binding after such conformational changes. However, the direct involvement in binding is not yet warranted, since, in general, dissociation constants for substrates are not equal to K_m values and their determination requires thorough kinetic analyses (Cleland, 1963). It is interesting to note that both Lys-263 and Lys-329 are not conserved in the corresponding regions of the slime mold enzyme (Ragheb & Dottin, 1987; Katsube et al., 1990), which has an about 10 times lower specific activity than the potato enzyme (Pannbacker, 1967). The low activity may be partly explained by the absence of Lys-263 and Lys-329 in the slime mold enzyme.

The Lys-409 → Gln and Lys-410 → Gln mutant enzymes had steady-state kinetic constants almost identical with those of the wild-type enzyme, indicating that both Lys-409 and Lys-410 do not participate in catalysis. However, this does not necessarily show that these two lysyl residues are located apart from the active site.

In conclusion, among the five lysyl residues identified by the affinity labeling studies to be located at or near the active site, Lys-367 was found to be essential for the catalytic activity of UDP-glucose pyrophosphorylase. Although it is unknown how Lys-367 functions in the catalysis, the amino group of its side chain may interact with a phosphate group of the substrate during the transition state as has been shown by an NMR study of adenylate kinase (Fry et al., 1986), in which the amino group of Lys-21 may interact with the α-phosphate group of the bound substrate MgATP.

Registry No. Lys, 56-87-1; PP_i, 14000-31-8; UDP-glucose phosphorylase, 9026-22-6; glucose-1-P, 59-56-3.

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Metabolism of Low-Density Lipoprotein Free Cholesterol by Human Plasma Lecithin–Cholesterol Acyltransferase†

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ABSTRACT: The metabolism of cholesterol derived from [^{3}H]cholesterol-labeled low-density lipoprotein (LDL) was determined in human blood plasma. LDL-derived free cholesterol first appeared in large α-migrating HDL (HDL_2) and was then transferred to small α-HDL (HDL_3) for esterification. The major part of such esters was retained within HDL of increasing size in the course of lecithin–cholesterol acyltransferase (LCAT) activity; the balance was recovered in LDL. Transfer of preformed cholesteryl esters within HDL contributed little to the labeled cholesteryl ester accumulating in HDL_2. When cholesterol for esterification was derived instead from cell membranes, a significantly smaller proportion of this cholesteryl ester was subsequently recovered in LDL. These data suggest compartmentation of cholesteryl esters within plasma that have been formed from cell membrane or LDL free cholesterol, and the role for HDL_2 as a relatively unreactive sink for LCAT-derived cholesteryl esters.

High-density lipoprotein (HDL) is now recognized to be highly heterogeneous in size as well as lipid and apoprotein composition. An important part of HDL metabolism involves transfers of lipids, particularly free cholesterol, between different HDL subfractions (Eisenberg, 1984; Patsch & Gotto, 1987).

There are two major sources for the free cholesterol appearing in HDL: the surface of secreted lipoproteins, particularly very low and low-density lipoproteins (VLDL and LDL), and cell membranes. An earlier report showed that different HDL subfractions were active in the transport of free cholesterol from these sources (Miida et al., 1990). Cellular cholesterol was initially transferred to the pre-β-migrating HDL whose protein moiety contained only apolipoprotein A-I. LDL free cholesterol was transferred mainly to the major

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α-migrating HDL fraction, which contained both apo A-I and apo A-II.

The independent metabolism of cell- and lipoprotein-derived free cholesterol could allow regulation of cellular cholesterol levels regardless of the presence of high plasma lipoprotein cholesterol concentrations. Recent studies have provided information on the metabolism of cell-derived cholesterol by plasma LCAT (Castro & Fielding, 1988; Francone et al., 1989). The HDL species involved in the esterification of LDL-derived free cholesterol in plasma, and the transfer of this cholesteryl ester to other lipoproteins, are not well understood, nor are the physical processes involved in the initial transfers of cholesterol between HDL species.

It has been shown that part of HDL cholesteryl ester is transferred to VLDL and LDL in a reaction catalyzed by the cholesteryl ester transfer protein (CETP). The pool within HDL from which this is derived is not known. It is also unclear which factors determine the distribution of LCAT-derived cholesteryl esters within the subfractions of HDL.

In the present study, the movement of labeled cholesteryl esters generated from LDL-derived [^{3}H]cholesterol has been followed as these transfer between lipoprotein subfractions in native plasma.

Experimental Procedures

Materials. Heparin–agarose was purchased from Pharmacia–LKB (Piscataway, NJ). Human serum albumin was obtained from Sigma (St. Louis, MO), and agarose beads (Affi-gel 10) were from Bio-Rad (Richmond, CA). [1,2-^{3}H]Cholesterol was obtained from New England Nuclear (Boston, MA). Nitrocellulose membranes (Sartorius, 0.45 μm) were obtained from West Coast Scientific (Oakland, CA).

Preparation of Plasma and LDL. Blood was drawn from healthy normolipemic donors after an overnight fast, and was collected into ice-cooled tubes containing streptokinase (150 IU/mL) as anticoagulant (Miida et al., 1990). Plasma was obtained after centrifugation (2000*g*, 30 min) at 0 °C.

