The rate of sphingomyelin synthesis *de novo* is influenced by the level of cholesterol in cultured human skin fibroblasts

Petra LEPPIMÄKI¹, Robert KRONQVIST and J. Peter SLOTTE

Department of Biochemistry and Pharmacy, Åbo Akademi University, P.O. Box 66, FIN-20521 Turku, Finland

Plasma membrane sphingomyelin (SM) is known to affect the cellular distribution of cholesterol. The aim of this work was to examine how SM homeostasis in human skin fibroblasts is affected by alterations in the level of cholesterol in the cell. The cellular cholesterol level was decreased by exposing cells to 2-hydroxypropyl- β -cyclodextrin, and increased by exposing cells to cholesterol-methyl- β -cyclodextrin inclusion complexes. A lowering of the cellular unesterified cholesterol content by 20 % was shown to increase the incorporation of [¹⁴C]palmitic acid into SM by 70 %. Subsequently, the cellular SM mass was shown to be increased (24 % increase after a 24 h period). Since L-cycloserine completely abolished the increased incorporation of [¹⁴C]palmitic acid into SM in cholesterol-depleted cells, we concluded that the *de novo* synthesis of the sphingosine backbone

of SM was activated in cholesterol-depleted cells. This conclusion was further verified by performing a cell-free assay of serine C-palmitoyltransferase (SPT) in cholesterol-depleted cells, which showed that the activity of the enzyme was increased by 30 % after cholesterol depletion. Most of the newly synthesized SM in cholesterol-depleted cells was susceptible to degradation by sphingomyelinase, indicating that it was transported efficiently to the cell surface. Loading of fibroblasts with cholesterol had essentially the opposite effects on SM homeostasis to those of cholesterol depletion, i.e. 20–30 % decreased incorporation of [¹⁴C]palmitic acid into SM and decreased activity of SPT. The results of this study show that cellular cholesterol levels have marked effects on the homeostasis of SM.

INTRODUCTION

Sphingomyelin (SM) is an important structural and functional component of cellular membranes. Depending on the cell type examined, 40–90 % of the total amount of SM in the cell is reported to be localized in the plasma membrane (PM) [1–4], and especially in its external leaflet [5–7]. Different studies have shown that cholesterol also is distributed unevenly between the cellular membranes, with the bulk of cholesterol residing in the PM [2–4,8]. The co-localization of SM and cholesterol to the PM could be due to the fact that cholesterol appears to interact more favourably with SM than with other phospholipid classes [9–11]. Previous studies from our laboratory and others [5,12–14] have shown that a lowering of the content of SM in PMs dramatically alters the cellular cholesterol distribution, by mobilizing PM cholesterol for esterification [5,12], decreasing sterol biosynthesis [5,13], and by activating the synthesis of steroid hormones in steroidogenic cells [14].

The synthesis of SM has been reported to occur in either the *cis*- or medial-Golgi network [15–18], from where newly synthesized SM is transported to the PM by a vesicular transport system [19,20]. SM synthesis has also been reported to occur in endosomes, from where SM can enter directly the PM by fusion of the endosomal membrane with the PM [21,22]. Serine C-palmitoyltransferase (SPT) initiates the biosynthesis of sphingolipids in the endoplasmic reticulum (ER) [23] by catalysing the condensation of serine and palmitoyl-CoA, to yield 3-oxosphinganine [24–26]. The step catalysed by SPT is thought to be both a regulatory and rate-limiting step in the synthesis of sphingolipids in brain and liver tissues [27,28]. The product 3-oxosphinganine is then further metabolized through sphinganine

and dihydroceramide to ceramide [29,30], which is subsequently incorporated into more complex sphingolipids.

The aim of the present work was to investigate how the homeostasis of SM is affected by changes in the cholesterol content of human skin fibroblasts (HSFs). We altered cellular cholesterol levels by depletion or loading using cyclodextrins (CyDs), and measured both SM mass and synthesis rates after treatments. In addition, we measured the activity of the rate-limiting enzyme, SPT, in cell-free homogenates of CyD-treated cells.

EXPERIMENTAL

Materials

[1 α ,2 α -³H]Cholesterol (48 Ci/mmol), [1-¹⁴C]palmitic acid (57 mCi/mmol) and [9,10-³H]oleic acid (10 Ci/mmol) were purchased from Amersham. The reagents used for cell cultures were obtained from Gibco Laboratories (Paisley, Renfrewshire, Scotland, U.K.). Ham F-12 nutrient mixture, lipids, 2-hydroxypropyl- β -cyclodextrin (2OHp- β CD), methyl- β -cyclodextrin, cycloserine and sphingomyelinase (SMase; from *Staphylococcus aureus*) were from Sigma (St. Louis, MO, U.S.A.). L-Serine and formaldehyde were obtained from Fluka (Buchs, Switzerland).

