Regulation of plasma lecithin:cholesterol acyltransferase in man. III. Role of high density lipoprotein cholesteryl esters in the activating effect of a high-fat test meal

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Abstract Plasma lecithin:cholesterol acyltransferase (LCAT) activity is increased during the clearance phase of alimentary lipemia induced by a high-fat test meal in normal subjects. Ultracentrifugal fractionation of high density lipoproteins (HDL) into HDL_2, HDL_3, and very high density (VHD) subfractions followed by analyses of lipid and protein components has been accomplished at intervals during alimentary lipemia to seek associations with enzyme changes. HDL_2 lipids and protein increased substantially, characterized primarily by enrichment with lecithin. HDL_3, which contain the main LCAT substrates, revealed increased triglycerides and generally reduced cholesteryl esters which were reciprocally correlated, demonstrating a phenomenon previously observed in vitro by others. Both changes correlated with LCAT activation, but partial correlation analysis indicated that ester content is primarily related to triglycerides rather than LCAT activity. The VHD cholesteryl esters and lysolecithin were also reduced. Plasma incubation experiments with inactivated LCAT showed that alimentary lipemic very low density lipoproteins (VLDL) could reduce levels of cholesteryl esters in HDL by a nonenzymatic mechanism. In vitro substitution of lipemic VLDL for postabsorptive VLDL resulted in enhanced reduction of cholesteryl esters in HDL_3 and VDH, but not in HDL_2, during incubation. Nevertheless, augmentation of LCAT activity did not result, indicating that cholesteryl ester removal from substrate lipoproteins is an unlikely explanation for activation. Since VHD and HDL_3, which contain the most active LCAT substrates, were also most clearly involved in transfers of esters to VLDL and low density lipoproteins, the suggestion that LCAT product lipoproteins are preferentially involved in nonenzymatic transfer and exchange is made. The main determinant of ester transfer, however, appears to be the level of VLDL, both in vitro and in vivo. **Rose, H. G., and J. Juliano.** Regulation of plasma lecithin: cholesteryl acyltransferase in man. III. Role of high density lipoprotein cholesteryl esters in the activating effect of a high-fat test meal. *J. Lipid Res.* 1979. **20:** 399–407.

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Plasma lecithin:cholesterol acyltransferase (EC 2.3.1.43) activity is increased in normal subjects 5–10 hr after ingestion of a high-fat test meal (1–3), temporally coinciding with the clearance phase of alimentary lipemia. Activation of LCAT has been related to maximal increases in plasma triglycerides (1, 2) and does not occur if fat is removed from the test meal (1). In addition to elevation of plasma chylomicrons and VLDL, fat feeding produces alterations in HDL. Havel (4) reported elevations of HDL phospholipids which tended to persist even after 12 hr, with less consistent increases in cholesterol and protein (4). In more recent studies in which HDL_2 and HDL_3 were isolated (5), increases in HDL_2 total cholesterol, phospholipids, and protein were noted. HDL_3 total cholesterol fell, while there was an increase in phospholipids which persisted even 10 hr after feeding. Similarly, Nichols and coworkers (6) reported elevation of $S_{f(1.20)}$ 4–8 and $S_{f(1.20)}$ 0–4 fractions 5 hr after oil ingestion. HDL glycerides and phospholipids were elevated and the fatty acid composition of the glycerides reflected the composition of the fed oil. HDL_3 and very high density lipoproteins (VHDL, $1.21 < d < 1.25$ g/ml) are LCAT substrates (7–9) while HDL_2 is nearly inactive (7) and perhaps inhibitory (3). According to Wallentin and Vikrot (2) the elevation of HDL phospholipids after fat ingestion correlates with LCAT response. Taken together, these observations point to the possibility that fat-induced alterations in levels or composition of

Abbreviations: HDL_3, high density lipoproteins$_3$; HDL_2, high density lipoproteins$_2$; VHD, very high density plasma fraction ($d > 1.220$ g/ml); LDL low density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; VLDL, very low density lipoproteins; TG, triglycerides; UC, unesterified cholesterol; EC, ester cholesterol; PC, phosphatidylcholine; LyPC, lysophosphatidylcholine.

LCAT substrates and/or products within the HDL are related to the activation phenomenon. We have accordingly isolated HDL_2, HDL_3, and a very high density fraction (d > 1.22 g/ml) at intervals after fat feeding in order to relate changes to activation of the enzyme. More specifically, we sought answers to the following questions. *1*) Would activation prove to be correlated with new lipoprotein substrates appearing in HDL_2 and/or HDL_3 during lipolysis? *2*) Would the lipid composition of HDL_2 and HDL_3 be changed during alimentary lipemia in a direction suggesting better substrate performance, i.e., enrichment in substrate lipids [unesterified cholesterol (UC) and phosphatidylcholine (PC)] and reduction in product lipids [ester cholesterol (EC) and lysophosphatidylcholine (LyPC)]? *3*) Could those HDL lipid changes that correlate with LCAT augmentation in vivo be duplicated in vitro with resulting activation of the enzyme?

