Regulation of the selective uptake of cholesteryl esters from high density lipoproteins by sphingomyelin

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Abstract Although sphingomyelin (SM) is a major phospholipid in lipoproteins as well as in the membrane rafts where the scavenger receptor class B type I (SR-BI) is localized, its possible role in the selective uptake of cholesteryl ester (CE) by the SR-BI-mediated pathway is unknown. We investigated the effect of SM in lipoproteins and cell membranes on the selective uptake in three different cell lines: SR-BI-transfected CHO cells, hepatocytes (HepG2), and adrenocortical cells (Y1BS1). Incorporation of SM into recombinant high density lipoprotein (rHDL) containing labeled CE resulted in up to 50% inhibition of the selective uptake of CE in all three cell lines. This inhibition was completely reversed by treatment of rHDL with sphingomyelinase (SMase). Selective uptake from plasma HDL was activated by 22–72% after treatment of HDL with SMase. In addition, pretreatment of the cells with SMase resulted in stimulation of CE uptake from rHDL by CHO and Y1BS1, although not by HepG2. Incorporation of ceramide into rHDL resulted in up to 2-fold stimulation of CE uptake, although pretreatment of cells with egg ceramide had no significant effect. These results show that SM and ceramide in the lipoproteins and the cell membranes regulate the SR-BI-mediated selective uptake of CE, possibly by interacting with the sterol ring or with SR-BI itself. —Subbaiah, P. V., L. R. Gesquiere, and K. Wang. Regulation of the selective uptake of cholesteryl esters from high density lipoproteins by sphingomyelin. J. Lipid Res. 2005. 46: 2699–2705.

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Plasma HDL concentration is negatively correlated with atherogenic risk; therefore, the regulatory factors that are involved in HDL metabolism are of great scientific and clinical interest. An important determinant of plasma HDL concentration is scavenger receptor class B type I (SR-BI), a membrane receptor that facilitates the selective uptake of cholesteryl ester (CE) from HDL (1). SR-BI is predominantly expressed in the liver and steroidogenic tissues and serves as a docking receptor for HDL, facilitating the selective uptake of CE from HDL without the simultaneous uptake of HDL protein. It is an N-glycosylated and farnesylation protein that is localized in the cholesterol- and sphingomyelin (SM)-rich domains of plasma membrane (rafts) (2–4). In some cases, the receptor has been shown to be enriched in the caveola (2), although the presence of caveolae is not absolutely essential either for its raft localization (5) or for its function in selective uptake (6). The physiological importance of this receptor in lipoprotein metabolism is evident from the abnormalities that occur in SR-BI knockout mice, which exhibit marked increases of plasma HDL levels, depletion of adrenal cholesterol, and diminished secretion of bile acids and cholesterol in the bile (1). In contrast, transgenic mice overexpressing SR-BI have significantly lower HDL levels, accelerated clearance of HDL and non-HDL cholesterol (7), and increased biliary secretion of cholesterol (1). Despite its negative effects on plasma HDL concentration, SR-BI protects against atherosclerosis because of its overall stimulation of reverse cholesterol transport (8).

The mechanism by which SR-BI facilitates the selective uptake of CE from HDL is not completely understood. An endocytic recycling of HDL particles, accompanied by the selective loss of CE as a result of cellular hydrolisis, may operate in some cells (9), whereas surface transfer of HDL CE to the plasma membrane (10) may take place in others. It is also possible that a combination of the two pathways occurs in many cells. In support of the surface uptake pathway, Rodrigueza et al. (11) suggested the formation of a hydrophobic channel between the membrane and the lipoprotein. It was proposed that the hydrophobic CE molecule moves down the concentration gradient in this channel, from the lipoprotein to the cell membrane. The surface

Abbreviations: ACTH, adrenocorticotropic hormone; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; FC, free cholesterol; PC, phosphatidylcholine; rHDL, recombinant high density lipoprotein; SM, sphingomyelin; SMase, sphingomyelinase; SR-BI, scavenger receptor class B type I.

