

Cholesterol–sphingomyelin interaction in membrane and apolipoprotein-mediated cellular cholesterol efflux

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Abstract Helical apolipoproteins interact with cellular surface and generate high density lipoprotein (HDL) by removing phospholipid and cholesterol from cells. We have reported that the HDL is generated by this reaction with the fetal rat astrocytes and meningeal fibroblasts but cholesterol is poorly available to this reaction with the astrocytes (Ito et al. 1999. *J. Neurochem.* 72: 2362–2369). Partial digestion of the membrane by extracellular sphingomyelinase increased the incorporation of cholesterol into thus-generated HDL from both types of cell. This increase was diminished by supplement of endogenous or exogenous sphingomyelin to the cells. The sphingomyelinase treatment decreased cholesterol in the membrane mainly in the detergent-resisting domain. The intracellular cholesterol used by acylCoA:cholesterol acyltransferase increased by the sphingomyelinase treatment in the absence of apoA-I, more remarkably in the fibroblast than in the astrocytes. ApoA-I suppressed this increase completely in the astrocytes, but only partially in the fibroblast. The effect of the sphingomyelin digestion was more prominent for the apolipoprotein-mediated reaction than the diffusion-mediated cellular cholesterol efflux. Thus, cholesterol molecules restricted by sphingomyelin in the domain of the plasma membrane appear to be primarily used for the HDL assembly upon the apolipoprotein–cell interaction.—Ito, J., Y. Nagayasu, and S. Yokoyama. Cholesterol–sphingomyelin interaction in membrane and apolipoprotein-mediated cellular cholesterol efflux. *J. Lipid Res.* 2000. 41: 894–904.

Supplementary key words apolipoprotein • cholesterol • Sphingomyelin • HDL • membrane

Apolipoprotein–cell interaction and subsequent generation of high density lipoprotein (HDL) from the cellular lipid (1) constitute one of the major pathways for the release of cellular cholesterol, an important component of the cellular and body sterol homeostasis (2). This reaction requires several cellular elements including an apolipoprotein interaction site on cell surface (3), assembly of HDL by apolipoprotein with membrane phospholipid (4), signaling to initiate intracellular cholesterol mobilization and specific intracellular cholesterol trafficking for the assembly of the HDL (5). Many cells are equipped with this system, but some may lack a part of the system,

such as vascular smooth muscle cells lacking the signal to mobilize intracellular cholesterol for the HDL assembly (6). Recently, mutations were identified in ATP-binding cassette transporter protein 1 to cause the impairment of this pathway and consequently to result in the HDL deficiency including Tangier disease (7–11).

We recently reported that rat fetal astrocytes interact with extracellular human apolipoprotein (apo)A-I and generate HDL, in addition to the release of HDL with endogenously synthesized apoE (12). Interestingly, the availability of the cellular cholesterol diminishes as the reaction with the exogenous apolipoprotein proceeds resulting in generation of cholesterol-poor HDL, while the HDL formed by the same cells with endogenous apoE is rich in cholesterol in contrast. The intracellular cholesterol pool available for the acylCoA:cholesterol acyltransferase (ACAT) mildly but rapidly decreased in response to apoA-I indicating that the intracellular cholesterol mobilization system for the apolipoprotein-mediated HDL generation itself is active. By using this model, we intended to investigate the local environment of cholesterol molecules in the plasma membrane that may regulate cholesterol availability for the HDL assembly by apolipoproteins.

Many reports indicate that cholesterol is tightly associated with sphingomyelin rather than other phospholipids in the plasma membrane (13–15). Such an interaction seems to be one of the key factors for the construction of the membrane microdomain rich in cholesterol and sphingomyelin that may exhibit many of the communicative cellular functions (16, 17). Accordingly, the treatment of the cells with sphingomyelinase (SMase) was shown to induce cholesterol redistribution in the plasma membrane (18, 19), while digestion of phosphatidylcholine has no effect (20). Sphingomyelin digestion also results in the increase of intracellular cholesterol esterification by ACAT

Abbreviations: HDL, high density lipoprotein; apo, apolipoprotein; ACAT, acylCoA acyltransferase; SMase, sphingomyelinase; LDL, low density lipoprotein; FCS, fetal calf serum; PBS, phosphate buffered saline (10 mm sodium phosphate buffer, pH 7.4, containing 0.15 m NaCl); TLC, thin layer chromatography.

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(18, 21) and the decrease of cholesterol biosynthesis (22), indicating the relocation of cholesterol to the regulatory compartment for intracellular sterol homeostasis perhaps in the endoplasmic reticulum.

We therefore studied the effect of the digestion of the cellular sphingomyelin on the apolipoprotein-mediated HDL assembly, focusing the cholesterol incorporation into the HDL. We demonstrated that this treatment lead to the increase of cholesterol in the HDL generated with extracellular apolipoprotein. The results thus suggest that cholesterol molecules utilized for the HDL assembly are in the sphingomyelin-rich domain. Cholesterol in this domain of the astrocytes is less relocatable into the intracellular compartment than that of the fibroblasts by the sphingomyelin digestion suggesting the less active intracellular cholesterol trafficking in this type of the cell (12).

MATERIALS AND METHODS

Fetal rat astrocytes and fibroblasts

Monolayer culture of astrocytes was prepared as described previously (23). Briefly, the cerebra were obtained from 17-day fetuses of Wister rats, and the surface blood vessels and meningeal layers were carefully removed. The tissue was dissected into about 2-mm cubes and treated with the 0.1% trypsin solution in the cell buffer of 0.36 mM sodium phosphate-bicarbonate, pH 7.4, containing 135 mM NaCl, 2.7 mM KCl, and 5.5 mM glucose, at room temperature for 5 min. After the one-week primary culture in the 10% fetal calf serum in F-10 media (10% FCS/F-10) and the same trypsin treatment, the cells were cultured in 10% FCS/F-10 in a 6-well multiple tray (3-cm culture plates, Coster 3516) for one week. Meningeal fibroblasts were obtained by the same culture method with a starting material of the meningeal layer (12).

Lipoproteins and apolipoproteins

Low density lipoprotein (LDL) and HDL were isolated by ultracentrifugation from fresh human plasma as density ranges 1.006–1.063 and 1.063–1.21 g/mL, respectively. Lipid microemulsion in the LDL size was prepared from triolein (Sigma Chemical Co., St. Louis, MO) and egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) with the starting weight ratio of 1:1 by sonication and gel permeation chromatography (24). To incorporate [1, 2-³H]cholesteryl oleate (Amersham, Buckinghamshire, UK), LDL was incubated with the lipid microemulsion composed of triolein, egg phosphatidylcholine and [³H]cholesteryl oleate in the presence of human plasma protein fraction containing cholesteryl ester transfer protein at 37°C for 48 h and the labeled LDL was re-isolated (25). All lipoprotein preparations were dialyzed against 10 mM sodium-phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS).

ApoA-I was isolated from human HDL according to the previously described method (26). Recombinant human apoE3 was kindly provided by Mitsubishi Chemical Corporation (Yokohama, Japan). Apolipoprotein solution was prepared by dissolving in 6 M guanidine-HCl and then thoroughly dialyzed against PBS.

