Size transformations of intermediate and low density lipoproteins induced by unesterified fatty acids

Thomas A. Musliner,[1] Michael D. Long, Trudy M. Forte, and Ronald M. Krauss

Research Medicine and Radiation Biophysics Division, Lawrence Berkeley Laboratory, University of California, Donner Laboratory, Berkeley, CA 94720

Abstract Plasma from individiual human subjects is known to contain multiple discrete subpopulations of low (LDL) and intermediate (IDL) density lipoproteins that differ in particle size and density. The metabolic origins of these subpopulations are unknown. Transformation of IDL and larger LDL to smaller, denser LDL particles has been postulated to occur as a result of the combined effects of triglyceride hydrolysis and lipid transfer. However, the presence of multiple small LDL subspecies has been described in patients lacking cholesteryl ester transfer protein. We have characterized an alternative pathway in which size decrements in IDL or LDL are produced in the presence of unesterified fatty acids and a source of apolipoprotein (apo) A-I. Incubation of IDL or LDL subfractions with palmitic acid and either high density lipoproteins (HDL), apoHDL, or purified apoA-I gives rise to apoA-I, apoB-containing complexes that can dissociate into two particles, an apoB-containing lipoprotein with particle diameter 10–30 Å smaller than the starting material, and a still smaller species (apparent peak particle diameter 140–190 Å) containing lipid and apoA-I but no apoB. The newly formed IDL or LDL are depleted in phospholipid and free cholesterol with no change in apoB-100 as assessed by SDS gel electrophoresis. We hypothesize that this reaction may contribute to the formation of discrete IDL and LDL subpopulations of varying size during the course of hydrolysis of triglyceride-rich lipoproteins in plasma. —**Musliner, T. A., M. D. Long, T. M. Forte, and R. M. Krauss.** Size transformations of intermediate and low density lipoproteins induced by unesterified fatty acids. *J. Lipid Res.* 1991. **32:** 903–915.

Supplementary key words apoA-I • fatty acids • HDL • IDL • LDL • VLDL • lipoprotein heterogeneity • lipolysis

Low density lipoproteins (LDL) from normal as well as hyperlipidemic subjects have been shown to contain multiple particle subpopulations heterogeneous with respect to size and density (1–5). Nondenaturing gradient gel electrophoresis has proved particularly useful for characterizing lipoprotein heterogeneity and has been used to resolve up to five discrete LDL subspecies within the plasma of a single individual, each LDL band differing by as little as 5–10 Å in peak particle diameter (5). Similar banding of at least two components has been observed in studies of intermediate density lipoproteins (IDL), while very low density lipoproteins (VLDL), although distributing in two broad size distributions identifiable by gradient gel electrophoresis, generally do not exhibit the sharp size discontinuities observed for LDL or IDL with this technique (6). There is evidence that specific subpopulations of IDL and LDL may be associated with altered atherosclerotic risk (7).

The origins of discrete size heterogeneity within IDL and LDL populations are incompletely understood. Deckelbaum et al. (8) have postulated that transformation of IDL and larger LDL to smaller, denser LDL subspecies can result from the combined effects of triglyceride hydrolysis and lipid transfer. However, it is unclear why this mechanism, to the extent that it occurs in vivo in normal plasma at all, should produce the discontinuous size differences observed within LDL, as opposed to a more uniform variation in LDL size distribution. Moreover, this mechanism cannot account for the observed presence of multiple distinct LDL subspecies in the plasma of patients lacking cholesteryl ester transfer protein (CETP) (9). In vivo studies of the metabolism of human VLDL and IDL subfractions in a rat model system (10) have suggested pathways for production of two of the major LDL subclasses, LDL-I and LDL-II (5, 7). Small VLDL and the larger IDL species (IDL-1) are specifically converted by lipolysis in the absence of CETP activity to particles of size and density similar to LDL-II (5, 7), while IDL-2 appear to be precursors of larger, more buoyant LDL-I particles. However, it is not known how the two major, discrete IDL size subspecies arise themselves.

Abbreviations: LDL, low density lipoproteins; IDL, intermediate density lipoproteins; apo, apolipoprotein; HDL, high density lipoproteins; VLDL, very low density lipoproteins; CETP, cholesteryl ester transfer protein; FFA, free fatty acid; SDS, sodium dodecyl sulfate; LCAT, lecithin:cholesterol acyl transferase.

[1]To whom reprint requests should be addressed at: Lawrence Berkeley Laboratory, Donner Laboratory, 1 Cyclotron Road, Berkeley, CA 94720.

Downloaded from www.jlr.org by guest, on June 18, 2012

We have recently described the occurrence of size transformations of narrow, ^{125}I-labeled LDL density fractions incubated in hypertriglyceridemic plasma containing lipoprotein lipase (11) or in recombinant mixtures containing VLDL, high density lipoproteins (HDL), and lipoprotein lipase in the absence of albumin as free fatty acid (FFA) acceptor (12). In the latter case, the size changes observed were shown to be accompanied by the binding of apolipoprotein (apo) A-I to LDL. In the present study, the mechanism responsible for LDL size changes under these conditions was further investigated. It is shown that in the presence of sufficient FFAs and a source of apoA-I, LDL or IDL particles undergo size transformations resulting from the dissociation of complexes containing apoA-I, FFAs, and surface lipid. We hypothesize that this reaction contributes to the formation of discrete IDL and LDL size subclasses in vivo. This report focuses on the nature of the size transformations that are produced in apoB-containing lipoproteins by this mechanism, while a companion paper describes the properties of the apoA-I-containing dissociation complexes whose formation accompanies these size changes, and which appear to represent precursors of HDL.

METHODS

Isolation, subfractionation, and radiolabeling of lipoproteins

Blood was collected from normolipidemic or hyperlipidemic volunteers, after a 12–14 h fast, in tubes containing disodium ethylene diaminetetraacetate (EDTA) at a final concentration of 1.5 mg/ml. The VLDL ($d < 1.006$ g/ml), LDL (d 1.019–1.063 g/ml), and HDL (d 1.063–1.21 g/ml) fractions were isolated by preparative ultracentrifugation under standard conditions (13). LDL was further subfractionated by density gradient ultracentrifugation as described by Shen et al. (4). Contaminating Lp[a] was minimized in HDL preparations by isolation of the d 1.085–1.21 g/ml fraction by preparative ultracentrifugation. VLDL and IDL subfractions were isolated by nonequilibrium density gradient ultracentrifugation as previously described (6). Where necessary, lipoprotein solutions were concentrated by ultrafiltration using Amicon (Danvers, MA) XM50 or PM10 filters. Lipoproteins were iodinated using the iodine monochloride method (14, 15). Free iodine was removed by chromatography on Sephadex G25 (Pharmacia) followed by sequential dialysis against 0.1 M potassium iodide in 0.15 M sodium bicarbonate and then normal saline containing 0.01% EDTA, pH 7.4. Approximately 95% of the label remained bound to protein after delipidation. Lipoproteins were delipidated using methanol and diethylether as previously described (16). Radiolabeled palmitic or oleic acids were purchased from New England Nuclear (Cambridge, MA).

Incubation conditions

Lipoprotein incubations were carried out in covered polypropylene or glass tubes in a shaking water bath at 37°C. Unless otherwise specified, incubation mixtures included 0.01% EDTA and 0.2 M Tris buffer, pH 7.5. "Essentially" fatty acid-free bovine and human serum albumin and nonradioactive fatty acids were purchased from Sigma. Unless otherwise specified, incubation mixtures contained 0.01% EDTA and 0.2 M Tris buffer, pH 7.5. In experiments in which purified FFAs were included in incubations in the absence of albumin, an FFA solution in heptane was first added to the tubes, and the solvent was evaporated under a stream of nitrogen leaving a film of FFA at the bottom of the tube. Incubation buffer and other incubation constituents were subsequently added. Where indicated, sodium palmitate rather than palmitic acid was added directly to incubation mixtures as a 0.25 mM solution buffered to pH 7.5 with 0.2 M Tris. Solutions of albumin containing bound palmitic acid were prepared by incubating sodium palmitate with 20% FFA-free human serum albumin in 0.1 M Tris, (final pH 7.5) for 3 h at 37°C in a shaking water bath. The solution was filtered through a 0.45 μm Millipore filter, yielding an optically clear solution. The final concentration of FFAs was measured using NEFA kits (Wako, Japan).