MATERIALS AND METHODS

In vivo studies

Eight normal young men (mean age, 24 yr), free of known systemic disease, served as subjects for fat-loading studies. All had normal plasma cholesterol and triglycerides, using age-adjusted values of Fredrickson, Levy, and Lees (10) and normal lipoprotein electrophoretic patterns by agarose electrophoresis (Pfizer Diagnostics, NY). Informed consent was obtained from each in a manner approved by the Human Studies Subcommittee of this hospital, according to regulations established by the Veterans Administration.

Duplicate venous blood samples (15–30 min apart) were obtained at 8–9 AM from subjects after a 12–14 hr fast and immediately chilled in ice water in tubes containing 1.0 mg of Na_2EDTA/ml blood. The subjects then ingested a liquid high-fat test meal over approximately 10 min. The composition of the test meal was 36% cream, 4 ml; skimmed milk, 4 ml; sucrose, 0.25 g; and cocoa, 0.12 g. Sufficient volumes were mixed and blended to allow feeding at 1.5 g fat per kilogram body weight. A 70-kg subject would receive 105 g of fat, 47 g of carbohydrate, and 19 g of protein. A 2.0 g/kg dose was fed to obtain lipoprotein fractions for in vitro experiments. Blood samples were drawn at 2.5-hr intervals up to and including the 10th hour postfeeding. No additional food was allowed during the test.

Chemical methods

Chilled blood samples were centrifuged at 4°C to remove cellular elements. Aliquots of plasma were taken for determination of *a*) triglycerides by an enzymatic method (Boehringer-Mannheim, NY), *b*) cholesterol using the colorimetric method of Rudel and Morris (11), and *c*) LCAT using a method previously described and validated (12). Precision of the LCAT method is ±16% (2 SD). For analysis of lipoprotein fractions, diethyl *p*-nitrophenylphosphate (Paraoxon, K&K Labs, Plainview, NY) was added to blood (final concentration, 1.0 mM) to inhibit LCAT and lipase activities during handling. Aliquots of plasma (5 ml) were adjusted to 1.063 g/ml with NaBr–NaCl (350:150, w/w) and centrifuged for 30 hr at 40,000 rpm in a 40.3 rotor at 6°C with an L3-50 ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, CA). The top fraction, VLDL plus low density lipoproteins (LDL), was removed by tube slicing and the bottom fraction was raised to a solvent density of 1.125 g/ml, using solid salts. This fraction was then centrifuged for 30 hr in the 40.3 rotor. The top fraction, HDL_2 (d 1.063–1.125 g/ml), was removed by slicing approximately 0.7–0.9 cm below the meniscus. The subnatant solution was raised to 1.220 g/ml with solid salts and 4.5–5.0 ml of the solution was layered under 1.0–1.5 ml of 1.220 g/ml salt solution using a syringe and long needle. After 30 hr of centrifugation the top fraction, HDL_3, (d 1.125–1.220 g/ml) was removed by tube slicing. The subnatant fraction was designated very high density plasma fraction (VHD). The lipids in this fraction were those of very high density lipoprotein and albumin (LyPC and free fatty acids).

Purity of HDL_2, HDL_3, and VHD fractions was tested by lipoprotein and protein electrophoresis on agarose. HDL_2 contained only alpha-1 lipoprotein and a thin band in the pre-beta region (sinking pre-beta). HDL_3 yielded only a single alpha-1 lipoprotein band. Neither HDL subfraction contained detectable albumin by immunoelectrophoresis or by diffusion on Ouchterlony plates. However, HDL_3 proteins did contain 0.7–1.1% of albumin as judged by quantitative radial immunodiffusion (LC Partigen, Behring Diagnostics, Somerville, NJ). Both HDL fractions reacted with commercial anti-HDL antisera (Behring Diagnostics) on immunodifussion and immunoelectrophoresis, forming one arc when stained for lipid or protein. VHD contained only traces of lipid in the albumin region on electrophoresis and did not react with anti-HDL antisera.

The protein content of isolated lipoproteins was determined by a modification of the method of Lowry et al. (13). Lipid extracts, prepared by extraction with chloroform–methanol 2:1 (v/v) as described by Folch, Lees, and Sloane Stanley (14), were separated on micro-columns of silicic acid essentially as described by Barron and Hanahan (15). Fractions containing cho-

lesteryl esters, triglycerides plus unesterified cholesterol, and phospholipids were eluted sequentially. The purity of each fraction was assessed by thin-layer chromatography and showed negligible cross contamination. Mean recovery of ester and free cholesterol for 28 samples was 102 ± 8% (SD). The TG content of the second fraction was determined; lipid phosphorus in the third fraction was estimated by the Bartlett method (16). The major individual choline-containing phospholipids were determined after separation on Adsorbosil-5 plates using chloroform–methanol–water 75:25:4 as the developing solvent mixture.

Inasmuch as the experimental design employed duplicate postabsorptive sampling and analysis, it was possible to appraise the variability of the lipoprotein fractionation by comparing duplicate results for total cholesterol content of lipoprotein subfractions. Biologic variation during the short period between bleedings is probably small. After lipoprotein separation, additional analytic error arises from lipid extraction, separation procedures, and chemical methods. For 14 sets of duplicates, mean coefficients of variation (±SD) were as follows: HDL_2, total cholesterol, 3.8 ± 2.4%; HDL_3, total cholesterol, 5.0 ± 3.1%; HDL_2-EC, 3.4 ± 2.7%; HDL_3-EC, 4.6 ± 2.7%; HDL_2-TG, 11.2 ± 8.5%; HDL_3-TG, 8.0 ± 7.9%; HDL_3-PC, 8.5 ± 7.0%; VHD-LyPC 8.8 ± 5.3%.

