HDL and Plasma Phospholipids in Coronary Artery Disease

Friedl Kunz, Christoph Pechlaner, Reinhold Erhart, Falko Fend, Volker Mühlberger

Abstract Lipid fractions of native plasma and of high-density lipoprotein (HDL) were analyzed, and the clotting times of native platelet-rich and -poor plasma were recorded in patients with coronary artery disease and age-matched control subjects not taking any medication known to alter plasma lipid levels, coagulation, or platelet aggregation. Patients with coronary artery disease had lower HDL cholesterol and particularly HDL phospholipids but elevated HDL triglycerides, plasma triglycerides and diglycerides, and fibrinogen. Plasma lyssolecithin was diminished. Accelerated coagulation was observed in native plasma and may be related to these changes in plasma lipids. The HDL content in cholesterol may be less relevant than that in phospholipids, which, because of their amphiphilic properties, may be essential for the removal and transport of hydrophobic cholesterol. The lower lyssolecithin levels also suggest diminished esterification of cholesterol.

High-density lipoprotein cholesterol (HDL-C) has been examined in coronary artery disease (CAD) in countless investigations, but there are only few and, because of differences in methods and medication, contradictory reports on HDL content in phospholipids. This small number of publications is surprising considering the amphiphilic properties of phospholipids, which might play a mediating role in the transport and removal of hydrophobic neutral lipids, in particular of cholesterol esters, from vessel walls. Accordingly, HDL with reduced phospholipid content has been found to be a poor receptor of cell cholesterol. Because of their surface-active properties, phospholipids also play an important role at all stages of coagulation and therefore are probably essential for interactions between nonpolar lipids and polar coagulation proteins. Such interactions may be important in view of the contribution of thrombosis to arteriosclerosis, of the thrombotic origin of myocardial infarction and unstable angina, and of the poor lysability of platelet-rich thrombi.

Apart from fibrin, these thrombi also contain considerable amounts of both phospholipids derived from platelet material and cholesterol and triglycerides of plasmatic origin. Therefore, the evolution of platelet-rich and thus phospholipid-rich thrombi may be determined by the capacity to remove not only fibrin but also lipids interacting with the hemostatic system. To find out more about these interrelations and the reasons for the discrepant reports on HDL phospholipids, we examined the main lipid fractions of plasma and HDL, particularly phospholipids, together with hemostatic parameters in CAD patients and healthy control subjects, without interference from medication.

Methods

Patients and Control Subjects

The selection criteria used have been previously described. One additional patient and two control subjects were included, resulting in a total of 38 CAD patients and 41 control subjects. Subjects who suffered from any consuming, infectious, or metabolic disease and who had taken any medication known to influence coagulation, platelet function, or plasma lipids (for details see Reference 10) were excluded from the study. All patients had either significant coronary stenosis demonstrated by angiography or had suffered myocardial infarction 3 or more months previously. Informed consent was obtained from all participants. Physical activity was classified as follows: 1, hardly any; 2, very little; 3, little; 4, moderate; 5, much; and 6, very much.

Preparation of Plasma and HDL

Native blood was collected by puncture of an antecubital vein, using a 1.2-mm needle and polyethylene syringes. No anticoagulant was added. Until the end of sample collection the blood was cooled at 4°C and then immediately centrifuged for 10 minutes at 10°C and 2000g to yield native platelet-poor plasma and at 400g and 10°C for 5 minutes. The supernatant platelet-rich plasma was gently pipetted into plastic tubes. Platelets were counted in platelet-poor and platelet-rich plasma, which were then mixed to give 3 mL of plasma with a final concentration of 250,000 platelets per microliter (=native platelet-rich plasma). Plastic tubes with 3 mL of platelet-poor...
or platelet-rich plasma were put into a water bath at 37°C, and the time at which the first visible clot appeared was recorded. HDL was prepared according to Burstein et al.11

Lipid Extraction, Chromatography, and Estimation

Lipid extraction, chromatography, and estimation were carried out as described.10 Lipids were extracted according to Folch et al.12 from native platelet-poor plasma and HDL. All extraction procedures were started immediately after preparation, because allowing HDL to stand for more than 2 hours and even storing plasma for several hours before preparation of HDL resulted in degradation, as revealed by thin-layer chromatography and loss of phospholipids. Similarly, deep-frozen storage of plasma and HDL over years resulted in the degradation of phospholipids. Therefore, reproducibility was tested by measuring plasma and HDL phospholipids on different days and of phosphorus over several years. Day-to-day variance was less than 5%. During extraction and phase separation, samples were kept at 4°C. Of the lipid phase of the extracts, 2 mL was taken for estimation of total phospholipids using a modification13 of the original method.14 After evaporation of the solvent the lipid extracts were stored in glass tubes at 4°C and dissolved in approximately 0.1 mL hexane. Thin-layer chromatography and elution of neutral lipids was carried out using a modification15 of the original method.16 The eluates were dried, and analyses of cholesterol,17 glycerides,18 and free fatty acids19 were performed in the respective fractions. Phospholipid fractions were separated by one-dimensional thin-layer chromatography on silica gel as described,20 scraped into tubes, and measured using a modification13 of the original method.14

Coagulation Tests

Platelets were counted with a Coulter counter. Measurement of fibrinogen21 and thrombelastography22 were carried out as described. Tests for prothrombin time (Boehringer), partial thromboplastin time (Cephaloplastin Dade), and anti-thrombin III (chromogenic substrate, Boehringer) were carried out according to manufacturers’ instructions.