Lipoprotein and apolipoprotein analyses

Nonequilibrium density gradient ultracentrifugation for lipoprotein separation was performed using an SW-41 rotor (Beckman Instruments) and discontinuous NaBr salt gradients of density generally ranging from 1.010 to 1.15 g/ml. Centrifugation was carried out for 40 h at 40,000 rpm at 22°C in a Beckman ultracentrifuge. Background densities resulting from these centrifugations were determined from control tubes containing no lipoprotein samples using a DMA 46 Mettler/Paar density meter.

Nondenaturing polyacrylamide gradient gel electrophoresis was performed using 2–16% or 4–30% gels (Pharmacia PAA 2/16 and 4/30) as described in detail elsewhere (5, 17). Gels were stained for protein or lipid, calibrated, and scanned by densitometry as previously described (18). Electrophoretic blotting of gradient gels onto nitrocellulose paper, autoradiography of gel blots, and standardization and densitometric scanning of autoradiograms were also carried out using previously described methods (18). Where indicated, nitrocellulose blots derived from nondenaturing gradient gels were subjected to immunostaining using a 5% (w/v) nonfat milk powder/0.2% (v/v) Nonidet P-40 containing blocking buffer, monospecific sheep antisera directed against apoA-I or apoA-II, and affinity-purified, alkaline phosphatase-linked second antibody. Gradient gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed using 4–30% polyacrylamide gradient gels in a buffer system containing 0.04 M Tris, 0.02 M sodium acetate, 2 mM

Downloaded from www.jlr.org by guest, on June 18, 2012

EDTA, and 0.2% SDS, pH 7.4. The sample buffer consisted of 10 mM Tris, 1 mM EDTA, pH 8.0, containing 2.5% SDS, and 5% β-mercaptoethanol. Lipoproteins were delipidated prior to electrophoresis (16), dissolved in the sample buffer, and heated for 5 min at 100°C prior to application to the gel. Electrophoretic blotting of SDS gels and immunostaining of apolipoproteins on nitrocellulose blots were performed as described by Towbin, Staehelin, and Gordon (19), using monospecific sheep antisera directed against apoA-I and apoA-II. Bound antibody was detected through the use of an affinity purified, alkaline phosphatase-linked second antibody.

Extraction of FFAs from lipoprotein fractions was carried out as previously described (20). In brief, the lipoprotein solution in 0.5 ml was agitated with 2 ml of isopropanol, 1 ml of distilled water, and 2.5 ml of hexane. The hexane phase (2.5 ml) was removed and mixed with 0.5 ml of 0.1 M potassium hydroxide and the mixture was agitated for 10 min. The hexane and aqueous phases were separated and aliquots were counted on a Packard liquid scintillation counter after addition of 10 ml of Econogel. In some experiments total FFA levels were directly quantified using NEFA kits (Wako, Japan).

Protein and lipid measurements

Protein concentrations were determined by the Lowry procedure modified to include SDS (21). Phospholipid was determined by the method of Bartlett (22). Total cholesterol, free cholesterol, and triglyceride concentrations were measured using enzymatic methods on a System 3500 Gilford Computer-Directed Analyzer (Gilford Instruments, Oberlin, OH). Free cholesterol and cholesteryl esters were also determined by gas–liquid chromatography using a Hewlett-Packard 5830A gas chromatograph (23).

Electron microscopy

Lipoprotein fractions were dialyzed against 0.13 M ammonium acetate, pH 7.4, containing 26 mM EDTA. Samples were negatively stained with 2% sodium phosphotungstate (pH 7.4) and immediately examined in the electron microscope as previously described (24).

RESULTS

Fatty acid-induced LDL size transformations in the presence of HDL, apoHDL, or purified apoA-I

Recent studies of interactions of HDL with apoB-containing lipoproteins during lipolysis (12) demonstrated that incubation of narrow density subfractions of ^{125}I-labeled LDL with hypertriglyceridemic VLDL, HDL, and lipoprotein lipase resulted in the formation of two different sized ^{125}I-labeled LDL bands, readily distinguishable by 2–16% gradient gel blot autoradiography. The larger of the two bands had peak particle diameter 10–20 Å greater than the starting LDL, while the second band was 5–10 Å smaller than the starting fraction. A similar phenomenon was observed in mixtures in which delipidated rather than native HDL were incubated with VLDL, LDL, and lipase. It was also shown that lipid products of lipolysis as well as purified FFAs stimulate the association of apoA-I with both VLDL and LDL under these conditions.

The following experiment was performed to determine whether FFAs released during VLDL lipolysis were directly responsible for the observed size transformations of LDL, and whether apo-I was involved in this process. A narrow density fraction of LDL (d 1.033–1.039 g/ml, 0.65 mg/ml protein) was incubated with ^{125}I-labeled native HDL (d 1.085–1.21 g/ml, 2.3 mg/ml protein) and varying amounts of palmitic acid, ranging from 0 to 1.0 μmol in a final volume of 160 μl. Not all of the added palmitic acid was solubilized under these conditions in the incubation buffer at pH 7.5 (see below). After incubation for 2 h at 37°C, the LDL was analyzed on duplicate 2–16% polyacrylamide gradient gels, one of which was stained for protein (**Fig. 1,** left panel) while the other was electrophoretically blotted onto nitrocellulose paper and subjected to autoradiography (Fig. 1, right panel). While minimal label associated with the LDL band in the absence of added palmitic acid (Fig. 1A), as the amount increased progressively more ^{125}I-labeled HDL-derived label was seen to associate with the LDL band (Fig. 1B and 1C). With the addition of up to 0.5 μmol palmitate, the size of the major LDL band remained in the range of 260 Å, although smaller amounts of protein were seen in a peak at 316 Å (probably representing LDL dimer) and a very small peak at 241 Å. At still higher palmitic acid concentrations, about 50% of the LDL protein moved to this smaller peak, with the remainder in a peak similar in size to the starting LDL (Fig. 1D). In this case, a smaller, broader protein band also appeared in the size range between the LDL and the leading shoulder of the ^{125}I-labeled HDL, with apparent peak particle diameter of approximately 147 Å. Autoradiograms of corresponding blotted 2–16% gradient gels (Fig. 1, right panel) demonstrated that the HDL-derived label preferentially associated with the larger of the two LDL-sized bands as well as with the new band at 147 Å (Fig. 1D). The particles comprising the latter band have been designated "dissociation complexes." Their properties are described in detail in the accompanying paper (25).

Size reductions in LDL particle diameter were also observed when delipidated apoHDL was used in place of native HDL. In **Fig. 2A–C,** representative analyses are shown from an experiment in which LDL (d 1.033–1.039 mg/ml, 46 μg of protein) were incubated with 0.5 μmol palmitic acid and ^{125}I-labeled apoHDL in amounts ranging from 0 to 120 μg. Densitometric scans of Coomassie

Downloaded from www.jlr.org by guest, on June 18, 2012

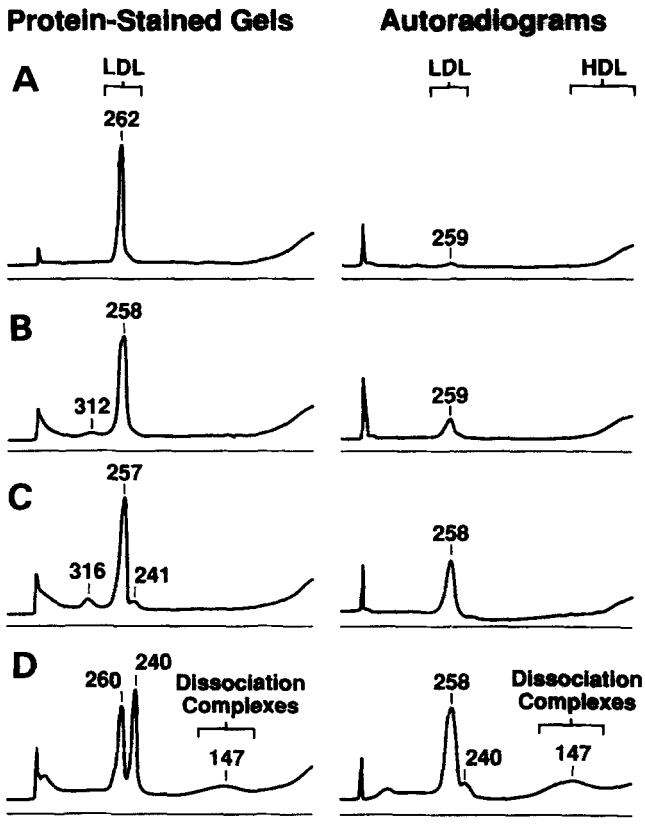

Fig. 1. Size changes in LDL and associated redistribution of ^{125}I-labeled apoHDL observed during incubation of LDL (d 1.033–1.039 g/ml, 104 μg protein) with ^{125}I-labeled native HDL (d 1.085–1.21 g/ml, 370 μg protein) and palmitic acid in amounts ranging from 0 to 1 μmol in a total volume of 160 μl, for 2 h at 37°C. The curves in the left panel represent densitometric scans of 2–16% polyacrylamide gradient gels stained for protein with Coomassie blue; the curves in the right panel represent densitometric scans of autoradiograms of corresponding gels electrophoretically blotted onto nitrocellulose paper. A. absence of palmitic acid; B. 0.25 μmol palmitic acid; C. 0.50 μmol palmitic acid; and D. 1.0 μmol palmitic acid. Particle diameters are shown in Å. The brackets at the top of the figure demarcate the regions on the gels where the specified lipoprotein species migrate; the HDL region represents only the larger end of the HDL spectrum of particles, as smaller species migrate off the end of the 2–16% gels.

