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Very low density lipoprotein subfraction abnormalities in IDDM patients: any effect of blood glucose control?

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Summary Normolipidaemic insulin-dependent diabetic (IDDM) patients are characterized by an increase in the smaller VLDL particles, considered to be the most atherogenic. Since blood glucose control is one of the main regulators of lipid metabolism in diabetic patients, it could influence the shift in the distribution of VLDL subfractions towards smaller particles. To evaluate this possibility, VLDL subfractions, post-heparin lipoprotein lipase and hepatic lipase activities have been evaluated in male IDDM patients with either unsatisfactory blood glucose control (group 1, $HbA_{1c} > 8\,\%$, $n = 18$) or good blood glucose control (group 2, $HbA_{1c} < 8\,\%$, $n = 16$) and in 16 normoglycaemic individuals. The three groups were comparable for sex, age, body mass index, and plasma lipid levels. Three VLDL subfractions (large, Svedberg flotation unit (Sf) 175–400; intermediate, Sf 100–175; small, Sf 20–100) were separated by density gradient ultracentrifugation and analysed for cholesterol, triglyceride, and phospholipid levels. When compared to control subjects both groups of IDDM patients showed a clear shift in VLDL subfraction distribution with a significant increase in the proportion of small VLDL (group 1; $49 \pm 2\,\%$; $p < 0.005$; group 2: $51 \pm 3\,\%$, $p < 0.01$; control subjects $40 \pm 2\,\%$) (mean ± SEM) in relation to total VLDL. By contrast, the absolute lipid concentration of small VLDL was higher only in group 1, compared to control subjects (35 ± 4 vs 27 ± 3 mg/dl, $p = 0.05$). Post-heparin hepatic lipase activity was significantly reduced in both IDDM groups (group 1: 254 ± 19 mU/ml, $p < 0.05$; group 2: 202 ± 19 mU/ml, $p < 0.005$; control subjects 317 ± 31 mU/ml). In conclusion, normolipidaemic IDDM patients show an increase in the smallest VLDL, whatever their degree of blood glucose control. However, this abnormality may be clinically relevant only in patients with unsatisfactory blood glucose control, since absolute lipid concentration of these potentially atherogenic particles is only increased in this group. [Diabetologia (1995) 38: 1419–1424]

Key words Lipoproteins, VLDL subfractions, insulin-dependent diabetes mellitus, blood glucose control, lipid concentration, lipolytic enzymes.

Insulin-dependent diabetic (IDDM) patients present a very high cardiovascular risk, even in the absence of frank hyperlipidaemia [1]. Therefore, other cardiovascular risk factors must play an important role and, among them, abnormalities in the composition of lipoproteins and in the distribution of their subfractions have received increasing attention in the last few years [2–8]. In particular, our group has found that normolipidaemic IDDM patients are characterized by a shift in the distribution of VLDL subfractions, consisting in a significant increase in the proportion of the smallest particles [9], which are considered to be more atherogenic.

Blood glucose control is one of the main regulators of lipid metabolism in the diabetic state and, in fact,

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; VLDL, very low density lipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase.

good blood glucose control is generally able to correct, at least in IDDM patients, lipoprotein concentration abnormalities secondary to diabetes [2, 10–12]. However, it is not known whether blood glucose control optimization has the same beneficial effect on more subtle lipoprotein abnormalities. In fact, almost nothing is known about the relationship between the degree of blood glucose control and the abnormal distribution of VLDL subfractions in IDDM patients. In particular, it is not known whether the concentration of small VLDL, which are considered to be the most atherogenic, is influenced by unsatisfactory blood glucose control, a condition unfortunately found frequently in diabetic patients.

Therefore, the aim of our study was to evaluate whether the abnormal VLDL subfraction distribution is influenced by the degree of blood glucose control in IDDM patients.

Since hepatic lipase (HL) and lipoprotein lipase (LPL) play a major role in regulating the distribution of VLDL subfractions, the second aim of our study was to evaluate post-heparin plasma lipase activities at different levels of blood glucose control.

