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Effects of sphingomyelin degradation on cell cholesterol oxidizability and steady-state distribution between the cell surface and the cell interior

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This study addresses questions related to (i) the distribution of cholesterol between the cell surface and intracellular membranes in cultured fibroblasts and (ii) the effects of plasma membrane sphingomyelin on this distribution. Cholesterol oxidase (*Streptomyces* sp.) converts cell cholesterol to cholestenone and was used in this study to probe the cellular distribution of cholesterol. The availability of cell cholesterol for oxidation by cholesterol oxidase was markedly influenced by the presence of sphingomyelin. In native, glutaraldehyde-fixed fibroblasts only about 20% of the cell cholesterol was oxidized under our experimental conditions. However, degradation of cell sphingomyelin with sphingomyelinase (*Staphylococcus aureus*) markedly enhanced the oxidation of cell surface cholesterol in glutaraldehyde-fixed fibroblasts. About 90% of the total unesterified cholesterol could be oxidized to cholestenone in confluent, sphingomyelin-depleted fibroblasts. These results suggest that about 90% of the unesterified cholesterol was at the cell surface in these cells. It was also observed that degradation of cell sphingomyelin exerted a dramatic effect on the distribution of cell cholesterol between the cell surface and intracellular membranes. Within 90 min after hydrolysis of cell sphingomyelin, about 30% of the total cell-associated unesterified cholesterol was transported from a cholesterol oxidase-susceptible pool to an oxidase-resistant pool. Together with the redistribution of cell cholesterol after sphingomyelin degradation, a marked enhancement of the endogenous cholesterol esterification reaction was observed. We conclude that the degradation of plasma membrane sphingomyelin resulted in a new apparent steady-state distribution of cellular cholesterol, with less cholesterol in the plasma membrane and more in intracellular membranes. It therefore appears that sphingomyelin is a major determinant of the distribution of cholesterol in intact cells.

Introduction

Unesterified cholesterol is a major structural and functional component in many types of biological membranes. During recent years a great deal of research has been focused on questions related to the intracellular and intermembrane distribution of unesterified cholesterol [1–7]. Experimental data suggest that a substantial fraction of the cellular unesterified cholesterol is located in plasma membranes, although there are controversies about the interpretation of some of the data [8,9]. The distribution of phospholipid classes between cellular membranes [2–4] and also within the plasma membrane bilayer itself [1] is asymmetric. In many cell types phosphatidylcholine and sphingomyelin are enriched in plasma membranes and are found in lower abundances in endomembranes, with aminophospholipids making up the balance [1,4,10]. Some evidence exists to indicate that sphingomyelin might to some extent affect the distribution of unesterified cholesterol within the cells [3,4,7,11,12].

In a recent paper it was shown that degradation of fibroblast sphingomyelin led to an upregulation of the activity of acetyl-CoA : cholesterol acyltransferase (ACAT), thereby increasing the esterification of cholesterol [7]. The degradation of sphingomyelin in fibroblasts also led to a decreased incorporation of [^{14}C]acetate into C_{27}-sterols, indicating that this treatment resulted in a net flow of plasma membrane cholesterol into the putative intracellular regulatory pool of cholesterol [7].

The objective of this study was to measure how much plasma membrane cholesterol was lost from the cell surface after the degradation of cell sphingomyelin with

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sphingomyelinase. We used the enzyme cholesterol oxidase to probe the distribution of cholesterol between the oxidase-susceptible pool (i.e., the cell surface) and the oxidase-resistant pool (undefined intracellular compartments) [8,13]. Since the degradation of plasma membrane sphingomyelin was assumed to have marked effects on cholesterol dynamics in the membrane bilayer, we also characterized the effects of this treatment on the availability of cholesterol for oxidation by cholesterol oxidase.

Experimental

Materials

[^{3}H]Cholesterol (more than 98% pure; 60 Ci/mmol) and [^{3}H]oleic acid (9 Ci/mmol) were obtained from Du Pont New England Nuclear. Cholesterol (99% +), oleic acid (99%), sphingomyelin (99%), dipalmitoylphosphatidylcholine (DPPC; 99%), dipalmitoylphosphatidic acid (DPPA; 99%), *p*-hydroxyphenylacetic acid, phosphorylcholine, horseradish peroxidase, and sphingomyelinase (*Staphylococcus aureus*) were from Sigma, St. Louis, MO. Cholesterol oxidase (*Streptomyces* sp.) was purchased from CalBiochem, La Jolla, CA. Glutaraldehyde (ultra pure) was obtained from Merck, F.R.G. Dulbecco's modified Eagle medium (MEM) and growth supplements were obtained from NordVacc (Stockholm). Fetal calf serum was purchased from Gibco. All other chemicals were of reagent grade.

