Cholesterol Esterification Rates in Very Low Density Lipoprotein– and Low Density Lipoprotein–Depleted Plasma

Relation to High Density Lipoprotein Subspecies, Sex, Hyperlipidemia, and Coronary Artery Disease

Milada Dobiasova, Jarmila Stribraa, Daniel L. Sparks, P. Haydn Pritchard, and Jiri J. Frohlich

The fractional rate of cholesterol esterification in very low density lipoprotein– and low density lipoprotein–depleted plasma (FER_HDL) was studied in normolipidemic subjects and in individuals with hyperlipidemia or proven coronary artery disease (CAD). The FER_HDL was significantly higher than the FER in whole plasma and was significantly higher in normal men than in normal women. In addition, men and women with primary hyperlipidemia had significantly higher FER_HDL values relative to their sex-matched controls. The most significant increases in FER_HDL values, however, were observed in individuals with CAD. In all patient groups, FER_HDL was positively correlated with plasma triglyceride concentration. In addition, FER_HDL was negatively related to plasma high density lipoprotein (HDL) cholesterol concentration in all groups except in men with CAD and in normolipidemic women. The gradient gel electrophoretic pattern of HDL from individuals with either low or high FER_HDL values indicated an inverse relation between this activity and the relative amount of HDL particles. FER_HDL likely reflects the metabolic properties of the heterogeneous population of HDL particles in the plasma and may be a function of the relative content of larger and smaller HDL particles. It appears to be a sensitive and reliable functional measure of the particle size distribution in the HDL pool and one of potential clinical value in the assessment of risk for CAD. (Arteriosclerosis and Thrombosis 1991;11:64–70)

Sperri1 described esterification of cholesterol in plasma in 1935, and Glomset2 elucidated much of what we know about the enzyme lecithin: cholesterol acyltransferase (LCAT). Presently, the activity of LCAT is measured using exogenous proteoliposome substrates,3 and this activity correlates well with the mass of the enzyme measured by immunoassays.4–6 Alterations in LCAT activity and/or mass have been reported in primary4–7 and secondary8 LCAT deficiency.

Many investigators have also measured the fractional rate of esterification of plasma cholesterol (FER). This activity reflects the interaction of LCAT with its endogenous substrates and is usually measured by incubation of plasma, followed by determination of the decrease in concentration of free cholesterol9 or by the rate of esterification of radioactive unesterified cholesterol.10–11 Changes in FER have been noted in numerous conditions (for a review, see Reference 12), but the findings have been inconsistent. This may have been due to the variety of assay methods employed, the difficulty in evaluating the effect of substrate abnormalities, the presence of potential activators and inhibitors of the enzyme, and changes in the LCAT enzyme mass. Thus, the clinical usefulness of FER has not been accepted.

Most of the cholesterol esterification in plasma takes place in the high density lipoprotein (HDL) fraction2–13; the smaller (HDL2) particles appear to be a preferred substrate, and the larger particles (HDL3), a product of the reaction.13–16 Therefore, we decided to estimate the FER in very low density lipoprotein (VLDL)– and low density lipoprotein...
Dobiasova et al  Cholesterol Esterification Rates in HDL 65

(LDL)-depleted plasma. The removal of these potential sources of free cholesterol for the LCAT reaction in VLDL and LDL assures that only the unesterified cholesterol in HDL serves as a substrate. We have studied cholesterol esterification in plasma from patients with hyperlipidemia and atherosclerosis as well as from normal individuals. Our results suggest that the FER in this fraction of plasma reflects the relative content of HDLα in the HDL pool and that it may be a useful predictor of the risk for atherosclerosis.

Methods

Materials

[3H]cholesterol and [14C]cholesterol were purchased from Amersham Corp., Arlington Heights, Ill. Total and unesterified cholesterol and triacylglycerol determination reagent kits were purchased from Boehringer, Mannheim, F.R.G. All other chemicals were of analytical grade.

Subjects

Thirty-five apparently healthy, normolipidemic men and women, 65 hyperlipidemic patients, and 27 patients with angiographically proven coronary artery disease (CAD) were studied. Hyperlipidemic patients and the patients with CAD were recruited from the University Hospital Lipid Clinic in Vancouver, the Institute of Clinical and Experimental Medicine in Prague, and the angiocatheterization laboratory of University Hospital and Vancouver General Hospital, Vancouver, Canada. None of the patients from Prague was on any drug treatment during this investigation, whereas almost all the Vancouver patients were treated with a variety of drugs, including lipid-lowering agents and β-blockers.

