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Reversible effects of sphingomyelin degradation on cholesterol distribution and metabolism in fibroblasts and transformed neuroblastoma cells

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Plasma-membrane sphingomyelin appears to be one of the major determinants of the preferential allocation of cell cholesterol into the plasma-membrane compartment, since removal of sphingomyelin leads to a dramatic redistribution of cholesterol within the cell [Slotte & Bierman (1988) Biochem. J. **250**, 653–658]. In the present study we examined the long-term effects of sphingomyelin degradation on cholesterol redistribution in cells and determined the reversibility of the process. In a human lung fibroblast-cell line, removal of 80% of the sphingomyelin led to a rapid and transient up-regulation (3-fold) of acyl-CoA:cholesterol acyltransferase (ACAT) activity, and also, within 30 h, to the translocation of about 50% of the cell non-esterified cholesterol from a cholesterol oxidase-susceptible compartment (i.e. the cell surface) to oxidase-resistant compartments. At 49 h after the initial sphingomyelin degradation, the cell sphingomyelin level was back to 45% of the control level, and the direction of cell cholesterol flow was toward the cell surface, although the original distribution was not achieved. In a transformed neuroblastoma cell line (SH-SY5Y), the depletion of sphingomyelin led to a similarly rapid and transient up-regulation of ACAT activity, and to the translocation of about 25% of cell-surface cholesterol into internal membranes (within 3 h). The flow of cholesterol back to the cholesterol oxidase-susceptible pool was rapid, and a pretreatment cholesterol distribution was reached within 20–49 h. Also, the re-synthesis of sphingomyelin was faster in SH-SY5Y neuroblastoma cells and reached control levels within 24 h. The findings of the present study show that the cellular redistribution of cholesterol, as induced by sphingomyelin degradation, is reversible and suggest that the normalization of cellular cholesterol distribution is linked to the re-synthesis of sphingomyelin.

INTRODUCTION

Non-esterified cholesterol plays a pivotal role both as a structural component and as a regulator of enzymic activities in cellular membranes [for reviews, see Green (1977) and Yeagle (1985)]. Although the quantitative distribution of non-esterified cholesterol between various cellular organelles has been a subject of argument (Lange & Ramos, 1983; van Meer, 1987), many lines of evidence suggests that the bulk of non-esterified cellular cholesterol is located in plasma membranes (Lange & Ramos, 1983; J. P. Slotte, G. Hedström & M. I. Pörn, unpublished work).

The asymmetric distribution of cholesterol between membranes of intact cells may be related to the similarly asymmetric distribution of different phospholipid classes, since sphingomyelin, which is predominantly located at the cell surface (Steck & Wallach, 1970; Colbeau *et al.*, 1971), appears to interact more favourably with cholesterol than the other phospholipid classes (Wattenberg & Silbert, 1983; Lund-Katz *et al.*, 1988).

Experimental evidence has been produced to support the view that sphingomyelin may dramatically affect the cellular distribution of cholesterol. Gatt & Bierman (1980) showed elegantly that the introduction of sphingomyelin mass to cultured cells markedly increased the rate of sterol biosynthesis, suggesting that sphingomyelin effectively removed cholesterol from the intracellular regulatory pool. It has further been shown that degradation of sphingomyelin from cells leads to an equally dramatic decrease in the rate of sterol biosynthesis (Slotte & Bierman, 1988).

It was also found that sphingomyelin degradation led to a marked increase in the endogenous esterification of plasma-membrane-derived cholesterol (Slotte & Bierman, 1988). The increased up-regulation of esterification activity was a result of the flow of cholesterol from plasma membranes into intracellular compartments, including the endoplasmic reticulum. Within 2 h of sphingomyelin degradation, about 30% of the non-esterified cholesterol in fibroblasts had been translocated from a cholesterol oxidase-susceptible compartment (i.e. proximal to the cell surface) to a resistant pool (i.e. intracellular pools), suggesting that sphingomyelin degradation led to a general redistribution of cholesterol within the cell (Slotte *et al.*, 1989*a*,*b*).