Cell culture

HSFs (GM 8333A) were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.). The

Abbreviations used: apoA-I, apolipoprotein A-I; CyD, cyclodextrin; chol β CyD, complex of cholesterol and methyl- β -cyclodextrin; ER, endoplasmic reticulum; HSF, human skin fibroblast; HDL, high-density lipoprotein; 2OHp β CD, 2-hydroxypropyl- β -cyclodextrin; PC, phosphatidylcholine; PM, plasma membrane; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine C-palmitoyltransferase.

¹ To whom correspondence should be addressed (e-mail pholmber@abo.fi).

cells were cultured continuously in Dulbecco's modified Eagle's medium supplemented with 12% (v/v) foetal bovine serum, 8 mM Hepes, pH 7.4, 2 mM L-glutamine, 1 mM non-essential amino acids, 100 units/ml penicillin and 100 µg/ml streptomycin. For experiments, the cells were seeded in 35-mm or 100-mm cell-culture dishes.

Manipulation of cellular cholesterol levels with CyDs

The cells were grown for 3–4 days in 35-mm culture dishes. After rinsing the confluent cells with PBS, they were incubated for 1 h in serum-free Ham F-12 nutrient mixture. To deplete the cells of cholesterol, 20HpβCD in Ham F-12 was added to the cell dishes (5 mM final concentration), and the cells were incubated for 60 min at 37 °C. To load the cells with cholesterol, a complex of cholesterol and methyl-β-cyclodextrin (cholβCD) was prepared by dissolving 960 mg of methyl-β-cyclodextrin in water and adding this solution to a thin film of cholesterol (40 mg) in a tube. The fibroblasts were then incubated for 60 min at 37 °C with 50 µg/ml (cholesterol) of the cholβCD complexes in Ham F-12.

Metabolic labelling of cellular SM, phosphatidylcholine (PC), ceramide and triglycerides

After the indicated pretreatments (cholesterol depletion or cholesterol loading) of cells, the rates of SM, PC and ceramide synthesis were determined using [¹⁴C]palmitic acid as a metabolic precursor. Triglyceride synthesis was measured by the incorporation of [³H]oleic acid. To label the cellular lipids, the cells were rinsed three times with PBS, and then 1 µCi/ml [¹⁴C]palmitic acid (57 mCi/mmol) or [³H]oleic acid in serum-free Ham F-12 was added. [³H]oleic acid was diluted with unlabelled oleic acid to the specific radioactivity used (57 mCi/mmol). Both precursors were complexed with BSA, as described by Oram [31]. After labelling, cells were rinsed with PBS, extracted with hexane/2-propanol, and analysed for lipid radioactivity after isolation of lipid classes by TLC.

Preparation of apolipoprotein A-I (apoA-I)/ 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles

ApoA-I was isolated from high-density lipoprotein (HDL) that had been purified by ultracentrifugation from fresh human plasma [32,33]. The proteins (10–20 mg) in HDL were precipitated with ice-cold ethanol (5 ml) and delipidated by extraction with 4 × 4 ml of hexane/2-propanol and 4 × 4 ml of chloroform/methanol (2:1, v/v). The remaining proteins were dissolved in a buffer containing 10 mM Tris and 6M urea, pH 8.0, and apoA-I was purified by gel-filtration chromatography (column dimensions 2.5 cm × 100 cm) with Sephacryl S-200 (Pharmacia LKB, Uppsala, Sweden). The fractions containing apoA-I were dialysed against 10 mM ammonium bicarbonate, pH 8.0, and concentrated by freeze-drying.

To form POPC vesicles, the phospholipid was first dried on to the wall of a glass tube, then hydrated in Ham F-12 nutrient mixture, and finally the tube was vortex-mixed for 1 min and sonicated for 15 min with a Braun titanium probe ultrasonicator (with cooling). After the sonication and removal of shed titanium particles, apoA-I was added to the tube containing the POPC vesicles to reach a final concentration of 20 µg/ml apoA-I and 100 µg/ml POPC. The apoA-I/POPC vesicles were prepared in sterile tubes, using sterile medium, and were kept at room temperature for 30 min before adding them to the cells.

Efflux of cellular [³H]cholesterol

To label cells with [³H]cholesterol, subconfluent cells were incubated for 72 h with [³H]cholesterol in foetal bovine serum [5 µCi/ml in serum, 10% (v/v) serum in Dulbecco's modified Eagle's medium]. The solvent of [³H]cholesterol was evaporated and then [³H]cholesterol was solubilized in ethanol (1 mCi/ml). The ethanol solution was then added to foetal bovine serum. The labelled cells were incubated for 1 h in Ham F-12, before starting the experiments. [³H]Cholesterol-labelled, confluent fibroblasts were treated for the times indicated with 20HpβCD (5 mM) or for 17 h with apoA-I/POPC vesicles (20 µg of protein/ml) in Ham F-12 medium. The efflux medium was removed from the cells and centrifuged (1 min, 10000 g) to remove detached cells. Aliquots (0.5 ml) of the medium were placed into scintillation vials, together with 3.5 ml of scintillation cocktail. The radioactivity in the medium was measured by scintillation counting. [³H]Cholesterol remaining in cells was extracted and analysed by TLC. The spots were revealed by staining with iodine and cut into vials, after which the radioactivity was measured by scintillation counting.

