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

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Abstract Incubation of low (LDL), intermediate (IDL), or very low density lipoproteins (VLDL) with palmitic acid and either high density lipoproteins (HDL), delipidated HDL, or purified apolipoprotein (apo) A-I resulted in the formation of lipoprotein particles with discoidal structure and mean particle diameters ranging from 146 to 254 Å by electron microscopy. Discs produced from IDL or LDL averaged 26% protein, 42% phospholipid, 5% cholesteryl esters, 24% free cholesterol, and 3% triglycerides; preparations derived from VLDL contained up to 21% triglycerides. ApoA-I was the predominant protein present, with smaller amounts of apoA-II. Crosslinking studies of discs derived from LDL or IDL indicated the presence of four apoA-I molecules per particle, while those derived from large VLDL varied more in size and contained as many as six apoA-I molecules per particle. Incubation of discs derived from IDL or LDL with purified lecithin:cholesterol acyltransferase (LCAT), albumin, and a source of free cholesterol produced core-containing particles with size and composition similar to HDL_{2b}. VLDL-derived discs behaved similarly, although the HDL products were somewhat larger and more variable in size. When discs were incubated with plasma d>1.21 g/ml fraction rather than LCAT, core-containing particles in the size range of normal HDL_{2a} and HDL_{3a} were also produced. A variety of other purified free fatty acids were shown to promote disc formation. In addition, some mono and polyunsaturated fatty acids facilitated the formation of smaller, spherical particles in the size range of HDL_{3c}. Both discoidal and small spherical apoA-I-containing lipoproteins were generated when native VLDL was incubated with lipoprotein lipase in the presence of delipidated HDL. We conclude that lipolysis product-mediated dissociation of lipid–apoA-I complexes from VLDL, IDL, or LDL may be a mechanism for formation of HDL subclasses during lipolysis, and that the availability of different lipids may influence the type of HDL-precursors formed by this mechanism. —**Musliner, T. A., M. D. Long, T. M. Forte, A. V. Nichols, E. L. Gong, P. J. Blanche, and R. M. Krauss.** 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.* 1991. **32:** 917–933.

Supplementary key words HDL • IDL • LDL • VLDL • lipoprotein heterogeneity • lipid transfer • lipolysis

Surface lipids and apolipoproteins are known to transfer from triglyceride-rich lipoproteins to the high density lipoprotein (HDL) fraction in the course of intravascular lipolysis (1–3). The physical basis for these transfers is poorly understood. Some in vitro studies suggest that lipid transfers from triglyceride-rich lipoproteins may contribute to conversion of HDL_3 to more buoyant HDL_2 species during lipolysis, through reactions that may involve lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) (4, 5). Conversion of HDL_3 to HDL_2-like particles has also been demonstrated in vitro after incubations of very low density lipoproteins (VLDL), HDL_3, LCAT, and CETP in the absence of lipase (6). The extent to which any of these conversions occur in vivo is unknown. The possibility that HDL species may be generated de novo from triglyceride-rich lipoprotein-derived lipid and a source of HDL apolipoprotein has not been explored.

We have previously reported (7) that lipolysis of VLDL in the presence of low density lipoproteins (LDL) and HDL, under conditions favoring the accumulation of lipolysis products, results in the binding of HDL-derived apolipoprotein (predominantly apoA-I) to apoB-containing lipoproteins. Lipid extracts of lipolyzed VLDL or purified free fatty acids (FFAs) also induced association of apoA-I with VLDL or LDL in the absence of lipase, suggesting

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

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that they were involved in the apoA-I binding reaction. Furthermore, limited VLDL lipolysis in the presence of delipidated ^{125}I-labeled HDL resulted in the appearance of newly formed particles in the HDL size range. Taken together, these findings suggested a potential pathway for the generation of HDL subclasses in which FFAs or other lipolysis products initially stimulate the association of apoA-I (and possibly other apolipoproteins) with apoB-containing particles, with subsequent dissociation of lipid-protein complexes representing HDL precursors. The studies reported here support such a pathway and demonstrate that lipoproteins resembling HDL_{2b}, as well as certain smaller HDL subspecies, can be produced in vitro by this mechanism.

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), IDL (d 1.006–1.019 g/ml), and LDL (d 1.019–1.063 g/ml) fractions were isolated by preparative ultracentrifugation under standard conditions (8). HDL was isolated at d 1.085–1.21 g/ml in order to minimize contaminating Lp[a]. LDL was further subfractionated by density gradient ultracentrifugation as described by Shen et al. (9). VLDL and IDL subfractions were isolated by nonequilibrium density gradient ultracentrifugation as previously described (10). Where necessary, lipoprotein solutions were concentrated by ultrafiltration using Amicon (Danvers, MA) XM50 or PM10 filters. Lipoproteins were iodinated using the iodine monochloride method (11, 12). 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 (13). Radiolabeled palmitic or oleic acids were purchased from New England Nuclear (Cambridge, MA).

Purification of lipoprotein lipase, LCAT, and apolipoproteins

Lipoprotein lipase was purified from bovine milk by the method of Bengtsson and Olivecrona (14), except that a gradient of 0.5 M to 2.0 M NaCl was used for the second heparin-Sepharose affinity chromatography step. The specific activity of the purified enzyme ranged from 3,000 to 6,000 units/mg, where 1 unit = 1 μmol of fatty acid liberated per h at 27°C, using the Triton X-100-stabilized triolein emulsion assay of Krauss, Levy, and Fredrickson (15). ApoA-I and apoA-II were purified from human HDL as previously described (16, 17). Partially purified LCAT was prepared from the plasma d>1.21 g/ml ultracentrifugation fraction as previously described (16). The purification was approximately 3000-fold and LCAT preparations were shown to be free of CETP activity. LCAT was added to incubation mixtures to an activity level of 160 units/ml, where 1 unit represents 1 nmol of cholesteryl ester formed per h using the proteoliposome assay method of Chen and Albers (18), to approximate the activity in normal plasma.

Incubation conditions

Fatty acid-free bovine and human serum albumin and nonradioactive purified fatty acids were purchased from Sigma. Lipoprotein incubations were carried out in covered polypropylene or glass tubes in a shaking water bath at 37°C. 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. Unless otherwise specified, incubation mixtures contained 0.01% EDTA and 0.2 M Tris buffer, pH 7.5. Solutions of albumin containing bound palmitic acid were prepared by incubating palmitic acid in the form of the sodium salt 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 and the final concentration of FFA was measured with the use of NEFA kits (Wako, Japan). In incubation studies in which the sodium salt of palmitic acid was used, it was added in solution in 0.2 M Tris buffer, pH 7.5.

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 which, unless otherwise specified, ranged in density 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 centrifuge. Background densities resulting from these centrifugations, as determined from control tubes containing no lipoprotein samples with density directly measured using a DMA 46 Mettler/Paar density meter, are indicated in relation to specific experiments.

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 (19, 20). Gels were stained for protein or lipid, calibrated, and scanned by densitometry as previously de-

scribed (21). 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 (21). 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 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, 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 (22), using monospecific sheep or goat 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 (23). 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 (24). Phospholipid was determined by the method of Bartlett (25). 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 (26). Crosslinking studies for the determination of the number of apoA-I molecules per lipoprotein particle were performed according to the method of Swaney and O'Brien (27).

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 (28).

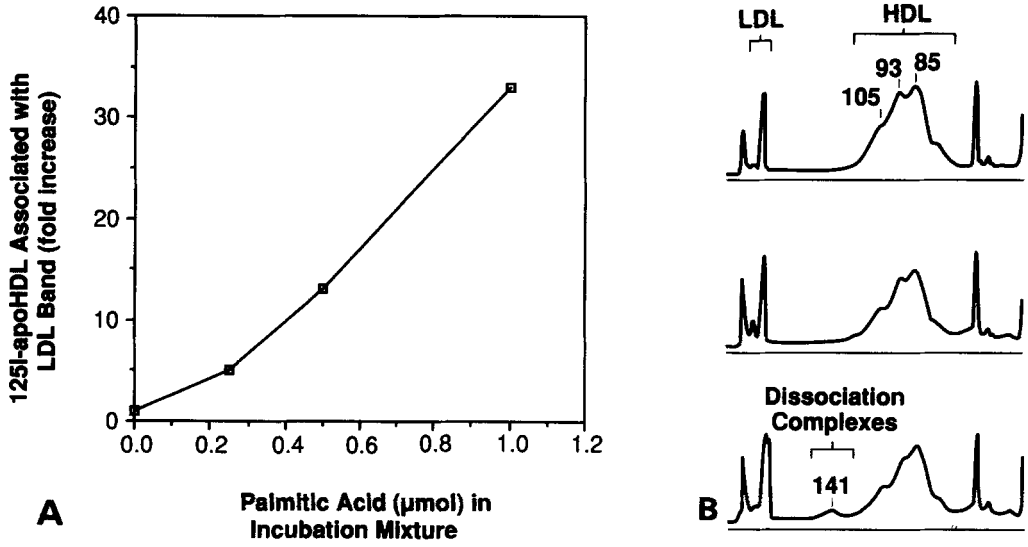


Fig. 1. Incubation of LDL (d 1.033–1.039 g/ml; 0.65 mg/ml protein) with native ^{125}I-labeled HDL (d 1.085–1.21 g/ml; 2.3 mg/ml protein) and varying amounts of palmitic acid for 2 h at 37°C. Panel A shows the relative increase in ^{125}I-labeled HDL associated with the LDL band as a function of palmitic acid concentration, estimated from densitometric scans of autoradiograms of 2–16% gradient gel blots. Panel B represents densitometric scans of 4–30% gradient gels (stained for protein) of corresponding incubation mixtures containing 0 μmol (top), 0.5 μmol (center), or 1 μmol (bottom) of palmitic acid in a final volume of 0.16 ml. The newly formed "dissociation complex" band with peak particle diameter of 141 Å is seen in the bottom curve.

