Localization of cholesterol in sphingomyelinase-treated fibroblasts

M. Isabella PÖRN*† and J. Peter SLOTTE

Department of Biochemistry and Pharmacy, Åbo Akademi University, BioCity, Turku, Finland

The distribution of cellular unesterified cholesterol was studied in fibroblasts, which had been depleted of plasma membrane sphingomyelin by exposure to exogenous sphingomyelinase. This treatment has previously been shown to induce an increase in cholesterol esterification, a decrease in the biosynthesis of cholesterol, and a decreased susceptibility of cell cholesterol to oxidation with cholesterol oxidase. When the cellular localization of cholesterol was studied with fluorescent filipin staining, sphingomyelin depletion did not cause any visible changes in the filipin–cholesterol staining pattern, suggesting that the major part of cellular cholesterol was retained in the plasma membrane after sphingomyelinase treatment. After the oxidation of cell-surface cholesterol with cholesterol oxidase, the plasma membrane was no longer stained by filipin, but the plasma membrane cholesterol of sphingomyelin-depleted cells appeared to be resistant to oxidation with cholesterol oxidase when sphingomyelinase was used as an oxidation-promoting agent. However, the use of hypotonic buffer or phosphatidylcholine-specific phospholipase C together with cholesterol oxidase resulted in a

complete oxidation of the cell-surface cholesterol in sphingomyelin-depleted cells, as evidenced by the filipin–cholesterol staining pattern. Similar results were obtained when [³H]cholesterol-labelled fibroblasts were used for determination of the susceptibility to cholesterol oxidation. The kinetics of [³H]cholesterol oxidation in sphingomyelin-depleted cells with cholesterol oxidase in hypotonic buffer indicated that ~85% of the cellular cholesterol still resided in the plasma membrane after sphingomyelin depletion. These results are contradictory to earlier reports on sphingomyelinase-induced changes in cellular cholesterol distribution and suggest that minor changes in the kinetics of cholesterol transport from the plasma membrane to the endoplasmic reticulum may be responsible for the sphingomyelinase-induced changes in the rates of cholesterol metabolism. Whereas the use of phospholipases to promote the oxidation of cholesterol in some instances might lead to misinterpretations, the use of hypotonic buffer together with cholesterol oxidase proved to be a more reliable method for the determination of cellular cholesterol distribution.

INTRODUCTION

The bulk of cellular cholesterol and sphingomyelin appears to be concentrated to the cell surface in most cell types studied, and in fibroblasts approximately 90% of both the cholesterol and sphingomyelin has been reported to reside in the plasma membrane [1–4]. This co-localization has been suggested to result from the greater affinity of cholesterol for sphingomyelin compared with other phospholipids [5–7].

The role of sphingomyelin in cellular cholesterol homeostasis has been investigated thoroughly in recent years [3,8–15]. Degradation of plasma membrane sphingomyelin with exogenous sphingomyelinase has been shown to induce increased intracellular esterification of cholesterol [8], decreased biosynthesis of cholesterol [8,12], and stimulated steroidogenesis in steroidogenic cells [11]. The specific degradation of cell-surface phosphatidylcholine does not induce any such effects on the cellular cholesterol homeostasis [14]. These alterations in intracellular cholesterol metabolism are thought to result from the loss of interactions between sphingomyelin and cholesterol in the plasma membrane upon sphingomyelin depletion, which could lead to the translocation of plasma membrane cholesterol into intracellular regulatory pools of cholesterol metabolism. The results in previous studies have suggested that up to 50% of the plasma membrane cholesterol could be transferred to intracellular compartments, based on the decreased susceptibility of cell cholesterol to oxidation with cholesterol oxidase [3,9].

In the present study we have attempted to further clarify the fate of plasma membrane cholesterol upon sphingomyelin degra-

dation. The distribution of unesterified cellular cholesterol was studied by use of the fluorescent sterol-binding polyene, filipin, and the effects of sphingomyelin depletion and cholesterol oxidase treatments were monitored by fluorescence microscopy. In addition, the effects of different oxidative mixtures on the oxidation of cell-surface cholesterol with cholesterol oxidase were studied. Our results suggest that the major part of plasma membrane cholesterol remains at the cell surface even after sphingomyelin depletion. The requirements for an optimal oxidative mixture appear to change, however, after pretreatment with sphingomyelinase.

