

# Spontaneous reconstitution of discoidal HDL from sphingomyelin-containing model membranes by apolipoprotein A-I

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**Abstract** Nascent HDL is known to be formed by the interaction of apolipoprotein A-I (apoA-I) with transmembrane ABCA1, but the molecular mechanism by which nascent HDL forms is less well understood. Here, we studied how reconstituted high density lipoprotein (rHDL) forms spontaneously on the interaction of apoA-I with model membranes. The formation of rHDL from pure phosphatidylcholine (PC) large unilamellar vesicles (LUVs) proceeded very slowly at 37.0°C, but sphingomyelin (SM)-rich PC/SM LUVs, which are in a gel/liquid-disordered phase ( $L_d$  phase) at this temperature, were rapidly microsolvubilized to form rHDL by apoA-I. The addition of cholesterol decreased the rate at which rHDL formed and induced the selective extraction of lipids by apoA-I, which preferably extracted lipids of  $L_d$  phase rather than lipids of liquid-ordered phase. In addition, apoA-I extracted lipids from the outer and inner leaflets of LUVs simultaneously. These results suggest that the heterogeneous interface of the mixed membranes facilitates the insertion of apoA-I and induces  $L_d$  phase-selective but leaflet-nonselective lipid extraction to form rHDL; they are compatible with recent cell works on apoA-I-dependent HDL generation.—Fukuda, M., M. Nakano, S. Sriwongsitanont, M. Ueno, Y. Kuroda, and T. Handa. Spontaneous reconstitution of discoidal HDL from sphingomyelin-containing model membranes by apolipoprotein A-I. *J. Lipid Res.* 2007. 48: 882–889.

**Supplementary key words** ATP binding cassette transporter A1 • cholesterol • lipid efflux • liposome • liquid-disordered phase • liquid-ordered phase • phosphatidylcholine • high density lipoprotein

HDLs transport excess cholesterol (Chol) from cells in the periphery to the liver, where Chol is metabolically converted to bile and removed from the body (1, 2). This pathway, termed the reverse cholesterol transport pathway, has been a target of basic research for the development of new drugs for arteriosclerosis because of the correlation between high circulating levels of HDL and a

lower risk of cardiovascular disease (3). The antiatherogenic properties of HDL also have been demonstrated directly with the observation that increasing HDL levels in mice reduce the sizes of atherosclerotic lesions (4–6).

Apolipoprotein A-I (apoA-I), the unique protein component of nascent HDL called pre $\beta$ -HDL or discoidal HDL, also has antiatherogenic properties because of its crucial role in reverse cholesterol transport. In nascent HDL, two apoA-I molecules surround the hydrophobic edge of the lipid bilayer like a belt (7–9). In recent years, it has been demonstrated that nascent HDL is formed by the interaction of apoA-I with transmembrane ABCA1 (5, 6, 10, 11). ABCA1-mediated lipid efflux is not specific for apoA-I. Other apolipoproteins with amphipathic helices and synthetic amphipathic helical peptides have been shown to efflux lipids from cells (12–14), indicating that the amphipathic helical structure is the most important factor for the interaction with ABCA1. The significance of ABCA1 in the neogenesis of HDL is demonstrated by the fact that mutations in the ABCA1 gene lead to Tangier disease, which is characterized by low plasma HDL levels (15). The macrophage-specific loss of ABCA1 expression accelerates atherosclerosis in vivo (16, 17). It is well established that ABCA1 transports phospholipids (PLs) and free (unesterified) Chol to lipid-free apoA-I, triggering the formation of nascent HDL (18), but the steps by which apoA-I accepts PLs and Chol and the nascent HDL is formed are less well understood. A number of mechanisms for ABCA1-mediated lipid efflux to apoA-I have been pro-

Abbreviations: apoA-I, apolipoprotein A-I; Chol, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene;  $L_d$  phase, liquid-disordered phase;  $L_o$  phase, liquid-ordered phase; LUV, large unilamellar vesicle; NBD-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); NBD-PC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PL, phospholipid; pyrene-PC, 1-hexadecanoyl-2-(1-pyrenehexanoyl)-*sn*-glycero-3-phosphocholine; rHDL, reconstituted high density lipoprotein; SM, sphingomyelin.

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posed, including the direct binding of apoA-I to ABCA1 or binding to ABCA1-perturbed plasma membrane that stimulates apoA-I binding and lipid efflux (18). It has been suggested that Chol is transferred by the aqueous diffusion mechanism to fully lipidated apoA-I formed by ABCA1 (19); alternative models suggest that PLs and Chol are transported simultaneously to apoA-I (20, 21) or that Chol is transported by another transporter, such as ABCG1 (22).