LDL was separated from plasma by sequential affinity chromatography; 8 mL of plasma was applied to a heparin–agarose column (2 × 10 cm) equilibrated in 0.15 M NaCl–0.01% w/v Na_2EDTA, pH 7.4 (saline–EDTA) (Ishikawa et al., 1988). The retained fraction, containing VLDL and LDL, was eluted with 3 M NaCl, 10 mM Tris-HCl, and 0.01% Na_2EDTA (pH 7.4). To remove the VLDL containing apo E in this fraction, the eluate was immediately applied to an immunoaffinity column of rabbit anti-human apo E IgG immobilized on agarose and equilibrated with saline–EDTA (Fielding & Fielding, 1981a). LDL without apo E was recovered in the unretained eluate.

The LDL was labeled with [^{3}H]cholesterol by equilibration with [^{3}H]cholesterol–human serum albumin complex. Human albumin covalently bound to agarose (Porath et al., 1967) was incubated at 37 °C for 60 min with 0.5–1.0 mCi of [^{3}H]-cholesterol. The cholesterol–albumin complex was washed 3 times with saline–EDTA. Affinity-purified LDL was added to the complex and incubated at 37 °C for 60 min. LDL labeled with [^{3}H]cholesterol was obtained in the supernatant following mild centrifugation (0 °C, 800*g*, 20 min). Final specific activity was (1–3) × 10^7 dpm/μg (Miida et al., 1990).

Cell Culture. Normal human skin fibroblasts were grown to near-confluency in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. In some experiments, the cells were labeled with [^{3}H]cholesterol by preincubation for 48 h with the same medium containing 0.5–1.0 mCi of [^{3}H]cholesterol complexed with fetal calf serum (Castro & Fielding, 1988). Before each experiment, the fibroblasts were washed 4 times with phosphate-buffered saline, pH 7.4.

Determination of ^{3}H-Free and Esterified Cholesterol Transfer. To investigate the metabolism of LDL-derived free cholesterol, unlabeled fibroblasts were incubated at 37 °C with plasma containing [^{3}H]cholesterol-labeled LDL. In parallel experiments, labeled fibroblasts were incubated at 37 °C with unlabeled plasma to determine the transfer of cell-derived cholesterol among plasma lipoproteins. During incubations of from 1 to 60 min at 37 °C, plasma samples were taken and immediately cooled to 0 °C prior to fractionation by nondenaturing two-dimensional electrophoresis, as described below. Two types of experiments were performed. In the first, LCAT activity continued during the incubation, so that labeled free cholesterol transferred from cell membranes or LDL was esterified by LCAT and the cholesteryl esters produced transferred, as a function of time, among different plasma lipoproteins. In the second type of experiment, after incubation and LCAT activity had generated labeled cholesteryl esters for a period of 20 min, the synthesis of further labeled esters by LCAT was inhibited by the addition of a final concentration of 2 mM dithiobis(2-nitrobenzoic acid) (DTNB) (Stokke & Norum, 1971). Further incubation under these conditions then followed the transfer of preformed labeled esters among plasma lipoprotein fractions, since CETP-catalyzed cholesteryl ester transfer is not blocked by DTNB (Fielding & Fielding, 1981b).

Electrophoretic Fractionation. [^{3}H]Cholesterol-labeled plasma fractions were separated first by agarose gel electrophoresis and then, in most experiments, in a second dimension, by nondenaturing polyacrylamide gradient gel electrophoresis, as previously described in detail (Francone et al., 1989). In brief, 20 μL of plasma was applied to 0.75% w/v agarose strips in 50 mM barbital buffer (pH 8.6). Electrophoresis was carried out at 0 °C, 200 V. In some experiments, following electrophoresis, 0.5-cm transverse fractions of the gel were cut and added to 1 mL of 0.15 M NaCl containing 2.0 mM DTNB to inhibit any further esterification. The gel pieces were dissolved at 100 °C and labeled lipids extracted with 2 mL of a chloroform–methanol mixture (1/1 v/v) (Bligh & Dyer, 1959).

For further fractionation, in most experiments two longitudinal pieces (0.5 × 7 cm) were cut from the developed agarose gel strips and placed end-to-end on top of a gradient (14 × 15 cm) of polyacrylamide (2–15% w/v). Electrophoresis was carried out in 0.025 M Tris-HCl–0.2 M glycine (pH 8.3) at 0 °C, 400 V for 5 h. Following electrophoresis, a half of the gel (containing a single complete separation) was used for transfer, while the other half was kept at 4 °C for subsequent extraction and analysis.