Subjects and methods

Patients. Thirty-four young male IDDM patients (diagnosed according to the World Health Organization (WHO) criteria) [13] and sixteen healthy male control subjects participated in the study. Patients were recruited from our diabetic clinic on the basis of normal plasma lipid values (cholesterol < 5.7 mmol/l; triglyceride < 2.30 mmol/l). None of the patients had clinically manifest kidney disease (albuminuria > 0.5 g/24 h and/or glomerular filtration rate < 80 ml/min), hypertension, coronary heart disease or any disease known to affect lipid metabolism. Patients followed a standard isoenergetic diet, resembling the composition of the habitual diet in southern Italy: 53 % carbohydrates, 17 % protein, 30 % fat, most of which was monounsaturated (12–15 %). They were all treated by intensified insulin therapy consisting of three injections of regular insulin before meals plus intermediate insulin before dinner. No medications other than insulin were taken. According to their glycated haemoglobin (HbA_{1c}) value patients were divided into two groups, one with HbA_{1c} greater than 8 % (the group with unsatisfactory blood glucose control; average HbA_{1c} 9.0 ± 0.1 %, mean ± SEM) and the other with HbA_{1c} less than 8 % (the group with good blood glucose control; average HbA_{1c} 6.9 ± 0.2 %). Control subjects were recruited from personnel and students at the Institute of Internal Medicine and Metabolic Diseases in Naples. Control subjects were matched with patients according to age, body mass index (BMI) and plasma lipid levels. The main characteristics of the patients and control subjects are shown in Table 1. All participants gave their informed consent and the protocol was approved by the local ethical committee.

Lipoprotein separation. After an overnight fast, blood samples were taken from all subjects by venipuncture without stasis. After 30 min at room temperature, to allow blood clotting, serum was recovered by low-speed centrifugation (3000 rev/min) for 10 min, then added to merthiolate (final concentration 0.01 %), EDTA disodium salt and sodium azide (final concentration 0.05 %). For lipoprotein analysis, VLDL were isolated at density *(d)* 1.006 g/ml by preparative ultracentrifugation [14]; HDL were precipitated by dextran sulphate magnesium chloride [15]; LDL were calculated from the difference [16].

Table 1. Clinical characteristics of subjects

	IDDM patients ($n = 18$) $HbA_{1c} > 8$ %	IDDM patients ($n = 16$) $HbA_{1c} < 8$ %	Control subjects ($n = 16$)
Age (years)	28 ± 3	32 ± 2	28 ± 2
BMI (kg/m^2)	23.8 ± 0.6	23.9 ± 0.5	24.5 ± 0.5
HbA_{1c} (%)	9.0 ± 0.1	6.9 ± 0.2	–
Insulin dosage ($IU \cdot kg^{-1} \cdot day^{-1}$)	0.61 ± 0.04	0.63 ± 0.03	–
Diabetes duration (years)	12 ± 2	12 ± 2	–
Plasma cholesterol (mmol/l)	4.48 ± 0.23	4.40 ± 0.18	4.25 ± 0.13
Plasma triglyceride (mmol/l)	0.89 ± 0.09	0.79 ± 0.04	0.81 ± 0.08

Mean ± SEM

VLDL subfractions were isolated at *d* 1.006 g/ml by discontinuous density gradient ultracentrifugation [17, 18] as previously described [9]. Briefly, this method is based on a four-step density gradient consisting of plasma with a density increased to *d* 1.10 g/ml with solid NaBr (0.1268 g/ml) and of three different NaBr solutions of decreasing density (*d* 1.065, 1.020, 1.006 g/ml, respectively). Three VLDL subfractions of increasing density, (lighter, intermediate and denser) were isolated. These three subfractions correspond to particles of decreasing size (large 50–75 nm; intermediate 37–50 nm; small 20–37 nm) as reported by Redgrave and Carlson [18]. Centrifugations were carried out in a Beckman SW 40 Ti rotor (Palo Alto, California, USA) at 20 °C on an Ultracentrifuge Centrikon T 2060 (Kontron Instruments, Zurich, Switzerland) with operating mode preselection keys set at "vertical on-off". After correction for acceleration and deceleration forces, three consecutive runs were made at 16 °C: 1) 105 min at 40000 rev/min (large VLDL, Svedberg flotation unit (Sf) 175–400); 2) 80 min at 40000 rev/min (intermediate VLDL, Sf 100–175); 3) 18 h at 37000 rev/min (small VLDL, Sf 20–100).