MATERIALS AND METHODS

Materials

[1 α ,2 α -³H]Cholesterol (48.4 Ci/mmol) and [*methyl*-³H]choline chloride (80 Ci/mmol) were obtained from Amersham. Cell culture media, supplements and fetal calf serum (FCS) were purchased from GIBCO. Cell culture dishes were from Nunc (Denmark). Phosphatidylcholine-specific phospholipase C (PC-PLC; EC 3.1.4.3, *Bacillus cereus*, Grade I) was obtained from Boehringer-Mannheim. Ham F-12 nutrient mixture, sphingomyelinase (EC 3.1.4.12, *Staphylococcus aureus*), filipin III, and phospholipids were purchased from Sigma. Cholesterol oxidase (EC 1.1.3.6, *Brevibacterium* sp.) was from Beckman Instruments (Carlsbad, CA, U.S.A.) and Mowiol 40-88 was obtained from Aldrich Chemie (Steinheim, Germany). Low-density lipoprotein

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; PC-PLC, phosphatidylcholine-specific phospholipase C.

* Present address: Institute of Environmental Medicine, Division of Toxicology, Karolinska Institute, Box 210, S-17177 Stockholm, Sweden.

† To whom correspondence should be addressed at the above address.

Figure 1 Cytochemical examination of the distribution of unesterified cholesterol in native and phospholipase-treated fibroblasts

Cells were seeded on glass coverslips and grown for 24–48 h before filipin staining. (a), Cells grown in 12% FCS; (b), LPDS treatment for 24 h; (c), LDL treatment for 24 h; (d), cells grown in 12% FCS and treated with 50 units/ml of sphingomyelinase for 30 min; (e), cells grown in 12% FCS and then treated with 1.0 unit/ml of PC-PLC for 30 min. Bar = 50 μ m.

(LDL) and lipoprotein-deficient serum (LPDS) were prepared from fresh human plasma (4 mM EDTA) by sequential ultracentrifugation [16,17]. After dialysis, LDL and LPDS were sterilized and stored in the dark at 4 °C. The units of the enzymes used in this study are defined as μ mol/min.

Cell culture

Normal human skin fibroblasts (line GM 8333) were purchased from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A. They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12% (v/v) FCS, 100 units of penicillin/ml, 100 μ g of streptomycin/ml, and 1 mM non-essential amino acids. Cell cultures were maintained in 75-cm² culture flasks, and cells for isotope experiments were seeded in 35-mm-diam. culture dishes and grown to confluency. Cells for filipin staining were seeded on coverslips in 35-mm-diam. culture dishes, 24–48 h before experiments. For depletion of cholesterol, cells were rinsed with serum-free DMEM and incubated in LPDS [(10%, v/v) in serum-free DMEM]. After 6 h cells were then rinsed and received new LPDS, and the incubation was continued for 18 h. For enrichment of cell cholesterol, cells were pretreated with LPDS for 6 h in order to induce LDL receptor expression, and then incubated with LDL (300 μ g/ml) for 24 h. Compound 58-035 (Sandoz), was included in the medium in order to avoid re-esterification of the cholesterol released from internalized LDL.

Labelling of cells with [³H]cholesterol

Subconfluent cells were incubated for 48 h with [³H]cholesterol in FCS (5.0 μ Ci/ml serum; 9% serum in DMEM). Prior to experiments cells were incubated in serum-free DMEM for 3 h.

Degradation of [³H]choline-labelled choline phospholipids

The choline phospholipids of subconfluent fibroblasts were labelled with 2.0 μ Ci/ml of [*methyl*-³H]choline chloride for 48 h. For degradation of cellular phosphatidylcholine, cells were rinsed twice with PBS, and then exposed to PC-PLC for 30 min at 37 °C. In some experiments, cholesterol oxidase (1.5 units/ml) was included in the solution, since the degradative effect of PC-PLC was shown to change in the presence of cholesterol oxidase.