Cell culture

Human skin fibroblasts were obtained from skin biopsies of healthy volunteers (kindly provided by the Department of Medical Chemistry, University of Turku). Cells were cultured in Dulbecco's modified Eagle medium (Dulbecco's MEM) supplemented with 10% fetal calf serum. Cells for experiments were seeded in 30 mm diameter cell culture dishes (at about 50000 cells/dish) and were grown to confluency or pre-treated prior to experiments as described separately in the legends to the figures.

Labeling of the cellular cholesterol pool with [^{3}H]-cholesterol

Human skin fibroblasts in 30 mm diameter dishes were grown in Dulbecco's MEM supplemented with 10% fetal calf serum for 4 days. Cells were then kept for 2 days in a growth medium containing 10% fetal calf serum with unesterified [^{3}H]cholesterol (5–10 μCi/ml serum). The cells were finally incubated for 24 h in a serum-free Dulbecco's MEM prior to the experiments. With this labeling procedure, cells contained less than 2% esterified [^{3}H]cholesterol at the start of the experiment.

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

Cells to be used for [^{3}H]oleic acid incorporation into cholesterol esters were kept for 24 in serum-free Dulbecco's MEM prior to the experiments. Cells were incubated for 30 min at 37°C in 1 ml/dish serum-free HAM's F-12 medium (with 20 mM Hepes, pH 7.2) supplemented with 0.5 μCi [^{3}H]oleic acid (20 μM, complexed to bovine serum albumin; see Ref. 14) and sphingomyelinase (0–100 mU/ml). The incubation was stopped by rapidly chilling the cells on ice. The dishes were then rinsed with ice-cold phosphate-buffered saline (pH 7.4) and stored frozen (−20°C) until lipid analysis.

Oxidation of cell cholesterol

After treatment of cells with sphingomyelinase (varying concentrations or time), the plasma membrane cholesterol was oxidized by treatment of cells with cholesterol oxidase. The cells were first rinsed with ice-cold phosphate-buffered saline, kept on ice and fixed for 10 min (4°C) with 1% glutaraldehyde in phosphate-buffered saline [8,13]. The fixative was removed and the cells rinsed (2 × 2 ml) with ice-cold phosphate-buffered saline. Then 1.0 ml phosphate-buffered saline containing 1 U/ml cholesterol oxidase and 0.1 U/ml sphingomyelinase was added and each dish was incubated for 30 min at 37°C on a water bath. At the end of the incubation, the dishes were chilled, rinsed with phosphate-buffered saline and stored frozen (−20°C) until further analysis.

Determination of the release of cellular phosphorylcholine

Cells labeled with [^{3}H]cholesterol were kept in serum-free Dulbecco's MEM 24 prior to the experiments. To determine the release of phosphorylcholine and the change in cholesterol oxidizability after treatment with sphingomyelinase, the dishes were rinsed extensively with chilled phosphate-buffered saline (3 × 2 ml) and the cells incubated for different periods of time at 37°C with 1.0 ml phosphate-buffered saline containing 4 mU of sphingomyelinase. At indicated time intervals, the medium containing released phosphorylcholine was removed and saved, whereas the cells (on ice) were washed once with phosphate-buffered saline containing 5 mM EDTA. The oxidation of cell [^{3}H]cholesterol was performed as described previously.

The phosphorylcholine released into the medium (phosphate-buffered saline) was converted to choline by treatment with alkaline phosphatase. In short, 0.1 ml 100 mM glycine buffer with 1 mM $MgCl_2$ (pH 10.2) was added to 0.5 ml of the medium together with 1 U/ml of alkaline phosphatase and the reaction mixture was incubated at 37°C for 30 min. Then the pH was lowered to 8.0 by addition of 0.5 ml 0.1 M Tris-HCl buffer containing 100 mU/ml choline oxidase, 1 U/ml horseradish peroxidase and 0.63 mg/ml *p*-hydroxy-

phenylacetic acid. The oxidation of choline was allowed to proceed at 37°C for 60 min. The fluorescence of the conjugated form of *p*-hydroxyphenylacetic acid was determined with exitation at 325 nm and emission at 415 nm on a Hitachi F-4000 spectrofluorimeter. Fluorimetric read-outs were converted to concentrations from a standard curve prepared from choline chloride (or phosphorylcholine) treated exactly as described for the samples. With these reaction conditions more than 95% of the phosphorylcholine was converted to free choline and was subsequently oxidized by choline oxidase. Values are expressed as nanomoles of phosphorylcholine released from cells per mg cell protein.

