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Parinaroyl and pyrenyl phospholipids as probes for the lipid surface layer of human low density lipoproteins

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A simple protocol employing lipid transfer proteins was developed to label human low density lipoprotein (LDL) in a controlled manner with parinaroyl and pyrenyl phosphatidylcholines. In order to study the lipid fluidity in the surface lipid layer of LDL, the temperature-dependence of both polarization (parinaroyl probes) and excimer to monomer (E/M) intensity ratio (pyrenyl probes) were analyzed. A series of pyrenyl phosphatidylcholines containing a pyrenyl fatty acid varying from 6 to 14 carbons in length at the *sn*-2 position were inserted into LDL to investigate the lateral distribution of different phosphatidylcholines in the lipoprotein surface at 37°C. Both polarization and E/M vs. temperature plots displayed discontinuities in the region of 22–32°C, which coincides with the melting of the neutral lipid core, indicating that the latter induces an ordered to more disordered phase transition in the surface lipid layer. Determination of the E/M intensity ratio as a function of pyrene lipid concentration in LDL showed a linear relationship for the pyrenyl hexanoate and octanoate species, whereas a slope discontinuity was observed for the lipids containing a longer pyrenyl chain. These data suggest that two lipid domains with distinct properties exist in the surface layer and secondly, pyrenyl lipids partition between these domains in a chainlength-dependent manner. This is consistent with measurement of the tryptophan to pyrene energy transfer efficiency vs. pyrenyl lipid concentration, which showed a biphasic relationship for the long-chain pyrenyl lipids. These measurements further indicate that two surface lipid domains correspond to the protein-lipid boundary and the bulk lipid phase, respectively. The fact that relatively small changes in chainlength have a marked influence on the partitioning of pyrenyl lipids between the boundary and the bulk phase suggests also that native phospholipid species may not be randomly distributed in the surface lipid layer of LDL.

Introduction

LDL represents the end product of the intravascular lipoprotein lipase-catalyzed degradation of very low density lipoprotein particles secreted from the liver [1]. LDL is the major carrier of plasma cholesterol and its concentration in plasma is positively correlated with the incidence of coronary heart disease [2]. The LDL particle consists of an apolar core of cholesterol esters and triacylglycerol surrounded by a monolayer of phospholipids and free cholesterol as well as incorporated protein [3,4]. The major phospholipids in LDL are phosphatidylcholine (65%) and sphingomyelin (25%) and the ratio of cholesterol to phospholipid is close to unity in the surface layer [5]. The protein moiety of LDL consists of a single, high-molecular weight (M_r 512.937) glycoprotein [6,7], apoB-100, which probably interacts with the lipid components through several amphipathic peptide domains [8–11].

Despite extensive studies, the structures and interactions between the molecular components of LDL are not fully understood. For instance, it remains controversial whether the thermotropic transition of the neutral lipid core occurring at around 30°C [12,13] is reflected in structural and dynamic properties of the

Abbreviations: apoB-100, apolipoprotein B-100; LDL, human low density lipoprotein; PC, phosphatidylcholine; *cis*-PnAPC, 1-palmitoyl,2-*cis*-parinaroyl (*cis,trans,trans,cis*-9,11,13,15-octadecatetraenoic acid) phosphatidylcholine; *trans*-PnAPC, 1-palmitoyl,2-*trans*-parinaroyl (all-*trans*-9,11,13,15-octadecatetraenoic acid) phosphatidylcholine; Pyr_xPC, 1-palmitoyl,2-pyrenyl (C_6–C_{14} acyl) phosphatidylcholine; SM, sphingomyelin; DSC, differential scanning calorimetry; PC-TP, phosphatidylcholine-specific transfer protein; E/M, excimer-to-monomer fluorescence intensity ratio; RET, resonance energy transfer; HPLC, high-pressure liquid chromatography; PA, phosphatidic acid; TPE, trinitrophenylphosphatidylethanolamine.

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surface lipid layer [14–17]. Furthermore, the lateral organization of the various lipid species in this layer remains unclear.