Previous studies on the effects of sphingomyelin degradation on cell cholesterol homoeostasis have been short-term experiments and have mainly focused on the acute effects of the treatment. The objective of the present study was to examine long-term effects of sphingomyelin degradation on cell cholesterol distribution and metabolism. For a comparison, we have performed the studies on two widely different cell types. The human fibroblast was used as a cell type with a fairly low turnover rate with regard to cholesterol metabolism, whereas a human neuroblastoma tumour-cell line (SH-SY5Y) was chosen for its higher rate of cholesterol biosynthesis and turnover.

EXPERIMENTAL

Cell culture

Human lung fibroblasts were obtained from the Statens Bakteriologiska Laboratorium (Stockholm, Sweden). Human neuroblastoma cells (SH-SY5Y) were generously given to our Department by the Department of Pathology, University of

Abbreviation used: ACAT, acyl-CoA: cholesterol acyltransferase.

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Uppsala, Uppsala, Sweden. Both cell lines were cultivated in Dulbecco's modified Eagle medium supplemented with 9% (v/v) fetal-calf serum, 10 mM-Hepes, pH 7.4, 2 mM-L-glutamine, 1 mM non-essential amino acids, penicillin (100 i.u./ml) and streptomycin (100 μg/ml). Cells for experiments were plated in 35 mm-diameter cell-culture dishes, with incubation media volumes between 1.0 and 1.5 ml/dish.

Incorporation of [^{3}H]oleic acid into cholesterol [^{3}H]oleate

The incorporation of [^{3}H]oleic acid (du Pont–New England Nuclear; 9 Ci/mmol) into cholesteryl [^{3}H]oleate in lung fibroblasts and SH-SY5Y cells was monitored either during a continuous exposure of cells to [^{3}H]oleic acid (up to 90 min) or in 30 min pulses at various times after sphingomyelinase treatment.

In continuous exposures, cells were incubated for up to 90 min at 37 °C with or without sphingomyelinase (50 munits/ml), together with [^{3}H]oleic acid (0.5 μCi/ml) (complexed to albumin) in Hams F12 medium without serum.

With the pulse protocol, cells were exposed for 60 min to sphingomyelinase (50 munits/ml) (in Hams F12 medium without serum), rinsed once with Dulbecco's phosphate-buffered saline, pH 7.5, containing 5 mM-EDTA, and twice with plain phosphate-buffered saline, and finally post-incubated for up to 49 h in serum-free Dulbecco's medium. At various time intervals during the post-incubation, sphingomyelinase-treated cells were pulsed for 30 min with 2.0 μCi of [^{3}H]oleic acid/ml. The incorporation of tracer into cholesteryl [^{3}H]oleate was determined from the cell lipid extract.

Labelling of cells with [^{3}H]cholesterol

Human lung fibroblasts or SH-SY5Y neuroblastoma cells in 35 mm-diameter dishes were cultured in appropriate growth medium for 4 days. Cells were then incubated for 2 days in growth medium supplemented with 9% fetal-calf serum containing [^{3}H]cholesterol (dissolved in ethanol; 5–10 μCi/ml of serum; du Pont–New England Nuclear; 60 Ci/mmol). The cells were finally incubated for 3–5 h in serum-free Dulbecco's medium before the experiments were performed. With this labelling procedure, fibroblasts and SH-SY5Y cells contained less than 3 and 7% of esterified [^{3}H]cholesterol respectively.

Oxidation of cell cholesterol

The distribution of non-esterified cell [^{3}H]cholesterol between cholesterol oxidase-susceptible and -resistant pools was determined in cells at various time intervals after the degradation of cell sphingomyelin with sphingomyelinase. Cells, pre-labelled with [^{3}H]cholesterol, were exposed for 60 min to 50 munits of sphingomyelinase/ml, rinsed, and post-incubated for up to 48 h in serum-free Dulbecco's medium. At various time intervals, cells were taken to determine the fraction of cell [^{3}H]cholesterol that was available for oxidation by cholesterol oxidase (from *Streptomyces* sp., Calbiochem). Cells were fixed (10 min at 4 °C) with 1% glutaraldehyde in phosphate-buffered saline, rinsed extensively, and exposed for 45 min at 37 °C to 0.4 unit of cholesterol oxidase/ml. The extent of [^{3}H]cholesterol oxidation to [^{3}H]cholestenone was determined from the cell lipid extract.