Lipid Analyses

Blood specimens were collected into EDTA-containing tubes after a 12-hour, overnight fast. The blood was placed on ice and centrifuged within 2 hours at 1,750g for 10 minutes to separate plasma. Plasma was analyzed within 48 hours if kept on ice or within 1 week if stored at −20°C. Total and free cholesterol and triglycerides were estimated enzymatically.17,18 VLDL/LDL-depleted plasma was prepared by precipitation of apolipoprotein B-containing lipoproteins with phosphotungstate-MgCl2.19,20 One hundred microliters phosphotungstate solution (4 g phosphotungstic acid, 16 ml 1 M NaOH in 100 ml deionized water) was added to 1 ml plasma, followed by addition of 25 μl MgCl2 solution. After the solution was mixed and allowed to stand for 30 minutes at 4°C, the suspension was centrifuged at 12,000 rpm at 4°C. This method does not interfere with the enzymatic analysis of HDL unesterified cholesterol (HDL-FC), the estimation of FER, or the gradient gel electrophoretic pattern of HDL subclasses.

Determination of Esterification Rates

The molar and fractional esterification rates (MERs and FERs) were estimated by measuring the rate of esterification of [14C]cholesterol or [3H]cholesterol as described previously.11 Briefly, [3H]cholesterol or [14C]cholesterol was incorporated onto filter paper disks, which were subsequently incubated overnight with 200 μl plasma or VLDL/LDL-depleted plasma at 4°C. The labeled plasma or VLDL/LDL-depleted plasma samples were incubated for 30 minutes at 37°C. Samples of 50 μl were taken before and after incubation and extracted with 1 ml ethanol. After 2 hours, the protein precipitate was removed by centrifugation (10 minutes, 3,000 rpm), the supernatant was evaporated, and the free and esterified cholesterol fractions were separated by thin-layer chromatography. Radioactivity was measured by scintillation counting, and the FER was calculated as the difference between the percentage of radioactive cholesterol esterified before and after incubation. MER was calculated from FER estimations and HDL-FC levels, as previously described.10 For each experimental series, an aliquot of frozen, normolipidemic plasma was used for quality control. The interassay coefficient of variation for FERHDL was 5.6% (n=8) and for the HDL-FC assay was 5.7% (n=8) on aliquots of plasma stored at −70°C for 1 month. Negligible changes in both FERHDL and HDL-FC were observed during this period.

Gradient Gel Electrophoresis

Precast 4/30 polyacrylamide gradient gels (Pharmacia, Uppsala, Sweden) were electrophoresed in Tris/borate/EDTA buffer, pH 8.35, as described by Nichols et al.21 High molecular weight calibration proteins (Pharmacia) were used as reference standards to evaluate the electrophoretic mobility of HDL. Plasma proteins were removed from unfrozen plasma or VLDL/LDL-depleted plasma samples by ultracentrifugation at a final d=1.21 g/ml. Ultracentrifugation was performed with 75Ti rotor in a Beckman 8-L
Results

Figure 1 shows the time course for the FER in the VLDL/LDL-depleted plasma (FER\textsubscript{HDL}) of two individuals with a similar concentration of HDL-FC. As in whole plasma, the esterification was linear for at least 30 minutes, and the rate did not level off for at least 5 hours despite the fact that 60% of HDL cholesterol was esterified in subject 1 and 30% was esterified in subject 2. In a preliminary study, esterification rates in plasma and LDL/VLDL-depleted plasma were compared in 24 men and 20 women (17 men and 10 women of whom were hyperlipidemic). As observed for any other lipid parameters. Of the patients with identifiable genetic disorders of lipid metabolism, nine men with familial combined hyperlipidemia had the highest FER\textsubscript{HDL} values (Table 2). No such systematic difference between groups was observed for other lipid parameters. Of the patients with identifiable genetic disorders of lipid metabolism, nine men with familial combined hyperlipidemia had the highest FER\textsubscript{HDL} values (Table 2). No such systematic difference between groups was observed for other lipid parameters.
Several patients whose blood lipid levels were lowered to desirable levels by treatment with lipid-lowering drugs had $\text{FER}_{\text{HDL}}$ values that were similar to those of the group of normal subjects; conversely, those whose lipid levels remained high despite drug treatment had higher-than-normal $\text{FER}_{\text{HDL}}$ values. An arbitrary risk factor score (RFS) was determined for each hyperlipidemic subject, which reflected the relative risk of having CAD. Risk factors taken into account were age, smoking, hypertension, diabetes, obesity, and familial history of vascular disease. This RFS was used to segregate the hyperlipidemic subjects into a high- and a low-risk subgroup. While no significant differences were observed in total or unesterified cholesterol levels of HDL for these two groups, both hyperlipidemic men and women with high RFSs had significantly higher $\text{FER}_{\text{HDL}}$ and $\text{MER}_{\text{HDL}}$ values ($p<0.01$) than their sex-matched, low-risk groups (data not shown).