In vitro net transfer experiments

The capacity of VLDL present in plasma 5 hr after fat ingestion to reduce HDL-EC levels by nonenzymatic transfer (17, 18) was assessed in five studies with three normal and two primary hypertriglyceridemic male subjects. The latter patients were mildly hypertriglyceridemic (type IV patterns) without demonstrable etiology. In each study postabsorptive and alimentary lipemic plasma samples were incubated at 37°C for 3 hr to allow determination of LCAT. Paraoxon (2 mM) was then added to inhibit LCAT during an ensuing 12-hr transfer period. Samples at onset, 3 hr, and 15 hr time points were taken for determination of plasma LCAT, UC, and EC. Enzyme inhibition was nearly complete during the transfer period, since further consumption of UC was not observed. Duplicate 4.0-ml aliquots, obtained at 3 and 15 hr, were layered under 2.0 ml of phosphate-buffered saline (pH 7.4) for ultracentrifugal isolation of VLDL ($d < 1.006$ g/ml). After slicing the centrifuge tube to remove the VLDL, the subnatant solution was adjusted to 1.063 g/ml with concentrated salt solution for isolation of LDL and the $d > 1.063$ g/ml fraction. The latter was designated HDL. Isolated lipoprotein solutions were extracted with chloroform–methanol 2:1 (v/v) and the lipid extracts were fractionated by thin-layer chromatography using hexane–ether–acetic acid 90:10:1 for separation of EC and UC. After elution of the lipids from the plate with chloroform, portions of the eluate were used for cholesterol determination.

In vitro pre-incubation experiments

Studies were performed with plasmas from five normal subjects and one with primary type IV hyperlipoproteinemia in order to compare depletion of cholesteryl esters in HDL subfractions by pre-incubation with postabsorptive and alimentary lipemic VLDL and the effect, if any, on LCAT activity. In these experiments VLDL ($d < 1.006$ g/ml) was isolated by ultracentrifugation from fasting and lipemic plasmas obtained 5 hr post feeding from the same individual. Fasting and lipemic plasmas were then reconstituted at the concentration of original unfractionated plasma. In order to test the effect of alimentary lipemic VLDL, a third sample consisted of lipemic VLDL mixed with the $d > 1.006$ g/ml fraction from postabsorptive plasma. Prior to recombination, each $d > 1.006$ g/ml fraction was labeled with [^{3}H]cholesterol as described previously (19, 20). 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB, K&K Laboratories) was added to a concentration of 1.4 mM (21) in order to inhibit LCAT. Plasma samples were then pre-incubated for 5 hr at 37°C. At that time mercaptoethanol (11.8 mM) was added to activate the enzyme (21). Aliquots were taken for plasma lipids, radioactivity, and ultracentrifugal isolation of HDL_2, HDL_3, and VHD as previously described. Incubations were continued for 3 hr and the reaction was terminated by extraction of an aliquot with isopropanol and addition of Paraoxon (2 mM) to another aliquot prior to untracentrifugation. After lipoprotein fractionation, solutions were extracted with chloroform–methanol 2:1 and ester and free cholesterol were separated on thin-layer plates for quantitation of mass and radioactivity.

RESULTS

Plasma LCAT and TG responses to fat ingestion

Mean postabsorptive plasma lipid levels for the group of eight normal subjects were as follows: total cholesterol, 192 ± 13.0 (SEM) mg/dl; UC, 53.1 ± 3.6 mg/dl; EC, 139 ± 9.6 mg/dl; and TG, 75.8 ± 13.5 mg/dl. Peak plasma TG response after fat ingestion was at 5 hr, averaging 108 ± 30% (SEM) for the group. At 10 hr TG had fallen to below basal levels. Plasma LCAT was increased above the mean basal level of 61.3 ± 4.2 (SEM) μmol/l per hr at 2.5 hr, reached a broad plateau between 5 and 7.5 hr, and fell slightly