Effect of drugs on SM synthesis in cholesterol-depleted fibroblasts

To inhibit the SPT, unlabelled cells were rinsed with PBS and then incubated for 1 h at 37 °C with 105 µg/ml L-cycloserine [34,35]. The cells were then exposed to 105 µg/ml cycloserine and 5 mM 20HpβCD in Ham F-12 for 1 h to induce cholesterol depletion. After these treatments, the rate of SM synthesis was determined by incubating treated cells with Ham F-12 containing 105 µg/ml cycloserine and 1 µCi/ml [¹⁴C]palmitic acid for 1 h. The activity of [¹⁴C]SM was determined by scintillation counting after TLC.

To inhibit the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, fibroblasts were incubated for 1 h with 20 µM mevinnolin [35]. Cellular cholesterol depletion was then effected by a 1 h exposure of cells to 5 mM 20HpβCD in Ham F-12, which also contained 20 µM mevinnolin. The rate of SM synthesis was determined by incubating treated cells with Ham F-12 containing 20 µM mevinnolin and 1 µCi/ml [¹⁴C]palmitic acid for 1 h. The radioactivity of [¹⁴C]SM was determined by scintillation counting after TLC.

To study the effect of monensin on the synthesis of SM in control and cholesterol-depleted cells, these were first exposed to 1 µM monensin in Ham F-12 for 30 min. Then cells were treated with 1 µM monensin and 5 mM 20HpβCD for 30 min, after which the activity of SM synthesis was determined by labelling the cells for 1 h with 1 µCi/ml [¹⁴C]palmitic acid in Ham F-12, which also contained 1 µM monensin. The activity of [¹⁴C]SM was determined by scintillation counting after TLC.

Cellular distribution of newly synthesized [¹⁴C]SM

To determine the cellular distribution of newly synthesized [¹⁴C]SM, its susceptibility to degradation by SMase was determined in fixed cells. Unlabelled cells were rinsed with PBS and incubated for 1 h in Ham F-12 containing either 5 mM 20HpβCD (cholesterol depletion) or 50 µg/ml cholβCD (cholesterol loading). After these treatments, cells were exposed for 2 h to 1 µCi/ml [¹⁴C]palmitic acid in Ham F-12. After rinsing the cells with PBS, these were fixed with 3.7% (v/v) formaldehyde for 15 min at 4 °C. The fixative was removed by extensive rinsing, after which the cells were exposed for 45 min to 0.1 unit/ml SMase. Control cells did not receive any SMase, but were incubated in serum-free medium for the same time as

the SMase-treated cells. The extent of [^{14}C]SM degradation was determined after TLC analysis and scintillation counting.

Analysis of cellular lipids

Lipids were extracted from the cell dishes with hexane/2-propanol (3:2, v/v). After evaporation of the solvent, the lipids were dissolved in hexane/2-propanol and separated on silica-gel TLC plates. Phospholipids were eluted by chloroform/methanol/acetic acid/water (25:15:2, by vol.). The neutral lipids were eluted by hexane/diethyl ether/acetic acid (65:15:1, by vol.). The solvent system for separating ceramides was chloroform/methanol (93:7, v/v). Lipid spots were revealed by staining with iodine, and identified from standards run in parallel. The SM, PC, ceramide or cholesterol spots were scraped into scintillation vials. Scintillation cocktail (2.5 ml) was added and the radioactivity was measured with a liquid scintillation counter.

To determine the masses of SM, PC and cholesterol, these were separated from other lipid classes on high-performance TLC plates. The lipid spots were revealed by spraying the plates with cupric acetate (3%, w/v) in *o*-phosphoric acid (8%, v/v) and heating the plates for 15 min at 150 °C. Lipid masses were then quantified with a Camag Automated TLC Scanner and compared with standards run in parallel.

SPT assay

Confluent cells were incubated for 1 h at 37 °C in Ham F-12 (control), or in Ham F-12 containing either 5 mM 2OH β CD or 50 $\mu\text{g}/\text{ml}$ chol β CD. The activity of the enzyme SPT was then measured as described by Merrill [36]. The cells were scraped from 100-mm culture dishes into 0.5 ml of a buffer containing 50 mM Hepes, 10 mM EDTA and 5 mM dithiothreitol. After a brief sonication of the cell suspension, 50 μl of the cell homogenate (150–200 μg of protein) was added to 50 μl of a reaction mixture containing 200 mM Hepes, 5 mM EDTA, 10 mM DTT and 50 μM pyridoxal 5-phosphate. The tubes were then incubated for 10 min at 37 °C. The reaction was started by the addition of palmitoyl-CoA (0.2 mM final concentration) and [^3H]serine (1 mM final concentration, 130000 d.p.m./nmol). After a 10 min incubation, the reaction was stopped by the addition of 1.5 ml of chloroform/methanol (1:2, v/v), 0.5 ml of 0.5 M NH_4OH and 25 μg of sphingosine (as the carrier). The products were extracted, and the organic phases were then washed twice with water and evaporated, before products were analysed by scintillation counting.

