the pathogenesis of Alzheimer's disease as well as more general implication in mechanisms regulating the physiological metabolism of many other membrane proteins [9].

EXPERIMENTAL

Materials

Tissue culture plastics were from Corning. All culture media and supplements were obtained from Gibco Life Technologies (Paisley, Scotland, U.K.). Fetal calf serum (FCS) was purchased from Mascia Brunelli Biolife (Milano, Italy). [^{14}C]Oleic acid (60 mCi/mmol), [^3H]cholesteryl oleate and [^3H]cholesterol were from Amersham International (Amersham, Bucks., U.K.). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA, U.S.A.). All other reagents were of the highest grade available and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise specified.

Cell culture

COS-1 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, penicillin/streptomycin, non-essential amino acids and Tricine buffer (20 mM, pH 7.4) at 37 °C in 5% CO_2 /95% air. Experiments were performed at 37 °C (except where indicated) in serum-free MEM. In experiments conducted at 15 °C, bicarbonate and Tricine buffer were replaced with 25 mM Hepes.

Lipoprotein preparation

Rabbit β -very-low-density lipoprotein (β -VLDL) and human low-density lipoprotein (LDL) were isolated by sequential ultracentrifugation, as previously described [26]. β -VLDL was raised in plasma of adult male New Zealand White rabbits (Charles River, Calco, Italy) maintained on a 1% cholesterol diet for 6 weeks.

Experimental treatments

Confluent monolayers of cells were washed twice with PBS and once with serum-free culture medium before undergoing different treatments. Lipoproteins were dispersed in serum-free medium and added to the cell monolayers. Incubation was continued for 2 h at 37 °C. In experiments including chloroquine, the drug from a 100 mM stock solution in water was added at a final concentration of 50 μM . Non-esterified cholesterol was dissolved in ethanol and diluted into serum-free medium at the appropriate concentrations. The final concentration of ethanol never exceeded 0.5% (v/v). Methyl- β -cyclodextrin-cholesterol complexes were prepared in stock solution by dissolving cholesterol in a 300 mM buffered solution of methyl- β -cyclodextrin to a final molar ratio of 1:30. Controls with vehicle alone were included in all experiments. For progesterone experiments, cells were kept at 15 °C for 10 min and then surface-enriched by incubation with 30 $\mu\text{g}/\text{ml}$ non-esterified cholesterol as methyl- β -cyclodextrin-cholesterol complex in serum-free MEM/25 mM Hepes for 30 min. Cells were then rinsed and incubation was continued for 2 h at 37 °C in fresh serum-free medium containing [^{14}C]oleic acid (0.68 $\mu\text{Ci}/\text{sample}$) complexed with BSA with or without progesterone (4 $\mu\text{g}/\text{ml}$).

Cell viability

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [27] and lactate

dehydrogenase (LDH) leakage into the culture medium. MTT salt was dissolved in serum-free medium to a final concentration of 0.75 mg/ml and added to the cells at the beginning of the different treatments. The cellular formazan precipitate formed during the treatment was extracted with 1 M HCl/propan-2-ol (1:24, v/v). Absorbance of the samples was read at 560 nm on a Multiscan reader. Leakage of cellular LDH was measured as enzyme activity (units/litre) in the medium by using a commercial kit (Merck, Darmstadt, Germany).

Harvesting of the cells and preparation of conditioned medium

Conditioned medium was collected after 2 h of incubation and centrifuged at 13000 g for 5 min to remove detached cells. Proteins in the conditioned medium were quantitatively precipitated by the deoxycholate/trichloroacetate procedure described by Löffler and Huber [28]. Cell monolayers were washed twice with ice-cold PBS and lysed on the tissue culture dish by addition of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X100) and scraped with a rubber spatula. An aliquot of the cell lysate was used for protein analysis with the Bio-Rad Bradford kit for protein quantification.

