ABSENCE OF LIQUID CRYSTALLINE TRANSITIONS OF CHOLESTEROL ESTERS IN RECONSTITUTED LOW DENSITY LIPOPROTEINS

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1. Introduction

A method for reconstitution of plasma low density lipoproteins (LDL) has been reported [1]. The procedure is based on extraction of cholesterol esters in apolar solvent, followed by replacement with exogenous cholesterol esters. The reconstituted LDL (r-LDL) has a composition similar to native LDL [1]. Importantly, r-LDL binds to the LDL cell surface receptor, is internalized and regulates intracellular cholesterol metabolism [1].

Native LDL consists of a core of apolar lipids (cholesterol esters and small amounts of triglyceride), surrounded by a shell of more polar constituents (phospholipids, unesterified cholesterol). Studies of native LDL by differential scanning calorimetry (DSC) and X-ray scattering have shown that it undergoes reversible thermal transitions near body temperature, due to a change of its core cholesterol esters from a smectic-like (layered) to a more disordered state [2,3]. Here, we have performed DSC on r-LDL, containing exogenous cholesterol linoleate (CL) or cholesterol oleate (CO) introduced from heptane. r-LDL does not display liquid crystalline transitions of its cholesterol esters, probably due to retention of substantial amounts of heptane.

2. Materials and methods

Human plasma LDL was isolated from the blood of normal fasting donors, by preparative ultracentrifugation at densities 1.025–1.050 g/ml. The reconstitution procedure [1] employed dialysis of LDL, lyophilization in the presence of potato starch, extraction of endogenous neutral lipids with heptane, introduction of exogenous CL or CO (both containing [^{3}H]CL) dissolved in heptane, evaporation of heptane at 20°C under a stream of N_2 for 45 min and solubilization of the r-LDL in aqueous buffer. LDL protein concentration was determined by the method in [4] and lipids by quantitative thin-layer chromatography [5]. Recoveries of protein and cholesterol ester were similar to those in [1]. The cholesterol esters, CL and CO, were obtained from Nu-Chek Prep and the [^{3}H]CL from New England Nuclear.

DSC of r-LDL was performed with a Perkin-Elmer DSC-II, calibrated as in [6]. The temperature programming used to characterize the crystal and liquid crystal transitions of cholesterol esters was similar to that in [2,7]. Samples of r-LDL were also examined by negative stain electron microscopy and polarized light microscopy. r-LDL was negatively stained with Na phosphotungstate on Formvar coated Cu grids and examined with an Hitachi 11C electron microscope, operated at 75 kV. Polarized light microscopy was performed with a Leitz Ortholux Pol-II microscope, fitted with a heating and cooling stage. The crystalline and liquid crystalline phases of cholesterol esters were recognized from their thermal and optical properties [7].

3. Results and discussion

The compositions of r-LDL, prepared from 3 different ratios of cholesterol ester to protein, are shown in table 1. LDL reconstituted from a 3/1 (w/w) ratio

Table 1
Composition (percentage) of reconstituted LDL[a]

Cholesterol ester/protein[b]	Protein	Cholesterol	Cholesterol ester	Triglyceride	Lysolecithin	Sphingomyelin	Lecithin	Diam.[c] (Å)
3.0	36	0	33	0	0	12	21	164
2.0	38	0	24	0	0	13	25	150
1.0	37	0	14	0	0	17	33	135

[a] Results shown are means of duplicate experiments for LDL reconstituted with cholesterol linoleate
[b] Mass ratio of exogenous cholesterol ester/LDL protein
[c] Mean diameters (n = 100 particles) from negative stain electron microscopy

of cholesterol ester to protein had a high content of cholesterol ester, as in [1]. With decrease in the ratio of exogenous cholesterol ester/protein, there was progressively less cholesterol ester in the r-LDL. Negative stain electron microscopy of r-LDL showed a fairly uniform population of spherical particles. Particles reconstituted from higher ratios of cholesterol ester/protein had larger diameters (table 1) but were still somewhat smaller than native LDL (diam. 220–240 Å). Thus, the reconstitution procedure resulted in formation of cholesterol ester-rich lipoproteins which were similar but not identical to native LDL. The diminished size of r-LDL would not be expected per se to affect its cholesterol ester liquid crystalline transitions, since lipoprotein particles of comparable size (HDL_c, diam. 150–190 Å) display cholesterol ester transitions similar to those of native LDL [8].