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composition of the lipoprotein may have a strong influence on the selective uptake reaction, because the solubilization of some CE in lipoprotein surface lipids may be necessary for its transfer to the cell membrane. The major lipid components of the HDL surface are phosphatidylcholine (PC), SM, and free cholesterol (FC). Previous studies by Yancey et al. (12) showed that the SM/PC ratio of HDL significantly affects the bidirectional flux of FC that is mediated by SR-BI. The PC in HDL was shown to increase the efflux of cellular cholesterol, whereas SM inhibited the influx of HDL cholesterol into the cell. The possible effects of HDL phospholipids on the physiologically more important selective uptake of CE, however, have not been investigated. We have previously shown that the lipoprotein SM inhibits the synthesis of CE by the LCAT reaction (13) and inhibits the hydrolysis of phospholipids by the secretory phospholipases (14). Furthermore, SM is a strong inhibitor of lipoprotein oxidation (15). In this study, we investigated the effect of SM and its metabolite ceramide on the SR-BI-mediated selective uptake of CE; we found that the uptake by different cell lines is inhibited by HDL SM but activated by HDL ceramide. The plasma membrane SM of the cells also inhibited the selective uptake, but this effect varied among different cells.

**MATERIALS AND METHODS**

**Materials**

\[^{3}H\]cholesterol oleate (cholesterol 1,2,6,7,14H; 75 Ci/mmol) and \[^{3}H\]sucrose (600 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Egg PC, egg SM, and egg ceramide were obtained from Avanti Polar Lipids (Alabaster, AL). Apolipoprotein A-I (apoA-I) was purified from HDL concentrated in DMEM containing 15% horse serum, 2% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The HepG2 cells were maintained in Ham's F12 medium containing 10% FCS and 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The HepG2, Y1BS1, or CHO-SR-BI cells were plated on 35 mm six-well plates at a density of 1.0–1.5 × 10^5 cells/well and allowed to grow in their respective media for 24 h. In the case of Y1BS1 cells, the cells were washed three times with serum-free F10 medium and incubated for 24 h in F10 medium containing 5 mg/ml BS A and 10^-7 M adrenocorticotropic hormone (ACTH). The cells were then washed, and labeled plasma HDL (100 µg protein/ml) or rHDL (20 µg protein/ml) was added in F10 medium containing 10^-7 M ACTH and incubated for 18 h. After removing the medium, the cells were washed with phosphate-buffered saline (three times) and lysed in 1 ml of 0.1 N NaOH. Aliquots of the medium and cell lysate were counted for \[^{3}H\] and \[^{14}C\] in a Beckman liquid scintillation counter using the dual-channel win-

**Cell culture**

CHO cells stably expressing SR-BI were obtained from Dr. Theodore Mazzone (University of Illinois at Chicago). The expression of SR-BI in these cells is constitutively driven by a cytomegalovirus promoter (18). They were maintained in Ham’s F12 medium containing 10% FBS and 1% geneticin. Y1BS1 cells, the mouse adrenocortical tumor cells, were kindly provided by Dr. Bernard Schimmer (University of Toronto). These cells were maintained in DMEM containing 15% horse serum, 2% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Human hepatoma cells (HepG2) were purchased from the American Type Culture Collection (Rockville, MD). The HepG2 cells were maintained in DMEM containing 4 mM L-glutamine, 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