Immunodetection of sAPP

Samples of conditioned medium standardized to lysate protein concentration were subjected to SDS/PAGE (10% gel) and then transferred to nitrocellulose membrane (Costar, Cambridge, MA, U.S.A.). For the detection of secreted APP, the monoclonal antibody 1G5 [29] was used, and the blots were incubated overnight at room temperature. Detection was carried out by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.) for 1 h. The blots were then washed extensively and sAPP visualized using an enhanced chemiluminescence method (Amersham).

sAPP is secreted into the medium of COS cells as a protein with an apparent molecular mass of 120 kDa. Since the same immunoreactive band was also detected by using the antibody 22C11 (Boehringer-Mannheim, Mannheim, Germany) and the antiserum ER3 β 1-16 (not shown), which recognizes epitopes in the first 16 amino acids of βA4 (M. Racchi, unpublished work) which also constitutes the C-terminus of α -secretase-cleaved APP, the identified band can be assumed to be authentic sAPP.

Lipid and protein quantification

The lipid composition of the lipoprotein preparations (total cholesterol, non-esterified cholesterol, phospholipids and triacylglycerols) was determined by enzymic assay kits from Boehringer-Mannheim. Protein was determined by the method of Lowry et al. [30].

Determination of non-esterified and esterified cholesterol in COS cells

At the end of an incubation, cells were washed three times with PBS and extracted with hexane/propan-2-ol (3:2, v/v). Lipid-free cell residues remaining in the culture wells were digested in 1 ml of 1 M NaOH for protein determination by the method of Lowry et al. [30]. The lipid extracts were evaporated under a stream of nitrogen and resuspended in propan-2-ol for total cholesterol determination by an enzymic assay kit from Boehringer-Mannheim [31]. [^3H]cholesteryl oleate or [^3H]cholesterol was added, where appropriate, as internal standard during extraction procedures. Under our conditions, cholesteryl ester determined after TLC of lipid extracts was

about 1 and 3 % for untreated and cholesterol-loaded cells respectively.

Cholesterol esterification assay

Cholesterol esterification was measured after the addition of [$1\text{-}^{14}\text{C}$]oleic acid ($0.68\text{ }\mu\text{Ci/sample}$) complexed with BSA, during the 2 h of incubation in the presence or absence of progesterone as indicated in the Results section, and subsequent determination of radioactivity associated with cellular cholesteryl esters [32].

Quantitative densitometry and statistics

Quantitative analysis of Western blots was performed by calculating the relative density of the immunoreactive bands after acquisition of the blot image through with a Nikon CCD videocamera module and analysis by means of the Image 1.47 program (Wayne Rasband, NIH, Research Service Branch, NIMH, Bethesda, MD, U.S.A.). Statistical analysis of the data was performed using the statistical package CSS-Statistica for Windows 4.0. Analysis of variance followed by the *post hoc* Tukey HSD test was performed. A $P < 0.05$ was considered significant.

RESULTS

Effect of lipoprotein non-esterified cholesterol on sAPP secretion from COS cells

The effect of membrane cholesterol enrichment on sAPP release was first investigated using lipoproteins as an exogenous source of non-esterified cholesterol. Rabbit β -VLDL, which is very rich in non-esterified cholesterol, and human LDL, which is relatively rich in non-esterified cholesterol, were used. β -VLDL isolated from cholesterol-fed rabbits inhibited in a dose-dependent manner the release of sAPP from COS cells within 2 h of incubation in serum-free medium (Figure 1). The effect correlated with the lipoprotein non-esterified cholesterol concentration, and resulted in 70 % inhibition of basal sAPP secretion at a β -VLDL non-esterified cholesterol concentration of $10\text{ }\mu\text{g/ml}$

Figure 1 Dose-response effect on sAPP release from COS cells by lipoprotein-delivered non-esterified cholesterol