Figure 1 shows the DSC heating curves of native LDL (A,B) and of r-LDL (C–H). On heating native LDL (A) there was a major transition between 20–40°C and a second, smaller transition at higher

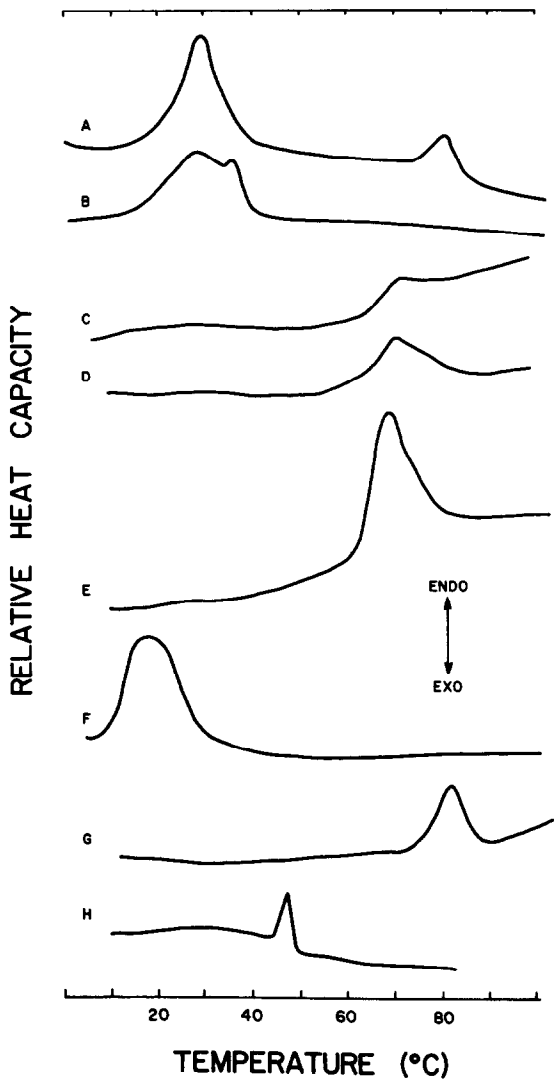

Fig.1. Differential scanning calorimetry heating curves of plasma LDL (A,B) and reconstituted LDL (C–H). LDL was reconstituted with cholesterol linoleate/protein of weight ratios 3/1 (C), 2/1 (D) or 1/1 (E); (F) shows the 3/1 sample following thermal denaturation and cooling to −60°C. (G) shows LDL reconstituted with 3/1 cholesterol oleate/protein and (H) shows a similar sample following vacuum drying. Samples were concentrated by vacuum dialysis to 50–150 mg/ml and sealed in 75 μl capsules. The samples were heated at 5°C/min. with instrument sensitivities of 0.2 or 0.4 mcal/s. *Endo* and *Exo* show, respectively, the directions of endothermic and exothermic transitions.

temperature (peak temperature, T_m = 81°C). The former is due to a reversible change of cholesterol esters from a smectic-like (layered) state to a more disordered state, while the high temperature transition is due to irreversible lipoprotein denaturation [2]. Following lipoprotein denaturation (fig.1B), the cholesterol ester transition becomes double-peaked and is increased in enthalpy [2]. In addition, if the heat denatured LDL is cooled to −60°C and then re-heated, a very large, irreversible (i.e., monotropic [7]) transition occurs at $T_m \sim 30$°C (not shown), due to the crystal melt of cholesterol esters [2]. The large enthalpy (ΔH = 8 cal/g) of the latter transition allows detection of very small amounts of cholesterol esters in heat denatured LDL samples.

