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# Effects of sphingomyelin degradation on cholesterol mobilization and efflux to high-density lipoproteins in cultured fibroblasts

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**The hydrolysis of sphingomyelin from cellular plasma membranes imposes many consequences on cellular cholesterol homeostasis by causing a rapid and dramatic redistribution of plasma membrane cholesterol within the cells (Slotte, J.P. and Bierman, E.L. (1988) Biochem. J. 250, 653–658). The objective of this study was to examine the effects of an extracellular cholesterol acceptor on the directions of the sphingomyelinase-induced cholesterol flow in cultured fibroblasts. We have used $HDL_3$ as a physiological acceptor for cholesterol, and measured the effects of sphingomyelin hydrolysis on efflux and endogenous esterification of cellular [$^3$H]cholesterol. Treatment of cells with sphingomyelinase did induce a dramatically increased esterification of plasma-membrane-derived [$^3$H]cholesterol. The presence of $HDL_3$ in the medium (100 $\mu$g/ml) did not prevent or reduce the extent of the sphingomyelinase-induced cellular esterification of [$^3$H]cholesterol. Degradation of cellular sphingomyelin (75% hydrolysis) also did not enhance the rate of [$^3$H]cholesterol efflux from the plasma membranes to $HDL_3$. In addition, we also observed that the degradation of sphingomyelin in the $HDL_3$ particles (complete degradation) did not change the apparent rate of [$^3$H]cholesterol transfer from $HDL_3$ to the cells. These findings together indicate that hydrolysis of sphingomyelin did not markedly affect the rates of cholesterol surface transfer between $HDL_3$ and cells. By whatever mechanism cholesterol is forced to be translocated from the plasma membranes subsequent to the degradation of sphingomyelin, it appears that the sterol flow is specifically directed towards the interior of the cells.**

## Introduction

The cellular and substructural distribution of lipid classes is known to be highly asymmetric. In many different cell types, both cholesterol, sphingomyelins, and phosphatidylcholines are distributed in favor of the plasma membrane compartment, whereas aminophospholipids are preferentially enriched in endomembranes [1–6].

This asymmetric distribution of neutral and polar lipids within the structures of intact cells certainly implies an important albeit a largely unknown physiological function of the selective lipid distribution. It is further known that the balance of the lipid distribution is highly delicate. An induced substitution of one lipid class for another may have dramatic effects on the distribution of one or several of the other lipid classes. An example of this is the observation that a partial removal of sphingomyelin from the plasma membrane of intact fibroblasts leads to a dramatic change in the cellular distribution of cholesterol [7].

The sphingomyelinase-induced redistribution of cell cholesterol eventually results in a net flow of cholesterol mass from the cell surface into the cells, leading to (i) upregulation of the endogenous esterification activity and to an increased formation of cholesterol ester mass, (ii) downregulation of cholesterol biosynthesis, and (iii) to the transfer of a large fraction of the cellular unesterified cholesterol from a cholesterol oxidase susceptible compartment to an oxidase-resistant pool [7–9].

Since the initial objective of the studies on the sphingomyelin-dependent changes in sterol distribution were focused on intracellular events, conditions were usually chosen so that sterols could not escape (efflux) from cells to the medium (serum-free). It is therefore not known how a sphingomyelinase-treatment of cells would affect the distribution of cholesterol between the cellular and the extracellular compartment, if an extracellular acceptor of cholesterol (e.g., $HDL_3$) would be available. The objective of the present study was to examine the effects of $HDL_3$ on the distribution of plasma-membrane-derived [$^3$H]cholesterol subsequent to the degradation of plasma membrane sphingomyelin.

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## Experimental

*Cells.* Human skin fibroblasts (GM 8333) were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Nord-Vacc, Stockholm) supplemented with 15% fetal calf serum (GIBCO), L-glutamine, penicilline and streptomycine, and non-essential amino acids (all from Nordvacc). Cells for experiments were seeded in 35 mm diameter cell culture dishes (at about 50000 cells/dish) and were grown to confluency and pre-treated prior to experiments as described separately.