Cells normally growing in 100 mm Petri dishes were incubated in serum-free medium for 2 h in the absence (lane 1) or presence of 1, 5 or $10\text{ }\mu\text{g/ml}$ β -VLDL non-esterified cholesterol, corresponding to 0.5, 2.5 or $5\text{ }\mu\text{g/ml}$ β -VLDL protein (lanes 2–4), or the presence of $10\text{ }\mu\text{g/ml}$ LDL non-esterified cholesterol corresponding to $20\text{ }\mu\text{g/ml}$ LDL protein (lane 5). Values of non-esterified cholesterol/phospholipid ratio (FC/PL) for β -VLDL and LDL are reported. For Western-blot analysis of sAPP secreted into the culture medium, the volume of the sample loaded was normalized to the protein concentration of the corresponding cell lysate. Densitometric analysis of Western blots was performed as described in the Experimental section. Quantification gave the following results expressed as percentage of control (means $\pm$ S.D. for three independent determinations): β -VLDL ($1\text{ }\mu\text{g/ml}$) 112 ± 8 ; β -VLDL ($5\text{ }\mu\text{g/ml}$) $57 \pm 5^{**}$; β -VLDL ($10\text{ }\mu\text{g/ml}$) $27 \pm 13^{**}$; LDL ($10\text{ }\mu\text{g/ml}$) $65 \pm 22^{**}$. ** Significantly different ($P < 0.01$) from control.

Figure 2 Inhibition of cholesteryl ester hydrolysis by chloroquine does not affect β -VLDL inhibition of sAPP secretion

COS cells normally growing in 100 mm Petri dishes were incubated in serum-free medium for 2 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of $10\text{ }\mu\text{g/ml}$ β -VLDL non-esterified cholesterol, corresponding to $5\text{ }\mu\text{g/ml}$ β -VLDL protein. Simultaneously, samples in lanes 2 and 4 were treated with $50\text{ }\mu\text{M}$ chloroquine (chlor.). Treatment with chloroquine alone did not affect basal sAPP secretion (lane 2) nor the inhibitory effect of β -VLDL (lane 4). For Western-blot analysis, the volume of the sample loaded was normalized to the protein concentration of the corresponding cell lysate.

which corresponds to a protein concentration of $5\text{ }\mu\text{g/ml}$ (Figure 1). Treatment of cells with human LDL resulted in a similar, but less pronounced, inhibition of sAPP secretion when equal concentrations of non-esterified cholesterol were added (Figure 1, lanes 4 and 5). The quantitative difference between the two lipoprotein treatments probably relates to the different non-esterified cholesterol/phospholipid ratio (FC/PL) (see Figure 1), which is known to determine the cholesterol-releasing ability of a lipoprotein [33,34]; indeed, another preparation of β -VLDL with a FC/PL ratio (0.69) similar to that of LDL produced a quantitatively similar effect (results not shown). Simultaneous treatment of the cells with β -VLDL and chloroquine to inhibit hydrolysis of cholesteryl esters and transport of cholesterol from intracellular storage to the plasma membrane did not modify the inhibitory effect of the lipoprotein (Figure 2).

Cholesterol inhibition of sAPP secretion inversely correlates with cell cholesterol content

To rule out the possibility that lipoprotein components other than non-esterified cholesterol could modulate sAPP release from cells, we investigated the effect of changing cholesterol membrane content by direct cholesterol addition to the incubation medium. For this purpose, membrane cholesterol enrichment was achieved by adding non-esterified cholesterol solubilized in either ethanol (final concentration 0.5 %, v/v) or as cholesterol-methyl- β -cyclodextrin complex (1:30 molar ratio).

Increasing cell cholesterol concentration with cholesterol dissolved in methyl- β -cyclodextrin resulted in a dose-dependent lowering of sAPP appearance in the medium. sAPP release decreased progressively with cholesterol concentration of 10, 30 and $60\text{ }\mu\text{g/ml}$ (Table 1). In contrast, by depleting plasma membrane cholesterol, methyl- β -cyclodextrin alone elicited a slight but detectable increase in sAPP in the culture medium (Table 1). Cholesterol cell membrane assessment inversely correlated with sAPP secretion: a significant and dose-dependent enrichment of cholesterol mass was detected when cells were incubated with increasing concentrations of cholesterol-methyl- β -cyclodextrin complex (Table 1), whereas a net decrease in cell cholesterol was observed when COS cells were incubated with methyl- β -cyclodextrin alone. The effect of ethanol-solubilized cholesterol was similar (results not shown).

