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Reverse cholesterol transport is regulated by varying fatty acyl chain saturation and sphingomyelin content in reconstituted high-density lipoproteins

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Abstract

Because phospholipid composition of high-density lipoprotein (HDL) plays a vital role in its reverse cholesterol transport (RCT) function, we studied RCT in vitro (uptake and efflux) with reconstituted HDLs (rHDLs) containing phosphatidylcholine (PC) with fatty acids of increasing saturation levels (stearic, oleic, linoleic, linolenic) and without or with sphingomyelin (SM). Uptake significantly increased from basal value when the PC component included up to 50 mol % of oleic or linolenic acid, but did not change with linoleic acid. Increasing oleic and linoleic acids to 100 mol % significantly decreased uptake, but increasing linolenic acid to the same value did not affect it. Sphingomyelin in rHDL significantly decreased uptake, but only with PC-containing unsaturated fatty acids, and not with saturated fatty acid. Efflux was not affected in a dose-dependent manner when oleic or linoleic acid content was increased, but was significantly increased with levels of linolenic acid up to 25 mol % in PC, and was dramatically lowered with higher levels. Sphingomyelin in rHDL (PC/SM, 20:80, mol/mol) significantly increased efflux only with oleic or linoleic acid–containing rHDLs, compared with efflux without SM. In conclusion, enrichment of PC component up to 25 mol % as linolenic acid has a beneficial effect on RCT, whereas a higher percentage of it or other unsaturated fatty acids seems to be detrimental. In addition, high SM content decreases uptake with rHDL-containing unsaturated fatty acids, whereas it increases efflux for rHDL-containing oleic or linoleic acid. These results show for the first time the importance of SM in RCT in a well-defined in vitro system.

1. Introduction

Excess cholesterol is removed from peripheral tissue by reverse cholesterol transport (RCT), a process in which cellular cholesterol is removed by high-density lipoprotein (HDL) (ie, cholesterol efflux) and transported to the liver (ie, cholesterol uptake) for metabolic transformation into bile acid and steroid hormones [1-3]. Two proteins have been shown to be involved in the cholesterol efflux process, the adenosine triphosphate–binding cassette transporter 1 (ABCA1) and the scavenger receptor class B type I (SR-BI). Both ABCA1 and SR-BI are expressed by macrophages and parenchymal liver cells. ABCA1 facilitates cellular cholesterol and phospholipid efflux, and SR-BI binds HDL with high affinity [4-7]. Factors that affect the RCT function of HDL are cholesterol and phospholipid content, lecithin cholesterol acyltransferase activity, and fatty acid saturation levels.

With regard to the latter factor, we showed in a previous investigation that the RCT function of HDL is affected by the saturation level of dietary fat intake. Our previous studies of oral feeding of alcohol diet for 8 weeks according to Lieber's method, and fish oil fat (only 2.8% of total dietary energy as ω-3 fatty acids [n-3FAs]; α-tocopherol, 120 IU/L of diet), resulted in lower plasma and liver lipid levels [8]. Furthermore, the same low level of dietary n-3FA restored the decreased apolipoprotein (apo) E content in HDL due to ethanol. In addition, we have shown that the presence of as low as 2.8% of total dietary energy as n-3FA

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in the alcohol diet not only can decrease the damage done by alcohol to the RCT mechanism, but also can enhance by several fold the ability of HDL to promote RCT, even in control rats [9]. We also showed that there was no interaction between the effect of n-3FA and the effect of alcohol on hepatocytes in terms of RCT function [9].

In addition to being modulated by HDL fatty acid components, cholesterol efflux is positively correlated with sphingomyelin (SM) content in HDLs in humans with high serum HDL cholesterol levels (>67.7 mg/dL) [10]. Thus, factors that regulate local modification of HDL phospholipid composition may have a large impact on RCT. It has been demonstrated that SR-BI–mediated free cholesterol flux is very sensitive to the phosphatidylcholine (PC) and SM composition of HDL and that the phospholipid composition of HDL alters the steady-state distribution of cholesterol between cells expressing SR-BI and HDL. In particular, SM enrichment of HDL enhances the net efflux of cholesterol from SR-BI–expressing COS-7 cells by decreasing the influx of HDL cholesterol [11].

The SM content of the cell membrane is thought to contribute to the maintenance of cellular cholesterol homeostasis because the cholesterol content of the cell membrane is positively correlated with SM content [12]. The interaction between cholesterol and SM is the basis for the formation and maintenance of cholesterol/sphingolipid-enriched nano- and microdomains (referred to as membrane "rafts") in the plane of plasma and other organelle (ie, Golgi) membranes [13]. SM is thought to bind cholesterol with high affinity and inhibit its efflux from the plasma membrane by preventing cholesterol desorption [14]. In addition, SM prevents the exchange of cholesterol between the plasma membrane and intracellular pools [15]. However, the role of SM in the function of circulating lipoproteins remains unclear.

Numerous studies on RCT reported how various well-defined plasma components, particularly reconstituted HDL (rHDL) particles with a variety of compositions, regulate cholesterol efflux from cells [16-18]. However, there is no information on the combined role of the fatty acid saturation levels of the phospholipids, and the SM levels, when both are varying in individual HDL particles.