Measurement of cholesterol and phospholipid release

The details of the method are described elsewhere (12). The confluent rat astrocytes and meningeal fibroblasts in 3-cm culture plates were washed twice with the cell buffer, and then cultured in 1 mL of the F-10 medium containing 0.1% bovine serum albumin (0.1% BSA/F-10) for 24 h. After refreshing the

medium, the cells were incubated with LDL containing [³H]cholesteryl ester at the concentration of 25 μg protein/mL with or without 0.5 μCi/mL of [methyl-¹⁴C]-choline chloride (NEN Life Science, Boston, MA) for 24 h. The cells were further washed with PBS three times and incubated for 24 h in 1 mL of 0.1% BSA/F-10. After the replacement of the medium by 1 mL of 0.02% BSA/F-10, the cells were incubated for 8 h for the apolipoprotein-mediated release of cholesterol and phospholipid from the cells. For the digestion of sphingomyelin in the plasma membrane, the cells were pre-incubated with SMase (Sigma) at various concentrations for 1 h and washed prior to the incubation with apolipoprotein. Sphingomyelin was given to the cells as the vesicles by injecting its ethanol solution into the culture medium to make the final ethanol concentration 0.1%. Lipid was extracted with hexane–isopropanol 3:2 (v/v) and chloroform–methanol 2:1 (v/v) from the cells and conditioned medium, respectively. Radioactivity of cholesterol, cholesteryl ester, phosphatidylcholine, and sphingomyelin were measured by a Liquid Scintillation counter after the separation by thin layer chromatography (TLC). Total and unesterified cholesterol and choline-phospholipid were also measured by enzymatic colorimetric method with commercial assay kits for the cellular lipid extracts. Esterified cholesterol was then calculated by subtracting unesterified cholesterol from total cholesterol. The relative mass amount of phosphatidylcholine and sphingomyelin was determined by a TLC/FID analyzer (Iatroscan MK-5) at the Iatron Laboratories Inc., Tokyo, (Tokyo, Japan), and each mass amount was calculated by proportionating the choline-phospholipid mass. The release of lipid from the cell was expressed as either the percentage for the total count of the respective lipid in the medium and cell, or the mass of lipid calculated from the specific radioactivity of cellular lipid and the count of the lipid in the medium, except for that directly measured. Cellular protein was measured using the method of Lowry et al. (27).

Pulse labeling of cellular phospholipid

The astrocytes in the 3-cm culture plates were incubated with 1 μCi/ml of [methyl-¹⁴C]choline chloride for 1 h. The cells were washed and treated with SMase for 1 h, and the washed cells were incubated in 0.02% BSA/F-10. After certain periods of the incubation, lipid was extracted with the organic solvent from the cells and the radioactivity in sphingomyelin and phosphatidylcholine was analyzed after separation by TLC.

Preparation of detergent-resisting membrane fraction

Cholesterol and phosphatidylcholine of the astrocytes were labeled by incubating with the LDL containing [³H]cholesteryl ester and [methyl-¹⁴C]choline chloride as described above. Plasma membrane was prepared from the cells by the method of Thom et al. (28). The plasma membrane fraction was then treated in 0.1% Triton X-100 in PBS containing 1 mM benzamidine and 1 mM PMSF on ice for 20 min. The mixture was centrifuged in 30% sucrose at 90,000 rpm for 1 h to remove the membrane protein-lipid complex as a pellet. The supernatant was diluted by three folds with PBS containing same protease inhibitors to make the sucrose concentration 10% and recentrifuged at 90,000 rpm for 1 h. The Triton X-100 “insoluble” fraction was obtained as a pellet. Lipid was extracted from the membrane fractions and analyzed by TLC for the radioactivity.

Intracellular cholesterol esterification

To analyze an intracellular free cholesterol pool available for the esterification by ACAT, incorporation of [¹⁴C]oleic acid (0.45 μCi/ml, Amersham) into cholesteryl ester was determined (29). After incubation of the cells with apolipoprotein for 2 h, the cells were incubated with [¹⁴C]oleic acid for 1 h and the

incorporation of the radioactivity to cholesteryl ester fraction was measured.

RESULTS

Figure 1 shows the change by the SMase treatment in the radioactivity count of the choline-labeled sphingomyelin and phosphatidylcholine in the astrocytes preconditioned by incubating with LDL (25 μg protein/mL for 24 h) to match the cholesterol release experiments. The two thirds of sphingomyelin was selectively digested by the extracellular enzyme while phosphatidylcholine was largely intact. **Figure 2** shows the time-dependent change of cellular sphingomyelin and phosphatidylcholine mass

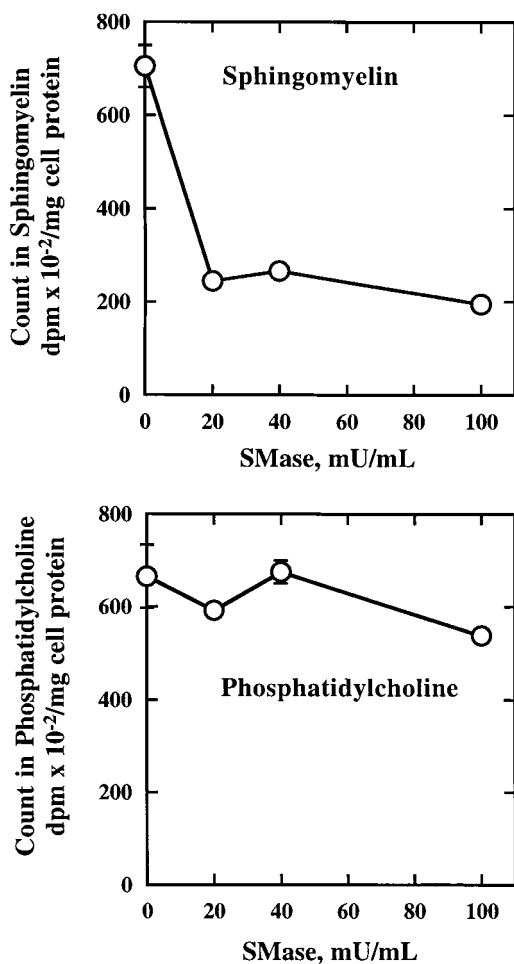


Fig. 1. Digestion of sphingomyelin in the rat astrocytes by extracellular SMase. The astrocytes in the 3-cm culture plates (74.5 ± 20.8 μg protein/plate) were labeled with 0.5 $\mu\text{Ci}/\text{mL}$ of [methyl-¹⁴C]-choline chloride for 24 h in the presence of LDL 25 $\mu\text{g}/\text{mL}$, as described in Materials and Methods. After washing with the cell buffer and subsequent culture for 24 h, the cells were treated with SMase (0, 20, 40, or 100 mU) for 1 h. Lipid was extracted with the organic solvent from the cells and the radioactivity count in sphingomyelin (upper panel) and phosphatidylcholine (lower panel) was analyzed after the separation by TLC. Each data point represents the average and standard error of the triplicated samples.