Plasma post-heparin lipolytic activities. Blood samples for the measurement of LPL and HL activities were collected in tubes containing EDTA, 15 min after intravenous administration of 50 U heparin per kg of body weight. Plasma was immediately separated by centrifugation at 4 °C and stored at – 70 °C. LPL and HL activities were determined according to Nilsson-Ehle and Ekman [19], using as substrate a (^3H) trioleoylglycerol emulsion stabilized by dioleoyl phosphatidyl choline. Specific measurements of the two lipases are based on differences in pH, NaCl molarity and on the presence of serum in the incubation mixture, as well as on the addition of albumin to the substrate emulsion either before or after sonication. For technical reasons, LPL and HL activities were not measured in one patient and three control subjects. Samples from the diabetic patients were measured in the same assay as their respective matched control subjects. Coefficients of variation were 6.3 and 2.4 % (intra-assay) and 8.9 and 4.6 % (inter-assay) for LPL and HL, respectively.

Other measurements. Total and non-esterified cholesterol, triglyceride, and phospholipids were assayed in serum, isolated lipoproteins and VLDL subfractions on an autoanalyser COBAS-MIRA (Roche, Basilea, Switzerland) by enzymatic colorimetric methods using commercially available kits (Boehringer Mannheim, Mannheim, Germany) [20–23] modified in order to obtain the highest sensitivity for the lowest concentrations. Esterified cholesterol was calculated as the difference between total and non-esterified cholesterol. Quality control of lipid analysis is regularly ensured by the WHO Prague Reference Centre [24]. Recovery of single constituents in VLDL (sum of concentration in VLDL subfractions as percent of concentration in total VLDL) was 96.6 ± 1.2 % for total cholesterol, 96.9 ± 1.2 % for non-esterified cholesterol, 94.3 ± 1.2 % for triglyceride and 94.1 ± 0.97 % for phospholipids, with no difference among the three groups.

HbA_{1c} was measured by high-pressure liquid chromatography [25]: normal values in our laboratory are less than 6.7 %.

Statistical analysis

Values in the text are presented as mean ± SEM. Comparisons between diabetic patients and control subjects were made using the Student's unpaired *t*-test [26]. Pearson's correlations were performed between lipolytic enzyme activities and VLDL subfractions expressed as a percentage of the total VLDL lipid concentration [26]. The level of statistical significance was set at $p < 0.05$ for a two-tailed distribution. Variables not uniformly distributed were log-transformed.

Results

Although total plasma lipid levels were similar between diabetic patients and control subjects, the distribution of triglyceride in plasma lipoproteins was slightly different (Table 2). In particular, diabetic patients with good blood glucose control showed a significant reduction in VLDL triglyceride, with respect to control subjects (0.37 ± 0.04 vs 0.55 ± 0.07 mmol/l, $p < 0.025$), while no difference in this parameter was observed between diabetic patients with unsatisfactory blood glucose control and control subjects (0.55 ± 0.08 vs 0.55 ± 0.07 mmol/l). LDL triglyceride tended to increase in both groups of diabetic patients, but the differences were not statistically significant (0.19 ± 0.02 mmol/l for patients with $HbA_{1c} > 8$ %, 0.18 ± 0.02 mmol/l for patients with $HbA_{1c} < 8$ %, 0.14 ± 0.02 mmol/l for control subjects). No difference was observed in HDL triglyceride level. Moreover, cholesterol distribution did not change among lipoprotein classes (Table).

