Proatherogenic Role of Elevated CE Transfer From HDL to VLDL₁ and Dense LDL in Type 2 Diabetes

Impact of the Degree of Triglycerideremia

Maryse Guérin, Wilfried Le Goff, Taous S. Lassel, Arie Van Tol, George Steiner, M. John Chapman

Abstract—Plasma cholesteryl ester transfer protein (CETP) facilitates intravascular lipoprotein remodeling by promoting the heteroexchange of neutral lipids. To determine whether the degree of triglycerideremia may influence the CETP-mediated redistribution of HDL CE between atherogenic plasma lipoprotein particles in type 2 diabetes, we evaluated CE mass transfer from HDL to apoB-containing lipoprotein acceptors in the plasma of type 2 diabetes subjects (n=38). In parallel, we investigated the potential relationship between CE transfer and the appearance of an atherogenic dense LDL profile. The diabetic population was divided into 3 subgroups according to fasting plasma triglyceride (TG) levels: group 1 (G1), TG<100 mg/dL; group 2 (G2), 100<TG<200 mg/dL; and group 3 (G3), TG>200 mg/dL. Type 2 diabetes patients displayed an asymmetrical LDL profile in which the dense LDL subfractions predominated. Plasma levels of dense LDL subfractions were strongly positively correlated with those of plasma triglyceride (TG) (r=0.471; P=0.0003). The rate of CE mass transfer from HDL to apoB-containing lipoproteins was significantly enhanced in G3 compared with G2 or G1 (46.2±8.1, 33.6±5.3, and 28.2±2.7 μg CE transferred·h⁻¹·mL⁻¹ in G3, G2, and G1, respectively; P<0.0001 G3 versus G1, P=0.0001 G2 versus G1, and P=0.02 G2 versus G3). The relative capacities of VLDL and LDL to act as acceptors of CE from HDL were distinct between type 2 diabetes subgroups. LDL particles represented the preferential CE acceptor in G1 and accounted for 74% of total CE transferred from HDL. By contrast, in G2 and G3, TG-rich lipoprotein subfractions accounted for 47% and 72% of total CE transferred from HDL, respectively. Moreover, the relative proportion of CE transferred from HDL to VLDL₁ in type 2 diabetes patients increased progressively with increase in plasma TG levels. The VLDL₁ subfraction accounted for 34%, 43%, and 52% of total CE transferred from HDL to TG-rich lipoproteins in patients from G1, G2, and G3, respectively. Finally, dense LDL acquired an average of 45% of total CE transferred from HDL to LDL in type 2 diabetes patients. In conclusion, CETP contributes significantly to the formation of small dense LDL particles in type 2 diabetes by a preferential CE transfer from HDL to small dense LDL, as well as through an indirect mechanism involving an enhanced CE transfer from HDL to VLDL₁, the specific precursors of small dense LDL particles in plasma. (Arterioscler Thromb Vasc Biol. 2001;21:282-288.)

Key Words: cholesteryl ester transfer protein ▪ reverse cholesterol transport ▪ lipoprotein subfractions ▪ type 2 diabetes ▪ phospholipid transfer protein

The most common alterations in lipid and lipoprotein profile in type 2 diabetes involve an elevation in both postprandial and fasting plasma triglyceride (TG) and VLDL concentrations, a dense LDL phenotype, and low levels of HDL cholesterol.¹ Hypertriglycerideremia contributes significantly to the increased risk for premature cardiovascular disease in type 2 diabetes.² There is a strong positive correlation between plasma concentrations of TG and small dense LDL in nondiabetic subjects, suggesting that plasma TG concentrations influence LDL subclass distribution.³ The particle size of newly secreted VLDL of hepatic origin represents a major factor in determining the extent to which VLDL particles are remodeled intravascularly to LDL.⁴ In this context, it has been proposed that the large VLDL₁ subfraction preferentially generates small dense LDL subspecies, whereas VLDL₂ and IDL induce the formation of larger LDL subfractions of light and intermediate density.⁴ Cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) are key factors in the reverse cholesterol transport system, a metabolic pathway responsible for the removal of free cholesterol from peripheral tissues and its transport back to the liver for excretion in the bile.⁵ We previously demonstrated that CETP is implicated in the intravascular formation of small dense LDL in combined hyperlipidemia through an indirect mechanism involving an elevated rate of CE transfer from HDL to VLDL.⁶ More
recently, we observed that large VLDL₁ particles, the key precursors of small dense LDL in plasma, represent the major CE acceptors both in normolipidemic subjects ⁷ and in combined hyperlipidemic patients displaying a type IIb phenotype.⁸

