Apolipoprotein-mediated Cellular Lipid Release Requires Replenishment of Sphingomyelin in a Phosphatidylcholine-specific Phospholipase C-dependent Manner*

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When sphingomyelin is digested by sphingomyelinase in the plasma membrane of rat astrocytes, productions of sphingomyelin, diacylglycerol, and phosphatidylcholine are stimulated. D609, an inhibitor of phosphatidylcholine-specific phospholipase C, suppressed these effects. Similarly, when apolipoprotein A-I removed cellular cholesterol, phosphatidylcholine, and sphingomyelin to generate high density lipoprotein, cholesterol synthesis from acetate subsequently increased, and sphingomyelin synthesis from acetate and ceramide also increased. D609 inhibited these effects again. D609 also inhibited the cholesterol removal by apoA-I not only from the astrocytes but also from BALB/3T3 and RAW264 cells. D609 decreased cholesterol synthesis, although D609 did not directly inhibit hydroxy methylglutaryl-CoA reductase. ApoA-I-stimulated translocation of newly synthesized cholesterol to cytosol was also decreased by D609. A diacylglycerol analog increased the apoA-I-mediated cholesterol release, whereas ceramide did not influence it. We concluded that removal of cellular sphingomyelin by apolipoproteins is replenished by transfer of phosphatidylcholine from phosphatidylcholine to ceramide, and this reaction may limit the removal of cholesterol by apoA-I. This reaction also produces diacylglycerol that potentially triggers subsequent cellular signal cascades and regulates intracellular cholesterol trafficking.

Plasma high density lipoprotein (HDL) is mainly generated by the interaction of helical apolipoproteins with cells to remove their phospholipid and cholesterol (1). This reaction requires a key membrane protein, ATP-binding cassette transporter A1 (ABCA1) (2–5). The mechanism for this HDL assembly with cellular lipid and apolipoproteins is not fully understood, and a role of ABCA1 in this reaction is also unclear at the current stage. Most of somatic cells are capable of generating HDL by this pathway, and this is one of the main mechanisms for exporting cholesterol of peripheral cells to the liver, its major catabolic site to bile acids. However, the principal organ of the production of HDL by this mechanism also seems to be the liver (6).

On the other hand, astrocytes generate HDL in vitro with apolipoprotein (apo) E endogenously synthesized and with other exogenously given helical apolipoproteins such as apoA-I (7). The HDL particles thus produced are thought to function as a main extracellular lipid carrier in the brain. HDL production with endogenous apoE is up-regulated by acidic fibroblast growth factor released presumably by the astrocytes themselves potentially by an autocrine mechanism upon the requirement such as the case of brain injury (8).

Whereas HDL assembled with endogenous apoE is rich in cholesterol, exogenous apolipoproteins generate HDL relatively cholesterol-poor (7). Digestion of sphingomyelin in the cell surface results in enrichment with cholesterol of the HDL assembled with exogenous apoA-I, and the replenishment of sphingomyelin restored the original profile of the production of cholesterol-poor HDL (9). Therefore, the restriction of cholesterol molecules by sphingomyelin in the membrane can be one of the factors to regulate the release of cell cholesterol by an apolipoprotein-mediated pathway. Sphingomyelin interacts with cholesterol in the membrane and forms a cluster domain rich in cholesterol and sphingolipids (10–12). This is a basic driving force to create a domain structure called rafts or caveolae in the plasma membrane, which are thought to participate in various specific cellular functions such as signal transduction and cholesterol trafficking (13, 14). The results above indicated that the cholesterol molecules assembled to HDL with exogenous apolipoproteins are also provided from these domains. The apolipoprotein-cell interaction removes phosphatidylcholine and sphingomyelin as major phospholipids to generate HDL. Removal of sphingomyelin was parallel with the cholesterol removal under certain conditions (15, 16).

The other important factor of this reaction is involvement of intracellular signal transduction to mobilize cell cholesterol for incorporation into the HDL. Protein kinase C is indicated as one of the signal mediators for this pathway, but the details of this aspect remain unclear (17, 18).