Because more energy is needed to transport PLs from membrane to bulk ( $\sim 15$  kcal/mol) than for ATP hydrolysis (7.3 kcal/mol), it cannot be assumed thermodynamically that nascent HDL containing hundreds of PLs is formed via a mechanism whereby ABCA1 transports lipids to apoA-I one by one. Therefore, we assume that disruption of the plasma membrane by ABCA1 spontaneously induces the extraction of lipids and the formation of nascent HDL by apoA-I. Here, we report the physicochemical mechanism behind the spontaneous formation of discoidal reconstituted high density lipoprotein (rHDL) by the interaction of apoA-I with model membranes at physiological temperature, aiming at the differentiation and elucidation of the spontaneous and energy-dependent process by which nascent HDL forms.

## EXPERIMENTAL PROCEDURES

### Materials

Guanidine hydrochloride and heptaethylene glycol monododecyl ether were purchased from Wako (Osaka, Japan). Egg yolk phosphatidylcholine (PC), egg yolk sphingomyelin (SM), Chol, 1,6-diphenyl-1,3,5-hexatriene (DPH), and sodium hydro-sulfate (dithionite) were purchased from Sigma. 1-Hexadecanoyl-2-(1-pyrenehexanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) and 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC) were from Molecular Probes. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DPPE) was from Avanti Polar Lipids. ApoA-I was isolated from pig plasma using procedures described previously (23–25). The protein was further purified by affinity column chromatography (HiTrap Blue affinity column; Pharmacia Biotech) to remove trace amounts of pig serum albumin. In all experiments, apoA-I was freshly denatured at concentrations of  $\leq 1$  mg/ml in a 6 M guanidine hydrochloride solution and refolded by slow removal of the denaturing agent by dialysis. The protein concentration was determined by the method of Lowry et al. (26) using BSA (Pierce) as the standard.

### Liposome preparation

To prepare large unilamellar vesicles (LUVs) with a specific lipid composition, the required amounts of a chloroform-methanol solution of PLs, Chol, and fluorescent probes were mixed in a round-bottomed glass flask. The organic solvent was removed by evaporating, and the residue was dried overnight under vacuum. The dried lipids were dispersed in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 0.01 %  $\text{NaN}_3$  by vortexing. After seven rounds of freeze-thawing, the suspension was extruded through a 100 nm pore size polycarbonate filter. The concentrations of PLs and Chol were determined using an enzymatic assay kit for choline from Wako.

### Fluorescence polarization

Fluorescence measurements were performed on a Hitachi F-4500 spectrofluorimeter equipped with a sample heater/cooler. The fluorescence polarization of DPH, which partitions equally into gel, liquid-ordered ( $L_o$ ), and liquid-disordered ( $L_d$ ) phases (27), was used to evaluate the fluidity of lipid bilayers in LUVs with different lipid compositions. The concentration of PLs was 100  $\mu\text{M}$ , and the probe/PL molar ratio was 1:200. The excitation and emission wavelengths were 360 and 434 nm, respectively. The fluorescence polarization [ $\langle r \rangle_{\text{DPH}}$ ] was calculated as  $\langle r \rangle_{\text{DPH}} = (I_{\text{VV}} - GI_{\text{VH}}) / (I_{\text{VV}} + 2GI_{\text{VH}})$ , where  $I_{\text{VV}}$  and  $I_{\text{VH}}$  are the intensities of vertically and horizontally polarized fluorescent light, respectively, when excitation light is vertically polarized.  $G = I_{\text{HV}}/I_{\text{HH}}$  is the correction factor, where  $I_{\text{HV}}$  and  $I_{\text{HH}}$  represent the intensities of vertically and horizontally polarized light, respectively, when excitation light is horizontally polarized. A slight amount of a methanol solution of DPH was added to the LUVs and incubated at 50.0°C for 1 h to incorporate the DPH into the vesicles. The temperature was increased from 10.0 to 50.0°C in increments of 2°C, and the sample was equilibrated for 2 min before polarization was measured. The temperature profile was fitted by the following sigmoidal function (28, 29):

$$\langle r \rangle_{\text{DPH}} = r_0 + a/[1 + \exp(-(T - T_M)/b)] \quad (\text{Eq. 1})$$

where  $\langle r \rangle_{\text{DPH}}$  is the polarization at a given temperature ( $T$ ) and  $r_0$ ,  $a$ ,  $b$ , and  $T_M$  are constants.  $T_M$  is the midpoint temperature of the phase transition from the gel to  $L_d$  phase, which corresponds to a 50% change in the polarization.

