Effect of cholesterol on apolipoprotein A-I binding to lipid bilayers and emulsions

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Abstract The effects of cholesterol (Chol) on the interaction of apolipoprotein A-I (apoA-I) with phospholipid bilayer vesicles and lipid emulsions were investigated. ApoA-I bound to phosphatidylcholine (PC) vesicles with higher affinity and lower capacity compared to triglyceride-PC emulsions. An increase in surface Chol in triglyceride-PC emulsions decreased the binding capacity without changing the binding affinity. In contrast, addition of Chol to PC vesicles caused a marked increase in capacity and decrease in affinity for apoA-I binding. ApoA-I caused a large release of entrapped aqueous dye, calcein, from PC vesicles, whereas this apoA-I-induced leakage was relatively small in the vesicles containing Chol. The incorporation of phosphatidylethanolamine into the vesicles also exerted effects similar to those of Chol on apoA-I binding and calcein leakage. The shifts of fluorescence emission maximum of dansyl lysine, probing the surface region of membranes, indicated that Chol as well as phosphatidylethanolamine increased the headgroup space of the vesicles. The binding maximum of apoA-I was closely correlated with the emission maximum of dansyl lysine, not with the fluorescence anisotropy of 1-[4-(trimethylamino)phenyl]phenylhexatriene, suggesting that the binding capacity of apoA-I to the bilayer surface was modulated by the headgroup space rather than the acyl chain fluidity. These results show that Chol affects the bilayer surface so as to allow more apoA-I to bind to bilayers and may suggest the possibility of the interaction of apoA-I with Chol-enriched membrane domains.—**Saito, H., Y. Miyako, T. Handa, and K. Miyajima.** Effect of cholesterol on apolipoprotein A-I binding to lipid bilayers and emulsions. *J. Lipid Res.* 1997. **38:** 287–294.

Supplementary key words apolipoprotein A-I • cholesterol • emulsions • bilayers • headgroup space

Apolipoprotein A-I (apoA-I) is the major protein component of high density lipoproteins and plays a role in reverse cholesterol transport (1–4). ApoA-I possesses multiple tandem repeating 22-mer amphipathic α-helices, which are thought to be responsible for the association with lipids (5, 6). Although plasma apoA-I is mostly lipid bound, lipid-poor or lipid-free forms of apoA-I, which may interact with cells, could exist (7–10). The importance of lipid-free apoA-I in the reverse cholesterol transport has been established in studies showing that apoA-I promotes the release of phospholipid and cholesterol (Chol) from cultured cells (11–14). In addition, this process is significantly enhanced in the cells enriched with Chol, suggesting that the interaction of apoA-I with the cells may be controlled by the level of Chol in the cells (15, 16).

Differences in surface lipid compositions of plasma lipoproteins could be an important factor influencing the distribution of exchangeable apolipoproteins among lipoprotein particles. Several studies have shown that the surface content of Chol affects the binding of apoA-I and other apolipoproteins to lipid emulsions (17–20). Chol decreases the adsorption of apoA-I to egg phosphatidylcholine (PC) and lipoprotein surface lipid monolayers (21, 22), and also changes the conformation of apoA-I in reconstituted discoidal lipoprotein particles (23–25). These effects of Chol on the interaction of apoA-I with phospholipids are presumably due to the modulation of the lipid packing because apoA-I has preferential affinity to PC rather than Chol (26, 27). However, Chol has also been suggested to exert its effect directly by binding to apoA-I (25, 28).

In contrast to the effects of Chol on the association and organization of apoA-I on the lipoprotein surface, how Chol affects the binding behavior of apoA-I to the cell surface remains less understood. In the present study, we investigated the effect of Chol on apoA-I binding to bilayer vesicles, used as a model membrane, and compared it with lipid emulsions using a centrifugation assay. Addition of Chol markedly increased the binding capacity of apoA-I to the vesicles with decreasing binding affinity. Fluorescence studies using the membrane surface probe in the vesicles, together with the results of an aqueous dye leakage from the vesicles, suggested

Abbreviations: apoA-I, apolipoprotein A-I; PC, egg yolk phosphatidylcholine; PE, egg yolk phosphatidylethanolamine; Chol, cholesterol; TG, triglyceride; CO, cholesteryl oleate; TMA-DPH, 1-[4-(trimethylamino)phenyl]phenylhexatriene.