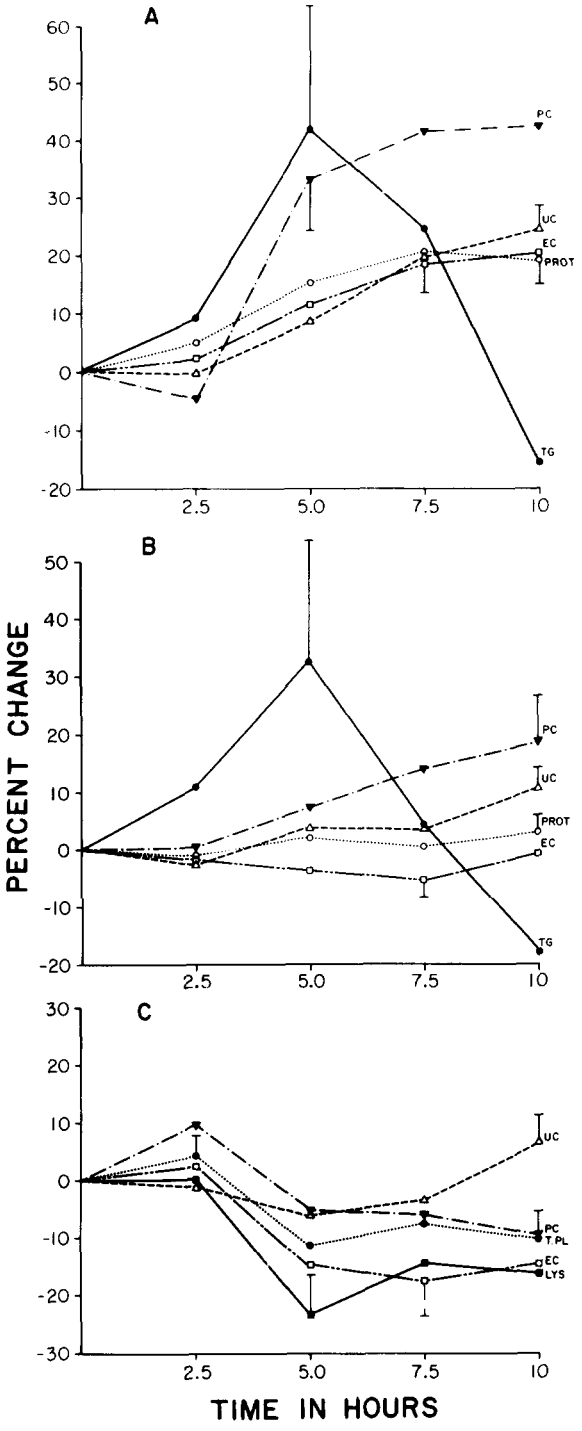

Fig. 1. (*A*) Changes in high density lipoprotein$_2$ (HDL_2) components during the 10-hr period following ingestion of high-fat test meal in eight normal male subjects. Mean percent changes are shown at each time point along with one representative SEM for each component. Abbreviations are: TG, triglycerides; PC, phosphatidylcholine; UC, unesterified cholesterol; EC, esterified cholesterol; Prot, lipoprotein protein. (*B*) Changes in high density lipoprotein$_3$ (HDL_3) components during the 10-hr period following ingestion of a high-fat test meal in eight normal male subjects. Mean percent changes are shown at each point along with one representative SEM for each component. Abbreviations as in Panel *A*. (*C*) Changes in very high density plasma fraction (VHD) lipids during the 10-hr period following ingestion of a high-fat test meal in eight normal male subjects. Legends and abbreviations as in Panel A. Additional abbreviations are: T.P.L., total phospholipids, and LYS, lysophosphatidylcholine.

at 10 hr although it was still significantly above baseline. The group mean increase at 5 hr was 33.8 ± 5.8% (SEM), while the mean of maximal individual responses was 39.8 ± 5.1% (SEM). These results are virtually identical to those of our original report (1). Wallentin and Vikrot (2) have reported a similar temporal response pattern but appreciably smaller enzyme elevations.

HDL changes during alimentary lipemia

Fig. 1 summarizes changes in HDL_2, HDL_3, and VHD constituents during the 10 hr after fat ingestion. Basal levels of HDL_2 components were as follows: UC, 7.2 ± 1.1 (SEM) mg/dl; EC, 16.4 ± 2.6 mg/dl; PC phosphorus, 0.75 ± 0.10 mg/dl; protein, 46.6 ± 6.1 mg/dl; and TG, 3.2 ± 0.8 mg/dl. HDL_2-TG generally paralleled plasma TG, rising to a peak value at 5 hr and falling below baseline at 10 hr (Fig. 1, panel *A*). Lipoprotein TG responses were more variable, since three of eight subjects displayed either negative responses or no change during the 2.5- to 7.5-hr primary response period. Negative responses occurred in those subjects exhibiting the smallest elevations of plasma TG. Cholesteryl esters increased by 5 hr, with a broad peak at 7.5–10 hr. Increases also occurred in proteins, UC, total phospholipids, PC, and the molar ratio of PC/UC. The mean percentage increase in PC was approximately double that of the other components exclusive of TG.

Fig. 1, panel *B*, shows changes in HDL_3 during the period of alimentary lipemia. Basal levels of HDL_3 components were as follows: UC, 7.0 ± 0.7 (SEM) mg/dl; EC, 21.1 ± 2.2 mg/dl; PC phosphorus, 1.25 ± 0.11 mg/dl; protein, 102 ± 6.6 mg/dl; and TG, 3.7 ± 1.3 mg/dl. HDL_3-TG followed a pattern similar to that of plasma and HDL_2. As with HDL_2-TG, both positive (5/8 subjects) and negative (3/8 subjects) responses occurred. Individual HDL_3-TG changes, however, did not correlate significantly with plasma TG changes. Cholesteryl esters showed a small mean reduction greatest at 7.5 hr (−5.4%). Fig. 1, panel *B*, also depicts the pattern of change in other HDL_3 components. Proteins and UC showed little change except for a small UC rise at 10 hr. Phosphatidyl choline showed a slow rise beginning at 5 hr with a 10-hr peak. The PC/UC molar ratio did not change significantly.

