Persistent Abnormalities in Lipoprotein Composition in Noninsulin-dependent Diabetes after Intensive Insulin Therapy

John D. Bagdade, Wilfred E. Buchanan, Timo Kuusi, and Marja-Ritma Taskinen

To determine whether rigorous insulin therapy, which normalized the routinely measured plasma lipids, also reversed qualitative abnormalities in the composition of lipoproteins in noninsulin-dependent diabetes mellitus (NIDDM), we studied 18 NIDDM patients (eight men and 10 women) before and 2 months after intensive insulin therapy. Glycosylated hemoglobin levels (11.7% vs. 8.7%), plasma triglyceride (TG) (250±91 vs. 164±56 mg/dl, p<0.001), and cholesterol (214±43 vs. 198±31 mg/dl, p<0.025) all fell, and both HDL cholesterol and HDL cholesterol increased (59.1% and 10.9%, respectively, p<0.001). However, abnormalities in two indices of lipoprotein surface constituents, which were present before insulin therapy, remained so thereafter. The first of these, the new cardiovascular risk factor, the plasma free cholesterol/lecithin ratio, which was increased before treatment, fell only slightly after therapy (pre-therapy 1.02±0.29 vs. post-therapy 0.90±0.17, p<0.4; reference group, 0.83±0.14), and remained elevated in very low density lipoprotein (VLDL) and low density lipoprotein (LDL). Secondly, the sphingomyelin/lecithin ratio, an index of the surface rigidity of lipoproteins, was abnormal before treatment in VLDL, HDL, and HDL, and this alteration persisted after insulin therapy (p<0.001). Lipoprotein core lipid abnormalities were also present before treatment: the TG/cholesterol ester ratio was reduced in VLDL and increased in LDL, HDL, and HDL, and the apo A-I increased significantly (p<0.005), and apo A-II and apo E were unchanged; in contrast, in HDL, apo A-I was unchanged, and apo A-II and apo E both decreased (p<0.005) after insulin therapy. Since lipoproteins with altered surface and core lipids have been shown to have an impaired capacity to transfer their constituents to other lipoproteins and cells and compromised participation in reverse cholesterol transport, persistence of these qualitative changes may sustain the increased cardiovascular risk in NIDDM even when clinical control is excellent and the routinely measured plasma lipids appear normal. (Arteriosclerosis 10:232-239, March/April 1990)
This group was seen two to three times weekly to assess patients spent 3 to 4 days each week during the period of the heparin test for lipoprotein lipase (LPL) measurement for urinary albumin. The mean serum creatinine of the group was 87±12 μmol/l (range 71 to 118 μmol/l). Ten patients were mildly hypertensive, and six had a history of prior myocardial infarction or coronary heart disease. Four patients were taking nitrates; five, antihypertensives; seven others, diuretics; and six, beta blockers. These medications were continued at the same dose throughout the study.

**Study Design**

While in hospital on a metabolic ward after entry into the study, all patients received a weight-maintaining sucrose-free diet (35% fat, 45% carbohydrate, 20% protein), and their oral medications were continued. After this 3-day period of stabilization, adipose tissue biopsy and the heparin test for lipoprotein lipase (LPL) measurement as previously reported were performed on consecutive mornings after an overnight fast of 10 to 12 hours. Lipoprotein analyses were performed on blood samples drawn before the heparin test was performed. After these pretreatment studies were obtained, oral antidiabetic agents were discontinued, and insulin therapy was initiated. These same tests were repeated under identical conditions 7 weeks later. All patients were instructed to continue their usual level of physical activity and to daily record their body weights.

**Insulin Therapy**

Diabetic control was achieved by two or three injections daily with regular insulin alone (Actrapid, Novo Industri A/S) or in conjunction with intermediate acting insulin (Protaphan, Novo Industri A/S). Therapy was initiated with 30 to 40 units of intermediate insulin per day injected before breakfast and at 9:00 P.M. Additional regular insulin was added before breakfast and dinner to achieve good glycemic control (fasting glucose less than 140 mg/dl and postprandial glucose levels less than 180 mg/dl). Ten days to 2 weeks were required before insulin doses and glucose levels were stabilized.