Esterification of [^{3}H]cholesterol

The esterification of membrane-derived [^{3}H]cholesterol in sphingomyelinase-treated lung fibroblasts and SH-SY5Y cells was determined from the lipid extract of the same cells that were used for cholesterol-oxidation studies (detailed in the previous paragraph).

Degradation of [*choline*-^{3}H]sphingomyelin

To determine the amount of cell sphingomyelin that was degradable by exogenously added sphingomyelinase, cellular sphingomyelin and phosphatidylcholine were labelled with [*methyl*-^{3}H]choline chloride (Amersham International; 75 Ci/mmol) as follows. Confluent cells were exposed to 2 μCi of [*methyl*-^{3}H]choline chloride/ml (28 μM final choline concentration) for 48 h. Cells were then rinsed (3 × 2 ml) with phosphate-buffered saline and fixed for 10 min at 4 °C with 1% glutaraldehyde in phosphate-buffered saline. After the fixation, cells were rinsed (3 × 2 ml) and exposed to 50 munits of sphingomyelinase/ml (from *Staphylococcus aureus*; Sigma) in phosphate-buffered saline for up to 40 min. The cellular content of [^{3}H]sphingomyelin in control and treated cells was determined by t.l.c. and densitometric scanning of the total lipid extract.

Phospholipid analysis

Total cell lipids from untreated or sphingomyelinase-treated fibroblasts or SH-SY5Y neuroblastoma cells were extracted with hexane/propan-2-ol (3:2, v/v; 2 × 2 ml/dish for 20 min each). The organic solvent was evaporated and the total lipids dissolved in chloroform and spotted on to heat-activated Kiselgel 60 t.l.c. plates (Merck). The plates were developed with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.; Skipski *et al.*, 1964), air-dried and stained with cupric acetate (3%, w/v) in phosphoric acid (8%, v/v). The colour was developed by heating the plates for 30 min at 150 °C. Standards of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were run on parallel lanes. The absorbances of the three major phospholipid classes on the plates were determined with an LKB laser scanning densitometer.

Cholesterol analysis

^{3}H-labelled sterols from cell extracts were separated on normal-phase t.l.c. sheets (Kodak Chromagram), using hexane/diethyl ether/acetic acid (260:60:3, by vol.) as developing solvent. Lipid spots were detected with I_2 staining. Spots for [^{3}H]cholesterol (R_F 0.15–0.20), [^{3}H]cholestenone (R_F 0.25–0.35) and [^{3}H]cholesterol esters (R_F 0.91–0.95) were identified from standards run in parallel. The appropriate spots were marked, the I_2 stain was removed and the spots were cut into scintillation vials. The radioactivity was counted in an LKB RackBeta liquid-scintillation counter.

The mass of free and esterified cholesterol in total lipid extracts from cells was determined fluorimetrically as described by Heider & Boyett (1978).

Protein

Cell protein mass in unfixed cells was determined by the method of Lowry *et al.* (1951), as described previously (Slotte & Bierman, 1988), with BSA as standard.

RESULTS

Cellular cholesterol mass and phospholipid composition

A characteristic feature of many tumour cell lines is the unregulated and high rate of cholesterol biosynthesis (Siperstein, 1970; Chen *et al.*, 1978). The neuroblastoma cells used in this study had a 4-fold higher rate of [^{14}C]acetate incorporation into [^{14}C]sterols synthesized *de novo* under serum-free conditions as compared with the fibroblast-cell line (results not shown). The masses of cellular total sterols in these two cell lines were also quite different, with SH-SY5Y neuroblastoma having considerably less sterol mass (20 ± 3 μg) per mg of cell protein than the fibroblasts (38 ± 4 μg/mg). About 6 and 9% of the cholesterol

Table 1. Phospholipid distribution among the major phospholipid classes in human lung fibroblasts and SH-SY5Y neuroblastoma cells

Confluent cells in 35 mm-diameter cell-culture dishes were kept in serum-free Dulbecco's medium for 4–6 h before extraction of total lipids. The polar lipids were separated by t.l.c., and the relative concentrations of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were measured by laser scanning densitometry. Values are averages (±S.D.) from two different determinations with $n = 4–6$.