RESULTS

Free fatty acid-induced generation of apoHDL-containing lipoproteins from VLDL, IDL, and LDL

In a previous report (7) we showed that FFAs stimulate the binding of HDL-derived apoA-I to LDL in vitro. In subsequent studies, described in the accompanying paper (29), we observed that apoA-I-containing LDL, IDL, and VLDL formed in this manner are subject to size transformations when sufficient quantities of FFA are present. As described further in this report, these size changes are accompanied by the appearance of newly formed, apoA-I-containing lipoproteins. An example is shown in **Fig. 1**, from an experiment in which an LDL subfraction (d 1.033–1.039 g/ml) was incubated with native ^{125}I-labeled HDL (d 1.085–1.21 g/ml) and palmitic acid in amounts ranging from 0 to 1 μmol for 2 h at 37°C. As the amount of palmitic acid present increased, the association of HDL-derived label with the LDL band on nondenaturing polyacrylamide gradient gels increased up to 33-fold above baseline (Fig. 1A). As seen in Fig. 1B, the highest palmitic acid concentration was associated with the appearance of a new band on gradient gel electrophoresis, migrating in the size range between LDL and HDL with apparent peak particle diameter of 141 Å. Oil Red O-stained gels demonstrated the presence of lipid in this band, indicating the presence of lipoprotein particles. These newly generated lipoproteins were designated "dissociation complexes." Concomitant with the formation of the latter new peak, the LDL decreased in particle diameter, as described in the companion paper (29). The profile of the HDL did not significantly change.

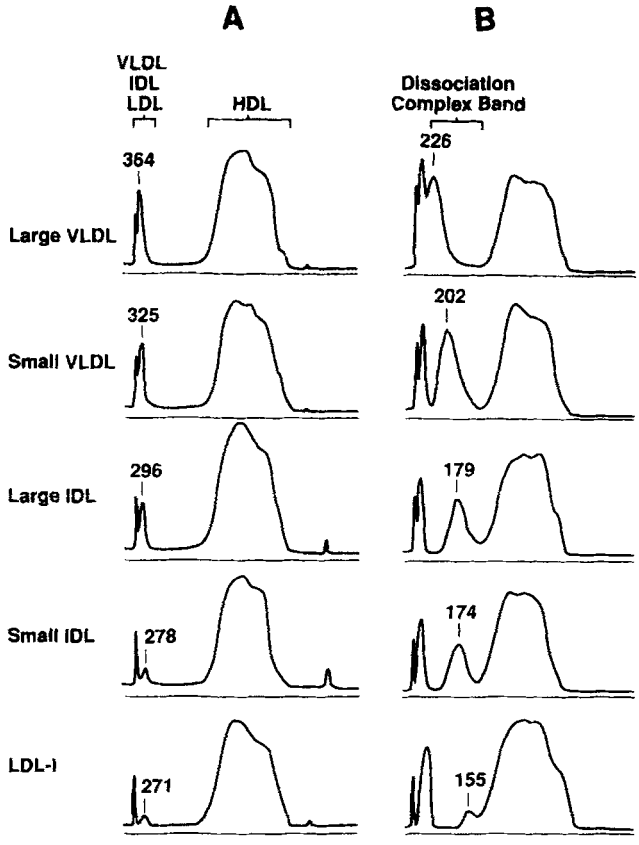


Fig. 2. Incubation of subfractions of VLDL, IDL, and LDL with native ^{125}I-labeled HDL in the presence or absence of palmitic acid for 2 h at 37°C. The incubation mixtures included the following components in a final volume of 80 μl: VLDL, IDL, or LDL subfractions, 13 μg protein; native ^{125}I-labeled HDL, 38 μg protein; palmitic acid, 0 or 0.3 μmol. A. Native ^{125}I-labeled HDL, absence of palmitic acid. B. Native ^{125}I-labeled HDL, 0.3 μg palmitic acid. The curves represent densitometric scans of autoradiograms of 4–30% gradient gel blots. Particle diameters are shown in Å, with the values for bands >250 Å taken from corresponding 2–16% gels for greater accuracy.

The formation of dissociation complexes was further investigated using a series of subfractions of VLDL, IDL, or LDL isolated from normolipidemic plasma by density gradient ultracentrifugation as outlined under Methods. Equivalent protein concentrations of each subfraction were incubated with native ^{125}I-labeled HDL in the presence or absence of added palmitic acid. The redistribution of HDL-derived ^{125}I-labeled apolipoprotein was measured by autoradiography of gradient gel blots (**Fig. 2**). In the absence of palmitic acid, small amounts of ^{125}I-labeled apoHDL associated with the different apoB-containing lipoproteins to varying degrees (29), but there was otherwise no significant change in the gradient gel profiles. In the presence of palmitic acid, however, a new ^{125}I-labeled apoHDL-containing band was formed in each case, migrating between the apoB-containing lipoprotein and the migration position of normal HDL_{2b}.

Dissociation complexes in the same general size range were generated in incubations in which delipidated rather than native ^{125}I-labeled HDL was used (**Fig. 3**). The reaction was observed at somewhat lower HDL protein concentrations with delipidated compared to native HDL, and in some cases (e.g., compare dissociation complex bands formed from LDL-I in Fig. 2 and 3) the dissociation complex bands formed from delipidated versus native HDL were of slightly larger apparent particle diameter. Larger apoB-containing lipoproteins also yielded dissociation complexes with larger apparent particle diameter at similar palmitic acid concentrations compared to smaller donor apoB-containing lipoproteins, when either native or delipidated HDL were used. In some cases there was the suggestion of particle size heterogeneity within the dissociation complex band (e.g., Fig. 3B, top panel). When LDL was excluded from the incubation mixture, no ^{125}I-labeled apoHDL label was seen in particles greater in apparent particle diameter than the albumin standard (71 Å) and what label was retained on the gel migrated primarily as a band at the bottom of the gel, seen both in the presence or absence of palmitic acid, probably representing aggregated apoA-I and/or apoA-II.

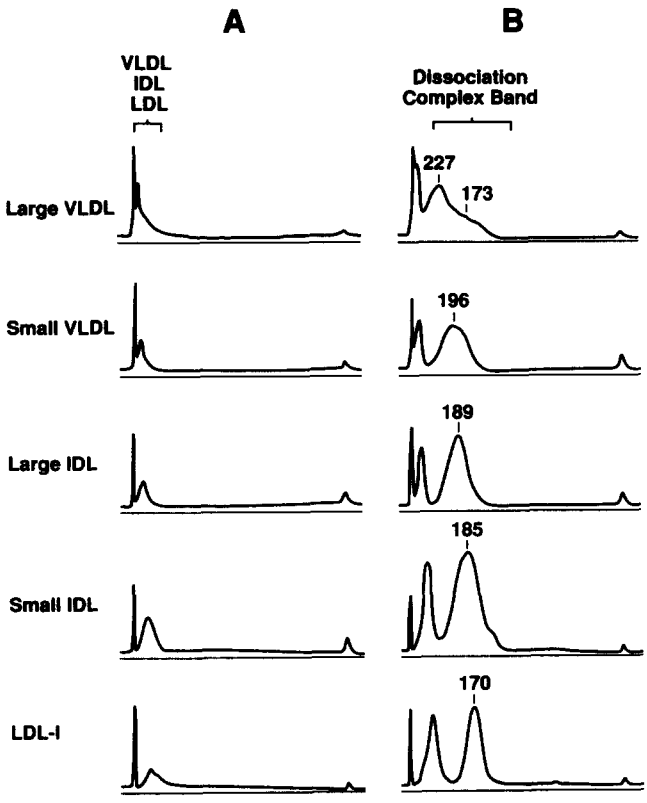


Fig. 3. Incubation of subfractions of VLDL, IDL, and LDL with delipidated ^{125}I-labeled HDL in the presence or absence of palmitic acid for 2 h at 37°C. The incubation mixtures included the following components in a final volume of 80 μl: VLDL, IDL, or LDL subfractions, 13 μg protein; delipidated ^{125}I-labeled HDL, 22 μg protein; palmitic acid, 0 or 0.3 μmol. The curves represent densitometric scans of autoradiograms of 4–30% gradient gel blots. A. Delipidated ^{125}I-labeled HDL, absence of palmitic acid. B. Delipidated ^{125}I-labeled HDL, 0.3 μmol palmitic acid. Particle diameters are shown in Å.