Thus, in the work presented herein, our objective was to investigate the RCT function of HDL, namely, cholesterol uptake and efflux, by varying both the saturation levels of the fatty-acid chain of the PC component and the levels of SM in various rHDL species.

2. Materials and methods

2.1. Materials

Materials were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM), glutamine, and penicillin/streptomycin, Invitrogen (Carlsbad, CA); fetal calf serum (FCS), Life Technologies (Gaithersburg, MD); bovine brain SM and egg yolk PC (EYPC), Sigma-Aldrich (St Louis, MO); [^{3}H]cholesteryl oleate ([^{3}H]CO), Amersham Biosciences (Piscataway, NJ); tissue culture plastic ware, Falcon (Lincoln, NJ); wild-type HepG2 cells and mouse macrophages J774, American Type Culture Collection (Rockville, MD); insulin, Eli Lilly (Indianapolis, IN); 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, 18:0), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, 18:1, n-9FA), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DLOPC, 18:2, n-6FA), and 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (DLNPC, 18:3, n-3FA), Avanti Polar Lipids (Alabaster, AL).

2.2. Preparation of HDL fraction

High-density lipoprotein was isolated by sequential flotation from human pooled plasma [19] and its protein fraction was delipidated in 20 volumes of chloroform-methanol (1:1, vol/vol) for 2 hours at 4°C, followed by centrifugation at 2000 rpm (Beckman J6 centrifuge with 4.2 rotor). The HDL pellet was homogenized in 5 volumes of 25 mmol/L ammonium bicarbonate, 10 mmol/L sodium decyl sulfate, and 0.1% (vol/vol) β-mercaptoethanol and solubilized overnight at 4°C under gentle agitation. The HDL solution was then dialyzed in 25 mmol/L ammonium bicarbonate, "0.1% (vol/vol) β-mercaptoethanol to remove sodium decyl sulfate, and its protein content was measured by the Bradford method with bovine serum albumin as a standard [20]. Apo A-I content was determined by densitometry analysis of the HDL fraction separated by denaturing electrophoresis on a 4% to 20% (wt/vol) linear gradient acrylamide gel and stained with SYPRO Ruby protein stain (Bio-Rad, Hercules, CA). The delipidated HDL fraction was used to reconstitute HDL as described below, taking into account its apo A-I content and the apo A-I molecular weight of 28 000 Da.

2.3. Liposome preparation

The rHDL complexes were prepared by sodium cholate dispersion according to previously reported methods

Table 1
Composition of rHDL species (without SM)

rHDL species	Molar ratio
EYPC	EYPC only
DSPC (18:0)	DSPC only
DSPC/DOPC (18:0/18:1)	75:25
	50:50
	25:75
	0:100
DSPC/DLOPC (18:0/18:2)	75:25
	50:50
	25:75
	0:100
DSPC/DLNPC (18:0/18:3)	75:25
	50:50
	25:75
	0:100

The chemical composition of the various phospholipids DSPC, DOPC, DLOPC, and DLNPC is given in the Materials and methods section.

Table 2
Composition of rHDL species (with SM)

rHDL species	Molar ratio	PC/SM molar ratio	DS/(DO,DLO, or DLN) molar ratio
DSPC/SM (18:0/SM)	PC only	PC only	DSPC only
	200:50	80:20	
	150:100	60:40	
	50:200	20:80	
DSPC/DOPC/SM (18:0/18:1/SM)	PC only	PC only	75:25
	150:50:50	80:20	
	112.5:37.5:100	60:40	
	37.5:12.5:200	20:80	
DSPC/DLOPC/SM (18:0/18:2/SM)	PC only	PC only	75:25
	150:50:50	80:20	
	112.5:37.5:100	60:40	
	37.5:12.5:200	20:80	
DSPC/DLNPC/SM (18:0/18:3/SM)	PC only	PC only	75:25
	150:50:50	80:20	
	112.5:37.5:100	60:40	
	37.5:12.5:200	20:80	

The chemical composition of the various phospholipids DSPC, DOPC, DLOPC, and DLNPC is given is the Materials and methods section.