Transfer to a nitrocellulose membrane was carried out at 0 °C in 0.025 M Tris-HCl–0.2 M glycine, pH 8.3–20% methanol v/v at 30 V for 17 h (Towbin et al., 1979). The nitrocellulose membranes were then immunoblotted with rabbit polyclonal anti-human apo A-I or with site-directed antibodies to LCAT and CETP as previously described (Francone et al., 1989). The membranes were then reacted with a second antibody of ^{125}I-labeled goat anti-rabbit IgG which had been preadsorbed against human IgG. Reactive complexes were detected by autoradiography, using Kodak X-OMAT AR radiographic film. With the developed film as template, lipoprotein areas were identified on the remaining half-gel, excised, and eluted with saline–EDTA containing 2 mM DTNB, and then the lipids were extracted with chloroform–methanol (2/1 v/v). When individual HDL fractions recovered from polyacrylamide were electrophoresed again in the same system, no detectable contamination with other

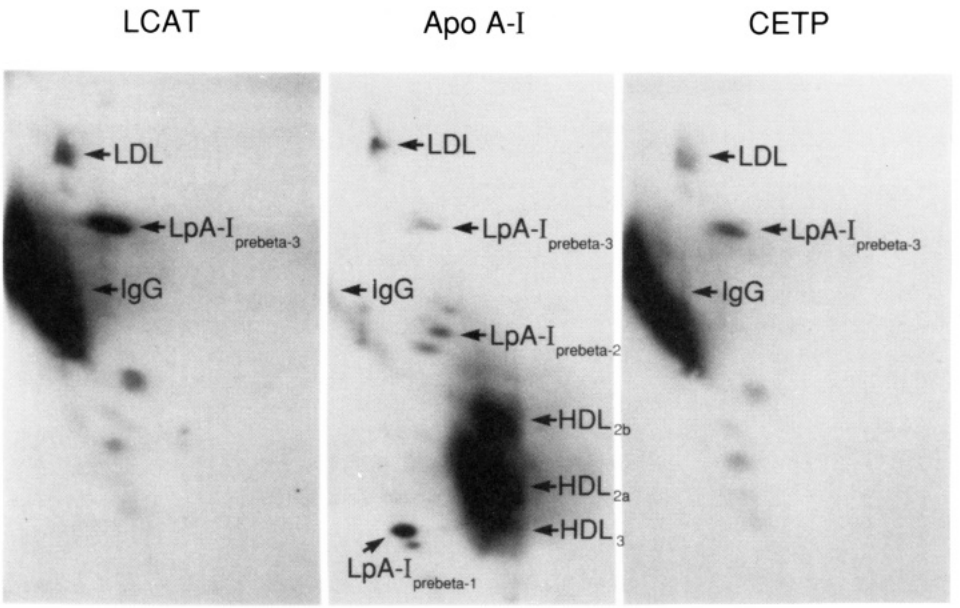

FIGURE 1: Distribution of LCAT, apo A-I, and CETP proteins determined by nondenaturing two-dimensional electrophoresis and immunoblotting of the same normal plasma sample. Electrophoresis was carried out as described under Experimental Procedures. Agarose electrophoresis is from left to right and polyacrylamide gradient gel electrophoresis from top to bottom. The separated proteins were transferred to nitrocellulose, and the distribution of antigens was determined by reaction with the corresponding unlabeled rabbit anti-human antibody, followed by ^{125}I-labeled goat anti-rabbit IgG and autoradiography. The arc in the upper left-hand quadrant is IgG from residual cross-reactivity with the labeled second antibody. The nomenclature of HDL subspecies is the same as that used in Francone et al. (1989).

fractions was evident (Miida et al., 1990).

In the absence of the first antibody, there was no detectable reaction on nitrocellulose between lipoprotein complexes and the second antibody, ^{125}I-labeled goat anti-rabbit IgG. The same pattern (except for the cross-reactivity with human IgG) was obtained when the first anti-LCAT or anti-CETP antibody was labeled directly, as when unlabeled first antibody was followed with labeled goat anti-rabbit IgG.

Labeled free and esterified cholesterol in the extracted lipids from the experiments described above was fractionated by thin-layer chromatography on silica gel layers (Merck, Darmstadt, FRG) developed in hexane–diethyl ether–acetic acid (83/16/1 v/v). Radioactivity in the esterified and free cholesterol fractions (R_f 0.9–0.95 and 0.1–0.15, respectively) was quantitated by liquid scintillation spectrometry.

Free and esterified cholesterol mass in lipoproteins or plasma was determined enzymatically with an automated Roche Mira analyzer (Allain et al., 1974). LCAT activity was determined as the rate of decrease in plasma free cholesterol with time (Miida et al., 1990).

Results

Distribution of LCAT and CETP among HDL Subfractions. Subfractions of pre-β- and α-HDL were separated by nondenaturing two-dimensional electrophoresis. After electrotransfer to nitrocellulose, the distribution of apo A-I, LCAT, and CETP was visualized by reaction with specific antibodies (Figure 1). LCAT and CETP comigrated with apo A-I in the largest pre-β-HDL component (LpA-I$_{\text{pre-}\beta\text{-3}}$) but were absent in the two smaller components. In addition, both LCAT and CETP radioactivities were identified at the left edge of each of the major α-migrating HDL fractions. The distribution of these antigens quantitated from the distribution of ^{125}I-labeled antibody is shown in Table I.