In this paper, we have investigated a role of sphingomyelin and its metabolism in the release of cholesterol by the apolipoprotein-mediated HDL assembly pathway. The experimental data implicated that the compensatory synthesis of sphingomyelin for its removal is required for continuous release of cholesterol by apolipoprotein, and this reaction produces diacylglycerol from phosphatidylcholine, which can be a potential trigger of intracellular signal transduction.
Experimental Procedures

Cell Culture and Conditioning—Rat fetal astrocytes were prepared as described elsewhere (19). The cerebra without surface blood vessels and meningeal layers were obtained from 17-day fetal Wistar rats. They were dissected into about 2-mm cubes and were treated with 0.1% trypsin solution in Dulbecco's phosphate-buffered saline containing 0.15% glucose (DPBS/G) at room temperature for 5 min. The cells were seeded in 10% fetal calf serum (FCS) in F-10 medium and cultured for 1 week (primary culture). After the treatment with 0.1% trypsin in DPBS/G again, the cells were cultured in 10% FCS/F-10 in a 24-well multiple tray (3-cm culture plates, Costar 3516) for 1 week. BALB/3T3 and RAW264 cells were obtained from the ATCC and maintained as described in the text. After separating the lipid by TLC, radioactivity in sphingomyelin (A), phosphatidylcholine (B), and diacylglyceride (C) was determined. The data represent the average ± S.E. of triplicate assays. * and ** indicate p < 0.05 and 0.01 from the cells without the SPMase treatment.

Preparation of LDL Containing Radiolabeled Cholesterol Ester—Lipoproteins of the plasma from a healthy volunteer were labeled with [1,2-3H]cholesteryl oleate (Amersham Biosciences), and the LDL fraction was isolated by ultracentrifugation and dialyzed against 5 mM NADP, 8 mM dithiothreitol, 200 mM KCl, and 0.25% Brij 97, containing 50 mM imidazole, 5 mM EDTA, 200 mM KCl, and 0.25% Brij 97, according to the method described previously (24).

Cellular Lipids Metabolism—Rat astrocytes at the confluent stage in 3-cm culture plates were washed three times with DPBS/G and then cultured in 1 ml of F-10 medium containing 0.02% bovine serum albumin (BSA) for 24 h. After replacement with 1 ml of the fresh medium, the cellular lipids were labeled by incubating for 1–24 h with 20 or 40 μCi/ml of [3H]acetate (PerkinElmer Life Sciences) for cholesterol, phosphatidylcholine, sphingomyelin, and diacylglyceride, and 5 or 10 μCi/ml of [1,3-3H]glycerol (Amersham Biosciences) for diacylglyceride, 1 μCi/ml of 1-[14C]serine (Amersham Biosciences) for ceramide and sphingomyelin, or 25 μg of protein/ml of the labeled LDL for cholesterol. Lipid was extracted with hexane/isopropanol alcohol (3:2, v/v) or chloroform/methanol (2:1, v/v) from the cells or the conditioned medium, respectively. Radioactivity of cholesterol, phosphatidylcholine, sphingomyelin, diacylglyceride, and ceramide was measured by a liquid scintillation counter after separation by TLC with the solvents of diethyl ether, benzene, ethanol, and acetic acid (40:50:2:0.2) for separation of cholesterol and diacylglyceride; chloroform, methanol, acetic acid, and water (250:150:40:10) for phosphatidylcholine and sphingomyelin; 2-butanol, acetic acid, and water (3:1:1) for ceramide. Cellular protein was determined by the method of Lowry et al. (25). The same data were displayed as a percent decrease (F). The data represent the mean ± S.E. of triplicate assay.

Assay of HMG-CoA Reductase Activity—HMG-CoA reductase assay was carried out according to the method described elsewhere (26). Briefly, the cell pellets were harvested by scraping with a rubber policeman and centrifugation at 1,000 × g for 10 min. The cell extracts were prepared by the treatment of the cell pellet with the buffer containing 50 mM imidazole, 5 mM EDTA, 200 mM KCl, and 0.25% Brij 97, pH 7.4, at 37 °C for 10 min and centrifugation at 12,000 × g for 15 min. The aliquot of the detergent-solubilized extract was incubated with 0.2 mM potassium phosphate buffer containing 40 mM potassium 6-phosphate, 5 mM NADP, 9 mM dithiothreitol, 10 μunits/ml glucose-6-phosphate dehydrogenase, and 2 μCi/ml 1-3H-glycerol (3-oxo-3-[3H]hydroxy-2-methylglutarate-CoA (American Radiolabeled Chemicals, Inc.) at 37 °C for 2 h. The reaction was terminated with 0.2 mM HCl, and the mixture was incubated at 37 °C for 15 min to allow lactonization of [3H]mevalonic acid. The labeled mevalonolate was identified by TLC by using acetone/benzene (1:1, v/v).
Apom-mediated Cell Lipid Release Requires SPM Replenishment

Cytosol Preparation—The cytosol of rat astrocytes was prepared according to the method of Thom et al. (27) and Io et al. (28). Briefly, the cells were harvested with a rubber policeman after washing four times with DPBS/G. The cell pellet prepared by centrifugation at 1,000 rpm for 10 min was treated with cold extract solution composed of 0.02 M boric acid, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine, pH 10.0, for 15 min with 25° strong agitation per 10 s every 5 min. The suspension was neutralized with 0.5 × HCl and then centrifuged at 3,000 rpm for 10 min. After removal of nuclei and cell debris, the suspension was centrifuged at 300,000 × g for 30 min at 4 °C to obtain supernatant as a cytosol fraction.