In the present study we describe in detail a method to introduce fluorescent phospholipid analogues into LDL in order to investigate the properties of the surface lipid layer. PC and SM analogues containing either a *cis*- or *trans*-parinaroyl chain (*cis*-PnAPC and *trans*-PnAPC) or a pyrenyl acyl chain of variable length (Pyr_xPCs and Pyr_{10}SM) were used to probe the surface lipid layer of LDL, because (i) these probes have been extensively characterized in model systems [18–26]; (ii) they are expected to mimic closely the behavior of their natural counterparts and (iii) their spectroscopic properties make them suitable for study, e.g., dynamics and lateral distribution of phospholipids. The results show that melting of the neutral-lipid core decreases the order and/or increases the dynamics abruptly in the surface lipid layer, probably by inducing a phase transition. Secondly, the behavior of the pyrenyl lipids indicate that two distinct domains exist in the surface layer and these domains most likely represent the annulus around the lipid-associating segments of apoB-100 and the bulk lipid phase, respectively. The fact that relatively small structural differences markedly influence the partition of the probes between the domains suggests that the natural phospholipid species may also tend to segregate in surface lipid layer of LDL.

Materials

Preparation of LDL

Human LDL (density = 1.019–1.063 g/ml) was obtained from the plasma of a fasted normolipemic single donor by sequential ultracentrifugation followed by gel chromatography on a Bio-Gel A15-m column as previously described [6]. Using sodium dodecylsulfate-polyacrylamide gel electrophoresis of the LDL preparation, apoB-100 migrated as a single band. Electron micrographs of negatively stained LDL preparation displayed a homogeneous population of particles with mean diameter of 25 nm. LDL contained protein (22% of dry weight), phospholipid (22%), free cholesterol (8%), cholesterol ester (37%), and triacylglycerol (11%). LDL was stored at +4°C in the dark and used within 4 days after isolation.

Lipids

Trinitrophenylphosphatidylethanolamine (TPE) was obtained from Sigma. *cis*-Parinaric acid was isolated from the seed kernels of *Parinarum glaberrimum* and *trans*-parinaric acid was either prepared from *cis*-parinaric acid, as described by Sklar et al. [27], or was obtained from Molecular Probes. 1-Palmitoyl, 2-oleoylphosphatidylcholine and phosphatidylcholines containing either a *cis*- or *trans*-parinaroyl or pyrenyl acyl (5–13 methylene units) moiety in the *sn*-2 position were synthesized from 1-palmitoyl-*sn*-glycero-3-phosphocholine and the corresponding fatty acid anhydrides as described previously [23,28]. The lipids were purified with HPLC using a Merck Lichrospher silica gel column and, when necessary, were further purified with reverse-phase HPLC [28]. PA was prepared from egg PC by phospholipase-D-catalyzed hydrolysis [30].

Transfer proteins

PC-TP and the nonspecific transfer protein from bovine liver were provided by Dr. K.W.A. Wirtz and his colleagues (Department of Biochemistry, University of Utrecht). The proteins were stored at −20°C in 50% glycerol solution.

Methods

Preparation of donor vesicles. Negatively charged donor vesicles consisted of Pyr_xPC or PnAPC (50 mol%), PA (40 mol%), and TPE (10 mol%). TPE was used as a quencher of pyrene and parinaric acid fluorescence [31]. Small unilamellar donor vesicles were prepared by injection of the lipids dissolved in ethanol into the buffer with a spring-loaded syringe [32]. The volume of ethanol was always less than 1% of the final volume.

Labeling of LDL with fluorescent phospholipids. Quenched donor vesicles containing up to 20 nmol of Pyr_xPC and PnAPC species were prepared in 1 ml of buffer containing 20 mM Tris-HCl, 90 mM NaCl, 1 mM EDTA (pH 7.4). After incubation for 10 min at 37°C, 1.8–3.7 μg of PC-TP and LDL (150 nmol of phospholipid) were included and the incubation was continued for 1–2 h at 37°C. This was sufficient to equilibrate the fluorescent lipids between the donor vesicles and LDL as evidenced by a leveling off of the pyrene or the parinaric acid fluorescence intensity (see below). Pyr_{10}SM-labeled LDL was prepared similarly, but the nonspecific transfer protein (3–5 μg) was used instead of PC-TP and cardiolipin replaced PA in the donor vesicles.