The $\text{FER}_{\text{HDL}}$ was strongly correlated with plasma concentration of triglycerides ($r=0.464-0.761$) in all groups of subjects (Table 3). Negative correlations were found between $\text{FER}_{\text{HDL}}$ and plasma levels of HDL cholesterol ($r=-0.553$ to $-0.799$) in all groups except in men with CAD and in normolipidemic women ($r=-0.042$ and $-0.235$, respectively). The small group of six women with CAD also showed a positive correlation between $\text{FER}_{\text{HDL}}$, age, and body mass index (Table 3). $\text{FER}_{\text{HDL}}$ did not correlate with the level of plasma cholesterol except in the group of hyperlipidemic men.

The electrophoretic pattern of HDL on gradient gel electrophoresis was identical when either whole plasma or VLDL/LDL-depleted plasma was used as a starting material. Thus, it is unlikely that the precipitation procedure caused major compositional alterations of HDL. A comparison of HDL mobility on gradient gel electrophoresis between individuals with low and high $\text{FER}_{\text{HDL}}$ values is shown in Figure 2. Pairs of individuals with similar HDL-FC concentrations (within 15% of each other) were compared, and their individual data are pre-

TABLE 3. Correlation of Fractional Esterification Rate in Very Low Density Lipoprotein/Low Density Lipoprotein-Depleted Plasma of High Density Lipoprotein With Other Parameters

<table>
<thead>
<tr>
<th>Patients/subjects</th>
<th>$n$</th>
<th>Age</th>
<th>BMI</th>
<th>TC</th>
<th>TG</th>
<th>HDL-TC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>16</td>
<td>0.097</td>
<td>0.382</td>
<td>0.306</td>
<td>0.508*</td>
<td>-0.553*</td>
</tr>
<tr>
<td>Hyperlip</td>
<td>34</td>
<td>-0.123</td>
<td>...</td>
<td>0.551†</td>
<td>0.761†</td>
<td>-0.619†</td>
</tr>
<tr>
<td>CAD</td>
<td>21</td>
<td>-0.345</td>
<td>0.194</td>
<td>0.180</td>
<td>0.706‡</td>
<td>-0.042</td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>19</td>
<td>...</td>
<td>0.477</td>
<td>0.218</td>
<td>0.464*</td>
<td>-0.235</td>
</tr>
<tr>
<td>Hyperlip</td>
<td>32</td>
<td>-0.017</td>
<td>...</td>
<td>-0.479*</td>
<td>0.530†</td>
<td>-0.673†</td>
</tr>
<tr>
<td>CAD</td>
<td>6</td>
<td>0.763*</td>
<td>0.705</td>
<td>-0.227</td>
<td>0.477</td>
<td>-0.799*</td>
</tr>
</tbody>
</table>

BMI, body mass index; TC, total cholesterol; TG, total triglycerides; HDL, high density lipoprotein; Hyperlip, hyperlipidemic; CAD, coronary artery disease.

Significance of difference from normal: *$p<0.05$, †$p<0.01$, ‡$p<0.001$. 