Oxidation of cell-surface cholesterol with cholesterol oxidase

Confluent cells were rinsed twice with PBS and appropriate dishes of cells were pretreated with sphingomyelinase (50 units/ml) for 30 min in Ham F-12 nutrient mixture. Cells were then fixed with 1% glutaraldehyde on ice for 15 min, and thoroughly rinsed with PBS. The oxidation of cell cholesterol was performed with 1.5 units/ml of cholesterol oxidase in Ham F-12 at 37 °C for 30 min. Sphingomyelinase (50 units/ml) or PC-PLC (different concentrations) was included in the oxidation mixture in order to facilitate the oxidation. Alternatively, the cholesterol oxidase was dissolved in hypotonic PBS (diluted 1:10) according to the method of Hokland et al. [18], without addition of any phospholipase.

Fluorescent cytochemical staining of cholesterol and microscopic analysis

Fibroblasts on coverslips were rinsed with PBS and pretreated with appropriate enzymes as described above. Cells were then fixed with 1% glutaraldehyde on ice for 15 min (except for the

cells which had already been fixed and treated with cholesterol oxidase), and rinsed three times with PBS. The fluorescent staining of unesterified cell cholesterol was performed according to the method developed in the laboratories of H. S. Kruth and P. G. Pentchev [19,20], with minor modifications. Filipin was dissolved in tetrahydrofuran (2.5 mg/ml) and then diluted in PBS. The final concentrations of filipin and tetrahydrofuran were 50 μ g/ml and 2% (v/v) respectively. After addition of filipin to the cells, dishes were kept for 30 min at room temperature. Following filipin staining, cells on coverslips were rinsed thoroughly and mounted in glycerol containing 0.1 g/ml of Mowiol 40-88. Cells were viewed with a fluorescence microscope (Olympus IMT-2) using a UV filter set and photographed.

Lipid analysis

Total lipids were extracted from dishes of cells with hexane/2-propanol (3:2, v/v). Phospholipids were separated on silica-gel TLC plates (Merck) using chloroform/methanol/acetic acid/water (25:15:4:2, by vol. [21]). Phospholipid spots were visualized by staining with iodine and identified from standards run in parallel. Sphingomyelin and phosphatidylcholine spots were scraped into scintillation vials together with 3 ml of scintillation cocktail, and radioactivity was counted. Neutral lipids from [3 H]cholesterol-labelled cells were separated on silica-gel TLC sheets (Kodak) using hexane/diethyl ether/acetic acid (65:15:1, by vol.) as solvent. Sheets were stained with iodine, and spots of [3 H]cholesterol, [3 H]cholestenone and [3 H]cholesteryl esters were cut into scintillation vials for counting of radioactivity.

RESULTS

Fluorescence microscopy examination of fibroblasts grown in 12% (v/v) FCS revealed intense intracellular filipin-cholesterol staining, as well as a weaker, but clearly defined, staining of the plasma membrane (Figure 1a). The intracellular filipin-cholesterol staining has been shown to correspond to lysosomes and the Golgi complex [22]. After treatment with lipoprotein-deficient serum for 24 h the intracellular filipin-cholesterol staining was less intense, whereas the plasma membrane was clearly stained (Figure 1b). In fibroblasts treated with LDL for 24 h the intracellular filipin-cholesterol staining was even more intense than in cells cultured with FCS (Figure 1c). We have previously shown that the oxidizability of cell-surface cholesterol with cholesterol oxidase decreases dramatically in fibroblasts treated with sphingomyelinase for degradation of cell-surface sphingomyelin [3,9]. This was interpreted as a major flow of plasma membrane cholesterol into intracellular compartments due to the loss of molecular attractions between sphingomyelin and cholesterol, especially since a simultaneous increase in the esterification and decrease in the biosynthesis of cholesterol were observed. In Figure 1(d), fibroblasts had been cultured in FCS-containing DMEM and pretreated with sphingomyelinase before filipin staining. Surprisingly enough, the filipin-cholesterol staining of the sphingomyelin-depleted plasma membrane did not appear to differ markedly from the staining of the plasma membranes of control cells. Sphingomyelinase treatment induced shape changes in the exposed cells, which were observed also during routine light microscopy examinations in all experiments. In spite of these morphological changes the filipin-cholesterol staining at the cell surface was clearly visible. Figure 1(e) shows the filipin-cholesterol staining of fibroblasts treated with PC-PLC for degradation of cell-surface phosphatidylcholine. This treatment, which has previously been shown not to affect the