When purified apoA-I replaced delipidated apoHDL in similar incubations, formation of dissociation complexes was again observed. This is shown in **Fig. 4** where large VLDL, small VLDL, and IDL were incubated with purified apoA-I in the presence or absence of palmitic acid. Products were analyzed on 2–16% gradient gels. The Coomassie blue-stained gels (Fig. 4A) demonstrated the appearance of small dissociation complex bands in the size range similar to those observed above. Immunostaining of corresponding gradient gel blots using a monospecific polyclonal antiserum (Fig. 4B) confirmed the presence of apoA-I in the dissociation complex band, as well as associated with the VLDL and IDL bands, described in more detail in the companion paper (29). The immunoblots again suggested particle size heterogeneity, particularly among the dissociation complexes formed from the VLDL subfractions. Immunostaining using a monospecific, polyclonal antibody directed against apoB, in contrast, failed to detect apoB antigenic sites in the dissociation complex bands (data not shown). Dissociation complexes were not formed when purified apoA-II was used alone in place of apoA-I in incubations with LDL and palmitic acid.

As shown in the companion paper (29), an inverse relationship was observed between the size of the dissociation complex formed under varying conditions and the calculated volume reduction in the donor apoB-containing lipoprotein (r = 0.8). It is also demonstrated that dissociation complexes are formed when palmitate is added as the sodium salt (0.2 mM in 0.2 M Tris, pH 7.5) or when palmitate is present in fully solubilized form in the presence of FFA-free human serum albumin at a molar ratio of 5:1. In the experiments reported here, albumin was excluded from the incubation mixtures to eliminate potential effects due to apolipoproteins or other contaminants that may be present in commercial albumin preparations (30, 31).

Physical characterization of dissociation complexes

Dissociation complexes formed from apoB-containing lipoproteins, a source of apoA-I, and palmitic acid were separated from other components in the incubation mixture by density gradient ultracentrifugation (see Methods). The material corresponding to the dissociation complex

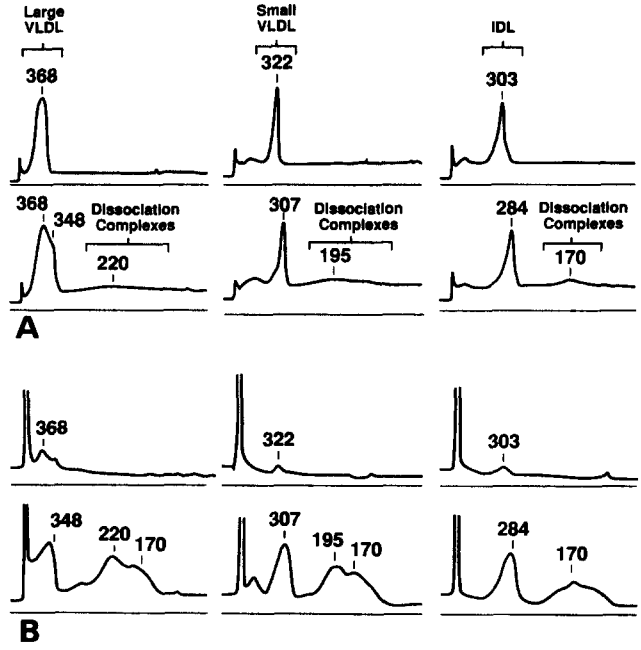
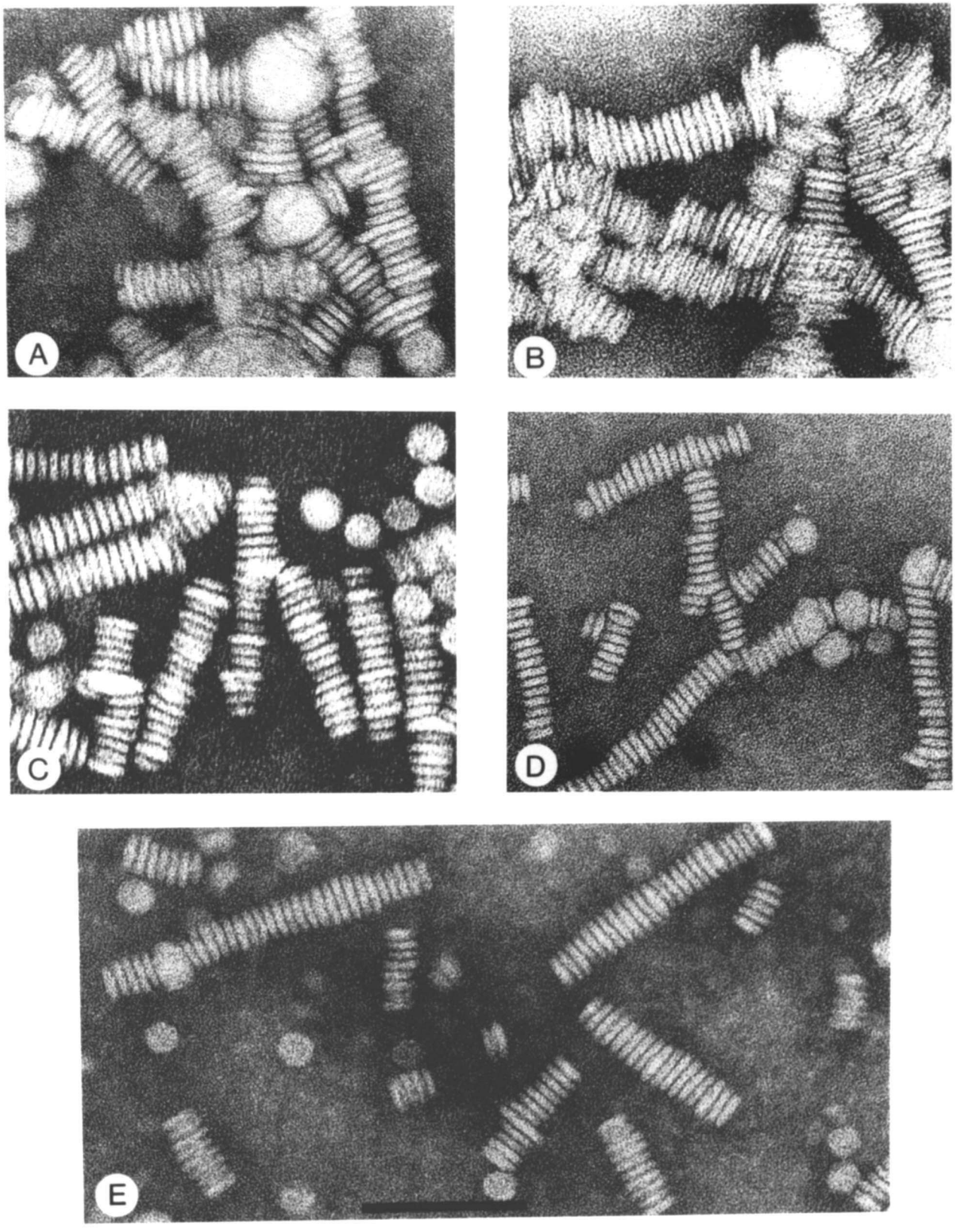


Fig. 4. Incubation of large VLDL (53 μg protein), small VLDL (12 μg protein), and IDL (14 μg protein) with purified apoA-I (21 μg protein) for 2 h at 37°C in the absence or presence of palmitic acid (0.4 μmol) in a final volume of 60 μl. The curves in A represent densitometric scans of 2–16% gradient gels stained for protein with Coomassie G-250, while the curves in B represent densitometric (reflectance) scans of 2–16% nitrocellulose gradient gel blots immunostained for apoA-I. The top panels of both A and B are from incubations performed in the absence of palmitic acid, while palmitic acid was present in the incubations shown in the corresponding bottom panels.



band formed from small VLDL, IDL, or LDL was found in fractions of density ranging from approximately 1.040 to 1.085 g/ml. When FFA-free human serum albumin was added (molar ratio of palmitic acid:albumin = 4:1) and the incubation was continued for an additional 30 min prior to centrifugation, the dissociation complex peak shifted to a slightly higher density range (1.060 to 1.100 g/ml, for LDL-derived complexes). Although the buoyant density of the material centrifuged without prior removal of excess FFA overlapped the conventional LDL density range, it could be cleanly separated from LDL in such incubation mixtures when relatively buoyant (d 1.025–1.033 g/ml) LDL subfractions were used for their preparation or by increasing the density difference between the two species by brief incubation with FFA-free albumin prior to centrifugation.

Representative electron micrographs of reisolated dissociation complexes generated from incubations of large VLDL, small VLDL, IDL, or large LDL with palmitic acid and either apoHDL or purified apoA-I are shown in **Fig. 5.** The predominant species in all cases had discoidal morphology with mean dimensions of the long axis ranging from 146 to 265 Å, and the short axis 43 to 48 Å. Discoidal dissociation complexes deriving from LDL or IDL (Fig. 5C, D, and E) were remarkably uniform in size and appearance. Dissociation complexes derived from VLDL (Fig. 5A and B) were larger and displayed greater variability of the length of the long axis; in addition, these preparations contained occasional large, round profiles which probably represent core-containing particles. After incubation of LDL-apoHDL-derived dissociation complexes with human FFA-free albumin as described above, [^{3}H]palmitic acid comprised <2% of their total dry weight; however, most of the particles remained discoidal as judged by electron microscopy.