### Kinetics of the microsolubilization of LUVs by apoA-I

The kinetics of the microsolubilization of LUVs having various lipid compositions by apoA-I was measured as the time-dependent decrease of turbidity followed by right-angle light scattering. The reduction in right-angle light scattering was attributable to the transformation of the LUVs ( $d \sim 120$  nm) to small discoidal rHDL ( $d \sim 10$  nm). LUVs and apoA-I, which were preequilibrated at the reaction temperature, were mixed to final concentrations of 100 and 5  $\mu\text{M}$ , respectively, in a final volume of 300  $\mu\text{l}$ , and the change in the right-angle light scattering intensity was monitored on an F-4500 spectrofluorimeter for 1 h using excitation and emission wavelengths of 650 nm. The intensity [ $I(t)$ ] was normalized by the initial intensity before the addition of apoA-I ( $I_0$ ), which was corrected for the effect of volume change by the addition of apoA-I. The data were analyzed by the two-exponent decay model to estimate the rate of microsolubilization:

$$I(t)/I_0 = C_1 \exp(-k_1 t) + C_2 \exp(-k_2 t) + (1 - C_1 - C_2) \quad (k_1 > k_2) \quad (\text{Eq. 2})$$

### Electron microscopy

A mixture of PC/SM = 10:90 LUV (300  $\mu\text{M}$ ) with apoA-I (5  $\mu\text{M}$ ) was incubated at 37.0°C for 12 h. The samples were negatively stained with 2% ammonium molybdate. Electron micrographs were obtained with a JEOL JEM-200 CX electron microscope.

### Domain selectivity on disc formation

LUVs containing 0.5 mol% pyrene-PC, which tends to be distributed to the  $L_d$  phase (30), and 0.5 mol% NBD-DPPE, which is distributed to the  $L_d$  phase and the gel phase indifferently or prefers the gel phase slightly (31), were mixed with apoA-I to a final concentration of 300  $\mu\text{M}$  total lipids and 0.12–5  $\mu\text{M}$  apoA-I and incubated at 37.0°C for 1 or 5 h. To standardize the pro-

portion of discoidal rHDL formed among LUVs with different lipid compositions, the apoA-I concentration and incubation time were altered. After the addition of 100  $\mu$ l of 50% (w/w) sucrose to 400  $\mu$ l of sample to adjust specific gravity to 1.037–1.047, the mixture was ultracentrifuged (78,000  $g$ ) at 37.0°C for 2 h to separate the generated discoidal rHDL from LUVs. The discoidal rHDL fraction was collected from the bottom (200  $\mu$ l) and solubilized in 50  $\mu$ l of 10% (v/v) heptaethylene glycol monododecyl ether. The fluorescence intensity of pyrene-PC ( $F_{\text{py in Disc}}$ ) and NBD-DPPE ( $F_{\text{NBD-DPPE in Disc}}$ ) was measured on a F-4500 spectrofluorimeter with excitation/emission wavelengths of 342/377 nm and 470/530 nm, respectively. The percentage of discoidal rHDL formed was given by ( $F_{\text{py in Disc}}/F_{\text{py in LUV}}$ ). The fluorescence intensity for LUV without incubation with apoA-I ( $F_{\text{py in LUV}}$  and  $F_{\text{NBD-DPPE in LUV}}$ ) was also measured after solubilization of the LUVs by 2% heptaethylene glycol monododecyl ether. Selective extraction of lipids by apoA-I was evaluated with the following index:

$$S = (F_{\text{py in Disc}}/F_{\text{NBD-DPPE in Disc}})/(F_{\text{py in LUV}}/F_{\text{NBD-DPPE in LUV}}) \quad (\text{Eq. 3})$$

Values greater than/less than 1 represent the selective extraction of lipids by apoA-I from the  $L_d$  phase/ $L_o$  phase.

### Inner/outer leaflet selectivity on disc formation

LUVs containing 0.5 mol% pyrene-PC and 1.0 mol% NBD-PC were prepared, and the NBD group of the probe distributed in the outer leaflet of the LUVs was selectively quenched by adding a reducing agent, ditionite (32). NBD-PC was used instead of NBD-DPPE because of the short half-time of the flip-flop of NBD-DPPE. After the agent was removed by gel filtration, LUVs were mixed with apoA-I to a final concentration of 300  $\mu$ M total lipid and 3.5–5  $\mu$ M apoA-I and incubated at 37.0°C for 2 h. After the incubation, the generated discoidal rHDL was separated and solubilized as described above. Leaflet selectivity of lipid extraction by apoA-I was evaluated by the following index:

$$S = (F_{\text{py in Disc}}/F_{\text{NBD-PC in Disc}})/(F_{\text{py in LUV}}/F_{\text{NBD-PC in LUV}}) \quad (\text{Eq. 4})$$