*Isolation of $HDL_3$.* High density lipoproteins ($HDL_3$, $d = 1.12–1.21$ g/ml) were prepared from fresh human plasma (EDTA 4 mM) by sequential ultracentrifugation [10,11]. The dialysed $HDL_3$ stock solution (about 16–20 mg protein/ml) was stored sterile in the dark at 4°C and was used within 3–4 weeks of preparation. For some experiments, $HDL_3$ devoid of sphingomyelin was used as a cholesterol acceptor. For this, $HDL_3$ (10 mg) was exposed to 0.1 U/ml of sphingomyelinase (*Staphylococcus aureus*; Sigma Chemicals) for 30 min at 37°C. This-procedure resulted in a complete degradation of $HDL_3$ sphingomyelin, as verified by thin-layer chromatography of the total lipid extract from sphingomyelin-depleted $HDL_3$.

*Labeling of $HDL_3$ with [$^3$H]cholesterol.* $HDL_3$ was labeled with free [$^3$H]cholesterol (60 Ci/mmol, Du Pont NEN) as described previously [12]. The [$^3$H]UC-$HDL_3$ was stored at 4°C and used within 2–4 days of preparation.

*Labeling of cells with [$^3$H]cholesterol.* Cells in 35 mm diameter dishes were cultured in growth medium for 3–4 days. Cells were then incubated for 48 h in growth medium with 9% fetal calf serum containing unesterified [$^3$H]cholesterol (5 μCi/ml serum). The cells were finally incubated for 3–5 h in a serum-free Dulbecco's MEM prior to the experiments.

*Incubation protocol.* Confluent cells, labeled with [$^3$H]cholesterol (in some experiments unlabeled cells were used), were rinsed once with phosphate-buffered saline (pH 7.4) and then 1.0 ml serum-free HAM F12 medium was added together with indicated (0–200 μg/ml) amounts of $HDL_3$. Cells were exposed to $HDL_3$ for an indicated period of time (0 to 4 h) at 37°C. Appropriate dishes received 100 mU/ml of sphingomyelinase after the first hour of efflux incubation, without removal of the medium. The efflux of cell-derived [$^3$H]cholesterol was determined by counting the radioactivity from an aliquot (0.1–0.25 ml) of the medium.

*Assay procedures.* Labeled sterols in extracts from cells were separated on normal phase thin-layer chromatography (Kodak silica gel sheets) 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 and [$^3$H]cholesterol esters 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 2 ml of a xylene-based scintillation cocktail in a LKB RackBeta liquid scintillation counter.

The protein content of the lipoprotein batches was determined according to Lowry et al. [13], with bovine serum albumin as standard.

## Results

One of the responses to sphingomyelin degradation in plasma membranes of cultured cells is the increased flow of cholesterol from the plasma membrane into the cells, which conveniently can be observed as an increased formation of [$^3$H]cholesterol esters [7,8]. In this study we have determined the effects of $HDL_3$, as an extracellular cholesterol acceptor, on the sphingomyelinase-induced cholesterol redistribution in cultured fibroblasts.

First the effects of $HDL_3$ on the sphingomyelinase-induced endogenous esterification of plasma-membrane-derived [$^3$H]cholesterol was measured. In control cells, with no $HDL_3$ in the incubation medium, the degradation of plasma membrane sphingomyelin resulted in an increased esterification of cell [$^3$H]cholesterol, the rate of which amounted to about 3.8%/h (Fig. 1). With $HDL_3$ present in the incubation medium (100 μg/ml), cellular sphingomyelin degradation led to a rate of endogenous [$^3$H]cholesterol esterification that