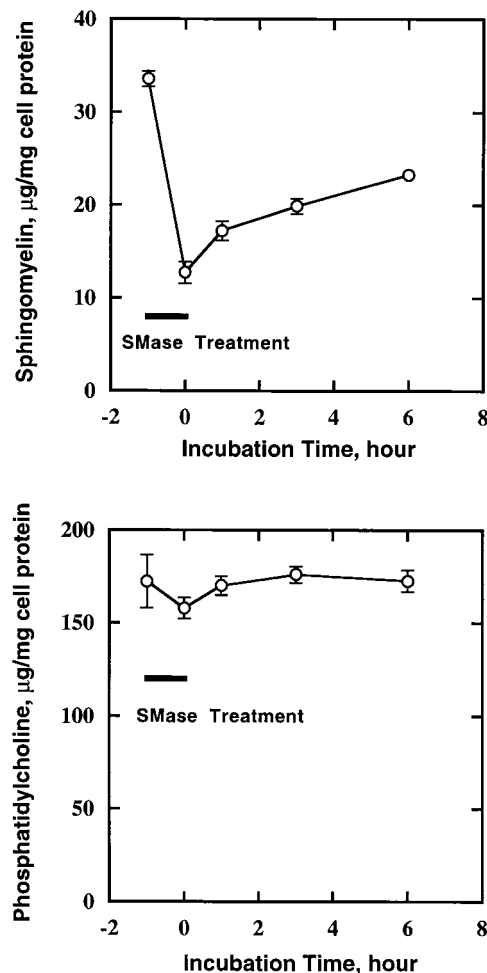


Fig. 2. Change in the mass of cellular phospholipid by the SMase treatment. The astrocytes (88.9 ± 1.2 μg protein/plate) were treated by SMase (100 mU) for 1 h and washed. Cellular lipid was extracted at the time point indicated in the figure. Total choline-phospholipid was measured by an enzymatic colorimetric method and relative mass amount of phosphatidylcholine and sphingomyelin was determined by using a TLC/FID analyzer for each sample. Mass of each lipid was estimated accordingly. The data points represent the mean \pm standard error of the triplicated samples.

caused by the SMase treatment. After the selective decrease by the digestion, sphingomyelin gradually increased to recover a substantial part of the loss during the 6 h. Phosphatidylcholine largely remained the same except for slight decrease immediately after the digestion, which may represent the rapid synthesis of sphingomyelin from phosphatidylcholine by choline transfer during this phase (mentioned later). In this condition, the cell viability remained unchanged as monitored by the trypan blue uptake.

The apoA-I-mediated cholesterol release was demonstrated from the rat astrocytes loaded with LDL containing [³H]cholesteryl oleate by measuring cholesterol mass (**Table 1**). The release was increased by pretreatment of the cells with SMase (100 mU), and cellular cholesterol reciprocally decreased mainly in esterified cholesterol. No significant difference was noticed in cholesterol specific

TABLE 1. Change in cholesterol mass in cells and medium by the apoA-I-induced cellular cholesterol release

| SMase | ApoA-I | Cells | | | | Medium | |
|-------|--------|--------------------------------------|--------------------------|--------------------------------------|--------------------------|--------------------------------------|--------------------------|
| | | Unesterified | | Esterified | | Unesterified | |
| | | Cholesterol | Specific Radioactivity | Cholesterol | Specific Radioactivity | Cholesterol | Specific Radioactivity |
| | | $\mu\text{g}/\text{mg cell protein}$ | $\text{dpm}/\mu\text{g}$ | $\mu\text{g}/\text{mg cell protein}$ | $\text{dpm}/\mu\text{g}$ | $\mu\text{g}/\text{mg cell protein}$ | $\text{dpm}/\mu\text{g}$ |
| - | - | 21.4 ± 0.9 | 949 ± 107 | 8.06 ± 1.07 | 788 ± 150 | 2.16 ± 0.26 | 818 ± 126 |
| - | + | 22.4 ± 0.3 | 828 ± 45 | 6.36 ± 0.17 | 1042 ± 272 | 5.21 ± 0.02 ^a | 1042 ± 94 |
| + | - | 23.9 ± 0.9 | 1054 ± 94 | 7.49 ± 0.59 | 831 ± 26 | 2.14 ± 0.05 | 986 ± 45 |
| + | + | 20.0 ± 1.3 | 923 ± 41 | 6.22 ± 0.4 ^b | 870 ± 171 | 6.82 ± 0.54 ^c | 1143 ± 123 |

The astrocytes in the 3-cm culture plates ($76.7 \pm 0.7 \mu\text{g protein}/\text{plate}$) preloaded with LDL $25 \mu\text{g}/\text{mL}$ containing [^3H]cholesteryl ester were treated by SMase (100 mU) for 1 h, and incubated with apoA-I, $10 \mu\text{g}/\text{mL}$, for 8 h. Lipid was extracted from the medium and cells, and unesterified and esterified cholesterol were determined by an enzymatic colorimetric method. Cholesterol radioactivity was counted for each fraction. The values represent the mean ± standard error of the triplicated experimental data.

^a $P < 0.001$ from SMase (-)/apoA-I (-).

^b $P < 0.05$ from SMase (-)/apoA-I (-) and SMase (+)/apoA-I (-).

^c $P < 0.001$ from all others.

radioactivity among its cellular and extracellular compartments by exposure of the cells either to SMase or apoA-I (Table 1). Therefore, the release of cellular cholesterol was estimated by measuring radioactivity in the medium and specific radioactivity of cholesterol in the cells for further experiments.

The release of cholesterol was induced either by apoA-I or apoE at $5 \mu\text{g}/\text{mL}$ (Fig. 3A). Release of cholesterol by apoA-I or apoE was moderate, and the pretreatment of the cells with SMase (100 mU) enhanced both the apolipoprotein-mediated cholesterol releases. In contrast, the lipid microemulsion caused substantial cellular cholesterol efflux via a diffusion-mediated pathway, and its relative increase by the SMase treatment was only slightly though the increment seemed similar to that in the apolipoprotein-mediated release (Fig. 3B). Figure 4 illustrates a typical dose-dependent profile of the apoA-I-mediated and the lipid emulsion-mediated cholesterol release from the cells pretreated with SMase. The increase of the cholesterol release was shown with the wide range of apoA-I and microemulsion concentration.

In contrast to the cholesterol, neither sphingomyelin nor phosphatidylcholine release was significantly influenced by the SMase treatment of the astrocytes either to apoA-I or the lipid microemulsion (Fig. 5). The cellular phospholipid was also unchanged in these experiments. These data indicate that the cellular system for the assembly of HDL with cellular phospholipid by apoA-I is kept functional even after the sphingomyelin digestion and the HDL generated by this reaction became cholesterol-rich after the SMase treatment.