Apolipoprotein and lipid composition of dissociation complexes

Dissociation complexes produced by incubating LDL with delipidated ^{125}I-labeled HDL and palmitic acid were reisolated by density gradient ultracentrifugation and analyzed for apolipoprotein content by SDS polyacrylamide gradient gel electrophoresis after delipidation.

Fig. 5. Electron microscopy of dissociation complexes. ApoHDL-containing dissociation complexes generated during incubation of different apoB-containing lipoproteins with apoHDL or purified apoA-I in the presence of palmitic acid were isolated by density gradient ultracentrifugation and analyzed by electron microscopy. All micrographs are 300,000 × magnification; the bar represents 1000 Å and applies to all micrographs. The sources of dissociation complexes were: A. large VLDL, apoHDL; B. small VLDL, apoHDL; C. IDL, apoHDL; D. LDL, apoHDL; and E. LDL, purified apoA-I. Large round particles that are apparently not discoidal are seen in A and B. The dimensions of the long axis of the discoidal particles were: A. 254 ± 52 Å; B. 265 ± 41 Å; C. 222 ± 36 Å; D. 146 ± 17 Å; and E. 215 ± 25 Å. The disc short axis ranged from 43 ± 5 to 48 ± 5 Å.

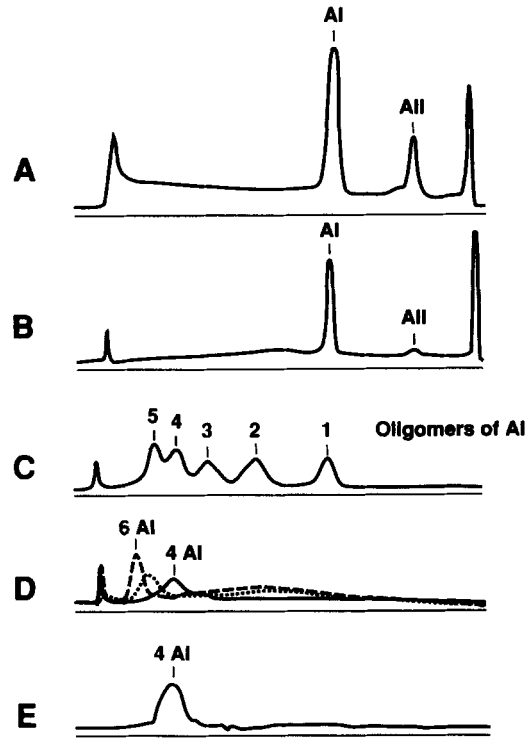


Fig. 6. Apolipoprotein content of dissociation complexes derived from incubations of LDL with apoHDL or purified apoA-I. The curves represent densitometric scans of 4–30% SDS gradient gels. A. Starting apoHDL fraction; Coomassie blue R-250-stained gel. B. Delipidated dissociation complexes derived from incubation of LDL (5.4 mg protein), apoHDL (4.4 mg protein), and palmitic acid (52 μmol) for 2 h at 37°C in a final volume of 4.2 ml, followed by isolation of dissociation complexes by density gradient ultracentrifugation as described in Methods; Coomassie R-250-stained gel. C. Crosslinked apoA-I; Coomassie blue R-250-stained gel. D. Delipidated, crosslinked dissociation complexes derived from incubation of either LDL (solid line) (1.8 mg protein), large VLDL (dashed line) (d<1.003 mg/dl; 2.6 mg protein) or small VLDL (dotted line) (d 1003–1.006 g/ml; 0.6 mg), with apoA-I (1.1 to 1.5 mg protein), and palmitic acid (15 to 20 μmol), in a final volume of 2.0 to 3.0 ml, followed by isolation of dissociation complexes by density gradient ultracentrifugation; Coomassie blue R-250-stained gel. E. Delipidated, crosslinked dissociation complexes derived from incubation of LDL (0.38 mg protein), ^{125}I-labeled apoHDL (0.84 mg protein), and palmitic acid (8 μmol) for 2 h at 37°C in a final volume of 1.3 ml, followed by isolation of dissociation complexes by density gradient ultracentrifugation as described in Methods; densitometric scan of autoradiogram.

Coomassie blue-stained gels shown in **Fig. 6A and B** demonstrate that the dissociation complex fraction was considerably enriched in apoA-I relative to apoA-II in comparison with the starting apoHDL fraction. When quantitated by densitometric scanning of autoradiograms of SDS gel blots, the ratio of A-I:A-II was approximately 10:1. Crosslinking analyses of LDL-apoHDL or LDL-apoA-I-derived dissociation complexes were performed as outlined in Methods. SDS gradient gels of the crosslinked samples (Fig. 6C–6E) revealed a predominant species with molecular weight equivalent of four apoA-I per particle in both cases. IDL-derived dissociation complexes also contained predominantly four apoA-I per particle, while

the larger dissociation complexes derived from VLDL were more heterogeneous in their apoA-I content, with crosslinking analyses yielding values ranging from four to six molecules per particle (Fig. 6D).

Chemical composition analyses, summarized in **Table 1,** were performed on dissociation complexes prepared from different apoB-containing lipoproteins under varying conditions. Protein, phospholipid, and free cholesterol were the major constituents. Smaller amounts of cholesteryl esters and triglycerides were present, with the relative amounts varying among different preparations. Dissociation complexes derived from VLDL contained larger amounts of triglyceride, possibly associated with the apparent core-containing larger round particles observed by electron microscopy (Fig. 5A). The amount of palmitic acid contained in the dissociation complex fractions was considerable in the absence of FFA-sequestering agents and also varied greatly (from 6 to 46% of total dry weight) among different preparations. Greater than 95% of the palmitic acid was removed from LDL-derived dissociation complex fractions after 30 min incubation with FFA-free human serum albumin and reisolation by density gradient ultracentrifugation. This treatment also resulted in a reduction of cholesteryl ester content and relative increase in phospholipids (Table 1).

Conversion of dissociation complexes to lipoproteins resembling HDL subclasses in the presence of LCAT

LDL was incubated with ^{125}I-labeled apoHDL and palmitic acid for 2 h at 37°C, after which FFA-free human serum albumin was added (final concentration 6%) together with partially purified LCAT at a concentration of 160 units/ml. The incubation was then continued for a total of 24 h. A control mixture was incubated similarly in the absence of added LCAT and albumin. The density gradient ultracentrifugation profile of the two mixtures is shown in **Fig. 7,** demonstrating a shift to higher density of the dissociation complex fraction in the incubation mixture that contained albumin and LCAT (compare fractions A and B). Gradient gel electrophoresis demonstrated a reduction in apparent peak particle diameter from 169 Å to 123 Å in the albumin/LCAT-treated sample (**Fig. 8**). Electron microscopy revealed conversion of the discoidal dissociation complexes (mean diameter 204 Å) to mostly round, core-containing particles with mean diameter 112 Å (Fig. 8). The latter were within the size range of normal HDL_{2b} (21).

Additional incubations were performed using dissociation complexes that had been isolated by density gradient ultracentrifugation prior to exposure to purified LCAT. Either LDL or washed human erythrocytes were added as a source of free cholesterol. LCAT-induced changes of isolated dissociation complexes derived from LDL were similar to those described above. **Fig. 9** shows examples for dissociation complexes derived from small VLDL and IDL. The complexes from both sources (Fig. 9A) showed a degree of size heterogeneity, with a major peak at 190–194 Å, flanked by peaks of much smaller mass with apparent diameters of 225–230 Å and 162 Å, respectively. After treatment with LCAT in the presence of albumin and human erythrocytes (Fig. 9B), this material was converted to smaller, broader bands with apparent mean peak particle diameters in the range of 120–130 Å (as measured by 4–30% gradient gel electrophoresis). The band derived from the IDL-derived dissociation complexes was slightly sharper and smaller, but in both cases the largest amount of material was seen at the larger end

TABLE 1. Chemical composition[a] of apoHDL-containing dissociation complexes generated from LDL, IDL, or VLDL in the presence of palmitic acid

Incubation Constituents	Protein	Cholesteryl Esters	Free Cholesterol	Triglyceride	Phospholipid
LDL + apoHDL	28.3	11.9	22.9	1.8	35.2
LDL + apoHDL, with subsequent incubation with FFA-poor albumin	28.2	4.4	23.1	2.7	41.6
LDL + apoA-I	31.2	11.1	15.5	4.0	38.2
IDL + apoHDL	20.5	2.6	23.9	4.1	48.9
IDL + apoA-I	20.1	0.0	33.5	4.5	41.9
Small VLDL + apoHDL	17.0	3.1	24.1	11.1	44.6
Small VLDL + apoA-I	15.1	0.0	39.0	9.8	36.1
Large VLDL + apoHDL	17.1	9.9	20.3	17.3	35.5
Large VLDL + apoA-I	14.9	3.7	21.0	21.0	39.3

[a]Percent dry weight, excluding free fatty acids. The palmitic acid content of the discoidal particles varied widely (from 6 to 46% of dry weight) under different conditions. Following 30-min incubation with fatty acid-poor human serum albumin (at a molar ratio of palmitic acid:albumin of 3:1), the palmitic acid content of reisolated discs was reduced to <0.7%. Incubations were otherwise carried out for 2 h at 37°C. The apoB-containing lipoproteins used in these incubations included subfractions in the following density ranges: LDL, d 1.025–1.039 g/ml; IDL, d 1.006–1.019 g/ml; small VLDL, d 1.003–1.006 g/ml; and large VLDL, d<1.003 g/ml. Dissociation complexes were separated from other incubation components prior to compositional analysis by density gradient ultracentrifugation as described in Methods. Data represent means of duplicates from individual experiments.