Recovery of Cholesteryl Esters in LDL. The labeled free cholesterol initially located in LDL was effectively utilized by LCAT for the synthesis of cholesteryl esters in human plasma. As shown in Figure 2, the addition of [^{3}H]cholesterol-labeled autologous LDL to native plasma was followed by the rapid

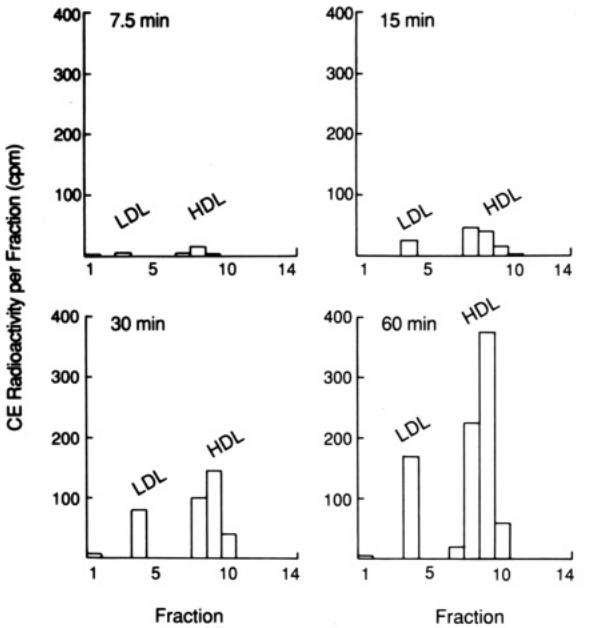

FIGURE 2: Time course of the appearance of labeled cholesteryl ester in HDL and LDL derived from the reaction of plasma LCAT with [^{3}H]cholesterol-labeled LDL. After incubation at 37 °C for the time indicated, plasma (20 μL) was fractionated by agarose gel electrophoresis and radioactivity in the cholesteryl ester moiety of HDL and LDL determined. Incubation times shown (from top left) are 7.5, 15, 30, and 60 min. HDL free cholesterol mass was 120 ± 1 μg/mL. Free cholesterol specific activity in HDL was 1.8×10^3, 2.7×10^3, 3.7×10^3, and 5.5×10^3 cpm/μg after 7.5, 15, 30, and 60 min of incubation with [^{3}H]cholesterol-labeled LDL, respectively. Total HDL cholesteryl ester radioactivity was 1.7×10^3, 6.4×10^3, 18.4×10^3, and 43.2×10^3 cpm/mL of plasma, and LDL cholesteryl ester was 20%, 21%, 22%, and 23% of total cholesteryl ester label, at the same incubation times.

appearance of labeled cholesteryl esters in both HDL and LDL, when these were separated by agarose gel electrophoresis.

A part of LCAT protein in human plasma is associated with LDL (Chung et al., 1982; Francone et al., 1989; Table I

Table I: Distribution of Apo A-I, LCAT, and CETP within HDL[a]

fraction	% apo A-I	% LCAT	% CETP
pre-β-3	0.7 ± 0.6	50.5 ± 12.7	45.5 ± 10.7
α-HCL_{2b}	23.8 ± 6.2	17.3 ± 7.0	8.8 ± 5.1
α-HCL_{2a}	39.0 ± 2.3	9.5 ± 5.6	31.4 ± 8.0
α-HDL_3	26.3 ± 6.2	17.5 ± 7.7	5.3 ± 4.9

[a] Values are means ± 1 SD of the percent distribution of each antigen in 12 independent experiments. The balance of apo A-I is present without LCAT or CETP in other pre-β-migrating HDL species (6, 7). LCAT is also present in LDL (range 0–24%, mean 6.3% of total reactivity). CETP is also present in LDL (range 0–11%, mean 2.6% of total reactivity).

legend). In porcine plasma, this LCAT has been implicated in the local synthesis of cholesteryl esters, that is, the synthesis of cholesteryl esters from LDL free cholesterol without the involvement of HDL (Knipping et al., 1987). In the present study, to determine whether the labeled cholesteryl ester recovered in human LDL was synthesized there, [^{3}H]cholesterol-labeled LDL was incubated with plasma at 37 °C for 5 min. A sample of the mixture was then chilled, and an aliquot was fractionated by agarose gel electrophoresis. The LDL fraction was eluted from the gel strip and incubated, together with its associated LCAT and apo A-I in medium containing 1% human serum albumin in 0.15 M NaCl–0.01 M Tris-HCl buffer (pH 7.4) for 60 min at 37 °C. A second aliquot of the same plasma was directly incubated for 60 min at 37 °C. This plasma was then also fractionated by agarose gel electrophoresis. The LDL was recovered from the gel as described above, and the cholesteryl ester label was quantitated.

When whole plasma was incubated, labeled cholesteryl ester was generated and transferred in part to LDL, as seen in the experiment shown in Figure 2. However, when LDL was first isolated and then incubated, the cholesteryl ester radioactivity recovered was less than 1% (0%, 0.90%; $n = 2$) of that found in whole plasma. These data support the conclusion that the labeled cholesteryl ester which was recovered in LDL following incubation of native plasma was first synthesized within HDL and subsequently transferred to LDL. In human plasma at least, the direct local synthesis of LDL cholesteryl ester appears to be negligible.

Synthesis of Cholesteryl Esters in HDL. As shown in Figure 1, LCAT is present in each major fraction of α-migrating HDL, as well as in pre-β-migrating $LpA\text{-}I_{pre\text{-}\beta\text{-}3}$ fraction. The LCAT present in $LpA\text{-}I_{pre\text{-}\beta\text{-}3}$ was implicated in an earlier study in the esterification of cholesterol derived from cell membranes (Francone et al., 1989). To determine whether the same pathway was used for the synthesis of cholesteryl esters derived from LDL cholesterol, the time course of the appearance of label in HDL was first determined (Figure 2).

