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Metabolism Clinical and Experimental 55 (2006) 879–884

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Intensive insulin therapy reduces small dense low-density lipoprotein particles in patients with type 2 diabetes mellitus: relationship to triglyceride-rich lipoprotein subspecies

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Received 29 August 2005; accepted 3 February 2006

Abstract

It remains unclear whether insulin improves dyslipidemia in patients with type 2 diabetes mellitus. Small dense low-density lipoprotein (sd-LDL) particles are recognized as a powerful risk factor for coronary heart disease and are often elevated in type 2 diabetes mellitus. We examined the effect of intensive insulin therapy on sd-LDL particles and triglyceride (TG)–rich lipoprotein subspecies. Intensive insulin therapy (insulin aspart [NovoRapid, Tokyo, Japan] before each meal and isophane insulin suspension at bedtime) was given to poorly controlled type 2 diabetic patients (n = 46) who were on high doses of sulfonylureas. Fasting serum samples were collected before and 14 days after the commencement of insulin therapy. Low-density lipoprotein size was measured by gradient gel electrophoresis, and the small dense LDL cholesterol (sd-LDL-C) concentration was measured by a new precipitation method. Chylomicrons (Svedberg flotation unit >400), very low-density lipoprotein 1 (VLDL1) (Sf, 60-400), and VLDL2 (Sf, 20-60) were separated by ultracentrifugation. Serum apolipoprotein B-48 and lipoprotein lipase levels were measured by the enzyme immunoassay method. Serum glucose and glycoalbumin levels were substantially decreased by insulin treatment. The LDL size increased (25.8-26.0 nm, $P < .05$) and the sd-LDL-C level was significantly reduced (44-34 mg/dL, $P < .005$). Apolipoproteins B-48 and C-III were decreased, whereas lipoprotein lipase was increased. Triglyceride levels in chylomicrons, VLDL1, and VLDL2 all showed a decrease. Changes of sd-LDL-C or LDL size were associated with changes of the TG levels in the major TG-rich lipoprotein subspecies. These results suggest that intensive insulin therapy decreases atherogenic sd-LDL particles by reducing TG in TG-rich lipoproteins. We did not find any specific relationship between VLDL1 and sd-LDL during insulin treatment.
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1. Introduction

Type 2 diabetes mellitus is a common disorder that is accompanied by numerous metabolic abnormalities leading to a high risk of coronary heart disease (CHD). The United Kingdom Prospective Diabetes Study confirmed that intensive glycemic control delays the onset and retards the progression of microvascular disease, and possibly CHD, in patients with type 2 diabetes mellitus [1]. Insulin resistance plays a major role in the development of hyperglycemia and dyslipidemia in type 2 diabetes mellitus. However, when pancreatic beta-cell function declines, insulin therapy is required to achieve optimal glycemic control in type 2 as well as type 1 diabetes mellitus. Although many patients with type 2 diabetes mellitus are on insulin therapy, few studies have investigated the influence of insulin therapy on dyslipidemia in type 2 diabetes mellitus. It is important to investigate how insulin therapy affects lipid metabolism in patients with type 2 diabetes mellitus because there is a possibility that it could cause hyperinsulinemia, which has the potential to exacerbate lipid abnormalities.

An increase in triglyceride (TG), decrease in high-density lipoprotein cholesterol (HDL-C), and increase in small dense low-density lipoprotein (sd-LDL) particles represent a common dyslipidemic pattern in individuals who have insulin resistance, such as patients with type 2 diabetes mellitus, obesity, and metabolic syndrome [2]. In particular, sd-LDL has attracted attention as a very strong risk factor for CHD beyond LDL cholesterol (LDL-C) [2,3]. The recent Quebec cardiovascular study clearly demonstrated that an increase of sd-LDL-C predicts a higher risk of CHD events,

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doi:10.1016/j.metabol.2006.02.014