Figure 2 Fluorescence microscopy of fibroblasts treated with cholesterol oxidase under different conditions

All cells were grown in 12% (v/v) FCS before the enzyme treatments. (a), Cholesterol oxidase plus sphingomyelinase for 30 min; (b), cholesterol oxidase in hypotonic PBS for 30 min; (c–e), sphingomyelinase pretreatment for 30 min and then cholesterol oxidase + (c) sphingomyelinase, (d) hypotonic PBS, or (e) PC-PLC for 30 min. Same magnification as in Figure 1.

Table 1 Dependence of cholesterol oxidation on cell-surface phospholipid content

Confluent fibroblasts, labelled with [^3H]cholesterol for 48 h, were rinsed and incubated in serum-free DMEM 3 h prior to experiments. One set of dishes were incubated with sphingomyelinase (Smase; 50 units/ml) for 30 min. Control cells received serum-free Ham F-12 medium only. Cells were then fixed and exposed to cholesterol oxidase (COase; 1.5 units/ml) with sphingomyelinase, with PC-PLC (2.0 units/ml), or in hypotonic PBS. Values are given as percentages of oxidized cholesterol, which was defined as $[\text{oxidized } ^3\text{H}] \text{cholesterol} / ([\text{oxidized } ^3\text{H}] \text{cholesterol} + [\text{oxidized } ^3\text{H}] \text{cholestenone}) \times 100\%$. The values represent means $\pm$ S.D. of three independent experiments.

Oxidative mixture	Pretreatment	
	Control	Smase
COase + Smase	86.1 $\pm$ 1.7	29.9 $\pm$ 7.4
COase + PC-PLC	17.8 $\pm$ 1.0	81.0 $\pm$ 0.9
COase in hypotonic PBS	84.6 $\pm$ 0.5	86.8 $\pm$ 0.4

oxidizability of cell cholesterol [14], did not appear to change the pattern of filipin-cholesterol staining.

The oxidation of cell-surface cholesterol with cholesterol oxidase requires the presence of either sphingomyelinase or hypotonic buffer in order to be complete [3,23]. In fibroblasts where the cell-surface cholesterol had been oxidized, the plasma membrane was no longer stained by filipin (Figures 2a and 2b), since filipin is unable to form fluorescent complexes with the oxidation product, cholest-4-en-3-one [24]. The intracellular filipin-cholesterol staining remained intense even after treatment of cells with cholesterol oxidase. There was no visible decrease in plasma membrane filipin-cholesterol staining upon treatment of fibroblasts with cholesterol oxidase plus PC-PLC (results not shown).

In cells pretreated with sphingomyelinase, an incubation with cholesterol oxidase plus sphingomyelinase no longer resulted in the disappearance of the plasma membrane filipin-cholesterol staining (Figure 2c). There appeared to be enough cholesterol left at the cell surface for a positive filipin staining, although this cholesterol seemed to have become resistant to oxidation. The use of hypotonic buffer with cholesterol oxidase after sphingomyelinase pretreatment, however, resulted in a filipin-cholesterol staining pattern typical of cells with oxidized plasma membrane cholesterol (Figure 2d). Also the use of PC-PLC (1.0 unit/ml) instead of sphingomyelinase in the oxidative mixture promoted oxidation of cell-surface cholesterol (Figure 2e).

Taken together, the micrographs in Figures 1 and 2 suggest that sphingomyelin depletion at the cell surface did not result in any major redistribution of cell cholesterol. The oxidation of cell-surface cholesterol, however, was no longer facilitated by sphingomyelinase, but needed either hypotonic buffer or PC-PLC to reach completion. This could be due to an increased attraction between cholesterol and phosphatidylcholine following depletion of sphingomyelin.