Separation of labeled LDL from the negatively charged donor vesicles was accomplished by modifying a method previously used to incorporate fluorescent phospholipids to transfer proteins [33]. 1 ml of the incubation mixture was applied on to a DEAE-cellulose column (0.5 × 1.5 cm) and eluted with 3 ml of the incubation buffer at 22°C. The concentration of Pyr_xPC or PnAPC in the eluted LDL fraction was assayed from the probe fluorescence intensity after addition of sodium deoxycholate (final concn. 1.0%, w/w). The LDL concentration was determined by tryptophan fluorescence intensity measurement after sodium deoxycholate addition. Control experiments showed that solubilization of LDL abolishes quenching of apoB-100 tryptophan fluorescence by the fluorescent lipids. The recovery of

LDL is dependent on the donor vesicle concentration in a manner suggesting 1 : 1 binding of LDL to the charged vesicles and consequent trapping to the column. This makes the present method unsuitable for extensive replacement of the LDL phospholipids by exogenous ones, since the requisite donor/LDL ratios would result in low recovery of LDL. Based on the determination of the relative binding constants for the probes and LDL PC indicated that less than 0.2% of the probe molecules are bound to the eluted transfer protein and thus their contribution to the total fluorescence signal is considered negligible.

Fluorescence measurements. Fluorescence intensities were obtained using a Hitachi F 4000 spectrofluorometer equipped with a cuvette holder thermostated to 37 °C and excitation and emission slits adjusted to 1.5 and 10 nm, respectively. Excitation wavelengths were 343, 322 or 318 nm for Pyr_xPCs, *cis*- and *trans*-PnAPC, respectively. The emission of PnAPC was recorded at 420 nm and that of pyrene monomer (M) and excimer (E) at 378 and 500 nm, respectively. Both the fluorescence polarization values of the PnAPC and the monomer and excimer fluorescence intensities of PyrPC were corrected for a small background due to the LDL particles and buffer. The buffer was pre-equilibrated at 37 °C with air (for Pyr_xPCs) or with argon (for PnAPCs). ApoB-100 tryptophans were excited at 290 nm and the emission intensity was measured at 330 nm.

The efficiency (T_r) of resonance energy transfer (RET) from apoB-100 tryptophan donors to the pyrenyl acceptors was determined from the increased excitation intensity of the Pyr_xPCs when incorporated to LDL [34]:

$$T_r = [I_{290}/I_{343} - \epsilon^A_{290}/\epsilon^A_{343}] \cdot [\epsilon^A_{343}/\epsilon^D_{290}]$$

where I is the fluorescence intensity of pyrene. I was measured at two wavelengths, i.e., 343 nm at which wavelength tryptophan does not absorb, and 290 nm, which is close to the absorption maximum of tryptophan. ϵ^A and ϵ^D represent the molar absorption coefficients of pyrenyl and tryptophan residues. Values of 3469, 42000 and 3925 were used for ϵ^A_{290}, ϵ^A_{343} and ϵ^D_{290}, respectively.

Differential scanning calorimetry. A Privalov DASM-4 differential scanning calorimeter at full-scale sensitivity of 0.2 mcal/s and heating rate of 1.0 C °/min was used. 1 ml samples contained 4.4 mg of LDL in the buffer mentioned above.

Chemical assays. Phosphate was determined as described by Bartlett [35] and protein was measured according to Lowry et al. [36] in the presence of 0.1% (w/w) sodium dodecyl sulfate.

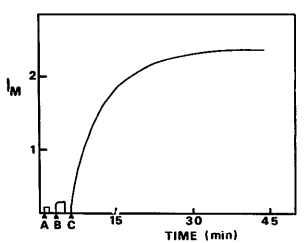

Fig. 1. Kinetics of transfer of PyrPC from quenched donor vesicles to LDL. Insertion of pyrenyl phospholipid (Pyr_{10}PC) into LDL was followed by measurement of pyrene monomer fluorescence intensity at 378 nm using 343 nm for excitation. Fluorescence of (A): quenched donor vesicles (Pyr_{10}PC/egg PC/PA/TPE, 4 : 14 : 15 : 3.3 nmol) in the presence of (B): LDL (150 nmol phospholipid) and (C): PC-TP (1.4 μg). The temperature was 37 °C.

Results

Labeling of LDL with fluorescent phospholipids

Transfer of the probes from the quenched donor vesicles can be readily followed as the probe fluorescence increases typically 20–50-fold upon insertion to LDL (Fig. 1). Under the conditions used, the equilibrium was practically reached after 30–60 min incubation as evidenced by leveling off of pyrene monomer fluorescence intensity. Labeled LDL can be easily separated from the negatively charged donor vesicles on a small anion-exchange column. Fig. 2 shows that trapping of the donor vesicles efficiently to the column, while maintaining a good recovery of LDL, was achieved with a NaCl concentration of 90–100 mM. Accordingly, NaCl concentration was adjusted to 90 mM in the labeling experiments proper. Some pyrene fluorescence eluted with LDL at low NaCl concentrations (Fig. 2). This is probably due to a small number of donor vesicles leaking from the column. It was, however, calculated that less than 1 donor vesicle per 1000 LDL particles elutes from the column with the 90 mM NaCl buffer so that the donor vesicle contamination of the LDL fraction is negligible.