In the present study, we evaluate the potential relationships between CETP activity and the quality and quantity of the major apolipoprotein (apoB-containing lipoprotein acceptors) as previously described.⁸ For that purpose, CE transfer from HDL to individual apoB-containing lipoprotein subfractions, notably small dense LDL and TG-rich VLDL, in plasma from type 2 diabetes patients was compared with that in nonobese, normolipidemic control subjects by a physiological assay.⁸ Our data demonstrate that elevated plasma CETP activity in normocholesterolemic type 2 diabetes patients is associated with the expression of an atherogenic lipoprotein profile characterized by a predominance of small dense LDL. Two pathways contribute to dense LDL formation: an indirect mechanism involving preferential CE transfer from HDL to large precursor VLDL₁ particles and a direct mechanism implicating increased CE transfer from HDL to small dense LDL subspecies.

**Methods**

**Subjects**

We recruited 38 type II diabetics (27 men and 11 women) who were under treatment with either diet alone or a combination of diet and oral hypoglycemic drugs, but not with insulin. Patients were stable, and their glycemia was moderately well controlled (mean glycated hemoglobin, Hb A₁c, 7.5±0.2%; normal value, <4.9%) (Table 1). Patients with hepatic, renal, or thyroid disease were excluded. Familial hypercholesterolemia was also excluded in all patients, and none were taking medications known to affect lipid metabolism. The diabetic population was divided into 3 groups according to fasting plasma TG levels: group 1 (G1; n=11), TG<100 mg/dL; group 2 (G2; n=14), 100<TG<200 mg/dL; and group 3 (G3; n=13), TG>200 mg/dL. Fourteen healthy normolipidemic nondiabetic and nonobese volunteers (10 men and 4 women) served as control subjects (group 0, G0; n=14). Blood samples were collected into sterile EDTA-containing tubes (final concentration, 1 mg/mL) after an overnight fast. Plasma was immediately separated from blood cells by low-speed centrifugation at 2500 rpm for 20 minutes at 4°C and frozen at −80°C until used.

**Isolation and Chemical Analysis of Plasma Lipoprotein Subfractions**

Subfractions of TG-rich lipoproteins (TRLs), ie, VLDL₁ (Sf 60 to 400), VLDL₂ (Sf 20 to 60), and IDL (Sf 12 to 20), were isolated from plasma (2 mL) by nonequilibrium density-gradient ultracentrifugation as previously described.⁸ LDL and HDL subfractions were isolated from a second aliquot of plasma (3 mL) by density-gradient ultracentrifugation by a slight modification of the method of Chapman et al⁹ as previously described.⁸

**Measurement of CE Transfer From HDL to ApoB-Containing Lipoproteins (Endogenous Assay)**

The physiological rate of CE transfer from HDL donor particles to apoB-containing lipoprotein acceptors was assayed in each plasma by a slight modification of the method of Guérin et al.⁶ The total VLDL and IDL fraction (d<1.019 g/mL) was isolated from an aliquot of the incubated plasma (0.5 mL) by ultracentrifugation at 45 000 rpm for 24 hours. The total LDL fraction (1.019<d<1.063 g/mL) was subsequently isolated by ultracentrifugation at 45 000 rpm for 24 hours, and the total HDL fraction (1.063<d<1.21 g/mL) after ultracentrifugation at 45 000 rpm for 48 hours. After incubation, a second aliquot of plasma (0.5 mL) was used to isolate VLDL₁, VLDL₂, and IDL as described above. Finally, a third aliquot of the same incubated plasma (0.5 mL) was used to isolate LDL subfractions as described above. The radioactive CE content of each isolated lipoprotein fraction was quantified by liquid scintillation spectrometry with a Rack Beta 1209. The rate of CE transfer was calculated from the known specific radioactivity of radiolabeled HDL-CE after its addition to plasma and is expressed in μg CE transferred·h⁻¹·mL plasma⁻¹.