COS cell accumulation of cholesterol did not lead to cell toxicity. Our measure of toxicity was based on the established

Table 1 Inverse correlation between cell cholesterol content and sAPP secretion

COS cells were treated with increasing concentrations of cholesterol–cyclodextrin complexes for 2 h as described in the Experiment section. Cholesterol mass determination and sAPP release was performed on the same set of cells. Data are expressed as percentage of control and represent the mean $\pm$ S.D. for three independent determinations. In all experiments the percentage of cholesteryl esters measured did not exceed 3% of total cholesterol mass. * Significantly different ($P < 0.05$) from control; ** significantly different ($P < 0.01$) from control.

Treatment	Cell cholesterol (% of control)	sAPP (% of control)
Control	100 $\pm$ 3	100 $\pm$ 6
Cyclodextrin (5 mM)	64 $\pm$ 12*	106 $\pm$ 7
Cholesterol–cyclodextrin		
10 μ g/ml	183 $\pm$ 11**	58 $\pm$ 6**
30 μ g/ml	221 $\pm$ 13**	43 $\pm$ 3**
60 μ g/ml	256 $\pm$ 24**	25 $\pm$ 7**

methods of monitoring LDH activity and MTT reduction (Table 2).

Cholesterol modulation of APP secretion may involve the plasma membrane

In order to determine whether the effect of cell cholesterol enrichment on sAPP secretion involved the plasma membrane, cells were incubated with progesterone (4 μ g/ml) after a 30 min preloading with cholesterol–methyl- β -cyclodextrin complex at 15 °C. Preloading of cells with cholesterol at 15 °C permits enrichment of the plasma membrane without exchange with intracellular compartments. When progesterone was combined with cholesterol to inhibit transfer of plasma membrane cholesterol to intracellular pools, secretion of APP was inhibited to a similar extent to that in the presence of cholesterol alone (Figure 3A, compare lanes 3 and 4). Cholesterol shuttling from the plasma membrane was effectively inhibited (Figure 3B), as assessed by the activity of the microsomal enzyme acyl-CoA-cholesterol acyltransferase (ACAT), which is extremely sensitive to the smallest intracellular non-esterified cholesterol variation [32]. Cholesterol esterification in COS cells was reduced to basal levels by incubation with progesterone, whereas the presence of cholesterol alone elicited a significant increase in ACAT activity (Figure 3B).

Table 2 COS cell toxicity after incubation with increasing concentrations of cholesterol–cyclodextrin complexes

COS cells were cholesterol-loaded with increasing concentrations of cholesterol–cyclodextrin complexes for 2 h as described in the Experimental section. Cell toxicity was measured by MTT assay and LDH leakage into the culture medium. Results are expressed as percentage of the control for MTT (A_{560}) and units/litre for LDH. Values are means $\pm$ S.D. ($n = 6$).

Treatment	MTT activity (%)	LDH activity (units/litre)
Control	100 $\pm$ 11	38.3 $\pm$ 4.4
Cyclodextrin (5 mM)	106 $\pm$ 13	33.3 $\pm$ 1.7
Cholesterol–cyclodextrin		
10 μ g/ml	91 $\pm$ 12	36.7 $\pm$ 3.3
30 μ g/ml	92 $\pm$ 7	35.0 $\pm$ 2.9
60 μ g/ml	91 $\pm$ 5	36.7 $\pm$ 4.4

A **B** **Figure 3** Effect of plasma-membrane cholesterol enrichment on sAPP release from COS cells