[21-24]. All rHDL species were prepared with an initial PC (±SM)/apo A-I/CO ratio equal to 250:1.6:12.5. Various lipids (ie, PC, SM, CO, [^{3}H]CO) were mixed together from stock solutions in chloroform to obtain rHDL compositions indicated in Table 1A and B and then evaporated to dryness with a gentle flow of nitrogen or helium. Under these conditions, the PC/CO molar ratio was always kept at 20:1. Only those rHDLs that would be used for cholesterol uptake assay with hepatocytes would contain radiolabeled CO. Dispersion buffer (10 mmol/L Tris-HCl [pH 8.0], 154 mmol/L NaCl, 1 mmol/L NaN_3, 0.3 mmol/L EDTA) was then added to all lipid mixtures to obtain a final lipid concentration of 10 mg/mL. This was followed immediately by vortexing for 2 minutes, after which sodium cholate (30 mg/mL) was added to obtain a lipid-sodium cholate molar ratio of 1:1, followed by vortexing for 2 minutes and by 1-hour incubation at 4°C. Several cycles of 5-minute sonication and incubation at 37°C were necessary to obtain clear liposome suspensions. An apo A-I–containing protein fraction (5-10 mg/mL) was then added to the liposome suspension to obtain a PC (±SM)/apo A-I molar ratio of 156.25:1. Each sample mixture was then incubated for 1 hour at 4°C and dialyzed extensively against dispersion buffer after adjusting the volume to 0.5 mL with dispersion buffer. The homogeneity and size of the reconstituted lipoprotein complexes were assessed by size exclusion chromatography and native PAGE, as described below, and by negative staining electron microscopy [25]. Electron microscopy analysis of our rHDL preparation revealed characteristic disc-shaped structures (average diameter, 15 nm), similar to those found in the literature for rHDL [22,26-28].

2.4. Analysis of rHDL

Chemical analysis was performed by measuring protein [20], triglyceride [29], phospholipid [30], SM [31], and total cholesterol [32,33] content. Homogeneity and size analysis were performed by (*a*) size exclusion chromatography on a Biogel 0.5M column (27 × 1 cm) using an AKTA Purifier system (Pharmacia, Uppsala, Sweden) and (*b*) nondenaturing electrophoresis on an 8% to 25% gradiant gel using PhastSystem (Pharmacia) [34,35]. Molecular weight estimation was performed by analyzing the gels with a FluorChem 8800 densitometer loaded with AlphaEaseFC software (Alpha Innotech, San Leandro, CA). R_f values were exported into Excel spreadsheet software (Microsoft, Redmond, WA) and analyzed with

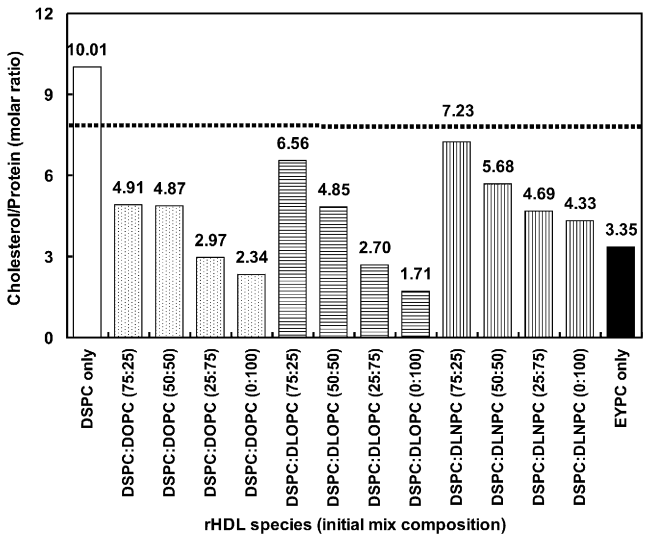

Fig. 1. Molar ratio of various rHDL species. At the end of the procedure for rHDL preparation, described in the Materials and methods section, each species was analyzed for its total cholesterol and protein content. Values are displayed on top of the corresponding bars for each rHDL species. rHDL species composition is expressed as molar percentage of each PC. The horizontal dotted line represents the calculated ratio based on the initial amount of cholesterol and protein added during the rHDL synthesis procedure.

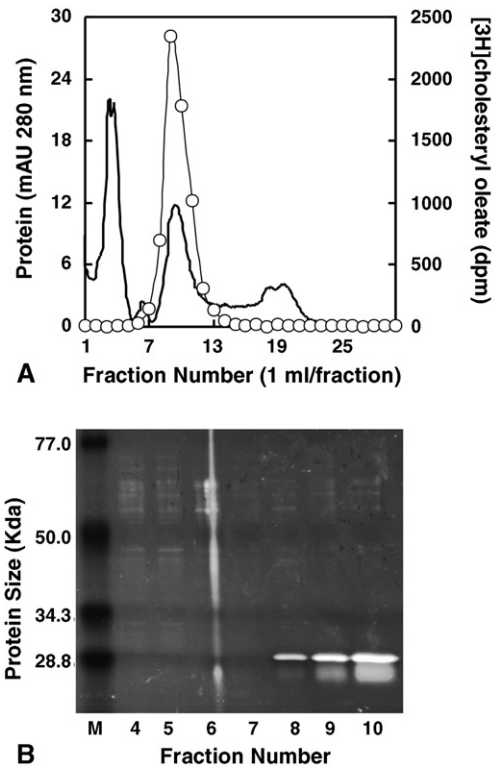

Fig. 2. Incorporation of cholesterol into apo A-I–containing rHDL. A representative set of data for rHDL species containing 50% DLNPC (see Table 1) and labeled with [^{3}H]CO is shown. A, Size exclusion chromatography profile on BioGel 0.5 mol/L of 100 μg protein of rHDL. Protein was continuously monitored spectrophotometrically at 280 nm (solid line), and radioactivity was measured by liquid scintillation on a 0.1-mL aliquot of each 2.5-mL fraction (thin line with open circles). B, An aliquot (12 μL) of each fraction 4 to 10 was analyzed by 4% to 15% gradient SDS-PAGE, followed by protein staining with SYPRO Ruby (Bio-Rad).

the power or exponential regression trend line function. Values listed in Table 3A and B are those obtained with the best correlation coefficient.