Since the evaluation of micrographs depicting cytochemical staining of cellular cholesterol is prone to subjectivity, the different types of cholesterol oxidation in [^3H]cholesterol-labelled fibroblasts before and after sphingomyelin depletion were also compared. As shown in Table 1, in native cells (not treated with sphingomyelinase) 85–90% of the cellular [^3H]cholesterol was oxidizable with cholesterol oxidase plus sphingomyelinase, or cholesterol oxidase in hypotonic buffer. These results are in good agreement with previous reports [3,9,23] suggesting that 80–90% of the fibroblast cholesterol mass resides in the plasma membrane.

Figure 3 Relationship between phosphatidylcholine degradation and oxidizability of cholesterol after sphingomyelin degradation

Fibroblasts for cholesterol oxidation were labelled with [^3H]cholesterol for 48 h, and incubated in serum-free DMEM prior to experiments for 3 h. Cells were then exposed to sphingomyelinase for 30 min and fixed with glutaraldehyde for 15 min. The oxidation with a mixture containing cholesterol oxidase (1.5 units/ml) and PC-PLC at various concentrations (▼) was allowed to proceed for 30 min. The oxidative effect of cholesterol oxidase + sphingomyelinase (○) or cholesterol oxidase in hypotonic PBS (●) have been included in the Figure for comparison. Cells for study of phosphatidylcholine degradation (□) were labelled with [^3H]choline chloride for 48 h and then incubated with serum-free DMEM for 3 h. The experimental design was the same as for cells in which the oxidation of cholesterol was measured. Cells were pretreated with sphingomyelinase and fixed, and then exposed to cholesterol oxidase + PC-PLC. The phosphatidyl[^3H]choline level of control cells (not exposed to any enzyme) was defined as 100%. Each point represents a mean value (S.D. < 5%) from an independent experiment.

In cells exposed to cholesterol oxidase plus PC-PLC (2.0 units/ml), however, only 18% of cellular [^3H]cholesterol was oxidized. We have previously shown that 2.0 units/ml of PC-PLC is sufficient for the degradation of cell-surface phosphatidylcholine [14]. The degradation of plasma membrane phosphatidylcholine apparently could not promote the oxidation of cholesterol, indicating a tight association between cholesterol and sphingomyelin. In fibroblasts depleted of sphingomyelin, the oxidation of cholesterol seemed to be more dependent on degradation of phosphatidylcholine by PC-PLC or treatment with hypotonic buffer (to loosen the lipid packing in the membrane). Treatment with cholesterol oxidase plus sphingomyelinase resulted in a 30% oxidation of cellular [^3H]cholesterol, as previously reported [10,14]. Taken together with the fluorescence micrograph for the same treatment (Figure 1d), and the ability of cholesterol oxidase in hypotonic buffer to induce 'optimal' oxidation of cholesterol, it appears that the major part of the plasma membrane cholesterol still resided at the cell surface (or in the immediate vicinity of the cell surface) after sphingomyelin depletion.

The subsequent degradation of both sphingomyelin and phosphatidylcholine in the plasma membrane of fibroblasts that were pretreated with sphingomyelinase and then exposed to cholesterol oxidase plus PC-PLC could be expected to render the membrane permeable to molecules such as cholesterol oxidase and PLC, and therefore we tested the effects of increasing concentrations of PC-PLC on phosphatidyl[^3H]choline degra-

Figure 4 Time course of cholesterol oxidation in hypotonic buffer for native and sphingomyelinase-treated cells

Experimental conditions were as in Figure 3. Cells with ^3H -labelled cholesterol pools were incubated with sphingomyelinase for 30 min. Control cells received Ham F-12 medium only. After fixation, cells were incubated with cholesterol oxidase in hypotonic PBS for indicated times. Values represent means from one representative experiment, which was repeated twice.