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that Chol affects not only the acyl chain fluidity but also the headgroup space of phospholipid, allowing more apoA-I to bind to the bilayer surface.

EXPERIMENTAL PROCEDURES

Materials

ApoA-I was isolated from pig plasma using the procedures described previously (27, 29). Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei Co. The purity (over 99.5%) was determined by thin-layer chromatography. Egg yolk phosphatidylethanolamine (PE), Chol, and cholesteryl oleate (CO) were obtained from Sigma and used without further purification. Soy bean triglyceride (TG) obtained from Nacalai Tesque was purified using a silicate (Wakogel C-200, Wako Pure Chemicals) column to remove fatty acids, diglycerides, and monoglycerides. Dansyl lysine and 1-[4-(trimethylamino)phenyl]phenylhexatriene (TMA-DPH) were purchased from Molecular Probes. All other chemicals were of special grade from Wako Pure Chemicals. Water was double distilled with a quartz still.

Preparation of emulsions and vesicles

TG-PC and CO-PC emulsion particles with a diameter of 100–130 nm (determined from quasi-elastic light scattering measurements, Photal LPA-3000/3100, Otsuka Electronic Co.) were prepared at 60–70°C as described (20, 30) using a high-pressure emulsifier (Nanomizer, Nanomizer Inc., Tokyo). Their TG (or CO): PC molar ratio was 85:15 for TG-PC and 87:13 for CO-PC emulsions. When Chol was added to the emulsions, PC was replaced by Chol at a given ratio before the preparation of emulsions. PC vesicles with a diameter of about 100 nm were prepared by an extrusion technique as described (20, 30).

NMR spectroscopy

^{31}P NMR spectra were obtained on a Brucker AC-300 spectrometer at 121.5 MHz by using 2-μs 45° pulses and application of a 50 kHz proton decoupling field during acquisition. A paramagnetic-shifting reagent, praseodymium (III) nitrate, was added into emulsion or vesicle samples (final concentration of 10 mM).

ApoA-I binding studies

ApoA-I binding assays were performed in 10 mM Tris-HCl buffer (pH 7.4), containing 150 mM NaCl, 1 mM EDTA, and 0.01% NaN_3. The mixtures (1.0 ml) of a constant amount of emulsions (or vesicles) and various amounts of apoA-I were incubated for 1 h at 25°C. When the mixtures contained vesicles, 3% sucrose was added to adjust the density. After incubation, the mixtures were placed in polycarbonate centrifuge tubes (11 × 34 mm, HITACHI) and ultracentrifuged in a HITACHI S120AT2 rotor at 46,000 rpm (78,000 *g*) for 1 h. The proportion of emulsions found in the bottom fraction was less than 1%. Although vesicles are more dense than emulsions, it was confirmed that the addition of 3% sucrose was sufficient for almost vesicles to distribute in the top fraction. After ultracentrifugation, the bottom fractions (400 μl) were collected and then 100 μl of 10% heptaethylene glycol dodecyl ether solution was added to each fraction. These samples were left overnight at 4°C to solubilize the few remaining lipids. The apoA-I concentration was determined by measurement of tryptophan fluorescence at 335 nm (excited at 280 nm). The lipid-bound apoA-I amount was calculated from the difference between the apoA-I concentration before and after ultracentrifugation.

Binding data were analyzed by a linearized plot of the equilibrium binding according to the following equation (31):

$$P_f = [PL]\ (P_f/P_b)\ N - K_d \qquad Eq.\ 1)$$

where P_f and P_b are free and bound protein concentration (μM), respectively, [PL] is the concentration of phospholipid (μM), N is the binding maximum (mol/mol), and K_d is the dissociation constant (μM). From a linear plot of P_f against [PL] (P_f/P_b), K_d and N can be estimated.