COS cells in replicate dishes were surface-enriched by incubation with 30 μ g/ml non-esterified cholesterol as methyl- β -cyclodextrin–cholesterol complex for 30 min in serum-free MEM/25 mM Hepes at 15 °C. The cells were rinsed and progesterone (4 μ g/ml) was added in fresh serum-free medium containing [14 C]oleic acid (0.68 μ Ci/sample) complexed with BSA. Different cell treatments are described in (A). Dishes were then incubated for 2 h at 37 °C. (A) Western-blot analysis of sAPP secreted into the culture medium from COS cells undergoing different treatments. (B) ACAT activity expressed as cholesteryl ester as a percentage of total cell cholesterol (%CE/TC) corrected for cell protein mass per h. Densitometric analysis of Western blots was performed as described in the Experimental section. Quantification gave the following results expressed as percentage of control (mean $\pm$ S.D. for three independent determinations): control + progesterone (Progest.), 115 $\pm$ 7; cholesterol, 54 $\pm$ 11*; cholesterol + progesterone, 48 $\pm$ 11*. * Significantly different ($P < 0.01$) from control.

DISCUSSION

The current data suggest a sterol-regulated proteolysis of APP processing. Membrane non-esterified cholesterol content regulates the transformation of APP membrane protein into its soluble non-amyloidogenic counterpart. When a net accumulation of non-esterified cholesterol in membrane of COS cells was induced, a significant decrease in sAPP release was observed. In contrast, the addition of cholesterol-acceptor molecules to the culture medium, by causing a selective loss of cellular cholesterol, slightly increased the release of sAPP. The ability of modulating APP processing is not related to cell toxicity and it is not shared by all sterols since another steroid, progesterone, did not affect sAPP secretion.

Membrane cholesterol enrichment was achieved by delivering cholesterol as a lipoprotein component or directly dissolved in either ethanol or cholesterol–methyl- β -cyclodextrin complex. The different methods employed for exposing cells to cholesterol produced similar results. Membrane cholesterol modulation of sAPP secretion appeared to be relatively specific, since COS cells metabolically labelled with [35 S]methionine did not show gross

alterations in the pattern of protein secretion after membrane cholesterol enrichment (results not shown).

Bodovitz and Klein [25] recently reported a similar modulation of sAPP secretion by cholesterol. They exposed HEK 293 cells overexpressing APP to extremely high cholesterol concentrations (range 0.4–2.4 mg/ml). Significant inhibition (50%) of sAPP secretion was observed at a cholesterol concentration of 0.6 mg/ml and after 8 h of treatment. Our results provide additional support for this modulation and further indicate that secretion of endogenously expressed APP may be strongly and rapidly inhibited at much lower cholesterol concentrations. The highest active cholesterol concentration used in this study (60 μ g/ml) is within the same order of magnitude as the concentration of free cholesterol found in human interstitial fluid (approx. 100 μ g/ml) as described previously [35].

Lipoproteins, the physiological cholesterol carriers, were very effective in inhibiting release of sAPP into culture medium. This may suggest that non-esterified cholesterol carried by lipoproteins is delivered to the cells in a more efficient way or that other lipids (namely cholesteryl esters, phospholipids or sphingomyelin) play a role in this metabolic modulation. However, the strong dependence of sAPP release on cholesterol is clear, since the specific alteration of COS membrane cholesterol content with water-soluble cholesterol–methyl- β -cyclodextrin complexes influenced sAPP release in the same way.