The total lipid concentration (sum of cholesterol, triglyceride and phospholipids) of VLDL and their subfractions for the three groups is shown in Table 3. In diabetic patients with unsatisfactory blood glucose control there was no change in the total lipid concentrations of VLDL, with respect to control subjects (78 ± 11 vs 76 ± 9 mg/dl). Similarly, no significant difference was observed between this group of patients and control subjects in relation to the total lipid concentrations of large (21 ± 3 vs 25 ± 4 mg/dl) and intermediate VLDL (19 ± 4 vs 20 ± 3 mg/dl) subfractions. Instead the total lipid concentration of small VLDL was increased in this group of patients compared to control subjects (35 ± 4 vs 27 ± 3 mg/dl, $p = 0.05$) (Table 3). This increase was due to a parallel increase in all lipid constituents of these particles (free and esterified cholesterol, triglyceride, phospholipids). Diabetic patients with good blood glucose control showed a significant reduction in total VLDL lipid concentration (53 ± 6 vs 76 ± 9 mg/dl, $p < 0.025$), with respect to control subjects. This difference was essentially due to a decrease in large (15 ± 3 vs 25 ± 4 mg/dl, $p < 0.025$) and intermediate (11 ± 2 vs 20 ± 3 mg/dl, $p < 0.01$) VLDL subfractions, while the total lipid concentration of small VLDL was not different in this group of patients compared to control subjects (24 ± 2 vs 27 ± 3 mg/dl) (Table 3).

To better quantify the relative contribution that each subfraction gave to the plasma concentration of total VLDL, the lipid concentration of each VLDL subfraction was expressed as a percentage of the total VLDL lipid concentration (Fig. 1). Compared to control subjects both groups of diabetic patients showed a significant increase (about 25 %) in the contribution of the smallest VLDL to the total mass of VLDL, and a reduction in both large ($p < 0.05$ for the group with unsatisfactory blood glucose control) and intermediate ($p < 0.01$ for the other group) particles.

The activity of lipolytic enzymes is shown in Table 4: plasma post-heparin LPL activity was similar

Table 2. Lipid concentration of plasma lipoproteins

	IDDM patients ($n = 18$) $HbA_{1c} > 8$ %	IDDM patients ($n = 16$) $HbA_{1c} < 8$ %	Control subjects ($n = 16$)
Cholesterol (mmol/l)			
VLDL	0.31 ± 0.05	0.24 ± 0.02	0.31 ± 0.05
LDL	2.77 ± 0.21	2.85 ± 0.16	2.72 ± 0.13
HDL	1.35 ± 0.10	1.32 ± 0.10	1.23 ± 0.08
Triglyceride (mmol/l)			
VLDL	0.55 ± 0.08	0.37 ± 0.04[a]	0.55 ± 0.07
LDL	0.19 ± 0.02	0.18 ± 0.02	0.14 ± 0.02
HDL	0.14 ± 0.01	0.20 ± 0.02	0.16 ± 0.02

[a] $p < 0.025$ vs control subjects. Mean ± SEM

Table 3. Lipid concentration (mg/dl) of total VLDL and their subfractions

	IDDM patients ($n = 18$) $HbA_{1c} > 8$ %	IDDM patients ($n = 16$) $HbA_{1c} < 8$ %	Control subjects ($n = 16$)
Total	78 ± 11	53 ± 6[b]	76 ± 9
Large	21 ± 3	15 ± 3[b]	25 ± 4
Intermediate	19 ± 4	11 ± 2[c]	20 ± 3
Small	35 ± 4[a]	24 ± 2	27 ± 3

[a] $p = 0.05$; [b] $p < 0.025$; [c] $p < 0.01$ vs control subjects. Mean ± SEM

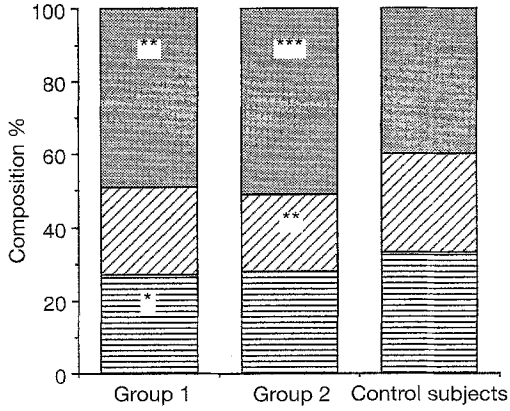

Fig. 1. Mean percentage contribution of each VLDL subfraction (▤, large VLDL; ▨, intermediate VLDL; ▦, small VLDL) to total VLDL lipid concentration in normolipidaemic IDDM patients with unsatisfactory blood glucose control (group 1, $n = 18$, $HbA_{1c} > 8\,\%$) and in IDDM patients with good blood glucose control (group 2, $n = 16$, $HbA_{1c} < 8\,\%$) and in sex-, age-, BMI-, and lipid-matched control subjects ($n = 16$). Significance vs control subjects: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$