blue-stained 2–16% gradient gels (left panel) showed the formation of two distinct LDL subspecies, with small amounts of protein migrating in the size range of the dissociation complex band identified in the previous experiment. Autoradiograms of corresponding nitrocellulose gel blots (right panel) demonstrated ^{125}I-labeled apoHDL label preferentially associating with the larger of the two LDL-sized bands, as well as with the dissociation complex band. At fixed concentrations of LDL and palmitic acid but increasing concentrations of ^{125}I-labeled HDL (Fig. 2A–C), there was paradoxically less formation of the smaller LDL subspecies (as well as the dissociation complex band). The transformation of LDL to the smaller species did not go to completion under these conditions or in other experiments in which palmitic acid was present at still higher concentrations or at varying ratios of LDL:apoHDL (data not shown). As described below, however, all LDL particles were reduced in size when certain other FFAs were present or when subfractions containing IDL or small VLDL were incubated with palmitic acid.

To analyze changes in apoB distribution accompanying FFA-mediated LDL size changes observed under these conditions, ^{125}I-labeled LDL were incubated with unlabeled apoHDL and palmitic acid under similar condi-

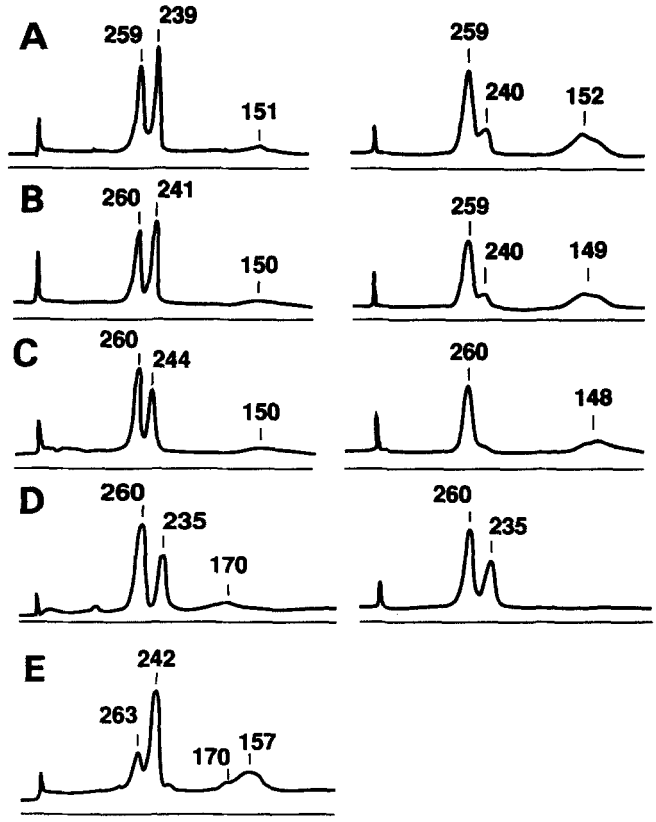

Fig. 2. Size changes of LDL (d 1.033–1.039 g/ml fraction) produced by incubation with palmitic acid and delipidated HDL or purified apoA-I for 2 h at 37°C. In A through C, the incubation mixture contained 46 μg of LDL, 0.5 μmol palmitic acid, and 30 μg (A), 60 μg (B), and 120 μg (C) of ^{125}I-labeled apoHDL protein in a final volume of 120 μl. The curves in the panel on the left represent densitometric scans of 2–16% polyacrylamide gradient gels stained for protein with Coomassie blue, while the curves in the right panel represent scans of autoradiograms of corresponding gels electrophoretically blotted onto nitrocellulose sheets. The starting LDL (not shown) migrated as a single sharp band similar to that shown in Fig. 1A. In D, the distribution of labeled apoB was tracked by incubating ^{125}I-labeled LDL (d 1.033–1.039 g/ml, 17 μg protein), unlabeled delipidated apoHDL (60 μg protein) and palmitic acid (0.4 μmol) in a final volume of 50 μl. The left panel shows a densitometric scan of a 2–16% gradient gel stained for protein with Coomassie blue, while the right panel illustrates a densitometric scan of an autoradiogram of a corresponding gradient gel-blot of the same mixture. In E the incubation mixture contained LDL (d 1.033–1.039 g/ml, 60 μg protein), palmitic acid (0.5 μmol), and purified apoA-I (52 μg) incubated in a final volume of 60 μl. The curve represents a densitometric scan of a 2–16% gradient gel stained for protein with Coomassie blue. Particle diameters in all curves are shown in Å.

Downloaded from www.jlr.org by guest, on June 18, 2012

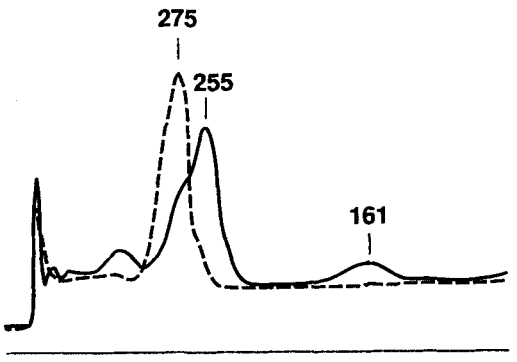

Fig. 3. Lipoprotein size changes after incubation of normal LDL (d 1.029–1.033 g/ml; 0.17 mg protein/ml) with apoHDL (0.42 mg/ml), and human serum albumin (10%) in the presence (solid line) or absence (dashed line) of palmitic acid (8 μmol/ml; palmitic acid:albumin molar ratio of 5:1). The lipoproteins were separated from albumin by ultracentrifugation after a 2-h incubation at 37°C. The curves represent densitometric scans of 2–16% gradient gels stained for protein with Coomassie blue; particle diameters are shown in Å.

tions (Fig. 2D). Comparison of a 2–16% gradient gel stained for protein with an autoradiogram of a corresponding nitrocellulose gel blot, revealed that ^{125}I-labeled apoB was present in the two LDL peaks, in proportion to the mass in each peak on the Coomassie-blue stained gel. ^{125}I-labeled apoB was not detected, however, in the region of the dissociation complex band. Direct analysis of apoB on Coomassie Blue-stained SDS gradient electrophoretic gels revealed no change in apoB-100 molecular weight or other evidence for degradation of apoB into smaller fragments under these conditions (data not shown).

The two LDL-size subspecies formed by this reaction were tested for stability under different conditions after separation from dissociation complexes by density gradient ultracentrifugation (see Methods and below). After incubation of a fraction of palmitic acid–apoHDL-modified ^{125}I-labeled LDL containing both the larger and smaller LDL bands with either normal whole plasma or lipoprotein-deficient (d > 1.21 g/ml) plasma fraction, small increases in size of the smaller band (5–7 Å) were observed, but the two sharp subspecies remained distinct (data not shown).

When purified apoA-I was used in place of delipidated apoHDL in incubations with LDL and palmitic acid, LDL size transformations of a similar nature were observed (Fig. 2E). Purified apoA-II alone failed to induce FFA-induced LDL size transitions at equivalent or even higher concentrations, and two other proteins that do not normally associate with lipoproteins (purified ovalbumin and chymotrypsin A) also did not facilitate FFA-mediated LDL size transformations under otherwise identical conditions.