During alimentary lipemia, HDL_2 maximal EC increments were highly reciprocally correlated with maximal HDL_2-TG changes ($r = -0.85$, $P = 0.01$). A similar inverse relationship existed in HDL_3 (r

$= -0.88, P = 0.01$). This relationship was also present at individual time points ($r = -0.54$, $P < 0.01$, $n = 32$). **Fig. 2** shows that slopes of the TG:EC interactions were similar for both HDL_2 and HDL_3. The molar changes represented by these regression lines indicated a reduction of EC of 2.4 mol in HDL_2 and 2.5 mol in HDL_3 for each mol increase of TG. These figures compare with a value of about 2 reported by Nichols and Smith (17) from incubation experiments with normal plasma. It should be noted that subjects (three out of eight) who exhibited reductions in lipoprotein TG levels (Fig. 2, solid triangles and circles) were those showing largest increases in EC levels. Also of note is that the intercept of the HDL_2 regression line on the x axis showed nearly 40% elevation of EC with no change in TG. This contrasted with the HDL_3 regression with an x-intercept near zero. Thus, TG changes appear to be prime correlates of EC levels in HDL_3, while an additional source must be invoked to explain the large ester elevation in HDL_2 unrelated to TG fluctuations. These esters might derive from lipolysis of VLDL and chylomicrons or by transfer from HDL_3 and VHD.

Fig. 1, panel *C*, illustrates the effect of fat feeding on the VHD fraction. Basal levels of VHD lipids were as follows: UC, 2.4 ± 0.2 (SEM) mg/dl; EC, 4.8 ± 0.5 mg/dl; PC phosphorus, 0.34 ± 0.02 mg/dl; and LyPC phosphorus, 0.33 ± 0.04 mg/dl. Two and a half hours after feeding, only PC showed a small increase, while total phospholipids, UC, EC, and LyPC were unchanged. By 5 hr all components had fallen below baseline and remained at low levels throughout the period, except for elevation at UC at 10 hr. The fall in EC, which averaged 17% at 7.5 hr, was observed in seven out of eight subjects (Fig. 2). Lysolecithin fell by 23% at 5 hr.

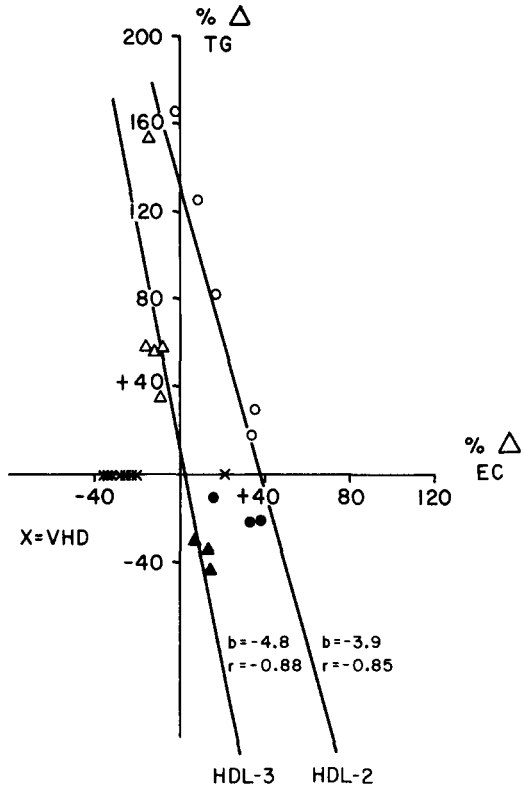

Fig. 2. Maximal percentage changes in HDL_3 and HDL_2 ester cholesterol as a function of maximal percentage changes in lipoprotein triglycerides during the 10-hr period after ingestion of a high-fat test meal. Individual values for each of eight normal male subjects are shown and the regression line was drawn by the least-squares method. Changes in the very high density plasma fraction (VHD, d > 1.220 g/ml) ester cholesterol are also shown on the horizontal axis (x axis) only since triglycerides could not be determined in this fraction. Abbreviations are: TG, triglycerides; EC, ester cholesterol; HDL_3, high density lipoprotein$_3$ (d 1.125-1.220 g/ml); and HDL_2, high density lipoprotein$_2$ (d 1.063-1.125 g/ml); *b*, slope of the regression lines and *r*, correlation coefficient. Solid symbols below the abscissa indicate individuals in whom lipoprotein TG levels fell below baseline. See Discussion.

