Increased cholesterylester transfer activity in complicated Type 1 (insulin-dependent) diabetes mellitus – its relationship with serum lipids

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Summary. In Type 1 (insulin-dependent) diabetes mellitus, macrovascular complications and the increased risk for cardiovascular disease in patients with microvascular complications may be related to alterations in plasma cholesterylester transfer. The activity of cholesterylester transfer protein, which mediates cholesterylester transfer between lipoproteins and lipoprotein lipid levels, was assessed in 7 normolipidaemic control subjects, 7 Type 1 diabetic control subjects without complications, 11 Type 1 diabetic patients with microvascular complications (retinopathy, incipient nephropathy) and in 7 Type 1 diabetic patients with macrovascular atherosclerotic lesions.

The cholesterylester transfer activity was 30% higher in the diabetic groups with macrovascular and microvascular lesions than in the 2 control groups. Very low + low density lipoprotein cholesterol was higher in the 3 diabetic groups than in the

non-diabetic control group. High density lipoprotein cholesterol was not different. The cholesterylester transfer activity was correlated positively with HbA1, urinary albumin excretion rate, serum cholesterol, very low + low density lipoprotein cholesterol and apolipoprotein B. The high density lipoprotein over very low + low density lipoprotein cholesterylester molar ratio was lower in the diabetic groups with micro- and macrovascular complications. A role for cholesterylester transfer activity in the lipoprotein abnormalities found in complicated Type 1 diabetes is suggested. A high cholesterylester transfer activity might be indicative of mechanisms which promote atherogenesis.

Key words: Type 1 (insulin-dependent) diabetes mellitus, cholesterol metabolism, cholesterylester transfer activity, microangiopathy, atherosclerosis.

The excess risk for cardiovascular disease in Type 1 (insulin-dependent) diabetes mellitus is observed predominantly in those patients who have developed diabetic nephropathy [1–3]. In association with atherosclerotic complications and with microvascular renal and retinal lesions increased levels of serum cholesterol, very low density lipoprotein (VLDL), low density lipoprotein (LDL) cholesterol and low levels of high density lipoprotein (HDL) cholesterol have been documented in Type 1 diabetes mellitus [4–8]. Among all other factors involved in lipoprotein metabolism, the process of cholesterylester transfer, mediated by the action of lipid transfer protein(s) will affect the distribution of cholesterylester between lipoproteins [9–12]. These lipid transfer protein(s) also catalyse the transfer and exchange of triacylglycerol and phospholipids [13–16].

In vitro the added activity of cholesterylester transfer protein (CETP) (denominated cholesterylester transfer activity (CETA)) and the composition and con-

centration of the lipoproteins involved are important in the process of cholesterylester transfer [12, 17–20]. As yet, the (patho)physiologic relevance of this process for cholesterol transport has only been partially elucidated. It may provide a mechanism by which free cholesterol originating from either peripheral cells or other lipoproteins, after esterification by the action of lecithine cholesterol acyltransferase (LCAT), is transported from HDL to lipoproteins of lower density classes which can be catabolised at hepatic degradation sites [9, 21], but which are also capable of depositing cholesterol in vascular tissue [10, 22].

CETA, determined in an assay system in which the activity is measured independent of plasma lipoproteins, was higher in hypercholesterolaemic and dysbetalipoproteinaemic subjects [18, 23]. No data are available on CETA in Type 1 diabetes mellitus. The present study emphasises the role of CETA in Type 1 diabetic patients without and with micro- or macrovascular lesions.