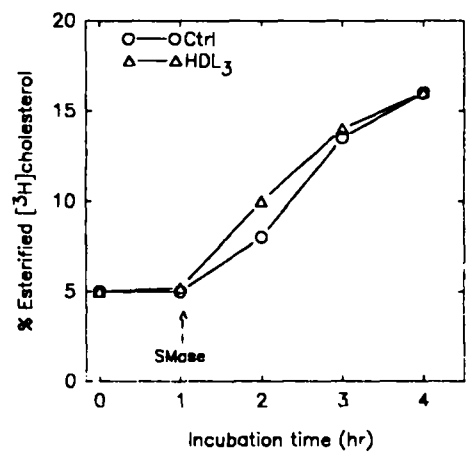


Fig. 1. Effects of sphingomyelin degradation on [$^3$H]cholesterol esterification in GM fibroblasts incubated with or without $HDL_3$. [$^3$H]Cholesterol-labeled, confluent fibroblasts in 35-mm dishes were incubated in 1.0 ml HAM F12 medium (serum-free) supplemented with either 0 or 100 μg/ml of $HDL_3$. After 1 h at 37°C, 100 mU/ml of sphingomyelinase (SMase) was added to the dishes and the incubation continued for another 3 h. The formation of cellular [$^3$H]cholesterol esters was determined. Values are pooled averages from two different experiments with duplicate dishes (range for duplicates less than 5%; range between experiments usually less than 10%).

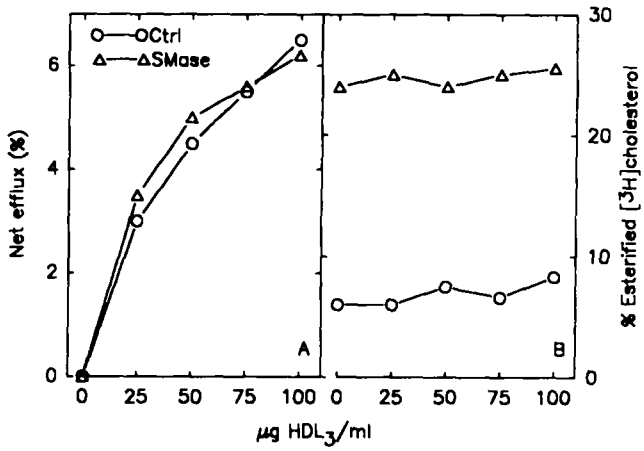


Fig. 2. Effects of sphingomyelin degradation on [$^3$H]cholesterol efflux and esterification in the presence and absence of $HDL_3$. [$^3$H]Cholesterol-labeled cells were exposed to indicated amounts of $HDL_3$ in 1.0 ml HAM F12 medium. After the first hour at 37°C, some dishes also received 100 mU/ml of sphingomyelinase (in 2–3 μl) and the incubation was continued for an additional 3 h. (Left panel) Net efflux of cell-derived [$^3$H]cholesterol to $HDL_3$. (Right panel) Cellular content of esterified [$^3$H]cholesterol. Values are averages from three separate experiments with duplicates ($n = 6$; range for duplicates less than 5%). The range for inter-experimental values of efflux % was less than 20%.

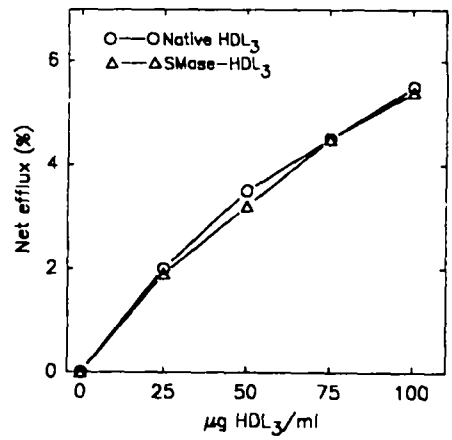


Fig. 3. Efflux of [$^3$H]cholesterol to native or sphingomyelin-depleted $HDL_3$. The net removal of cell-associated [$^3$H]cholesterol to indicated amounts of either native $HDL_3$ or sphingomyelin-depleted $HDL_3$ was determined during a 4 h efflux experiment. Values are averages from two separate experiments ($n = 4$). Inter-experimental variation less than 20% (duplicate range within 5%).

was not different from that observed in control cells (without $HDL_3$; Fig. 1). The presence of a cholesterol acceptor in the incubation medium apparently did not cause a removal of the liberated plasma membrane cholesterol, most of which eventually was transferred to the interior of the cells.