2.5. Cell culture systems

For uptake experiments, HepG2 cells were maintained in DMEM supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 10% (vol/vol) FCS, according to the instructions from the supplier. When needed, cells were subcultured and grown in a monolayer in 6-well culture dishes by culturing 1×10^6 cells per well containing 4 mL culture medium, followed by incubation at 37°C in 5% CO_2/air humidified atmosphere. Confluence of the cell layers was observed after 36 to 48 hours. All experiments were performed in the postconfluent proliferating phase of cells.

For efflux experiments, mouse macrophages J774 were maintained in DMEM supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, 10% (vol/vol) FCS, and 0.1 mU% insulin at 37°C in 5% CO_2/air humidified atmosphere. When needed, cells were split and grown in a monolayer in 6-well culture dishes (1×10^6 cells per well in 4 mL culture medium). Confluence of the cell layers was observed after 24 to 36 hours. All experiments were performed in the postconfluent proliferating phase of cells.

2.6. Esterified cholesterol uptake assay from labeled rHDL

An adequate volume of dialyzed rHDL was mixed with DMEM (4 mL final volume) and added as a sterile solution (by filtration through 0.22-μm sterile filters) to each culture dish containing the confluent HepG2 cells. An aliquot (0.1 mL) of the incubation medium was analyzed for the total esterified cholesterol radioactivity added to each dish. The uptake of labeled esterified cholesterol by the cells was determined after 24 hours by counting the cell-associated radioactivity as follows: the cell medium was completely removed and, after thorough washing of the cells 3 times with PBS to remove the medium's radioactivity, the cells were dissolved in 1 mL PBS containing 0.1% SDS and 1% Triton X-100. The dissolved cells were mixed with 4 mL of Packard's Ultima Gold scintillation cocktail (Perkin Elmer, Wellesley, MA) and analyzed for radioactivity in a Beckman model LS-6500 liquid scintillation spectrometer.

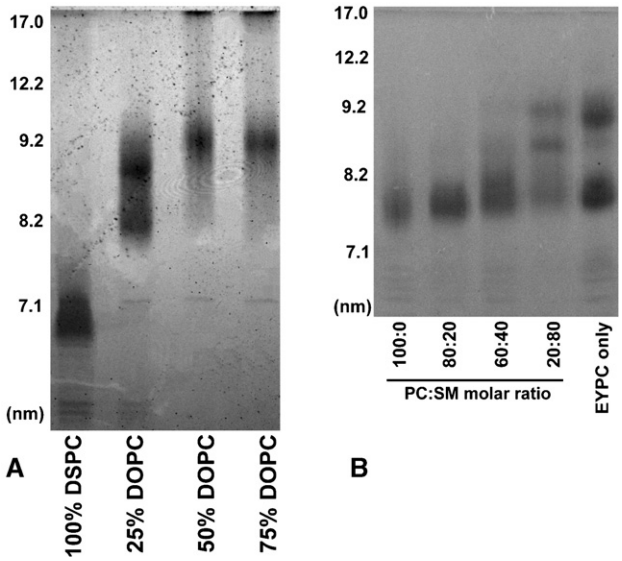

Fig. 3. Size analysis of rHDL. A. rHDL without SM. B. rHDL with SM. Each indicated rHDL species (approximately 1 μg protein) was subjected to electrophoresis on a 8 to 25% polyacrylamide native gel. Protein bands were revealed by Coomassie staining. Particle size markers were determined by densitometry analysis using a Fluorchem8800 gel analyzer (Alpha Innotech) loaded with AlphaEasyFC software for calculations. A, Representative gel for rHDL species containing DOPC. To minimize the graph's complexity, rHDL composition is displayed as molar percentage of the varying PC species. Thus, 100% DSPC, DSPC only; 25% DOPC, DSPC/DOPC (187.5:62.5); 50% DOPC, DSPC/DOPC (75:75); and 75% DOPC, DSPC/DOPC (62.5:187.5). B, Representative gel for rHDL species containing DLOPC and SM. rHDL species composition is displayed according to SM content only. Thus, 100:0 corresponds to DSPC/DOPC/SM (187.5:52.5:0), 80:20 to DSPC/DOPC/SM (150:50:50), 60:40 to DSPC/DOPC/SM (112.5:37.5:100), and 20:80 to DSPC/DOPC/SM (37.5:12.5:200). Data from densitometry measurements of the rHDL species particle size are reported in Table 3A and B. Particles size markers were bovine serum albumin (7.1 nm), lactate dehydrogenase (8.2 nm), thyroglobulin (9.2 nm), catalase (12.2 nm), and apoferritin (17.0).