Because the rate of equilibration of free cholesterol within HDL subfractions is much greater than the rate between LDL and HDL, or the rate of LCAT itself (Miida et al., 1990), the specific activity of free cholesterol substrate for LCAT will increase with time but be uniform within HDL. After 15 min, this increase becomes essentially linear (legend, Figure 2). The rate of appearance of cholesteryl ester can then be used to test the validity of the LDL free cholesterol label as a tracer in the LCAT reaction. For example, between 30 and 60 min, the mean free cholesterol specific activity of HDL was 4.6×10^3 cpm/μg (legend, Figure 2). The increase in total cholesteryl ester radioactivity over the same period (taken from Figure 2) was (863 cpm − 369 cpm) 494 cpm, and this represents an increase of 494 cpm/(4.6×10^3 cpm/μg) or 0.11

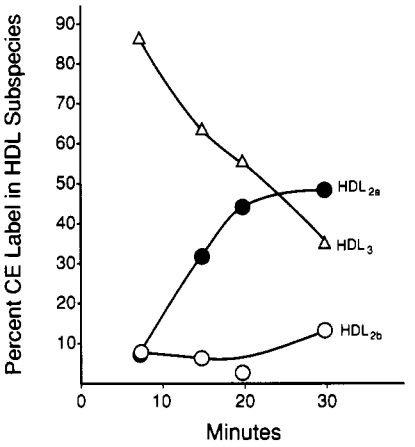

FIGURE 3: Time course of the appearance of labeled cholesteryl ester derived from [^{3}H]cholesterol-labeled LDL within subfractions of HDL. Plasma samples were taken during incubation at 37 °C at the indicated intervals after the addition of labeled LDL. The plasma was fractionated by two-dimensional electrophoresis as described under Experimental Procedures, and the radioactivity in the cholesteryl ester moiety of pre-β- and α-HDL subfractions was determined. No significant label was recovered in any pre-β-HDL fraction. Data are expressed as the proportion of total HDL cholesteryl ester label in each fraction. Total HDL cholesteryl ester label was 3.4×10^3, 12.9×10^3, and 28.3×10^3 cpm/mL of plasma at 7.5, 15, and 30 min of incubation, respectively.

μg of cholesteryl ester in the 20-μL sample over the 30-min incubation period, equivalent to 10.7 μg of cholesterol esterified (mL of plasma)$^{-1}$ h^{-1}. This can be compared with the rate of decrease in plasma free cholesterol mass [12 μg (mL of plasma)$^{-1}$ h^{-1}] determined for the same donor at the same time. This rate is also comparable to the mean LCAT rate previously reported (11.5 ± 1.8 μg mL^{-1} h^{-1}) (Miida et al., 1990). This comparison suggests that the LDL free cholesterol label, as transferred to HDL, provides an accurate measure of cholesterol metabolism in that fraction.

As shown in Figure 3, of the total cholesteryl ester formed 7.5 min following the addition of [^{3}H]cholesterol–LDL, most was present in the α-migrating HDL_3 fraction. There was no detectable label in the pre-β-HDL fraction containing LCAT protein ($LpA\text{-}I_{pre\text{-}\beta\text{-}3}$) and little or none in HDL_{2b} and HDL_{2a}, in spite of the LCAT protein also present in these fractions (Table I). During further incubation, the proportion of radioactivity in HDL_3 decreased while it increased in HDL_{2a} and to a lesser extent in HDL_{2b}. Over the time course shown, total cholesteryl ester label in HDL_3 decreased from 85% (7.5 min) to 63% (15 min) to 35% (30 min), while over the same time course the proportion of label in HDL_{2a} was 7%, 31%, and 50%, and label in HDL_{2b} was 7%, 5%, and 15%, respectively. These data suggest that the major site of early synthesis of cholesteryl ester was in HDL_3.

The distribution shown in Figure 3 could also have resulted from a preferential incorporation of free cholesterol into HDL_3, resulting in a higher specific activity in free cholesterol in HDL_3 (and therefore in cholesteryl ester synthesized there) compared to the other HDL fractions. To address this question, cholesteryl ester radioactivity was expressed as a proportion of free cholesterol radioactivity in each HDL fraction (Figure 4). An even more marked predominance of esterification of cholesterol in HDL_3 was evident, because of the early appearance of high levels of isotopic free cholesterol in HDL_{2b} and then HDL_{2a} (Miida et al., 1990). On average, 30 ± 10% of total cholesterol label was esterified in HDL_3, compared to only 7 ± 3% in HDL_{2a} and <2% in HDL_{2b}. These data confirm that the major part of cholesteryl ester synthesis occurs in HDL_3. The initial rapid appearance of

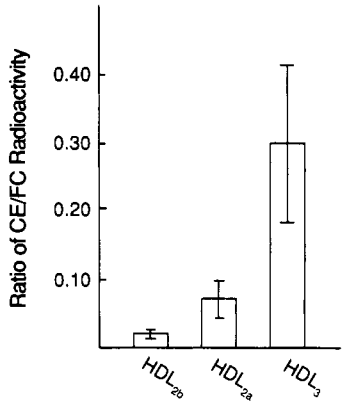

FIGURE 4: Ratios of the radioactivity in free and esterified cholesterol in HDL subfractions from plasma incubated for 15 min at 37 °C. Plasma samples were fractionated and component lipoproteins extracted as described under Experimental Procedures. Free and ester cholesterol label was fractionated by thin-layer chromatography. Values shown are the means ± 1 SD (n = 3) of these ratios.

cholesteryl ester radioactivity in HDL_3 seen in Figure 3 must therefore result from a greater reactivity of LCAT with free cholesterol in this HDL fraction.