Table 1
General profile of type 2 diabetic subjects before commencement of intensive insulin therapy

n	46 (F/M, 29/17)
Age (y)	63 ± 2
BMI	23.4 ± 0.5
HbA_{1c} (%)	10.4 ± 0.3
FPG (mg/dL)	221 ± 9.3
2-h Glucose (mg/dL)	328 ± 16
Fasting C-peptide (ng/mL)	2.3 ± 0.2
2-h C-peptide (ng/mL)	4.9 ± 0.3
Fasting insulin (μU/mL)	6.1 ± 0.6

Data are expressed as mean ± SE. Glucose and C-peptide were measured 2 hours after breakfast. BMI indicates body mass index; HbA_{1c}, hemoglobin A_{1c}; FPG, fasting plasma glucose.

whereas a change of large buoyant LDL-C does not [4]. Griffin and Packard [5,6] demonstrated that large TG-rich very low-density lipoprotein 1 (VLDL1) is a precursor of sd-LDL particles, and VLDL1 is preferentially produced in the liver after insulin resistance developed. This explains the intimate association between sd-LDL particles and insulin resistance. Very recently, the same group reported that hyperglycemia stimulates VLDL1 production in type 2 diabetes mellitus [7]. Accordingly, we speculated that control of hyperglycemia with insulin could reduce sd-LDL particles by specifically suppressing VLDL1 secretion.

In the present study, we investigated how insulin therapy changes LDL size and the sd-LDL concentration in type 2 diabetes mellitus and explored the relationship between sd-LDL and TG-rich lipoprotein (TGRL) subspecies. We measured VLDL1 (large VLDL), VLDL2 (small VLDL), and chylomicrons (CMs) after separation by ultracentrifugation, as well as serum apolipoprotein (apo) B-48 (a marker of CMs and its remnants). Because CMs are not a precursor of LDL, it is interesting to investigate the relationship between sd-LDL particles and CMs to elucidate the specific association between VLDL1 and sd-LDL particles.

2. Methods

Forty-six patients with type 2 diabetes mellitus were enrolled in this study. All of the patients were admitted to the Showa University Hospital to receive insulin therapy. They had been treated with high doses of sulfonylureas (>7.5 mg glibenclamide or 4 mg glimepiride per day), but hemoglobin A_{1c} had remained more than 7.5% for at least 3 months before admission. Fourteen patients were being treated with α-glucosidase inhibitors in addition to sulfonylureas. Dietary therapy was supervised by a dietitian, and exercise therapy was prescribed by a physician for at least 3 months before and during the study according to the recommendations of the Japanese Diabetes Association. Table 1 shows the profile of the subjects on the first day of admission. Type 1 diabetes mellitus was excluded by no detention of glutamic acid decarboxylase (GAD) antibody and a significant increase of C-peptide at 2 hours after breakfast. Intensive insulin therapy (insulin aspart [NovoRapid, Tokyo, Japan] before each meal and isophane insulin suspension at bedtime) was given for 3 to 5 days after hospitalization. The total dose of insulin was 12 to 56 U/d (mean, 26 ± 11 U/d). All oral hypoglycemic agents were withdrawn 1 day before starting insulin administration. Ten patients were being treated with statins for hypercholesterolemia, and they were allowed to continue this medication during the study. Fasting serum samples were collected before and 14 days after the commencement of insulin therapy. Written informed consent was obtained from all subjects, and this study was approved by the ethics committee of the Showa University.

Low-density lipoprotein size was measured by gradient gel electrophoresis [8], and the sd-LDL-C concentration was measured by a newly developed precipitation method, as

Table 2
Changes in serum concentrations before and after intensive insulin therapy