Oxidation of [^{3}H]cholesterol in lipid vesicles

Mixed vesicles containing varying proportions of sphingomyelin and DPPC and with fixed amounts of [^{3}H]cholesterol and DPPA were prepared from lyophilized lipids by ultrasonic treatment in phosphate-buffered saline. Lipids from stock solutions in chloroform were pipetted into disposable glass tubes, the organic phase was evaporated under a stream of nitrogen at room temperature, and 1.0 ml phosphate-buffered saline was added. The tubes were mixed on a Vortex mixer and subjected to ultrasonic treatment in an MSE Ultrasonic Disintegrator supplied with a titanium probe. The lipid emulsion was sonicated for 10 min at about 45°C to yield a clear solution. The vesicles contained either 5 μM [^{3}H]cholesterol, 1 μM sphingomyelin, 19 μM DPPC and 1 μM DPPA (vesicle type I: cholesterol/sphingomyelin molar ratio 5:1); or 5 μM [^{3}H]-cholesterol, 20 μM sphingomyelin, and 1 μM DPPA (vesicle type II: cholesterol/sphingomyelin molar ratio 1:4).

To 0.1 ml of either type of vesicles was added 0.4 ml phosphate-buffered saline containing cholesterol oxidase (final concentration 10 mU/ml) and the reaction was allowed to proceed for 20 min at 37°C. The reaction was stopped by addition of 3 ml chloroform/methanol (2:1, v/v). The lipids were extracted and analyzed for [^{3}H]cholesterol and [^{3}H]cholestenone content. Values are averages from three different incubations with two different batches of vesicles.

Lipid extraction

Cellular neutral lipids were extracted with hexane/isopropanol (3:2, v/v) as previously described [7]. [^{3}H]Cholesterol and [^{3}H]cholestenone from the aqueous media were extracted with chloroform/methanol (2:1, v/v) as reported in Ref. 13.

Gas-liquid chromatography

Cell sterol mass in the neutral lipid extract was determined by gas-liquid chromatography. The sterols in the neutral lipid extract (one dish contained 2–3 μg unesterified sterol) were silylated in 100 μl bis(trimethylsilyl)trifluoroacetamide for 1 h at 70°C. One to two μl of the sample was injected on the Bonded SE 54 column and the chromatography was performed with hydrogen as the carrier gas, starting at a temperature of 270°C and ending at 295°C (temperature change 4 C°/min). 5-Cholesten-3β-ol-7-one (7-ketocholesterol) was used as an internal standard. The retention times were 7.6 min, 8.7 min and 11.1 min for cholesterol, cholestenone (4-cholesten-3-one), and 7-ketocholesterol, respectively. The integrated peak areas for each individual sterol species were calibrated against individual standards of known concentrations.

Thin-layer chromatography

^{3}H-labeled sterols from cell or phosphate-buffered saline extracts were separated by normal phase thin-layer chromatography (Kodak silica gel plates) using hexane/diethyl ether/acetic acid (130:30:1.5, v/v) 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]cholesteryl 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 cut into scintillation vials. The radioactivity was counted with 3 ml of a xylene-based scintillation cocktail in a LKB RackBeta liquid scintillation counter.

Protein

Cell proteins from unfixed-fixed cells were digested into 1.0 ml 0.1 M NaOH (60 min) and the concentration in an aliquot of the hydrolysate was determined according to Lowry et al. [15].