In order to examine the redistribution of cholesterol molecules after the SMase treatment, intracellular cholesterol was probed by measuring the incorporation of the labeled oleic acid into cholesteryl ester by the microsomal enzyme ACAT in the astrocytes (Fig. 6). The change was measured in the initial 2 h since it rapidly reaches the maximum in a few hours, indicating the involvement of the intracellular signal-mediated process (5, 6). The treatment of the astrocytes with SMase increased the esterification of intracellular cholesterol, suggesting the translocation of cholesterol from the compartment unavailable to ACAT to that available to this enzyme. Pre-incubation of the cells with apoA-I or apoE for 2 h caused the rapid decrease of the ACAT-available cholesterol indicating the translocation of cholesterol from this compartment perhaps

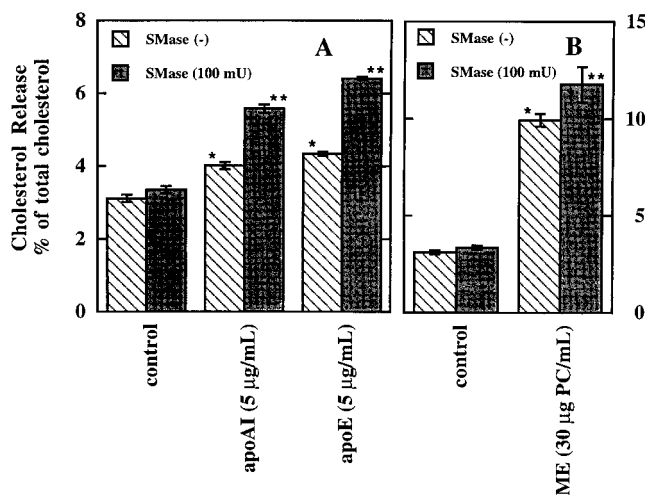


Fig. 3. The effect of the SMase treatment on the cholesterol release from the rat astrocytes mediated by apolipoprotein or microemulsion. The astrocytes were cultured in the 3-cm culture plates ($175.8 \pm 15.2 \mu\text{g protein}/\text{plate}$). LDL labeled with [^3H]cholesteryl oleate ($601,021 \text{ dpm}/20.8 \mu\text{g cholesterol moiety of cholesteryl ester}$) was added to the cells in a fresh 0.1% BSA/F-10, followed by the incubation at 37°C for 24 h. After washing with the cell buffer and replacement with 0.1% BSA/F-10, the cells were treated with 100 mU SMase for 1 h, and further incubated in the presence or absence of apoA-I, apoE, or microemulsion (ME) at the indicated concentration for 8 h. Lipid was extracted with the organic solvent from the cells and the conditioned medium and analyzed after separation by TLC as described in Materials and Methods. The data represents the average of the triplicated measurements and standard error of the triplicated measurements. The single asterisk indicates the significant difference from the respective "control" by $P < 0.01$, and the double asterisk indicates the difference from the respective SMase (-) by $P < 0.01$

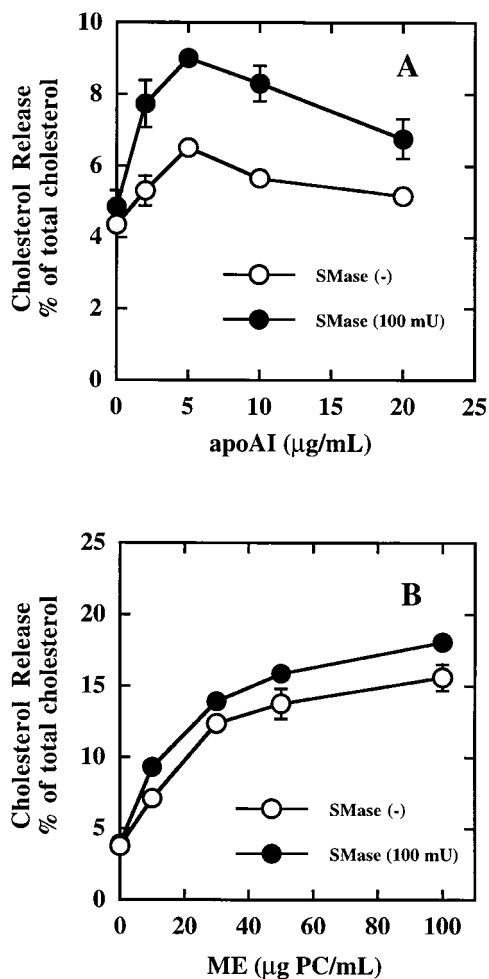


Fig. 4. The effect of the SMase treatment on the cholesterol release from the rat astrocytes. The astrocytes were cultured in 3-cm culture plates (134.5 ± 5.2 µg protein/plate). The cells were loaded with the labeled LDL with [^3H]cholesteryl oleate ($601,021$ dpm/ 20.8 µg cholesterol moiety of cholesteryl ester). After the 1-h treatment with 100 mU SMase, apoA-I or microemulsion (ME) was added to the medium at the various concentrations indicated and further incubated with the cells for 8 h. Closed circles represent the SMase-treated cells and open circles represent the SMase-untreated cells. The data represents the average and standard error of the triplicated measurements.

to the compartment utilized by the HDL assembly. Interestingly, the effect of the SMase treatment to increase the ACAT-available cholesterol pool was no longer observed in the astrocytes in the presence of apolipoproteins suggesting that more cholesterol was used for the HDL. Although a substantial amount of cellular cholesterol was removed by the lipid microemulsion (Figs. 3 and 4), no significant decrease of the ACAT-available pool was demonstrated and the SMase treatment did not influence this result either.

The rat fibroblasts were also examined for the effect of the SMase treatment on the apoA-I-mediated cholesterol release and the change (Fig. 7) in the ACAT-available intracellular compartment (Fig. 8). The treatment enhanced the apoA-I-mediated cellular cholesterol release, and also the lipid microemulsion-mediated release, but to

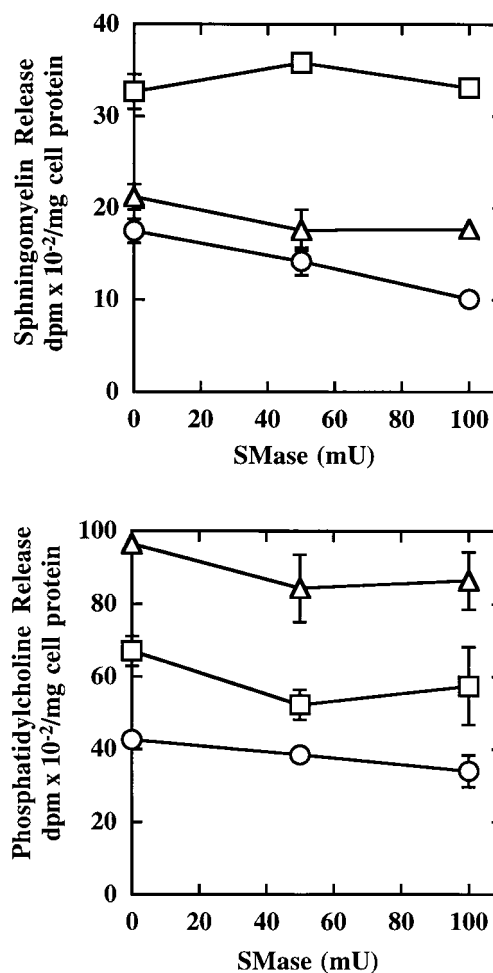


Fig. 5. Release of sphingomyelin and phosphatidylcholine from the rat astrocytes treated with SMase. The astrocytes in the 3-cm culture plates (74.5 ± 20.8 µg protein/plate) were labeled with 0.5 µCi/mL of [methyl- ^{14}C]choline chloride for 24 h in the presence of LDL 25 µg/mL as shown in Fig. 1. The cells were incubated with the blank medium (circles), apoA-I (triangles), or microemulsion (squares) at the indicated concentration for 8 h after the 1-h treatment with 100 mU SMase. After the lipid extraction from the culture medium, the radioactivity in sphingomyelin (upper panel) and phosphatidylcholine (lower panel) were analyzed after separation by TLC. The data represents the average and standard error of the triplicated measurements.