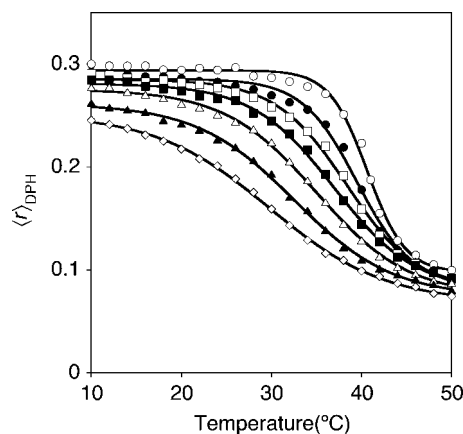
where the fluorescence intensity of NBD-PC ( $F_{\text{NBD-PC in Disc}}$  and  $F_{\text{NBD-PC in LUV}}$ ) was measured with excitation/emission wavelengths of 470/530 nm. Thus, values  $> 1$  represent the selective extraction of lipids from the outer leaflet of LUVs, and values close to 1 indicate that apoA-I extracts lipids from both the outer and inner leaflets.

## RESULTS

### Fluorescence polarization of DPH in PC/SM LUVs

In the PC/SM binary system, the variation in  $\langle r \rangle_{\text{DPH}}$  with temperature is used to determine the phase-transition temperature ( $T_M$ ) from the gel phase to the  $L_d$  phase (Fig. 1). In the case of pure SM LUVs, a sharp decrease in  $\langle r \rangle_{\text{DPH}}$  was observed at  $\sim 41^\circ\text{C}$ , in good agreement with a previous report (33).

This sharp change in  $\langle r \rangle_{\text{DPH}}$  shifted to lower temperatures and became broader as the PC molar fraction in PC/SM LUVs increased, suggesting a decrease in the cooperativity of the transition and an increase in the temperature range of the gel/ $L_d$  phase. As shown in Fig. 1, the data were well fitted by a sigmoidal curve expressed as equation 1.



**Fig. 1.** Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene [DPH;  $\langle r \rangle_{\text{DPH}}$ ] in phosphatidylcholine/sphingomyelin (PC/SM) large unilamellar vesicles (LUVs), with molar ratios of PC/SM of 0:100 (open circles), 5:95 (closed circles), 10:90 (open squares), 15:85 (closed squares), 20:80 (open triangles), 30:70 (closed triangles), and 40:60 (open diamonds), as a function of temperature. The phospholipid (PL) concentration was 100  $\mu$ M, and the probe/PL molar ratio was 1:200. The temperature profile was fitted by the sigmoidal function (equation 1).

The obtained  $T_M$  value, which represents a midpoint temperature of the transition, is listed in Table 1. The  $T_M$  value was shown to decrease with an increase in the PC molar fraction and to be close to 37.0°C and 33.0°C for LUVs with molar ratios of 15:85 and 30:70, respectively.