Fluorescence measurements

Steady-state fluorescence anisotropy of TMA-DPH was measured as previously described (30). For the vesicles labeled with dansyl lysine, lipids and dansyl lysine stocks were mixed in chloroform–methanol at a lipid: probe molar ratio of 100:1 before the preparation of vesicles. Fluorescence measurements of dansyl lysine were performed using a HITACHI F-4500 spectrofluorometer at 25°C. Excitation was at 340 nm and emission spectrum was measured from 480 to 600 nm with a scan rate of 60 nm/min.

Calcein leakage from vesicles

For leakage measurements, an aqueous fluorescence dye, calcein (Dojindo) was entrapped in vesicles. The dried lipid film was hydrated with 70 mM calcein solution. Then the suspension was vortexed, followed by an extrusion procedure as described (30). Untrapped calcein was removed by gel filtration through a Sephadex G-50 (Pharmacia) column eluted with the buffer. An apoA-I solution was added to the calcein-entrapped vesicles at 25°C. Fluorescence emission intensity was measured at 520 nm using an excitation wavelength of 490

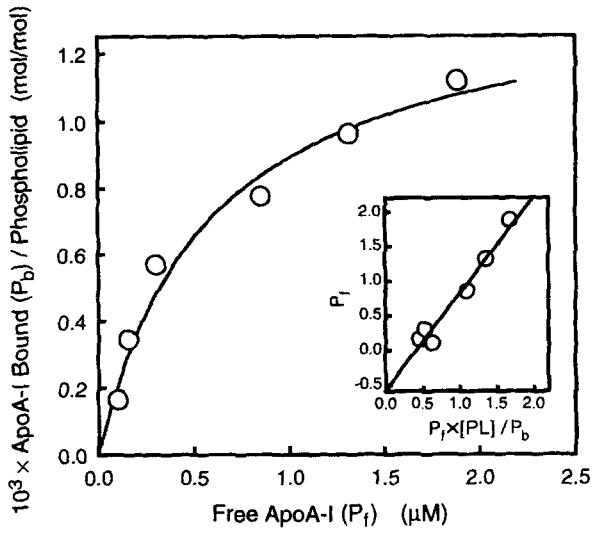

Fig. 1. Binding profiles of apoA-I to TG-PC emulsions without Chol. Each point represents the mean of three experiments. The solid line is a theoretical isotherm curve calculated using K_d and N values listed in Table 1. Inset, linearized plot of apoA-I binding data according to *Eq. 1*.

nm. The percent leakage of calcein was determined using the fluorescence intensity corresponding to 100% leakage obtained in the presence of 0.3% of Triton X-100.

Lipid and protein analysis

Phospholipid concentration was determined by the phosphorus assay according to the method of Bartlett (32). The concentrations of other lipids were determined using enzymatic assay kits for TG, free and total Chol, purchased from Wako Pure Chemicals. The protein concentration was determined by the method of Lowry et al. (33), using bovine serum albumin (Bio-Rad) as standard.

RESULTS

ApoA-I binding to emulsion particles

Figure 1 shows the typical binding profile of apoA-I to TG-PC emulsions. The amount of apoA-I bound to the emulsions increased with the apoA-I concentration. The linearized plot of the binding data according to equation 1 showed reasonably good fit to a straight line (Fig. 1, inset), indicative of an equilibrium binding of apoA-I to the emulsion surface. From the slope and the ordinate intercept of the straight line, binding parameters, the dissociation constant K_d and the binding maximum N were obtained (**Table 1**). Although addition of Chol to the emulsion surface did not change the K_d value, N was significantly decreased only in the presence of 40 mol% of surface Chol (Table 1). This delayed effect of surface Chol on the binding capacity of apoA-I to the emulsion surface is similar to the Chol-induced change in fluorescence anisotropy of TMA-DPH in TG-PC emulsions (30), and consistent with the results of human apoA-I binding to emulsions as observed by Derksen and Small (18). As shown in Table 1, replacing of core TG with CO, which reduces the mobility of core lipids because of the smectic-like ordered structure of CO, also reduced the binding capacity of apoA-I without changing the affinity. A similar effect of the core replacement on apolipoprotein binding was observed for the binding of apolipoproteins C-II and E to the emulsion surface in human serum (20).