**Recombinant high density lipoprotein preparations**

Recombinant high density lipoprotein (rHDL) preparations containing labeled apoA-I and labeled CE were prepared by sonication of a modification of the method described by Pittman and colleagues (19, 20). Chloroform solutions of egg PC (3 mg), unlabeled cholesteryl oleate (3 mg), \[^{3}H\]cholesterol oleate (1.8 µCi), and FC (0.5 mg) were mixed in a glass tube with varying amounts of SM and ceramide, where indicated. The solvent was evaporated under nitrogen, and the sample was sonicated in 10 ml of 10 mM Tris/0.15 M NaCl buffer, pH 8.0, in a Sonics Vibra-cell sonicator at 52°C at a setting of 80 W under a stream of nitrogen. The sonication was performed for a total of 30 min, with intermittent cooling of the tip. The temperature was then decreased to 42°C, \[^{14}C\]sucrose-labeled apoA-I (2 mg) was added in 1.85 ml of 8 M urea in small aliquots, and sonication was continued for another 15 min. The clear suspension was then centrifuged at 15,000 g for 20 min to remove the titanium particles, concentrated to ~0.5 ml by ultrafiltration, and chromatographed on a Superdex 200 HR 10/30 column (Pharmacia) to separate any unincorporated lipids and protein. Fractions containing both \(^3\)H and \(^14\)C were pooled, dialyzed against 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA, and concentrated to 1 ml final volume using Centricon filters. Aliquots were taken for the determination of cholesterol, phospholipids, and radioactivity. In general, the ratio of SM to PC was within 10–15% of the expected value (Table 1), although the net recovery of lipids varied from experiment to experiment.

**Labeling of native HDL**

HDL was prepared from normal human plasma obtained from the blood bank. The plasma was first incubated with 1 mM DTNB for 15 min at 37°C, made to 1.063 g/ml density with solid KBr, and centrifuged at 100,000 g for 18 h at 10°C. VLDL and LDL in the top were removed by tube-slicing. The bottom fraction containing HDL and CE transfer protein was incubated with \[^{3}H\]cholesterol oleate (15 µCi), which was added in a small volume of ethanol (50 µl in 30 ml), for 24 h at 37°C. The density of the sample was then adjusted to 1.21 g/ml with solid KBr, and the sample was centrifuged at 100,000 g for 44 h at 10°C. The HDL fraction was recovered by tube-slicing and dialyzed extensively against 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA. The sample was then labeled with \[^{14}C\] sucrose as described by Pittman et al. (17). Aliquots of HDL were taken for the determination of radioactivity, protein, free and total cholesterol, PC, and SM.

**Selective uptake measurements**

HepG2, Y1BS1, or CHO-SR-BI cells were plated on 35 mm six-well plates at a density of 1.0–1.5 × 10^5 cells/well and allowed to grow in their respective media for 24 h. In the case of Y1BS1 cells, the cells were washed three times with serum-free F10 medium and incubated for 24 h in F10 medium containing 5 mg/ml BS A and 10^-7 M adrenocorticotropic hormone (ACTH). The cells were then washed, and labeled plasma HDL (100 µg protein/ml) or rHDL (20 µg protein/ml) was added in F10 medium containing 10^-7 M ACTH and incubated for 18 h. After removing the medium, the cells were washed with phosphate-buffered saline (three times) and lysed in 1 ml of 0.1 N NaOH. Aliquots of the medium and cell lysate were counted for \[^{3}H\] and \[^{14}C\] in a Beckman liquid scintillation counter using the dual-channel win-

**Table 1. Composition of recombinant high density lipoprotein particles prepared with different amounts of SM (weight% of total)**

| SM % | Protein | Cholesterol | Ester PC | SM/PC | SM/PC (Expected) |
|------|---------|-------------|----------|-------|------------------|
| 0    | 31.0    | 9.2         | 15.8     | 44.0  | 0.00             |
| 7.5  | 32.0    | 9.0         | 14.0     | 40.4  | 0.69             | 0.08 |
| 15   | 32.0    | 11.9        | 18.1     | 32.0  | 6.0              | 0.19 |
| 25   | 31.0    | 11.1        | 16.9     | 31.8  | 9.2              | 0.29 |
| 35   | 34.0    | 11.2        | 16.8     | 25.3  | 12.5             | 0.49 |

PC, phosphatidylcholine; SM, sphingomyelin. The values shown are from the analysis of samples from one typical experiment.

* SM as a percentage of total phospholipid (theoretical) corresponding to the values shown in Fig. 2.
was converted into equivalents of CE, based on the ratio of \(^{3}H\) and \(^{14}C\) in the original rHDL preparation. This value was subtracted from the amount of \(^{3}H\)CE taken up, to calculate the selective uptake of CE. The uptake was expressed as nanomoles of CE taken up per milligram of cell protein. The uptake in CHO-SR-BI and in HepG2 cells was determined by the same procedure described for Y1BS1 cells, except that ACTH was not included in the reaction mixture.