The apparent particle diameter of the newly formed LDL subspecies varied to a small degree depending on the incubation conditions. At higher ratios of palmitic acid to apoHDL, slightly greater reductions in LDL size were observed, in conjunction with the formation of dissociation complexes of larger apparent size, as measured by gradient gel electrophoresis. The calculated volume change in the apoB-containing particle (assuming sphericity—see electron microscopic studies below) correlated well with the apparent particle diameter of the newly formed dissociation complex band (r = 0.8, n = 25; $P < 0.0001$).

FFA–apoA-I-induced LDL size transformations also occurred when palmitate (0.20 mM) was added to incubation mixtures (buffered to pH 7.5) in the form of the sodium salt (data not shown) or when palmitate was present in fully solubilized form in the presence of FFA-free human serum albumin. In the latter case, although size transformations were not observed in vitro at FFA:albumin molar ratios typically seen in normal, equilibrated whole plasma (i.e., <1.0), LDL size transformations were readily produced using optically clear solutions of palmitate and human serum albumin at molar ratios in the range of 5:1. **Fig. 3** is taken from an experiment in which LDL (d 1.029–1.033 g/ml) was incubated with apoHDL and varying amounts of a 5:1 molar ratio palmitate:albumin solution. After incubation for 2 h at 37°C, the d < 1.21 g/ml lipoproteins were reisolated by ultracentrifugation and analyzed by gradient gel electrophoresis. Stimulation of apoHDL binding to LDL was observed at palmitic acid concentrations of 1–2 μmol/ml, and LDL size transitions were observed to increasing degrees when the palmitic acid concentration reached 4.8 μmol/ml or higher. In most of the experiments in this report, however, albumin was not included, in order to exclude potential effects of apolipoproteins (particularly apoA-I) or other contaminants that are often present in commercial albumin preparations (26, 27). When added to incubation mixtures in the form of the acid in the absence of albumin or lipoproteins, the amount of [^{3}H]palmitic acid solubilized in the incubation buffer after 2 h at 37°C was roughly proportional to the amount of solid palmitic acid present, with the level of soluble palmitic acid achieved representing <7% of the total added under these conditions. When lipoproteins were present in the incubation mixtures, solubilization of palmitic acid increased to varying degrees as a consequence of lipoprotein binding.

Fatty acid-induced size transitions of apoB-containing lipoprotein of varying size

Subfractions of LDL of progressively smaller size and increasing density were compared for susceptibility to FFA-induced size transformations. The results presented in **Fig. 4** include studies using three LDL subpopulations corresponding to density gradient ultracentrifugation fractions of d 1.025–1.029 g/ml, 1.033–

Downloaded from www.jlr.org by guest, on June 18, 2012

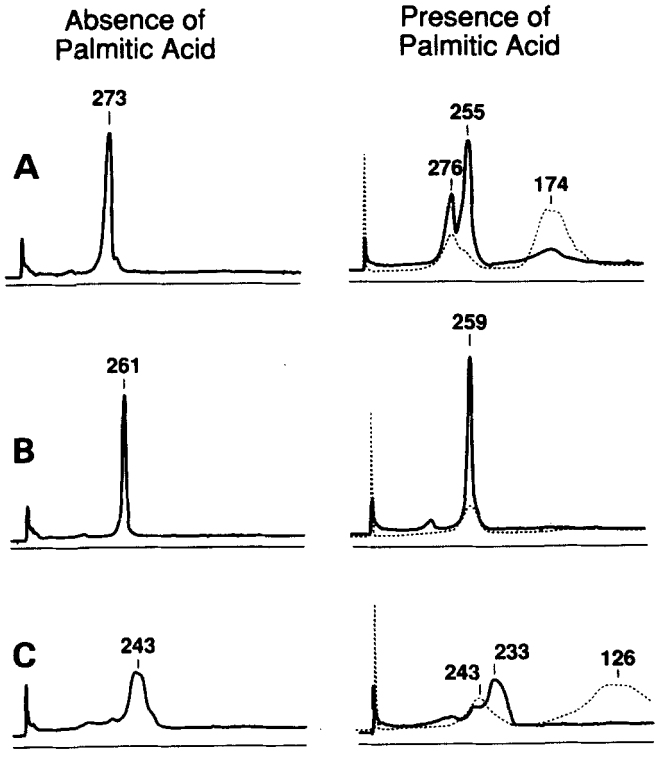

Fig. 4. Variation in susceptibility of different LDL size subpopulations to palmitic acid-apoHDL-induced size transitions. All of the fractions were isolated from normolipidemic plasma by density gradient ultracentrifugation using the method of Shen et al. (4), and were incubated for 2 h at 37°C at equivalent concentrations (25 μg protein) with ^{125}I-labeled apoHDL (60 μg protein) in the absence (left panel) or presence (right panel) of 0.2 μmol palmitic acid in a final volume of 80 μl. The curves represent densitometric scans of 2–16% gradient gels stained for protein with Coomassie blue (solid lines) or scans of corresponding gel-blot autoradiograms (dotted lines). Particle sizes are shown in Å. Panel A. LDL of d 1.025–1.029 g/ml; panel B. LDL of d 1.033–1.039 g/ml; and panel C. LDL of d 1.045–1.054 g/ml.

1.039 g/ml, 1.044–1.054 g/ml isolated from the plasma of the same normal subject by the method of Shen et al. (4). Equivalent protein concentrations of each fraction were incubated with ^{125}I-labeled apoHDL, in the presence or absence of palmitic acid. Particle size changes were analyzed by gradient gel electrophoresis. Under these conditions, greater than 50% of d 1.125–1.029 g/ml LDL was converted to a subspecies 21 Å smaller in particle diameter, while d 1.033–1.039 g/ml LDL showed negligible size reduction at this palmitic acid concentration (compare Figs. 4A and 4B). At two- to fourfold higher palmitic acid concentrations, however, size changes were readily produced in the latter LDL subfraction (e.g., see Fig. 2). The requirement for higher palmitic acid concentrations to produce size reductions in intermediate sized and density LDL was confirmed in several experiments. LDL subfractions of d 1.044–1.054 g/ml, in contrast, underwent partial size reduction at the same lower palmitic acid concentration as observed with the most buoyant LDL subfraction (compare Figs. 4A and 4C). Although the formation of a new dissociation complex in conjunction with the observed changes in denser LDL was not always evident on the protein-stained gels, it could be readily detected by gel-blot autoradiography when ^{125}I-labeled apoHDL was used (Fig. 4C).

Size changes were similarly investigated in series of VLDL and IDL subfractions isolated from the same normolipidemic subject using nonequilibrium density gradient ultracentrifugation (6). Equivalent quantities of protein of each fraction were incubated with ^{125}I-labeled apoHDL in the presence or absence of varying concentrations of palmitic acid and the products were analyzed on 2–16% gradient gels (**Fig. 5**). In the presence of palmitic acid, size reductions in the range of 17–23 Å were observed for all of the VLDL and IDL subspecies, but with some differences. The largest VLDL fraction showed a small size reduction of the major peak as well as a broadening of the size spectrum of particles in the smaller range (Fig. 5A). Size reductions of the sharper small VLDL and large IDL fractions were more pronounced and, in contrast to LDL, formed a single product without the presence of a clearly identifiable residual lipoprotein band comparable in size to the starting material (Fig. 5B–5C). With progressively smaller IDL species, however, partial size transformations of their bands was observed at the lower palmitic acid concentrations, with less conversion to the smaller subspecies as IDL size decreased (Fig. 5D–5F). At higher palmitic acid levels, however, complete transformation to smaller diameter bands was observed even for the densest IDL fraction studied (Fig. 5F), in contrast to the findings with LDL (see above). These observations suggest that small VLDL and IDL are more susceptible to FFA-apoA-I-induced size reductions than LDL, and that among IDL, larger subspecies are more susceptible than smaller ones. Similar size transformations of VLDL and IDL were observed in incubations in which native ^{125}I-labeled HDL replaced delipidated ^{125}I-labeled apoHDL. The size changes among VLDL and IDL produced in the presence of palmitic acid were accompanied by the appearance of dissociation complexes of varying size, the properties of which are described in the companion paper (25).