Although a contribution of cholesterol derived via specific receptors cannot be excluded, these pathways are unlikely to represent a major source of membrane cholesterol enrichment. The inhibition of sAPP release by lipoproteins was evident in a relatively short incubation period (as early as 30 min), whereas delivery of cholesterol via the lipoprotein receptor pathway requires a series of metabolic steps including binding of lipoprotein to a specific receptor (LDL and/or LDL-receptor related protein receptors), lipoprotein delivery to lysosomes, lysosomal hydrolysis of lipoprotein-cholesteryl esters and cholesterol transport to the plasma membrane. The experiment with chloroquine showing that the lysosomal inhibitor does not influence the effect of lipoprotein treatment further supports a negligible influence of the receptor-mediated pathway. In addition, the inhibition of sAPP secretion was found to be maximal for protein concentrations of β -VLDL and LDL and higher than those saturating the receptor-mediated uptake (2.5 μ g/ml and 10 μ g/ml respectively). Finally, the inhibition of sAPP release could be reproduced by incubating COS cells directly with non-esterified cholesterol, in the absence of any apolipoprotein.

Membrane association and co-localization of enzyme and substrate (APP) appears to be required for the activity of α -secretase [36–38]. However, neither the precise cellular localization nor the identity of the enzyme have been elucidated [36,39]. Thus cholesterol, which moves rapidly among cell membranes, may affect APP metabolism at several steps along the secretory pathway, possibly by interfering with the protease at the plasma membrane, according to data supporting such a localization [36], or by interfering with the movement of secretory vesicles from the *trans*-Golgi network to the plasma membrane, according to other models for the mechanism of sAPP secretion [39]. Progesterone has been shown previously to inhibit transfer of cholesterol from the plasma membrane to intracellular membranes [40,41]. This inhibition demonstrated here by the low ACAT activity in cholesterol-preloaded cells incubated with progesterone did not alter the cholesterol-inhibitory effect on sAPP secretion. This result allows us to hypothesize that cholesterol inhibits sAPP release at the plasma-membrane level.

Cholesterol may act as a regulator of protease access to its substrate or as an inert structural membrane component of the

lipid bilayer which alters the activity of embedded proteins by modulating membrane fluidity. In addition, it may perturb membrane protein function by a mechanism independent of its membrane-ordering effect [42]. As a relevant example [8], it has recently been reported that the activity of SREBP-1 is modulated by membrane cholesterol content. The activity of SREBP-1 is inhibited by cholesterol by a mechanism that involves inhibition of the proteolytic conversion of the SREBP-1 membrane-bound precursor into the soluble active moiety. The proposed mechanism involves a conformational change in either the protease or its substrate, or interference with the accessibility of the substrate to the enzyme because of the effect of sterols on membrane fluidity.

APP exists in two forms: as a protein resident on the cell surface of brain and peripheral tissues, and as a soluble component, resulting from cleavage by a protease known as α -secretase [14]. Another pathway cleaves APP to release β A4, the major protein component of cerebral amyloid deposits in patients with Alzheimer's disease [20]. The association of APP and α -secretase with cell membrane appears to be essential for correct enzyme activity [36]. Current thinking suggests that accumulation of β A4 results from aberrant processing of membrane APP [20]. Therefore any abnormality in the production of sAPP membrane protein by α -secretase could lead to Alzheimer's disease. In the present study, plasma-membrane cholesterol enrichment slows the rate of sAPP secretion, possibly by limiting α -secretase access to its substrate. Indeed, it has been recently reported that rabbits fed on a cholesterol-rich diet develop brain amyloidosis [43]. Thus cholesterol modulation of sAPP secretion may actually have clinical relevance.

