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Small-Angle X-Ray Scattering Study of Human Serum Low-Density Lipoproteins With Differential Reactivity for an Arterial Proteoglycan

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The structure and thermal behavior of human serum low-density lipoproteins showing either a high or a low reactivity against a proteoglycan isolated from human arteries have been found to be different from each other. It is suggested that modifications in the lipoprotein surface structure induced by the physical state of the neutral lipids could modulate the affinity of the macromolecule for the arterial component.

Key words: lipoprotein structure, x-ray scattering, thermal transitions, interaction arterial proteoglycans

Although several workers have demonstrated that low-density lipoproteins (LDL) from hypercholesterolemic subjects (type II) have differences in composition and in physicochemical parameters when compared to LDL from apparently healthy controls (1–6), very little structural work (7) has been carried out with LDL involved in pathologic processes. Also, little is known on the possible relationships between changes in the LDL structure and interaction of this molecule with arterial wall components.

Evidence has been presented indicating that serum LDL forms insoluble aggregates in the presence of macromolecular complexes isolated from extracts of arterial intima-media (8–10). Some of these arterial components, which are basically made of proteoglycans (11), react specifically with LDL. It was also found that more than 30% of acute ischemic heart disease patients have in their serum LDL with high reactivity towards the arterial factor; in contrast only 10% of apparently healthy controls show this highly reactive LDL. From the above results and quantitative analyses LDL has been operationally grouped into two classes: high-reacting (HR-LDL) and low-reacting (LR-LDL). The potential relationship of the above findings to the atherogenic process prompted an investigation of the molecular properties of these two classes of particles.

In previous work (12, 13) we have shown that in the resolution range above $(50\ \text{Å})^{-1}$, HR-LDL and LR-LDL exhibit clear differences in their x-ray scattering diagrams. In this paper we report a study on the temperature-dependent small-angle x-ray scattering of both HR-LDL and LR-LDL and on the neutral lipids derived from these particles.

Our results will show that the structure and the thermal behavior of HR-LDL and LR-LDL are different from each other and that the difference depends on the relative

Abbreviations: LDL–low density lipoprotein; HR-LDL–high reacting, low density lipoprotein; LR-LDL–low reacting, low density lipoprotein.

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content of cholesteryl esters and triglycerides in these particles. It is felt that the physical state of the neutral lipids could induce a modification of the LDL surface structure which may account for the selective affinity of these particles for the arterial factor.

METHODS

Lipoprotein Preparation

LR-LDL and HR-LDL of d = 1.006 to 1.063 were prepared from fresh sera obtained from apparently healthy adult male donors. The method of isolation and purification has been previously described (12, 13). All the samples were assayed for the precipitation test with the arterial factor (12, 13), which permits the identification of the low- and high-reacting species.

Lipids

LDL total lipids were extracted by the method of Folch et al. (14). From these extracts the neutral lipids (cholesteryl esters, free cholesterol and triglycerides) were separated by silicic acid column chromatography.

Samples of neutral lipids containing increasing amounts of triglycerides were prepared by addition of weighted amounts of a mixture of more than 98% pure triglycerides (fatty acid composition: C16:0, 10%; C18:0, 4%, C18:1, 41% C18:2, 45%). The lipids were solubilized in chloroform and the solvent was evaporated at 50°C, first under a nitrogen stream and then under vacuum. These mixtures were used for the x-ray experiments.

Analytical Procedures

The methods of analysis for protein, phospholipid, triglyceride, and free and esterified cholesterol have been previously described (12, 13).

X-ray Scattering Techniques

X-ray scattering experiments were performed with an Elliott GX6 rotating-anode tube and a focusing camera utilizing a nickel-coated bent glass mirror operating with linear collimation. The scattering intensity was recorded with a position-sensitive proportional detector and stored in an on-line computer. In the LDL experiments, the interparticle correlation effects were eliminated by comparing the x-ray scattering curves recorded at decreasing dilutions. The concentration-corrected curves were subsequently transformed to point collimation (15).

RESULTS

Chemical Composition of HR-LDL and LR-LDL

The chemical composition of the HR-LDL and LR-LDL is shown in Table I. The main differences between the two classes of macro-molecules are apparent in their neutral lipid components: The HR-LDL has a larger relative content of cholesteryl ester (CE) and a decrease in triglycerides (T). The molecular ratio (CE/T) is 14 for HR-LDL and 6 for LR-LDL.