Thermotropic properties of the surface lipid layer

Prior to the study of the thermotropic phenomena in LDL surface, DSC of the isolated LDL was carried out. DSC measurement showed (Fig. 3) an endotherm between 24 and 39 °C with a midpoint at 32 °C and an enthalpy of 0.68 cal/g of LDL cholesterol esters, which are in agreement with previously published data [13]. Plots of emission polarization ratio vs. temperature for

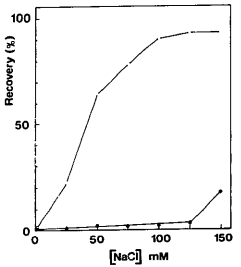

Fig. 2. Separation of LDL from negatively charged donor vesicles. LDL (150 nmol phospholipid) was incubated at 22°C for 5 min with small unilamellar $Pyr_{10}PC$/egg PC/PA (1:59:40, molar, ratio) donor vesicles (10 nmol phospholipid) in 1 ml of a buffer containing 25 mM Tris-HCl, 1 mM EDTA (pH 7.4) and varying concentrations of sodium chloride. The samples were then chromatographed on a small DEAE-column and the recovery of LDL (open circles) and donor vesicles (solid circles) in the eluent was determined by tryptophan and pyrene fluorescence, respectively, as specified under Methods. Data from a single experiment are shown but practically identical profiles were obtained reproducibly.

cis- and *trans*-PnAPC in LDL are shown in Fig. 4A. The *trans*-PnAPC plot displays two slope discontinuities at 22 and 32°C while a single breakpoint is found at 24°C for *cis*-PnAPC. The E/M intensity vs. temperature plot for $Pyr_{10}SM$, $Pyr_{10}PC$ (Fig. 4B) and other Pyr_xPC probes (data not shown) displayed a slope discontinuity close to 32°C. These findings indicate that an abrupt change(s) in the order and/or dynamics of the surface lipids of LDL takes place at 22–32°C, which coincides with the thermotropic transition of the neutral lipid core determined by DSC. The results suggests that the change detected in the surface layer by the

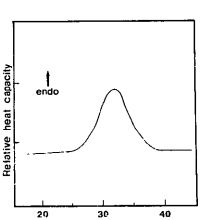

Fig. 3. Differential scanning calorimetry of LDL. The endothermic transition shows melting of LDL core cholesteryl esters with peak transition at 32°C. The transition enthalpy is 0.68 cal/g of cholesteryl ester. Heating rate was 1 C°/min.

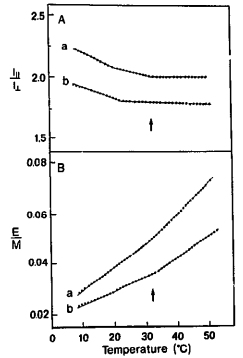

Fig. 4. Temperature dependence of polarization ratio of PnAPC and E/M intensity ratio of PyrPC probes in LDL. Fluorescent phospholipid probes were incorporated into LDL by PC-TP mediated exchange as described under Methods. After separation of the labeled LDL from the donors, each sample was cooled to 10°C and then heated at a rate of 1.5 C°/min to 50°C. Each sample contained 120 nmol LDL phospholipid in 3 ml of the buffer. The probe concentration in LDL was approx. 1 mol% of total phospholipid. (A): The polarization ratio (0/90°) of *trans*-PnAPC (a) and *cis*-PnAPC (b) fluorescence. (B): E/M fluorescence intensity ratio for $Pyr_{10}PC$ (a) and $Pyr_{10}SM$ (b). The arrows indicate the peak of the endothermic transition of the core lipids determined by DSC. The lines through data points represent the best fits of regression analysis with correlation coefficient $r \geq \pm 0.995$.

phospholipid probes is a consequence of the core melting. This conclusion is consistent with the absence of slope discontinuities in E/M vs. temperature plots for $Pyr_{10}PC$ in vesicles of LDL phospholipids and cholesterol in 3:2 molar ratio (data not shown).