Cholesterol is carried between tissues by lipoproteins. Apolipoprotein E plays a central role in plasma lipoprotein metabolism and cholesterol homeostasis by redistributing lipids among cells of different organs and within a tissue [44]. In the nervous system, apolipoprotein E is synthesized and secreted by astrocytes and serves as the only lipid-transport apolipoprotein mediating cellular 'in and out' cholesterol exchange [45,46]. A link between apolipoprotein E isoforms and neurodegenerative disorders such as Alzheimer's disease has been observed [47]. Three major forms of this apolipoprotein exist, the most common of which is apoE3; apoE2 and apoE4 are less widespread [46]. The apoE4 isoform is a risk factor for Alzheimer's disease, whereas apoE2 is thought to have a protective role [45,46,48]. Although our experiments would exclude the involvement of receptor-mediated mechanisms in the effect of lipoprotein-carried cholesterol, it is noteworthy that the plasma of individuals homozygous for apoE4 is far less efficient than plasma from E3/E3 individuals in extracting cholesterol from cell membranes [49]. On the other hand, apoE2, which possesses a very low affinity for the LDL receptor compared with the other isoforms [50], may accumulate in the cerebral extracellular space, actively promoting cellular cholesterol efflux. Therefore the observation that both apolipoprotein E and cholesterol are involved in processes related to Alzheimer's disease may not be casual. A cholesterol efflux defect in E4/E4 subjects could channel APP processing toward aberrant β A4 production, whereas an efficient efflux in E2/E2 subjects could preferentially direct membrane APP processing toward sAPP release.

The results reported here demonstrate that cleavage of APP from the cell surface by α -secretase is susceptible to control by cholesterol. Secretion of APP follows a pathway similar to that of many other membrane-bound proteins. The similarity between APP metabolism and that of many other proteins with different cell specificity and different biological activity [9] supports the existence of a widespread cellular mechanism [51] that regulates

the conversion of membrane-anchored protein into soluble and potentially bioactive factors. Cholesterol regulation of such mechanisms may actually be an important controller of cellular function of fundamental significance for cell biology or, in some instances, pathophysiology.