a lesser extent, essentially in the same manner as it did for the astrocytes. The exogenous apoA-I or apoE, but not the lipid microemulsion, induced the decrease of the ACAT-available cholesterol compartment. The increase of this cholesterol compartment by the SMase treatment was observed in all these conditions in the fibroblasts and more prominent than in the astrocytes. In contrast to the astrocytes, this increase was still obvious even after the apolipoproteins reduced this compartment. The finding suggested the cholesterol molecules in the fibroblasts tend to be more easily translocated from the cell surface to the intracellular compartment by the relief of the sphingomyelin-restriction than those in the astrocytes.

As described above, the treatment of rat astrocytes with SMase resulted in digestion of two-thirds case of the cellular

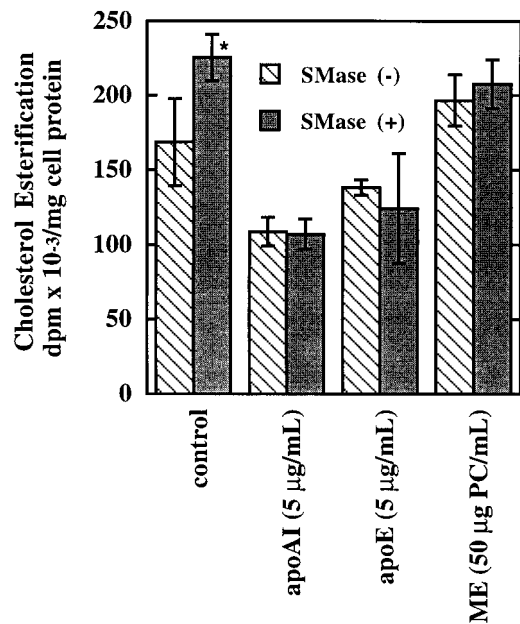


Fig. 6. The effect of the SMase treatment on redistribution of cellular cholesterol in the astrocytes. Incorporation of [¹⁴C]oleate into cholesteryl ester by the astrocytes pretreated with SMase and then apoA-I. After loading with LDL as 25 µg protein/mL for 24 h, the cells in the 3-cm plates were incubated for 24 h in fresh 0.1% BSA/F-10. The cells were treated with 100 mU SMase for 1 h and incubated with apoA-I (5 µg/ml), apoE (5 µg/ml), or microemulsion (50 µg PC/ml) for 2 h after washing. After washing again, the cells were incubated with [¹⁴C]oleate for 1 h and the radioactivity in cholesteryl ester was determined. The data represents the average and standard error of the triplicated measurements. The asterisk indicates the significant difference from the SMase (-) by $P < 0.01$.

sphingomyelin and thereafter it was gradually recovered (Fig. 2). The change in the de novo synthesis of sphingomyelin during this process was examined after the treatment by a pulse-chase type experiment (Fig. 9). Incorporation of [³H]choline into sphingomyelin was enhanced by the treatment. The choline uptake by phosphatidylcholine also increased perhaps indicating that the rescue synthesis of sphingomyelin is done mainly by the transfer of phosphorylcholine. The transient decrease of phosphatidylcholine mass shown in Fig. 2 may reflect this process. The peak of the appearance of the pulse label was at around 2 h after the completion of the sphingomyelin digestion and 3 h after the end of the pulse labeling, suggesting the compensatory supplement of sphingomyelin should be effective after this period.

The 8-h efflux experiments represent the overall integrated effect of the sphingomyelin digestion and subsequent recovery demonstrated in Fig. 2. During this period, the SMase treatment did not influence the baseline cholesterol release which perhaps represents its secretion with endogenous apoE and non-specific release by diffusion (Figs. 3 and 4). The effect of the SMase treatment on the apoA-I-mediated efflux was therefore observed for the segments of this period by measuring the 2-h efflux (Fig. 10). Because the recovery of sphingomyelin after the digestion was rapid (Fig. 2), the time segments examined in-

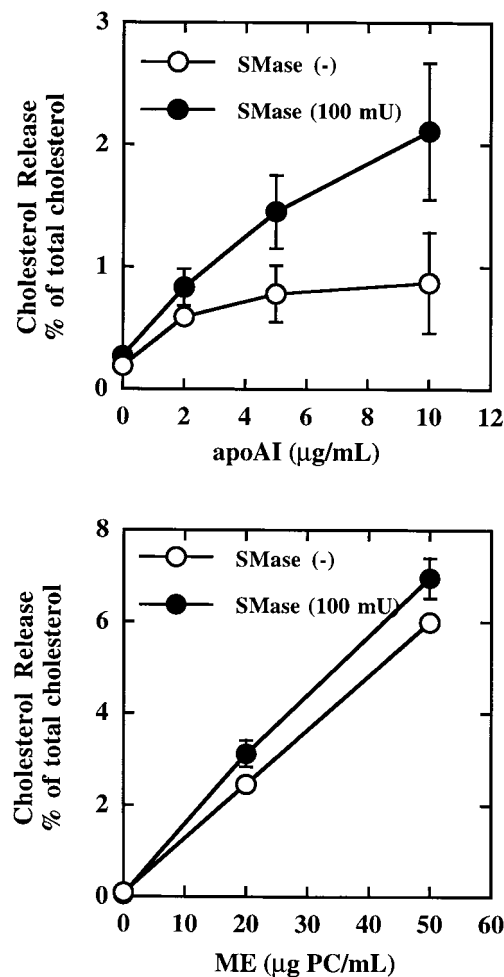


Fig. 7. ApoA-I-mediated cholesterol release from the SMase-treated rat fibroblasts. Rat fibroblasts were cultured in the 3-cm culture plates (146.8 ± 2.1 µg protein/plate) and labeled with LDL containing [³H]cholesteryl oleate (579,213 dpm/8.8 µg cholesterol moiety of cholesteryl ester). After the incubation with (closed circle) or without (open circle) 100 mU SMase for 1 h, the cells were incubated for 8 h with apoA-I (upper panel) or microemulsion (lower panel) at the indicated concentration. The lipid was extracted from the medium and the cells and counted for the radioactivity in cholesterol. The data represents the average and standard error of the triplicated measurements.

cluded those during and after the digestion at the time points indicated (Fig. 10). The cholesterol efflux kinetics had an initial slow phase (12); percent increase by the SMase treatment was somewhat lower than the 8-h experiments. The effect of the sphingomyelin digestion on the apoA-I-mediated cholesterol release did not last longer than 2 h after its completion.