Statistical Analyses

Statistical analyses were conducted as described.10 Most of the data were not normally distributed, so the Mann-Whitney U test was applied throughout for comparison between the two groups. Significance levels for Pearson correlation coefficients were calculated using standard formulas23; two-way frequency tables were analyzed using the Pearson χ2 test.

Correlations

Fibrinogen and coagulation time of clots and thrombi, thrombus-forming time, and maximal amplitude of the thrombelastogram were correlated with phospholipids and total HDL-C and with the main lipid fractions of plasma. Only correlations with probability values less than or equal to .001 were classified as significant. These retain statistical significance even after Bonferroni correction.

Results

Comparisons

Weight, height, age, sex, physical activity, and existing smoking habits did not differ between the groups. However, body mass index (24.8±2.7 versus 23.6±2.3, CAD patients versus control subjects, P=.03) and the number of exsmokers were significantly greater in the CAD group (18/38 versus 7/41, P=.005).

Free (11.7±3.7 versus 14.4±2.7 mg/dL, P=.028), esterified (34.4±8.1 versus 45.7±14.8 mg/dL, P=.0006), and total (48.2±13.9 versus 59.5±17.7 mg/dL, P=.002) cholesterol and phospholipids (83.3±16.4 versus 109.8±24.1 mg/dL, P=.0000) of HDL were lower in CAD patients (Figure), in contrast to higher levels of triglycerides in plasma and HDL (11.9±4.9 versus 8.6±3.7 mg/dL, P=.0031). Plasma lysolecithin was decreased, but diglycerides (Table 1) and the molar ratios of plasma triglycerides to free fatty acids (7.10±5.28 versus 4.33±4.69, P=.018) and plasma triglycerides to monoglycerides (61.8±53.4 versus 28.1±18.5, P=.0006) were higher in CAD patients.

Lysolecithin and free fatty acids of HDL are not presented because they are mainly bound to albumin rather than HDL.

Fibrinogen and the maximal amplitude of the thrombelastogram were higher in CAD, whereas the clotting times of both native platelet-poor and platelet-rich plasma and the thrombus-forming time of the thrombelastogram were shorter (Table 2).

Correlations

In control subjects, HDL phospholipids correlated directly with plasma phospholipids, physical activity, clotting time of platelet-poor plasma, and thrombus-forming time and inversely with both the maximal amplitude of the thrombelastogram and fibrinogen (Table 3). HDL-C correlated directly with physical activity and thrombus-forming time and inversely with fibrinogen. No correlation between these variables was found in CAD patients.

Discussion

We excluded from this study subjects with interfering medication, which most coronary patients receive. Therefore, although the investigation was carried out over more than 6 years, the number of eligible patients was comparatively small. This disadvantage appears to be more than compensated for by the advantage of
TABLE 1. Plasma Lipid Levels in Study Patients and Control Subjects

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Control Subjects (n=41)</th>
<th>CAD Patients (n=38)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids, mg/dL</td>
<td>205.7±31.52</td>
<td>208.9±40.75</td>
<td>NS</td>
</tr>
<tr>
<td>Monoglycerides, mg/dL</td>
<td>4.216±2.439</td>
<td>3.649±2.663</td>
<td>NS</td>
</tr>
<tr>
<td>Diglycerides, mg/dL</td>
<td>4.662±3.554</td>
<td>5.961±2.783</td>
<td>.0139</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>91.71±51.11</td>
<td>139.6±51.84</td>
<td>.0000</td>
</tr>
<tr>
<td>Free cholesterol, mg/dL</td>
<td>62.28±16.15</td>
<td>68.09±26.11</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol esters, mg/dL</td>
<td>152.2±24.65</td>
<td>173.2±51.87</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>214.5±33.87</td>
<td>240.6±70.14</td>
<td>NS</td>
</tr>
<tr>
<td>Free fatty acids, μmol/dL</td>
<td>358.7±194.0</td>
<td>304.0±157.3</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>134.1±33.50</td>
<td>162.4±69.95</td>
<td>.0332</td>
</tr>
<tr>
<td>Lysolecithin, %</td>
<td>6.090±1.813</td>
<td>5.039±0.9130</td>
<td>.0226</td>
</tr>
<tr>
<td>Sphingomyelin, %</td>
<td>16.10±3.001</td>
<td>16.07±2.267</td>
<td>NS</td>
</tr>
<tr>
<td>Lecithin, %</td>
<td>71.72±2.543</td>
<td>72.22±2.530</td>
<td>NS</td>
</tr>
</tbody>
</table>

CAD Indicates coronary artery disease; LDL, low-density lipoprotein. Values are mean±SD. Statistical significance is described by probability values derived from nonparametric tests (Mann-Whitney U test).