Results

Effects of sphingomyelinase treatment on cell cholesterol oxidation

Cholesterol oxidase was used in this study to probe the relative distribution of unesterified cholesterol between the cell surface (the cholesterol oxidase-susceptible pool) and intracellular sites (oxidase-resistant pools) in normal confluent fibroblasts. In agreement with previously published reports [16,17], we found that cholesterol in native, unfixed cells was not readily available for oxidation by externally added cholesterol oxidase (data not shown). When the cell monolayers were fixed in isotonic buffer with 1% glutaraldehyde and then subjected to oxidation by cholesterol oxidase, about 20% of the total cell unesterified cholesterol mass was oxidized to cholestenone (Table I). However, in cells treated with sphingomyelinase a much greater portion of the unesterified cholesterol was available for oxidation. Usually about 90% of the cell unesterified cholesterol was oxidizable in sphingomyelinase-treated cells (Table I). This finding is consistent with reports by

TABLE I

Susceptibility of cell cholesterol to oxidation by cholesterol oxidase

Human skin fibroblasts were fixed for 10 min in 1% glutaraldehyde at 4°C and were then exposed for 30 min at 37°C to either cholesterol oxidase alone (1 U/ml) or to cholesterol oxidase (1 U/ml) and sphingomyelinase (0.1 U/ml). The conversion of cholesterol to cholestenone was determined by gas-liquid chromatography of the neutral lipid extract. Values are given as averages of triplicate dishes from one representative experiment ± S.D.

Treatment	% Oxidation [a]
Fixed cells, no cholesterol oxidase	0 [b]
Fixed cells + cholesterol oxidase (1 U/ml)	20.4 ± 1.6
Fixed cells + cholesterol oxidase (1 U/ml) and sphingomyelinase (0.1 U/ml)	93.3 ± 0.5

[a] (mass of cholestenone/mass of cholestenone + cholesterol) × 100.
[b] Cholestenone not detectable.

Barenholz, Wagner and co-workers [16,18], who showed that phospholipase C-treatment of biological membranes renders their cholesterol available for oxidation by cholesterol oxidase. Since sphingomyelinase is a sphingomyelin-specific phospholipase C, it is likely that the effect on oxidation we observe in our system is similar to the observations made by Barenholz and co-workers in theirs.

The relationship between the amount of sphingomyelinase added and the amount of cell [^{3}H]cholesterol oxidized was not linear (Fig. 1). The results in Fig. 1 shows that only small amounts of sphingomyelinase (less than 10 mU/ml) was needed to achieve a dramatic effect on the oxidizability of cholesterol by cholesterol oxidase, suggesting that only partial degradation of sphingomyelin was needed to achieve this effect. This interpretation of the data was further substantiated in the time-course study shown in Fig. 2. In these experiments cells were exposed for different periods of time to a relatively low concentration of sphingomyelinase and the effects of sphingomyelin degradation on cholesterol oxidation was determined. It was observed that the degree of cholesterol oxidation increased most during the degradation of the first 7.5 nmol/mg of cell sphingomyelin, this representing about or slightly less than half of the total cell sphingomyelin (Fig. 2; cf. also Ref. 7).

Oxidation of cholesterol in model membranes with different phospholipid composition

Since the introduction of sphingomyelin degradation products (e.g., ceramides) into the bilayer might affect the availability of cholesterol for oxidation by cholesterol oxidase, the direct effects of sphingomyelin on cholesterol oxidation was examined in model membranes of known lipid compositions. Vesicles were prepared by sonication and contained [^{3}H]cholesterol in an either predominantly sphingomyelin (molar ratio of cholesterol to sphingomyelin 1:4) or DPPC-rich environment (Chol/Spm 5:1 molar ratio). The molar ratio of cholesterol to total phospholipids was the same in both vesicle types (1 to 4.2). The [^{3}H]cholesterol in sphingomyelin-rich vesicles was a poor substrate for cholesterol oxidase (27.5 ± 2.6% oxidation) whereas the [^{3}H]cholesterol in sphingomyelin-poor and DPPC-rich vesicles was highly susceptible for oxidation by the cholesterol oxidase (90.0 ± 2.5% oxidation). These results suggest that sphingomyelin by itself may prevent the oxidation by cholesterol oxidase of cholesterol in lipid bilayers.

Having established the relationship between the cholesterol–sphingomyelin interaction and the susceptibility of cholesterol for oxidation, we went further to actually determine the effects of plasma membrane sphingomyelin degradation on the distribution of unesterified cholesterol between cholesterol oxidase-susceptible and oxidase-resistant pools.