		Composition (% of the total of the three phospholipid classes)	
Phospholipid class	Cell line...	Human lung fibroblasts	SH-SY5Y cells
Sphingomyelin		15±1	5±1
Phosphatidylcholine		45±3	55±4
Phosphatidylethanolamine		40±3	40±3

mass was esterified in SH-SY5Y neuroblastoma cells and fibroblasts respectively.

The overall phospholipid compositions of these two cell lines were further compared. The phospholipid distribution among the three major phospholipid classes (i.e. phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) was determined, and these classes comprised more than 90% of the total phospholipid mass in the cells. In both cell types phosphatidylcholine and phosphatidylethanolamine made up the bulk of the phospholipids (Table 1). The sphingomyelin content was found to be markedly higher in fibroblasts compared with SH-SY5Y neuroblastoma cells, being 15 and 5% (w/w), respectively (Table 1).

Sphingomyelin degradation

In order to measure accurately the extent of sphingomyelin degradation in cells after exposure to exogenous sphingomyelinase, the cellular choline-containing phospholipids were labelled to a constant specific radioactivity (48 h) with [*methyl*-^{3}H]choline before the experiments. A 40 min exposure of glutaraldehyde-fixed cells to 50 munits of sphingomyelinase/ml led to degradation of approx. 60% of the [^{3}H]sphingomyelin in neuroblastoma cells and 80% in fibroblasts (Fig. 1). The extent of sphingomyelin degradation was similar in unfixed cells (results not shown). The lesser degradation of sphingomyelin observed in neuroblastoma cells would suggest that a larger fraction of the cellular sphingomyelin in SH-SY5Y neuroblastoma cells was distributed among intracellular membranes. By analogy, it appears that most of the sphingomyelin in fibroblasts was located at the cell surface, consistent with previous reports on sphingomyelin distribution in fibroblasts (Lange *et al.*, 1989).

Effects of sphingomyelin degradation on cholesterol esterification

We have previously shown that degradation of plasma-membrane sphingomyelin in fibroblasts results in a dramatic and rapid activation of the endogenous cholesterol esterification reaction (Slotte & Bierman, 1988; Slotte *et al.*, 1989*a*,*b*). To compare the sphingomyelinase-induced increases in cholesterol ester formation in human lung fibroblasts and neuroblastoma cells, confluent cells were incubated with [^{3}H]oleic acid and the formation of cholesterol [^{3}H]oleate was determined. The sphingomyelinase-induced acute increase in [^{3}H]oleic acid incorporation into cholesterol [^{3}H]oleate was about 4-fold higher (90 min after

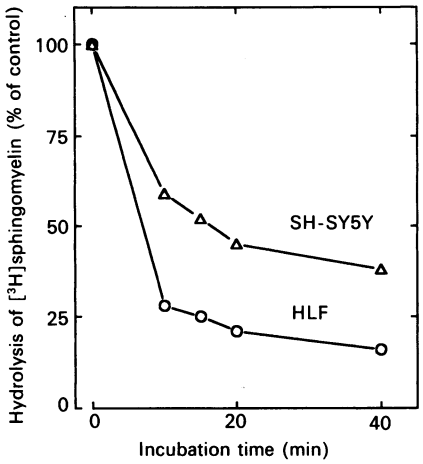

Fig. 1. Degradation of [*choline*-^{3}H]sphingomyelin by sphingomyelinase in fixed cells

The choline-containing phospholipids in lung fibroblasts (HLF, ○) or SH-SY5Y neuroblastoma (△) cells were labelled with [^{3}H]choline for 48 h. After a post-incubation period in serum-free media, the cells were fixed (1% glutaraldehyde) and exposed to 50 munits of sphingomyelinase/ml for the indicated periods of time. The content of [*choline*-^{3}H]sphingomyelin in the cellular total lipid extract was determined, and values for treated cells are expressed relative to the control cells [control values: 2000 and 12000 c.p.m. (±10%)/dish for fibroblasts and neuroblastoma cells respectively]. Values presented are means for duplicate dishes (± ⩽ 10%) from one representative experiment out of three.