2.7. Cholesterol efflux assay

Macrophages, confluent in 6-well plates, were first incubated with [^{3}H]CO-labeled acetylated low-density lipoprotein (0.06 μCi and 115.5 μg of low-density lipoprotein per well) in 4 mL of culture medium for 24 hours. After washing 3 times with PBS (pH 7.4), the macrophages were then incubated with 75 μg protein of indicated rHDLs per well in culture medium without FCS for 6 hours. At the end of the incubation period, radioactivity in the medium was measured, as described above. Efflux was expressed as the percentage of the initial radioactivity loaded in the macrophages that is found in the medium after 6 hours. In these conditions, approximately 45% of labeled CO is hydrolyzed to free cholesterol, and more than 90% of the released cholesterol appeared as unesterified in the culture medium [36].

2.8. Statistical analyses

The significance of the various effects was evaluated by a 1-way analysis of variance (ANOVA) with post hoc Tukey test.

3. Results

3.1. rHDL characterization

To carry out the measurement of cholesterol transport with HDLs of various lipid compositions, we made rHDL particles from individual purified components, namely, cholesteryl ester, PC, SM, and HDL fraction. Tables 1 and 2 summarize the composition of the various rHDL species used in this study.

For all rHDL species the molar ratio for total PC/apo A-I/cholesteryl ester was 250:1.6:12.5. The protein fraction was obtained from delipidated human HDL that was isolated by sequential flotation, and apo A-I content was determined by densitometry analysis of SDS-PAGE profile (results not shown). Analysis of the cholesterol to apo A-I molar ratio showed that values differed from the calculated value of 7.8. As shown in Fig. 1, increasing unsaturated PC content in rHDLs decreased their cholesterol content in a dose-dependent manner. It appears that adding linolenic FA–containing PC (DLNPC, 18:3[n-3]) to rHDL minimized the loss of cholesterol, compared with linoleic FA (DLOPC, 18:2)– or oleic FA

Table 3
Particle size of rHDL species

A. Size distribution of rHDL without SM			B. Size distribution of rHDL with SM		
rHDL species	*% PC*	*nm*	*rHDL species*	*PC:SM ratio*	*nm*
DSPC only	100% DSPC	6.9	**DSPC/SM**		
DSPC/DOPC			**250:0**	DSPC only	6.9
187.5:62.5	25% DOPC	9.1, 8.1	**200:50**	80:20	6.9
75:75	50% DOPC	9.3	**150:100**	60:40	6.8
62.5:187.5	75% DOPC	9.3	**50:250**	20:80	6.9, 7.4
0:250	100% DOPC	9.4	**DSPC/DOPC/SM**		
DSPC/DLOPC			**187.5:62.5:0**	100:0	6.9, 7.7
187.5:62.5	25% DLOPC	8.8	**150:50:50**	80:20	6.9, 7.8
75:75	50% DLOPC	9.1	**112.5:37.5:100**	60:40	7.7
62.5:187.5	75% DLOPC	9.0	**37.5:12.5:200**	20:80	7.8
0:250	100% DLOPC	7.5	**DSPC/DLOPC/SM**		
DSPC/DLNPC			**187.5:62.5:0**	100:0	7.6
187.5:62.5	25% DLNPC	8.7, 7.5	**150:50:50**	80:20	7.6
75:75	50% DLNPC	7.7, 6.3	**112.5:37.5:100**	60:40	7.6
62.5:187.5	75% DLNPC	7.8, 6.4	**37.5:12.5:200**	20:80	7.8, 8.6, 8.9
0:250	100% DLNPC	8.0, 6.4	**DSPC/DLNPC/SM**		
			187.5:62.5:0	100:0	7.4
			150:50:50	80:20	7.4
			112.5:37.5:100	60:40	7.4
			37.5:12.5:200	20:80	9.4
			EYPC/SM		
			250:0	EYPC only	8.9, 7.7
			200:50	80:20	8.9, 7.7
			150:100	60:40	9.0, 7.8
			50:250	20:80	8.9, 7.8

rHDLs were subjected to native gel electrophoresis, and sizes were measured by densitometry as described in the Materials and methods section. rHDL species in A and B, whose sizes are listed in grayed areas, have their corresponding electrophoretic profile displayed in Fig. 3A and B, respectively.