Correlations between HDL changes and plasma LCAT responses

Although large changes occurred in HDL_2 during assimilation of dietary fat, including increases in the LCAT substrate lipids, PC and UC, there was no correlation between LCAT responses and any change in this subfraction. Similarly, there was no relationship between LCAT and any change in the VHD fraction. Maximal LCAT responses, however, were positively correlated with maximal changes in HDL_3-TG ($r = 0.74$, $P < 0.05$) and negatively with maximal changes in EC ($r = -0.80, P < 0.05$). As described in the preceding section, maximal EC and TG changes were inversely correlated ($r = -0.88$, $P = 0.01$). In order to separate the contribution of each LCAT correlate arising from each variable, partial correlation coefficients were calculated, alternately holding the third variable constant. The TG:EC correlation, with LCAT held constant, was reduced only modestly to $r = -0.71$. The LCAT correlation with TG, with EC changes held constant, was greatly reduced to $r = 0.13$. Thus, the possibility of an independent effect on LCAT through TG changes seems small. Similarly, the LCAT correlation with EC, with TG held constant, was reduced from $r = -0.80$ to -0.47. This residual correlation, although still substantial, was not statistically significant. It should be pointed out that three of eight subjects who exhibited negative TG responses and increased EC levels in their HDL_3 nevertheless had significant elevations of plasma LCAT, although less than the mean response. This might indicate that the activation mechanism is not removal of inhibition by cholesteryl esters, as suggested

by in vitro studies of Fielding, Shore and Fielding (22). However, flotation at d 1.125 g/ml does not discretely separate two lipoprotein components. Consequently, LCAT substrates in HDL_3 could have experienced reduced EC levels which would be obscured by contamination with HDL_2 esters. In view of this uncertainty, in vitro experiments were next employed to test directly the possibility that activation of the enzyme was due to decreased inhibition as a result of EC depletion.

In vitro nonenzymatic net transfer experiments

Nonenzymatic net transfer of HDL-EC to VLDL in apparent exchange for triglycerides has been described by Nichols and Smith (17), and Glomset, Norum, and King (23) using both normal and endogenous hyperlipemia plasmas. To test for this phenomenon in alimentary lipemic plasmas, both fasting and alimentary lipemic (5 hr post feeding) plasma samples from the same individuals were incubated for 3 hr to allow measurement of LCAT activity. This was followed by a 12-hr transfer period during which LCAT was inhibited by Paraoxon. During this transfer period an absolute reduction in HDL-EC occurred in all samples, ranging from 2.9 to 12.6 mg/dl in postabsorptive plasmas and 4.3 to 13.8 mg/dl in lipemic samples. Paired t tests confirmed greater absolute EC decreases during the transfer period in HDL from alimentary lipemic plasmas compared with fasting (mean decrement, 8.5 ± 3.9 mg/dl and 6.9 ± 3.8 mg/dl respectively, $P < 0.01$). The difference in EC reduction between groups was 23.2%. This decrement did not correlate with pretransfer period LCAT activity ($r = 0.53$, $P > 0.05$), but did correlate inversely with VLDL cholesterol levels as determined after isolation from samples at 3 hrs of incubation ($r = -0.75$, $P < 0.05$), immediately after the addition of Paraoxon. Very low density lipoprotein cholesterol also correlated with LCAT activity ($r = 0.86$, $P < 0.01$). During incubation VLDL cholesteryl esters increased in all postabsorptive samples, but greater elevations in corresponding post-feeding samples were demonstrable only in those subjects who exhibited the largest post-feeding increases in VLDL.

In vitro pre-incubation experiments

The results of the transfer experiments indicated that increased VLDL, resulting from fat ingestion, would increase transfer of cholesteryl esters from the HDL to VLDL. Since depletion of HDL_3 esters might activate LCAT, the possibility of in vitro activation by this mechanism was tested. The experimental design employed a 5-hr pre-incubation period in the presence of an LCAT inhibitor and radioisotopic dispersion, since this period is similar to the lag period for peak activation observed in vivo. Comparisons were made by replacement of postabsorptive VLDL with alimentary lipemic VLDL from the same subject. After the pre-incubation period, enzyme was activated for 3 hr by addition of mercaptoethanol and the activity was determined. Prior to these experiments, two experiments were performed replacing fasting with alimentary lipemic VLDL and one testing a lipemic VLDL plus LDL fraction (d < 1.063 g/ml), but without pre-incubations. No changes in LCAT resulted from these replacements.

In plasmas from five normal subjects and one primary hypertriglyceridemic subject, the effects of replacement by alimentary lipemic VLDL on postabsorptive HDL_2, HDL_3, and VHD-EC content were determined. The results (**Table 1**) revealed significantly greater reduction of HDL_3 esters ($P < 0.02$) after 5 hr of pre-incubation with lipemic VLDL. The mean of individual reductions was 10.3%, ranging from 23.2% to +1.2%. The mean maximal HDL_3-EC change observed post feeding in vivo was −3.4%, ranging from −16.0 to 14.0%. Of those displaying decreases (five out of eight, Fig. 2), the mean decrease was 11.9%. Thus, in vitro pre-incubation produced depletion of a similar magnitude to that noted in vivo. This change was not associated with in vitro activation of LCAT during the following period of enzyme activity (Table 1). However, some LCAT comparisons in lipemic plasmas exceeded the 16% limit previously established as a significant change (12), but the precision of the determination is probably reduced by processing, including use of an inhibitor and activator. Analysis by paired t test showed no significant activation. In contrast, addition of alimentary lipemic VLDL to the d > 1.006 g/ml fraction from lipemic plasma, which reconstitutes the lipemic plasma as obtained 5 hrs post feeding, did result in significantly increased activity ($P < 0.025$), even though the hypertriglyceridemic patient (exp. 4) did not respond to fat loading with increased activity. LCAT response to fat feeding in hyperlipemic subjects is variable and follows different temporal patterns compared to normals.[1]

Table 1 also shows that lipemic VLDL failed to deplete postabsorptive HDL_2 esters. A mean individual reduction of 5.0%, (range, −14.8% to +3.6%) was found ($P > 0.05$), but the hyperlipemic VLDL (exp. 4) did yield the largest individual reduction. Like HDL_3, VHD esters were reduced (−0.6 mg/dl, −12%, $P < 0.05$).