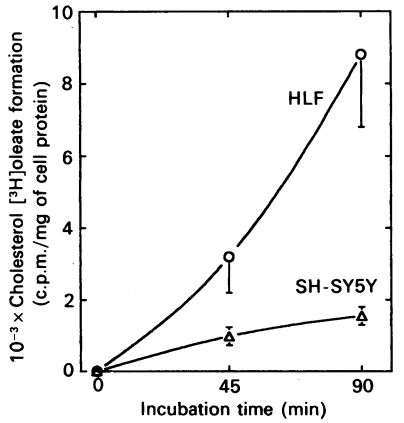

Fig. 2. Effects of sphingomyelin degradation on [^{3}H]oleic acid incorporation into cholesterol [^{3}H]oleate

Confluent lung fibroblasts (HLF, ○) or SH-SY5Y neuroblastoma cells (△) were exposed at 37 °C to 0.5 μCi of [^{3}H]oleic acid/ml± 50 munits of sphingomyelinase/ml and incubated for up to 90 min. The incorporation of [^{3}H]oleic acid into cholesterol [^{3}H]oleate was determined from the neutral-lipid extract. Values represent net increases of tracer incorporation in sphingomyelinase-treated cells (tracer incorporation in untreated cells has been subtracted for each time point). The values were further normalized to cell protein content (averages+S.D., with $n = 6$).

sphingomyelin degradation) in fibroblasts compared with SH-SY5Y neuroblastoma cells (Fig. 2). A similar difference in rates of cholesterol esterification was observed when the esterification of plasma-membrane-derived [^{3}H]cholesterol was determined in fibroblasts and SH-SY5Y neuroblastoma cells under comparable conditions (curves not shown).

The long-term effects of sphingomyelinase treatment on the esterification of plasma-membrane-derived [^{3}H]cholesterol were

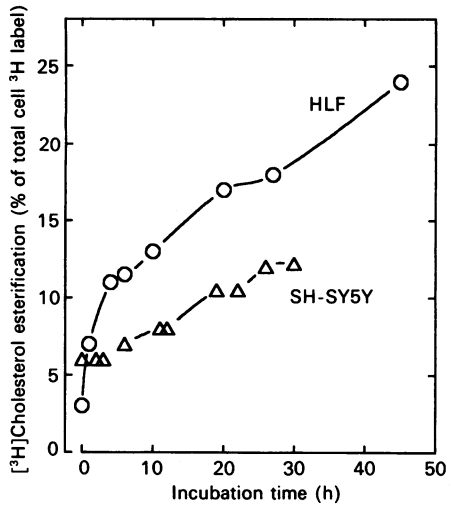

Fig. 3. Effects of sphingomyelin degradation of long-term esterification of membrane-derived [³H]cholesterol

Confluent human lung fibroblasts (HLF, ○) and SH-SY5Y neuroblastoma cells (△) were labelled with [³H]cholesterol as described in the text. Cells were then exposed for 60 min to 50 munits of sphingomyelinase/ml, rinsed, and post-incubated in serum-free Dulbecco's medium for up to 45 h. At time intervals, cells were taken for lipid analysis. The conversion of [³H]cholesterol into [³H]cholesterol ester was determined. Values from one representative experiment (out of five) are presented. Total [³H]sterol represented about 10^5 c.p.m./dish. Each point is the mean for duplicate dishes (range less than 5%).

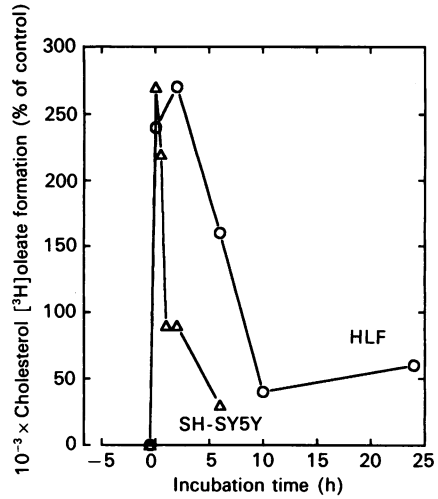

Fig. 4. Apparent ACAT activity in cells at various times after sphingomyelin degradation