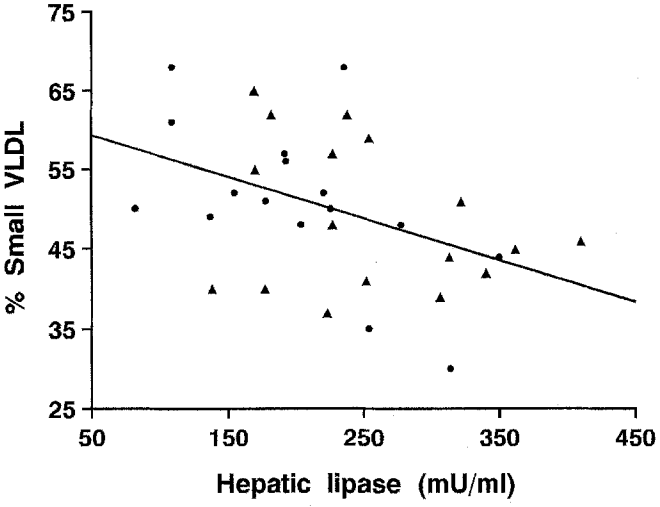

Fig. 2. Relationship between HL activity and percentage contribution of small VLDL subfraction ($y = 61.96–0.052\ x$, $r = -0.43$, $p < 0.025$) in normolipidaemic IDDM patients ($n = 33$) both with unsatisfactory (▲) and good (●) blood glucose control

in the two groups of patients and in the control group. On the other hand, HL activity was significantly reduced in both groups of diabetic patients compared to control subjects (254 ± 19 vs 317 ± 31 mU/ml, $p < 0.05$ for the group with $HbA_{1c} > 8\,\%$; 202 ± 19 vs 317 ± 31 mU/ml, $p < 0.005$ for the group with $HbA_{1c} < 8\,\%$). No significant difference was observed between the two groups of IDDM patients.

Correlation analysis between lipolytic enzyme activities and VLDL subfractions, expressed as a percentage of the total VLDL, showed that HL activity was inversely related to the percentage of small VLDL in diabetic patients ($r = -0.43$, $p < 0.025$) (Fig. 2).

Discussion

The most important results of our study are as follows:

1) normolipidaemic IDDM patients, whatever their degree of blood glucose control, reveal a significant relative increase (about 25 %) in the smallest VLDL particles compensated by a relative decrease in the large and/or intermediate VLDL subfractions; 2) HL activity is also reduced in IDDM patients, independently of the degree of blood glucose control; 3) in IDDM patients with good blood glucose control the increase in small VLDL is only relative (since VLDL of large and intermediate size are reduced) but the absolute concentration of these particles is similar to that observed in non-diabetic control subjects. Conversely, in IDDM patients with unsatisfactory blood glucose control the absolute amount of small VLDL is also increased as compared to control subjects.

These results suggest that all IDDM patients, whatever their blood glucose control, have a common metabolic abnormality causing a slowdown in the catabolic pathway of the smaller VLDL particles. Very likely, this abnormality is represented by low HL activity that was, in fact, significantly reduced in both groups of diabetic patients. Moreover, an inverse correlation existed between HL activity and the percentage of small VLDL. The reduction in HL activity in IDDM patients might be secondary to a relative hepatic hypoinsulinaemia. The peripheral site of insulin administration is not, in fact, able to completely restore normal hepatic insulin levels (hepatic insulin levels are one of the main regulators of HL activity). In support of this interpretation was the demonstration that intraperitoneal insulin therapy is able to normalize the reduced HL activity observed in IDDM patients and, at the same time, to re-