FIGURE 2. Diagrams of gradient gel electrophoresis of d>1.21 g/ml plasma lipoprotein fractions in individuals with similar HDL-free cholesterol levels but different $\text{FER}_{\text{HDL}}$ values (see Table 4 for individual [1-8] biochemical data). Scans are demarcated into HDL subfractions as described by Nichols et al.21 HDL, high density lipoprotein; FER, fractional esterification rate.
sent in Table 4. It is apparent that individuals with high \( \text{FER}_{\text{HDL}} \) values had few or no particles with the mobility of HDL\(_2\), and an increased amount of the smaller HDL\(_3\) particles (Figure 2). Correlation analysis revealed a significant inverse relation \( (r = -0.78) \) between the estimated \( \text{FER}_{\text{HDL}} \) and the relative amount of HDL\(_2\) (Figure 3).

**Discussion**

Differences between the FER and MER in plasma and those in LDL/VLDL-depleted plasma observed in this study clearly outline the inherent problems in estimating esterification rates in plasma. In particular, the assumption that all the equilibrated radioactive cholesterol or the total FC pool is available for esterification by LCAT seems questionable. The increased FER in the LDL/VLDL-depleted sample, as compared with that of plasma, suggests that much more of the labeled cholesterol was available for esterification. In addition, the apparent increase in MER\(_\text{plasma} \) relative to MER\(_{\text{HDL}} \) may be artifactual if the total FC pool in plasma is not available for esterification. Therefore, we propose that the estimation of both FER and MER after precipitation of LDL and VLDL is a closer approximation of the actual esterification rate in plasma.

In this study, we have identified an inverse relation between the relative plasma HDL\(_2\) content and \( \text{FER}_{\text{HDL}} \). This indicates that the cholesteryl ester-generating capacity of the HDL pool may be a function of the relative HDL particle size distribution. Previous studies by ourselves\(^{22}\) and Barter et al\(^{23}\) suggest that esterification rates may be independent of HDL cholesteryl ester content and may instead be directly related to HDL particle size. Investigations by Fielding and Fielding,\(^{16}\) however, have suggested that cholesteryl ester may be a feedback inhibitor of LCAT. In the present study, no relation between \( \text{FER}_{\text{HDL}} \) and HDL cholesteryl ester content was observed. Alternatively, the increased \( \text{FER}_{\text{HDL}} \) observed in hyperlipidemic subjects was shown to be associated with a change in particle size distribution of HDL, caused by a reduction in the number of HDL\(_2\) particles and an increase in the number of very small HDL particles. It is unclear, however, whether this increased esterification rate is entirely due to the lack of HDL\(_2\), which is capable of inhibiting LCAT, or to the presence of a small subset of HDL\(_3\) which is an excellent substrate for LCAT. Studies by Barter et al\(^{24-25}\) suggest that a combined effect may have resulted in the elevated esterification rates we have observed. Investigations in their laboratory have shown that HDL\(_2\) may be a competitive inhibitor of LCAT\(^{24}\) and that plasma of hypertriglyceridemic subjects may have an increased content of small HDL\(_3\), which have a greater-than-normal reactivity with LCAT.\(^{25}\)

The consensus of these studies is that the smaller (HDL\(_3\)) particles are the best substrate for the enzyme and that HDL\(_2\) is a competitive inhibitor of this reaction.\(^{22-25}\) While we have recently shown that small HDLs are preferred LCAT substrates in vitro and that an inverse, linear relation exists between the size of an HDL particle and its ability to interact with LCAT,\(^{22}\) it is still unclear which factors lead to changes in HDL particle size distribution. In the present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\) present study, subjects with the highest \( \text{FER}_{\text{HDL}} \) had practically no HDL\(_2\) particles. Low levels of HDL\(_2\)
found in these individuals could be due to either impaired synthesis or to increased catabolism. Eisenberg\textsuperscript{12} has suggested that the interconversion of HDL\textsubscript{2} and HDL\textsubscript{3} probably requires the coordinated action of LCAT, cholesteryl ester transfer protein, and the triglyceride lipases. However, while numerous studies have shown that HDL\textsubscript{2} can be generated from HDL\textsubscript{3}\textsuperscript{26-28} and can also be converted back to HDL\textsubscript{3},\textsuperscript{15} the factors that regulate their interconversions and determine the level of HDL\textsubscript{2}, relative to HDL\textsubscript{3} remain unclear.