To support the hypothesis that the sphingomyelin content in the cell is a direct regulating factor of the cell cholesterol release by apolipoprotein, sphingomyelin was directly provided to the cells after the digestion (Fig. 11). The rat astrocytes loaded with the labeled LDL (25 µg/ml) were treated with SMase at 50 mU for 2 h, and then sphingomyelin was added to the medium before the induction of apoA-I-mediated cell cholesterol release. Non-specific re-

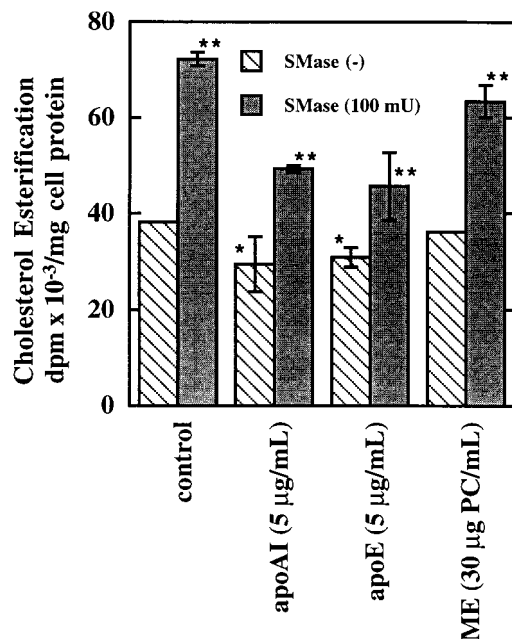


Fig. 8. The effect of the SMase treatment on redistribution of cellular cholesterol in the rat fibroblasts. Incorporation of [^{14}C]oleate into cholesteryl ester by the fibroblasts pretreated with SMase and then apoA-I. Rat fibroblasts in 3-cm culture plates ($131.3 \pm 16.1 \mu\text{g}$ protein/plate) were treated according to the same manner as described in Fig. 6. The data represent the average and standard error of the triplicated measurements. The single asterisk indicates the difference from the "control" by $P < 0.05$, and the double asterisk indicates the difference from the respective SMase (-) by $P < 0.001$.

lease of cellular cholesterol to the sphingomyelin vesicle ($5 \mu\text{g}/\text{mL}$ for 1 h) was estimated negligible ($<0.1\%$ of cellular cholesterol) during this treatment. The cellular uptake of sphingomyelin was approximately $1 \mu\text{g}/\text{mg}$ cell protein when $5 \mu\text{g}/\text{mL}$ sphingomyelin was incubated, accounting for the 8% increase of its cellular level immediately after the digestion (Fig. 2). Pre-incubation with sphingomyelin reversed the effect of the SMase treatment on the apoA-I-mediated cellular cholesterol release while it had no effect on the endogenous apoE-mediated cholesterol release.

Finally, the membrane domain was examined for the response to the SMase treatment (Fig. 12). The plasma membrane was prepared from the astrocytes prelabeled with the LDL containing [^3H]cholesteryl ester and [^{14}C]choline, and digested by SMase. The cholesterol count in the detergent-resisting fraction of the plasma membrane markedly decreased by the SMase treatment while it hardly decreased in the 30% sucrose pellet fraction. No significant change was found in the count of the phosphatidylcholine either in the detergent-resisting or the 30% sucrose pellet fraction.

DISCUSSION

The results are summarized as follows. 1) The treatment of the cells with SMase enhanced the apolipoprotein-mediated cholesterol release from the cell in a manner of increasing cholesterol incorporation into the HDL to gener-

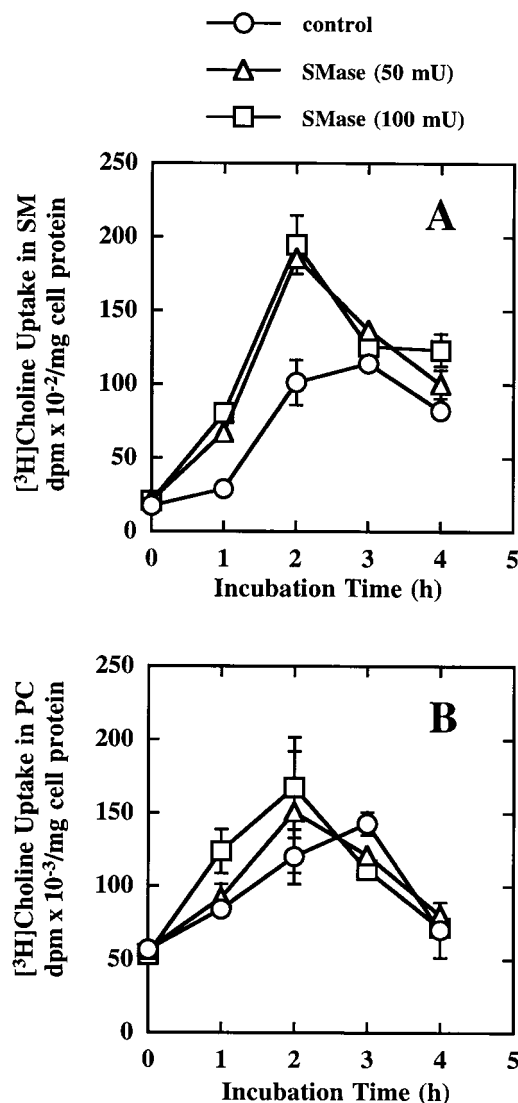


Fig. 9. Enhancement of phospholipid biosynthesis in the astrocytes shown by pulse labeling. The astrocytes in the 3-cm culture plates ($73.6 \pm 4.6 \mu\text{g}$ protein/plate) were incubated with $1 \mu\text{Ci}/\text{ml}$ of [methyl- ^{14}C]choline chloride for 1 h. After washing and treatment with SMase (0 mU, circles; 50 mU, triangles; 100 mU, squares) for 1 h, the washed cells were incubated in 0.02% BSA/F-10 for the indicated time. Lipid was extracted with organic solvent from the cells and the radioactivity in sphingomyelin (upper panel) and phosphatidylcholine (lower panel) was analyzed after separation by TLC. The data represents the average and standard error of the triplicated measurements.

ate cholesterol-rich HDL. 2) This effect was related to the decrease of cellular sphingomyelin, and therefore supplementation with sphingomyelin endogenous or exogenous reversed the effect of the digestion. 3) The SMase treatment lead to the redistribution of cellular cholesterol and apoA-I counteracted to this effect by removing cholesterol from the cells. 4) These findings are largely selective for the apolipoprotein-mediated reactions and the diffusion-mediated cell cholesterol efflux may be indirectly influenced.