### Kinetics of the microsolvubilization of LUVs by apoA-I

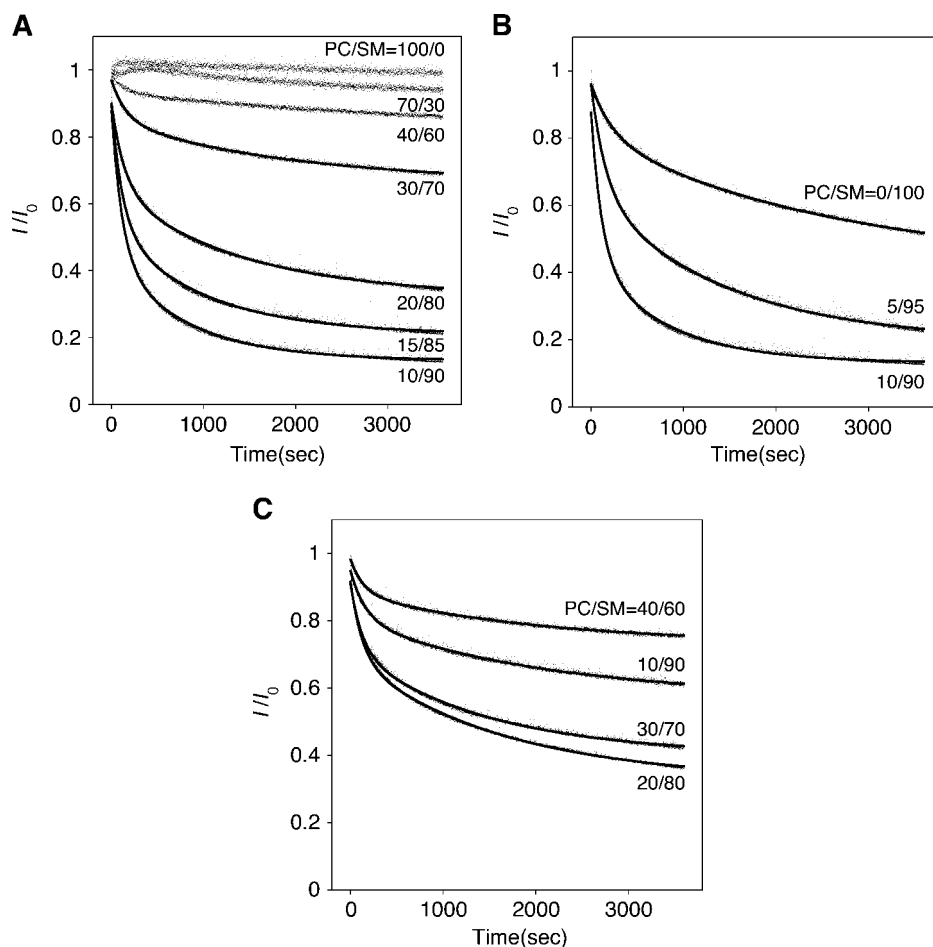
The effect of SM on the kinetics of the spontaneous solubilization of PC/SM LUVs by apoA-I was monitored by measuring right-angle light scattering at 37.0°C and 33.0°C. The solubilization of pure PC LUVs was very slow at 37.0°C, but the rate increased progressively with the addition of SM. Maximal solubilization at 37.0°C was found at PC/SM = 10:90 (Fig. 2A). Any further increase in SM, however, decreased the rate of solubilization (Fig. 2B), but pure SM LUVs were still solubilized to a certain degree. At 33.0°C, the rate was maximum at 20:80 PC/SM (Fig. 2C), which was a lower SM content than at 37°C.

To relate the phase behavior of LUVs with the kinetics of microsolvubilization by apoA-I, the rate constant  $k_1$  at 37.0°C and 33.0°C was determined by fitting the solubilization profile with equation 2 and plotted as a function of  $T_M$ . As shown in Fig. 3, a maximal value of  $k_1$  was obtained at a  $T_M$

TABLE 1. Gel-to-liquid-disordered  $T_M$  based on fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene

		PC/SM					
0:100	5:95	10:90	15:85	20:80	30:70	40:60	
40.6	39.5	38.4	36.9	35.0	32.6	29.8	

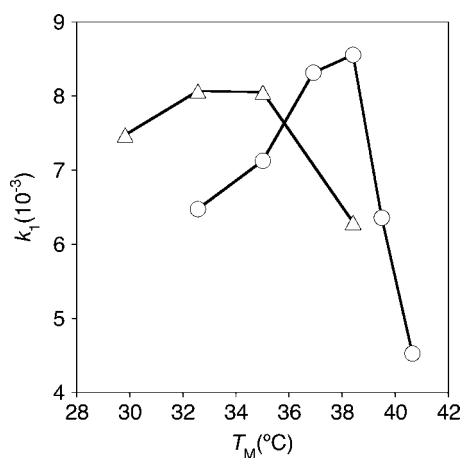
PC, phosphatidylcholine; SM, sphingomyelin;  $T_M$ , phase-transition temperature.  $T_M$  was obtained by fitting the anisotropy data shown in Fig. 1 with equation 1.



**Fig. 2.** Reduction in light-scattering intensity of PC/SM LUVs by apolipoprotein A-I (apoA-I) at 37.0°C (A, B) and 33.0°C (C). The PL and apoA-I concentrations were 100 and 5  $\mu$ M, respectively. The data were analyzed as a two-exponent decay model (equation 2).

close to the experimental temperatures of 33.0°C and 37.0°C. These results indicated that spontaneous solubilization of LUVs by apoA-I was accelerated when the membrane was in a state of two-phase coexistence.

To observe the effect of Chol, similar experiments were conducted with PC/SM/Chol LUVs at 37.0°C. The addition of Chol decreased the solubilization rate in a dose-dependent manner (**Fig. 4**).



**Fig. 3.** Kinetic constant ( $k_1$ ) for microsolvubilization of PC/SM LUVs as a function of the phase-transition temperature ( $T_M$ ) of each LUV: open circles, 37.0°C; open triangles, 33.0°C.