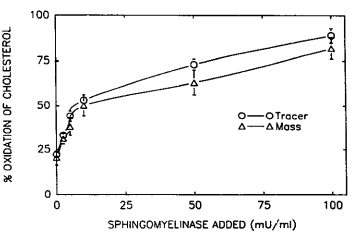

Fig. 1. Susceptibility of cell cholesterol for oxidation after treatment of cells with varying amounts of sphingomyelinase. Human skin fibroblasts in 30-mm diameter dishes were grown in Dulbecco's MEM supplemented with 10% fetal calf serum for 4 days. Cells to be labeled with [^{3}H]cholesterol were switched to a growth medium containing 10% fetal calf serum and unesterified [^{3}H]cholesterol (5–10 μCi/ml serum). Cells to be analyzed for sterol mass (gas-liquid chromatography) were switched to normal growth medium instead. After 48 h in the normal or [^{3}H]cholesterol supplemented growth medium the cells were incubated for 24 h in serum-free Dulbecco's MEM. Prior to the experiments cells were washed once with 2 ml phosphate-buffered saline, then 1.0 ml serum-free HAM's F-12 medium was added together with indicated amounts of sphingomyelinase. Cells were exposed to the sphingomyelinase for 20 min and the reaction stopped by wasing the cell monolayers with phosphate-buffered saline containing 5 mM EDTA. Cells were the fixed (10 min, 4°C, 1% glutaraldehyde in phosphate-buffered saline), rinsed (2×2 ml phosphate-buffered saline), and exposed for 30 min at 37°C to 1 U/ml of cholesterol oxidase in phosphate-buffered saline. The dishes were stored at −20°C until further analysis. Each point represents an average from at least six different dishes derived between at least two different and representative experiments (± S.D.).

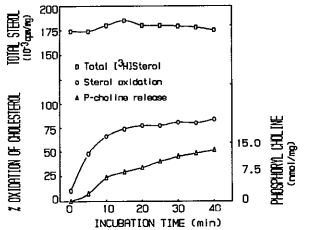

Fig. 2. Relationship between oxidation of cell cholesterol and release of phosphorylcholine after treatment with sphingomyelinase. Cells were grown in Dulbecco's MEM supplemented with 10% fetal calf serum for 4 days. The cells were then switched to a growth medium containing 10% fetal calf serum with unesterified [^{3}H]cholesterol (5–10 μCi/ml serum). After a 48 h incubation, the cells were kept in serum-free Dulbecco's MEM for another 24 h prior to the experiments. After rinsing the cells once with phosphate-buffered saline, 1.0 ml phosphate-buffered saline was added together with 4 mU/ml of sphingomyelinase. Cells were exposed to the sphingomyelinase for indicated time periods, then the reaction was stopped by rinsing cells with phosphate-buffered saline containing 5 mM EDTA. The incubation medium (phosphate-buffered saline) with the released phosphorylcholine was saved for further analysis whereas the cells were fixed (10 min, 4°C, 1% glutaraldehyde in phosphate-buffered saline), rinsed (2×2 ml phosphate-buffered saline), and exposed for 30 min at 37°C to 1 U/ml of cholesterol oxidase in phosphate-buffered saline. The dishes were stored at −20°C until lipid analysis. Values are averages ± S.D. of triplicate dishes from two independent experiments.

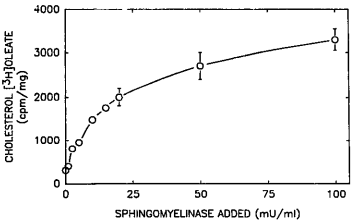

Fig. 3. Effects of sphingomyelinase on [^{3}H]oleic acid incorporation into cellular cholesteryl esters. Confluent cells were incubated for 24 h in serum-free Dulbecco's MEM prior to the experiment. After rinsing cells once with 2 ml phosphate-buffered saline, 1.0 ml serum-free HAM's F-12 medium was added together with indicated amounts of sphingomyelinase and 0.5 μCi [^{3}H]oleic acid (20 μM, complexed to bovine serum albumin). The cells were incubated for 30 min at 37°C, rinsed extensively with phosphate-buffered saline, and analyzed for the content of cholesteryl [^{3}H]oleate. Values are averages ± S.D. of triplicate dishes from a representative experiment.

Effects of sphingomyelinase treatment on the flow of cholesterol from the plasma membrane into the cell

It was previously demonstrated that treatment of cells with sphingomyelinase resulted in an increased incorporation of oleic acid into cellular cholesteryl esters [7]. We are now able to confirm those results in a different laboratory and with a different fibroblast cell type (Fig. 3). Addition of increasing amounts of sphingomyelinase to cultured fibroblasts in the presence of [^{3}H]oleic acid resulted in a 'dose'-dependent increase of [^{3}H]oleic acid incorporation into cellular cholesteryl [^{3}H]oleate. This finding again implies that addition of sphingomyelinase, via the degradation of sphingomyelin, resulted in a net flow of cholesterol from the cell surface into an intracellular site which provides ACAT with its sterol substrate.