There is a substantial amount of information that the level of sphingomyelin in the membrane regulates intra-

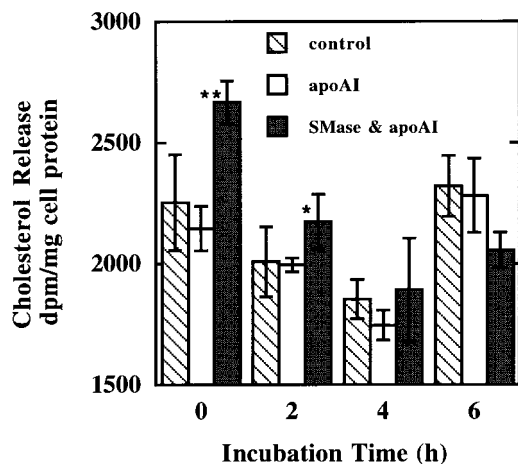


Fig. 10. Time-dependent change of the effect of the SMase treatment of the astrocytes on the apoA-I mediated cholesterol release. The apoA-I-mediated cholesterol efflux was measured for each 2-h segment of the 8-h period including the 2-h period of the incubation with the enzyme and 6-h period after washing the enzyme. The astrocytes were cultured in the 3-cm culture plates ($145.6 \pm 10.5 \mu\text{g}$ protein/plate). The cells were labeled by incubating with LDL containing ^3H -labeled cholesteryl oleate ($1,272,129 \text{ dpm}/65.8 \mu\text{g}$ cholesterol moiety of cholesteryl ester) for 24 h. The cells were then treated with SMase (0 or 50 mU) for 2 h after washing and medium replacement with 0.02% BSA/F-10. Cellular cholesterol release into the medium was measured in the presence of apoA-I ($5 \mu\text{g}/\text{ml}$) for the 2-h segment; the 2-h period during the digestion with SMase ($-2-0 \text{ h}$), and each 0–2, 2–4, and 4–6 h period after washing the enzyme. The abscissa indicates the timing of adding apoA-I. Lipid was extracted from the conditioned medium and the radioactivity was counted for cholesterol. The data represents the average and standard error of the triplicated measurements. The single asterisk indicates the difference from the “control” by $P < 0.05$, and the double asterisk by $P < 0.01$.

cellular cholesterol distribution. Depletion of sphingomyelin in plasma membrane by the SMase digestion induced the redistribution of cholesterol from the plasma membrane to the endoplasmic reticulum (18, 19, 30). The cholesterol molecules thus relocated to the endoplasmic reticulum was converted to their acylester by ACAT (18, 21, 30). The SMase treatment reportedly enhanced the vesiculation of plasma membrane (31). However, U18666A, a hydrophobic amine that inhibits specific cholesterol transport pathway, suppressed both the fundamental movement of plasma membrane cholesterol to the endoplasmic reticulum and the accelerated transport of cholesterol by the SMase treatment, but not plasma membrane vesiculation (32). From these findings, it is a widely accepted concept that cholesterol is strongly associated with sphingomyelin in plasma membrane and its movement is restricted by this interaction. The digestion of sphingomyelin decreases this restriction to cause the translocation of cholesterol molecules. The identification of the membrane “raft” rich in both cholesterol and sphingomyelin supported this view (16).

It was shown that cholesterol adsorption by 2-hydroxypropyl- β -cyclodextrin increased from the human skin fibroblasts whose sphingomyelin was digested approximately

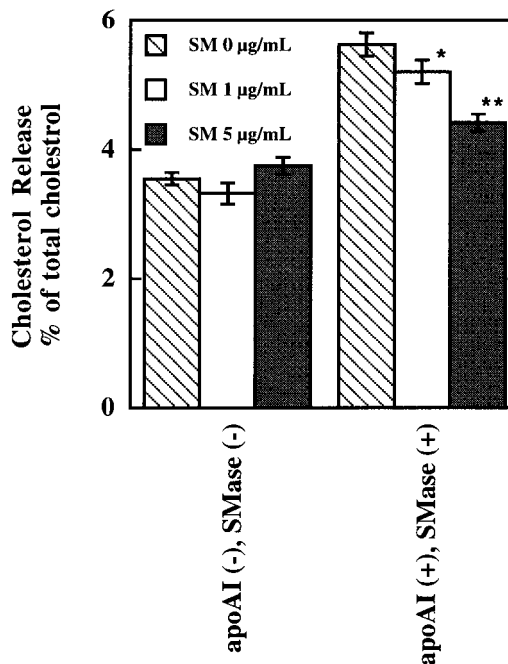


Fig. 11. The effect of sphingomyelin supply on the cholesterol release from the rat astrocytes pretreated with SMase. The astrocytes were cultured in the 3-cm culture plates ($158.6 \pm 12.5 \mu\text{g}$ protein/plate). The cells were labeled by incubating with LDL containing ^3H cholesteryl oleate ($601,021 \text{ dpm}/20.8 \mu\text{g}$ cholesterol moiety of cholesteryl ester) and incubated for 24 h. The cells were treated with SMase (0 or 100 mU) for 1 h and then washed with the cell buffer. After the medium replacement with 0.02% BSA/F-10, the cells were incubated for 1 h with sphingomyelin (SM) (0, 1, or 5 $\mu\text{g}/\text{mL}$) in an ethanol solution to make the ethanol concentration 0.1% in the medium, washed, and then with apoA-I (0 or 10 $\mu\text{g}/\text{mL}$) for 6 h. Lipid was extracted from the cells and the conditioned medium and the count in cholesterol and cholesteryl ester were analyzed after separation by TLC. The data represents the average and standard error of the triplicated measurements. The single asterisk indicates the difference from the “SM 0” by $P < 0.05$, and the double asterisk by $P < 0.01$.

50% by SMase (33). On the other hand, the HDL-mediated cellular cholesterol efflux was not influenced by sphingomyelin degradation in the cultured fibroblasts and HDL failed to inhibit the SMase-induced intracellular cholesterol esterification (34). Thus, it has not yet been clear if such cholesterol redistribution caused by the SMase treatment influences the cellular cholesterol removal or efflux.