TABLE 1. Binding parameters of apoA-I to emulsions at 25°C

	K_d (μM)	$N \times 10^3$ (mol/mol Phospholipid)
TG-PC emulsions		
0% Chol	0.58 ± 0.14	1.41 ± 0.13
20% Chol	0.59 ± 0.22	1.48 ± 0.23
40% Chol	0.46 ± 0.07	0.63 ± 0.09
CO-PC emulsions		
0% Chol	0.60 ± 0.16	0.92 ± 0.09

ApoA-I binding to vesicles

We also examined the effect of Chol on apoA-I binding to bilayer vesicles. As apoA-I is considered to bind to the outermost layer of vesicles, phospholipid molecules available for apoA-I binding were calculated from the lamellarity of vesicles determined by ^{31}P NMR spectra in the presence of praseodymium (20, 30). The addition of praseodymium causes a downfield shift of signals from PC headgroups in the outermost layer of vesicles and thereby gives the lamellarity of the vesicles. **Figure 2** shows the binding isotherms of apoA-I to PC vesicles with increasing amounts of Chol. In the absence of Chol, saturation of apoA-I binding to the vesicles was observed in the apoA-I concentration range studied. As also shown in Fig. 2, addition of Chol to the vesicles was found to increase the binding amount of apoA-I, in sharp contrast to the effect of Chol on apoA-I binding to the emulsions. In all vesicles, reproducible values of K_d and N were obtained from the linearized plots in a similar manner as the emulsions and listed in **Table 2.** Although both K_d and N of the vesicles without Chol were significantly smaller than those of TG-PC emulsions, progressive increases in K_d and N were observed in the Chol-enriched vesicles. Interestingly, the significant increases in K_d and N were also observed when PE was incorporated into the PC vesicles.

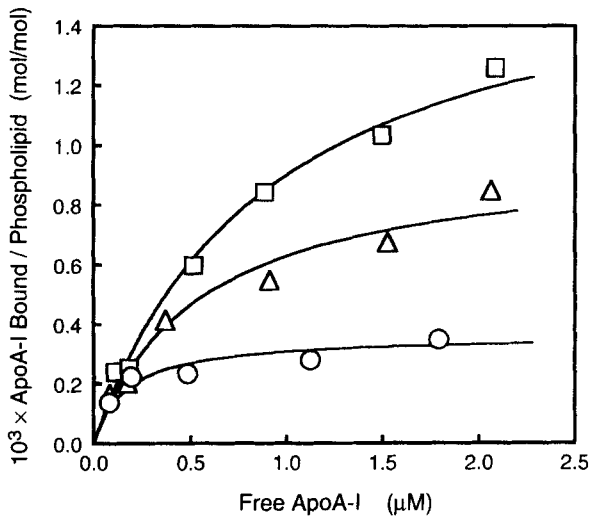

Fig. 2. Binding profiles of apoA-I to PC vesicles with increasing amount of Chol: (○) Chol = 0 mol%; (△) 20 mol%; (□) 40 mol%. Each point represents the mean of two or three experiments. The solid lines are theoretical isotherm curves calculated using K_d and N values listed in Table 2. Phospholipid molecules available for apoA-I binding were calculated from the lamellarity of vesicles determined by ^{31}P NMR.

Fluorescence measurements

To investigate the physical properties of bilayer membranes governing apoA-I binding, we measured the fluorescence emission spectrum of dansyl lysine for probing the surface region of membranes, and the fluorescence anisotropy of TMA-DPH for acyl chain order. The fluorescence emission of dansyl lysine is very sensitive to the solvent environment and thereby, reflects the lateral separation of the phospholipid headgroups because the increased separation of phospholipid headgroups elevates the hydration in the headgroup region (34). The emission maximum of dansyl lysine embedded in PC vesicles showed a large blue shift from that in buffer (data not shown). Addition of Chol to the vesicles caused a progressive red shift of the emission maximum of dansyl lysine (**Fig. 3A**), indicating that Chol increased separation (spacing) of the phospholipid headgroups (35). PE also showed the spacing effect of the headgroups as reflected by the red shift of dansyl lysine, and a close correlation between the binding maximum of apoA-I, N and the emission maximum of dansyl lysine ($r = 0.97$) was observed (Fig. 3A). In contrast, the fluorescence anisotropy of TMA-DPH seemed to be less correlated with N ($r = 0.81$) (Fig. 3B).