In incubations using native ^{125}I-labeled HDL, a degree of association of label with VLDL was observed in the absence of added FFA. Progressively less spontaneous association was seen with smaller apoB-containing particles such that there was minimal spontaneous binding to LDL. Approximately 60–70% of the native ^{125}I-labeled HDL-derived labeled protein spontaneously associating with VLDL consisted of apoA-I (as assessed by SDS-gradient gel electrophoresis) with the remainder migrating in the size range of apoA-II and apoCs (data not shown). This spontaneous association could be inhibited or reversed by incubation in the presence of 5% (w/v)

Downloaded from www.jlr.org by guest, on June 18, 2012

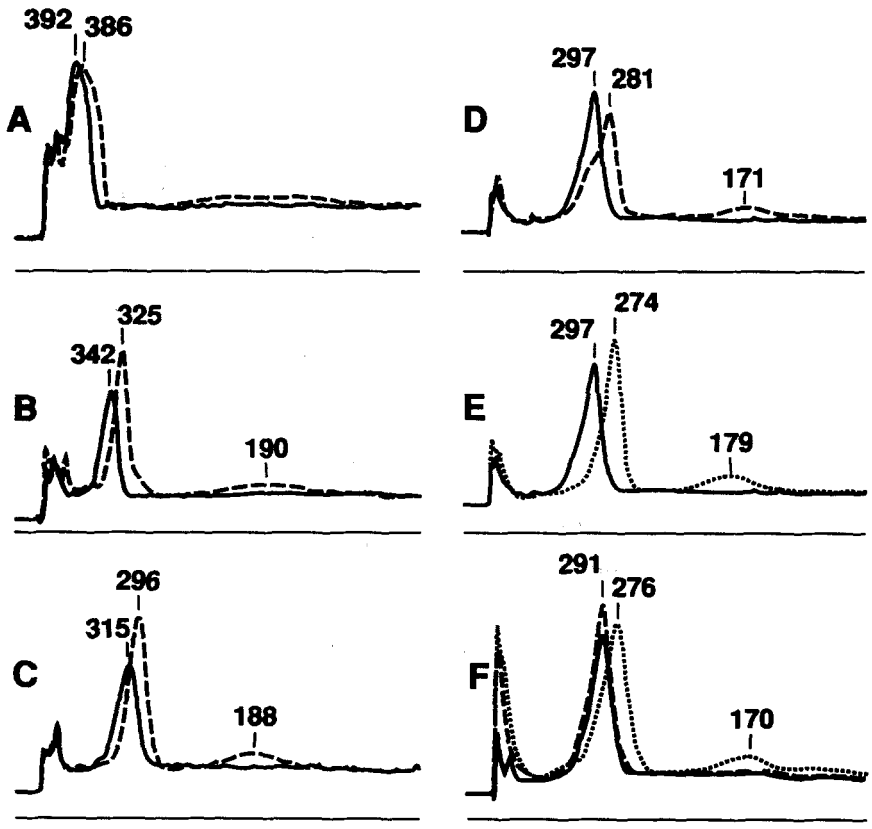

Fig. 5. Variation in susceptibility of different VLDL and IDL subpopulations to palmitic acid/apoHDL-induced size transitions. The VLDL and IDL subfractions were isolated from normolipidemic plasma by nonequilibrium density gradient ultracentrifugation as previously described (6). Equivalent amounts (13 μg protein) of each fraction were incubated with delipidated ^{125}I-labeled HDL (38 μg protein) in the absence (solid lines) or presence of palmitic acid (dashed lines, 0.1 μmol; dotted lines, 0.3 μmol) for 2 h at 37°C in a final volume of 80 μl. A. large VLDL (d<1.003 g/ml); B. small VLDL (mean density = 1.006 g/ml); C. large IDL (mean density = 1.010 g/ml); D. intermediate IDL (mean density = 1.013 g/ml); E. intermediate IDL (mean density = 1.013 g/ml) incubated with 0.3 μmol palmitic acid; and F. small IDL (mean density = 1.019 g/ml) incubated with 0.1 and 0.3 μmol palmitic acid. The curves represent densitometric scans of 2–16% gradient gels stained for protein with Coomassie blue; particle diameters are shown in Å.

FFA-free albumin, suggesting that endogenous FFAs or other lipids on the VLDL surface are required. Incubation of delipidated ^{125}I-labeled HDL with VLDL also resulted in binding of apoHDL; however, this reaction was less affected by albumin or added FFAs and involved association of larger amounts of apoA-II than was the case for incubations with native HDL (data not shown).

Physical and chemical properties of modified LDL and IDL subspecies

The two different sized LDL subspecies generated by incubation with palmitic acid and apoHDL were reisolated by density gradient ultracentrifugation as described in Methods. Only partial separation of these species could be achieved due to overlap in density, however the smaller of the two LDL subspecies had a mean buoyant density slightly lower than the larger species. For example, when the starting LDL fraction was a density fraction of 1.033–1.039 g/ml, the smaller species was found at highest concentrations in a fraction of density 1.037 g/ml compared with 1.047 g/ml for the larger subspecies. The lower apoHDL and phospholipid (see below) content of the smaller subspecies, as well as possible differences in FFA content, probably account for these density differences, which contrast with the usual LDL subspecies size-density relationships (4). By pooling fractions containing the peak and leading shoulder of the modified LDL band, it was possible to obtain preparations >90% free of the larger subspecies for compositional analyses. In the case of IDL, where there was nearly complete conversion of the starting fraction to a smaller subspecies, density gradient ultracentrifugation readily achieved isolation of a homogeneous fraction. Electron microscopy confirmed the spherical morphology of both IDL and LDL modified fractions. Chemical composition analyses of representative LDL and IDL fractions are presented in **Table 1**, with the

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 1. Chemical composition[a] of LDL and IDL subfractions modified by incubation with palmitic acid and a source of apoA-I

Lipoprotein	Protein	Cholesteryl Esters	Free Cholesterol	Triglyceride	Phospholipid
LDL-I (d 1.025-1.029 g/ml)	18.0	46.5	9.4	4.4	22.0
LDL-I:apoHDL/PA-treated	29.6	43.0	6.9	7.0	13.5
LDL-I:apoA-I/PA-treated	25.3	41.6	9.0	7.5	16.6
LDL-I:apoHDL/PA/HSA-treated	28.6	47.1	7.5	6.4	10.4
LDL-II (d 1.033-1.039 g/ml)	21.4	44.5	9.8	1.2	23.2
LDL-II:apoHDL/PA/HSA-treated	28.4	47.0	6.5	2.7	15.5
IDL (d 1.008-1.019 g/ml)	15.8	31.3	8.7	20.6	23.7
IDL:apoA-I/PA-treated	20.0	38.3	2.8	26.0	13.1
IDL (d 1.010-1.019 g/ml)	16.0	34.5	9.2	21.0	19.3
IDL:apoHDL/PA/HSA-treated	28.2	36.3	6.7	13.7	15.1

[a]Percent dry weight, excluding fatty acid. The palmitic acid (PA) content of the non-albumin-treated LDL fractions in these experiments varied from 1.5 to 10% of total dry weight. After 30-min incubation with fatty acid-poor human serum albumin (HSA) at a molar ratio of palmitic acid:albumin of 3:1, 92-98% of the LDL-associated palmitic acid was removed. Data represent means of duplicates from individual experiments.

compositions of the starting material shown for comparison. The values shown do not include bound palmitic acid, which varied from preparation to preparation, ranging from 1.5 to 10% of the total LDL dry weight. The presence of fatty acid probably contributed to the fact that the buoyant density of the modified LDL was comparable to that of the starting LDL, despite its protein enrichment. In experiments in which the LDL was exposed to FFA-free human serum albumin for 30 min at 37°C and subsequently reisolated by density gradient ultracentrifugation, 92-98% of the bound [^{3}H]palmitic acid was removed. The buoyant density of the LDL increased, but the changes in chemical composition exclusive of bound FFA (Table 1) were essentially the same. Based on the compositional changes observed in analyses from eight experiments employing LDL or IDL fractions, treatment with palmitic acid and a source of apoA-I produced a 33% decrease in percent phospholipid ($P < 0.0001$), a 30% decrease in free cholesterol ($P < 0.002$), and a 44% increase in protein ($P < 0.0004$). Percent cholesteryl esters did not change significantly.

Analysis of apolipoprotein composition of LDL modified by incubation with palmitic acid and ^{125}I-labeled HDL or apoHDL by SDS polyacrylamide gradient gel electrophoresis revealed apoA-I to be the predominant non-apoB protein present, with much smaller amounts of protein migrating in the size range of apoA-II monomer (**Fig. 6**). When the FFA-apoHDL-treated LDL was further incubated with FFA-free human serum albumin as described above, the association of apoA-I with the reisolated LDL was reduced by about 70%, while the amount of apoA-II binding was relatively unaffected (Fig. 6B). After treatment with albumin, apoA-I and apoA-II comprised <10% of the total protein present (the remainder consisting of apoB), compared to approximately 20% prior to this treatment.