The data presented in this paper demonstrated that apolipoprotein-mediated cholesterol release was more influenced by the SMase treatment rather than the diffusion-mediated cholesterol efflux. The intracellular redistribution of cholesterol induced by the sphingomyelin digestion was also more prominently influenced by the apoA-I-mediated cholesterol release, especially in the astrocytes. Therefore, it is conceivable that the liberation of cholesterol molecules from the restriction by the sphingomyelin digestion takes place mainly in the region related to the domain used by the apoA-I-mediated HDL generation. In the absence of the extracellular apolipoprotein, cholesterol molecules are relocated to the ACAT-available pool. If there is apolipoprotein

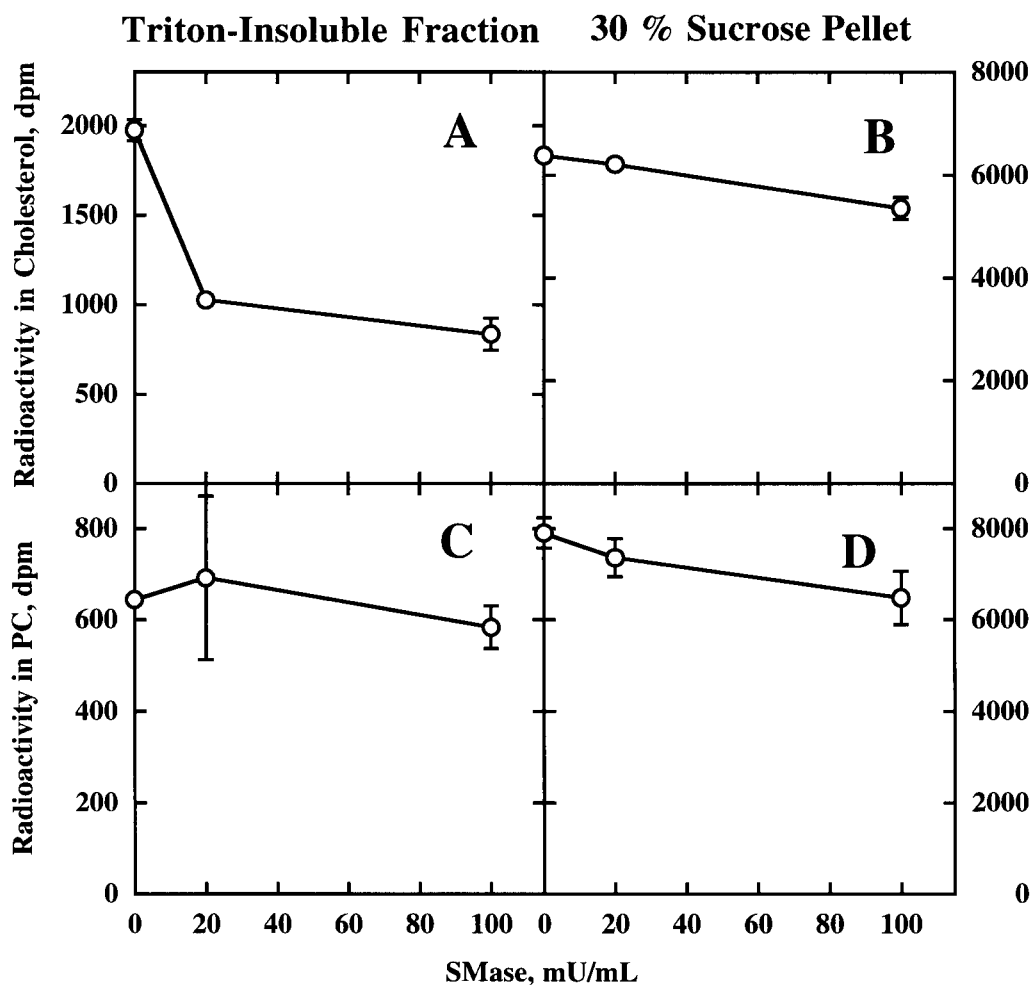


Fig. 12. Change by the SMase treatment of the cholesterol content in the membrane fractions of the rat astrocytes. The astrocytes were cultured in the 15-cm petri dish (2.89 ± 0.12 mg protein/20 ml of medium/dish). Cholesterol and phosphatidylcholine of the cells were labeled by incubating with the LDL containing [^3H]cholesteryl ester and [^{14}C]choline chloride as described for Fig. 2. The plasma membrane fraction was prepared according to the method of Thom et al. (28). After the treatment of this fraction with SMase (0, 20, or 100 mU) as 29.0 μg membrane protein/200 μL in Tris-buffered saline, pH 7.5, containing 1 mm benzamidine and 1 mm PMSF, TBS, for 1 h, the membrane fraction was recovered as a pellet of the centrifugation at 15,000 rpm for 1 h. After resuspending the pellet in 200 μL of TBS, it was treated with 0.1% Triton X-100 in PBS containing 1 mm benzamidine and 1 mm PMSF on ice for 20 min. Triton-"insoluble" fraction (or detergent-resisting domain) (panels A and C) and 30% sucrose pellet (panels B and D) were prepared as described in Materials and Methods. Lipid was extracted and the radioactivity count was analyzed for cholesterol (panels A and B) and phosphatidylcholine (panels C and D) for each membrane fraction. The data represents the average and standard error of the triplicated measurements.

tein, cholesterol is also used more for the HDL generation. The digestion of sphingomyelin in such a domain may also allow the lateral diffusion of the restricted cholesterol molecules in the plasma membrane leading to the moderate relative increase of the diffusion-mediated cholesterol efflux to the lipid microemulsion.

In the astrocytes, the cholesterol relocation by the SMase treatment to the ACAT-available compartment was canceled perhaps by changing its direction towards the apolipoprotein-mediated HDL-generating reaction. In contrast, cholesterol in the fibroblasts was relocated to the ACAT-available compartment more prominently by the SMase treatment and apolipoprotein did not cancel this change. Thus, there seems to be a cell-specific vector

of the membrane cholesterol translocation by the relief of sphingomyelin-restriction. This may simply be because more cholesterol molecules are relocated to the ACAT-available compartment in the fibroblasts.

The effect of the SMase treatment seems to be transient (Fig. 9). It was most remarkable when the enzyme treatment was ongoing during the incubation of the cells with apoA-I, while the 2-h incubation with apoA-I alone did not show the apparent cholesterol release (also refer to ref. 12). The effect became less after the completion of the SMase treatment and almost disappeared when apoA-I was added at 2 h after the treatment. The small increase of the nonspecific diffusion-mediated cholesterol efflux to the lipid microemulsion was also highest in the midst of

the SMase treatment (data not shown). This finding suggests that the loss of sphingomyelin is rapidly recovered at the site of the HDL assembly by de novo synthesis or by translocation. Indeed, sphingomyelin synthesis is already substantially stimulated at 1 h after completion of the 1-h SMase treatment, and reaches the maximal level at 2 h (Fig. 9). The recovery of the total cellular sphingomyelin was also already substantial within the initial few hours after the treatment (Fig. 2). Furthermore, extracellularly supplied sphingomyelin to the cells reversed the effect of the SMase treatment (Fig. 11).

Finally, as the initial attempt to identify the domain that supplies cholesterol for the apolipoprotein-mediated HDL assembly, the sensitive domain to the SMase treatment was surveyed. The membrane fraction isolated as the Triton X-100-"insoluble" fraction appeared to be more susceptible to the SMase treatment than other part of the plasma membrane with respect to the decrease in cholesterol. We believe this is an equivalent fraction to what is called a "detergent-resisting domain" defined as a cholesterol- and sphingomyelin-rich domain by many other research groups (17). There is no direct evidence yet that this is the domain where the apolipoprotein-mediated HDL generation takes place. However, the present paper provided a strong implication that cholesterol restricted in this domain by sphingomyelin is released for the use of the apolipoprotein-mediated HDL generation. ■

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