Lateral distribution of the PyrPC species in LDL

The E/M ratio as a function of probe concentration was determined to study the lateral distribution of the Pyr_xPC species incorporated in LDL surface at 37°C (Fig. 5). The E/M ratio increased linearly with probe concentration for Pyr_6PC and Pyr_8PC, while biphasic plots were obtained for $Pyr_{10}PC$, $Pyr_{12}PC$, and $Pyr_{14}PC$ probes. Since linear plots are expected in the case of random distribution [37], these results indicate that the long-chain pyrenyl lipids are not randomly distributed, but that they partition preferentially to one of at least two distinct phases existing in the surface layer of LDL. No discontinuities were observed in the E/M vs. Pyr_xPC concentration plots obtained for each probe in vesicles consisting of LDL phospholipids and cholesterol (3:2

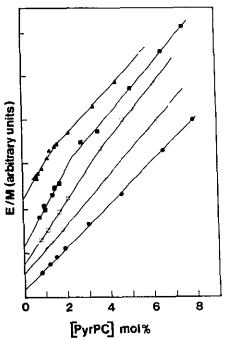

Fig. 5. E/M fluorescence intensity ratio vs. PyrPC concentration in LDL. LDL was labeled to a varying extent with Pyr_xPC species, separated from the donor vesicles and the E/M ratio was determined at 37°C as indicated in Methods. The plots intersect the y-axis at zero, but for clarity the plots have been displaced upwards by an arbitrary unit. Symbols: (●) Pyr_6PC; (○) Pyr_8PC; (□) $Pyr_{10}PC$; (■) $Pyr_{12}PC$; and (▲) $Pyr_{14}PC$. Each linear segment represents the best fit from a regression analysis with a correlation coefficient $r \geq 0.985$.

molar ratio; data not shown) suggesting that the heterogencity of the LDL surface lipid layer is related to the presence of the apoB-100 protein.

The lateral distribution of the probe phospholipids was further investigated by measuring the average efficiency of RET (T_s) from the tryptophan residues of apoB-100 to the pyrene moiety of the probes incorporated to LDL (Fig. 6). T_s was linearly dependent on Pyr_6PC and Pyr_8PC concentration, while biphasic plots were observed for $Pyr_{10}PC$, $Pyr_{12}PC$ and $Pyr_{14}PC$. This and the fact that the discontinuities coincide closely with those observed for the E/M plots suggest that two distinct lipid domains exist in the surface layer of LDL. In addition, because T_s is strongly dependent on the donor–acceptor distance [34], the RET plots indicate that one of the two phases correspond to the boundary around the lipid-interacting segments of apoB-100 (tryptophans) and the other bulk lipid surface, respectively. Secondly, the long-chain probes appear to partition preferentially to the boundary phase.

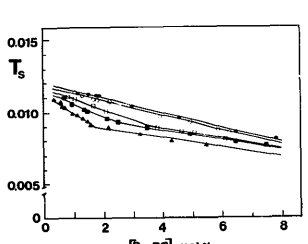

Fig. 6. Efficiency of sensitized energy transfer from apoB-100 tryptophans to PyrPCs in LDL. Average RET efficiency (T_s) per single tryptophan residue of apoB-100 vs. Pyr_xPC concentration in LDL was calculated as described under Methods. The temperature was 37°C. Symbols: (●) Pyr_6PC; (○) Pyr_8PC; (□) $Pyr_{10}PC$; (■) $Pyr_{12}PC$; and (▲) $Pyr_{14}PC$. Each linear segment represents the best fit from a regression analysis with a correlation coefficient $r \leq -0.986$.

Discussion

The rapid and simple protocol for labeling of LDL with PnAPCs and Pyr_xPCs developed here was based on the facts that LDL phosphatidylcholine molecules are readily available for PC-TP mediated exchange [38] and that PC-TP can efficiently transfer PnAPC and PyrPC species [22,31,39]. Minimal perturbation of the lipoprotein surface is likely to occur, since the probes are structurally similar to the endogenous phospholipids and the amount of LDL phospholipid remains unchanged because, when used in catalytical amounts, PC-TP mediates exchange rather than net transfer [40]. Also, the protein should release the labeled lipid only into domains where it can be exchanged for another phospholipid molecule.