	Pretreatment	Posttreatment	Difference	% Change
FPG (mg/dL)	207 ± 12	126 ± 6.8	−86.8 ± 14****	−32.6 ± 7.6****
Glycoalbumin (%)	32.0 ± 1.7	26.1 ± 1.1	−5.7 ± 0.9****	−15.7 ± 1.9****
Triglyceride (mg/dL)	158 ± 12	123 ± 7.0	−38.7 ± 11***	−14.5 ± 4.6***
LDL-C (mg/dL)	140 ± 6.5	130 ± 6.1	−11.3 ± 4.5*	−5.8 ± 3.5
HDL-C (mg/dL)	51 ± 2.2	47 ± 1.9	−4.6 ± 1.3***	−7.0 ± 2.5**
ApoA-I (mg/dL)	130 ± 4.7	119 ± 4.0	−11.8 ± 3.3***	−7.7 ± 2.2***
ApoB (mg/dL)	114 ± 5.2	104 ± 4.5	−10.2 ± 3.3***	−6.7 ± 3.1*
ApoC-III (mg/dL)	8.7 ± 0.6	5.7 ± 0.5	−3.2 ± 0.6***	−30.0 ± 0.5***
ApoE (mg/dL)	4.8 ± 0.7	4.2 ± 0.6	−0.7 ± 0.2****	−16.1 ± 2.8****
ApoB-48 (μg/ mL)	6.1 ± 0.8	4.6 ± 0.5	−1.7 ± 0.5*	−13.8 ± 5.0
LPL (ng/mL)	31.8 ± 2.2	37.2 ± 1.7	5.4 ± 1.2**	17.1 ± 2.1*
sd-LDL-C (mg/dL)	43.4 ± 3.9	34.5 ± 3.1	−10.4 ± 3.0***	−14.7 ± 5.4***
Mean LDL diameter (nm)	258.2 ± 0.8	260.1 ± 0.8	1.5 ± 0.8*	0.6 ± 0.3*

Data are expressed as mean ± SE. Difference represents posttreatment minus pretreatment.
* $P < .05$.
** $P < .01$.
*** $P < .005$.
**** $P < .0001$.

Table 3
Changes in lipoprotein concentrations before and after intensive insulin therapy

	Pretreatment	Posttreatment	Difference	% Change
CM-TG (mg/dL)	23 ± 4	15 ± 1	−10.3 ± 3.6*	−15.2 ± 7.6
CM-C (mg/dL)	6 ± 0.9	5 ± 0.9	−1.2 ± 1.0	−10.6 ± 8.9
VLDL1-TG (mg/dL)	28 ± 3	21 ± 1	−7.3 ± 2.1**	−14.4 ± 5.0**
VLDL1-C (mg/dL)	9 ± 2	8 ± 2	−2.1 ± 1.2	−4.3 ± 4.4
VLDL1-apoB (mg/dL)	16 ± 1	14 ± 1	−2.0 ± 1.0	−4.0 ± 10.2
VLDL1–apoC-III(mg/dL)	0.7 ± 0.1	0.3 ± 0.07	−0.5 ± 0.1****	−52.1 ± 7.2****
VLDL2-TG (mg/dL)	20 ± 1	16 ± 0.8	−4.0 ± 1.4*	−10.1 ± 5.2
VLDL2-C (mg/dL)	8 ± 1	7 ± 1	−2.3 ± 0.9*	−5.0 ± 3.3
VLDL2-apoB (mg/dL)	11 ± 0.8	10 ± 0.6	−1.4 ± 0.5	−4.6 ± 5.9
VLDL2–apoC-III (mg/dL)	1.1 ± 0.1	0.6 ± 0.07	−0.5 ± 0.1****	−45.1 ± 6.1****
VLDL2-TG/VLDL1-TG	0.71 ± 0.03	0.78 ± 0.03	0.07 ± 0.03	6.6 ± 5.8

Data are expressed as mean ± SE. Difference represents posttreatment minus pretreatment.
* $P < .05$.
** $P < .01$.
*** $P < .005$.
**** $P < .0001$.