[1] Rose, H. G. Unpublished observations.

TABLE 1. Effects of depletion of post-absorptive high density lipoprotein$_3$ (HDL_3) cholesteryl esters by 5-hr preincubation with alimentary lipemic VLDL on plasma LCAT activity

Exp	HDL_3 EC			HDL_2 EC			LCAT			LCAT
	Fast VLDL	Lipemic VLDL	Difference	Fast VLDL	Lipemic VLDL	Difference	Fast VLDL	Lipemic VLDL	% Change	Lipemic Plasma
	mg/dl		*mg/dl*	*mg/dl*		*mg/dl*	*μmol/l/hr*			*μmol/l/hr*
1	22.9	19.1	−3.8	11.2	10.3	−1.4	54.2	52.0	−4.1	—
2	24.5	22.4	−2.1	16.2	15.6	−0.6	52.5	55.3	+5.3	77.4
3	14.5	14.7	+0.2	11.4	10.7	−0.7	49.9	59.1	+18.4	59.3
4	6.9	5.3	−1.6	10.1	8.6	−1.5	90.3	72.2	−20.0	94.6
5	29.9	27.3	−2.6	16.6	17.1	+0.5	45.6	55.9	+22.6	64.5
6	28.2	26.5	−1.7	11.0	11.4	+0.4	53.3	44.7	−16.1	80.8
Mean	21.2	19.2	−1.9	12.8	12.3	−0.6	57.6	56.4	+1.0	75.3
SD	8.8	8.3	1.3	3.8	3.3	0.8	16.3	9.1	17.6	14.0
P			<0.02			>0.05			>0.05	<0.025

VLDL (d < 1.006 g/ml) was removed from post-absorptive plasma from five normal subjects and one hypertriglyceridemic subject (exp. 4) and replaced with fasting VLDL or alimentary lipemic VLDL isolated 5 hr after fat ingestion. Concentrations are those in original unfractionated plasma. Mixtures were incubated for 5 hr in presence of DTNB and then activated for 3 hr by mercaptoethanol and LCAT was determined (μmol/l/hr). HDL_3 and HDL_2 ester cholesterol (EC) levels are in mg/dl as obtained at termination of the 5-hr pre-incubation period. The last column lists LCAT activity obtained by mixing lipemic VLDL with the d > 1.006 g/ml fraction from lipemic plasma and is, therefore, reconstituted lipemic plasma. *P* value is for difference with reconstituted fasting plasma.

In these experiments radioactive unesterified cholesterol had been added to samples and esterification was determined during the activation period. The distribution of radioactive esters was determined in HDL subfractions at the termination of the activation period. The order of specific activities relative to plasma in subfractions isolated from reconstituted postabsorptive plasmas was VHD > HDL_3 > HDL_2 (3.8/2.6/1.3). Similar ratios were obtained from fasting plasmas substituted with lipemic VLDL and reconstituted lipemic plasma. This order of labeling suggests that the VHD fraction contains the most reactive substrates (9). Fractional distribution of labeled esters in the fasting samples was HDL_2, 12.3 ± 2.3% (SD); HDL_3, 40.7 ± 8.3% (SD); and VHD, 13.3 ± 2.4% (SD). Pre-incubation with lipemic VLDL resulted in a shift in distribution significantly decreasing the fraction in HDL_3 and VHD to 38.0% and 12.1%, respectively, with no change in HDL_2. The fraction remaining in the total HDL was reduced from 66.3% to 62.7%, thereby indicating enhanced distribution of esters to lipoproteins of d < 1.063 g/ml during the period of enzyme activity.

DISCUSSION

Stimulation of LCAT activity during alimentary lipemia in man has been reported from three laboratories (1–3). This phenomenon is reproducible and associated with large changes in HDL_2 and HDL_3. These associations raise the possibility that new substrate lipoproteins of intestinal origin are responsible for the activation effect. HDL (24–26) and possibly LCAT (24) are synthesized by rat intestine. Furthermore, a substantial increase in HDL phospholipids, particularly lecithin, occurs during alimentary lipemia in man, which could reflect a characteristic of new substrates that would make for enhanced substrate reactivity. Thus, analysis of HDL subfraction changes that are simultaneous with LCAT measurements might reveal relationships with augmentation of enzymatic activity. We have performed such analyses during alimentary lipemia in eight normal male volunteers. Although the largest changes in protein and lipid content were observed in the HDL_2, none was significantly correlated with LCAT response. This was true even though the HDL_2 were greatly enriched in lecithin, which is a substrate lipid, along with another substrate lipid, free cholesterol. The HDL_3 also exhibited enrichment in lecithin, but once again this failed to correlate with LCAT response. However, the temporal pattern of lecithin increase in HDL_2 and HDL_3 generally paralleled the LCAT response pattern. In view of the report of Wallentin and Vikrot (2) that fat-induced LCAT responses correlated with HDL phospholipid changes and that phospholipid dispersions activate the enzyme in vitro, direct testing of substrate reactivities with purified enzyme will be needed to clarify the significance of this relationship.