Fatty acid specificity for induction of LDL size transformation

A variety of purified FFAs of varying chain length and degrees of saturation were compared for their ability to induce LDL size changes. The FFAs tested included myristic, myristoleic, palmitic, palmitoleic, oleic, linoleic,

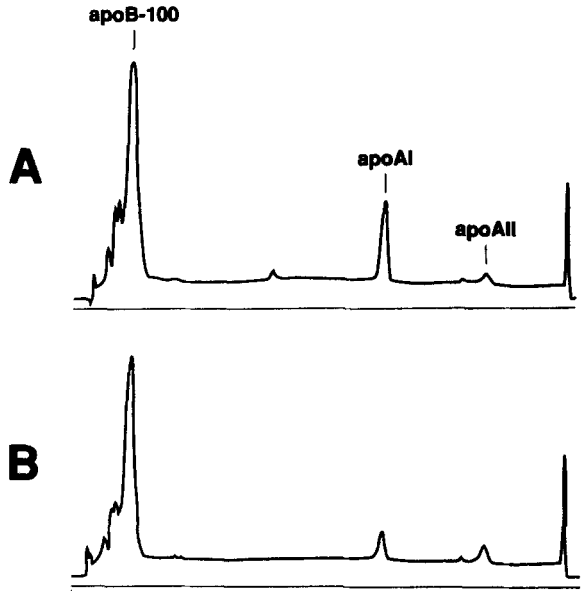

Fig. 6. SDS polyacrylamide gradient gel electrophoresis (4-30%) of palmitic acid-apoHDL-treated LDL. The starting LDL fraction (d 1.033-1.039 g/ml) was incubated under conditions that resulted in a 23 Å size reduction of approximately 65% of the starting material. FFA-free human serum albumin (10% final concentration; molar ratio of albumin:palmitic acid = 1:3) was then added to half of the mixture and buffer to the remaining half. The incubation was continued for an additional 30 min at 37°C, after which the LDL fractions were reisolated by density gradient ultracentrifugation as described in methods. The curves represent densitometric scans of SDS gels (stained for protein with Coomassie blue) of the delipidated LDL fractions. A. absence of albumin; B. presence of albumin.

Downloaded from www.jlr.org by guest, on June 18, 2012

linolenic, stearic, arachidic, and eicosapentaenoic acids. Incubations were carried out for 2 h at 37°C in the presence of the different FFAs in amounts ranging from 0.0 to 0.2 μmol, using LDL (d 1.029–1.033 g/ml, 50 μg protein), and ^{125}I-labeled apoHDL (72 μg protein) in a final volume of 80 μl. LDL size reductions were observed with saturated fatty acids as short as myristic acid (C14:0) and as long as stearic acid (C18:0), with the shorter chain FFAs inducing size changes at lower concentrations. The reaction was not observed under the above conditions with arachidic acid (C20:0); however, the highly unsaturated eicosapentaenoic acid (C20:5) did induce size transformations at equivalent molar concentrations. Comparing different C18 fatty acids of varying degrees of saturation, the order of effectiveness in producing size reductions in LDL was stearic acid (C18:0) < oleic acid (C18:1) < linoleic acid (C18:2) < linolenic acid (C18:3). Moreover, stearic acid, like palmitic acid (see above) induced size reduction of only a portion of the LDL in the incubation mixture, while its monounsaturated or polyunsaturated counterparts facilitated conversion of the entire LDL band to a smaller product in the presence of apoHDL, similar to the type of change produced by palmitic acid acting on IDL subfractions (see Fig. 5). Palmitoleic acid behaved similarly to oleic acid in this respect. Monoglycerides (1-monopalmitoyl-glycerol and 2-monopalmitoyl-glycerol) did not induce LDL size changes (data not shown).

DISCUSSION

The existence of multiple distinct subclasses of IDL and LDL has been recognized for some time, but the origins of this heterogeneity are not well understood. In the present report it is shown that in vitro incubations of IDL and LDL subfractions with FFAs and a source of apoA-I induce sharp size discontinuities in these particles that resemble those observed among IDL and LDL in human plasma. This has led us to hypothesize that FFA/apoA-I-mediated reactions may contribute to this heterogeneity in vivo.

The size transformations observed during incubation of IDL or LDL with FFAs and a source of apoA-I result from a process that begins with FFA-induced binding of apoA-I to apoB-containing lipoproteins, a phenomenon observed either in the setting of hydrolysis of triglyceride-rich lipoproteins or upon addition of exogenous FFAs (12). The data presented in this and the companion paper (25) indicate that when lipoprotein surface FFA levels are sufficiently high, the apoB–apoA-I-containing lipoprotein intermediate thus formed is capable of releasing an apoA-I/lipid-containing "dissociation complex," resulting in a size decrement of the starting IDL or LDL of 10–30 Å as measured by nondenaturing gradient gel electrophoresis. A mechanism involving dissociation is supported not only by the simultaneous appearance of the two new species, but also by the inverse relationship between the change in the size of the apoB-containing particle and the apparent size of the newly formed dissociation complex, as well as the complementary nature of the lipid changes in the apoB-containing lipoprotein and the lipids that appear in the dissociation complex fraction (see Table 1 and the data presented in the companion paper (25)).

The size changes produced in LDL subfractions by exposure to exogenously added purified FFAs (e.g., Fig. 1) differed from the transformations previously reported when LDL was present in mixtures of VLDL undergoing lipolysis (12). With added FFA, the larger of the two bands formed was similar in size to the starting LDL, while the second LDL band was considerably smaller than that of the starting component (e.g., Fig. 1). With VLDL lipolysis, the diameters of both LDL products were significantly larger than in the case of added FFA, with the bigger species of greater particle diameter than the starting LDL, and the second species somewhat smaller (12). It is likely that the greater increase in LDL particle size seen in the presence of VLDL undergoing lipolysis results from transfer of other lipid components from VLDL under these conditions (11, 12), with consequent particle enlargement. This is currently under further investigation.

Native HDL, delipidated HDL, or purified apoA-I were all effective in inducing size reductions in IDL or LDL in the presence of FFAs. However, the size reductions occurred at lower HDL protein concentrations when delipidated rather than native HDL was used (compare Fig. 1 and Fig. 2). It may be that native HDL binds FFAs in a way that limits their availablity for interaction with LDL or, alternatively, that apoA-I on native HDL is less accessible than in delipidated form for interaction with LDL. ApoA-II and several proteins that are not normally associated with plasma lipoproteins were ineffective alone in inducing LDL and IDL size changes, but other apolipoproteins that associate with HDL or triglyceride-rich lipoproteins (e.g., apoE, C-apolipoproteins) remain to be studied in this regard.

The amounts of palmitic acid required to induce reductions in particle size were lowest for lipoproteins within the VLDL–IDL spectrum and were generally higher for IDL and LDL particles of decreasing size (Fig. 4 and 5). Among LDL from normolipidemic subjects, however, particles at both ends of the LDL size and density spectrum (i.e., d 1.025–1.029 or d 1.045–1.054 g/ml, respectively) appeared to undergo size reductions at lower palmitic acid concentrations than particles in the mid-range (d 1.033–1.039 g/ml). These differences in susceptibility to palmitic acid-induced size reduction require further investigation to determine whether they reflect differences in availability of removable surface lipid on the apoB-containing particle, differences in binding capacity for FFAs, or other factors.

Downloaded from www.jlr.org by guest, on June 18, 2012

JOURNAL OF LIPID RESEARCH ASBMB

Susceptibility to size transformation also varied depending on the specific FFAs present. While palmitic acid induced size reductions in only a portion of the LDL particles, the mono- or polyunsaturated C16 or C18 FFAs tested were capable of converting LDL virtually completely to a size-reduced product. Moreover, with C18 FFAs, the reaction moved to completion at progressively lower fatty acid concentrations the more highly unsaturated the fatty acid. Size transformations readily occurred with the short chain (C-14) myristic acid, but not with the long-chain C-20 arachidic acid at equivalent molar concentrations.