Some studies have suggested the HDL\textsubscript{2} levels may be directly affected by the duration and magnitude of triglyceridemia.\textsuperscript{19,20} Investigators have demonstrated a marked decrease in the number of HDL\textsubscript{2} particles in hypertriglyceridemic subjects\textsuperscript{29} and also in subjects in the postprandial state.\textsuperscript{30} In addition, a significant inverse relation between plasma triglyceride levels and the levels of HDL\textsubscript{2a} and HDL\textsubscript{2b}, as estimated by gradient gel electrophoresis, has been demonstrated.\textsuperscript{31} In the present study, we also observed an inverse relation between triglyceride levels and plasma HDL\textsubscript{2a} content. These observations are expected, since HDL\textsubscript{2} levels have been shown to be strongly correlated with the activity of lipoprotein lipase and inversely related to the activity of hepatic lipase.\textsuperscript{29,30} Therefore, since we have observed that elevated triglyceride levels are associated with a decrease in HDL\textsubscript{2} cholesterol levels and with an increase in the rate of cholesterol esterification in HDL\textsubscript{2}, it seems possible that the low HDL\textsubscript{2} levels observed in hypertriglyceridemia may be partially due to an increased transfer of cholesteryl esters from the HDL pool to lower-density lipoproteins. This is, in fact, what we have observed in other investigations in which hyperlipidemic patients had impaired equilibration of cholesteryl esters within their HDL pool, resulting in their increased transfer to apo B-containing lipoproteins.\textsuperscript{32} It is of interest that low levels of HDL\textsubscript{2a} cholesterol have also been associated with the increased ratio of waist-to-hip circumference, plasma insulin levels, and glucose intolerance.\textsuperscript{33} These findings agree with our observations of increased FER\textsubscript{HDL} values in patients with an increased number of risk factors for CAD.

This study suggests that the FER\textsubscript{HDL} value may reflect the capacity of the HDL pool to synthesize cholesteryl esters and that this may be related to the ratio of HDL\textsubscript{2} to HDL\textsubscript{3}. Thus, the determination of FER\textsubscript{HDL} may have important diagnostic merit since the low ratio of HDL\textsubscript{2} to HDL\textsubscript{3} appears to be the best indicator of the presence of coronary atherosclerosis.\textsuperscript{34} In our study, dyslipidemic patients with a high risk of developing CAD had significantly higher FER\textsubscript{HDL} values. Patients with proven CAD were shown to have significantly higher FER\textsubscript{HDL} values than do normal subjects. We suggest that there are several points that indicate that the diagnostic potential of this assay may be superior to the determination of HDL\textsubscript{2} to HDL\textsubscript{3} cholesterol levels. The FER\textsubscript{HDL} gives very reproducible values when measured in the same patient over several years (data not shown), and the coefficient of variation for this assay is markedly better than that for the HDL\textsubscript{2}/HDL\textsubscript{3} cholesterol determinations. In addition, the differences in FER\textsubscript{HDL} between men and women and in subjects with or without CAD are more much marked than the differences in their HDL\textsubscript{2}/HDL\textsubscript{3} ratios.\textsuperscript{35} The dual-precipitation procedure for the estimation of HDL\textsubscript{3} has been recently reviewed by Cleo and Bachorik,\textsuperscript{36} who concluded that problems with this methodology must be solved before its introduction into clinical laboratories.

We propose, therefore, that determination of FER\textsubscript{HDL} may represent the relative ability of LCAT to preferentially use HDL of different sizes and, as such, may be a sensitive way to characterize the metabolic properties of a very heterogeneous population of HDL particles.

Acknowledgments

Benny Bar Shlomo and Donald Ricci provided us with specimens from patients with coronary artery disease. We would like to thank Marie Schutzova, Cathy McGuiness, and Lida Adler for their invaluable assistance with this project.

References


30. Patsch JR, Prasad S, Gotto AM, Patsch W: Relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. *J Clin Invest* 1987;80:341–347


**KEY WORDS** • lecithin:cholesterol acyltransferase • high density lipoproteins • coronary artery disease • hyperlipidemia
Cholesterol esterification rates in very low density lipoprotein- and low density lipoprotein-depleted plasma. Relation to high density lipoprotein subspecies, sex, hyperlipidemia, and coronary artery disease.

M Dobiasova, J Stribrna, D L Sparks, P H Pritchard and J J Frohlich

Arterioscler Thromb Vasc Biol. 1991;11:64-70
doi: 10.1161/01.ATV.11.1.64
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/11/1/64

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/