TABLE I. Chemical Composition of the LR-LDL and HR-LDL Particles*

	Low-reacting (LR-LDL)		High-reacting (HR-LDL)	
	Weight %	Molecules per particle	Weight %	Molecules per particle
Protein	24.7 (0.2)		22.9 (0.9)	
Phospholipids	15.9 (0.3)	474 (8)	17.3 (1.0)	515 (29)
Free cholesterol	9.6 (1.0)	542 (56)	10.3 (0.5)	583 (28)
Cholesteryl esters (CE)	40.8 (1.2)	1,388 (42)	45.4 (1.1)	1,542 (36)
Triglycerides (T)	9.1 (0.4)	234 (11)	4.3 (0.9)	110 (23)
CE/T	4.5 (0.1)[a]	5.9 (0.2)[b]	10.7 (1.5)[a]	14.0 (2.3)[b]

*The analyses were carried out using procedures described in previous reports (11, 12). Figures represent the mean of four different samples. CE/T is the mean ratio of cholesteryl esters to triglycerides in the four samples. Standard deviations of the mean measured values are given in parentheses following the value. Statistical differences between CE/T ratios in percent weight ([a]) and in molecules per particle ([b]) are significant to $p < 0.001$.

Thermal Transitions in Intact HR-LDL and LR-LDL

Small-angle x-ray experiments were performed between 3°C and 37°C on several lipoproteins from different donors. The x-ray patterns of single samples of HR-LDL and LR-LDL are shown in Fig. 1. One feature common to all the scattering curves is the presence of fringes separated by low minima whose position and intensity do not change (or change very little) with temperature. This observation indicates that the lipoprotein particles are quite homogeneous and that in the explored temperature range their structure is spherically symmetric (or quasi-spherical) (16, 17).

In addition to the small-angle fringes, the x-ray diagrams display a temperature-dependent strong band centered around $s = (36\ \text{Å})^{-1}$. Deckelbaum et al (18) have reported that this band is present at 10°C and is totally absent at 45°C; they have associated its disappearance to a smectic to isotropic phase transition of cholesteryl esters within the particle. Our experiments show that the thermal phenomenon is progressive: Upon heating, the intensity of the band in each lipoprotein decreases continuously until complete disappearance. In addition. LR-LDL and HR-LDL had a different thermal dependence. At low temperature (3°C) the strong band centered at $(36\ \text{Å})^{-1}$ was present in the x-ray diagrams of the two lipoproteins (see Fig. 1). By increasing the temperature the intensity of the band began to decrease in both cases. However, at room temperature (24°C) whereas in LR-LDL the band disappeared completely, it only displayed about 50% decrease of intensity in HR-LDL. At nearly-physiologic temperature (37°C) the band was completely absent in both x-ray diagrams.

The intensity of the band permits estimation of the occurrence of the thermal transition in each lipoprotein sample. In Fig. 2 we have plotted the band areas as a function of temperature for all experimental intensities presented in Fig. 1 (see legend). The curves differ for each lipoprotein in that for LR-LDL the onset and the end of the transition are shifted to lower temperatures. This phenomenon was found to be completely reversible, as indicated by the progressive reappearance of the band with the same intensity on cooling of the sample, and by the identical changes which were observed on repeated thermal cycles between 3 and 37°C.