### Electron microscopy

To confirm the formation of a disc-like complex with the microsolvubilization of LUVs, the morphology of a LUV(PC/SM = 10:90)/apoA-I mixture was observed by negative staining electron microscopy. Electron micrographs showed the presence of single-disc particles along with their rouleaux (**Fig. 5**), which could be an artifact of the negative staining process (34). These discs had a thickness of  $\sim$ 5 nm and a diameter of  $\sim$ 12 nm. These findings are consistent with a previous report (35).

### Selective extraction of lipids by apoA-I

Because the two-phase state was found to facilitate the microsolvubilization by apoA-I, we next examined whether apoA-I selectively extracts lipids from either phase. LUVs containing two fluorescent probes were used: pyrene-PC



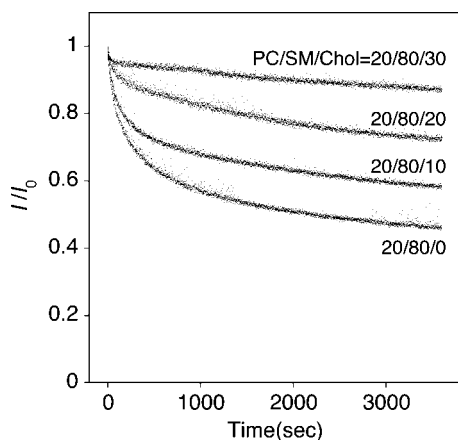


Fig. 4. Reduction in light-scattering intensity of PC/SM/cholesterol (Chol) LUVs (100  $\mu$ M total lipid) by apoA-I (5  $\mu$ M) at 37.0°C.

has a bulky fluorescent group at a short acyl chain and tends to be distributed in the high fluid phase (30), whereas NBD-DPPE is distributed to the  $L_d$  phase and the gel phase indifferently or prefers to be in the low-fluid phase slightly because of its saturated acyl chains (31). Among PC/SM/Chol LUVs with different compositions, the apoA-I concentration was arranged to maintain the proportion of discoidal rHDL at <1%, which corresponds to the formation of a few discoidal rHDLs per LUV (Table 2). For LUVs with PC/SM = 20:80, the value of selectivity (S) was approximately equal to 1 in the absence of Chol, but the addition of Chol dramatically increased the value (Table 2), suggesting that apoA-I preferably extracts pyrene-PC rather than NBD-DPPC. Thus, it can be concluded that apoA-I has difficulty accessing the  $L_o$  phase and that discoidal HDL selectively consists of lipids of the  $L_d$  phase. A similar phenomenon was observed for LUVs with PC/SM = 70:30 (Table 2), but the rate of increase of S with Chol was much less prominent than that for LUVs with PC/SM = 20:80.

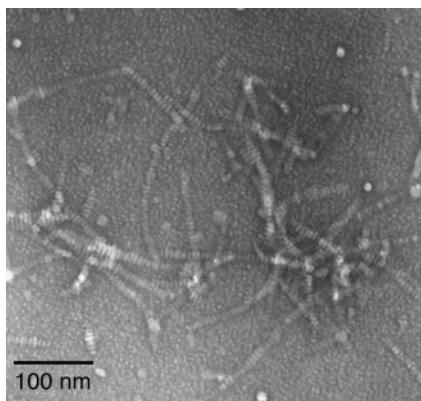


Fig. 5. Electron micrograph of a negatively stained sample from the mixture of PC/SM = 10:90 LUVs (300  $\mu$ M) and apoA-I (5  $\mu$ M). The sample was incubated at 37.0°C for 12 h and then negatively stained with ammonium molybdate.

TABLE 2. Selective lipid extraction from PC/SM/Chol LUVs by apoA-I

PC/SM/Chol	ApoA-I <sup>a</sup> $\mu$ M	Disc Formation <sup>a</sup> %	S <sup>b</sup>
20:80:0	0.12	0.70	1.02 $\pm$ 0.01
20:80:10	0.60	0.65	1.56 $\pm$ 0.07
20:80:20	2.5	0.99	2.50 $\pm$ 0.12
20:80:30	3.0	0.67	7.22 $\pm$ 0.62
20:80:40	4.0	0.73	10.00 $\pm$ 1.07
70:30:0	1.8	0.77	1.72 $\pm$ 0.09
70:30:10	2.8	0.72	2.07 $\pm$ 0.04
70:30:20	4.0	0.71	2.57 $\pm$ 0.03
70:30:30	4.5	0.71	3.00 $\pm$ 0.14
70:30:40	5.0	0.70	2.92 $\pm$ 0.11

apoA-I, apolipoprotein A-I; Chol, cholesterol; LUV, large unilamellar vesicle.