Table 1. Clinical characteristics of the 4 groups of subjects

	C ^a (n=7)	DC ^b (n=7)	D Micro ^c (n=11)	D Macro ^d (n=7)	Statistical differences
Age (years)	24 ± 2	31 ± 12	39 ± 12	57 ± 5	4 > 1, 2, 3; <i>p</i> < 0.01 3 > 1; <i>p</i> < 0.01
Duration of disease (years)	0	13 ± 13	19 ± 9	31 ± 13	2, 3, 4 > 1; <i>p</i> < 0.01 4 > 2; <i>p</i> < 0.05
Body mass index (kg/m ²)	21.50 ± 1.33	23.00 ± 2.18	24.60 ± 2.17	25.20 ± 1.10	3, 4 > 1; <i>p</i> < 0.01 4 > 2; <i>p</i> < 0.05
Mean arterial pressure (mm Hg)	94 ± 6	90 ± 5	94 ± 8	93 ± 7	NS
HbA1 (%)	5.12 ± 0.44	7.14 ± 0.83	7.87 ± 1.18	8.21 ± 0.78	2, 3, 4 > 1; <i>p</i> < 0.01 4 > 2; <i>p</i> < 0.05
Blood glucose (mmol/l)	4.50 ± 0.39	11.50 ± 2.17	12.13 ± 1.83	11.56 ± 1.39	2, 3, 4 > 1; <i>p</i> < 0.01
Serum creatinine (μmol/l)	84 ± 6	81 ± 13	85 ± 10	81 ± 16	NS
Glomerular filtration rate (ml/min/1.73 m ²)	n.d.	123 ± 6	125 ± 34	116 ± 32	NS
Urinary albumin excretion rate ^e (μg/min)	2.98 (1.26–4.61)	4.65 (1.83–6.80)	30.58 (7.20–145.21)	17.90 (7.87–54.28)	3, 4 > 1, 2; <i>p</i> < 0.01
Insulin dose (U/day)	0	45 ± 10	59 ± 13	54 ± 27	2, 3, 4 > 1; <i>p</i> < 0.01 3 > 2; <i>p</i> = 0.05
Retinopathy (number)	0	0	7	7	
Incipient nephropathy (n)	0	0	6	2	
Coronary heart disease (n)	0	0	0	6	
Peripheral vascular disease (n)	0	0	0	3	

Results are given as mean ± SD except n.d. denotes: not determined. ^a Control subjects; ^b Type 1 (insulin dependent) diabetic patients without complications; ^c Type 1 diabetic patients with microvascular lesions; ^d Type 1 diabetic patients with macrovascular lesions; ^e median (range)

Subjects and methods

Subjects

All subjects consented to the procedure after explanation of the purpose of the study. Four groups of subjects were investigated (Table 1): seven healthy control subjects (designated C), 7 Type 1 diabetic patients without complications (DC), 11 Type 1 diabetic patients with microvascular complications, either retinopathy, incipient nephropathy or both (D Micro) and 7 Type 1 diabetic patients with macrovascular complications, either coronary heart disease, peripheral vascular disease or both (D Macro).

Male subjects only participated in the study thus avoiding any effect of the menstrual cycle on lipid levels [24]. No subject had arterial hypertension (systolic blood pressure > 160 mm Hg, diastolic blood pressure > 90 mm Hg), diabetic nephropathy (defined as persistent proteinuria > 500 mg/24 h) or overt hyperlipidaemia (defined as cholesterol > 8 mmol/l and/or triacylglycerol > 2.5 mmol/l). All diabetic patients suffered from ketosis prone diabetes mellitus and were deemed insulin dependent on clinical grounds. The diabetic patients were recruited from the out-patient clinic and were in a stable metabolic condition by the use of multiple daily insulin injections. None of the subjects used other medication than insulin or oral anticoagulant drugs (D Macro). The last administration of insulin was 12 h and of anticoagulant drugs 24 h preceding the blood sampling.

Blood pressure was measured using a sphygmomanometer (Baumanometer, W.A. Baum Inc., New York, NY, USA). Korotkoff phase 5 was taken as the diastolic blood pressure. Mean arterial pressure (in mm Hg) was calculated as $0.67 \times$ diastolic pressure + $0.33 \times$ systolic pressure.

Retinopathy was assessed by fundoscopy through dilated pupils (range: background-proliferative). Incipient nephropathy was defined as a mean urinary albumin excretion of more than 30 and less than 200 μg/min in 3 consecutive overnight urine collection periods obtained just prior to the study. Urinary albumin excretion rates of this magnitude represent a risk factor associated with future development of diabetic nephropathy [25]. In D Macro coronary heart disease was documented by positive exercise testing, angiography or myocardial infarction (at least 6 months preceding the study). Peripheral vascular disease was assessed by positive exercise testing or angiography. Clinically relevant coronary heart disease and peripheral vascular disease were excluded by exercise testing in the 3 other groups. Serum creatinine was normal in all subjects. The glomerular filtration rate (GFR) was assessed in the diabetic patients with micro- or macrovas-

cular lesions and in 2 diabetic control subjects using a previously described method [26]. The GFR was greater than 85 ml/min/1.73 m² in all tested subjects. No subject suffered from thyroid or liver disease as determined by routine thyroid and liver function tests.

As is shown in Table 1, the diabetic patients with macrovascular complications were older than the other subjects and had suffered from diabetes for a longer period of time than the diabetic patients without complications. These differences could be expected since atherosclerotic complications are related to age and duration of disease. The body mass index (kg/m²) was higher in D Macro. The metabolic control was adequate. The mean HbA1 was higher in D Macro than in DC. The dose of insulin was larger in D Micro than in DC. There were no differences in serum creatinine, GFR and mean arterial pressure between the groups.