Protein determination and cell viability

The amount of cellular protein was determined by the method of Lowry et al. [37], with BSA as standard. Trypan Blue exclusion was measured in cells that had been exposed to either 2OH β CD or chol β CD. Cells were resuspended in PBS and Trypan Blue was added. The suspension was then analysed microscopically for uptake of dye and for the number of cells.

RESULTS

Effect of cellular cholesterol level on the incorporation of [^{14}C]palmitic acid into SM, PC and ceramide

To investigate whether the rate of SM synthesis in cells was responsive to their level of cholesterol, we manipulated cellular cholesterol levels using 2OH β CD (depletion) or chol β CD (loading), and determined the rate of SM synthesis using [^{14}C]palmitic acid as precursor. The 2OH β CD and chol β CD treatments did not result in any loss of cell number or viability, as

determined by Trypan Blue exclusion. The number of cells was $590000 \pm 24400/\text{dish}$ (mean $\pm$ S.D.), and the percentage of cells permeable to Trypan Blue was less than 4%, irrespective of the treatment. When [^3H]cholesterol-labelled fibroblasts were exposed to 2OH β CD (5 mM) for different periods of time (Figure 1), there was a substantial efflux of cell-derived [^3H]cholesterol to the medium. After 1 h of depletion, as much as 24% of the total cell-associated [^3H]cholesterol was removed. The gradual depletion of cellular [^3H]cholesterol also led to a gradual stimulation of [^{14}C]palmitic acid incorporation into SM, as shown in Figure 1(B). The depletion of about 24% of cellular [^3H]cholesterol led to a 70–75% increase in [^{14}C]palmitic acid incorporation into SM, as compared with control cells. A similar increase in the rate of SM synthesis was observed in cholesterol-depleted cells when [^3H]serine was used as the metabolic precursor instead of [^{14}C]palmitic acid (results not shown). Depletion of cellular cholesterol did not result in an increased incorporation of [^{14}C]palmitic acid into [^{14}C]ceramides (Table 1) or into neutral glycosphingolipids (results not shown), but it decreased [^{14}C]palmitic acid incorporation into [^{14}C]PC [Table 1; qualitatively similar results for PC synthesis were seen when [^3H]oleic acid was used as precursor instead of [^{14}C]palmitic acid (results not shown)]. Cholesterol depletion of the cells had no effect on the rate of incorporation of [^3H]oleic acid into triglycerides (Table 1). As a result of the increased synthesis of SM in cholesterol-depleted fibroblasts, the mass of cellular SM was increased by about 24% 24 h after the 2OH β CD treatment (Table 2).

In order to load the cells with cholesterol, these were incubated with chol β CD inclusion complexes. Cells exposed for 60 min to chol β CD (50 $\mu\text{g}/\text{ml}$ cholesterol) contained 42% more cholesterol than control cells (Table 2). When the rate of SM synthesis was determined in cholesterol-loaded cells using either [^{14}C]palmitic acid (Table 1) or [^3H]serine (results not shown), SM synthesis was reduced by 20–30%, as compared with control cells. The incorporation of [^{14}C]palmitic acid into [^{14}C]ceramide and [^3H]oleic acid into [^3H]triglycerides was also decreased in cholesterol-loaded cells (Table 1). Cholesterol loading stimulated both [^{14}C]palmitic acid and [^3H]oleic acid incorporation into PC (results not shown for latter), and slightly increased PC mass in the cells (Table 2).

Rate of SM synthesis in cells exposed to apoA-I/POPC vesicles

Exposure of cells to apoA-I/POPC vesicles is known to induce cholesterol efflux [38]. To test whether cholesterol depletion induced by exposure of cells to apoA-I/POPC also led to activation of SM synthesis, the following experiment was performed. [^3H]Cholesterol-labelled fibroblasts were exposed for 17 h to 20 μg of protein/ml of apoA-I/POPC vesicles. After this treatment, cells were pulsed for 3 h with [^{14}C]palmitic acid. About 17% of the cell-associated [^3H]cholesterol was found in the medioperiod (Table 3). Exposure of cells to apoA-I/POPC vesicles stimulated [^{14}C]palmitic acid incorporation into SM by about 31% (Table 3). A parallel experiment using 2OH β CD to induce cholesterol efflux (1 h exposure) led to 25% efflux of [^3H]cholesterol, and to a stimulation of SM synthesis by 56% (Table 3). This experiment shows that cholesterol efflux from cells leads to the activation of SM synthesis, irrespective of the means of cholesterol removal from the cells.