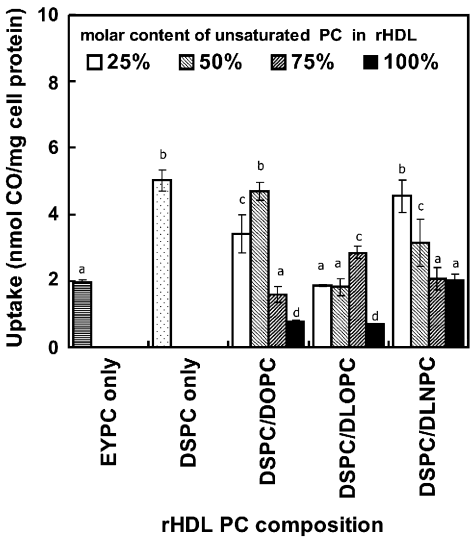

Fig. 4. Cholesterol uptake function of rHDL species that contain various amounts of PC with increasing saturation levels in the FA chain. There are 5 major groups of rHDL, as indicated in the abscissa. rHDLs belonging to "EYPC only" and "18:0 only" groups have only one source of PC, namely, EYPC and DSPC (or 18:0 FA–containing PC), respectively. Other rHDL groups contain 2 sources of PC, namely, DSPC in all of them and an "unsaturated PC," ie, DOPC (18:1), DLOPC (18:2), or DLNPC (18:3). These groups are divided into 4 rHDL subgroups, each with various molar ratios of DSPC and "unsaturated PC," as indicated in the frame of the graph. Detailed rHDL compositions are given in Table 1. Data are the means ± SD of triplicate measurements. For all rHDL groups, bars with a different superscript letter are significantly different, $P < .05$ (ANOVA with post hoc Tukey test).

(DOPC, 18:1)–containing species. rHDL species with only saturated PC (DSPC, 18:0) had, however, a cholesterol/apo A-I ratio higher than 7.8, namely, 10.1. There was no correlation between cholesterol/apo A-I ratio and particle size.

To verify the homogeneity of freshly prepared rHDL species, their protein and cholesterol profiles were analyzed with species containing [^{3}H]CO. As shown in Fig. 2A, a representative size exclusion chromatography profile indicates that apo A-I–containing rHDL elutes as a single peak that overlaps with the [^{3}H]CO profile. The absorbance peak appearing in the early fractions (2 to 5) does not contain apo A-I and does not appear to indicate the presence of a significant amount of protein, as shown by SDS-PAGE analysis of the eluted fractions. In addition, cholesteryl ester is exclusively bound with apo A-I–containing particles (Fig. 2B).

After verifying the size homogeneity of each rHDL, we investigated size variation among the various rHDL species. Thus, size analysis was performed on various rHDL species by nondenaturing gel electrophoresis as described in the Materials and methods section. Fig. 3A and Table 3A show that the rHDL species containing only saturated PC (ie, DSPC) has the smallest size (6.9 nm).

Analysis of the data listed in Table 3A indicates that higher levels of unsaturated FA slightly increase the size of rHDLs that contain DOPC (ie, 18:1), but decrease the size of rHDLs that contain the highest level of DLOPC (ie, 18:2), as well as the size of rHDLs that contain DLNPC (ie, 18:3). Analysis of the data listed in Table 3B indicates that SM does not affect the size of rHDLs, except at its highest content (80 mol %) with rHDL containing DLNPC.

3.2. Cholesterol uptake

As described in the following sections, we measured cholesterol uptake with rHDLs whose phopholipid composition had (*a*) PC with fatty acids of various levels of saturation and (*b*) various levels of SM. In addition, all rHDL species were radioactively labeled with [^{3}H]CO.

3.2.1. Effect of fatty acid composition of PC

As shown in Fig. 4, uptake of cholesterol in the hepatocyte system had a basal level when EYPC was used as the sole source of phospholipid in rHDL. Uptake was the highest when the rHDL PC component contained only saturated (18:0) fatty acid (DSPC only). When the proportions of DOPC (18:1) or DLNPC (18:3[n-3]) in rHDLs were increased up to 50 mol % of total PC content, uptake was significantly higher than basal value. However, the same increase in DLOPC (18:2) showed no change in cholesterol uptake. In contrast, uptake of cholesterol was significantly decreased below basal level when the proportion of DOPC or DLOPC in rHDL was increased to 100 mol

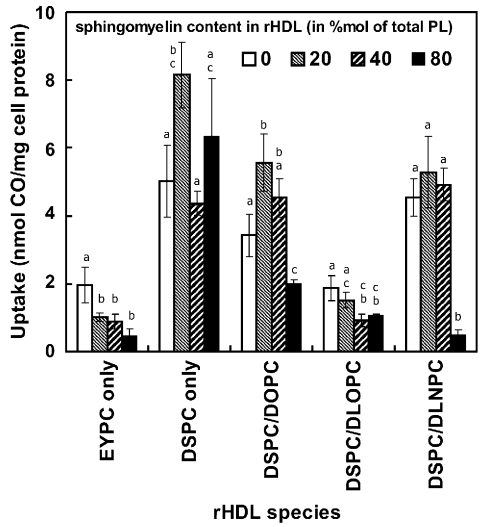

Fig. 5. Cholesterol uptake function of rHDL species that contain various amounts of SM and various saturation levels in the PC FA chain. There are 5 major groups of rHDL, as indicated in the abscissa. rHDLs belonging to "EYPC only" and "18:0 only" groups have only one source of PC, namely, EYPC and DSPC (or 18:0 FA–containing PC), respectively. Other rHDL groups contain 2 sources of PC, namely DSPC in all of them and an "unsaturated PC," ie, DOPC, DLOPC, or DLNPC, at a constant molar ratio. All groups are divided into 4 rHDL subgroups, each with various molar ratios of PC and SM, as indicated in the frame of the graph. SM content is expressed as a molar percentage of total phospholipids (PL). Detailed composition of rHDL is given in Table 2. Data are the means ± SD of triplicate measurements. Bars within each rHDL group that do not have an identical superscript letter are significantly different, $P < .05$ (ANOVA with post hoc Tukey test).