**Sphingomyelinase C treatment**

Native HDL and rHDL preparations were treated with bacterial sphingomyelinase (SMase) C from *Staphylococcus aureus*, as described previously (13, 14). The amount of SM hydrolyzed by the enzyme was determined from the loss of SM, which was determined by lipid phosphorus after TLC separation.

**Analytical**

Protein was determined by the method of Markwell et al. (21) using BSA as the calibration standard. The absorbance was measured on a 96-well plate reader at 660 nm. FC was determined using a kit from Wako Chemicals (Richmond, VA), whereas total cholesterol was determined with a kit from Sigma Chemical Co. CE was calculated as the difference between total cholesterol and FC. Phospholipids were separated on silica gel TLC plates using chloroform-methanol-water (65:25:4, v/v) as the solvent, and the spots were visualized by exposure to iodine vapors and identified with the help of standards run on the same plate. Spots corresponding to PC and SM were scraped, and lipid phosphorus was determined with a modified Bartlett procedure (22).

**RESULTS**

**Effect of SM incorporation into rHDL**

rHDLs containing labeled apoA-I, labeled CE, egg PC, FC, and varying amounts of egg SM were prepared by sonication, and the selective uptake of labeled CE was determined in CHO cells stably transfected with SR-BI. As shown in Fig. 1, the presence of SM (25% of phospholipid) inhibited the uptake of CE by the cells in a time-dependent manner. The uptake in untransfected CHO cells was ~25% of the rate observed with the transfected cells, and this was also inhibited by SM.

Figure 2 shows the effect of varying SM concentrations on the selective uptake of CE by CHO cells. In these studies, the total phospholipid content of rHDL was kept constant by substituting various amounts of SM for the egg PC. The percentage composition of rHDL particles from one set of preparations is shown in Table 1. The SM/PC ratio was within 10–15% of the expected value for all samples. The selective uptake was inhibited by >50% when 25% of the PC was replaced by SM, or at the SM/PC ratio of 0.33. This ratio is within the physiological range for human plasma lipoproteins, although the value for HDL fractions ranges from 0.1 to 0.25 (13). The effect of SM on the uptake of labeled apoA-I is shown in the inset in Fig. 2. Protein uptake from the SM-free rHDL, which was ~15% of CE uptake, was inhibited to a lesser extent by SM, and the inhibition showed no correlation with SM concentration. This shows that the inhibitory effect of SM is specific for CE uptake. It should be pointed out that the values shown for CE uptake in this and other figures have been corrected for the protein uptake in each reaction.

The effect of SM was then investigated on the selective uptake by three different cell lines representing functionally distinct tissues: the SR-BI-transfected CHO cells used in Figs. 1, 2; the steroidogenic murine adrenocortical cell line Y1BS1, which has been used extensively to characterize SR-BI-mediated uptake (11, 20); and the hepatocytes (HepG2), which represent the last step in the reverse cho-
lesterol transport pathway. As shown in Fig. 3, SM (25% of phospholipid) inhibited the selective uptake of CE in all cell lines, showing that the effect of SM is universal.

**Reversibility of SM effect**

We next studied the effect of the depletion of SM from SM-containing rHDL by the bacterial SMase C, which hydrolyzes SM to ceramide and phosphorylcholine, to determine whether the inhibitory effect is reversible. As shown in Fig. 4, the degradation of SM completely reversed the inhibition of CE uptake in all cell lines. Uptake from the SM-treated rHDLs was equal to or greater than that found with control rHDL (no SM). Treatment of control rHDL with SMase C did not affect the selective uptake from these particles (results not shown). These results show that the inhibition of uptake is specific for SM and not attributable to changes in the other components of rHDL.