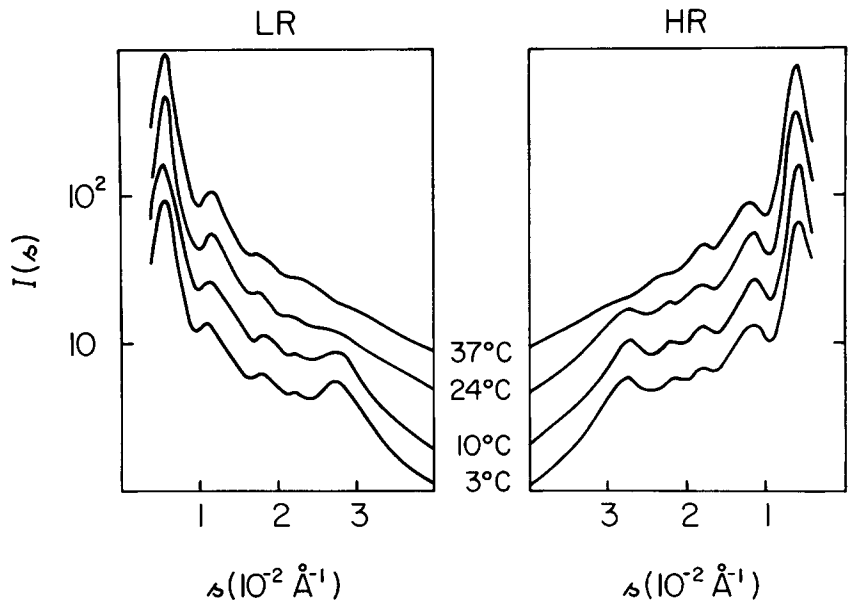

Fig. 1. Logarithmic plot of LR-LDL and HR-LDL x-ray scattering curves recorded at different temperatures with the position-sensitive detector over counting periods of 30 min. The relative scale is arbitrary. The temperature was increased after each recording and the sample was allowed to reach thermal equilibrium during 15 min. Note that at room temperature (24°C) the HR-LDL curve displays a strong band centered at $s = 2.8 \times 10^{-2}$ Å^{-1} (36 Å)$^{-1}$, whereas at the same temperature it is practically absent in LR-LDL. $s = 2 (\sin \theta)/\lambda$ (2θ, scattering angle; λ, wavelength).

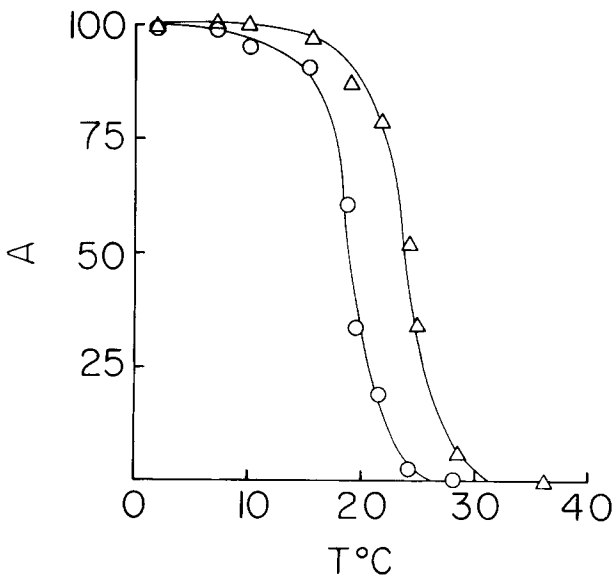

Fig. 2. Integrated intensity of the 2.8×10^{-2} Å^{-1} band plotted as a function of temperature for the experimental curves presented in Fig. 1. (○), LR-LDL; (△), HR-LDL. Each curve has been normalized to the maximum area. $A = [\int_{s_1}^{s_2} I(s)ds/(\int_{s_1}^{s_2} I(s)ds)_{max}] \times 100$. s_1 and s_2 correspond to values of s in the reciprocal space where the integration has been done. ($s_1 = 2.4 \times 10^{-2}$ Å^{-1}; $s_2 = 3.6 \times 10^{-2}$ Å^{-1}).

The above thermal dependence of the $(36\ Å)^{-1}$ band was consistently found in the four HR-LDL and four LR-LDL studied (not shown in the figures).

Thermal Transitions in Neutral Lipids Derived From HR-LDL and LR-LDL

In previous works (12, 13) we have reported that at room temperature the neutral lipids derived from either HR-LDL or LR-LDL produce very similar x-ray diffraction patterns: Both displayed a sharp and strong reflection centered at $(34.5\ Å)^{-1}$, which is characteristic of the smectic phase of cholesteryl esters. We have also reported that the addition of triglycerides to these neutral lipid mixtures resulted in a decrease up to complete disappearence of this reflection.

