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Presence of very low density lipoprotein compositional abnormalities in Type 1 (insulin-dependent) diabetic patients; effects of blood glucose optimisation

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Summary. Plasma lipoprotein compositional abnormalities were investigated in eight normolipidaemic (plasma cholesterol <5.70 mmol/l; triglyceride <2.03 mmol/l) young male Type 1 (insulin-dependent) diabetic patients (before and after a short period of optimised blood glucose control) and in nine healthy control subjects, matched for sex, age and body mass index. Free and esterified cholesterol, triglyceride, phospholipids were assayed in all lipoprotein classes (VLDL, IDL, LDL) and in HDL subclasses (HDL2 and HDL3); apoB was measured only in very low density lipoproteins (VLDL). All VLDL constituents were increased in the diabetic group, the differences being more striking for apoB (6.0 ± 1.1 mg/dl vs 2.0 ± 0.1 mg/dl, $p<0.02$), free cholesterol (0.27 ± 0.04 mmol/l vs 0.13 ± 0.02 mmol/l, $p<0.02$) and esterified cholesterol (0.32 ± 0.08 mmol/l vs 0.13 ± 0.01 mmol/l, $p<0.05$). Also HDL subfractions showed differences between the two groups: all HDL2 constituents were increased, while in HDL3 only triglyceride was significantly increased (0.11 ± 0.01 mmol/l vs 0.08 ± 0.004 mmol/l, $p<0.02$). After two weeks of optimised blood glucose control all VLDL constituents were reduced and particularly: esterified cholesterol (-39%, $p<0.02$), free cholesterol (-37%, $p<0.05$), apoB (-35%, $p<0.05$). Expressing each VLDL constituent as percent of the total lipoprotein mass, it was evident that the diabetic VLDL was rich in cholesterol both esterified ($8.4 \pm 1.0\%$ vs $5.4 \pm 0.5\%$, $p<0.02$) and free ($8.5 \pm 0.7\%$ vs $5.5 \pm 0.3\%$, $p<0.001$), apo B ($5.1 \pm 0.6\%$ vs $2.6 \pm 0.3\%$, $p<0.001$) and depleted in triglyceride ($57.0 \pm 1.7\%$ vs $64.1 \pm 1.7\%$, $p<0.001$). Two weeks of optimised blood glucose control were not able to correct the abnormal composition of VLDL. In conclusion, Type 1 (insulin-dependent) diabetic patients, although normolipidaemic, show an abnormal VLDL composition suggesting an increased prevalence of smaller and, possibly, more atherogenic VLDL particles. This abnormality is not corrected by a short period of blood glucose optimisation.

Key words: Lipoproteins, Type 1 (insulin-dependent) diabetes, blood glucose control, lipoprotein composition, atherosclerosis.

Atherosclerotic cardiovascular disease incidence and mortality are known to be increased in diabetic patients [1, 2]. This is only partly explained by the major atherosclerosis risk factors which are present in some but not in all diabetic patients experiencing a major cardiovascular event [1, 3]. In particular, an excess cardiovascular risk is reported in Type 1 (insulin-dependent) diabetic patients although in this category of patients plasma lipid concentrations are often normal [2, 4]. This justifies the efforts to find out more subtle metabolic abnormalities which predispose Type 1 diabetic patients to atherosclerosis.

Recently, it has been proposed that not only gross elevations of plasma lipid concentrations, but also faint abnormalities of lipoprotein composition should be considered as predictors of cardiovascular disease [5]. In particular, cholesterol rich VLDL lipoproteins have been shown to contribute to the process of atherosclerosis [6, 7].

Therefore, we have undertaken this study in order to evaluate the plasma lipoprotein composition in a group of normolipidaemic young male Type 1 diabetic patients. Hyperlipidaemic patients have been excluded since the hyperlipidaemic status has, per se, profound effects on plasma lipoprotein composition [8]. Moreover, we have investigated in these patients the effects of a short period of optimisation of blood glucose control on plasma lipoprotein composition.