RESULTS

Rat astrocytes were treated with sphingomyelinase for 1 h, and incorporation of [3H]acetate into sphingomyelin and phosphatidylcholine and incorporation of [3H]glycerol into diacylglyceride were monitored in a time-dependent manner. Syntheses of all of these lipids were increased by enzyme treatment (Fig. 1). Increase was apparent with sphingomyelin (Fig. 1A) and diacylglyceride (Fig. 1C) at 3 h after the treatment, but it took over 5 h before the increase of phosphatidylcholine synthesis became detectable (Fig. 1B). After the cell was labeled with [3H]serine, rat astrocytes were treated with sphingomyelinase and then incubated with D609 (Fig. 2, A and B). Radioactivity in sphingomyelin slightly decreased after 3 h perhaps reflecting the metabolic turnover. When sphingomyelin was digested, the count somewhat recovered after 3 h, and D609

Fig. 3. Effect of D609 on production of diacylglyceride and sphingomyelin in rat astrocytes when cellular lipid is removed by apoa-I. A, the cells were labeled by incubating in 0.1% BSAF-10 with [14C]serine (2 μCi/ml) for 16 h. The cells were washed and then incubated with (dark bars) or without (light bars) 5 μg/ml apoa-I for 2 h in a fresh 0.02% BSAF-10, followed by washing and the incubation with D609 (50 μg/ml) for 3 h. Lipid was extracted, and radioactivity was determined for sphingomyelin fraction. B, the cells were labeled in 0.1% BSAF-10 with [3H]acetate (20 μCi/ml) for 3 h, washed, and incubated in a fresh 0.02% BSAF-10 with (dark bars) or without (light bars) apoa-I (5 μg/ml) for 2 h in the presence of D609 (0, 20, and 50 μg/ml). Lipid was extracted, and radioactivity in diacylglyceride was determined. C, the cells were incubated with (dark bars and closed circles) and without (light bars and open circles) apoa-I (5 μg/ml) in the presence of D609 (0, 20, and 50 μg/ml) for 2 h and then washed. The cells were then labeled with [3H]acetate (20 μCi/ml) for 2 h in fresh 0.02% BSAF-10 in the presence of D609 at the same concentration as the pretreatment. Radioactivity in cellular sphingomyelin was determined. D, the data in C are displayed as a percentage of the control. The data represent the mean ± S.E. of triplicate assays.

Fig. 4. Effect of D609 on apoa-I-mediated cholesterol release of rat astrocytes treated with SPMase. Rat astrocytes were incubated in a fresh 0.1% BSAF-10 for 24 h. The cells were labeled with [3H]acetate (40 μCi/ml) for 3 h in 0.02% BSAF-10, washed, and treated with SPMase (0 or 100 milliunits) in the presence (dark bars) or absence (light bars) of 50 μg/ml of D609 for 1 h in a fresh 0.02% BSAF-10. The cells were further incubated with apoa-I (5 μg/ml) in the presence or absence of 50 μg/ml of D609 for 4 h after washing. Lipid was extracted from the conditioned medium, and radioactivity in sphingomyelin (A) and cholesterol (B) was determined. The data represent the mean ± S.E. of triplicate assays.