Cholesterol depletion does not stimulate SM synthesis in L-cycloserine-treated cells

L-Cycloserine is an effective inhibitor of SPT, the first enzyme in the pathway leading to SM synthesis [34,35]. Since the synthesis

Figure 1 $[^3\text{H}]$ Cholesterol efflux and SM synthesis in fibroblasts exposed to 20Hp β CD

HSFs, prelabelled with $[^3\text{H}]$ cholesterol, were exposed to 20Hp β CD (5 mM) for the indicated periods of time. $[^3\text{H}]$ Cholesterol efflux to the medium was measured by scintillation counting of the cell-free medium (**A**). The amount of cell-associated $[^3\text{H}]$ cholesterol was determined from the total cellular lipid extract. To study SM synthesis, the cells were exposed to 20Hp β CD for the indicated periods of time, and then pulse-labelled with 1 $\mu\text{Ci}/\text{ml}$ $[^{14}\text{C}]$ palmitic acid for 6 h. The formation of $[^{14}\text{C}]$ SM (**B**) was determined from the total cellular lipid extracts by TLC and scintillation counting. In (**B**), 100% is equivalent to 949 d.p.m./mg of protein per h. Values are averages $\pm$ S.D. from three experiments.

Table 1 Effect of cholesterol depletion or loading on lipid synthesis

Cells were treated for 1 h in serum-free medium with or without 5 mM 20Hp β CD (cholesterol depletion) or 50 $\mu\text{g}/\text{ml}$ chol β CD (loading). After treatment, the rate of SM, PC and ceramide synthesis was determined by pulsing the cells for 6 h with 1 $\mu\text{Ci}/\text{ml}$ $[^{14}\text{C}]$ palmitic acid in serum-free medium. The rate of triglyceride synthesis was determined by pulsing the cells with $[^3\text{H}]$ oleic acid in serum-free medium for 3 h. The samples were analysed by TLC and scintillation counting. Values shown are percentages of the control (d.p.m./mg of protein per h) and are averages $\pm$ S.D. from three experiments, and significance was determined by Student's *t* test (*, $P < 0.05$).

	$[^{14}\text{C}]$ Ceramide	$[^{14}\text{C}]$ SM	$[^{14}\text{C}]$ PC	$[^3\text{H}]$ Triglyceride
Control	100 $\pm$ 6 (675)	100 $\pm$ 7 (886)	100 $\pm$ 10 (5273)	100 $\pm$ 6 (7172)
20Hp β CD	103 $\pm$ 3	170 $\pm$ 2*	80 $\pm$ 7*	91 $\pm$ 6
Chol β CD	61 $\pm$ 5*	77 $\pm$ 3*	144 $\pm$ 5*	59 $\pm$ 2*

Table 2 The effect of CyD treatment on SM, PC and cholesterol mass

Confluent fibroblasts were incubated with or without 20Hp β CD or chol β CD for 1 h. Total cellular SM and PC mass were determined by TLC and scanning densitometry in cells that were incubated for 24 h in Ham F-12 containing 2% (v/v) serum after the depletion/loading treatment. The mass of cholesterol was determined by TLC and scanning densitometry in extensively rinsed cells immediately after the depletion/loading treatment. Data shown are nmol/mg of protein, percentages of control values are given in parentheses, and are means from three different experiments $\pm$ S.D. Significance was determined by Student's *t* test (*, $P < 0.05$).

	Cholesterol mass	SM mass	PC mass
Control	77 $\pm$ 1 (100)	17 $\pm$ 1.2 (100)	98 $\pm$ 4 (100)
20Hp β CD	64 $\pm$ 4 (83)*	21 $\pm$ 1 (124)*	89 $\pm$ 5 (91)
Chol β CD	109 $\pm$ 2 (142)*	16 $\pm$ 1.1 (94)	111 $\pm$ 7 (113)*

of SM was increased in cholesterol-depleted cells, we examined if treatment of cells with L-cycloserine could abolish completely the activation of SM synthesis. The cells were incubated for 1 h with or without L-cycloserine before and during treatment with

Table 3 Effect of cholesterol depletion on SM synthesis in fibroblasts

Cells were cholesterol-depleted with or without 5 mM 20Hp β CD for 1 h or 20 μg of protein/ml of apoA-I/POPC vesicles for 17 h. Control cells were incubated for the same time under the same conditions as the treated cells. The synthesis of SM was measured by pulsing the cells for 3 h with 1 $\mu\text{Ci}/\text{ml}$ $[^{14}\text{C}]$ palmitic acid after the depletion period. The extent of $[^3\text{H}]$ cholesterol depletion (given as % efflux to the medium) was determined in cells that had been prelabelled with $[^3\text{H}]$ cholesterol for 72 h (total radioactivity in cholesterol and medium was 448 980 $\pm$ 30 100 d.p.m./dish). Values are averages $\pm$ S.D. from three experiments. Significance was determined with Student's *t* test (*, $P < 0.05$).