Not one of 10 different preparations of r-LDL showed any liquid crystalline transitions of cholesterol ester between 10–50°C, whether prepared from CL (fig.1C–F) or from CO (fig.1G). In these experiments similar amounts of LDL were present to the native LDL shown in fig.1A,B, so that failure to detect the transitions did not result from lack of instrumental sensitivity. When r-LDL was heated to higher temperatures, there was an irreversible denaturation endotherm. LDL reconstituted from CL was denatured at lower temperature (fig.1C, T_m = 72 ± 0.6°C) than LDL reconstituted from CO (fig.1G, T_m = 83 ± 0.5°C) or native LDL (fig.1A, T_m = 81 ± 0.8°C). In studies of monkey LDL containing cholesterol esters of variable fatty acid composition (CO/CL ratio from 1.0–3.5) [9], the T_m of the denaturation endotherm was significantly correlated with the CO/CL ratio [9]. Thus, the thermal stability of LDL is influenced by the fatty acid composition of its cholesterol esters. Also, the denaturation of r-LDL was influenced by the ratio of cholesterol ester/protein. With decreasing ratio of CL/protein (fig.1C–E), there was a small decrease in T_m (72 → 70°C) and a large increase in ΔH from 0.9 cal/g protein (reconstitution ratio = 3/1) to 2.4 cal/g (2/1) to 3.8 cal/g (1/1). The denaturation enthalpy of r-LDL (3/1) was similar to that of native LDL (1 cal/g protein [2]).

Following heat denaturation, r-LDL displayed no liquid crystalline transitions of cholesterol ester between 10–50°C. However, following cooling to −60°C, a broad endotherm, of large enthalpy (6 cal/g cholesterol ester) was observed between 5 and 30°C (fig.1F). This endotherm was associated with melting of birefringent crystals (see below) with similar properties to pure CL, except that their T_m was ~25°C lower. Also, when LDL reconstituted with CO was cooled to −60°C, then heated, a broad melt of T_m = 30°C occurred, about 20°C below the melt of pure CO crystals. Thus, although cholesterol esters of r-LDL did not form liquid crystals in the temperature range of interest, they were able to form crystals with markedly depressed melting temperatures. These results suggest the presence of an impurity, probably heptane, in the cholesterol esters of r-LDL. Therefore, the effects of vacuum drying of r-LDL and of adding heptane to pure cholesterol esters were examined.

In the vacuum drying experiments, duplicate samples of r-LDL were placed in DSC pans. One sample was sealed and the other dried under vacuum (30 mTor) for 24 h, re-hydrated and then sealed. Whereas the sample sealed immediately resembled the previous preparations of r-LDL, the vacuum dried sample showed an endotherm of T_m = 48°C and enthalpy 7 cal/g CO (fig.1H), resembling the crystal melt of pure CO (fig.2A). On heating to higher temperatures the vacuum dried r-LDL showed no lipoprotein denaturation endotherm. Similar results were obtained with r-LDL prepared from CL. These results suggest that vacuum drying removed a volatile impurity, presumably heptane, from r-LDL, but destroyed the lipoprotein structure.

In fig.2 are shown the effects of adding heptane to pure CO. Pure CO displayed characteristic crystalline (fig.2A) and liquid crystalline transitions (fig.2B, shown on cooling) [7]. On addition of heptane there was marked depression of the T_m and broadening of the crystal melt, without major change in its ΔH (fig.2C,E). The liquid crystal transitions (shown on cooling in fig.2D) were merged into a single peak and depressed in temperature, or with addition of more heptane, as in the sample shown in fig.2E, completely absent. In a series of experiments the depression of the crystal melting temperature (ΔT_m) produced by heptane was found to conform (to within ± 2°C) to the equation describing the freezing point depression (ΔT) of solvents produced by addition of solutes:

$$\Delta T = RT_0{}^2 X_2 / \Delta H_f$$

where R is the universal gas constant, T_0 is the melting

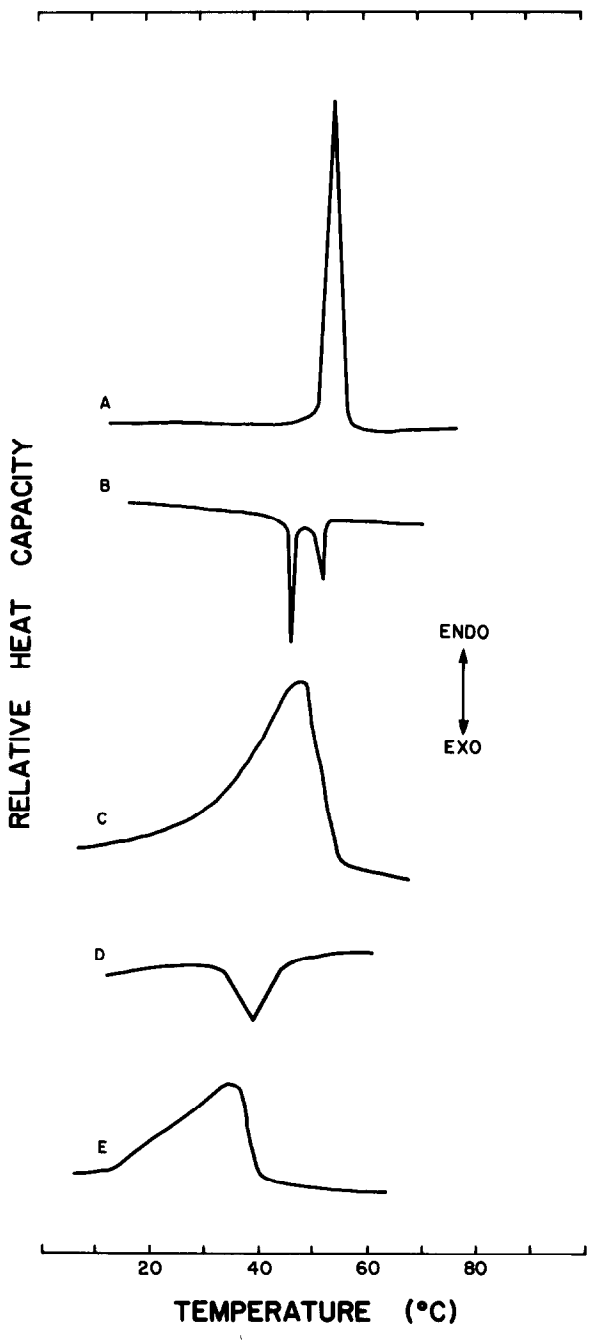

Fig.2. Differential scanning calorimetry curves of cholesterol oleate (A,B) and of cholesterol oleate with added heptane (C–E). The heating curves in (A), (C) and (E) show the crystal melt and the cooling curves in (B) and (D) the liquid crystalline transitions. The mole fraction of heptane was 0.3 in (C) and (D) and 0.6 in (E).

temperature of the pure solvent (CO crystal), X_2 is the mole fraction of the solute (heptane), and ΔH_f is the heat of fusion of the solvent. From the ΔT_m ($\sim$20°C) of the crystal melt of cholesterol ester observed experimentally in r-LDL (e.g., fig.1F), we can therefore calculate the mole fraction of heptane in r-LDL. The ΔT results show that r-LDL contained an amount of heptane approximately equimolar with the cholesterol ester ($X_2 = 0.67$).

Polarized light microscopy was used to establish the identity of transitions observed by DSC. The solutions of r-LDL were isotropic. Following heating to 100°C an oily phase separated out, reflecting release of cholesterol esters upon lipoprotein denaturation [2]. Upon cooling to ~5°C, these droplets failed to show liquid crystalline transitions. However, after freezing the droplets formed birefringent crystals. On heating, the crystals melted over a broad temperature range, similar to that observed by DSC (5–30°C for CL (cf. fig.1F)). The vacuum-dried LDL contained crystals of cholesterol ester without prior heating of the sample. The melt of these crystals was broad and slightly depressed in temperature (40–49°C for r-LDL (CO)), compared to the pure cholesterol ester.

In summary, r-LDL does not display liquid crystalline transitions of its cholesterol esters, due to the presence of an impurity, probably heptane. Future studies of the core structure of LDL should attempt to use solvents which can be completely removed from r-LDL without producing denaturation. (Preliminary results suggest that CCl_4 can be removed more readily from r-LDL.) The thermal stability of r-LDL is influenced by the fatty acid composition of its cholesterol esters and by the ratio of cholesterol ester/protein, suggesting the existence of thermodynamically important interactions between the cholesterol ester fatty acyl chains and the phospholipid–protein surface of the LDL particle.

Acknowledgements

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