TABLE 2. Binding parameters of apoA-I to PC vesicles at 25°C

	K_d (μM)	$N \times 10^3$ (mol/mol Phospholipid)
PC-Chol vesicles		
0% Chol	0.17 ± 0.10	0.36 ± 0.03
20% Chol	0.55 ± 0.17	0.98 ± 0.10
40% Chol	0.91 ± 0.18	1.72 ± 0.16
PC-PE vesicles		
33% PE	1.02 ± 0.29	1.18 ± 0.17

Calcein leakage from vesicles

We further examined the interaction of apoA-I with bilayer vesicles by measuring the release of entrapped fluorescence dye from the vesicles. **Figure 4** shows the calcein leakage from the vesicles with or without a saturated binding amount of apoA-I (4.0 μM). In the absence of apoA-I, no release of calcein was observed in either PC or PC with 40 mol% Chol vesicles, but the vesicles containing 33 mol% PE were somewhat leaky during the incubation. ApoA-I caused a significant release of calcein from the PC vesicles, whereas this apoA-I-induced leakage was relatively small in the vesicles containing Chol or PE. These results indicated that the binding of apoA-I caused a structural perturbation in bilayer vesicles but this effect was inhibited by the incorporation of Chol or PE into PC vesicles. During incubation, particle size of vesicles did not change and no detectable discs were observed by ultrafiltration, indicating that the binding of apoA-I to the vesicles did not alter the structural integrity of the vesicles.

DISCUSSION

Lipid-free apoA-I interacts with cultured cells to promote efflux of Chol and phospholipid (11–14), and this process is enhanced when the cells are enriched with Chol (15, 16). Two mechanisms by which free apoA-I mediate cellular lipid efflux are proposed; one is that apoA-I interacts with specific surface proteins and another is that apoA-I is associated with phospholipid-Chol domains of the cells (15). The fact that apoA-I-mediated Chol efflux is enhanced by the enrichment of Chol in the cells appears to be in agreement with the increased association of apoA-I with lipid domains. However, Li, Czarnecka, and Yokoyama (14) have recently shown that the trypsin-susceptible cellular surface factor(s) is required for the interaction of the cells with apoA-I.

The present study using bilayer vesicles as a model membrane clearly demonstrated that the increasing Chol content in bilayers caused a marked increase in the capacity for apoA-I binding to the bilayer surface.

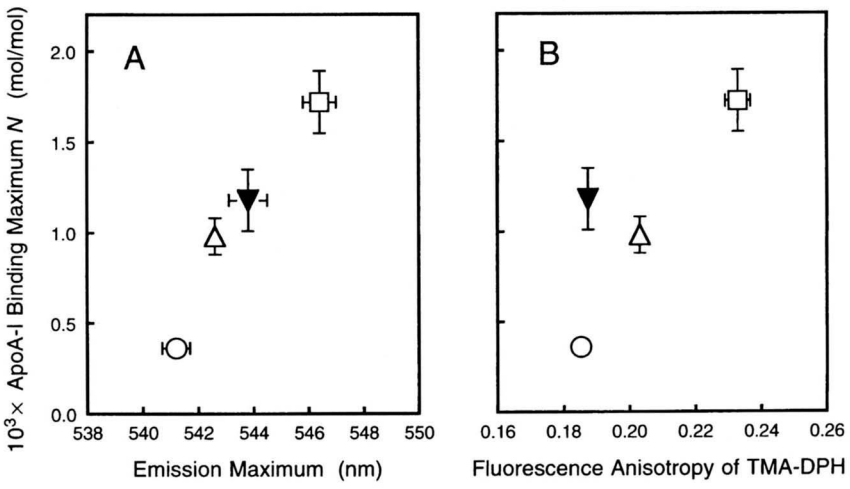

Fig. 3. Relationship of apoA-I binding maximum N with emission maximum of dansyl lysine (A) and fluorescence anisotropy of TMA-DPH (B). The emission spectra of dansyl lysine were obtained at the lipid concentration of 1–3 mM. PC vesicles used are: (○) Chol = 0 mol%; (△) 20 mol%; (□) 40 mol%; (▼) PE = 33 mol%.