To actually measure the amount of removal of cell [$^3$H]cholesterol after the degradation of cell sphingomyelin, efflux experiments with $HDL_3$ as a cholesterol acceptor were performed. Incubation of [$^3$H]cholesterol-labeled fibroblasts for 4 h with varying amounts of $HDL_3$ resulted in a net efflux of about 6% (at 100 μg/ml $HDL_3$) of the cell-associated [$^3$H]cholesterol (Fig. 2a). A rapid degradation of cellular sphingomyelin (with sphingomyelinase at 100 mU/ml) one hour into the efflux period did not further affect the removal of cellular [$^3$H]cholesterol to $HDL_3$ (Fig. 2a). The amount of cell [$^3$H]cholesterol released to the efflux medium without $HDL_3$ was negligible and unaffected by the sphingomyelinase treatment (data not shown). The degradation of cell sphingomyelin did, however, increase the cellular content of esterified [$^3$H]cholesterol to about 4-fold over control, even though $HDL_3$ was present in the incubation medium. Again, the presence of a cholesterol acceptor in the extracellular medium failed to prevent or reduce the sphingomyelinase-induced cellular esterification of plasma-membrane-derived [$^3$H]cholesterol (cf. Fig. 1).

Our experimental format was such that the sphingomyelinase enzyme, which was added to degrade cellular sphingomyelin, also hydrolyzed sphingomyelin in the $HDL_3$ particle. To make certain that sphingomyelin degradation in the $HDL_3$ particle did not interfere with the cholesterol exchange properties of the lipoprotein particle, two different types of control experiments were performed. First, efflux of cellular [$^3$H]cholesterol to native $HDL_3$ and sphingomyelin-depleted $HDL_3$ was performed. It was observed that during a 4 h efflux experiment, both acceptor types at varying concentrations (between 25 and 100 μg protein/ml) were equally effective in promoting the removal of cellular [$^3$H]cholesterol (Fig. 3).

Secondly, when [$^3$H]cholesterol-labeled $HDL_3$ was used to measure the transfer of [$^3$H]cholesterol from $HDL_3$ into unlabeled cells, it was again observed that sphingomyelin depletion of $HDL_3$ (and cells) did not alter this process (Fig. 4). Hence it appears that under

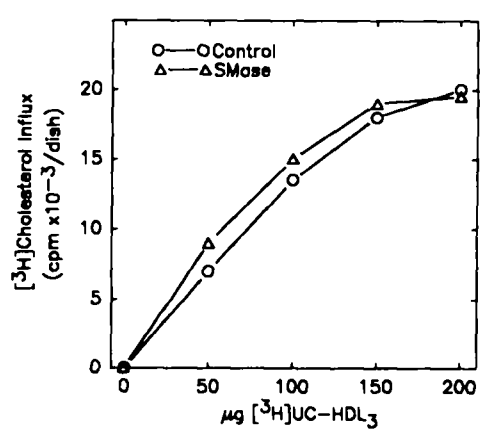


Fig. 4. Effects of sphingomyelin degradation in cells and $HDL_3$ on the transfer of [$^3$H]cholesterol from $HDL_3$ to cells. Unlabeled fibroblasts were incubated for 4 h with indicated amounts of [$^3$H]cholesterol-labeled $HDL_3$. Control cells received solvent alone, whereas the treated cells received 100 mU/ml of sphingomyelinase. Values are averages from two separate experiments with duplicates ($n = 4$). The range of individual paired values was within 10%.

the conditions used, $HDL_3$ devoid of sphingomyelin behaved like native $HDL_3$ with respect to its capacity to either accept or donate [$^3$H]cholesterol from/to cells.