described previously [9]. Chylomicrons (Svedberg flotation unit [Sf] >400), VLDL1 (Sf, 60-400), and VLDL2 (Sf, 20-60) were separated by ultracentrifugation (Hitachi CP-56G; Hitachi, Tokyo, Japan) with a swing rotor (P40ST, Hitachi) according to the method of Karpe et al [10]. The serum apoB-48 level was measured by the enzyme immunoassay method (Lumipulse apoB-48, Fujirebio, Tokyo, Japan) according to the method of Sakai et al [11]. The serum lipoprotein lipase (LPL) mass was measured by the enzyme-linked immunosorbent assay (LPL ELISA, Daiichi Pure Chemical, Tokyo, Japan) according to the method of Kobayashi et al [12]. ApoA-I, apoB, apoC-III, and apoE were measured by immunoturbidometry (Daiichi Pure Chemical). LDL-C and HDL-C were measured by direct assay using commercially available kits (Cholestest LDL and Cholestest N-HDL; Daiichi Pure Chemical). Immunoreactive insulin and C-peptide were measured by enzyme immunoassay using a commercially available test kit (Shionogi Pharmaceutical, Osaka, Japan). Glycoalbumin was measured by enzyme immunoassay (Lusica GA-L, Daiichi Pure Chemical). Lipoprotein separation by ultracentrifugation was performed immediately after the blood sampling, concurrently with the laboratory assays.

The significance of differences between before and after the treatment with insulin was determined by the paired Student t test. Correlations between 2 variables were calculated by Pearson simple linear regression analysis. Multiple regression analyses were performed to assess the independent effects of VLDL1 and VLDL2 on sd-LDL. Statistical significance was accepted at $P < .05$.

3. Results

Table 2 shows the serum levels of various parameters before and after commencement of intensive insulin therapy. As expected, hyperglycemia was significantly improved by insulin treatment, that is, the fasting serum glucose level was almost halved and glycoalbumin was reduced by 15%. Triglycerides and LDL-C were both reduced, but HDL-C was also unexpectedly reduced. Apolipoprotein B (almost equal to apoB-100), apoC-III, and apoE were decreased. Apolipoprotein B-48 was significantly decreased, and apoA-I was decreased as well as HDL-C. Lipoprotein lipase was significantly increased by insulin treatment. The LDL size increased significantly, so the prevalence of the sd-LDL phenotype (pattern B) [13] was markedly reduced from 33% to 11%. Insulin therapy significantly reduced the sd-LDL-C level, whereas the level of large buoyant LDL-C (calculated as total LDL-C minus sd-LDL-C) was not altered (98-97 mg/dL).

Table 3 shows serum lipoprotein levels before and after intensive insulin therapy, as well as the percent changes during insulin treatment. Although these were fasting serum samples, considerable amount of CM-lipid levels was

Table 4
Correlation coefficients (r) by simple regression analysis between sd-LDL-C and other parameters

	Pretreatment	Change during treatment	Posttreatment
CM-TG	0.37*	0.38*	0.32*
VLDL1-TG	0.44***	0.38*	0.48***
VLDL2-TG	0.47***	0.40**	0.52***
VLDL1–apoC-III	0.46***	0.53***	0.44***
VLDL2–apoC-III	0.30*	0.59****	0.22, NS
LDL-C	0.55****	0.48***	0.63****
HDL-C	−0.07, NS	0.14, NS	−0.08, NS
TG	0.50***	0.50***	0.44***
ApoB	0.68****	0.63****	0.71****
ApoC-III	0.56***	0.54***	0.30, NS
ApoB-48	0.34*	0.46**	0.001, NS
FPG	0.05, NS	0.21, NS	0.10, NS
Glycoalbumin	0.28, NS	0.002, NS	0.34*
LPL	0.26, NS	0.01	0.17, NS

NS indicates not significant ($P > .05$).
* $P < .05$.
** $P < .01$.
*** $P < .005$.
**** $P < .0001$.

detected in our poorly controlled diabetic subjects. Unlike CM-lipids, only traces of apoB (total) and apoC-III were detected in the CM fraction. Insulin treatment significantly decreased CM-TG without any change of chylomicron-cholesterol (CM-C). Insulin therapy also decreased VLDL1-TG and VLDL2-TG to similar extent, but did not significantly reduce the cholesterol and apoB levels in these VLDL subspecies. Unlike apoB, apoC-III levels in VLDL1 and VLDL2 were halved by insulin therapy.