Laboratory methods

Venous blood was drawn after a 12 h fast. Blood for lipid and CETA determinations was collected on ice and separated from erythrocytes immediately at 3000 rev/min for 15 min. Samples were frozen at –20°C and assayed within 4 weeks. The lipid and CETA assays were performed in duplicate in one run.

Cholesterylester transfer activity assay

CETA was assayed in serum using a previously described method [27]. By this method, CETA is determined after removal of endogenous VLDL and LDL by polyethylene glycol precipitation and under conditions of LCAT inhibition. CETA was calculated as the bidirectional transfer of cholesterylester between [1-¹⁴C oleate]-cholesterylester labelled LDL and HDL [28]. Data are expressed as nmol cholesterylester transfer · ml serum^{–1} · h^{–1}. The within assay coefficient of variation was 2.7%.

Lipid assays

Lipids were measured in serum and in the HDL containing supernatant fraction after precipitation. VLDL + LDL lipids were calculated as the difference between whole serum and the HDL fraction. The

Table 2. Serum lipids and lipoproteins

	C ^a (n=7)	DC ^a (n=7)	D Micro ^a (n=11)	D Macro ^a (n=7)	Statistical differences
Serum total cholesterol (mmol/l)	4.53 ± 0.61	5.54 ± 0.87	5.54 ± 0.97	6.60 ± 1.11	4 > 1; <i>p</i> < 0.01 2 > 1; <i>p</i> < 0.05
Serum triacylglycerol (mmol/l)	0.78 ± 0.25	0.90 ± 0.75	0.95 ± 0.35	0.86 ± 0.52	NS
HDL cholesterol (mmol/l)	1.26 ± 0.18	1.22 ± 0.19	1.11 ± 0.42	1.20 ± 0.27	NS
VLDL + LDL cholesterol (mmol/l)	3.28 ± 0.59	4.32 ± 0.89	4.43 ± 0.98	5.40 ± 1.14	4 > 1; <i>p</i> < 0.01 2,3 > 1; <i>p</i> < 0.05
Apolipoprotein A1 (g/l)	1.47 ± 0.23	1.64 ± 0.20	1.53 ± 0.33	1.62 ± 0.08	NS
Apolipoprotein B (g/l)	0.67 ± 0.15	0.75 ± 0.16	0.82 ± 0.18	1.13 ± 0.25	4 > 1, 2, 3; <i>p</i> < 0.01

Results are given as mean ± SD. ^a see legends to Table 1

HDL fraction was obtained by precipitation of VLDL + LDL with sodium phosphotungstate and MgCl₂ for determination of cholesterol and triacylglycerol and by precipitation with polyethylene glycol-6000 for determination of phospholipids [29, 30]. Total cholesterol was measured by gaschromatography [31], free cholesterol by a modification of this method in which the hydrolysis step was omitted. Cholesterylester was calculated as the difference between total and free cholesterol. Triacylglycerol was measured enzymatically on a SMAC-II autoanalyser (Technicon Instruments Inc., Tarrytown, NY, USA) [32]. Phospholipids were determined spectrophotometrically [33]. Apolipoprotein A1 and B were determined by immunoturbidimetry, using commercially available kits (Orion Diagnostica, Espoo, Finland).

Other techniques

Creatinine was measured on a SMAC-II autoanalyser. HbA1 was determined by colorimetry [34]. Glucose was measured on a Yellow Springs glucose analyser (YSI model 23A, Yellow Springs Inc., Yellow Springs, Ohio, USA).

Statistical analysis

Results are expressed as mean ± SD. Since urinary albumin excretion rates are positively skewed, these values are expressed as median and range. Comparison of data was performed using analysis of variance [35]. Least squares linear regression analysis was used for testing relationships between parametrically distributed data [36]. Log₁₀ transformed values of urinary albumin excretion rates were used. A *p* value of less than 0.05 was considered to be significant.

Results

The levels of serum lipids, apolipoprotein A1 and B are given in Table 2. Serum total cholesterol concentration was higher in D Macro and DC than in C (*p* < 0.01 and *p* < 0.05, respectively). Between the 3 diabetic groups there were no significant differences in total cholesterol levels. The VLDL + LDL cholesterol concentration was higher in the 3 diabetic groups than in C (D Macro vs C: *p* < 0.01; D Micro and DC vs C: *p* < 0.05). Apolipoprotein B was higher in D Macro than in the 3 other groups (*p* < 0.01). There were no significant differences between the 4 groups with respect to HDL cholesterol, serum triacylglycerol and apolipoprotein A1 concentrations.