Table 1. Clinical features of the participants (M ± SEM)

	Type 1 (insulin-dependent) diabetic patients ($n=8$)	Control subjects ($n=9$)
Sex	male	male
Age (years)	28.2 ±2.6	28.7 ±2.4
Body mass index (kg/m2)	22.6 ±0.8	24.0 ±0.7
Diabetes duration (years)	6.9 ±1.0	–
Plasma cholesterol (mmol/l)	4.47 ± 0.002	4.05 ± 0.16
Plasma triglyceride (mmol/l)	1.38 ± 0.22	1.02 ± 0.09

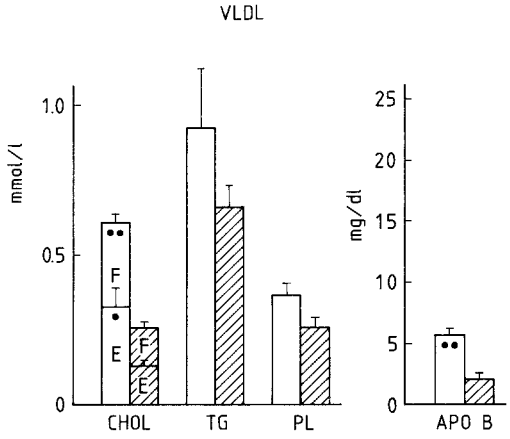

Fig. 1. VLDL lipid and apoB concentrations in a group of young male normolipidaemic Type 1 (insulin-dependent) diabetic patients (□) and in sex, age and BMI matched control subjects (▨). The results are expressed as mean ± SEM. Significance vs control subjects: • $p<0.05$; •• $p<0.02$. Abbreviations: CHOL = cholesterol; F = free; E = esterified; TG = triglyceride; PL = phospholipids

Subjects and methods

Patients

Eight young male patients with Type 1 (C-peptide negative) diabetes mellitus [9] participated in the study after giving their informed consent. They were recruited from our diabetic clinic on the basis of their blood glucose control (HbA1 > 10% during the last 3-6 months) and plasma lipid values (Cholesterol < 5.70 mmol/l; Triglyceride < 2.03 mmol/l). The main characteristics of the patients and their pre-study lipid levels are shown in Table 1. They were all treated with long or intermediate acting + regular insulins twice daily. Two patients had background retinopathy, but none had clinically manifest nephropathy (urinary protein greater than 0.5 g/24 h and/or glomerular filtration < 80 ml/min) or any other disease known to influence lipid metabolism. None of the patients was taking medications other than insulin.

Nine normolipidaemic healthy males, matched with patients for age and body mass index (BMI), volunteered to participate in the study as control subjects. Some relevant features of these subjects are listed in Table 1.

Study protocol

Type 1 diabetic patients were hospitalised and continued throughout the study to follow their habitual isoenergetic diet (CHO 53%, fat 30%, protein 17%, fibre 35 g) and their usual physical activity.

After a period of one week to get accustomed to the hospital life, they underwent the baseline lipoprotein assessment. Thereafter, an intensified insulin treatment was started in the attempt to achieve a near normal blood glucose control as soon as possible (within 2-4 days).

Insulin was given by conventional means as regular insulin before breakfast and lunch and as a mixture of regular + intermediate insulin before dinner.

Methods

Blood glucose was measured daily in the fasting state as well as before and 2 h after lunch and dinner on capillary blood, drawn by finger stick, using the dextrostix glucometer system (Miles Laboratory, Italy). Total glycosylated haemoglobin (labile + stable) was measured at baseline and after two weeks of optimised control by ion-exchange chromatography using prepacked microcolumns (Bio-Rad, Richmond, California, USA) [10]. Normal values in our laboratory are < 8% with an interassay coefficient of variation of 2.8 ± 0.2%.

At baseline (on two subsequent days) and after two weeks of optimised control, samples for lipoprotein analysis were drawn without stasis in the morning-after an overnight fast with the patient in the recumbent position. After centrifugation, serum, additioned with merthiolate (final concentration 0.01%), EDTA disodium salt and sodium azide (final concentration 0.05%), was stored at 4° C for lipoprotein analysis, which was performed within one week.