Composition analyses of size-reduced LDL and IDL demonstrated depletion of surface lipids (phospholipid and free cholesterol) with relative enrichment in protein. These changes were seen in conjunction with the appearance of phospholipid and free cholesterol in "dissociation complexes" produced simultaneously with the size reduction of the apoB-containing lipoprotein, as described in the accompanying report (25). The increase in percent protein in size-reduced LDL probably reflects a combination of a degree of binding of apoHDL to these lipoproteins, as well as relative protein enrichment due to lipid depletion. The fact that the relative content of core lipid changed little despite the decrease in particle size suggests that small amounts of core lipid (predominantly cholesteryl esters in the case of IDL and LDL) may also dissociate from these particles and contribute to their observed size reduction. Compositional analyses of dissociation complexes reported in the accompanying paper are consistent with this hypothesis (25). The electron microscopic images of size-reduced LDL or IDL indicate that spherical structures are preserved following this transformation, despite their relative depletion in surface lipid.

Relatively large amounts of FFAs associated with the LDL and IDL subfractions under the conditions of these experiments (up to 10% total dry weight for palmitic acid). Most of the bound FFAs (92–98%) could be removed by a 30-min incubation with FFA-free human serum albumin. Size-modified LDL retained their spherical shapes and compositional properties following this treatment. The two LDL size subspecies formed upon incubation with palmitic acid and apoHDL also remained distinct following incubation with whole plasma or $d > 1.21$ g/ml plasma fraction. The basis for the small size increase in the smaller subspecies following this treatment is not known. It is possible that since these particles contain increased amounts of apoA-I, they are substrates for the lecithin:cholesterol acyl transferase (LCAT) reaction and that consequent accumulation of core cholesteryl ester may account for the observed size increases. Alternatively, the presence of FFAs on these particles could stimulate CETP-mediated lipid transfers (28), or the surface-depleted state of the modified LDL could render these particles prone to acquire phospholipid or free cholesterol from other lipoproteins. These alternatives remain to be investigated, as well as the theoretic possibility that size-reduced IDL or LDL formed through the FFA–apoA-I-mediated pathway may have the capacity to participate in reverse cholesterol transport, since their surface-depleted state may predispose to the acquisition of free cholesterol from cell membrane surfaces as well as other lipoproteins.

The close resemblance of the LDL size subspeciation produced in vitro by FFA–apoA-I-mediated reactions to the sharp size discontinuities observed among LDL subspecies in human plasma raises the possibility that this reaction may contribute to this heterogeneity in vivo. Since the FFA:albumin ratios required to produce size changes in LDL in vitro are of the order of fivefold higher than those seen in the steady state in human plasma under normal conditions, the potential generation of subspeciation by this mechanism is based on the assumption that FFA:albumin ratios in this range are reached in the microenvironment of the capillary endothelial surface where rapid triglyceride hydrolysis occurs. Several lines of evidence makes this assumption plausible. Lipoproteins of all classes are known to have high affinity for FFA binding. Direct measurements of FFAs associated with the lipoprotein fraction even in whole plasma where there is a large molar excess of albumin have yielded values ranging from 5–10% to 13–21% of the total plasma pool (29, 30) and the proportion of FFAs bound to lipoproteins can increase further when total FFA or albumin levels are altered or when longer chain or saturated FFAs comprise a larger proportion of the FFAs present (29–32). Electron microscopic studies using cytochemical techniques applied to rat adipose tissue perfused with chylomicron and albumin-containing solutions have revealed FFA-containing lamellar forms associated with chylomicrons attached to endothelial cells (33, 34), lending support to the concept of local accumulation of high levels of FFAs during lipolysis.

Since FFAs bound to triglyceride-rich lipoproteins in vitro are known to partition primarily to the particle surface (35), it is possible that they may, in turn, accumulate on LDL particles as a result of simple proximity to lipoproteins undergoing lipolysis, particularly since FFAs on the surface of VLDL are known to stimulate VLDL–LDL physical complex formation (11). On the other hand, VLDL and IDL may be more likely candidates for FFA-induced size transformations under physiologic conditions in vivo, by virtue of the fact that size reductions can be produced in small VLDL and in IDL at lower concentrations than those required to induce the reaction in LDL and the greater likelihood for accumulation of triglyceride-derived FFAs at VLDL and IDL surfaces. In this case, LDL heterogeneity could result indirectly through the preservation of discontinuous size (and possibly conformation) changes created at the level of small VLDL or IDL by this mechanism and transmitted to the final LDL products

Downloaded from www.jlr.org by guest, on June 18, 2012

following further core depletion by triglyceride hydrolysis. In support of this concept, in vitro studies show that size differences among IDL and LDL species are preserved when they are exposed to core-depleting cycles of CETP-mediated triglyceride enrichment followed by lipolysis (R. M. Krauss and T. A. Musliner, unpublished observations). It is also possible that FFA-mediated size transformations occur at all levels of the VLDL–IDL–LDL cascade (probably to varying degrees), contributing to the multitude of IDL and LDL species that are frequently observed in human plasma.

Based on the recently reported apparent pK of oleic acid in solution with lipoproteins (35), it is probable that, in vivo, FFAs are present in both ionized and unionized forms. There is also evidence that fatty acid soaps and acid–soap complexes form bilayered lamellae observed in association with the chylomicron surface film during in vitro lipolysis or perfusion of rat fat pads (33, 36). In our in vitro studies, the subspeciation reaction could be demonstrated using palmitic acid added to incubation mixtures in different forms. The presence of micellar or lamellar FFA structures under some of these conditions is probable. However, it is unlikely that such forms were present in the millipore-filtered FFA–albumin solutions employed in the study shown in Fig. 3, making it improbable that the subspeciation phenomenon is dependent on the FFA being in micellar form. The albumin–FFA solution used in these experiments would be expected to contain small amounts of unbound FFAs in equilibrium with FFAs bound to albumin, and we cannot be certain which compartment (or both) is responsible for the reaction. However, the same type of equilibrium may exist in the vicinity of the surface of lipoproteins undergoing lipolysis. In vivo in the microenvironment of the capillary endothelial surface, the physical–chemical state of released FFAs has not yet been clearly established and may well include varied forms.

The chemical compositions of IDL or LDL subfractions that have undergone direct FFA–apoA-I-mediated size reductions (Table 1) differ from those reported for LDL size subpopulations of comparable size and density isolated from human plasma (4). If this mechanism in fact contributes to the formation of these LDL subpopulations in vivo, additional secondary modifications must be invoked to account for their final compositions. A variety of remodelling processes to which surface-depleted IDL and LDL may be subject are likely to contribute to the ultimate subspeciation pattern in the plasma of a given individual. Such processes may include CETP-mediated lipid transfers (also stimulated by FFAs (28)), possible non-CETP-mediated lipid transfers accompanying FFA-mediated VLDL–LDL complex formation (11), the LCAT reaction (particularly when these subspecies contain bound apoA-I), lipolysis (particularly by hepatic lipase (20)), and lipid uptake from other lipoprotein or membrane surfaces. The balance of these reactions would be anticipated to vary in different individuals with different lipid and apolipoprotein levels and differences in other conditions influencing lipoprotein metabolism. For example, in subjects with high triglycerides and low levels of apoA-I, one would expect stimulation of VLDL–LDL complex formation (11), stimulation of triglyceride transfer to IDL and LDL, greater degrees of association of FFAs with IDL and LDL subspecies, and a greater tendency for the occurrence of FFA-mediated size reductions. All of these processes would tend to promote the formation of the smaller, yet heterogeneous LDL size subpopulations observed in this setting. The fact that multiple smaller LDL subclasses with sharp size discontinuities are observed in hereditary plasma CETP deficiency (9) suggests that smaller LDL subpopulations or their precursors, formed by the FFA–apoA-I surface depletion pathway, may normally be recipients for CETP-mediated cholesteryl ester transfer, and that this process ordinarily maintains LDL in larger sized subclasses. Less extreme variations in CETP levels could similarly influence the final manifestations of FFA-mediated IDL or LDL size changes.

Evidence from both in vitro and in vivo studies indicates that differences among apoB-containing precursor particles contribute to LDL heterogeneity. Specifically, in vivo studies in the rat (a species lacking plasma CETP activity) have shown that small VLDL and IDL-1 are catabolized intravascularly to a discrete LDL product similar in size and density to the LDL-II (d 1.033–1.042 g/ml) found in human plasma (10). Denser, cholesterol-rich IDL, in contrast, yield larger, more buoyant LDL subspecies. It is not known, however, how these different IDL precursors arise. It is possible that FFA–apoA-I-mediated reactions play a role in their formation. On the other hand, differences in degrees of glycosylation between different LDL subpopulations (37) have raised the possibility that intrinsic differences in apoB-glycosylation contributes to LDL heterogeneity. Such a role would not be incompatible with the mechanism hypothesized here. Variation in IDL or LDL glycosylation could itself influence susceptibility to FFA–apoA-I-mediated size reductions, perhaps contributing to the multitude of final discrete subspecies that may be found in human plasma.