Fig. 5. Effect of D609 on the apoa-I-mediated cholesterol release from BALB/3T3 cells and RAW264 cells. BALB/3T3 cells were incubated with [3H]acetate (20 μCi/ml) for 16 h in 0.1% BSAF-10 then further incubated with (dark bars) or without (light bars) 5 μg/ml of apoa-I for 4 h in the presence of D609 (0, 20, or 50 μg/ml) in a fresh 0.02% BSAF-10. Lipid was extracted from the conditioned medium, and radioactivity was determined in cholesterol (A), phosphatidylcholine (B), and sphingomyelin (C). RAW264 cells were incubated with dibutyryl cAMP (0 or 300 μM) and [3H]acetate (20 μCi/ml) for 16 h in a fresh 0.1% BSAF-10, washed, and further incubated with (dark bars) or without (light bars) apoa-I (5 μg/ml) in the presence or absence of 50 μg/ml D609 for 4 h in 0.02% BSAF-10. Lipid was extracted from the conditioned medium, and radioactivity was determined in cholesterol (D), phosphatidylcholine (E), and sphingomyelin (F). The data represent the mean ± S.E. of triplicate assays.
inhibited this recovery (Fig. 2A). Radioactivity in ceramide reciprocally increased by D609 indicating that recovery reaction uses ceramide (Fig. 2B). D609 suppressed the incorporation of acetate into diacylglyceride, more greatly in the sphingomyelinase-treated cells than the untreated cells (Fig. 2C). Inhibition of sphingomyelin synthesis by D609 was also greater in the treated cells than the non-treated cells (Fig. 2, D–F). The results indicated that acute decrease of sphingomyelin by its digestion was rescued by the pathway including the transfer reaction of phosphorylcholine from phosphatidylcholine to ceramide. The decrease in diacylglyceride production by D609 was consistent with the notion that it inhibits phosphatidylcholine-specific phospholipase C, and the release of diacylglyceride was reduced.

When the cells were incubated with apoA-I to induce the removal of cellular lipid, incorporation of serine into sphingomyelin (Fig. 3, A, C, and D) and production of diacylglyceride (Fig. 3B) were all increased, and these increases were suppressed by D609. The responses were very similar to those after the sphingomyelinase treatment, indicating that the reduction of sphingomyelin after the removal by apoA-I is compensated by the same phosphorylcholine transfer pathway from phosphatidylcholine. Sphingomyelin synthesis was inhibited by D609 only to the extent to cancel the increase by apoA-I (Fig. 3, A and D), indicating that acute replenishment of sphingomyelin is dependent on phosphatidylcholine-specific phospholipase C, but the base-line synthesis is rather by other pathways. As shown in Fig. 4, the releases of cholesterol and sphingomyelin by apoA-I were both suppressed by D609 regardless of the pretreatment of the cells with sphingomyelinase. Inhibition of the apoA-I-mediated lipid release by D609 was observed in a similar manner with other cell lines such as BALB/3T3 and dibutylryl cAMP-treated RAW264 cell (Fig. 5). Thus, replenishment of sphingomyelin is required for continuation of cholesterol removal by apoA-I.

Inhibition of the cholesterol release by D609 resulted in suppression of cholesterol biosynthesis, not only at the step of mevalonate synthesis but also further downstream of the pathway (Fig. 6, A–C), although D609 does not show a direct inhibitory effect on HMG-CoA reductase (Fig. 6D). Thus, D609 indirectly influenced the cholesterol biosynthesis pathway through modulation of the apoA-I-mediated change of intracellular cholesterol homeostasis. Translocation of newly synthesized cholesterol to the cytosol was markedly inhibited by D609 along with significant suppression of the apoA-I-mediated translocation of sphingomyelin and phosphatidylcholine to the cytosol (Fig. 7).

It has been indicated that apolipoprotein-mediated cellular lipid release involves intracellular signal transduction (17, 18, 29–32). When sphingomyelin is removed by apoA-I, the replenishment reaction would modulate the levels of the lipid signal mediators by producing diacylglycerol and using ceramide. The direct effect of these compounds was therefore estimated. As shown in Fig. 8, C16-ceramide failed to decrease the apoA-I-mediated cholesterol release. On the other hand, the effect of phorbol 12-myristate 13-acetate (PMA) that mimics diacylglyceride was positive on the apoA-I-mediated cholesterol release (Fig. 8C). These findings suggested that the increase of diacylglyceride by D609 rather than consumption of ceramide may also be a factor that influences the apoA-I-mediated cholesterol release.

**DISCUSSION**

The results are summarized as follows. Digestion of sphingomyelin in plasma membrane stimulates the biosynthesis of sphingomyelin by the pathway of phosphorylcholine transfer from phosphatidylcholine to ceramide. The same cellular reaction is observed when apoA-I removes cellular sphingomyelin to produce HDL. This compensatory synthesis of sphingomyelin subsequently produces diacylglyceride. D609 has been first reported as an antiviral compound (20) and is widely accepted as a selective and competitive inhibitor of phosphatidylcholine-specific phospholipase C (21–23). This compound inhibited not only this pathway but also the apoA-I-mediated change in cellular cholesterol metabolism, its release, the increase of the biosynthesis, and its translocation to the cytosol.