Table 2: Coagulation Tests in Study Patients and Control Subjects

<table>
<thead>
<tr>
<th>Test</th>
<th>Control Subjects (n=41)</th>
<th>CAD Patients (n=38)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>259.5±53.77</td>
<td>311.2±75.92</td>
<td>.0022</td>
</tr>
<tr>
<td>Prothrombin time, %</td>
<td>100.4±15.72</td>
<td>97.66±13.42</td>
<td>NS</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>28.39±2.558</td>
<td>28.29±3.035</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet count, g/L</td>
<td>256.9±80.52</td>
<td>278.3±77.66</td>
<td>NS</td>
</tr>
<tr>
<td>Antithrombin III, %</td>
<td>98.09±11.14</td>
<td>103.6±15.12</td>
<td>NS</td>
</tr>
<tr>
<td>Clotting time of TEG, min</td>
<td>8.182±2.270</td>
<td>7.714±2.070</td>
<td>NS</td>
</tr>
<tr>
<td>Thrombus-forming time of TEG, min</td>
<td>7.515±2.210</td>
<td>6.533±2.609</td>
<td>.0382</td>
</tr>
<tr>
<td>Maximal amplitude of TEG, mm</td>
<td>45.83±8.733</td>
<td>51.94±10.33</td>
<td>.0114</td>
</tr>
<tr>
<td>Clotting time of PPP, s</td>
<td>1185±495.7</td>
<td>788.0±492.2</td>
<td>.0081</td>
</tr>
<tr>
<td>Clotting time of PRP, s</td>
<td>719.0±358.8</td>
<td>552.3±236.7</td>
<td>.0308</td>
</tr>
</tbody>
</table>

CAD Indicates coronary artery disease; aPTT, activated partial thromboplastin time; TEG, thrombelastogram; PPP, native platelet-poor plasma; and PRP, native platelet-rich plasma. Values are mean±SD. Statistical significance is described by probability values derived from nonparametric tests (Mann-Whitney U test).
stalant degradation of HDL phospholipids (see "Methods"). In another study the groups were not matched with respect to age and medication. The same objections also apply to an investigation in which HDL phospholipids were lower in CAD patients; most patients, but not the control subjects, received treatment modifying plasma or HDL lipids. These discrepant reports indicate that both methods adapted to the instability of phospholipids during preparation of HDL and selection criteria are critical in the evaluation of HDL phospholipids.

Even more contrasting than the reports about HDL phospholipids are those that show plasma lyssolecithin levels ranging from 3.7% to 12% of total phospholipids in CAD patients and from 3.4% to 8% in control subjects. Such enormous differences indicate technical problems in the separation of phospholipids. In contrast, we found normal percentages to be constant, at approximately 6%.

The decrease in this phospholipid fraction, also observed in other diseases associated with thrombosis, may also contribute to the development of CAD because lyssolecithin has been reported to relax smooth muscles and inhibit platelet aggregation.

Lower lyssolecithin levels in CAD also support the observation of reduced activity of lecithin:cholesterol acyltransferase, which is responsible for esterification and thus for the uptake of cholesterol by HDL. Therefore, decreased activity of this enzyme indicates both diminished removal of cholesterol and reduced degradation of lecithin and other glycerophospholipids, contributing 60% to platelet lipids. Their impeded deacylation may add to the poor lysability of platelet-rich and thus glycerophospholipid-rich thrombi, which also contain considerable amounts of triglycerides. Their removal is therefore also important for thrombolysis, particularly as triglyceride-rich LDLs have been shown to coat fibrin strands and therefore to limit their contact with fibrinolytic enzymes. Triglyceride degradation also may be reduced in CAD, as the ratio of triglycerides to monoglycerides and to free fatty acids was increased in CAD. Accordingly, lipoprotein lipase has been found to be decreased in CAD. Therefore, diminished lipolysis may be partly responsible for heavier thrombi containing more lipids and for the reported inhibition of fibrinolysis as indicated by higher levels of plasminogen activator inhibitor related to elevated triglyceride levels in CAD.

The higher fibrinogen levels that we found and the accelerated coagulation in native plasma aggravate these findings. These signs of a hypercoagulable state may be caused by a change in the reported thromboplastin-inhibiting properties of LDL, as a decrease in these has been found in triglyceride-rich LDL with an increased ratio of apoprotein B to phospholipids. This pattern is characteristic of small LDL augmented in CAD and associated with elevated plasma triglycerides and reduced HDL-C, as in our patients.

In addition, in CAD there may be a partial loss of the "protective" effect of HDL phospholipids; their direct correlation with clotting and thrombus-forming time as well as their inverse correlation with fibrinogen and maximal amplitude of the thrombelastogram were observed only in control subjects. Therefore, the interactions between lipids and hemostasis appear to be disturbed in CAD, possibly caused by alterations in platelets and plasma lipids. Notably, the decrease in HDL phospholipids may be related to a decreased capacity for reverse cholesterol transport, reduced lipid degradation, and enhanced thrombus formation and may therefore be significant for the development of CAD.

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