Table 4 shows the correlations between the sd-LDL-C concentration and various parameters before and after treatments, as well as the percent changes during insulin treatment. The sd-LDL-C level was significantly associated with TG in serum, VLDL1, VLDL2, and CMs before insulin treatment. Small dense LDL-C was also correlated with LDL-C, apoB, VLDL1–apoC-III, and VLDL2–apoC-III. All of the parameters were also significantly correlated in the posttreatment samples, with the exception of VLDL2–apoC-III. The sd-LDL-C level decreased with a decrease of serum TG and TGRL-TG, as well as a decrease of LDL-C and apoB. Changes of sd-LDL-C were correlated with the changes of apoB-48. The decreases of apoC-III in serum and VLDLs were substantially correlated with the decrease of sd-LDL-C. A decrease of sd-LDL-C was not associated with improved glycemic control or an increase of LPL mass.

Table 5 shows the correlations between LDL size and various parameters before treatment, as well as the changes during insulin treatment. Before treatment, LDL size showed a significant inverse correlation with TG in serum, CM, VLDL1 and VLDL2, and apoB-48. An increase of LDL size was associated with a decrease of VLDL1-TG, VLDL1–apoC-III, VLDL2-TG, and serum apoC-III. Changes of LDL size were not associated with improved glycemic control or an increase of LPL mass. Unlike the case in the pretreatment samples, the LDL size showed no significant correlations with the TG in serum, CM, VLDL1, VLDL2, or apoB-48 in the posttreatment samples. We performed multiple regression analyses to assess the independent effects of concentrations (TG, C, apoB, or apoC-III) in VLDL1 and VLDL2 on sd-LDL. As a result, we found that neither VLDL1 nor VLDL2 was independently associated with sd-LDL-C concentration or LDL size (data not shown).

Table 5
Correlation coefficients (r) between LDL size and other parameters

	Pretreatment	Change during treatment	Posttreatment
CM-TG	−0.34*	−0.25, NS	−0.05, NS
VLDL1-TG	−0.37*	−0.32*	−0.14, NS
VLDL2-TG	−0.38*	−0.49***	−0.25, NS
VLDL1–apoC-III	−0.48***	−0.42**	−0.23, NS
VLDL2–apoC-III	−0.11, NS	−0.27, NS	−0.09, NS
LDL-C	−0.07, NS	0.17, NS	0.05, NS
HDL-C	0.38*	0.39**	0.60****
TG	−0.40**	−0.32*	−0.21, NS
ApoB	−0.26, NS	−0.01, NS	−0.10, NS
ApoC-III	−0.65***	−0.42**	0.51, NS
ApoB-48	−0.44*	−0.31, NS	−0.26, NS
FPG	−0.99, NS	−0.17, NS	−0.29, NS
Glycoalbumin	0.03, NS	−0.007, NS	−0.06, NS
LPL	0.25, NS	0.01, NS	0.17, NS

* $P < .05$.
** $P < .01$.
*** $P < .005$.
**** $P < .0001$.

4. Discussion

According to investigations performed by Griffin and Packard [5,6] and Taskinen and colleagues [26], the liver produces and secretes both VLDL1 and VLDL2 subspecies, but only VLDL1 production is markedly affected by insulin resistance [5,6]. The same group has also reported that infusion of insulin for several hours directly suppresses VLDL1 production without affecting VLDL2 production [14]. Furthermore, Adiels et al [7] very recently reported that serum glucose, insulin, and free fatty acids together explain half of the variation of VLDL1 TG production, suggesting that hyperglycemia itself is the driving force that promotes overproduction of VLDL1 in type 2 diabetes mellitus. Griffin and Packard [5,6] also demonstrated by a kinetic study that VLDL1 is preferentially converted to sd-LDL particles. All of these findings led us to speculate that insulin treatment, especially intensive insulin therapy, could selectively suppress VLDL1 production and inhibit the generation of sd-LDL particles.