Fig. 1. Comparison of cholesterylester transfer activity (CETA) in 32 subjects. C: control subjects; DC: Type 1 (insulin-dependent) diabetic patients without complications; D Micro: Type 1 diabetic patients with microvascular lesions; D Macro: Type 1 diabetic patients with macrovascular lesions; *: D Micro > C, DC: *p* < 0.05; **: D Macro > C, DC: *p* < 0.01. Mean and SD values are indicated by bars

CETA was higher in D Macro and D Micro than in DC and C (D Macro vs DC and C: *p* < 0.01; D Micro vs DC and C: *p* < 0.05) (Fig. 1). No differences in CETA were observed between C and DC or between D Micro and D Macro. Analysis of variance was also carried out among the data of the 3 diabetic groups only. The difference in CETA between DC, D Micro and D Macro remained present (D Micro and D Macro vs DC: *p* ≤ 0.05). There was no significant correlation between CETA and age in the diabetic groups (*r*: 0.34: NS). In addition, CETA was determined in 11 other non-diabetic control subjects, aged 49 ± 8 years. In these subjects CETA was 108 ± 34 nmol cholesterylester transfer · ml serum⁻¹ · h⁻¹, comparable to the values

from C and DC and lower than the values from D Macro and D Micro.

The correlations of CETA and various other parameters are given in Table 3. There was a significant correlation between CETA and HbA1, both in all subjects ($p < 0.01$) and in the diabetic subjects only ($p < 0.05$) and between CETA and urinary albumin excretion rates, both in all subjects ($p < 0.01$) and in the diabetic subjects only ($p < 0.05$). There was no significant relationship between CETA and blood glucose levels. Significant positive correlations of CETA and serum total cholesterol (all subjects: $p < 0.01$; diabetic subjects only: $p = 0.05$), VLDL+LDL cholesterol (all subjects: $p < 0.01$; diabetic subjects only: $p < 0.05$) and apolipoprotein B (all subjects: $p < 0.05$; diabetic subjects only: $p = 0.05$) were observed. CETA did not correlate significantly to HDL cholesterol, serum triacylglycerol and apolipoprotein A1.

The distribution of cholesterylester between HDL and VLDL+LDL was compared in the 4 groups of subjects. The HDL over VLDL+LDL cholesterylester molar ratio was higher in C and in DC than in D Macro ($p < 0.01$ and $p < 0.05$, respectively) and was higher in C

than in D Micro ($p < 0.01$). Between C and DC no significant difference was observed (Table 4). In the HDL fraction, no significant differences with respect to free cholesterol, cholesterylester, triacylglycerol and phospholipids, expressed as mol% of total lipids, were observed (Table 4). In the combined VLDL+LDL fraction, there were no differences in free cholesterol and phospholipids content nor in the free cholesterol/phospholipids molar ratio. The cholesterylester content in D Macro was higher than in C ($p < 0.05$). The triacylglycerol content in D Macro was lower than in C ($p < 0.05$) (Table 4).

Discussion

The process of cholesterylester transfer mediated by CETP is supposed to be of major importance for the constellation of lipoproteins among the factors involved in lipoprotein metabolism [9–12, 37]. We report on an increased activity of CETP (CETA) in Type 1 diabetic patients in conjunction with micro- and macrovascular complications. The insulin-dependent state itself was not associated with alterations in CETA. It is very unlikely that the alterations in CETA could be attributed to differences in age among the groups of subjects studied, since there was no relation between CETA and age in the control subjects nor in the diabetic patients. The positive relations of CETA with serum total cholesterol, VLDL+LDL cholesterol, apolipoprotein B, HbA1 and urinary albumin excretion rate suggest an association of CETA with risk factors for the development of atherosclerosis, metabolic control and early renal lesions. In accordance, previous studies showed an effect of metabolic control and the presence of microvascular lesions on lipid levels [3–8, 38, 39]. These associations, however, do not provide evidence that in Type 1 diabetes, CETA itself is a cardiovascular risk factor. An increase in CETA has been reported in

Table 3. Relationships between cholesterylester transfer activity and various laboratory parameters