dation and the degree of cholesterol oxidation. The pretreatment of cells with sphingomyelinase apparently made them more susceptible to PC-PLC, resulting in the degradation of more than 90% of the total phosphatidyl[^3H]choline with 5.0 units/ml of PC-PLC (Figure 3). Without sphingomyelin depletion this treatment causes a 20% degradation of cellular phosphatidylcholine [14], which should correspond to the amount of phosphatidylcholine on the cell surface. Thus the PC-PLC clearly had penetrated the plasma membrane and was able to attack the phosphatidylcholine of intracellular membranes. Figure 3 shows that 70–80% of the cellular phosphatidyl[^3H]choline had to be degraded before the oxidation of cell cholesterol reached the level of hypotonic buffer-induced oxidation. The reported molecular mass for PC-PLC is $\sim 30\,000$ Da [25], whereas the molecular mass of cholesterol oxidase should be $\sim 55\,000$ Da [26]. It is possible that cholesterol oxidase also was able to penetrate the plasma membrane under these conditions, and therefore it is difficult to estimate the importance of phosphatidylcholine–cholesterol interactions after sphingomyelin depletion, based on these results.

When the kinetics of cholesterol oxidation with cholesterol oxidase in hypotonic buffer were examined, the results revealed that the cholesterol in sphingomyelin-depleted cells was oxidized with somewhat faster kinetics than cholesterol in native cells (Figure 4). This could be due to the sphingomyelinase-induced loosening of the lipid packing, and also indicates that the oxidase-susceptible cholesterol in sphingomyelin-depleted cells was located in a rapidly accessible compartment, presumably the plasma membrane. Furthermore, the portion of cellular cholesterol susceptible to a prolonged oxidation (45 min) proved to be $\sim 85\%$ in both native and sphingomyelin-depleted cells, which suggests that the cholesterol oxidase did not penetrate the plasma membrane under these conditions. The curves in Figure

4 thus support the notion that no significant amount of cholesterol was translocated from the cell surface upon sphingomyelin degradation.

DISCUSSION

Exogenously added sphingomyelinase has proved to be a good tool for a rapid stimulation of cholesterol esterification in all cell types studied so far [8–11]. The underlying mechanism for the stimulation of cholesterol esterification has been suggested to be an increased flow of substrate cholesterol to the endoplasmic reticulum, where acyl-CoA:cholesterol acyltransferase (ACAT), the enzyme responsible for cholesterol esterification, resides. This enzyme is thought to be regulated mainly through variations in substrate availability [27,28], and the depletion of plasma membrane sphingomyelin by treatment with exogenous sphingomyelinase is thought to induce an increased flow of cholesterol to the endoplasmic reticulum as a result of the lost interactions between cholesterol and sphingomyelin in the plasma membrane. The increase in cholesterol esterification, decrease in biosynthesis of cholesterol [8,12], and increased utilization of cholesterol for steroidogenesis [11], which occur upon degradation of sphingomyelin, are apparently not caused by degradation products such as ceramide and sphingosine [12,29].

The susceptibility of cell cholesterol to oxidation with cholesterol oxidase is dramatically decreased after sphingomyelin depletion of the plasma membrane, under conditions where sphingomyelinase is used as oxidation-promoting agent [3,9]. This has been interpreted to reflect a major translocation of plasma membrane cholesterol to intracellular, oxidase-resistant, membrane compartments. In the present study we have attempted to elucidate further the effects of sphingomyelin depletion on the distribution of cell cholesterol. Two different methodological approaches were used; (1) cellular unesterified cholesterol was stained with filipin, a polyene antibiotic which emits fluorescent light upon binding to unesterified cholesterol [24], and (2) [^3H]cholesterol-labelled fibroblasts were exposed to cholesterol oxidase under several different conditions. The staining of cell cholesterol with filipin appeared to give an accurate picture of the cholesterol content in cellular membranes, as determined from micrographs of fibroblasts with different levels of cholesterol saturation (Figure 1, a–c). Although the bulk of cellular cholesterol is thought to reside in the plasma membrane, one can observe a bright filipin–cholesterol fluorescence from intracellular organelles (cf. ref. [30]). This can perhaps be explained by the three-dimensional structure of the cell. In fibroblasts treated with sphingomyelinase, the plasma membrane was clearly stained by filipin–cholesterol complexes although this treatment previously has been thought to translocate $\sim 50\%$ of the plasma membrane cholesterol to intracellular compartments. While the plasma membranes of native cells were no longer stained by filipin after cholesterol oxidase treatment, the plasma membrane of sphingomyelin-depleted cells appeared to be resistant to cholesterol oxidase plus sphingomyelinase and was clearly stained by filipin. The use of hypotonic buffer together with cholesterol oxidase, on the other hand, resulted in the disappearance of filipin–cholesterol staining from sphingomyelin-depleted plasma membranes. Thus the micrographs of filipin-stained fibroblasts suggested a less dramatic effect of sphingomyelin depletion on the distribution of cell cholesterol, and experiments with [^3H]cholesterol-labelled cells gave further support to this theory. The oxidation of cholesterol with cholesterol oxidase in native cells was promoted by sphingomyelinase and hypotonic buffer, but not by PC-PLC. PLC has previously been reported to promote oxidation of cholesterol in vesicular stomatitis virus,