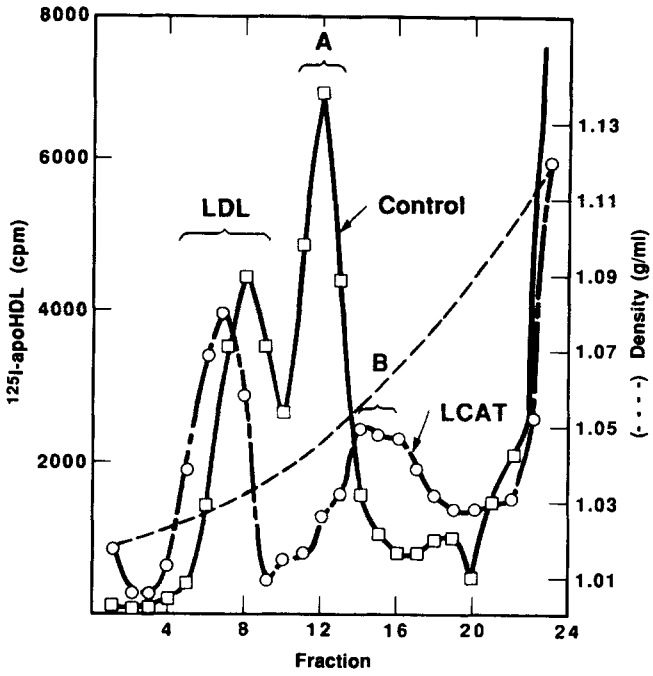


Fig. 7. Modification of LDL-derived dissociation complexes by LCAT. The initial incubation mixture included LDL (0.38 mg), ^{125}I-labeled apoHDL (0.84 mg), and palmitic acid (6.0 μmol) in a final volume of 1.3 ml. After 2 h at 37°C, FFA-free human serum albumin (final concentration 6%), and purified LCAT (to plasma concentration) were added (final volume 2.1 ml) and the incubation was continued for a total of 24 h. Buffer solution was added to the control incubation mixture in place of albumin and LCAT. Density gradient ultracentrifugation was performed as described in Methods. The distribution of ^{125}I-label among the density gradient fractions is shown for the control incubation (open squares) and for the incubation mixture that included albumin and LCAT (open circles). The fractions indicated by the brackets were pooled and further analyzed as described in the text and shown in Fig. 8 and Table 2.

of the HDL_{2b} (97–129 Å, (21)) size range. There was also the suggestion of a smaller band in the HDL_{2a} region (88–97 Å, (21)), with peak diameter of 91–92 Å. Electron microscopy demonstrated the production of core-containing particles in each case (not shown). When the same fractions were incubated in the presence of washed red blood cells and lipoprotein free plasma ($d > 1.21$ g/ml) rather than purified LCAT, considerably greater size reductions were observed (Fig. 9C), with the major band of the modified material now in the size range of normal HDL_{2a}, with peaks or shoulders of smaller mass in the HDL_{2b} and HDL_{3a} (82–88 Å, (21)) size regions. This suggests the potential involvement of other plasma factors besides LCAT in the transformations of these lipoproteins.

Chemical composition analyses of LDL-derived dissociation complexes reisolated by density gradient ultracentrifugation after incubation with LCAT, albumin, and a source of free cholesterol revealed large increases in cholesteryl ester content. The final composition (**Table 2**) of the LCAT-treated LDL-derived dissociation complexes was similar to that reported for the HDL_{2b} subfraction isolated from normolipidemic human subjects by Anderson et al. (32), although the amount of free cholesterol in these particles remained relatively high.

Differences among fatty acids in the generation of dissociation complexes from LDL

A variety of FFAs of varying chain length and degree of saturation were compared for their ability to generate dissociation complexes upon incubation with LDL and a source of apoA-I. The fatty acids tested included myristic, myristoleic, palmitic, palmitoleic, oleic, linoleic, linolenic, stearic, arachidic, and eicosapentaenoic acids. Incubation mixtures contained 0.0 to 0.2 μmol of FFA, 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. After 2 h at 37°C, the incubation mixtures were analyzed on 4–30% gradient gels stained for protein or blotted onto nitrocellulose paper and subjected to autoradiography. ApoHDL-containing dissociation complexes similar in size to those formed in the presence of palmitic acid were observed for myristic, stearic, myristoleic, palmitoleic, oleic, linoleic, linolenic, and eicosapentaenoic acids. The reaction was not observed under these conditions with arachidic acid or with the monoglycerides 1-monopalmitoyl-glycerol and 2-monopalmitoyl glycerol. When mono- or polyunsaturated FFAs were present (specifically oleic, palmitoleic, linoleic, linolenic, and eicosapentaenoic acids), a smaller ^{125}I-labeled apoHDL-containing species in the HDL density range was formed in addition to the typical, larger discoidal complexes observed with palmitic acid and the other FFAs listed above. **Fig. 10** shows an example for linoleic acid incubated with two different sized LDL subpopulations. The Coomassie blue-stained 4–30% gradient gels (Fig. 10A) revealed dissociation complex bands at 153 Å and 133 Å formed in the incubation mixtures containing the larger and smaller LDL species, respectively. Electron microscopy confirmed discoidal morphology of both of these species. An additional, sharper band, however, was seen at apparent peak diameter of 73–74 Å, just above the albumin standard, migrating in the lower end of the size range of normal HDL_{3c} (72–78 Å, (21)). Autoradiograms of corresponding gel blots (Fig. 10B) demonstrated the presence of ^{125}I-labeled apoHDL associated with both bands. Immunoblotting (Fig. 10C) confirmed the presence of apoA-I in these peaks. The anti-apoA-I immunoblots also suggested the presence of minor bands intermediate in size between the large dissociation complex band and the band at 73–74 Å. In control incubations in which LDL was excluded from the incubation mixtures, that portion of the apoHDL that was retained on 4–30% gels migrated below the albumin standard; Coomassie blue-stained gels in this case showed no bands larger than albumin (data not shown).