Confluent lung fibroblasts (HLF, ○) and SH-SY5Y neuroblastoma cells (△) were exposed for 60 min to 50 munits of sphingomyelinase/ml and post-incubated in serum-free Dulbecco's medium for up to 24 h. At indicated time intervals, cells were pulsed for 30 min with 2 μCi of [³H]oleic acid/ml and harvested. The incorporation of [³H]oleic into cholesterol [³H]oleate was determined as described in the text. The Figure shows values from one representative experiment (out of three), where each point is the mean for duplicate dishes. Values are given as percentage increases in [³H]oleic acid incorporation in sphingomyelinase-treated cells over control cells [total counts for control cells were about 500 and 1500 c.p.m. (±10%)/dish for neuroblastoma cells and fibroblasts respectively].

determined in cells prelabelled with [³H]cholesterol for 48 h. The amount of cell-associated [³H]cholesterol esters increased dramatically in fibroblasts during the first few hours after the degradation of sphingomyelin (Fig. 3). The rate of [³H]cholesterol ester formation decreased somewhat at later time points, but the amount of [³H]cholesterol esters continued to increase throughout the incubation period (Fig. 3). In neuroblastoma cells the sphingomyelin degradation led to a long-term increase in [³H]cholesterol ester formation that was markedly lower than that seen in fibroblasts, and the rate of which was unchanged over the entire incubation period.

To determine the long-term effects of sphingomyelin degradation on the apparent activity of acyl-CoA:cholesterol acyltransferase (ACAT), cells were shortly exposed to sphingomyelinase and then exposed to 30 min pulses of [³H]oleic acid at various time points during the subsequent post-incubation period. The initial degradation of cellular sphingomyelin led to rapidly increased [³H]oleic acid incorporation into cholesterol [³H]oleate, which was maximal 1 and 3 h after the onset of sphingomyelinase exposure in SH-SY5Y neuroblastoma and fibroblast cells respectively (Fig. 4). Within about 7 h of the initial exposure to sphingomyelinase, the apparent activity of ACAT in SH-SY5Y neuroblastoma cells had returned close to the pre-treatment basal level. In fibroblasts the decrease in the apparent ACAT activity was slower, and only approached the basal level 10 h into the post-incubation period (Fig. 4)

Effects of sphingomyelin degradation on cholesterol distribution within the cells

Degradation of sphingomyelin has previously been shown to induce a marked redistribution of cellular cholesterol from the cell surface to intracellular membranes (Slotte *et al.*, 1989*a*). The sphingomyelinase-induced translocation of cell-surface cholesterol to intracellular compartments is also consistent with the observable stimulation of the endogenous esterification reaction (cf. Slotte & Bierman, 1988; Slotte *et al.*, 1989*a*,*b*; the present study).

To test for the long-term effects of sphingomyelin degradation on cholesterol distribution in the two cell types the cholesterol oxidase susceptibility of membrane [³H]cholesterol was determined in cells exposed to sphingomyelinase and post-incubated without the enzyme for various time periods. Within 2 h of sphingomyelin degradation, the amount of [³H]cholesterol susceptible to oxidation by cholesterol oxidase had decreased by 25% in both cell types (Figs. 5 and 6). In human lung fibroblasts the fraction of cell [³H]cholesterol that was susceptible to oxidation continued to decrease for another 30 h before it started to increase again (Fig. 5). In SH-SY5Y neuroblastoma cells, however, the fraction of cell [³H]cholesterol that was susceptible to oxidation by cholesterol oxidase started to increase again after the first 2 h, and reached the original level after about 49 h of post-incubation in serum-free medium (Fig. 6).

The complete recovery of the original cellular cholesterol distribution in SH-SY5Y neuroblastoma cells was accompanied by a complete recovery of sphingomyelin mass (inset to Fig. 5). With fibroblasts, neither the recovery of oxidizability of cell cholesterol nor the recovery of sphingomyelin mass was complete at the end of the experimental period, although partial recovery of both parameters was observed (Fig. 6 and its inset).