**CETP-Dependent CE Transfer Assay (Exogenous Substrate Assay)**

The maximal rate of CETP-mediated transfer activity in plasma was assayed by a slight modification of the method of Ahnadi et al.¹¹ using an excess of an exogenous CE acceptor (d<1.063 g/mL apoB-containing lipoproteins) as previously described.⁸
**Results**

**Plasma Lipids and Clinical Characteristics of Type 2 Diabetic and Control Subjects**

The clinical features and plasma lipid profile of the type 2 diabetes patients and control subjects are shown in Table 1. No statistically significant difference in plasma lipid profile between subjects in the control group (G0) and type 2 diabetes patients from G1 was observed despite significant differences in fasting glucose levels and body mass index (BMI). Fasting plasma TG, total cholesterol, LDL cholesterol, apo B, and free fatty acid levels were significantly higher in type 2 diabetes patients with higher TG levels (G2 and G3) than in healthy subjects from the control group (G0). HDL cholesterol was significantly lower in both G2 and G3 subgroups of type 2 diabetes patients.

**Plasma Lipoprotein Mass Distribution in Type 2 Diabetic and Control Subjects**

Figure 1 represents the distribution of plasma TRL subfractions from type 2 diabetes patients and from control subjects. The total population of type 2 diabetes subjects displayed a marked elevation in the mean total plasma TRL mass (VLDL₁ + VLDL₂ + IDL) compared with control subjects (209 ± 98 and 101 ± 30 mg/dL, respectively, P = 0.0002). The concentrations of plasma TRL subfractions (VLDL₁ [Sf 60 to 400], VLDL₂ [Sf 20 to 60], and IDL [Sf 12 to 20]) in type 2 diabetes patients (G1 + G2 + G3) were significantly increased, by 2.3-fold (P = 0.0036), 1.8-fold (P = 0.0001), and 1.7-fold (P < 0.0001), respectively, relative to those of their counterparts in nondiabetic subjects. Plasma VLDL₁ concentrations were markedly increased, however, in G3 compared with type 2 diabetes subjects displaying lower plasma TG levels. A slight increase in VLDL₂ levels was observed in subgroups G3 compared with G2. Plasma VLDL₁ and VLDL₂ concentrations were strongly correlated with plasma TG levels (r = 0.813; P < 0.0001 and r = 0.575; P < 0.0001, respectively), whereas IDL levels were not significantly influenced by elevated concentrations of plasma TG at any tertile of TG in type 2 diabetes patients.

The distribution of LDL subfraction mass in plasmas of patients with type 2 diabetes and control subjects is shown in Figure 2. The mean plasma total LDL concentration was increased (mean +19%) in the whole population of type 2 diabetes patients compared with control subjects (336 ± 72 and 283 ± 43 mg/dL, respectively, P = 0.013). As reported earlier, normolipidemic subjects displayed a symmetrical LDL profile in which LDL particles of intermediate density (LDL₃, 1.029 < d < 1.039 g/mL) predominated. By contrast, in diabetic subjects, the LDL profile displayed a net asymmetry when the whole population of type 2 diabetes patients was considered. Indeed, the dense LDL subfraction (1.039 < d < 1.063 g/mL) in this population accounted for 43% of total LDL mass, whereas light and intermediate LDL subfractions accounted for 22% and 35%, respectively. The progressive shift of LDL profile toward denser LDL subfractions with increase in fasting TG level observed in type 2 diabetes subjects resulted mainly from a specific increase in plasma levels of dense LDL subfractions, LDL₄ (+60%; P = 0.0001) and LDL₅ (+51%; P = 0.0006), compared with
control subjects. Plasma levels of dense LDL subfractions were strongly correlated with plasma TG levels (r=0.471; P=0.0003), whereas LDL subfractions of intermediate density (LDLc) were inversely correlated with plasma TG levels (r=-0.345; P=0.012). Moreover, elevated plasma VLDL₁ and VLDL₂ levels, but not LDL, were significantly associated with the predominance of dense LDL subfractions in plasma (r=0.415; P=0.002 and r=0.424; P=0.002, respectively).