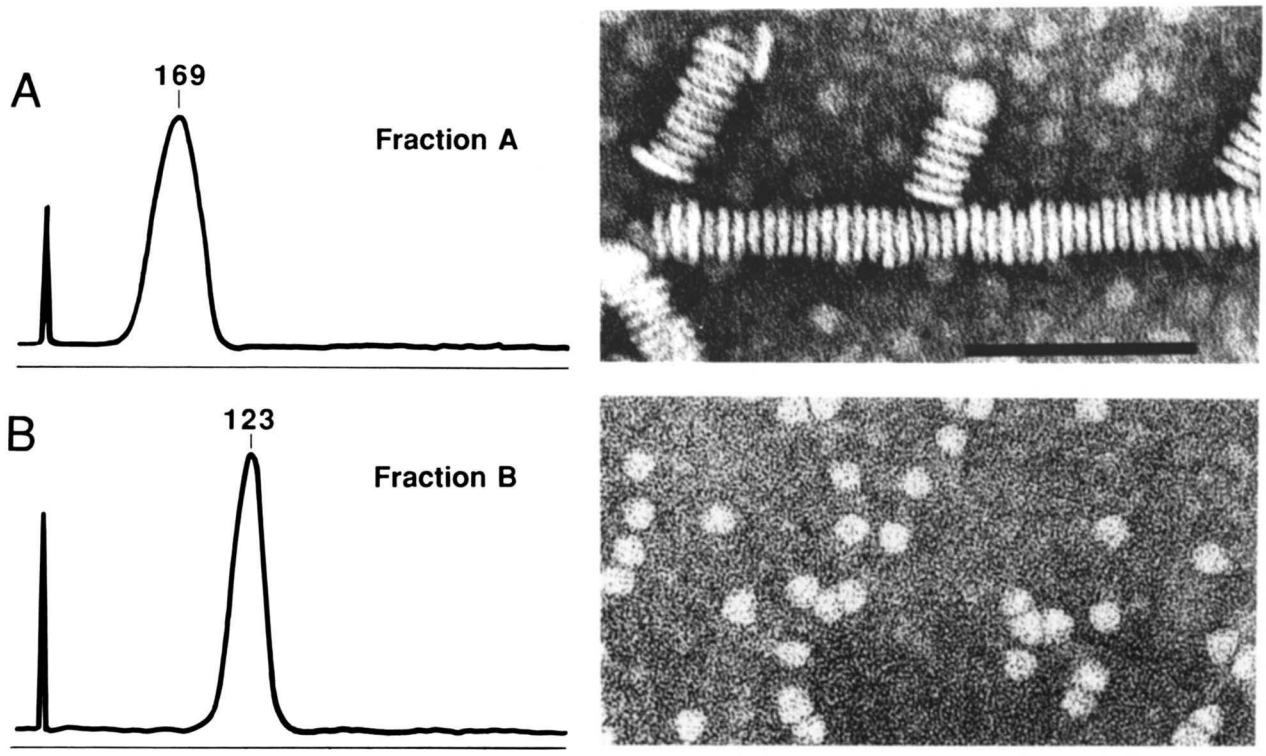


Fig. 8. Conversion of LDL-derived dissociation complexes to lipoproteins resembling HDL_2 in the presence of LCAT. The pooled fractions obtained by density gradient ultracentrifugation as shown in Fig. 7 were analyzed by gradient gel electrophoresis and electron microscopy. A. Densitometric scan of a 4–30% gradient gel blot autoradiogram of fractions A and B derived from the incubations and density gradient ultracentrifugation re-isolation procedure outlined in Fig. 7. B. Electron micrographs (300,000 × magnification) of the same fractions. The bar represents 1000 Å and applies to both micrographs.

The physical and chemical properties of the smallest sized species formed in the presence of LDL under these conditions were investigated using a preparation reisolated by density gradient ultracentrifugation after an incubation including ^{125}I-labeled apoHDL and oleic acid. The small particles were concentrated in the density range of 1.080–1.100 g/ml. This fraction contained approximately 30% unesterified oleic acid by weight. Chemical composition analysis (excluding bound FFA) otherwise revealed 66% protein, 18.4% phospholipid, 6.0% free cholesterol, 6.0% cholesteryl esters, and 3.9% triglycerides. ApoA-I comprised more than 90% of the protein present (as determined by SDS electrophoresis). Cross-linking analysis indicated the presence of both monomeric and dimeric apoA-I. Electron microscopy showed predominantly small spherical profiles with mean diameter of 67 ± 16 Å; small numbers of larger, discoidal particles were also present, presumably representing contaminating dissociation complexes.

Production of HDL-sized particles in incubations of VLDL, lipoprotein lipase, and apoHDL

Both small particles in the size range of 72–74 Å and larger lipoproteins with the characteristics of discoidal dissociation complexes were also observed by 4–30% gradient gel electrophoresis in incubation mixtures in which endogenous FFAs were generated by the action of bovine milk lipoprotein lipase on human VLDL in the presence of ^{125}I-labeled apoHDL. A representative experiment is shown in **Fig. 11.** In the absence of lipase, detectable ^{125}I-labeled apoHDL (probably in partially aggregated form under these nondenaturing conditions) migrated primarily as a band at the bottom of the gel, below the albumin standard at 71 Å (Fig. 11A). In the presence of lipase, the apoHDL shifted into larger sized particles with the presence of distinct heterogeneity. A broad band was seen in the size range just above that of normal HDL_{2b} (band marked 160 Å in Fig. 11B) in addition to multiple narrower and quantitatively larger bands with apparent peak particle diameters of 87 Å, 75 Å, 73 Å, and 71 Å. The use of ^{125}I-labeled apoHDL with subsequent gradient gel blot autoradiography demonstrated the presence of apoHDL in all of these bands and immunostaining confirmed the presence of apoA-I in the same bands (data not shown). Nonequilibrium density gradient ultracentrifugation was used to isolate the HDL-sized products, as described under Methods. They sedimented in the density range of 1.050–1.120 g/ml, with the more buoyant fractions containing the larger bands. Electron microscopy revealed the presence of both discoidal and small

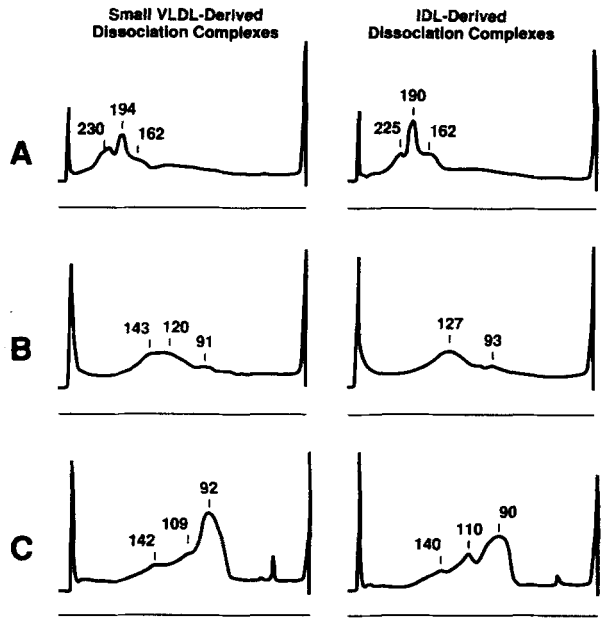


Fig. 9. Size transformations of dissociation complexes resulting from incubations with LCAT or lipoprotein-deficient human plasma. Dissociation complex fractions were generated by incubation of small VLDL (0.6 mg protein) or IDL (0.7 mg protein) with purified apoA-I (1.05 mg) and palmitic acid (20 μmol) in a final volume of 3.0 ml, and were separated from other incubation constituents by density gradient ultracentrifugation (see Methods). Aliquots were subsequently incubated with either purified LCAT (160 units/ml), FFA-free human serum albumin, and human erythrocytes or with d>1.21 g/ml plasma fraction and human erythrocytes, for 16 h at 37°C. The red blood cells were removed by low speed centrifugation, after which the lipoproteins were separated from other incubation protein components by ultracentrifugation (d 1.21 g/ml) at 40,000 rpm for 40 h at 10°C in an SW45 rotor. The lipoprotein products were subsequently analyzed on 4–30% gradient gels stained for protein with Coomassie blue. A. Small VLDL-derived (left panel) and IDL-derived (right panel) dissociation complexes incubated in the absence of other constituents. B. The same fractions following incubation with LCAT and human erythrocytes. C. The same fractions following incubation with d>1.21 g/ml plasma fraction and human erythrocytes. Densitometric scans were performed as described in Methods. Particle diameters are shown in Å.

spherical particles. Discs were the predominant particles in the more buoyant fractions (mean long axis 160 ± 40 Å), while small spherical particles (average diameter 68.1 ± 13.7 Å, within the size range of HDL_{3c}) were present in larger quantities in the denser fractions. When d>1.21 g/ml plasma fraction was included in similar incubations as a source of LCAT and other plasma proteins, the 73 Å HDL_{3c}-sized gradient gel band became the predominant product in the reisolated HDL, with minor bands at 93 Å and 78 Å (data not shown). Electron microscopy showed that nearly all of these particles were spherical.

When FFA-free bovine serum albumin was included in incubation mixtures of VLDL, lipase, and apoHDL in amounts capable of binding all released FFAs (triglyceride:albumin molar ratio of 0.67), apoA-I-containing particles in the size range similar to those formed in the absence of albumin (Fig. 11) were produced. Because of the presence of other protein bands, their presence on gradient gel profiles was obscured, but they were readily detectable by gel blotting with immunostaining for apoA-I, although overlapping protein bands produced some distortion of the profile. When d>1.21 g/ml plasma fraction substituted for albumin in similar incubations, dissociation complexes of similar appearance on gradient gels could be identified after ultracentrifugal reisolation, although the relative proportion of the smallest (HDL_{3c}) sized species was increased (data not shown).

DISCUSSION

The observations reported here suggest that FFA-induced apoA-I binding to triglyceride-rich lipoproteins may be an initial step in a process in which FFAs and surface lipids are removed from apoB-containing lipoproteins with the de novo generation of HDL precursors. The quantity of

TABLE 2. Changes in chemical composition[a] accompanying conversion of discoidal dissociation complexes to core-containing particles upon incubation with LCAT

Lipoprotein and Incubation Conditions	Protein	Cholesteryl Esters	Free Cholesterol	Triglyceride	Phospholipid
A. LDL-derived dissociation complexes (fraction A)	24.2	3.9	23.8	1.7	46.3
B. LDL-derived dissociation complexes exposed to LCAT and albumin (fraction B)	39.0	11.8	13.9	1.7	33.4
C. Isolated LDL-derived dissociation complexes incubated with LCAT, albumin, and RBCs	35.0	19.0	14.0	1.5	30.0
HDL_{2b}[b]	37.0	14.6	6.8	2.0	39.6

[a]Percent dry weight, excluding fatty acid. The lipoproteins analyzed in A and B represent fractions A and B prepared and isolated as outlined in the legends of Figs. 7 and 8. In C, the dissociation complexes were first separated from the LDL and apoHDL in the incubation mixture by density gradient ultracentrifugation (see Methods), after which they were incubated for 16 h at 37°C with purified LCAT (160 units/ml), human serum albumin, and washed human erythrocytes (RBCs). The final lipoprotein product was reisolated by ultracentrifugation at d 1.21 g/ml prior to analysis. Data represent means from duplicates of individual experiments.