DISCUSSION

The purpose of the present study was to elucidate the long-term effects of sphingomyelin degradation on cholesterol flow in two different cell types: the fibroblast-cell type and the transformed SH-SY5Y neuroblastoma-cell line. The SH-SY5Y neuroblastoma-cell type was chosen as a model for a cell with an elevated metabolic turnover rate. Its rate of cholesterol bio-

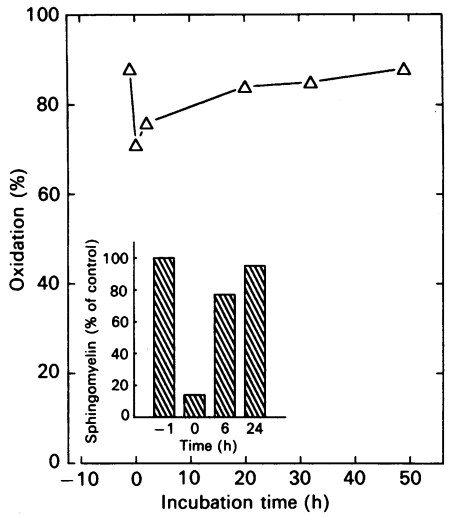

Fig. 5. Oxidation of [^{3}H]cholesterol and re-synthesis of sphingomyelin in SH-SY5Y cells after initial treatment with sphingomyelinase

Confluent SH-SY5Y neuroblastoma cells, prelabelled with [^{3}H]cholesterol, were exposed for 60 min to 50 munits of sphingomyelinase/ml, rinsed, and post-incubated in serum-free Dulbecco's medium for up to 49 h. The oxidizability of [^{3}H]cholesterol and the cellular content of sphingomyelin mass were determined at indicated time intervals as described in the text. Oxidation is defined as [^{3}H]cholestenone/[^{3}H]cholestenone plus [^{3}H]cholesterol × 100% [about (7–9) × 10^4 c.p.m./dish]. Each point represents the mean value for duplicate dishes (range less than 5%) from one representative experiment out of four. The mass of sphingomyelin (inset) is given relative to the amount present in untreated cells.

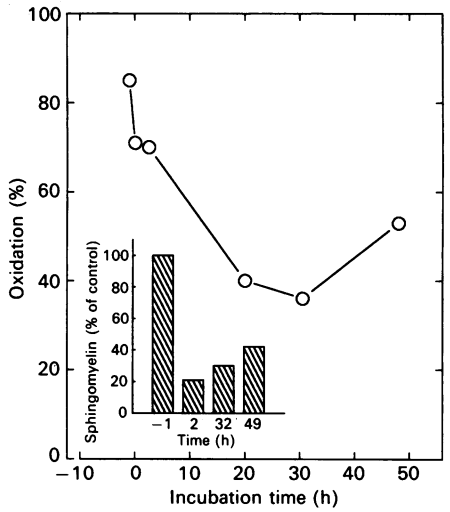

Fig. 6. Oxidation of [^{3}H]cholesterol and re-synthesis of sphingomyelin in human lung fibroblasts after initial treatment with sphingomyelinase

Confluent human lung fibroblasts, prelabelled with [^{3}H]cholesterol, were treated exactly as described for SH-SY5Y cells (Fig. 5), and the oxidizability of [^{3}H]cholesterol and sphingomyelin mass were determined at time intervals after the initial sphingomyelinase treatment. Each point is the mean value for duplicate dishes (range less than 5%) from one representative experiment out of three. The mass of sphingomyelin (inset) is given relative to the amount present in untreated cells.

synthesis from acetate was at least 4-fold higher than the comparable rate in fibroblasts.

Lipid analysis of the cells revealed that the SH-SY5Y neuroblastoma-cell type had a significantly lower sterol mass (per mg of protein) than the fibroblast-cell type. The relative sphingomyelin content was also only one-third of that found in fibroblasts. In SH-SY5Y neuroblastoma cells, about 60% of the cell sphingomyelin could be degraded by exogenously added sphingomyelinase, whereas the corresponding degradable fraction of sphingomyelin in fibroblasts was 80%. Since exogenously added sphingomyelinase degrades sphingomyelin at the cell surface, it can be concluded that a substantial fraction of the sphingomyelin in SH-SY5Y neuroblastoma cells was located in membranes of intracellular location. Our finding that 80% of the fibroblast sphingomyelin mass could be degraded by exogenously added sphingomyelinase is consistent with other reports showing that plasma membranes of fibroblasts contain about 90% of the total cell sphingomyelin mass (Lange *et al.*, 1989).