Total serum and all lipoprotein classes were assayed for their cholesterol, both free and total, triglyceride and phospholipid content by commercially available enzymatic methods (Boehringer Biochemia Robin Mannheim, West Germany [11-14]. Esterified cholesterol was calculated as the difference between total and free cholesterol. ApoB in VLDL was measured in a single run by an immunoenzymatic method (Elisa) [15]. The intrassay coefficient of variation for apoB on VLDL was 4%. Our laboratory participates in an international program for lipid quality control in connection with the Prague Reference Center of WHO [16].

For lipoprotein separation serum was firstly centrifuged (Beckman L5-65 with a fixed angle rotor 50.3 Ti) at a density of 1.006 g/ml (EDTA saline = 1.006 g/ml) for 16 h at 40,000 rpm and at 16° C to separate VLDL [17]. The bottom fraction was then adjusted to the density of 1.019 g/ml and IDL was separated after a centrifugation for 18 h at 40,000 rpm and at 16° C [17]. LDL in the infranate was separated from HDL by precipitation using a combination of dextran sulfate 500 (20 g/l) and magnesium chloride (2 mol/l) [18, 19]. For HDL3 separation serum density was increased at d = 1.125 g/ml and centrifuged for 48 h at 40,000 rpm and at 16° C. HDL2 lipid concentration was calculated as difference between total HDL and HDL3 concentration [20]. Recovery of total cholesterol, free cholesterol, triglyceride and phospholipids in lipoprotein fractions were respectively 104 ± 4%, 93 ± 3%, 103 ± 4%, 99 ± 4%). Interassay coefficient of variation ranged for trigylceride from 2.6% (IDL) to 4.1% (total HDL); for total cholesterol from 2.0% (HDL3) to 6.9% (IDL); for free cholesterol from 2.4% (IDL) to 5.6% (VLDL, HDL3); for phospholipids from 2.7% (VLDL) to 4.0% (total HDL).

Statistical analysis

Values in the text are given as mean ± SEM. At baseline, lipoproteins were measured in Type 1 diabetic patients and control subjects on two samples taken on subsequent days. Each value represents the average for the two days.

Statistical evaluation was performed according to standard procedures. In particular, the paired and unpaired t-test were utilised [21]. The level of statistical significance was set at $p<0.05$ for a two-tailed distribution. Log transformation was employed when appropriate (triglyceride).

Results

The composition of the different lipoprotein classes in young normolipidaemic male Type 1 diabetic patients and in age, sex and BMI matched control subjects is reported in Figures 1-3. All VLDL constituents were increased in the diabetic group; however, the differences were more striking for apoB (6.0 ± 1.1 vs 2.0 ± 0.1 mg/dl, $p < 0.02$), free cholesterol (0.27 ± 0.04 mmol/l vs 0.13 ± 0.02 mmol/l), $p < 0.02$) and esterified cholesterol (0.32 ± 0.08 mmol/l vs 0.13 ± 0.01 mmol/l, $p < 0.05$) while for triglyceride (0.91 ± 0.20 mmol/l vs 0.64 ± 0.08 mmol/l) and phospholipids (0.38 ± 0.09 mmol/l vs 0.25 ± 0.09 mmol/l) the differences did not reach the conventional level of statistical significance.

Also, the HDL subfractions were different in the two groups: all HDL2 components were increased in Type 1 diabetic patients, the difference being more evident for esterified cholesterol (0.45 ± 0.03 mmol/l vs 0.35 ± 0.02 mmol/l, $p < 0.02$). In HDL3, only triglyceride was significantly increased in the diabetic group (0.11 ± 0.01 mmol/l vs 0.08 ± 0.004 mmol/l, $p < 0.02$) (Fig. 2).

Both IDL and LDL showed no significant differences between diabetic patients and control subjects (Fig. 3).