In the native lipoproteins, the $(36\ Å)^{-1}$ band has been interpreted by Deckelbaum et al (18) as due to the particular packing of the cholesteryl esters in the core of the particle. Since the neutral lipids of HR-LDL and LR-LDL contain different proportions of these constituents, it became of interest to study the neutral lipids derived from both lipoproteins by x-ray diffraction over the same temperature ranges as for intact LDL. These results are shown in Fig. 3. As previously reported, the diffraction patterns obtained at room temperature displayed a sharp and strong reflection centered at $(34.5\ Å)^{-1}$, characteristic of the smectic phase of cholesteryl esters. This indicates that in spite of the differences in chemical composition, neutral lipid fractions from the two lipoprotein species exhibit the same structural organization when in free form. Upon heating from 20 to 45°C the $(34.5\ Å)^{-1}$ reflection is progressively substituted by a broad band centered at $(32\ Å)^{-1}$ (see Fig. 3), indicating that a phase transition from smectic liquid crystal to liquid isotropic has occurred. The fraction of cholesteryl esters in smectic state can be roughly approximated at each temperature by measuring the relative intensity of the $(34.5\ Å)^{-1}$ reflection. In Fig. 4 we have plotted the area of this reflection as a function of temperature. The plot indicates a difference in the thermal behavior of the two neutral lipid fractions, comparable to that found with the whole lipoproteins (see Fig. 2). The melting curve corresponding to the lipids derived from LR-LDL (which contain a higher

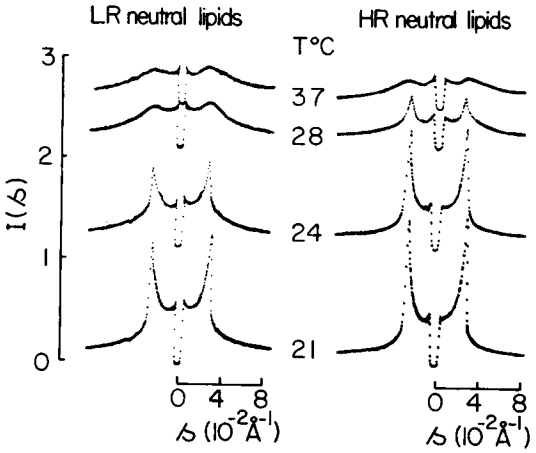

Fig. 3. Experimental x-ray diffraction patterns of the neutral lipids extracted from LR-LDL and HR-LDL recorded at different temperatures over a period of 15 minutes. The time for thermal equilibration was 15 minutes between temperature steps. At 21°C the organization of the lipids is smectic. At 37°C the lipids are arranged in a more disordered liquid-isotropic phase (see text).

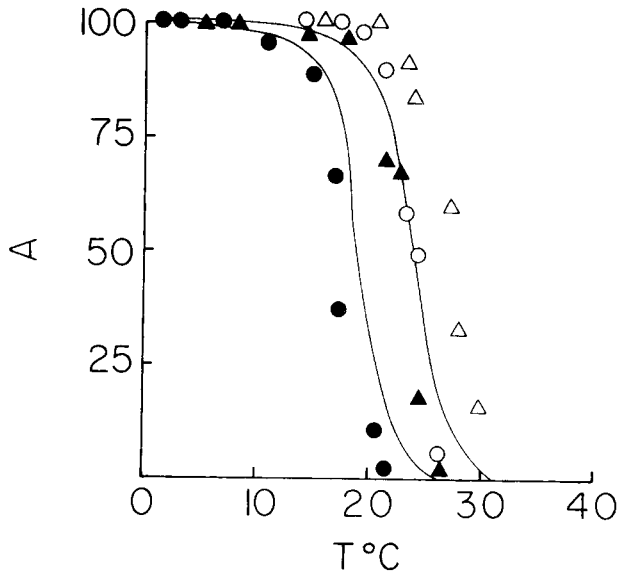

Fig. 4. Integrated intensity of the 2.9 × 10^{-2} A^{-1} (34.5 Å)$^{-1}$ reflection plotted as a function of temperature for the experimental recordings showed in Fig. 3. ○), LR-LDL neutral lipids; △), HR-LDL neutral lipids; ●), HR-LDL neutral lipids plus 34% triglycerides; ▲), HR-LDL neutral lipids plus 27% triglycerides. The continuous curves of LR-LDL and HR-LDL native particles in Fig. 2 are shown here for comparison (A as in Fig. 2).