Table 4. Plasma post-heparin lipase activities

	IDDM patients ($n = 17$) $HbA_{1c} > 8\,\%$	IDDM patients ($n = 16$) $HbA_{1c} < 8\,\%$	Control subjects ($n = 13$)
Lipoprotein lipase (mU/ml)	88 ± 7	86 ± 7	81 ± 11
Hepatic lipase (mU/ml)	254 ± 19^{a}	202 ± 19^{b}	317 ± 31

$^{a}\ p < 0.05$; $^{b}\ p < 0.005$ vs control subjects. Mean ± SEM

duce the levels of chylomicron remnants [27, 28]. Apparently not consistent with our hypothesis are data obtained in a very small group of NIDDM patients by hyperinsulinaemic clamp at different blood glucose levels [29].

Although the reduction of HL in our study is largely independent of blood glucose control, it cannot be completely excluded that blood glucose levels may also be involved in the regulation of HL activity. However, our study shows that the impact of the reduced HL activity on the absolute amount of small VLDL in blood is dependent on the degree of blood glucose control and, therefore, on the flux of VLDL entering the lipolytic cascade. In fact, in IDDM patients with unsatisfactory blood glucose control the synthesis of VLDL may be increased [12]. As a result, a higher number of VLDL particles are catabolized to VLDL of smaller size by LPL, but any further catabolic step is impaired by reduced HL activity. Therefore, small VLDL tend to accumulate and their plasma concentration increase. Conversely, in IDDM patients with optimal blood glucose control the synthesis of VLDL is reduced in comparison with normal individuals [30]. So, even if the smaller particles tend to accumulate in these patients (for the reduced HL activity), the absolute amount of small VLDL in plasma is not increased in comparison with normal individuals, since a minor number of VLDL particles enter the lipolytic cascade.

Of course, it is also possible that other metabolic abnormalities, such as a shift in the VLDL synthesis toward an increase in the smaller ones [31] and/or an enhanced cholesterol ester transfer protein activity may play a role in explaining our findings [32]. Whatever the mechanisms of our results, the increase in small VLDL particles may be relevant from a clinical point of view. Actually, both experimental and clinical data (in animals as well as in non-diabetic subjects) [33, 34] seem to suggest that small VLDL are more atherogenic, even if there is still no definitive evidence on the relationship between small VLDL and cardiovascular risk in diabetic patients. If the increase in small VLDL has to be considered a potential cardiovascular risk factor, of course IDDM patients with an unsatisfactory blood glucose control (which is the most frequent condition in these patients) are at a higher risk in comparison not only with control subjects, but also with IDDM subjects in optimised blood glucose control. In fact, even if optimised blood glucose control is unable to completely normalize the abnormal distribution of VLDL subfractions, the possible clinical relevance and the possible impact of the increase in small VLDL on atherogenic risk is surely reduced to a minimum, considering that, in this particular metabolic condition, small VLDL particles are not increased in absolute terms. Conversely, in patients with very poor metabolic control (not included in our study) the excess of small VLDL might be even greater and therefore, may have a more relevant clinical impact.

There are very few studies in the literature on VLDL subfractions in IDDM patients [35]. In the study by James and Pometta [35], IDDM patients with unsatisfactory blood glucose control had a significant increase in the total plasma concentration of small VLDL as compared to control subjects. Moreover, after a short period of blood glucose optimization, all VLDL subfractions declined, but the fall in large VLDL was more marked compared to small VLDL. This suggests that in their patients, as in ours, a relative increase of small VLDL is also present when blood glucose control is optimized.

In conclusion, normolipidaemic IDDM patients are characterized by an abnormal distribution of VLDL subfractions, consisting mainly of a relative increase in small VLDL, irrespective of their blood glucose control. This shift in the distribution of VLDL subfractions seems to be a consequence of a reduced HL activity due to a subnormal insulin concentration in the liver. However, the abnormal distribution of VLDL subfractions is more relevant clinically in patients with unsatisfactory blood glucose control, in whom the increase in small VLDL is not only relative, but also absolute. Therefore, this study once again reinforces the importance of optimizing blood glucose control in IDDM patients; even if it cannot normalize the abnormal distribution of VLDL subfractions, it can keep the concentration of small VLDL, considered to be potentially atherogenic, within the normal range.

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