We reported previously that sphingomyelin-cholesterol interaction in plasma membrane is one of the regulatory factors for the apoA-I-mediated release of cholesterol, especially in the astrocyte, by restricting cholesterol molecules (9). Massive
digestion of sphingomyelin increased the incorporation of cholesterol into the HDL generated by exogenous apoA-I. Sphingomyelin is rapidly recovered in the cells, and the effect of the digestion on the apoA-I-mediated cholesterol release gradually diminished as the recovery goes. Exogenous supply of sphingomyelin also reversed the effect of digestion.

The data presented in this work indicated that the replenishment of sphingomyelin is carried out mainly by the transfer reaction of phosphorylcholine from phosphatidylcholine to ceramide. Interestingly, the apoA-I-mediated generation of HDL, which accompanies substantial removal of cellular sphingomyelin, also stimulates this sphingomyelin replenishment pathway. In apparent contrast to the reactions after massive digestion of sphingomyelin, continuous cholesterol release by apoA-I seems to require this replenishment of sphingomyelin. Therefore, the effect of sphingomyelin digestion seems mainly due to the relief of the cholesterol molecule and induction of its intracellular redistribution. On the other hand, cholesterol and sphingomyelin may be removed together from the membrane by the apoA-I-mediated reaction, and the endogenous supply of sphingomyelin seems to be required for continuation of this reaction.

The apoA-I-mediated cellular lipid release and assembly of HDL are accompanied by the related intracellular events of cholesterol metabolism, such as translocation of cholesterol from the compartment used by acyl-CoA:cholesterol acyltransferase (32, 33), and to the cytosol to form lipid-protein complex particles and the increase of biosynthesis of cholesterol (28).

These related reactions are also suppressed by inhibiting the replenishment synthesis of sphingomyelin. The findings strongly implicate an essential role of sphingomyelin in the incorporation of cholesterol into the HDL generated by apolipoprotein-cell interaction. The intracellular cholesterol transport for the HDL assembly depends on the intracellular supply of sphingomyelin, so that formation of a cholesterol-sphingomyelin domain in the membrane may be important for the HDL assembly reaction.

It is indicated by a few authors including ourselves (17, 18, 29–32) that intracellular signaling is involved in activation of cholesterol trafficking for its incorporation into HDL. From this viewpoint, it is noteworthy that sphingomyelin synthesis reactions for the replenishment apparently involve lipid signal mediators. Phosphorylcholine is released from phosphatidylcholine by leaving diacylglycerol and is transferred to ceramide to produce sphingomyelin. The inhibition of apoA-I-mediated events by D609 can therefore be interpreted by a decrease in diacylglyceride or by an increase of ceramide. C2-ceramide, a ceramide analog, did not influence the apoA-I-mediated cholesterol release, implying that ceramide is not involved in direct regulation of these reactions. On the other hand, a functional analog of diacylglyceride, PMA, stimulated the apoA-I-mediated cholesterol release, suggesting that increase of diacylglycerol may be related to the enhancement of intracellular cholesterol trafficking. This is also consistent with the previous reports that protein kinase C activation can be involved in mobilization of cellular cholesterol molecules for assembly of HDL.
Inhibition of sphingomyelin synthesis by D609 markedly inhibited cholesterol synthesis in rat astrocytes treated with apoA-I. It is generally accepted that syntheses of cholesterol and sphingomyelin are regulated independently by such evidence that suicide inhibitors of serine palmitoyltransferase, β-chloroaalanine, and cycloserine do not alter cholesterol biosynthesis (34). However, some linkage between them can be considered as cholesterol synthesis was significantly increased when fibroblast membranes were enriched with sphingomyelin (35).

In the present work, suppression of cholesterol biosynthesis by inhibition of sphingomyelin synthesis did not accompany the decrease of HMG-CoA reductase. On the other hand, HMG-CoA reductase inhibitors did not influence phospholipid metabolism (data not shown). However, it is too premature to conclude about the mechanism for direct relationship between sphingomyelin metabolism and regulation of cholesterol homeostasis.

Luberto and Hannun (36) reported that D609 inhibited sphingomyelin synthase activity of SV40-transformed human lung fibroblasts but not phosphatidylcholine-specific phospholipase C activity, suggesting that the release of phosphorylcholine from phosphatidylcholine may be partially due to sphingomyelin synthase. Nevertheless, the present data suggested that the effect of D609 was achieved by inhibition of the release of phosphorylcholine from phosphatidylcholine no matter what the mechanism is.

For further confirmation of an important role of sphingomyelin replenishment in the apoA-mediated cholesterol release, more direct inhibition of sphingomyelin synthesis should be attempted.

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