In order to determine the amount of cell sterol that redistributed within the cell, subsequent to the degradation of sphingomyeline, the following approach was used. Native cells were exposed to 0.1 U/ml of sphingomyelinase for different periods of time (up to 4 h) and the distribution of unesterified cholesterol (or [^{3}H]cholesterol) between cholesterol oxidase susceptible and resistant pools was determined. The results are presented in Fig. 4. At time zero about 90% of the

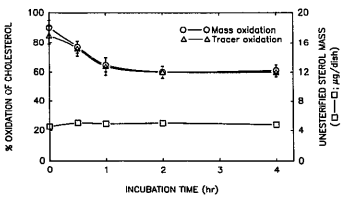

Fig. 4. Redistribution of cell surface cholesterol in cells treated with sphingomyelinase. Skin fibroblasts were grown in Dulbecco's MEM supplemented with 10% fetal calf serum for 4 days. Cells to be treated with radiolabeled cholesterol were switched to a growth medium containing 10% fetal calf serum with unesterified [^{3}H]cholesterol (5–10 μCi/ml serum). Cells to be analyzed for sterol mass (gas-liquid chromatography) were switched to normal growth medium instead. After 48 h in the regular or [^{3}H]cholesterol-supplemented growth medium the cells were incubated for another 24 h in serum-free Dulbecco's MEM. Prior to the experiments cells were washed once with 2 ml phosphate-buffered saline, then 1.0 ml serum-free HAM's F-12 medium was added together with 100 mU/ml sphingomyelinase. Cells were incubated for up to 8 h before the cell cholesterol was oxidized. At the end of each time period the cells were fixed (10 min, 4°C, 1% glutaraldehyde in phosphate-buffered saline), rinsed (2×2 ml phosphate-buffered saline), and exposed for 30 min at 37°C to 1 U/ml of cholesterol oxidase in phosphate-buffered saline. Lipids were extracted and analyzed for [^{3}H]cholesterol and [^{3}H]cholestenone (thin-layer chromatography), or for free cholesterol and cholestenone mass (gas-liquid chromatography). Each point represents an average from at least six different dishes divided between at least two different and representative experiments (± S.D.).

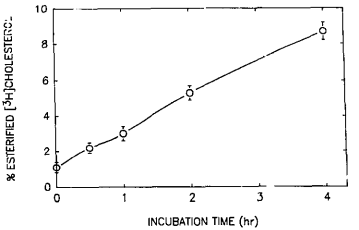

Fig. 5. Esterification of [^{3}H]cholesterol in cells treated with sphingomyelinase. The cells were labeled with [^{3}H]cholesterol and treated with sphingomyelinase as detailed in the legend to Fig. 4. The formation of [^{3}H]cholesteryl esters was determined from the lipid extract.

unesterified cholesterol was oxidized by cholesterol oxidase. The values for cholesterol oxidation were not significantly different whether they were derived from mass analysis of cholesterol and cholestenone (gas-liquid chromatography) or from tracer analysis of [^{3}H]cholesterol and [^{3}H]cholestenone.

With time the amount of cellular unesterified cholesterol that was oxidizable decreased in sphingomyelinase-treated cells, to reach a minimum of about 60% oxidation after 90 min (Fig. 4). The cellular distribution of cholesterol (or [^{3}H]cholesterol) between oxidase-susceptible and oxidase-resistant pools did not change further during prolonged incubations (up to 8 h; data not shown). The amount of cell-associated [^{3}H]sterol (data not shown) or the unesterified sterol mass did not change significantly during the experiment (Fig. 4), indicating that detectable amounts of sterols were not lost from cells during the experiments.

Treatment of [^{3}H]cholesterol-labeled cells with 0.1 U/ml of sphingomyelinase also led to a dramatically increased formation of [^{3}H]cholesteryl esters in these cells (Fig. 5). With the labeling procedure used in these experiments and with this particular cell type a linearly increasing incorporation of [^{3}H]cholesterol into [^{3}H]-cholesteryl esters was observed subsequent to the addition of sphingomyelinase.