## Discussion

The knowledge about how lipids in general and cholesterol in particular are transported to and from plasma membranes of intact cells is incomplete at best, and many pieces of information are lacking. Evidence exists to support the view that some of the lipid traffic from the sites of synthesis to the cell surface is mediated by vesicular transport [14]. On the other hand, a mediating role for intracellular lipid or sterol transport proteins in intracellular lipid transport has also been examined [15,16], although conclusive evidence for or against such a role is still lacking. Finally, at least individual cholesterol molecules are known to be transferred to and from the plasma membranes by a physico-chemical exchange mechanism which has been described in terms of an aqueous diffusion model [17]. Further, it has been demonstrated that cholesterol in plasma membranes can be transported spontaneously [18], or by manipulation [8] back into the cell where it participates in various metabolic pathways.

In the present study we have determined the capacity of $HDL_3$ to prevent or minimize the consequences of sphingomyelin degradation on intracellular cholesterol translocation. $HDL_3$ by itself is known to be able to remove cellular cholesterol from at least two different compartments, the plasma membrane compartment [19,20] and some less defined intracellular compartments [20,21]. It is further believed that the interaction of $HDL_3$ with its cell surface receptor promotes translocation of endogenously synthesized sterols to the cell surface [20,21]. The efflux format of the experiments in this study have been chosen so as to measure only the non-specific efflux of [$^3$H]cholesterol from plasma membranes, without largely involving possible receptor-mediated responses. These measures included the use of cells not enriched with unesterified cholesterol, and the long-term labeling (48 h) of cells with [$^3$H]cholesterol in serum to achieve uniform distribution of label among the cholesterol compartments of the cells [20].

The results of this study demonstrate that the non-specific and $HDL_3$-mediated removal of plasma-membrane [$^3$H]cholesterol from fibroblasts was not affected by the simultaneous degradation of cell sphingomyelin. This finding clearly suggests that cholesterol, which is liberated in the membrane by the disintegration of the putative sphingomyelin-cholesterol 'complex', does not desorb readily from the membranous bilayer. Otherwise one would expect that sphingomyelinase treatment would enhance the rate of cholesterol efflux to $HDL_3$. This finding was surprising, since it has been shown that sphingomyelin has the ability to retard the rate of cholesterol desorption from membranes [22,23]. Also, the presence of up to 100 $\mu$g/ml of $HDL_3$ outside the cells did not measurably reduce the extent of the sphingomyelinase-induced cholesterol redistribution within the cells, as observed from the endogenous esterification of plasma-membrane-derived [$^3$H]cholesterol.

These findings together imply that by whatever mechanism cholesterol is forced to be translocated from the membrane subsequent to the degradation of sphingomyelin, the flow of the sterol molecules is specifically directed towards the interior of the cells. The mechanism of the sphingomyelinase-induced rapid transport of cholesterol from the cell surface to intracellular sites, including the endoplasmic reticulum where the esterification reaction occurs, is unknown. The degradation of the putative sphingomyelin-cholesterol 'complex' and the concomitant introduction of ceramides into the bilayer structure could induce the formation of a new sterol partitioning in the plane of the membrane among lipid microdomains (lateral flow). This speculative sequence could be accompanied by an increased flow of cholesterol across the membrane bilayer ('flip-flop'). An enrichment of cholesterol in the endoleaflet could to a certain extent destabilize the structure and allow for blebbing of cholesterol-containing membranous material to the cytoplasm. This speculative sequence of events could involve endovesiculation on the cytoplasmic side of the membrane bilayer, which would not allow itself to be detected by the use of markers for fluid-phase pinocytosis. In agreement with this hypothesis we have observed that sphingomyelinase-treatment of fibroblasts did not increase the rate of fluid-phase pinocytosis as determined from [$^3$H] sucrose uptake [9]. In the erythrocyte model system, however, sphingomyelinase-treatment appears to induce both an endovesiculation and an increased uptake of fluid-phase markers [24].

A direct demonstration of a possible sphingomyelinase-induced blebbing of vesicular material from the endoleaflet of plasma membrane in native cells should be possible by the use of high-resolution fluorescence microscopy together with appropriate fluorescent sterol and phospholipid probes.

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