**Effect of SM in native lipoproteins**

The uptake of CE from HDL isolated from normal human plasma is shown in Fig. 5. The HDL was labeled with [3H]CE and [14C]sucrose as described in Materials and Methods, and an aliquot was treated with SMase C. The uptake of labeled CE from the native and SMase C-treated HDL was then determined in the three different cell lines. In all cases, the selective uptake of CE was significantly stimulated by SMase C treatment, showing that SM in the native lipoproteins has an inhibitory effect on selective uptake. In these studies, SMase C treatment resulted in the hydrolysis of ~65–75% of HDL SM, without any change in the PC content (results not shown).

**Effect of membrane SM**

SM is an important component of the plasma membrane and is critical for the structure of the membrane rafts (23, 24), where SR-BI is localized (2–4). Therefore, it is important to determine whether the plasma membrane SM plays any role in the activity of SR-BI. For this purpose, we treated the CHO-SR-BI cells with increasing amounts of SMase C for 1 h, and after washing out the enzyme, selective uptake was determined from rHDL containing no SM. As shown in Fig. 6, the hydrolysis of membrane SM resulted in up to 2-fold stimulation of uptake from the rHDL. The effect of SMase, however, appears to depend upon the cell type, because treatment of HepG2 cells did not stimulate the uptake from either SM-free rHDL or SM-containing rHDL (Fig. 7). Treatment of Y1BS1 cells with SMase, on the other hand, caused a 35% stimulation of uptake from the SM-free rHDL. Uptake from the SM-containing rHDL was not stimulated significantly by SMase treatment of either cell line.

**Effect of ceramide**

Because SMase treatment generates ceramide, which is known to have bilayer-disrupting properties (25), we tested...
the effect of the incorporation of ceramide into rHDL without SM. As shown in Fig. 8, the incorporation of 25 mol% egg ceramide (with respect to PC) enhanced the selective uptake of CE in all cell lines, although the stimulation in Y1BS1 cells did not reach statistical significance.

DISCUSSION

The results presented here show a novel function of SM in the lipoproteins, namely, the regulation of HDL metabolism through inhibition of the SR-BI-mediated selective uptake of CE. Although SM is the second most abundant phospholipid in the lipoproteins, its possible nonstructural roles in lipoprotein metabolism have not been appreciated until recently. Recent studies show that plasma SM may be an independent risk factor for atherosclerosis (26), although the underlying mechanisms for this are not clear. Our previous studies showed that SM regulates the esterification of cholesterol by the LCAT reaction (13) and the hydrolysis of lipoprotein phospholipids by the secretory phospholipases (14). It was also shown to inhibit the hydrolysis of triacylglycerol by lipoprotein lipase (27, 28) and to inhibit the oxidation of LDL (15). Although SM is

Fig. 5. Effect of treatment of plasma HDL with SMase C. HDL was prepared from normal human plasma and labeled with [14C]sucrose and [3H]CE, as described in the text. An aliquot of HDL was treated with SMase C, and the control and SMase C-treated HDL were used to determine selective uptake by the three cell lines. Approximately 75% of total SM was hydrolyzed under these conditions. Values shown are means ± SD of three experiments.

Fig. 6. Effect of SMase C treatment of CHO cells on selective uptake from SM-free rHDL. CHO cells transfected with SR-BI were treated with the indicated amount of SMase C for 90 min, and after removal of the enzyme and washing of the cells, selective uptake was determined in the presence of rHDL containing no SM. Results are from one typical experiment, with each point representing means ± SD of triplicate samples.

Fig. 7. Effect of SMase C treatment of HepG2 and Y1BS1 cells. The cells were treated with 50 mU/ml SMase C for 90 min, and the uptake of CE was determined in the presence of SM-free and SM-containing rHDL (25% phospholipid). Results are means ± SD of three separate experiments. ** P < 0.01 compared with untreated samples.

Fig. 8. Effect of the incorporation of egg ceramide into rHDL. Egg ceramide was incorporated into rHDL at a concentration of 25 mol% egg PC. The rHDL did not contain any SM. Selective uptake was determined in the three cell lines for 18 h in the presence of ceramide-free and ceramide-containing rHDL. *** P < 0.001 (n = 3).
known to inhibit the desorption of FC from the cell membranes, possibly because of its strong interaction with the sterol ring (29, 30), its possible role in the movement of CE between lipoproteins and cell membranes has not been investigated. Therefore, the regulation of the SR-BI-mediated CE uptake from lipoproteins represents a novel physiological function for this phospholipid.