% of the total PC content. Interestingly, the same change in DLNPC did not change uptake level from the basal value.

3.2.2. Effect of SM composition of PC

In this series of experiments, SM was added in increasing proportions into various rHDL species, whereas the ratio of saturated to unsaturated fatty acid was kept constant (3:1, as shown in Table 2). Results presented in Fig. 5 show that increasing SM content dramatically decreased the uptake of rHDL species containing EYPC or containing DOPC (18:1), DLOPC (18:2), and DLNPC (18:3[n-3]) ($P < .01$; compare 20% and 80% SM), and to a lesser extent also decreased the uptake of rHDL containing DSPC (18:0) only ($P < .05$, compare 20% and 40% SM). In addition, uptake was the lowest when EYPC was the only PC component of rHDL.

3.3. Cholesterol efflux

Similar to the cholesterol uptake experiments described above, we measured cholesterol efflux with rHDLs whose PC composition had (*a*) fatty acids with various levels of saturation and (*b*) various levels of SM, as described below. In this series of experiments, rHDL species were not radioactively labeled.

3.3.1. Effect of fatty acid composition of PC

The most striking observation in the results presented in Fig. 6 is the dramatic decrease of cholesterol efflux when rHDL DLNPC (18:3[n-3]) content was increased from 50% to 100% ($P < .01$). More importantly, when rHDL DLNPC (18:3[n-3]) content was only 25%, cholesterol efflux was significantly enhanced ($P < .01$) when compared with the

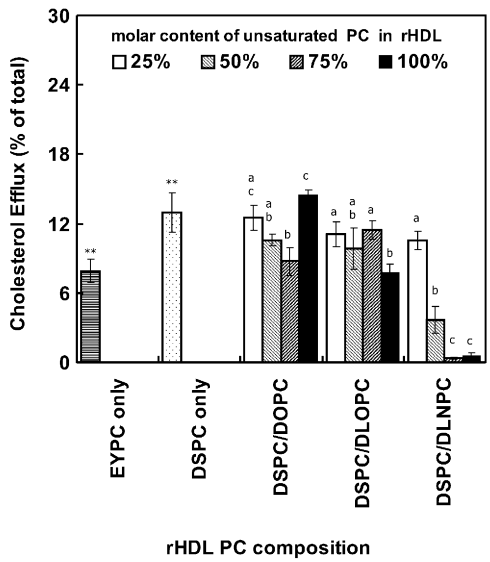

Fig. 6. Cholesterol efflux function of rHDL species that contain various amounts of PC with increasing saturation levels in the FA chain. Cholesterol efflux methodology is described in details in the Materials and methods section. Data are the means ± SD of triplicate measurements. Bars within each rHDL group that do not have an identical superscript letter are significantly different, $P < 0.05$ (ANOVA with post hoc Tukey test). Legend is otherwise identical to the legend of Fig. 4.

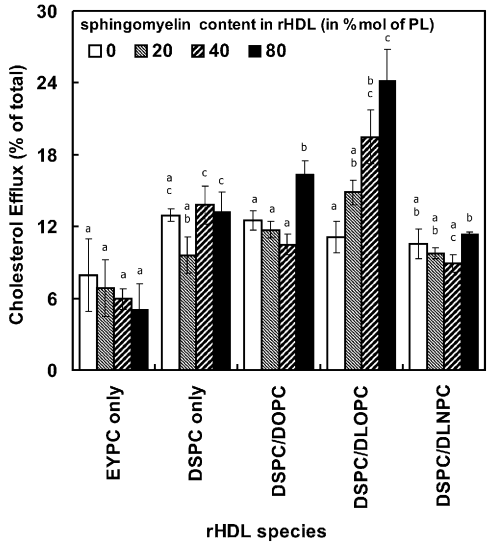

Fig. 7. Cholesterol efflux function of rHDL species that contain various amounts of SM and various saturation levels in the PC FA chain. Cholesterol efflux methodology is described in detail in the Materials and methods section. Data are the means ± SD of triplicate measurements. Bars within each rHDL group that do not have an identical superscript letter are significantly different, $P < 0.05$ (ANOVA with post hoc Tukey test). Legend is otherwise identical to the legend of Fig. 4.

basal value obtained with rHDL species whose sole source of phospholipids was EYPC. Other data from this set of experiments show that cholesterol efflux was not significantly affected, at least in a dose-dependent manner, whether DOPC (18:1) or DLOPC (18:2) was used to replace EYPC in rHDLs.