The enhancement of the binding capacity was also observed by addition of PE to the vesicles (Table 2). As we used vesicles with a diameter of about 100 nm in this study, the lipid packing appears arranged like a planar surface. In such planar bilayers, Chol (35, 36) as well as PE (34, 37) tends to increase the headgroup separation, resulting in an increase in the interfacial packing defects in the bilayers. Therefore, the close correlation of the emission maximum of dansyl lysine with apoA-I binding maximum (Fig. 3A) suggests that the binding capacity of apoA-I to the bilayer vesicles is increased by the interfacial packing defects (headgroup spaces) in the bilayers. Similar effects of Chol have been reported on protein kinase C activity in studies showing that protein kinase C activity, modulated by headgroup spacing effects such as increased acyl chain unsaturation and the presence of PE, is also increased by addition of Chol to PC vesicles (38, 39).

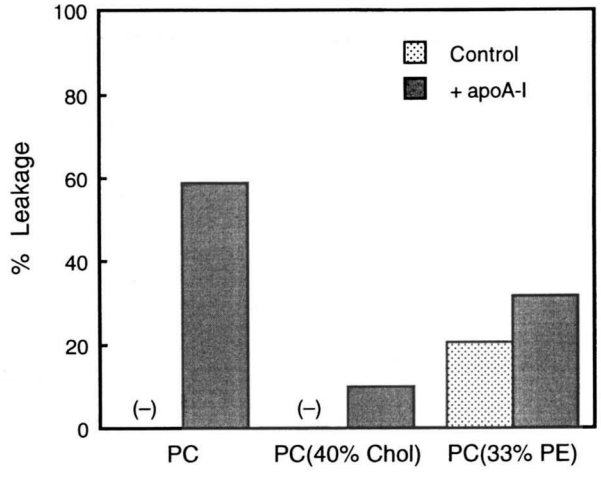

Fig. 4. Effect of apoA-I on leakage of calcein from PC vesicles. Vesicles were incubated in the absence (Control) or presence of 4.0 μM apoA-I for 1 h at 25°C; (−) indicates that no leakage of calcein was observed during the incubation.

ApoA-I is composed of several segments of class A amphipathic helices, which are characterized by the location of basic residues near the hydrophobic/hydrophilic interface and acidic residues opposite to the hydrophobic face (5, 40). This class A helix possesses high lipid binding affinity (41), and has been proposed to be associated with the outer surface of hydrated phospholipid such that its basic residues are extended toward the polar face of the helix, allowing its charged moieties to come in contact with the aqueous milieu (snorkel model) (40, 42). Based on the snorkel model, the class A helix has been suggested to have a wedge-shaped cross-section, which favors the highly curved structure of high density lipoproteins (42). Therefore, when apoA-I binds to the planar PC bilayers, it would create local packing defects in the hydrocarbon region, and thereby cause the large leakage of calcein from the vesicles (Fig. 4). The finding that apoA-I bound to PC vesicles with high affinity (K_d = 0.17 μM) but low capacity (about 2800 PC molecules per bound apoA-I) also appears to indicate that the high-affinity binding of apoA-I, such that its helices insert deeply into the hydrophobic interior of surface layers, may be unfavorable for maintaining the planar bilayers. Despite the calcein leakage, it was confirmed that the binding of apoA-I to the vesicles did not alter the structural integrity of the vesicles in our experimental condition.