We wish to thank Rosemarie Celli and Janet Selmek-Halsey for excellent technical assistance. This work was supported by NIH Program Project Grant HL-18574 from the National Heart, Lung, and Blood Institute of the National Institutes of Health, and was conducted at the Lawrence Berkeley Laboratory (Department of Energy contract DE-AC03-76SF00098 to the University of California).

Manuscript received 30 August 1990 and in revised form 12 March 1991.

Downloaded from www.jlr.org by guest, on June 18, 2012

REFERENCES

1. Lindgren, F. T., L. C. Jensen, R. D. Wills, and G. R. Stevens. 1972. Subfractionation of S_f 4-10^5, S_f 4-20 and high density lipoproteins. *Lipids.* **7:** 194-201.
2. Hammond, M. G., and W. R. Fisher. 1971. The characterization of a discrete series of low density lipoproteins in the disease, hyper-pre-betalipoproteinemia. *J. Biol. Chem.* **246:** 5454-5465.
3. Lee, D. M., and D. Downs. 1982. A quick and large-scale density gradient subfractionation method for low density lipoproteins. *J. Lipid Res.* **23:** 14-27.
4. Shen, M. M., R. M. Krauss, F. T. Lindgren, and T. M. Forte. 1981. Heterogeneity of serum low density lipoproteins in normal human subjects. *J. Lipid Res.* **22:** 236-244.
5. Krauss, R. M., and D. J. Burke. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23:** 97-104.
6. Musliner, T. A., C. Giotas, and R. M. Krauss. 1986. Presence of multiple subpopulations of lipoproteins of intermediate density in normal subjects. *Arteriosclerosis.* **6:** 79-87.
7. Musliner, T. A., and R. M. Krauss. 1988. Lipoprotein subspecies and risk of coronary disease. *Clin. Chem.* **34:** B78-B83.
8. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, E. Butbul, I. Sharon, and T. Olivecrona. 1982. Reversible modification of human plasma low density lipoproteins toward triglyceride-rich precursors. *J. Biol. Chem.* **257:** 6509-6517.
9. Yamashita, S., Y. Matsuzawa, M. Okazaki, H. Kako, T. Yasugi, H. Akioka, K. Hirano, and S. Tarui. 1988. Small polydisperse low density lipoproteins in familial hyperalphalipoproteinemia with complete deficiency of cholesteryl ester transfer activity. *Atherosclerosis.* **70:** 7-12.
10. Musliner, T. A., K. M. McVicker, J. F. Iosefa, and R. M. Krauss. 1987. Metabolism of human intermediate and very low density lipoprotein subfractions from normal and dysbetalipoproteinemic plasma. In vivo studies in rat. *Arteriosclerosis.* **7:** 408-420.
11. Musliner, T. A., K. M. McVicker, J. F. Iosefa, and R. M. Krauss. 1987. Lipolysis products promote the formation of complexes of very-low-density and low-density lipoproteins. *Biochim. Biophys. Acta.* **919:** 97-110.
12. Musliner, T. A., H. J. Michenfelder, and R. M. Krauss. 1988. Interactions of high density lipoproteins with very low and low density lipoproteins during lipolysis. *J. Lipid Res.* **29:** 349-361.
13. Lindgren, F. T., L. C. Jensen, and F. T. Hatch. 1972. The isolation and quantitative analysis of serum lipoproteins. *In* Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism. G. J. Nelson, editor. John Wiley and Sons, New York. 181-274.
14. MacFarlane, A. S. 1958. Efficient trace labelling of proteins with iodine. *Nature.* **182:** 53-57.
15. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Bophys. Acta.* **260:** 212-221.
16. Herbert, P. N., L. L. Bausserman, L. O. Henderson, R. J. Heinen, M. J. LaPiana, E. C. Church, and R. S. Shulman. 1978. Apolipoprotein quantitation. *In* The Lipoprotein Molecule. H. Peeters, editor. Plenum Publishing Co., London. 35-56.
17. Blanche, P. J., E. L. Gong, T. M. Forte, and A. V. Nichols. 1981. Characterization of human high-density lipoproteins by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **665:** 408-419.
18. Nichols, A. V., R. M. Krauss, and T. A. Musliner. 1986. Non-denaturing polyacrylamide gradient gel electrophoresis. *Methods Enzymol.* **128:** 417-431.
19. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350-4354.
20. Musliner, T. A., P. N. Herbert, and M. J. Kingston. 1979. Lipoprotein substrates of lipoprotein lipase and hepatic triacylglycerol lipase from human post-heparin plasma. *Biochim. Biophys. Acta.* **575:** 277-268.
21. Markwell, M. A. K., S. M. Hass, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206-210.
22. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466-468.
23. Hindriks, R. F., B. G. Wolthers, and A. Groen. 1977. The determination of total cholesterol in serum by gas-liquid chromatography compared to two other methods. *Clin. Chim. Acta.* **74:** 207-215.
24. Forte, T. M., and R. W. Nordhausen. 1986. Electron microscopy of negatively stained lipoproteins. *Methods Enzymol.* **128:** 442-457.
25. Musliner, T. A., M. D. Long, T. M. Forte, A. V. Nichols, E. L. Gong, P. J. Blanche, and R. M. Krauss. 1991. Dissociation of high density lipoprotein precursors from apolipoprotein B-containing lipoproteins in the presence of unesterified fatty acids and a source of apolipoprotein A-I. *J. Lipid Res.* **32:** 917-933.
26. Eisenberg, S., D. Feldman, and T. Olivecrona. 1981. Effects of two albumins and two detergents on the activity of bovine milk lipoprotein lipase against very low density and high density lipoprotein lipids. *Biochim. Biophys. Acta.* **665:** 454-462.
27. Fainaru, M., and R. J. Deckelbaum. 1979. Lipid binding protein (apolipoprotein A-I) contamination of high grade commercial albumins. *FEBS Lett.* **97:** 171-174.
28. Sammett, D., and A. R. Tall. 1985. Mechanisms of enhancement of cholesteryl ester transfer protein activity in lipolysis. *J. Biol. Chem.* **260:** 6687-6697.
29. Goodman, D. S., and E. Shafrir. 1959. The interaction of human low density lipoproteins with long chain fatty anions. *J. Am. Chem. Soc.* **81:** 364-370.
30. Shafrir, E., S. Gatt, and S. Khasis. 1965. Partition of fatty acids of 20-24 carbon atoms between serum albumin and lipoproteins. *Biochim. Biophys. Acta.* **98:** 365-371.
31. Shafrir, E. 1958. Partition of unesterified fatty acids in normal and nephrotic syndrome serum and its effect on serum electrophoretic pattern *J. Clin. Invest.* **37:** 1775-1782.
32. Gordon, R. S. 1955. Interaction between oleate and the lipoproteins of human serum. *J. Clin. Invest.* **34:** 477-484.
33. Blanchette-Mackie, E. J., and R. O. Scow. 1971. Sites of lipoprotein lipase activity in adipose tissue perfused with chylomicrons. *J. Cell Biol.* **51:** 1-25.
34. Scow, R. O., M. Hamosh, E. J. Blanchette-Mackie, and A. J. Evans. 1972. Uptake of blood triglycerides by various tissues. *Lipids.* **7:** 497-505.
35. Spooner, P. J. R., S. Bennett Clark, D. L. Gantz, J. A.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012

Hamilton, and D. M. Small. 1988. The ionization and distribution behavior of oleic acid in chylomicrons and chylomicron-like emulsion particles and the influence of serum albumin. *J. Biol. Chem.* **263:** 1444–1453.
36. Blanchette-Mackie, E. J., and R. O. Scow. 1976. Retention of lipolytic products in chylomicrons incubated with lipoprotein lipase: electron microscope study. *J. Lipid Res.* **17:** 57–67.
37. La Belle, M., and R. M. Krauss. 1990. Differences in carbohydrate content of low density lipoproteins associated with low density lipoprotein subclass patterns. *J. Lipid Res.* **31:** 1577–1588.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012