The mean plasma HDL concentration was reduced by 11% in type 2 diabetes patients compared with control subjects (301±39 and 338±63 mg/dL, respectively; P=0.014). Such a reduction was associated with a significant decrease (−12%) in plasma HDL₃ levels in the whole population of type 2 diabetes subjects compared with control subjects (146±22 and 167±35 mg/dL, respectively, P=0.014). When individual HDL particle subtypes were considered, significant reductions in both plasma HDL₁₀₃ (−14%; P=0.006) and HDL₁₀₅ (−15%; P=0.005) levels were observed in type 2 diabetes patients compared with control subjects. In addition, it is important to note that type 2 diabetes patients with elevated levels of plasma TG, ie, type 2 diabetes patients from G2 and G3, displayed a significant reduction in the levels of the HDL₃₀₃ subfraction (1.063<d<1.091 g/mL) compared with those of normotriglyceridemic type 2 diabetes patients from G1.

Endogenous and Exogenous Plasma CETP Activities in Type 2 Diabetic and Control Subjects

Using an endogenous and therefore physiological assay for the determination of plasma CETP activity, in which the transfer rate of CE is measured in the presence of the authentic plasma concentrations of lipoproteins in patients and control subjects, we observed a significant reduction (−28%; P<0.0001) in the transfer of radioactive CE in plasma from the total population of type 2 diabetes patients compared with control subjects (21.0±6.9% and 29.3±4.5%, respectively). The relative proportion of radioactive CE transferred from HDL to apoB-containing lipoproteins progressively increased in type 2 diabetes patients with increase in TG levels from G1 to G3. Both G1 and G2 displayed a significantly lower endogenous CETP activity than control subjects (G0) (−49%; P<0.0001 and −32%; P<0.0001 in G1 and G2, respectively), whereas a similar total CE transfer activity was observed in plasma from patients from G3 and G0.

Plasma CETP activity was also estimated by use of an exogenous assay of CETP activity that involves addition of excess exogenous acceptor particles and that reflects plasma CETP mass levels. No significant variation in CETP-dependent CE transfer activity was observed between subjects from G0 and type 2 diabetes patients irrespective of their plasma TG levels, indicating similar CETP mass in all subjects.

Cholesteryl Ester Transfer From HDL to ApoB-Containing Lipoproteins of Type 2 Diabetic and Control Subjects