The low relative content of sphingomyelin mass in SH-SY5Y neuroblastoma cells as well as the finding that a substantial fraction of the sphingomyelin was resistant to sphingomyelinase may explain the observation that sphingomyelin degradation led to such a small increase in cholesterol ester formation in this cell type. Although the transient up-regulation of ACAT activity was of a similar magnitude in SH-SY5Y neuroblastoma cells to that seen in fibroblasts, the activity of ACAT returned to the basal level more quickly and resulted in lower cholesterol ester formation.

The transient nature of the up-regulation of the activity of ACAT after the degradation of cell-surface sphingomyelin would indicate that the initial sphingomyelinase-induced cholesterol translocation into intracellular membranes was fast and rapidly completed. This interpretation seems to be true at least for the SH-SY5Y neuroblastoma-cell type, since both the transient ACAT up-regulation and the translocation of cell-surface cholesterol into the cells were reversed within about 3 h. In SH-SY5Y neuroblastoma cells a normal (i.e. pre-treatment level) distribution of cholesterol was achieved somewhere between 20 and 49 h after the initial sphingomyelinase treatment.

In the fibroblast model the equation seems to be more complicated. The transient up-regulation of the activity of ACAT peaked at about 3 h and approached the basal level after about 10 h. Cell-surface cholesterol still continued to flow into intracellular compartments up to about 30 h after the initial sphingomyelin degradation (as determined from the oxidizability of cell-surface cholesterol). The finding that the apparent activity of ACAT did not quite reach the basal level may result from the continuing flow of cell-surface cholesterol into the substrate pool of the enzyme.

In previous short-term incubations with fibroblasts, it was observed that a maximum of about 25–30% of the cell-surface cholesterol was translocated to intracellular membranes subsequent to the sphingomyelin degradation (Slotte *et al.*, 1989*a*,*b*). However, the present study shows that, if the cells are given enough time, at least the fibroblast cell type appears to be able to translocate about 50% of its non-esterified cholesterol to intracellular and cholesterol oxidase-resistant compartments. It is remarkable that the cell can redistribute such a marked amount of its cholesterol from the cell surface and still remain apparently viable over a long period of time in serum-free medium.

The kinetics of the processes that lead to the translocation of cell-surface cholesterol to intracellular membranes after the degradation of sphingomyelin were totally different in the fibroblasts and the neuroblastoma cells. This difference may relate to different capacities of the two cell lines to handle the rapid surge of cholesterol flow that is induced by the degradation of cell sphingomyelin. The differences in translocation kinetics may also relate to differences in membrane properties, since the rates of cholesterol release from plasma membranes to extra-

cellular acceptors is known to be cell-specific and dependent on the protein composition of the membranes (Bellini *et al.*, 1984). A similar difference in membrane protein composition of the endoleaflet of the plasma membrane may exist for the two different cell types.

Another important aspect of this study is the finding that the sphingomyelinase-induced cholesterol redistribution appears to be reversible within a reasonable time span. The recovery of a normal cholesterol distribution was faster and almost complete in the SH-SY5Y neuroblastoma-cell system, whereas the (partial) recovery in fibroblasts took longer. In both cell types the recovery of cholesterol distribution appeared to be linked to a resynthesis of sphingomyelin mass. This finding again points to the important role of sphingomyelin as a modulator of cholesterol distribution and metabolism in cells.

Many studies with model systems have demonstrated that there appears to exist a preferential interaction between sphingomyelin and cholesterol (Damen *et al.*, 1981; Wattenberg & Silbert, 1983; Clejan & Bittman, 1984; Fugler *et al.*, 1985; Yeagle & Young, 1986; Lund-Katz *et al.*, 1988), although contradictory evidence also has been reported (Calhoun & Shipley, 1979; Schroeder & Nemecz, 1989). Whether or not cholesterol in plasma membranes of intact cells interacts preferentially with sphingomyelin, it is quite clear from the present results and from previous reports on the subject (Gatt & Bierman, 1981; Slotte & Bierman, 1988; Slotte *et al.*, 1989*a*) that sphingomyelin is a major determinant of the apparent distribution of cholesterol in intact cells.

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