Unexpectedly, we found that intensive insulin therapy reduced VLDL1-TG and VLDL2-TG to a similar extent without affecting the apoB or cholesterol levels in these lipoproteins [15,16]. We also found that insulin treatment could significantly reduce CM-TG without affecting CM-C. A decrease of TG without any change of the apoB or cholesterol levels in lipoproteins suggests that insulin therapy only stimulates TG hydrolysis without affecting particle clearance. We did not measure the LPL activity of postheparin plasma in the present study, but we speculate that an increased serum LPL mass implies stimulation of the production of this enzyme by insulin, leading to increased lipolytic activity in the serum. We also found that the apoC-III concentrations in VLDL1 and VLDL2 were substantially reduced by insulin therapy. Because apoB was unaltered, insulin therapy seems to selectively reduce the apoC-III content of lipoprotein particles. Apolipoprotein C-III is known to suppress LPL activity or to interfere with the attachment of lipoproteins to this enzyme [17]. Thus, apoC-III–poor VLDLs are more easily hydrolyzed by LPL. Several lines of evidence suggest that the production of apoC-III is suppressed by insulin [18,19], and conversely, its production is stimulated in the insulin-resistant state [20]. Nagashima et al [21] recently reported that pioglitazone, an insulin sensitizer, decreases apoC-III production in type 2 diabetic patients, and they explained this change as being due to increased VLDL catabolism. Taken together, we

speculate that insulin treatment stimulates TG hydrolysis by suppressing apoC-III production and enhancing LPL production. Why did we fail to observe a reduction in only VLDL1 and no reduction in VLDL2 during insulin treatment? This was probably because our type 2 diabetic patients were neither obese nor hyperinsulinemic, so their insulin resistance was less severe than in Western populations. Thus, VLDL1 production may not be deeply involved in the mechanism that increases the serum TG concentration in nonobese type 2 diabetic patients.

The serum TG level is the most powerful determinant of LDL size [22]. Indeed, we always found a close relationship between the TG level in serum or lipoproteins and the LDL size or sd-LDL-C level (Tables 4 and 5). Mechanisms generating sd-LDL particles in patients with hypertriglyceridemia are multifactorial, but it is generally accepted that an increase of TGRLs facilitates lipid transfer between lipoproteins, and that lipid-poor LDL is finally produced by the action of hepatic lipase [23]. Several studies have shown that LDL size is not only regulated by fasting TG levels, but also by postprandial TG levels [24,25], suggesting that CMs could participate in this exchange of lipids among lipoproteins. The LDL size was increased and the sd-LDL-C level was significantly decreased by intensive insulin therapy. We observed that the changes of LDL size and sd-LDL-C concentration were not only regulated by changes of VLDL1-TG, but also of VLDL2-TG. In addition, a decrease of CM-TG or apoB-48 was also associated with a decreased sd-LDL-C level. Because CM is not a precursor of LDL, our results suggest that the actual TG concentration rather than a specific change of VLDL subspecies is more closely associated with the preponderance of sd-LDL particles. Serum apoB-48 is an excellent marker of CM remnants in the fasting state. Insulin treatment decreased the apoB-48 level and this change was significantly correlated with a decrease of sd-LDL-C, suggesting that an increase of TG in CM remnants is involved in the generation of sd-LDL particles.

We observed that the LDL-C and apoB levels were significantly decreased by intensive insulin therapy, but the mechanisms involved remain unknown. Low-density lipoprotein receptors might be activated by insulin treatment. High-density lipoprotein cholesterol and apoA1 levels were unexpectedly decreased by insulin, but HDL-C and apoA-I levels are reported to be high in insulin-dependent type 1 diabetic patients [26], so these reductions would probably be reversed during long-term insulin treatment.

In conclusion, intensive insulin therapy decreases atherogenic sd-LDL particles by reducing TG in major TGRLs, probably because of increased lipolysis, in patients with type 2 diabetes mellitus. We did not find a specific relationship between VLDL1 and sd-LDL during insulin treatment. The limitation of the present study is that the number of subjects is relatively small. Further studies will be required to compare the effect of intensive insulin therapy and conventional insulin therapy on lipid metabolism in a large number of subjects.

Acknowledgment

We are deeply indebted to Mrs Hiroko Takeuchi and Dr Hiroto Tajima for their excellent technical support.

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