Table 2 shows the transfer rates of CE from HDL to individual apoB-containing lipoproteins in plasmas of type 2 diabetes patient subgroups and control subjects. In normolipidemic subjects, the rate of CE transfer to LDL (35.2±4.9 μg CE transferred·h⁻¹·mL⁻¹) exceeded that to the total TRL subfractions (13.9±2.6 μg CE transferred·h⁻¹·mL⁻¹) by ≥2-fold, and thus, LDL represents the major CE acceptor. LDL particles equally represent the preferential CE acceptor in patients from G1 and accounted for 74% of total CE transferred from HDL. By contrast, in groups G2 and G3, TRL subfractions accounted for 47% and 72% of total CE transferred from HDL, respectively. When individual TRL subfractions were considered, VLDL₁ represented the major CE acceptor among the TRL subfractions in control subjects (G0). Thus, VLDL₁ accounted for 65% of total CE transferred from HDL to TRL, whereas VLDL₂ and IDL accounted for significantly less (16% and 19%, respectively, P<0.0005). The relative proportion of CE transferred from HDL to VLDL₁ in type 2 diabetes patients increased progressively
with increase in plasma TG levels. Indeed, the VLDL₁ fraction accounted for 34%, 43%, and 52% of total CE transferred from HDL to TRL in type 2 diabetes patients from G1, G2, and G3, respectively. Moreover, a concomitant reduction of the relative proportion of CE transferred to IDL occurred in the diabetic population. Indeed, the IDL fraction acquired 40%, 34%, and 26% of total CE transferred from HDL to TRL in type 2 diabetes patients from G1, G2, and G3, respectively. On a quantitative basis, CE transfer from HDL to IDL in the total population of type 2 diabetes patients was significantly lower than that observed in normolipidemic subjects (17.5±5.1 and 32.3±4.9 μg CE transferred·h⁻¹·mL⁻¹, respectively, P<0.0001). Total CE mass transferred from HDL to LDL in type 2 diabetes patients progressively decreased from G1 to G3 in parallel with elevation in plasma TG levels. We observed a marked difference between TG tertiles in the relative capacities of LDL subpopulations to act as acceptors of CE from HDL. Light LDL subspecies (LDL₁ and LDL₂) acquired an average of 24% and 38% of total CE transferred from HDL to LDL in type 2 diabetes patients (G1+G2+G3) and in control subjects, respectively (P<0.0001), whereas dense LDL subspecies (LDL₃ and LDL₄) acquired 45% and 27% of total CE transferred to LDL in diabetic and normolipidemic subjects, respectively (P<0.0001). In addition, an equivalent proportion of CE (≈35%) was transferred from the HDL to the LDL subfraction of intermediate density (LDL₃) in both type 2 diabetes patients (G1+G2+G3) and control groups.

### Plasma PLTP Activity in Type 2 Diabetic and Control Subjects

Plasma PLTP activity was not significantly different between the diabetic (G1+G2+G3) and control groups (12.0±2.7 and 10.1±3.5 μmol·mL⁻¹·h⁻¹, respectively; P=0.209). By contrast, PLTP activity progressively increased in type 2 diabetes subjects in parallel with increase in plasma TG levels. Indeed, we observed a significant elevation (+20%; P=0.027) in PLTP activity in plasma from type 2 diabetes subjects from G3 compared with those from G2 (13.5±2.9 and 11.2±1.8 μmol·mL⁻¹·h⁻¹ in type 2 diabetes patients from G3 and G2, respectively).

### Discussion

In the present study, we observed a reduced (−28%; P<0.0001) plasma CETP activity in our type 2 diabetes patients (G1+G2+G3) compared with normolipidemic subjects. Similar observations have been reported previously by Fielding et al.,14,15 who showed a reduced net CE mass transfer from HDL to apoB-containing lipoproteins in type 2 diabetes. Contrasting data have been reported by others, however, indicating either an unaltered16,17 or increased18–24 plasma CE transfer in type 2 diabetes. The severity of hypertriglyceridemia or variations in BMI may have been at the origin of these different observations.25 Indeed, in the present study, normotriglyceridemic control subjects displayed a significantly lower BMI than type 2 diabetic patients. The present data suggest that the apparently conflicting findings referred to above may result in part from differences in plasma TG levels in type 2 diabetes subjects. We demonstrate that plasma CETP activity is strongly related to plasma TG levels (r=0.745; P<0.0001). In type 2 diabetes patients with plasma TG levels <100 mg/dL, a 1.5-fold lower plasma transfer activity was noted than in those displaying elevated plasma TG levels (>200 mg/dL) (P<0.0005). These observations are consistent with earlier data, in which type 2 diabetes patients and normolipidemic subjects displaying similar plasma TG levels exhibited similar or reduced plasma CETP activity.14–17 By contrast, when plasma TG levels were increased in type 2 diabetes patients (≥2-fold compared with control subjects), a significant increase in plasma CETP activity was reported.18,22,24 In agreement with earlier studies,17,18,26 we failed to detect variation in plasma CETP activity between control and diabetic subjects by use of an exogenous substrate assay, which indirectly estimates CETP.