[b]From reference 32.

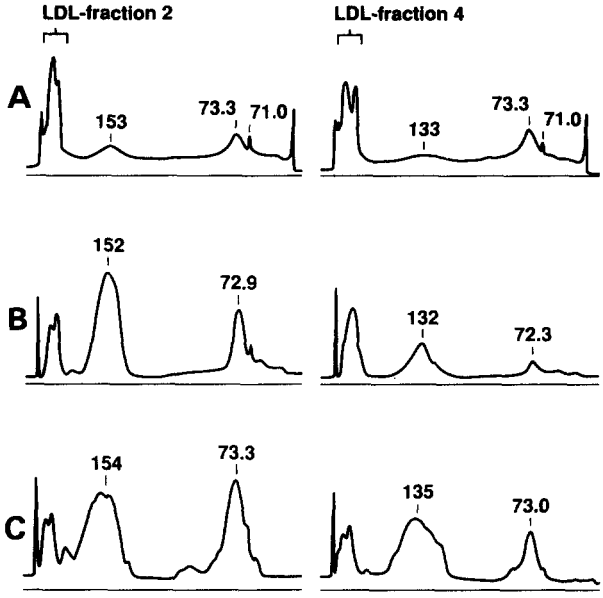


Fig. 10. Dissociation reactions produced by incubation of two density subclasses of LDL in the presence of apoHDL and linoleic acid. The incubation mixtures contained ^{125}I-labeled apoHDL (0.75 mg protein), linoleic acid (15 μmol), and either LDL-I (d 1.029–1.033 g/ml; 0.75 mg protein; left panel) or LDL-III (d 1.039–1.045; 0.80 mg protein; right panel) in a final volume of 3.0 ml. The incubation was carried out for 2 h at 37°C. A. Densitometric scans of Coomassie blue-stained 4–30% gradient gels. B. Densitometric scans of autoradiograms of gradient gel blots of the corresponding incubation mixtures. C. Densitometric (reflectance) scans of gradient gel blots of the same incubation mixtures immunostained with a monospecific, polyclonal antibody against apoA-I. Particle diameters are shown in Å.

apoA-I binding to apoB-containing lipoproteins under the conditions studied in these experiments increased as the amount of palmitic acid added to the incubation mixture increased (Fig. 1). At higher concentrations, a new lipoprotein species was formed in conjunction with a size decrement in the apoB donor lipoprotein (29). The newly generated species formed through this reaction were discoidal structures that contained apoHDL (predominantly apoA-I) but no apoB or apoB fragments. Their formation was observed when either native HDL, delipidated HDL, or purified apoA-I (but not purified apoA-II by itself) were used in these incubations. Although ^{125}I-labeled apoHDL was used in many of these experiments to aid in the tracking of newly formed dissociation complexes, the reaction occurred readily using unlabeled sources of apoA-I, excluding an artifactual effect introduced by the labeling procedure. Delipidated rather than native HDL were used in most of these studies to facilitate the reisolation of dissociation complexes which overlap in density with native HDL.

Several observations support a mechanism whereby these particles are generated by dissociation from apoA-I–apoB-containing lipoprotein intermediates. These include: *a*) the simultaneous appearance of apoA-I-containing dissociation complexes and a size-reduced, apoA-I-depleted IDL or LDL species (relative to the larger A-I, B-containing intermediate); *b*) the inverse relationship between the size of the dissociation complex and the size decrement in the apoA-I–apoB precursor lipoprotein, and *c*) simultaneous phospholipid and free cholesterol depletion of the residual apoB-containing lipoproteins (29) in conjunction with appearance of these lipids in dissociation complexes.

Formation of dissociation complexes was observed in conjunction with in vitro treatment of either VLDL, IDL, or LDL with FFAs and a source of apoA-I. At lower FFA concentrations the dissociation reaction occurred more readily with VLDL and large IDL than with small IDL or with large LDL (Fig. 2). These differences may reflect the availability of more dissociable surface lipid on the larger donor particles. Dissociation complexes formed from VLDL also tended to be bigger, more variable in size, and on the average contained more apoA-I per particle compared with complexes formed from IDL or LDL.

Although the composition of dissociation complexes formed under different conditions varied (Table 1), the major constituents were protein (predominantly apoA-I), phospholipid, and free cholesterol. Preparations derived from VLDL contained larger amounts of triglyceride, possibly associated with the apparent core-containing, larger round particles observed in these preparations by electron microscopy (Fig. 5A). These probably represented nondiscoidal lipoproteins that were either already present in the starting VLDL fraction or were formed during the incubation with palmitic acid, and subsequently co-isolated in the same density fraction as the more prevalent discoidal complexes. FFAs were a major but variable (6–46%) component of dissociation complex fractions prepared in the absence of albumin. The bulk of the FFAs could be re-

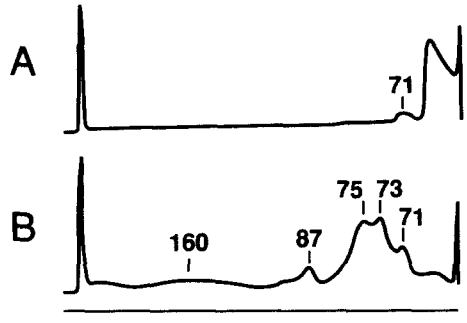


Fig. 11. Generation of HDL-sized particles during incubation of hypertriglyceridemic VLDL (1.2 mg protein) with apoHDL (1.7 mg) in the absence (A) or presence (B) of bovine milk lipoprotein lipase (28 units). The incubation mixture also contained 0.15 M NaCl and 0.2 M Tris buffer, pH 7.5, in a final volume of 800 μl. After incubation for 2 h at 37°C, samples from each mixture were analyzed by electrophoresis on 4–30% gradient gels. The curves represent densitometric scans of Coomassie blue-stained gels, with particle diameters shown in Å.

moved, however, by brief incubation with FFA-poor albumin, with only a small decrease in particle size and with preservation of the predominantly discoidal morphology as determined by electron microscopy. Dissociation complexes formed from LDL appeared to contain greater amounts of cholesteryl ester than those formed from larger apoB-containing lipoproteins. However, the reduction in cholesteryl ester content in purified fractions isolated after incubation with albumin suggests that small amounts of contaminating LDL (possibly in aggregated form) might have contributed to the higher cholesteryl ester levels detected in some LDL-derived fractions.

Incubation of dissociation complexes with LCAT and a source of free cholesterol resulted in large increases in cholesteryl ester content and transformation to core-containing particles by electron microscopy. The chemical composition as well as the size (measured by electron microscopy or gradient gel electrophoresis) of the final products formed from LDL-derived dissociation complexes was similar to that of HDL_{2b} isolated from normolipidemic plasma (Table 2) (32). The presence of four apoA-I molecules per precursor particle also corresponds with the known presence of four apoA-I per HDL_{2b} particle (34). Dissociation complexes derived from VLDL or IDL also formed core-containing, spherical particles during incubations with LCAT, somewhat larger in the case of the VLDL-derived product compared to the IDL- or LDL-derived products, overlapping the size range of HDL_1 and HDL_{2b}.

A variety of FFAs of varying chain length and degree of saturation were shown to be capable of producing discoidal dissociation complex formation from apoB-containing lipoproteins. In addition to inducing the formation of large amounts of discoidal dissociation complexes, mono or polyunsaturated FFAs gave rise to the formation of greater amounts of smaller particles in the size range of HDL_{3c}. Particles with similar mobility on gradient gels were also formed during incubations of native VLDL with lipoprotein lipase and delipidated HDL (Fig. 11). The smaller lipoproteins formed in both settings were shown by electron microscopy to represent small spherical particles. Larger discoidal particles were also produced during incubations of VLDL, apoHDL, and lipase, similar to those formed in the presence of added exogenous FFAs, along with a spectrum of additional subpopulations with varied apparent diameter, as judged by gradient gel electrophoresis. It is likely that these apoA-I-containing HDL-sized species represented dissociation complexes formed in conjunction with release of FFAs and possibly other lipolytic products of VLDL lipids. The reduction in VLDL core size during triglyceride lipolysis would be expected to result in excess surface lipid on the VLDL, which would theoretically foster FFA-mediated dissociation reactions. The formation of a broader spectrum of both small spherical and larger discoidal species by this mechanism would be consistent with the known presence of a variety of saturated, monounsaturated, and polyunsaturated fatty acids in VLDL triglycerides. Ongoing lipolysis (as well as the known phospholipase activity of lipoprotein lipase) might influence the properties of the dissociation complexes generated in this setting, possibly contributing to their smaller size, compared to those formed in incubations of VLDL with purified FFAs and a source of apoA-I in the absence of lipase.