	$[^3\text{H}]$ Cholesterol efflux (% of total d.p.m.)	Synthesis of $[^{14}\text{C}]$ SM (% of control (d.p.m./mg of protein per h))
Control	2.3 $\pm$ 0.18	100 $\pm$ 7 (907 $\pm$ 63)
20Hp β CD	24.9 $\pm$ 1*	162 $\pm$ 9*
Control	2 $\pm$ 0.1	100 $\pm$ 9 (949 $\pm$ 85)
ApoA-I/POPC	17.3 $\pm$ 1*	131 $\pm$ 9*

20Hp β CD (1 h, 5 mM). After cholesterol depletion, cells were pulse-labelled with $[^{14}\text{C}]$ palmitic acid (1 h) and the amount of label in SM was determined. Cells that received both L-cycloserine and 20Hp β CD had incorporated the same amount of $[^{14}\text{C}]$ palmitic acid into SM as cells that received only L-cycloserine (Table 4). Cells treated with only 20Hp β CD contained 70% more radioactive label in SM compared with non-depleted cells. This experiment shows that *de novo* synthesis of SM was activated in cholesterol-depleted cells, because inhibition of the formation of 3-oxosphinganine completely abolished the incorporation of $[^{14}\text{C}]$ palmitic acid into SM.

The activity of SPT is affected by the cellular cholesterol level

To investigate the activity of SPT in a cell-free homogenate, the assay developed by Merrill [36] was used. The incorporation of $[^3\text{H}]$ serine into 3-oxosphinganine was shown to be a curvilinear function of time during the first 20 min of the assay (Figure

Table 4 Effect of L-cycloserine on the activation of SM synthesis in cholesterol-depleted cells

Cells were cholesterol-depleted by exposure to 20Hp β CD (5 mM) for 1 h. Those cells that were treated with L-cycloserine (CS; 105 μ g/ml) received the drug 1 h before and during the cholesterol depletion. After the cholesterol depletion, cells were pulse-labelled with 1 μ Ci/ml [14 C]palmitic acid for 1 h (with or without cycloserine). The amount of [14 C]SM formed was analysed by TLC and scintillation counting. Control cells were incubated under the same conditions as the treated cells. Values are averages $\pm$ S.D. from three experiments, and significance was determined with Student's *t* test (*, *P* < 0.05).

	[14 C]SM formed [% of control (d.p.m./mg protein per h)]
Control	100 $\pm$ 6 (675 $\pm$ 42)
20Hp β CD	203 $\pm$ 3*
CS	59 $\pm$ 10*
CS + 20Hp β CD	53 $\pm$ 11*

2A). On the basis of this time course, we elected to use a 10 min assay period for further experiments. To study the effect of cellular cholesterol level on the activity of SPT, cells were first exposed to 20Hp β CD (cholesterol depletion) or chol β CD (chol-

esterol loading) for 1 h, and then the activity of SPT was measured in cell-free homogenates of the treated cells. In cholesterol-depleted cells, the activity of SPT was increased by 30 %, whereas the activity was decreased by 15 % in cholesterol-loaded cells (Figure 2B). These changes in the activities of SPT were in qualitative agreement with the observed rates of [14 C]-palmitic acid incorporation into SM in intact cells, after corresponding treatments.

The susceptibility of newly synthesized SM to degradation by SMase

To study the cellular distribution of the newly synthesized SM, its susceptibility to degradation by SMase in fixed cells was examined. In these experiments, the cells were first depleted of cholesterol (5 mM 20Hp β CD for 1 h) or loaded with cholesterol (50 μ g/ml chol β CD for 1 h), and then labelled with [14 C]palmitic acid for 2 h. After fixing the cells with 3.7 % (v/v) formaldehyde, SM was degraded by SMase (0.1 unit/ml, 45 min incubation). In control cells (no cholesterol depletion or loading), 60 % of the [14 C]SM could be degraded by SMase. In cholesterol-depleted cells, 76 % more [14 C]SM was formed in comparison with

Figure 2 Activity of SPT in cholesterol-depleted or cholesterol-loaded cells

The activity of SPT was determined in a cell-free homogenate assay. (A) shows the incorporation of [3 H]serine into [3 H]-3-oxosphinganine as a function of time in a control cell extract. Values are given as pmol of product formed per mg of total cell protein in the assay mixture. (B) shows the activity of SPT in cell homogenates from cholesterol-depleted or cholesterol-loaded cells. The cellular cholesterol content was altered by exposing the cells for 1 h to either 5 mM 20Hp β CD or 50 μ g/ml of cholesterol in the form of chol β CD. The SPT assay was performed for 10 min using 150 μ g of total cell protein. In (B), 100 % is equivalent to 7.5 pmol of product/min. Values are averages $\pm$ S.D. from three experiments.