<sup>a</sup>The apoA-I concentration was arranged to maintain the proportion of discoidal reconstituted high density lipoprotein (rHDL) formed, which was given by  $F_{py \text{ in Disc}}/F_{py \text{ in LUV}}$ , at <1%, corresponding to the formation of a few discoidal rHDLs per LUV.

<sup>b</sup>Given by equation 3. The data represent means  $\pm$  SD of three experiments.

### Simultaneous extraction of lipids from outer and inner leaflets of LUVs by apoA-I

To demonstrate whether apoA-I extracts lipids from the outer leaflet of LUVs selectively or from both the outer and inner leaflets together, selective lipid extraction experiments were conducted using "asymmetric" LUVs, which contained pyrene-PC on both leaflets and NBD-PC on the inner leaflet. Pyrene-PC and NBD-PC, having their bulky fluorescent group at a short acyl chain, are thought to localize to the high-fluid phase similarly (30, 36). To maintain the asymmetric distribution of NBD-PC during the experiments, the percentage of discoidal rHDL was kept at <1% (Table 3). The flip-flop of NBD-PC was confirmed not to take place during the experiment by a ditionite assay (data not shown). In pure PC LUVs and PC/SM = 70:30 LUVs, selectivity (S) was approximately equal to 1 (Table 3), suggesting nonselective extraction of the two probes. These results indicate that apoA-I extracts lipids from the outer and inner leaflets simultaneously. In PC/SM/Chol LUVs, the value of S was <1. This result was probably caused by the difference in the partition coefficients of the two probes between the  $L_d$  and  $L_o$  phases. The value of S in "symmetric" LUVs, which contained

TABLE 3. Leaflet selectivity of lipid extraction from asymmetric LUVs by apoA-I

PC/SM/Chol	ApoA-I <sup>a</sup> $\mu$ M	Disc Formation <sup>a</sup> %	S <sup>b</sup>
100:0:0	5.0	0.73	0.98 $\pm$ 0.01
70:30:0	3.5	0.82	1.03 $\pm$ 0.02
70:30:30	5.0	0.47	0.72 $\pm$ 0.01

<sup>a</sup>The apoA-I concentration was arranged to maintain the proportion of discoidal rHDL formed, which was given by  $F_{py \text{ in Disc}}/F_{py \text{ in LUV}}$ , at <1%, corresponding to the formation of a few discoidal rHDLs per LUV.

<sup>b</sup>Given by equation 4. The data represent means  $\pm$  SD of four experiments.

pyrene-PC and NBD-PC on both leaflets, was nearly equal to that in asymmetric LUVs, suggesting no leaflet selectivity (data not shown).

## DISCUSSION

Many previous studies using artificial membranes have reported the properties of heterogeneous membranes attributable to phase separation and the formation of phase boundary regions where membrane lattice defects occur. It is well established that the defects stimulate ion permeability (37, 38) and the rate of flip-flop of PLs (39). Similarly, the microsolubilization of multilamellar vesicles of dimyristoylphosphatidylcholine by apoA-I is accelerated at the  $T_M$  ( $\sim 24^\circ\text{C}$ ), at which the gel and  $L_d$  phases coexist and lattice defects occur (40–42). It has been established that PLs undergo phase transition in clusters, consisting of  $\sim 100$  PLs, and the size of these clusters is proportional to the cooperativity of the transition (43). The size of the lattice defect, in which the insertion of apoA-I is assumed to occur, is related to the size of these clusters (44). There is a possibility of the involvement of lattice defects in the formation of nascent HDLs. To test this possibility, our experiments were performed at physiological temperature using pig apoA-I and PLs isolated from a natural source. Pig apoA-I consists of 241 amino acids, which is 2 amino acids shorter than human apoA-I (45). The protein sequence, with a well-conserved secondary structural motif of the amphipathic helices, is very homologous (79%) to human apoA-I (45). Human apoA-I was shown to have slightly lower potency to induce the LUV solubilization than pig apoA-I (data not shown).

PC isolated from a natural source has a low  $T_M$  because of an unsaturated acyl chain at the *sn*-2 position. SM has a high  $T_M$  because of its mostly saturated acyl chain, where C16:0 is the main fatty acid residue for SM from chicken egg. SM is the most abundant sphingolipid in many tissues and is distributed predominantly in the outer leaflet of the plasma membrane. The main PL of the outer leaflet is PC, but in some cases, SM is the predominant PL [e.g.,  $\sim 75\%$  SM and  $\sim 25\%$  PC in the intestinal brush-border membrane (46)]. In the PC/SM binary system, we found that SM-enriched LUVs were microsolubilized spontaneously by apoA-I at  $37.0^\circ\text{C}$  and that these membranes were in gel/ $L_d$  phase at this physiological temperature. These results indicate the possibility that apoA-I is inserted in a lattice defect at the gel/ $L_d$  phase interface of the plasma membranes of cells. In the plasma membrane, however, the amount of SM is less than that of PC and it is difficult for the gel/ $L_d$  phase to occur. Furthermore, PC is the predominant lipid in HDL (47). These findings are inconsistent with the results of the present study. However, we should note that the formation of nascent HDL in vivo is a much slower phenomenon. An excess amount of SM is not necessary for a moderate amount of nascent HDL to form. The most important thing is that the heterogeneous interface of the membrane formed by PC and SM offers an advantage for the insertion of apoA-I and the spontaneous formation of discoidal rHDL.