Small HDL particles, similar in size to those produced by the mechanism described here, are of particular interest in view of evidence that they are involved in the uptake of free cholesterol from cells and its subsequent esterification by LCAT (35). Newnham and Barter (36) have recently reported that VLDL, CETP, and hepatic lipase have synergistic effects in stimulating the conversion of plasma HDL to a small (74 Å diameter, by gradient gel electrophoresis) subpopulation during in vitro incubations of plasma. These effects were observed under conditions in which the FFA binding capacity of albumin in the incubated plasma was exceeded, suggesting a role for lipoprotein-associated FFAs in the observed size changes. Since moderate amounts of small HDL were produced even in the absence of added CETP, it is possible that the dissociation reaction described in the present report was responsible in part for the generation of these particles, with the HDL pool present in the plasma serving as the source of apoHDL in the reaction with added VLDL. Our results suggest that CETP may not be required for the production of small HDL species, although its presence under the conditions studied by Newnham and Barter (36) clearly stimulated its formation. It is also possible that small amounts of contaminating CETP associated with VLDL in our lipase incubation studies contributed to the formation of the observed small HDL species. Our separate finding that discoidal, IDL- or LDL-derived dissociation complexes formed smaller core-containing species during incubations with $d > 1.21$ g/ml plasma fraction than with purified LCAT (Fig. 9) is consistent with the hypothesis that CETP may promote HDL size reduction, presumably by transfer of core cholesteryl ester to other lipoproteins or cell membranes.

Our findings also suggest that the recently described role of FFAs in stimulating the formation of small HDL in the presence of apoB-containing lipoproteins and CETP ("conversion factor" activity) (37) may involve the FFA-mediated dissociation pathway described here, rather than direct conversion of larger to smaller HDL species. Conceivably, FFA-mediated dissociation of small particles from larger HDL species could also occur in conversion factor reactions in which apoB-containing lipoproteins are not present (37, 38).

On the basis of the in vitro lipoprotein interactions reported here and previously (7, 29, 33) we propose that FFAs and apoA-I jointly influence and modulate the

metabolism of triglyceride-rich lipoproteins, LDL and HDL. In this model, hydrolysis of plasma triglycerides by lipases gives rise to the accumulation of FFAs (and other lipolysis products) on apoB-containing lipoproteins. Their presence stimulates two competing reactions: *1*) the association of apoA-I (and possibly other apolipoproteins) with VLDL, IDL, or LDL; and *2*) physical complex formation between apoB-containing lipoproteins, including VLDL and LDL (33). The latter phenomenon stimulates lipid and apolipoprotein transfers, the former through CETP- and possibly non-CETP-mediated reactions (33, 39). In the case of FFA-induced binding of apoA-I to apoB-containing lipoproteins, the reaction can proceed through a second step in which complexes of apoA-I, FFA, and surface lipid dissociate from the apoB-containing particles. Depending on the FFAs present, small spherical HDL may also be produced by this mechanism. Discoidal dissociation complexes formed by the dissociation reaction can be transformed to spherical particles resembling HDL_{2b}, and possibly other HDL subclasses. In effect, this pathway provides a mechanism whereby surface lipids and FFAs are removed from apoB-containing lipoproteins in the course of lipolysis, with resultant formation of HDL precursor particles that can acquire free and esterified cholesterol from other sources as they are converted to spherical HDL particles. The fact that different FFAs as well as different apoB-containing lipoproteins yield HDL precursors with varying characteristics suggests that FFAs may dictate the type of HDL subclasses formed during FFA-apoA-I-mediated surface depletion. The potential role of this process in the generation of IDL and LDL heterogeneity is discussed in the accompanying paper (29).

The in vitro dissociation reactions described in this report were observed at FFA:albumin molar ratios approximately fivefold greater than those seen in whole plasma under normal, steady-state conditions. Although it is not known whether such reactions occur in vivo, several arguments make their occurrence plausible. It is probable that FFA:albumin ratios higher than those seen in equilibrated whole plasma are transiently reached at the surface of triglyceride-rich lipoproteins during rapid lipolysis in the microenvironment of the capillary endothelium. 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 (40, 41), lending support to the concept of local accumulation of high levels of FFAs during lipolysis. Plasma lipoproteins are known to have high affinity for FFAs. Direct measurements of FFAs associated with the lipoprotein fraction even in whole plasma (where albumin is present in large molar excess) have yielded values ranging from 5–10% to 13–21% of the total plasma pool (42, 43). Moreover, the proportion of FFAs bound to lipoproteins relative to albumin 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 (42–45).

While it would be expected that transient accumulation of FFA would be most probable on triglyceride-rich lipoproteins undergoing lipolysis at sites of lipolysis, it is also possible that FFAs transiently bind in increased amounts to other apoB-containing particles or HDL in proximity. The fact that the accumulation of lipolysis products or FFAs on triglyceride-rich lipoproteins stimulates the physical binding of LDL or IDL to these lipoproteins (33) could provide a mechanism whereby FFAs accumulate on these particles as well. If the amounts of surface FFAs transiently localizing on lipoproteins are sufficient, it is possible that the reactions described in this report play a physiologic role in the generation of HDL subclasses as well as the remodeling of apoB-containing lipoproteins (29). While is is unlikely that most albumin-bound FFAs in whole plasma under equilibrium conditions participate in dissociation complex formation in vivo, it is possible that certain FFAs known to preferentially associate with lipoproteins (43) might also preferentially play a role in these reactions. As noted in the accompanying paper, the physical-chemical form in which FFAs are present at lipoprotein surfaces during lipolysis is uncertain, but may well be heterogeneous and include monomeric ionized and unionized forms, albumin-bound FFA, and micellar or lamellar forms containing FFA soap or acid-soap complexes (46). However, it is unlikely that the dissociation reaction is specifically dependent on FFAs in micellar form since such forms were presumably absent in the optically clear, Millipore-filtered FFA-albumin solutions (molar ratio 5:1) that readily facilitated the dissociation reaction (see Fig. 3 of the accompanying paper (29)).

It would be expected that in vivo an equilibrium exists among the multitude of reactions to which dissociation complexes may be susceptible, including transfer of FFAs to albumin, LCAT-mediated conversion of free to esterified cholesterol with associated loss of phospholipid, possible lipolysis by hepatic lipase, and lipid transfers (CETP- and possibly non-CETP-mediated) involving other lipoproteins and potentially cell membrane surfaces. The size and distributions of the final HDL products formed under specific circumstances would depend on the properties and lipid content of the apoB-containing lipoproteins from which they dissociate, as well as the balance of these secondary reactions.

A central feature of the model suggested by our findings is that FFA concentrations at the surface of apoB-containing lipoproteins constitute a pivotal modulator of both VLDL catabolism and HDL subspecies production. ApoA-I shares in this regulatory role since, as we have shown here, it facilitates the removal of surface lipids and FFAs from the surface of apoB-containing lipoproteins with the generation of HDL precursors. A variety of theoretical

implications follow that may help to explain known interrelationships between HDL and triglyceride-rich lipoproteins. For example, metabolic states in which apoA-I levels are high would be expected to result in efficient removal of FFAs and surface lipids from triglyceride-rich lipoproteins undergoing lipolysis, fostering efficient triglyceride hydrolysis and HDL_2 formation, while competing with VLDL-LDL complex formation and associated triglyceride-cholesteryl ester exchange. In states where apoA-I levels are low, in contrast, FFA and surface lipid removal from triglyceride-rich lipoproteins undergoing lipolysis would be diminished, HDL_2 generation would be lower, VLDL-LDL complex formation and triglyceride-cholesteryl ester transfers between them would be favored, and lower rates of lipolysis might result from inhibiting effects of excess FFAs and other surface lipids on lipoprotein lipase activity. In this model, a dual mechanism could contribute to the known inverse relationship between triglyceride and HDL or apoA-I levels (47, 48). On the one hand, higher VLDL-FFA levels resulting from low apoA-I may impede lipolysis through inhibition of lipases while, on the other, rapid clearance of apoA-I bound to FFA-enriched apoB-containing lipoproteins could enhance apoA-I turnover.

The phenomena reported in this and the accompanying paper, together with data recently brought forth from our own and other laboratories, point to a potential major role for FFA in a variety of reactions that influence lipoprotein metabolism. These reactions include lipoprotein binding interactions, interparticle transfers of apolipoproteins, stimulation of lipoprotein compositional changes (7, 33), stimulation of CETP-mediated reactions (39, 49), synergistic effects of FFAs and CETP in inducing particle size reductions in HDL (36, 37), redistribution of cholesteryl esters from high to low density lipoproteins (50), release of lipoprotein lipase from endothelial cells (51), and impairment of lipoprotein lipase interactions with glycosaminoglycans and apoC-II (52). Local generation and accumulation of FFAs at the capillary endothelial surface have also been implicated in release of lipoprotein lipase activity into the bloodstream after rapid in vivo infusions of triacylglycerol emulsions (53). In all of these cases, the described phenomena were demonstrated only when FFA:albumin molar ratios exceeded normal steady state whole plasma levels. Attention must now be directed at more clearly defining the extent to which FFAs and other lipolysis products accumulate on lipoprotein surfaces during physiologic lipolysis and establishing whether these phenomena can be demonstrated directly in vivo.

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