On the other hand, the incorporation of Chol or PE into PC vesicles is thought to allow more apoA-I to bind to the bilayer surface without strong membrane perturbation because the increased headgroup space of phospholipids will be favorable for the binding of the wedge-shaped helix of apoA-I to these vesicles. Indeed, the binding of apoA-I caused the relatively small calcein leakage from the vesicles containing Chol or PE despite the increased amount of apoA-I binding (Fig. 4). Derksen, Gantz, and Small (43) have recently shown that the binding of apoA-I to PC surface is mainly an entropically driven hydrophobic process. The low affinity for the apoA-I binding to these vesicles might thus reflect the weak hydrophobic interaction between apoA-I helix and PC molecule. The weak interaction between apoA-I and PC may suggest that apoA-I binds to the Chol-enriched bilayers primarily through insertion of amphipathic helical segments between the phospholipid headgroups and does not penetrate deeply into the hydrocarbon region, as compared to PC bilayers. Furthermore, this binding feature of apoA-I predicts the looser binding to Chol-enriched cells, and may be favorable for the reversible dissociation of apoA-I (13) promoting the removal of membrane segments of phospholipids and Chol (15).

The binding of apoA-I to the emulsions showed two major differences compared with that to the vesicles. One difference is that about 4-fold more apoA-I bound to TG-PC emulsions than to PC vesicles, and another is that Chol decreased the binding capacity to the emulsion surface even though Chol increased binding capacity to the vesicles. These differences could be partly explained by the interaction between surface and core lipids in the emulsions as suggested previously (20). Although binding of apoA-I to the emulsion surface may produce the packing defects in a similar manner as bilayers, the penetration of core lipids towards the surface monolayers could fill the packing defects, resulting in the increased amount of apoA-I binding to the emulsion surface. That is, a poor binding capacity of vesicles for apoA-I compared to emulsions seems to be due to the lack of core penetration in the vesicles. This explanation is supported by the finding that replacement of core TG with CO reduced the binding capacity of apoA-I probably due to the decreased mobility of core lipids (20).

Derksen and Small (18) have previously shown that Chol content of 34 mol% or larger in the emulsion surface started to decrease the binding capacity of apoA-I and E-3. In the present study, we also observed the similar effect of Chol on the apoA-I binding. That is, only when 40 mol% of Chol is present in the emulsion surface, does the binding capacity of ApoA-I begin to decrease without changing the affinity (Table 1). We have previously shown that surface Chol is located in the inner hydrocarbon region at a low Chol ratio (20 mol%), but is squeezed towards the outer surface region at a high Chol ratio (40 mol%) in TG-PC emulsions (20). Therefore, the binding capacity of apoA-I is considered to decrease only when Chol is squeezed towards the outer surface region in the emulsion surface layers. This surface-orientated Chol causes a condensation of the outer acyl chain region in the emulsion surface (20, 30), and is also likely to inhibit the penetration of core lipids into the surface layers, presumably resulting in the decrease in the binding capacity of apoA-I. In addition, the headgroup space of emulsion surface is considered to be smaller than that of the bilayer surface because the acyl chain region in the emulsion monolayers has a more ordered structure than that in the bilayers (20, 30). In such a condensed surface, the headgroup space caused by the incorporation of Chol would not be sufficient to increase the binding capacity of apoA-I to the emulsion surface. As a result, the increase in the surface Chol may completely block the insertion of apoA-I helix between phospholipids and exclude bound apoA-I from the emulsion surface. However, we could not identify the underlying mechanism because the headgroup space of the emulsion surface could not be estimated by the fluorescence technique used in this study due to the strong light scattering.

Physiological implications

Lipid-free apoA-I promotes efflux of phospholipid and Chol from Chol-enriched cells. This process is controlled by the level of Chol in the cells (16), but the mechanism of the enhanced lipid efflux by Chol remains unclear. In the present study, we demonstrated that the increase in Chol content in bilayers markedly increased the binding amount of apoA-I to the bilayer surface, indicating that more apoA-I can interact with Chol-enriched membrane domains. In addition, the lipid binding feature of apoA-I to Chol-enriched bilayers such that apoA-I does not penetrate deeply into the phospholipid hydrocarbon region may be favorable for the reversible dissociation. The ease of dissociation of apoA-I bound to Chol-enriched membranes will promote the removal of membrane segments of phospholipid and Chol.

ApoA-I is essentially found in high-density lipoproteins, but is also present in chylomicrons. The effect of Chol on ApoA-I binding to the emulsions indicates that the accumulation of Chol in the surface of chylomicrons facilitates the dissociation of bound apoA-I, which may be responsible for reverse cholesterol transport.

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