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REFERENCES

- Yeagle, P. L. (1991) *Biochimie* **73**, 1303–1310
- Bretscher, M. S. and Munro, S. (1993) *Science* **261**, 1280–1281
- Klein, U., Gimpl, G. and Fahrenholtz, F. (1995) *Biochemistry* **34**, 13784–13793
- Klein, I., Moore, L. and Pastan, I. (1978) *Biochim. Biophys. Acta* **506**, 42–53
- Sinha, A. K., Shattil, S. J. and Colman, R. W. (1977) *J. Biol. Chem.* **252**, 3310–3314
- Mitchell, D. C., Straume, M., Miller, J. L. and Litman, B. J. (1990) *Biochemistry* **29**, 9143–9149
- Criado, M., Eibl, H. and Barrantes, F. J. (1982) *Biochemistry* **21**, 3622–3629
- Wang, X., Sato, R., Brown, M. S., Hua, X. and Goldstein, J. L. (1994) *Cell* **77**, 53–62
- Ehlers, M. R. and Riordan, J. F. (1991) *Biochemistry* **30**, 10065–10074
- Ramchandran, R., Sen, G. S., Misono, K. and Sen, I. (1994) *J. Biol. Chem.* **269**, 2125–2130
- Pandiella, A. and Massagué, J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1726–1730
- Pandiella, A. and Massagué, J. (1991) *J. Biol. Chem.* **266**, 5769–5773
- Kahn, J., Ingraham, R. H., Shirley, F., Migaki, G. I. and Kishimoto, T. K. (1994) *J. Cell Biol.* **125**, 461–470
- Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D. and Ward, P. J. (1990) *Science* **248**, 1122–1124
- Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A. and Price, D. L. (1990) *Science* **248**, 492–495
- Kang, J., Lemaire, H., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987) *Nature (London)* **325**, 733–736
- Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F. and Neve, R. F. (1988) *Nature (London)* **331**, 528–530
- Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F. and Cordell, B. (1988) *Nature (London)* **331**, 525–527
- Kitaguchi, N., Takahaschi, Y., Tokushima, Y., Shiojiri, S. and Ito, H. (1988) *Nature (London)* **331**, 530–532
- Selkoe, D. J. (1994) *Annu. Rev. Neurosci.* **17**, 489–517
- Glenner, G. G. and Wong, C. W. (1984) *Biochem. Biophys. Res. Commun.* **120**, 885–890
- Haas, C., Koo, E. H., Mellon, A., Hung, A. Y. and Selkoe, D. J. (1992) *Nature (London)* **357**, 500–503
- Haas, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B. and Selkoe, D. J. (1992) *Nature (London)* **359**, 322–325
- Bergamaschi, S., Binetti, G., Govoni, S., Wetsel, W. C., Battaini, F., Trabucchi, M., Bianchetti, A. and Racchi, M. (1995) *Neurosci. Lett.* **201**, 1–4
- Bodovitz, S. and Klein, W. L. (1996) *J. Biol. Chem.* **271**, 4436–4440
- Mims, M. P., Soma, M. R. and Morrisett, J. D. (1990) *Biochemistry* **29**, 6639–6647
- Denizot, F. and Lang, R. (1986) *J. Immunol. Methods* **89**, 271–277
- Löffler, J. and Huber, G. (1993) *Biochem. Biophys. Res. Commun.* **195**, 97–103
- Henriksson, T., Barbour, R., Braa, S., Ward, P., Fritz, L. C., Johnson-Wood, K., Chung, H. D., Burke, W., Reinikainen, K. J. and Schenk, D. B. (1991) *J. Neurochem.* **56**, 1037–1042
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Bernini, F., Didoni, G., Bonfadini, G., Bellosta, S. and Fumagalli, R. (1993) *Atherosclerosis* **104**, 19–26
- Brown, M. S., Ho, Y. K. and Goldstein, J. L. (1980) *J. Biol. Chem.* **255**, 9344–9352
- Arbogast, L. Y., Rothblatt, G. H., Leslie, M. H. and Cooper, R. A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3680–3684
- Warner, G. J., Stoudt, G., Bamberger, M., Johnson, W. J. and Rothblatt, G. H. (1995) *J. Biol. Chem.* **270**, 5772–5778
- Wong, L., Curtiss, L. K., Huang, J., Mann, C. J., Maldonado, B. and Roheim, P. S. (1992) *J. Clin. Invest.* **90**, 2370–2375
- Sisodia, S. S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6075–6079
- Maruyama, K., Kametani, F., Usami, M., Yamao-Harigaya, W. and Tanaka, K. (1991) *Biochem. Biophys. Res. Commun.* **179**, 1670–1676
- Anderson, J. P., Esch, F. S., Keim, P. S., Sambamurti, K., Lieberburg, I. and Robakis, N. K. (1991) *Neurosci. Lett.* **128**, 126–128
- Sambamurti, K., Shioi, J., Anderson, J. P., Pappolla, M. A. and Robakis, N. K. (1992) *J. Neurosci. Res.* **33**, 319–329
- Lange, Y. (1994) *J. Biol. Chem.* **269**, 3411–3414
- Liscum, L. and Underwood, K. W. (1995) *J. Biol. Chem.* **270**, 15443–15446
- Schroeder, F., Jefferson, J. R., Kier, A. B., Knittel, J., Scallen, T. J., Gibson Wood, W. and Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med.* **196**, 235–252
- Sparks, D. L., Scheff, S. W., Hunsaker, III, J. C., Liu, H., Landers, T. and Gross, D. R. (1994) *Exp. Neurol.* **126**, 88–94
- Mahley, R. W. (1988) *Science* **240**, 622–630
- Weisgraber, K. H., Roses, A. D. and Strittmatter, W. J. (1994) *Curr. Opin. Lipidol.* **5**, 110–116
- Strittmatter, W. J. and Roses, A. D. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4725–4727
- Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S. and Roses, A. D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1977–1981
- Mahley, R. W., Nathan, B. P., Bellosta, S. and Pitas, R. E. (1995) *Curr. Opin. Lipidol.* **6**, 86–91
- Huang, Y., von Eckardstein, A., Wu, S. and Assmann, G. (1995) *J. Clin. Invest.* **96**, 2693–2701
- Weisgraber, K. H., Innerarity, T. L. and Mahley, R. W. (1982) *J. Biol. Chem.* **257**, 2518–2521
- Arribas, J. and Massagué, J. (1995) *J. Cell Biol.* **128**, 433–441