3.3.2. Effect of SM composition of PC

As described in the uptake experiment above, rHDL species with a constant ratio of saturated (DSPC, 18:0) to unsaturated fatty acid (DOPC [18:1], DLOPC [18:2], or DLNPC [18:3(n-3)]), namely, 3:1, and an increasing proportion of SM were used to assay the efflux transport of cholesterol. Results are plotted in Fig. 7 and show that SM does not significantly affect cholesterol efflux in the presence of rHDL species that contain EYPC, DSPC only, or DSPC/DLNPC (18:0/18:3[n-3]). However, SM increased significantly the cholesterol efflux with rHDL species containing DSPC/DOPC (18:0/18:1), but only at the highest proportion of SM, namely, 80 mol % of phospholipids. The most significant effect was obtained when efflux was carried out in the presence of rHDL containing DSPC/DLOPC (18:0/18:2), and there was an SM-dose–dependent increase of cholesterol efflux. In summary, cholesterol efflux was significantly increased with DSPC/DOPC (18:0/18:1) or DSPC/DLOPC (18:0/18:2) in rHDL species whose PC/SM ratio was 20:80, compared with efflux with rHDLs without SM.

4. Discussion

Overall, these results show a dose-dependent effect (positive and negative) of both unsaturated fatty acids and

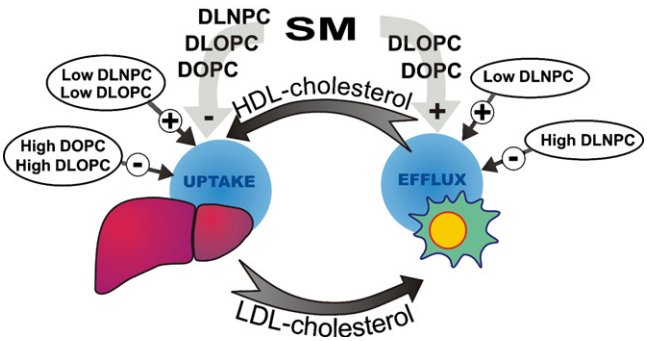

Fig. 8. Schematic representation of the interaction of various HDL phospholipid components with the RCT process. The liver is on the left side, indicating cholesterol uptake, and a macrophage is represented on the right side, indicating cholesterol efflux. RCT is the transport of cholesterol by HDL from peripheral tissues (eg, macrophages) to the liver. Fatty acid content of various PCs: DOPC, oleic acid (18:2); DLOPC, linoleic acid (18:2); DLNPC, linolenic acid (18:3[n-3]).

SM in the phospholipid component of rHDL on cholesterol uptake and cholesterol efflux. The effects of various HDL lipid components investigated in the present study on the RCT process are summarized in Fig. 8.

Enrichment of the rHDL PC component with a limited amount of DLNPC (18:3[n-3]) by up to 25 mol % of the total phospholipids has a beneficial effect on cholesterol uptake (Fig. 4). Any further increase in the DLNPC (18:3[n-3]) or other polyunsaturated fatty acid content seems to be detrimental to this function. These results are consistent with the observation that high levels of polyunsaturated fatty acids in the diet are associated with higher incidence of coronary artery disease, whereas lower amounts have cardioprotective effects, especially n-3FA.

Most studies on dietary oil supplement focus on the effect of the diet on lipid profiles. One work with monkeys described a direct correlation between HDL acyl chain composition and the level of dietary oil saturation in various regimens [17]. However, no subsequent effect on RCT was observed. In a recent study [37], HDL composition (ie, HDL cholesterol) and RCT function were investigated in humans fed diets containing various oils defined by their level of saturated or unsaturated fatty acids. Results showed that a diet rich in n-3FA (ie, 1.6% of total dietary energy as fish oil) improved RCT function. However, no analysis was made to determine HDL FA composition. In the present study, HDL composition is well defined and our results show that RCT is also increased in correlation with the addition of limited amounts of n-3FA in rHDL. Thus, we hypothesize that the improvement of RCT after dietary intake of fish oil is due to the enrichment of HDL with n-3FA. However, it is important to point out that the n-3FA in DLNPC (ie, 18:3) used in the present study is chemically different from n-3FA found in fish oil (ie, 20:5 and 22:6) because of the difference in carbon chain length and in the number of ethylene bounds.

When both RCT components are compared, our results show that cholesterol uptake by the liver is more sensitive to variation in the levels of unsaturated fatty acids in rHDL phospholipid composition than cholesterol efflux is. Indeed, cholesterol uptake is significantly increased from basal level by moderate enrichment of rHDL in DOPC (18:1) and DLNPC (18:3[n-3]), whereas efflux is significantly increased only with rHDL moderately enriched in DLNPC (18:3[n-3]). Conversely, cholesterol uptake is significantly decreased from basal level when rHDL is highly enriched in DOPC (18:1) and DLOPC (18:2) (Fig. 4), whereas efflux is significantly decreased only with rHDL highly enriched with DLNPC (18:3[n-3]) (Fig. 6).

With regard to the effect of SM on RCT, we showed that cholesterol uptake is significantly decreased by a high content of SM in rHDL. The reverse is true for cholesterol efflux: high levels of SM in rHDL significantly increase it. These findings are in accordance with the known high-affinity binding of SM with cholesterol, thus preventing its desorption from SM-rich rHDL in the uptake process and promoting desorption from macrophage membrane in the efflux process [13].

Acknowledgments

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