After two weeks of intensified insulin treatment, all parameters of blood glucose control improved significantly reaching a near normal level (Table 2). This was achieved by splitting the insulin dosage in more injections during the day without major changes in the total amount of insulin administered daily (39 ± 6 U vs 37 ± 5 U).

After two weeks of optimised blood glucose control, all VLDL constituents were reduced. The reduction was maximal for cholesterol, both esterified (-39%, $p < 0.02$) and free (-37%, $p < 0.05$) and for apoB (-35%, $p < 0.05$). It was less impressive for triglyceride (-25%, $p < 0.05$) and phospholipids (-23%, ns). Conversely, all other lipoprotein classes were not modified to any significant extent by the short period of optimised blood glucose control.

In order to better evaluate the abnormalities of VLDL composition in Type 1 diabetic patients, each lipoprotein constituent was expressed as percent of the total lipoprotein mass (Table 3). Apoprotein Cs and apoprotein E were not considered since they were not measured in this study.

It is evident that the diabetic VLDL has an abnormal composition since it is enriched in esterified cholesterol ($8.4 \pm 1.0\%$ vs $5.4 \pm 0.5\%$, $p < 0.02$), free cholesterol ($8.5 \pm 0.7\%$ vs $5.5 \pm 0.3\%$, $p < 0.001$), apoB ($5.1 \pm 0.6\%$ vs $2.6 \pm 0.3\%$, $p < 0.001$) and depleted in triglyceride ($57.0 \pm 1.7\%$ vs $64.2 \pm 1.7\%$, $p < 0.02$). Two weeks of optimised blood glucose control were not able to correct the abnormal composition of VLDL which remained rich in cholesterol ester ($7.4 \pm 0.6\%$ vs $5.4 \pm 0.5\%$,

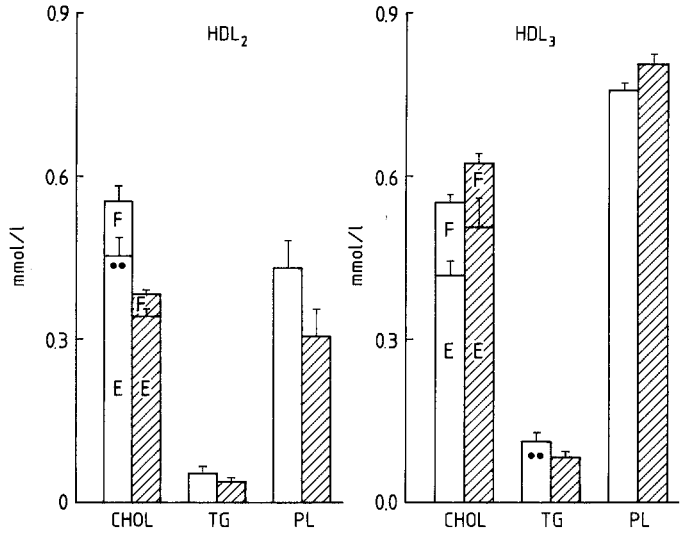

Fig. 2. HDL2 and HDL3 lipid concentrations in a group of young male normolipidaemic Type 1 diabetic patients (□) and in sex, age and BMI matched control subjects (▨). The results are expressed as mean ± SEM. Significance vs control subjects: •• $p < 0.02$ (Abbreviations see legend Figure 1)