Table 5 Susceptibility of newly synthesized SM to degradation with SMase

Cells were incubated in serum-free medium with or without 5 mM 20Hp β CD or 50 μ g/ml chol β CD for 1 h. After the CyD treatment, cellular lipids were labelled in serum-free medium containing 1 μ Ci/ml [14 C]palmitic acid for 2 h. After fixing the cells for 15 min with 3.7 % (v/v) formaldehyde in PBS they were exposed to 0.1 unit/ml SMase in Ham F-12 for 45 min. Control cells were incubated under the same conditions without SMase. The amount of labelled SM was determined by TLC and scintillation counting. Values are averages $\pm$ S.D. from three separate experiments. Student's *t* test was performed to determine the significance of the results (*, *P* < 0.05).

Treatment	[14 C]SM		
	Synthesized [d.p.m./mg of protein per h (% of control)]	Resistant (d.p.m.)	% degraded (d.p.m.)
Control	1012 $\pm$ 63 (100)	401 $\pm$ 21	60 (612)
20Hp β CD	1811 $\pm$ 84 (179)*	612 $\pm$ 63*	66 (1202)
Chol β CD	475 $\pm$ 2 (47)*	253 $\pm$ 33*	48 (232)

Table 6 Effects of drugs on SM synthesis in cholesterol-depleted cells

The effect of monensin and mevinolin on the activation of SM synthesis in cholesterol-depleted cells was determined. The cells were treated with or without mevinolin for 1 h or monensin for 30 min (in serum-free medium). Then cells were exposed to 5 mM 20Hp β CD and the drug [mevinolin (1 h) or monensin (30 min)]. SM was labelled with 1 μ Ci/ml [14 C]palmitic acid for 2 h (monensin-treated cells, 1 h) with the drug present during the pulse. Values, given as percentages of control values, are averages $\pm$ S.D. from three experiments. For control experiments, 100% is equivalent to 943 d.p.m./mg protein per h. Significance of results was determined by Student's *t* test (*, $P < 0.05$, compared with control value).

Drug	No treatment	20Hp β CD
Control	100 $\pm$ 3	176 $\pm$ 4*
Mevinolin (20 μ M)	101 $\pm$ 2	171 $\pm$ 9*
Control	100 $\pm$ 5	158 $\pm$ 0.5*
Monensin (1 μ M)	94 $\pm$ 7	106 $\pm$ 5

untreated cells, and about 66% of all newly synthesized [14 C]SM was degraded by the subsequent treatment with SMase (Table 5). Consequently, most of the newly synthesized [14 C]SM had been transported to the outer leaflet of the PM before the cells were fixed. In cholesterol-loaded cells, only 48% of the newly synthesized [14 C]SM was susceptible to degradation by SMase, suggesting either that less [14 C]SM was transported to the PM or that cholesterol enrichment of the PM somehow affected the susceptibility of SM to degradation by SMase.

Effect of mevinolin on the activation of the SM synthesis in cholesterol-depleted cells

Mevinolin is an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase [39], and it was used in this study to test whether synthesis of cholesterol is necessary for the activation of SM synthesis in cholesterol-depleted cells. Cells were first pre-incubated with 20 μ M mevinolin for 1 h, before the cells were depleted of cholesterol with 20Hp β CD. This concentration of mevinolin completely blocked the incorporation of [14 C]acetate into cholesterol (results not shown). After the depletion, the cells were pulse-labelled with [14 C]palmitic acid in the presence of mevinolin, and the formation of [14 C]SM was determined. As shown in Table 6, the inhibition of cholesterol synthesis with mevinolin did not affect the activation of [14 C]SM synthesis in cholesterol-depleted cells.

SM synthesis in cholesterol-depleted cells was affected by drugs known to interfere with Golgi function

Monensin is known to interfere with the vesicular transport through the Golgi apparatus [40,41]. In this study, monensin at 1 μ M concentration was shown to completely inhibit the activation of [14 C]SM formation in cholesterol-depleted cells (Table 6). Our finding that monensin inhibits SM formation in cholesterol-depleted cells agrees well with a previous report, which showed that monensin inhibits SM synthesis in baby hamster kidney cells [19].

DISCUSSION

In this study, we have used CyDs as tools to rapidly manipulate the level of cholesterol in cultured cells. Using 20Hp β CD, it was possible to induce a dramatic reduction in cell-surface cholesterol (24% in 60 min). Exposure of cells to CyDs is likely to also

remove lysophospholipids and fatty acids, if present in the membrane [42]. Using preformed chol β CD inclusion complexes, it was possible to induce cholesterol loading of cells. Fibroblasts exposed to 50 μ g/ml chol β CD for 60 min contained 42% more unesterified cholesterol than control cells. This cholesterol was most likely inserted initially into the PM, but it has been shown that it can be metabolized, i.e. esterified by the cells [43], and can also down-regulate cholesterol biosynthesis (P. Leppimäki and J. P. Slotte, unpublished observations).