but the PLC used in that study also showed sphingomyelin-degradative activity [31]. In sphingomyelin-depleted cells, however, oxidation was promoted by PC-PLC and hypotonic buffer, which both were able to promote an oxidation as extensive as in native cells. This could be explained in two ways; either the cholesterol remained in the plasma membrane after sphingomyelin depletion, or the cholesterol oxidase penetrated the plasma membrane and gained access to intracellular cholesterol. The use of PC-PLC in the oxidation solution after sphingomyelin depletion proved to be a problem, since the subsequent degradation of phosphatidylcholine and sphingomyelin apparently disturbed the plasma membrane structure and possibly made cells permeable to cholesterol oxidase at higher concentrations of PC-PLC. Exogenous PLC alone has previously been shown to render cells permeable to horseradish peroxidase (~40 000 Da) at low concentrations [32]. However, the enzyme used in that study was the *Clostridium perfringens* PLC, which is able to hydrolyse several different phospholipids [25].

The results from oxidation of cholesterol with cholesterol oxidase in hypotonic buffer, in our opinion, would suggest that the amount of cholesterol in the plasma membrane was approximately the same before and after sphingomyelin depletion, which also is consistent with the results from filipin-staining experiments.

In conclusion, the results in the present study suggest that the bulk of plasma membrane cholesterol is retained at the cell surface after depletion of plasma membrane sphingomyelin. These results are contradictory to earlier observations, where sphingomyelinase was used as the only oxidation-promoting agent together with cholesterol oxidase [3,9]. The results obtained in earlier studies suggested that the distribution of cholesterol changed dramatically after depletion of sphingomyelin at the cell surface. It appears that the use of phospholipases together with cholesterol oxidase in some cases can lead to misinterpretations, since the result may be highly dependent on the actual composition of the plasma membrane. The results in this study suggest that the use of cholesterol oxidase in hypotonic buffer is a more reliable alternative for measurement of the cellular cholesterol distribution.

It appears that the presence of sphingomyelin is not an absolute requirement for maintaining cholesterol in the plasma membrane, suggesting that other phospholipids are capable of accommodating the cholesterol after sphingomyelin depletion. This interpretation is in agreement with two recent papers by Tabas and co-workers [33,34], where it was shown that both sphingomyelin and phosphatidylcholine, and possibly also other phospholipids, were needed to accommodate excessive amounts of LDL-derived cholesterol in macrophages. However, the cellular sphingomyelin content appeared to be particularly important for the regulation of ACAT activity in macrophages [33]. It should be emphasized that the observed sphingomyelinase-induced increase in short-term cholesterol esterification in fibroblasts has represented < 5% of total cellular cholesterol mass, and the methods used in this study do not allow us to draw conclusions concerning the possible loss of 1–5% of plasma membrane cholesterol content upon sphingomyelin depletion. A recent paper by Lange and co-workers [28] suggests that plasma membrane cholesterol is continuously recycled to the endo-

plasmic reticulum and the level of ACAT activity may be regulated through the kinetics of cholesterol transport in and out of the endoplasmic reticulum. It is plausible that depletion of plasma membrane sphingomyelin with sphingomyelinase could induce changes in the kinetics of cholesterol recycling between the plasma membrane and the endoplasmic reticulum, and this in turn would result in increased cholesterol esterification, and decreased biosynthesis of cholesterol.

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