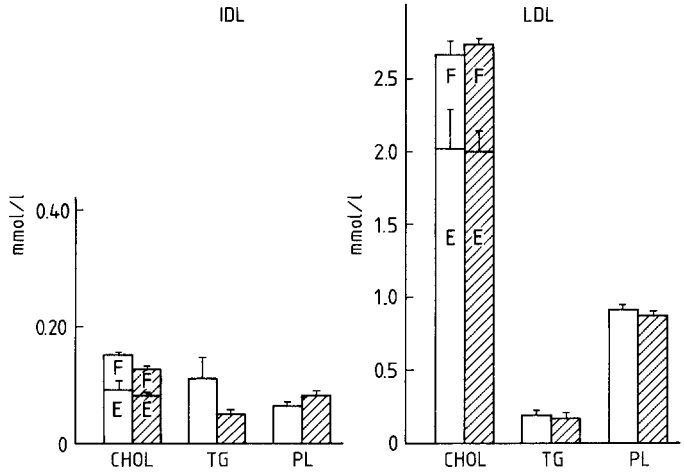

Fig. 3. IDL and LDL lipid concentrations in a group of young male normolipidemic Type 1 diabetic patients (□) and in sex, age and BMI matched control subjects (▨). The results are expressed as mean ± SEM. Abbreviations: see legend to Figure 1

Table 2. Blood glucose (BG) control and daily insulin dosage in Type 1 (insulin-dependent) diabetic patients at baseline and after two weeks of optimised blood glucose control (M ± SEM)

	Baseline	Optimised BG control
Fasting BG (mmol/l)	13.7 ± 2.0	6.9 ± 1.0[a]
2 h post-prandial BG (mmol/l)	20.8 ± 2.0	6.8 ± 1.3[a]
Average daily BG (mmol/l)	16.0 ± 0.8	6.9 ± 0.6[a]
Total HbA1 (%)	12.0 ± 1.5	9.8 ± 1.6[a]
Daily insulin dosage (U)	39.5 ± 6.2	36.7 ± 5.0

[a] $p < 0.001$ vs baseline

Table 3. VLDL composition[d] in Type 1 (insulin-dependent) diabetic patients at baseline and after two weeks of optimised blood glucose control and in control subjects (M ± SEM)

	Diabetic patients ($n=8$)		Control subjects ($n=9$)
	Baseline	Optimised BG control	
	(%)	(%)	(%)
ApoB	5.1 ± 0.6[c]	6.3 ± 1.6[b]	2.6 ± 0.3
Free cholesterol	8.5 ± 0.7[c]	6.6 ± 0.7	5.5 ± 0.3
Esterified cholesterol	8.4 ± 1.0[b]	7.4 ± 0.6[a]	5.4 ± 0.5
Triglyceride	57.0 ± 1.7[b]	58.4 ± 1.8[a]	64.2 ± 1.7
Phospholipids	21.0 ± 1.0	21.3 ± 1.4	22.2 ± 1.3

[a] $p<0.05$; [b] $p<0.002$; [c] $p<0.001$ vs control subjects; [d] Each VLDL component is expressed as percentage of the total lipoprotein mass

$p<0.05$) and apoB (6.3 ± 1.6% vs 2.6 ± 0.3%, $p<0.02$) and depleted in triglyceride content (58.5 ± 1.8% vs 64.1 ± 1.7%, $p<0.05$) in comparison with the control group.

Discussion

This study clearly demonstrates that in Type 1 diabetic patients abnormalities of VLDL composition exist which might be regarded as predisposing to atherosclerosis. These abnormalities were found despite the fact that all patients had normal plasma cholesterol and triglyceride levels. Furthermore, these abnormalities were not corrected by a short period of optimisation of blood glucose control. By contrast, in our group of Type 1 diabetic patients, other serum lipoproteins did not show major compositional abnormalities which might be considered atherogenetic.

Many studies have focused on lipoprotein metabolism in diabetic patients. In these studies, derangements of VLDL metabolism have been consistently demonstrated in patients with insulin-dependent diabetes [4, 22–24]. However, the interest has been, in general, limited to plasma VLDL concentrations without paying too much attention to the lipoprotein composition. The latter has become increasingly important once the evidence has been forwarded that abnormalities of VLDL composition might be related to the process of atherogenesis [5–7].

In the present study, the diabetic VLDL is enriched in cholesterol (both free and esterified) and apoB. For its composition, this lipoprotein closely resembles the VLDL remnant or β-VLDL which is able to produce cholesteryl ester deposition in macrophages and endotelial cells [6]. Moreover, in clinical studies cholesterol and apoB enriched VLDL, lipoproteins have been found to be associated with coronary and peripheral artery diseases [7].