Discussion

The molecular interactions between sphingomyelin and cholesterol is clearly different from the interaction between other classes of phospholipids and cholesterol. Although some controversy exists [19], several studies have implied that the cholesterol–sphingomyelin interaction is tighter or of a greater affinity than that of other cholesterol-phospholipid 'complexes' with comparable acyl chain compositions [3–5,20,21].

The results of the present study suggest that the cholesterol-sphingomyelin association in the plasma membranes of the fibroblast is so tight that it prevents or delays the oxidation of membrane-associated cholesterol by cholesterol oxidase. Only about 20% of the cellular cholesterol was oxidized in glutaraldehyde-fixed cells exposed for 30 min to a fairly high concentration of cholesterol oxidase. This protection against oxidation was lost when cells were exposed to sphingomyelinase, either prior to or simultaneously with exposure to the oxidase enzyme. More than 80% of the cell unesterified cholesterol was converted to cholestenone in sphingomyelin-depleted fibroblasts, suggesting that this portion of the total cell cholesterol was associated with the plasma membrane.

The same kind of 'protection' against oxidation that sphingomyelin appeared to exert towards fibroblast membrane cholesterol was also observed in model membranes. Cholesterol in sphingomyelin vesicles was a poor substrate for the cholesterol oxidase, whereas cholesterol in dipalmitoylphosphatidylcholine vesicles was readily available for oxidation by the enzyme. This finding is analogous to the reports by Moore et al. [18] and Patzer et al. [16] demonstrating that treatment of sphingomyelin-containing biological or model membranes with phospholipase C rendered the cholesterol in these membranes available for oxidation by cholesterol oxidase.

Lange and Ramos [8] have reported that more than 90% of the unesterified cholesterol in glutaraldehyde-fixed fibroblasts can be oxidized by cholesterol oxidase. We observed only about 20% oxidation of cholesterol in isotonically fixed fibroblasts. An almost complete oxidation of cholesterol was, however, obtained in sphingomyelin-depleted cells. It is therefore possible that the fixation of fibroblasts in hypotonic salt solution (as used by Lange and Ramos [8]) disrupts the cholesterol-sphingomyelin association in the plasma membranes of cells and thus makes the cholesterol available for oxidation, despite the presence of sphingomyelin in their cells.

In addition to having an effect on the availability of cholesterol for oxidation by cholesterol oxidase, sphingomyelinase-treatment of cells also had an effect on the distribution of cholesterol within the cell. Under normal growth conditions, the plasma membrane compartment is rich in cholesterol, despite the fact that plasma membrane sterols are continously transported from the cell surface into intracellular membranes [22]. However, when the association of cholesterol with sphingomyelin in the plasma membrane is disrupted (by sphingomyelinase treatment), the capacity of the plasma membrane to solubilize cholesterol decreased, and cholesterol started to flow from the plasma membranes into intracellular compartments (one of which was the endoplasmic reticulum). This was evidenced by the upregu-

lation of the ACAT activity and by the increased formation of cholesteryl ester mass (this study and Ref. 7). When the cellular distribution of cholesterol mass was probed with cholesterol oxidase, it was observed that within 90 min after sphingomyelinase treatment, about 30% of the cell cholesterol had been transferred from an oxidase-susceptible pool (i.e., plasma membrane) to an oxidase-resistant pool.

We do not have data which would allow us to draw conclusions about a possible mechanism that could explain how cholesterol was transferred from the plasma membrane into the intracellular organelles. The transfer could be due to an increased spontaneous desorption into and diffusion through the cytosol, perhaps mediated by cytosomal sterol carrier proteins [23,24], or it could be due to a sphingomyelinase-induced microvesiculation of the plasma membrane on the cytosolic side of the bilayer, as is suggested to occur in red blood cells with sphingomyelinase treatment [25]. Further work is clearly needed to elucidate the operating mechanism(s) in cultured cells.

It is evident that sphingomyelin, by its ability to strongly associate with cholesterol, at least partly can determine the distribution of cholesterol within the cell. Fairly small alterations in plasma membrane phospholipid composition may result in dramatic changes in the steady-state distribution of cholesterol within the cell, as this study showed. It is therefore plausible to assume that endogenous phospholipases in general and sphingomyelinases in particular could affect the fluxes of cholesterol between cell organelles and in so doing also regulate the metabolism and utilization of cholesterol in living cells.

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