It is well-established that the neutral lipid core of LDL undergoes a thermotropic transition between 20 and 40°C with the midpoint at 30–32°C [12,13]. Thus the DSC data reported here (Fig. 3) are consistent with the previous results. The slope discontinuities observed both in the polarization ratio and E/M vs. temperature plots (Fig. 4) clearly indicate that this transition has a marked influence on the order and/or dynamics of phospholipid acyl chains in the surface layer. One discontinuity was found for *cis*-PnAPC (at 24°C) and two for *trans*-PnAPC (at 22 and 32°C). This suggests that an ordered to more disordered transition occurs in the surface lipid layer as a consequence of core melting, since *cis*-PnAPC (and *cis*-PnA) and *trans*-PnAPC (and *trans*-PnA) are known to be sensitive to the onset of formation of a fluid phase, while the *trans*-derivatives also detect the disappearance of the last solid domains due to their preferential partitioning to the gel phase and the high quantum yield in such a phase [18–20,41].

Both the sphingomyelin and phosphatidylcholine pyrenyl probes display a single discontinuity at 30–32°C (Fig. 4B). This temperature is higher than expected, since at least $Pyr_{10}PC$ is known to partition preferentially to the fluid phase when fluid and solid phospholipid domains coexist [24]. Accordingly, one would presume that the pyrenyl probes respond mainly

to the onset of the transition similarly to *cis*-PnAPC which also shows a slight preference for the fluid phase [19,20]. However, the surface layer of LDL consists of a variety of phospholipid species, cholesterol and protein and thus the partitioning of the pyrenyl probes may be considerably different from that in simple phospholipid bilayers.

Previously, Laggner and Kostner [14] have detected an abrupt change in the order of the LDL surface at 28–30 °C by the use of spin-labeled fatty acids and suggested that a phase transition takes place in the surface layer as a consequence of the core melting. Also, Mn^{2+} binding to LDL displays discontinuities at 20 and 30 °C [42]. This supports the occurrence of a phase transition in the surface lipid layer, since ion binding to phospholipid surfaces is known to be sensitive to the phase state [43]. In contrast, *cis*- [16] and *trans*-parinaric [15,17] acid fluorescence polarization have been reported to be unresponsive to the melting of the core which may be due to location of these probes in lipid or even protein domains which are not affected by the transition of the LDL core. In some protein-lipid systems fatty acids (and sterols) have been shown to gain access to sites not available for phospholipids [44,45].

The present data (Figs. 5 and 6) strongly suggest that there are at least two distinct domains available for the Pyr_xPC probes in the surface layer of LDL. On the basis of the RET data (Fig. 6) we suggest that these domains correspond to the protein-lipid boundary and the bulk lipid, respectively. Partition of the probes between these phases is clearly chainlength-dependent, so that preference for the boundary phase increases with increasing length of the pyrenyl chain. Interestingly, it has been observed in model systems [46] that single-chain pyrenyl compounds distribute in a chainlength-dependent manner between the boundary of $(Ca^{2+} + Mg^{2+})$-ATPase and the bulk lipid phase; also, in this case, the long-chain compound showed much higher affinity to the boundary as compared to the short-chain ones. Thus, preferential association of long-chain pyrenyl compounds with protein boundaries may be a general phenomenon. The fact that relatively small changes in structure (chainlength) of pyrenyl phospholipid molecule can have a considerable effect on its partitioning suggests that the phospholipid-cholesterol surface of LDL may be laterally inhomogeneous because native phospholipid species have considerable structural variation [47].

The temperature-dependent core-surface interaction observed previously [14,42] and in this paper may have clinical significance because, as shown in animal [48–50] and human [51] studies, a diet rich in cholesterol and/or saturated fatty acids raises the melting temperature of LDL core, which may change the lipid order and dynamics in the lipoprotein surface even at physiological temperatures. Consequently, changes in the lipoprotein surface may disturb LDL metabolism, e.g., by decreasing the affinity of binding of LDL to its cellular receptor. In fact, it has been shown [52] in a human study that an increased proportion of saturated fatty acids in LDL cholesteryl esters decreases the fractional catabolic rate of apoB-100. The mechanism(s) by which a relatively more solid LDL surface might disturb LDL metabolism remains elusive. However, specific apoB-100–phospholipid interactions probably exist in LDL, evidenced by results that perturbation of the surface phospholipid monolayer by phospholipase A_2 treatment [53] changes the antigenic epitopes and tertiary structure of apoB-100. On the other hand, as suggested in the present study, LDL phospholipids may tend to segregate laterally on the lipoprotein surface. Thus, it is conceivable that a relatively more solid LDL core at physiological temperatures maintains the surface lipids in a more ordered state and affects their lateral distribution and lipid–lipid and lipid–apoB-100 interactions, which may indirectly alter apoB-100 conformation and consequently impair the recognition of the lipoprotein by its receptor.

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