Two weeks of blood glucose control optimisation reduced all VLDL constituents, but the relative contribution of each lipoprotein component to the overall VLDL mass remained unbalanced towards a cholesterol and apoB enrichment. This suggests that the optimisation of blood glucose control in Type 1 diabetic patients is able to reduce VLDL concentration but has no major influence on its composition. Of course, the short term nature of this study prevents one from drawing definitive conclusions on this aspect, but there are reasonable indications that, due to the rapid VLDL turnover rate, two weeks should be sufficient to reach a new steady state of VLDL metabolism [25]. Several studies have investigated the lipoprotein profile in Type 1 diabetic patients [4, 22–24], but no attempt has been made, so far, to evaluate the lipid composition of all lipoprotein classes including IDL and the HDL subfractions. We have investigated this aspect, but we did not find major compositional abnormalities in our Type 1 diabetic patients other than in VLDL. In particular, IDL and LDL concentration and composition were very similar in Type 1 diabetic patients and in normoglycaemic control subjects. This confirms that LDL metabolism is not deranged in Type 1 diabetic patients when insulin therapy is instituted [4, 23]. Although in our diabetic patients all HDL2 constituents were elevated, the overall HDL2 composition was similar to that found in normoglycaemic control subjects. Conversely, the HDL3 subfraction showed higher triglyceride levels in diabetic patients in comparison with the control group. Two weeks of blood glucose optimisation did not modify the concentration and the composition of these lipoproteins.

A metabolic interpretation of our findings is neither simple nor immediate. Three key metabolic processes regulate VLDL concentration and composition in the fasting state: (1) the hepatic synthesis, (2) the delipidation process activated by lipoprotein lipase, (3) the final catabolism through the metabolic cascade VLDL→IDL→LDL or by liver uptake.

VLDL synthetic rate has been reported to be normal in normolipidaemic Type 1 diabetic patients with a moderate degree of blood glucose control [26].

On the other hand, in this group of patients lipoprotein lipase is overstimulated by the elevated peripheral insulin levels [27]. Therefore, VLDL compositional abnormalities are, most likely, a consequence of the enhanced delipidation process regulated by lipoprotein lipase: the large, triglyceride enriched VLDL particles are readily transformed in smaller, denser, cholesterol and apoB enriched lipoproteins. The increased HDL plasma levels, which we have found in our group of patients, give further support to the hypothesis of an overactivation of lipoprotein lipase.

An alternative, although less plausible, explanation for the abnormal VLDL composition in Type 1 diabetic patients might be that in these patients the liver secretes an apoB VLDL that is relatively enriched with cholesterol.

In the presence of an abnormal lipoprotein, the limiting step of VLDL catabolism in Type 1 diabetic patients is the lipoprotein uptake at the liver level. In fact,

the other VLDL catabolic pathway, represented by the metabolic cascade VLDL→IDL→LDL, is probably not impaired in these patients, as demonstrated by the IDL and LDL normal concentration and composition.

The liver uptake of VLDL is receptor-mediated and is influenced by the lipid and apoprotein composition of VLDL. The cholesterol enrichment of this lipoprotein might impair this key catabolic step, thus leading to the accumulation of small VLDL particles [28]. In addition, an abnormal pattern of apoE isophorms in VLDL, as reported in some studies in diabetic patients, is also able to impair the liver removal of this lipoprotein [29, 30].

The optimisation of blood glucose control reduces the VLDL synthetic rate to values below normal thus lowering the VLDL concentration [26]. However, it cannot modify the abnormal VLDL composition which is, in our opinion, in large part a consequence of the lipoprotein lipase overstimulation due to the high peripheral insulin levels [27].

In conclusion, VLDL compositional abnormalities are present in Type 1 diabetic patients even in absence of frank hyperlipidaemia and are not corrected by a short period of optimised blood glucose control. They might contribute to the increased risk for cardiovascular disease of this group of patients.

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