Origin of Cholesteryl Esters in HDL_{2a} and HDL_{2b}. If the major part of cholesteryl ester is synthesized in HDL_3, two pathways are available to supply the radioactive cholesteryl ester label seen in HDL_2. These are the transfer of HDL_3 cholesteryl ester to HDL_2 through the action of CETP or the retention of cholesteryl esters within the HDL_3 particle, causing an increase in the lipid content and size of HDL. This would make the particle migrate more slowly during gradient gel electrophoresis, leading to the appearance of cholesteryl ester in the HDL_{2a} and then the HDL_{2b} size range.

These possibilities were distinguished by labeling HDL_3 cholesteryl ester in plasma. Incubation was carried out with [^{3}H]cholesterol-labeled LDL for 20 min at 37 °C. LCAT activity was then inhibited with DTNB. As shown in Figure 5, under these conditions there was little increase overall in cholesteryl ester label in HDL_2. On the other hand, labeled cholesteryl ester in HDL_3 decreased, and there was a significant increase in label in the LDL fraction. On average, 83 ± 8% of the cholesteryl ester radioactivity lost from HDL_3 was recovered in LDL in the presence of DTNB. The remainder was recovered in HDL_2.

The almost immediate reaction of LCAT with DTNB (Chong et al., 1983) precludes a contribution of labeled cholesteryl ester from a third pathway—the direct esterification of labeled free cholesterol already on HDL_2. DNTB does not inhibit cholesteryl ester transfer (Morton & Zilversmit, 1982).

Since there was only a modest increase in HDL_2 cholesteryl ester when LCAT was inhibited, these data suggest that the appearance of cholesteryl ester label in HDL_{2a} and HDL_{2b} when LCAT is active results mostly from a retention of cholesteryl ester within HDL as its size and lipid content increases, not by the transfer of cholesteryl ester from HDL_3 to HDL_2. The lack of transfer to HDL_2 that was observed in these experiments suggests that large HDL may be relatively poor acceptors of cholesteryl esters via the CETP pathway. This appears to be the case even though HDL_2 contains CETP protein. When the immunoblots of apo A-I and CETP were aligned, both HDL_{2a} and HDL_{2b} contained detectable CETP at their left-hand border (Figure 1, Table I). This distribution may result from an effect of the charge of the bound CETP protein itself, or from a change in the conformation of other HDL proteins when CETP binds to the particle. Since HDL_3 readily loses cholesteryl ester to LDL, the lack of transfer to

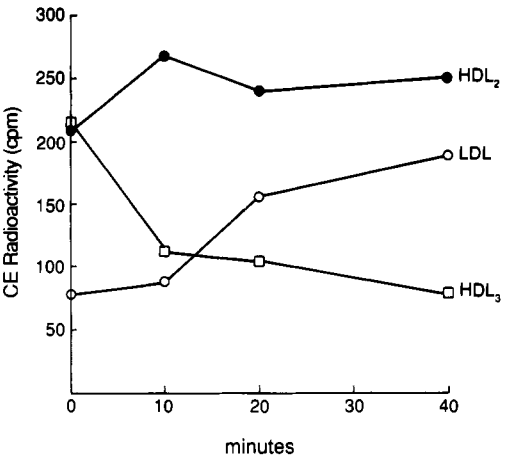

FIGURE 5: Effects of DTNB on the distribution of cholesteryl ester label within HDL. After incubation of plasma with [^{3}H]cholesterol LDL for 20 min at 37 °C to label HDL cholesteryl ester, DTNB (2.0 mM final concentration) was added to inhibit further LCAT activity; then the distribution of cholesteryl ester label within HDL determined as a function of time.

HDL_2 is unlikely to result from inactivity of the CETP in this donor. Because it is likely that the whole HDL cholesteryl ester is generated by the LCAT reaction (Chen et al., 1984), these data also imply that while LCAT activity was most effective in HDL_3, esterification continued, albeit at a reduced rate, as HDL_3 enlarged by increment of their cholesteryl ester content to HDL_{2a} and then HDL_{2b}.

In summary, of the LDL free cholesterol which appeared in HDL_3 and was esterified there, a part (about 30%) was transferred from that fraction to LDL; the remainder was retained in the HDL pathway leading to HDL_{2a} and HDL_{2b}.

Metabolism of LDL-Derived and Cell-Derived Cholesterol. LDL and cell membranes represent the two major sources of free cholesterol substrate for the LCAT reaction. To obtain further information on the relationship between these two pools, native plasma from the same donor was incubated under equivalent conditions with [^{3}H]cholesterol-labeled LDL in the presence of unlabeled cell monolayers, or with unlabeled LDL in the presence of [^{3}H]cholesterol-labeled cells. The subsequent distribution between LDL and HDL of the labeled cholesteryl ester formed was determined by agarose gel electrophoresis. The distribution of labeled cholesteryl ester within HDL was determined by nondenaturing two-dimensional electrophoresis, as described above.