Chol is also a major lipid component of plasma membranes. The tight packing of Chol with PLs having saturated acyl chains forms the  $L_o$  phase, which is a model for lipid rafts of the plasma membrane. Lipid rafts, which are commonly defined based on their insolubility in nonionic detergents, are domains rich in SM and Chol and certain types of membrane proteins involved in cell signaling. In the PC/SM/Chol tertiary system, which is a suitable model for raft-containing plasma membranes, we examined the effect of Chol and  $L_o$  phase on the microsolubilization of membranes by apoA-I. The results suggested that the addition of Chol decreased the rate of microsolubilization of LUVs (Fig. 4). It can be assumed that this result is caused by two factors. The first is the decrease in the cooperativity of the gel/ $L_d$  phase transition. It is known that the addition of Chol decreases the number of PL molecules that undergo phase transition in concert (48). Chol presumably reduces the sizes of the clusters and the lattice defects at which the insertion of apoA-I occurs. The second factor is the formation of the  $L_o$  phase. It could be difficult for apoA-I to access and microsolubilize the  $L_o$  phase domains. This study revealed that apoA-I has a higher affinity for the  $L_d$  phase than for the  $L_o$  phase and that discoidal rHDL selectively consists of lipids of the  $L_d$  phase.

The exclusion of apoA-I from the  $L_o$  phase may be physiologically important for the formation of discoidal rHDL. The increase in the Chol content of the plasma membrane promotes the formation of the  $L_o$  phase and the selective distribution of apoA-I to the  $L_d$  phase and, as a result, facilitates the interaction of apoA-I with ABCA1, which appears to be localized to Triton X-100-soluble fractions, the nonrafts (49). The Chol content of plasma membranes may regulate the formation of nascent HDL in this way. A similar mechanism has been proposed for the permeabilization of lipid vesicles by  $\delta$ -lysin, a 26 residue peptide with an  $\alpha$ -helical amphipathic structure (50). The peptide binds preferentially to the  $L_d$  phase and accumulates in these nonraft domains. The formation of discoidal rHDL predominantly consisting of  $L_d$  phase lipids may indicate that Chol, which is preferentially distributed to the  $L_o$  phase, is not transported simultaneously with PLs. It has been suggested that lipid efflux occurs sequentially: first, that PLs are transported to apoA-I by ABCA1 to generate nascent HDL; then, Chol is transported to this acceptor by another transporter, such as ABCG1 or scavenger receptor class B type I (51).

It is very interesting that apoA-I extracts LUV lipids from both outer and inner leaflets. Nascent HDL generated from cells has been reported to contain phosphatidylserine and phosphatidylethanolamine, which are preferentially distributed to the inner leaflet of the plasma membrane (47), suggesting the involvement of a mechanism by which apoA-I acquires plasma membrane lipids from the outer and inner leaflets.

The initial step in the lipidation of apoA-I and the formation of nascent HDL by ABCA1 is unknown; however, it can be assumed that the local environment around ABCA1 plays a crucial role in the membrane association and lipidation of apoA-I, because this process is not

apoA-I-specific (12–14) and the lipid affinity of an amphipathic helical protein positively correlates with its ability to remove cellular PL (52). A recent study has reported that ABCA1 expression results in a significant redistribution of SM and Chol from rafts to nonrafts (53). The compositional change in nonraft membranes caused by ABCA1 may induce a spontaneous extraction of lipids by apoA-I.

In summary, our experiments reveal that the PC/SM heterogeneous interface facilitates the insertion of apoA-I and the spontaneous formation of discoidal rHDL. This scenario implies that ABCA1 triggers the spontaneous formation of nascent HDL by changing the local environment (e.g., apoA-I-soluble microdomain formation) around ABCA1 and creating packing defects, where apoA-I can be deeply inserted to extract lipids from outer and inner leaflets simultaneously. Discoidal rHDL is formed by the lipids of the loosely packed  $L_d$  phase, consistent with the fact that nascent HDL is composed of the lipids of nonraft domains.

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