proportion of triglycerides) is shifted to low temperatures compared to the lipids extracted from HR-LDL. It may be noted, however, that in the native particles the transition occurred at comparatively lower temperatures. When the content of triglyceride was increased, the transition temperature of the artificial lipid mixture decreased. In Fig. 4 we show that the addition of 27% and 34% triglycerides to the neutral lipid fraction derived from HR-LDL resulted in melting curves very close to those corresponding to the whole lipoproteins. These results confirm our previous observation (12, 13) that the proportion of triglyceride to cholesteryl esters is important in determining the temperature of transition and suggest the possibility that these molecules have a nonhomogeneous distribution within each particle (see Discussion).

CONCLUSION AND DISCUSSION

One interesting conclusion of our work is that the structure and the thermal properties of lipoproteins from subjects with HR-LDL and LR-LDL differ from each other. Another result which extends previous observations from our laboratory (12, 13) is that triglycerides have a strong influence on the temperature-induced phase transition of cholesteryl esters. The decrease in the smectic liquid crystal to liquid isotropic transition temperature that was observed by increasing the amounts of triglycerides (see Fig.4) indicates that these molecules produce a disordering effect on the molecular packing of cholesteryl esters. A similar phenomenon was also observed with intact lipoproteins (see Fig.2). In LR-LDL where the content of triglycerides is about 5% higher than in HR-LDL, this

relatively small difference in chemical composition has the significant effect of lowering the temperature of disappearance of the $(36\ Å)^{-1}$ band by more than 5°C. This might be attributable to the disorder induced by triglycerides on the organization of cholesteryl esters within the native lipoproteins (see below).

We also found that in the whole LDL particles both onset and end of the thermal transition were shifted to temperatures lower than that exhibited by the neutral lipids which had been extracted from these particles. A plausible explanation for this thermal difference between LDL and extracted lipids is that in lipoproteins the cholesteryl esters are located in at least two different domains: one, rich in triglycerides, which behaves thermally as a pure cholesteryl ester-triglyceride mixture (transition shifted to lower temperatures by the higher concentration of triglyceride); the other containing cholesteryl esters strongly interacting with the paraffin chains of phospholipids and apoproteins. At present we cannot provide experimental support for this explanation, although it would seem logical that upon extraction the neutral lipids, regardless of population, would be pooled together. In consequence the potential difference between domains of cholesteryl esters and triglycerides would not be detected by the chemical analysis.

In a recent paper, Deckelbaum et al (18) have proposed on the basis of x-ray and differential scanning calorimetry (DSC) experiments, that at low temperatures, the organization of cholesteryl esters in LDL is similar to that of the smectic phase of pure cholesteryl esters. At body temperature these molecules would be arranged in more disordered liquid isotropic state; the $(36Å)^{-1}$ band was interpreted as directly originated by a concentric layer arrangement of cholesteryl esters within the core of the particle. We have recently shown (19) that the cooling of LDL solutions below 0°C causes the $(36\ Å)^{-1}$ band to disappear. In addition, by studying LDL prepared in solutions with different freezing points we have also found that the disappearance of the band occurs at various temperatures which closely correlate with the freezing of the solution in which LDL is immersed (Mateu, Kirchhausen, Camejo, manuscript submitted). The fact that the presence or absence of the $(36\ Å)^{-1}$ band strongly depends on the structure of the particle at the aqueous interface questions the interpretation by Deckelbaum et al. (18) that the $(36\ Å)^{-1}$ band arises from a multilayered concentric arrangement of the cholesteryl esters. We feel it unlikely that the freezing of the solvent could have affected the long-range organization of these lipid molecules, since they are sequestered from the aqueous environment in the lipoprotein core. Our low-temperature results (19) have shown that the $(36\ Å)^{-1}$ band strongly depends on a particular molecular arrangement at the lipoprotein surface (20, 21). It follows that the physical state of the neutral lipids may play an important role in modifying the surface structure of LDL as detected by the progressive disappearance of the $(36\ Å)^{-1}$ band at increasing temperatures (see Fig. 1).

It is possible that the observed phase behavior in LDL may relate to the high tendency of this particle to form complexes with the proteoglycans of the arterial wall.

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