	Correlation coefficients	
	Diabetic subjects	All subjects
HbA1	0.44 ^a	0.45 ^b
Blood glucose	0.08	0.27
Log ₁₀ urinary albumin excretion rate	0.41 ^a	0.45 ^b
Serum cholesterol	0.39 ^a	0.46 ^b
VLDL+LDL cholesterol	0.42 ^a	0.46 ^b
HDL cholesterol	-0.09	-0.06
Serum triacylglycerol	0.12	0.12
Apolipoprotein A1	-0.25	-0.07
Apolipoprotein B	0.39 ^a	0.41 ^a

Level of significance. ^a $p \leq 0.05$; ^b $p < 0.01$

Table 4. HDL and VLDL+LDL lipoprotein composition

	C ^e (n=7)	DC ^e (n=7)	D Micro ^e (n=10)	D Macro ^e (n=7)	Statistical differences
HDL					
FC ^a (mol%)	8.9 ± 1.5	8.4 ± 2.1	8.2 ± 2.0	7.6 ± 2.0	NS
CE ^b (mol%)	36 ± 4	35 ± 5	33 ± 5	36 ± 5	NS
TG ^c (mol%)	3.6 ± 0.8	4.0 ± 1.2	4.8 ± 1.5	3.9 ± 1.5	NS
PL ^d (mol%)	52 ± 5	52 ± 5	54 ± 5	53 ± 6	NS
VLDL+LDL					
FC ^a (mol%)	19 ± 2	20 ± 3	19 ± 2	19 ± 4	NS
CE ^b (mol%)	44 ± 6	45 ± 5	46 ± 4	49 ± 3	4 > 1; $p < 0.05$
TG ^c (mol%)	13 ± 4	11 ± 7	12 ± 4	9 ± 5	4 < 1; $p < 0.05$
PL ^d (mol%)	23 ± 5	25 ± 3	24 ± 3	23 ± 3	NS
FC/PL (mol/mol)	0.85 ± 0.20	0.82 ± 0.18	0.82 ± 0.11	0.84 ± 0.18	NS
HDLCE/(VLDL+LDL)					1 > 3, 4; $p < 0.01$
CE (mol/mol)	0.46 ± 0.15	0.34 ± 0.09	0.31 ± 0.17	0.26 ± 0.09	2 > 4; $p < 0.05$

Results are given as mean ± SD. FC^a: denotes free cholesterol; CE^b: denotes cholesterylester; TG^c: denotes triacylglycerol; PL^d: denotes phospholipids; ^e see legend to Table 1.

non-diabetic hyperlipidaemic and dysbetalipoprotein-aemic subjects [18, 23]. It should be noted that an increase in CETA could be the consequence of an increase CETP mass, but also of a decrease in possible inhibitor activity [40].

Little is known about the metabolic regulation of CETA. Diet studies showed that CETA increased after a single fat load in man and varied parallel with changes in VLDL and LDL cholesterol [18, 41–44]. In accordance with the proposed function of CETA to regulate cholesterylester distribution among lipoproteins [9–12, 21, 23], a decrease in the HDL over VLDL+LDL cholesterylester molar ratio was observed in complicated diabetic disease. The increase in cholesterylester and the decrease in triacylglycerol content in the combined VLDL+LDL fractions from patients with macrovascular complications should probably not be regarded as evidence for an effect of increase CETA on lipoproteins. Similar changes would be found with an increase in LDL mass per se, which suggested by the increase in apolipoprotein B in these patients.

A decrease or even reversal of cholesterylester net mass transfer from HDL to VLDL+LDL has been found in vitro in plasma from hypercholesterolaemic, dysbetalipoproteinaemic and Type 2 (non-insulin dependent) diabetic subjects [45, 46]. An increase in net mass transfer of cholesterylester in dysbetalipoprotein-aemic man has also been reported [18]. Net mass transfer of cholesterylester in vitro depends not only on CETA, but also on the concentration and composition of the lipoproteins involved [10–12, 17, 45]. In several conditions associated with an increased cardiovascular risk, an increase of free cholesterol and of the free cholesterol over phospholipids ratio, both in VLDL and LDL, was related to a decrease in cholesterylester net mass transfer into these lipoproteins [11, 45–47]. No alterations in these lipoprotein surface characteristics could be demonstrated in the combined VLDL+LDL fractions in the present study.

Apart from the process of cholesterylester transfer, many other important metabolic pathways such as hepatic cholesterol, triacylglycerol and apolipoprotein B production, lipoprotein and hepatic lipases and (non) receptor mediated lipoprotein uptake will affect the composition of lipoproteins [39]. We suggest that an increase in CETA may be involved in lipoprotein abnormalities found in complicated Type 1 diabetes. High CETA might be an early indication of an increased cardiovascular risk in this disease.

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