### Table 2. Rates of Cholesteryl Ester Transfer From HDL to ApoB-Containing Lipoproteins in Type 2 Diabetic Patients and Control Subjects

<table>
<thead>
<tr>
<th>Acceptor Lipoprotein Fraction</th>
<th>μg CE transferred·h⁻¹·mL plasma⁻¹</th>
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</thead>
<tbody>
<tr>
<td>Total apoB-containing lipoproteins</td>
<td>49.1±6.4</td>
</tr>
<tr>
<td>Total TRLs (SF 60–400)</td>
<td>13.9±2.6</td>
</tr>
<tr>
<td>VLDL₁ (SF 20–60)</td>
<td>9.0±1.6</td>
</tr>
<tr>
<td>VLDL₂ (SF 12–20)</td>
<td>2.2±0.8</td>
</tr>
<tr>
<td>Total LDL (d 1.019–1.063 g/mL)</td>
<td>35.2±4.9</td>
</tr>
<tr>
<td>LDL₁ (d 1.019–1.023 g/mL)</td>
<td>3.6±0.8</td>
</tr>
<tr>
<td>LDL₂ (d 1.023–1.029 g/mL)</td>
<td>9.9±1.7</td>
</tr>
<tr>
<td>LDL₃ (d 1.029–1.039 g/mL)</td>
<td>12.3±2.0</td>
</tr>
<tr>
<td>LDL₄ (d 1.039–1.050 g/mL)</td>
<td>6.0±0.9</td>
</tr>
<tr>
<td>LDL₅ (d 1.050–1.063 g/mL)</td>
<td>3.4±0.7</td>
</tr>
</tbody>
</table>

Rates of CE mass transfer were determined in normolipidemic subjects (G0, n=14) and in 3 subgroups of type 2 diabetes patients: G1, G2, and G3. ApoB-containing lipoproteins are defined as total d<1.063 g/mL lipoproteins and include VLDL₁, VLDL₂, IDL, and LDL subfractions. Values are mean±SD.

*P<0.05; †P<0.005, and ††P<0.0005 vs G0; §P<0.005, and ¶P<0.0005 vs G1; #P<0.05, **P<0.005, and †††P<0.0005 vs G2.
mass. Therefore, the reduced CE transfer from HDL to apoB-containing lipoproteins observed here in type 2 diabetes patients may result partially from alteration in the composition of CE donor or acceptor lipoprotein particles. The enhanced CE transfer from HDL to TRL subspecies observed in type 2 diabetes patients in relation to elevation in plasma TG levels results from an increase in TRL particle number, as well as from the higher relative capacity of these particles to act as CE acceptors in hypertriglyceridemic type 2 diabetes patients. It was previously demonstrated that nonesterified fatty acids bound to the surface of lipolysed VLDL might stimulate CETP-mediated CE transfer from HDL to TRL subfractions. Because we observed a significant increase in plasma nonesterified fatty acid concentration in type 2 diabetes patients (Table 1) as a function of elevation in plasma TG levels, the presence of nonesterified fatty acids on the surface of TRL particles would be predicted to facilitate electrostatic interactions between CETP and the negative charges of nonesterified fatty acids in these subjects.

We detected a significant increase in plasma PLTP activity in type 2 diabetes patients in relation to increases in plasma TG levels. These findings are consistent with data previously reported by one of us and others in which a higher plasma PLTP activity was detected in plasma from hypertriglyceridemic type 2 diabetes patients than in that from healthy control subjects. Considered together with data in the literature, our findings allow the proposal of an overall mechanism for reverse cholesterol transport in type 2 diabetes. Cellular free cholesterol removal from peripheral cells represents the first step of reverse cholesterol transport and is enhanced as a result of increased PLTP, hepatic lipase, and CETP activities. By contrast, another step of this pathway, which involves the CETP-mediated redistribution of CE between plasma lipoprotein particles, may display proatherogenic properties. Indeed, we demonstrate here that CETP induces the preferential transfer of CE from HDL to atherogenic small dense LDL subspecies and/or to their major precursors in plasma, i.e., large VLDL subparticles. In this regard, it is relevant that hypolipidemic drugs currently used in the treatment of diabetic dyslipoproteinemia significantly reduce plasma CETP-catalyzed cholesteryl ester transfer because of a marked reduction in the number of circulating lipoprotein acceptor particles of CE.

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