When the free cholesterol label originated in cell membranes, labeled cholesteryl ester in HDL increased as a function of time to contain on average 4.7×10^3, 13.5×10^3, and 38.4×10^3 cpm/mL of plasma after 7.5, 15, and 30 min of incubation, respectively. However, there was no change in the proportion of cholesteryl ester label in HDL_3, HDL_{2a}, and HDL_{2b} as a function of time. The proportion of total cholesteryl ester radioactivity in HDL_3 was 0.52 ± 0.07, 0.53 ± 0.09, 0.53 ± 0.07, and 0.52 ± 0.03 after 7.5, 15, 30, and 60 min of incubation (n = 3), respectively. The corresponding proportions for HDL_{2a} were 0.43 ± 0.05, 0.38 ± 0.01, 0.30 ± 0.13, and 0.38 ± 0.06, and for HDL_{2b}, they were 0.06 ± 0.10, 0.09 ± 0.08, 0.07 ± 0.06, and 0.08 ± 0.04. These results are in marked contrast to those obtained in the experiment when the labeled cholesterol originated in LDL (Figure 3). The data obtained with cellular cholesterol label suggest a transfer of cholesteryl esters synthesized in pre-β-HDL to α-HDL, consistent with the previous finding (Francone et al., 1990) that a major part of labeled α-HDL cholesteryl ester could be derived by transfer from a pre-β-HDL precursor

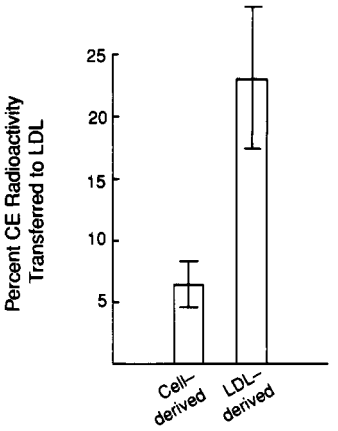

FIGURE 6: Relative rates of transfer to LDL of cholesteryl ester derived from cholesterol radiolabel in cultured fibroblast monolayers or in LDL. Values shown are the means ± 1 SD ($n = 9$) of the proportions of total plasma cholesteryl ester label in LDL, determined by agarose gel electrophoresis after 30-min incubation at 37 °C.

($LpA\text{-}I_{pre\text{-}\beta\text{-}3}$). These results suggest that HDL_3 may be the most effective acceptor of cholesteryl esters derived via pre-β-migrating HDL.

Another difference between the metabolism of LDL-derived and cell-derived cholesterol was found to lie in the proportion of α-HDL cholesteryl ester which was transferred to LDL. As shown in Figure 6, there was consistently a much greater transfer to LDL of cholesteryl ester generated within HDL from LDL-derived free cholesterol than was the case when the free cholesterol had been derived from cell membranes. On average 22.9 ± 5.9% of total cholesteryl ester synthesized from labeled LDL-derived free cholesterol was transferred back to LDL (Figure 6). The remainder was recovered in the α-migrating fractions of HDL.

When the labeled cholesterol originated from cell membranes, a much smaller proportion of the total cholesteryl ester formed was subsequently transferred to LDL under the same conditions (6.4 ± 1.8%) ($p < 0.02$ by paired t-test). This value is similar to the proportion (10–11%) obtained previously (Francone et al., 1990) in a comparison of three different fractionation techniques.

These results indicate that cholesterol of cellular and lipoprotein origin remains partially compartmentalized within plasma (and in particular within HDL) during esterification by LCAT, and during the subsequent transfer of LCAT-derived cholesterol esters.

Discussion

There is now considerable evidence that LCAT plays a role not only in the metabolism of cholesterol secreted with plasma lipoproteins but also in cellular cholesterol homeostasis in the peripheral tissues (Ray et al., 1980; Fielding & Fielding, 1981a; Davis et al., 1982; Chollet et al., 1988). LCAT itself has been detected immunologically in several lipoprotein fractions (Cheung et al., 1986; Francone et al., 1989). It is active enzymatically in different HDL species (Fielding & Fielding, 1971; Francone et al., 1989). The data in the present study suggest that this structural complexity may provide the physical basis for a compartmentation of LCAT activity that could explain the differential utilization of cell- and LDL-derived cholesterol previously observed (Francone et al., 1989).

In human plasma, free cholesterol levels (about 40 mg/dL) are high, particularly compared to those in most other mammalian species. Although cholesterol moves freely between lipid surfaces, the $t_{1/2}$ of transfer from confluent peripheral cells (such as endothelial cells or fibroblasts) (10–20 h) is much greater than that from either LDL (45 min) or HDL (2–3 min) (Fielding & Fielding, 1981a; Fielding et al., 1982; Lund-Katz et al., 1982; Miida et al., 1990). The plasma space is an open compartment to which free cholesterol is continually supplied via newly secreted lipoproteins. Without some process of effective compartmentation of substrate within the plasma, cells might supply little substrate cholesterol for esterification. The same continued input of new cholesterol prevents full equilibration between cholesterol and its esters in the different lipoprotein classes.