The results in this study show clearly that changes in cellular cholesterol levels had marked effects on the homeostasis of SM. When the cellular cholesterol level was reduced using 20Hp β CD, the incorporation of [14 C]palmitic acid into SM increased significantly. A similar enhanced incorporation of [14 C]palmitic acid into SM was seen when cholesterol depletion was effected using apoA-I/POPC vesicles, suggesting that the depletion vehicle did not make a difference. Cholesterol-depleted cells also showed an increase in SM mass 24 h after the depletion treatment, indicating that the activated synthesis also led to more SM mass in the cells. Since both [14 C]palmitic acid and [3 H]serine incorporation into SM were increased by cholesterol depletion, and because there was no additional [14 C]SM synthesized in cells pretreated with L-cycloserine, it was apparent that the *de novo* synthesis of SM was stimulated. This finding was further verified by the observation that the activity of SPT was increased in cell-free homogenates from cholesterol-depleted cells. It appeared that most, if not all, of the 3-oxosphinganine that was formed by the SPT reaction was shuttled into SM, since we observed no increased accumulation of [14 C]palmitic acid in neutral glycosphingolipids after cholesterol depletion. The activities of other enzymes in the SM synthesis cascade have not been examined in this study, therefore it is possible that an increased activity of enzymes other than SPT, e.g. acyl-CoA: sphingosine N-acetyltransferase and/or ceramide: phosphatidylcholine phosphocholinetransferase, could be partly responsible for the accumulation of SM in cholesterol-depleted cells. Because mevinolin, which is an inhibitor of β -hydroxy- β -methylglutaryl-CoA reductase [39], had no effect on the SM synthesis in cholesterol-depleted cells, we concluded that cholesterol synthesis was not necessary for the activation of SM synthesis. The observation that cellular cholesterol depletion did not increase the incorporation of fatty acids into other lipid classes, e.g. PC, glycosphingolipids and triglycerides, suggests that the effects observed were specific for SM synthesis.

In cells loaded with cholesterol, the rate of [14 C]palmitic acid incorporation into SM was decreased and the activity of SPT was diminished, as compared with cells with an unaltered cholesterol level.

Cholesterol loading also decreased the incorporation of [3 H]oleic acid into triglycerides, whereas the synthesis of PC was stimulated. Cholesterol loading is known to stimulate PC synthesis in macrophages [44–46] and, in this respect, our loading of fibroblasts with cholesterol from chol β CD inclusion complexes yielded similar results for PC synthesis. It is also known that cholesterol loading from liposomes stimulated [14 C]acetate incorporation into PC in rat smooth-muscle cells [47]. The decreased synthesis of triglycerides in cholesterol-loaded cells most probably resulted from a shortage of free fatty acids available for triglyceride synthesis, because both cholesterol esterification and PC synthesis were increased in these cells.

Although the enzyme initially activated by cholesterol depletion, SPT, has an ER location [23], SM is not synthesized in the same compartment. SM appears to be synthesized in at least two different compartments in cells, namely the *cis*- and medial-Golgi compartments [17,18], and in recycling endosomes [22].

SM is efficiently transported to the cell surface, where most of it appears in the exoleaflet of the PM [19,20]. In control cells, about 60 % of all cellular SM could be degraded using SMase. When SM synthesis was activated by cholesterol depletion (with 2OHp/CD), about 66 % of the newly synthesized [14 C]SM was susceptible to hydrolysis, suggesting that most of the newly made SM had been transported to the exoleaflet of the PM compartment. In cholesterol-loaded cells, a larger fraction of the synthesized SM was retained inside the cells, unavailable to SMase at the cell surface. These results suggest that manipulation of cholesterol levels in the PM not only affects the rate of SM synthesis in fibroblasts, but also affects the subcellular distribution of SM.

The univalent ionophore, monensin, which is known to interfere with vesicular transport through the Golgi apparatus, has been shown to inhibit the synthesis of SM in baby hamster kidney cells by up to 40 % [21]. We have shown in this study that monensin could completely block the activation of SM synthesis in cholesterol-depleted fibroblasts. This finding is consistent with the idea that monensin blocks the transport of ceramide through the Golgi apparatus [21,40,41], because synthesis of *de novo* SM would utilize ceramide produced in the ER.

In conclusion, our results show clearly that changes in the cellular content of cholesterol, as effected by CyDs, have dramatic effects on the synthesis rates of choline-containing phospholipids. Further studies are warranted to elucidate the mechanism(s) of regulation.

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