The mechanism by which SM regulates the selective uptake pathway is not clear. Based on the known effects of SM on FC movement between lipoproteins and cell membranes (12), the strong interaction of the sterol ring with SM could inhibit its release from the surface of the lipoproteins. It has been shown that the presence of SM in the lipoprotein inhibits the transfer of FC from the lipoprotein to the cell membrane, whereas the SM of the plasma membrane strongly inhibits the efflux of cholesterol from the cell to a lipoprotein acceptor (12). Unlike FC, however, CE does not have the free 3β-hydroxy group, which is critical for hydrogen bonding between cholesterol and phospholipids (31). On the other hand, the hydrophobic interactions between CE and SM may be stronger than those between SM and FC because of the presence of the long-chain acyl group in CE. It should be pointed out that the selective uptake of CE is inversely proportional to the chain length of the acyl group and directly proportional to its unsaturation (19), suggesting the role of acyl group interactions in the selective uptake. Although most of the CE in lipoproteins is in the core of the particles, up to 5% of neutral lipids may be present in the phospholipid monolayer, possibly in equilibrium with the core CE (32). This surface-localized CE may be the preferred form in the transfer reactions; therefore, the interaction of SM with this CE could be responsible for the inhibition of CE transfer to the cell membrane. It is also possible that SM affects either the conformation of apoA-I or the size of the rHDL particle, both of which have been shown to affect the binding of rHDL to SR-BI (33–35).

The role of membrane SM in the regulation of selective uptake may be related to the fact that SR-BI is concentrated in the SM-rich domains of the plasma membrane (3, 4). The effect of SMase C treatment of the cells, however, varied among the three cell lines used here. Although treatment of CHO and Y1BS1 cells stimulated the selective uptake, the effect on HepG2 was a slight (insignificant) inhibition. A recent report by Rhaïnds et al. (5) also found no effect of SMase C treatment on the selective uptake of HDL CE by HepG2, although it enhanced stimulation by cholesterol oxidase. Furthermore, they found that disruption of the raft structure in the cells inhibited the selective uptake from LDL but activated the uptake from HDL. The three cell lines used here represent functionally distinct tissues, and the SR-BI distribution in the plasma membrane may also be different among them. For example, HepG2 cells do not have caveolae (36), whereas Y1BS1 cells have few caveolae but contain extensive microvilli channels, which harbor SR-BI (37). CHO cells, on the other hand, have abundant caveolar structures (38). Furthermore, the overexpression of SR-BI in these cells may have resulted in a preferential localization in SM-rich domains, which would be sensitive to SMase C treatment. Therefore, the role of membrane SM in selective uptake may depend upon the cell type and function. It is of interest that SMase treatment of Y1BS1 cells did not stimulate the uptake from SM-containing liposomes, possibly because the cells replenished their SM by rapid uptake from the rHDL.

In contrast to SM, the presence of ceramide in the lipoprotein significantly stimulated the selective uptake of CE by CHO and HepG2 cells, although the effect on Y1BS1 was not statistically significant (Fig. 8). It is not clear whether this stimulation is the result of ceramide’s effect on lipoprotein structure or on SR-BI itself. It has been shown that ceramide displaces FC from the rafts (39) and induces nonbilayer structures in membranes (25). The effect of lipoprotein ceramide may also be attributable to its incorporation into cell membranes during the incubation and its subsequent modification of membrane structure. Our preliminary results show that treatment of CHO cells with 5–20 μM egg ceramide (added in ethanol) did not affect the selective uptake from rHDL (results not shown). This may be attributable, however, to the lack of cellular uptake of the long-chain ceramide, and ceramide may need to be generated in situ to be effective. In any case, the results presented here strongly suggest that both SM and ceramide regulate the SR-BI-mediated selective uptake of CE.

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