Two kinds of experimental evidence are now available to support the hypothesis that a metabolic pathway is present in native plasma to favor the utilization of cell membrane cholesterol. This pathway may be represented structurally by the pre-β fraction of HDL and, in particular, by its LCAT moiety. When plasma was incubated in the presence of [^{3}H]cholesterol-labeled fibroblast monolayers, the labeled cell cholesterol was esterified about 8-fold more efficiently than plasma lipoprotein cholesterol (Castro & Fielding, 1988). Second, an HDL fraction was identified within the pre-β region which efficiently processed cell-derived cholesterol (Francone et al., 1989). This contained a high concentration of LCAT molecules relative to its substrate, free cholesterol (about 50% of HDL LCAT associated with about 1% of HDL free cholesterol). Such a complex could efficiently convert diffusible free cholesterol to its nondiffusible cholesteryl ester product.

This situation is different from that in the main fraction of HDL (α-migrating HDL_3, HDL_{2a}, and HDL_{2b}). Here free cholesterol equilibrates rapidly (Miida et al., 1990) while the concentration of LCAT (relative to its substrate, free cholesterol) is much lower.

Earlier studies by several laboratories have provided evidence that HDL_3 are a significantly better substrate for LCAT than larger HDL (HDL_2) (Glomset et al., 1966; Fielding & Fielding, 1971; Jahani & Lacko, 1981). The present research confirms this conclusion with data from native human plasma. The appearance of cholesteryl ester formed from LDL free cholesterol within HDL_3 (measured as the ratio of cholesteryl ester to free cholesterol radioactivity) was much greater than within either HDL_{2a} or HDL_{2b}. Following the initial incorporation of [^{3}H]cholesterol-labeled LDL in plasma, almost all of labeled cholesteryl ester first appeared in HDL_3. There was an efficient transfer of labeled cholesteryl ester out of HDL_3 into LDL. These experiments suggest that HDL_2 functions as an end product whose cholesteryl ester is removed not by transfer but by endocytosis of intact particles (Blum et al., 1977) or by a disproportionate uptake of cholesteryl ester such as has been observed when HDL interact with hepatocytes and adrenal cells (Pittman et al., 1987; Gwynne & Mahaffee, 1989).

The same fate is likely to result for cholesteryl esters derived from cellular cholesterol, but here the mechanism must be different. The transfer of pre-β-HDL cholesteryl ester to α-HDL can occur as a result of CETP activity, since pulse–chase experiments have shown that transfer continues even when LCAT activity is inhibited (Fielding & Fielding, 1981b; Francone et al., 1989). When LCAT activity is ongoing, the major part of cell-derived cholesteryl ester appears in HDL_3, but there is no precursor–product relationship within HDL subspecies as was found with LDL cholesterol. The proportion of total cholesteryl ester label appearing in HDL_2 is instead largely unchanged over time. These experiments suggest that whether the cholesterol substrate for LCAT is derived from cell membranes or LDL, there is functional coordination between LCAT and CETP activities.

The concept that LCAT and CETP may associate on the same HDL particles to coordinate the metabolism of lipoprotein cholesterol is consistent with the presence of these antigens in each of the major α-migrating HDL species, as well as in pre-β-HDL (Figure 1). It is also consistent with studies from other laboratories (Dieplinger et al., 1989; Nishida et al., 1990; Schmitz, 1990). All of these studies suggest a heterogeneous structural association of LCAT and CETP in HDL. One laboratory has reported that most of plasma CETP is present in a single HDL fraction with a total molecular weight of 145K (Marcel et al., 1990), i.e., an HDL net molecular weight of about 80K, assuming one CETP (molecular weight 74K) per particle (Jarnagin et al., 1987). This 145-kDa HDL may be a product of the larger pre-β-3 lipoprotein (Fielding & Fielding, 1981a; Francone et al., 1989) which contained the largest part of LCAT and CETP in the present study.

A final significant difference in the metabolism of LDL- and cell-derived cholesterol appears in its transfer to LDL. A much lower proportion of cell-derived free cholesterol (compared to that from LDL) appeared in LDL as cholesteryl ester, and a proportionately higher amount was retained in HDL.

The data in this paper and that published earlier (Francone et al., 1989) are most consistent with the hypothesis that LCAT in the pre-β-HDL fraction represents enzyme largely active in processing cholesterol of cellular origin. LCAT in the α-HDL fraction (particularly that in HDL_3) would then represent enzyme whose major substrate is cholesterol derived directly from plasma lipoproteins (particularly LDL). Further research will be required to determine whether differences in cholesterol processing of the kind described here can explain the differences in plasma cholesterol metabolism found under pathological conditions in which the proportions of HDL species are perturbed (Ishida et al., 1987). Such changes are also found during postprandial lipemia, when the utilization of cell-derived cholesterol, as well as total LCAT activity, is transiently increased (Marcel & Vezina, 1973; Castro & Fielding, 1985). It does seem clear that the structural heterogeneity of HDL, already well recognized, is associated with a metabolic heterogeneity reflected in an effective compartmentation of free and ester cholesterol between plasma lipoprotein species. This may have important implications for the regulation of plasma cholesterol transport.

Registry No. LCAT, 9031-14-5; cholesterol, 57-88-5.

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