Changes in triglyceride levels in the HDL_3 did, however, correlate with LCAT response while cholesteryl esters were inversely related. However, weak correlations between these individual lipid levels and enzyme

response by partial correlation analysis suggest that neither lipid alone is instrumental in regulating activity. The ester response assumes mechanistic importance, since Fielding et al. (22) found reactivities of lecithin:free cholesterol dispersions to be strongly inhibited by the addition of cholesteryl esters. Thus, relief from product inhibition by increased removal of cholesteryl esters from LCAT substrate lipoproteins is a potential explanation for enzyme activation. Partial correlation analysis, however, did not support this possibility, since the correlation with LCAT was substantially diminished if HDL_3 triglycerides were held constant. The main correlate of cholesteryl ester content in HDL_3 is triglyceride content, as shown by a strong inverse relationship between these two lipid constituents (Fig. 2). The mechanism is speculative, but the phenomenon seems analogous to the nonenzymatic TG:EC interchange reported in vitro by Nichols and Smith (17) and Rehnborg and Nichols (18). In the experiments of these workers VLDL-TG transferred to the HDL and HDL-EC transferred to VLDL. This observation has been confirmed by Glomset et al. (23), who also noted that LCAT activity stimulated accumulation of TG in the HDL. Although the phenomenon is nonenzymatic, that is, it proceeds while LCAT is inhibited, it is nevertheless enhanced in the presence of active enzyme (17, 18, 23).

In vivo we have also shown that VHD cholesteryl esters fall during lipemia, but triglycerides in this fraction could not be measured. Interestingly, the lysolecithin content of the VHD also fell during lipemia. The explanation might be related to an observation of Minari and Zilversmit (27), who noted that dog chylomicrons lose phospholipids to the HDL during in vitro incubations, but chylomicron lysolecithin increases. In reverse binding experiments, lysolecithin has been reported to preferentially bind to lipoproteins rather than to apoproteins or albumin (28). This suggests a role for lipids in binding affinity. Since lysolecithin is a potent inhibitor of LCAT activity (22), accelerated removal by binding to chylomicrons and VLDL, leading to reduced levels in substrate lipoproteins, is a possible explanation for activation.

In vitro experiments were performed in order to distinguish between two possible explanations for in vivo associations between LCAT and HDL lipids. *1*) LCAT activation is a result of depletion of HDL_3 and VHD cholesteryl esters by transfer to lipemic VLDL. *2*) Transfer of esters to lipemic VLDL reduced HDL_3 and VHD ester levels, but without influence on enzymatic rate. A related question is whether the extent of ester transport to VLDL by a nonenzymatic mechanism, whatever its effect on LCAT activity, is influenced by ambient levels of enzyme activity. In the first experiment it was necessary to demonstrate that alimentary lipemic VLDL, like hyperlipemic VLDL (17, 23), would reduce HDL cholesteryl esters and that newly synthesized radioactive cholesteryl esters formed in LCAT substrates within HDL would participate in transfer to the VLDL and LDL. The positive results of this experiment extend similar observations of Nichols and Smith (17), Glomset et al. (23), and Akanuma and Glomset (8) to alimentary lipemic VLDL. Both of these phenomena were observed and, in addition, partial correlation analysis suggests that LCAT levels do not condition the extent of subsequent EC transfers, which are primarily related to VLDL and plasma TG levels. A similar conclusion was also reached by partial correlation analysis of our in vivo HDL_3-EC content data.

The second in vitro experiment utilized the demonstrated capacity of alimentary lipemic VLDL to reduce HDL cholesteryl esters. Cholesteryl ester levels were decreased during a 5-hr pre-incubation by the nonenzymatic mechanism in the HDL_3 and VHD fractions. Similar depletion of HDL_2 esters could not be demonstrated (Table 1). Likewise, radioactive cholesteryl esters formed during the activation period were reduced in HDL_3 and VHD, but not in HDL_2. These results favor the conclusion that endogenous LCAT substrate esters are among those removed. Although reduction of the HDL_3 esters was comparable to the largest reductions observed in vivo, in vitro activation of LCAT did not result. This corroborates negative reports by Rose and Juliano (12) and Wallentin and Vikrot (2) in experiments that did not employ preincubation and provides evidence against a modulating role for endogenous substrate cholesteryl ester content in the regulation of LCAT activity. The seeming paradox that increased LCAT activity is associated with reductions rather than increases of cholesteryl esters and lysolecithin in lipoprotein fractions containing substrate macromolecules may be explained by increased removal of product lipids and perhaps lipoproteins through transfer to VLDL, which are at elevated levels during the period of enzyme activation. It is possible that transfer of product cholesteryl esters to VLDL prevents accumulation of esters in substrate lipoproteins thereby permitting augmentation of enzymatic activity. Even though correlation analysis did not show a significant LCAT correlation with transfer in vitro or with changes in HDL_3 and VHD-EC levels in vivo, there exists the possibility that LCAT product lipoproteins constitute a pool of reactive lipoproteins that interact with VLDL.

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