All patients employed home glucose monitoring (Hypo- count MX glucometer; Oriola, Helsinki, Finland). Eight patients spent 3 to 4 days each week during the period of insulin therapy on the metabolic ward; 10 were followed as outpatients only after glycemic control had been achieved. This group was seen two to three times weekly to assess their home blood glucose profiles. Daily caloric intake was adjusted according to the disappearance of glucosuria to assure that a stable body weight was maintained.

**Glycemic Control**

The adequacy of diabetic control was monitored by twice daily blood glucose measurements obtained at 7:30 A.M. (fasting) and at 4:00 P.M. and with 24-hour urinary glucose determinations. Meals were served at 7:30 A.M., 11:30 A.M., and 4:30 P.M., and snacks, at 10:00 A.M., 2:00 P.M., and 8:00 P.M.

**Plasma Lipids and Lipoprotein Analysis**

Lipoprotein fractions were separated by sequential flotation in an ultracentrifuge (Beckman L8-70, Beckman, Palo Alto, CA). First, chylomicrons were isolated from fresh plasma by centrifugation for 30 minutes at 18,000 rpm at 4°C in a T.50.3 Beckman rotor. The infranatant then was overlaid with 0.16 M NaCl and 1 mM of ethylenediaminetetraacetic acid (d=1.006 g/ml) and VLDL was isolated by tube slicing following an 18-hour spin at 38,000 rpm. The density of the remaining plasma was then adjusted to 1.019 g/ml with a mixture of KBr (353 g/l) and NaCl (183 g/l), and intermediate density lipoproteins (IDL) were then isolated by ultracentrifugation for 24 hours at 38,000 rpm at 4°C. The density of the IDL bottom fraction was then raised to 1.063 g/ml with the same KBr-NaCl mixture (d=1.335 g/ml) utilized in a previously reported study. LDL was isolated by spinning for 24 hours at 38,000 rpm. HDL₃ and HDL₂ were isolated by differential precipitation with dextran sulfate from previously unfrozen fresh plasma. The HDL fractions isolated by this technique have been shown to correspond closely to those separated by ultracentrifugation. The following analyses of lipoprotein composition were performed on all fractions with the exception of IDL.

**Assay of Lipolytic Enzymes**

Heparin-releasable LPL was estimated in needle aspirates of gluteal subcutaneous adipose tissue by using labeled triolein as substrate as previously described. LPL and hepatic lipase (HL) activities were measured immunochromally in plasma at 5 and 15 minutes after the bolus injection of heparin (100 IU/kg body weight, Vitrum, Stockholm, Sweden) by utilizing a specific antiserum against HL in the assay of LPL. The HL activity was estimated with a substrate containing 1 M NaCl to inactivate the LPL.

**Analytical Methods**

All the following analyses with the exception of previously estimated HbA₁c were performed in this laboratory in the same assay on samples of whole plasma and lipoprotein fractions isolated in Helsinki that had been kept frozen (−70°C) and on the HDL fractions isolated in this laboratory by precipitation from the whole plasma samples. Plasma glucose was measured with a glucose oxidase method. HbA₁c (reference range, 8.0% to 8.5%) was assayed with a chromatographic microcolumn method (Isolab Incorporated, Akron, OH). Cholesterol and TG were measured in whole plasma and in the
Table 1. Changes in Whole-Plasma Lipids in 18 Non-Insulin-dependent Diabetic Patients before and after Intensive Diabetic Management

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Before</th>
<th>After</th>
<th>Reference group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>231.0±45.0</td>
<td>185.3±36.7</td>
<td>95.9±22</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>11.8±1.3</td>
<td>10.6±0.5</td>
<td>6.0 to 8.5</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>248.5±91.3</td>
<td>163.3±56.1†</td>
<td>94.8±39.4</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>213.8±43.1</td>
<td>197.7±30.8†</td>
<td>185.3±36.7</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>35.6±6.4</td>
<td>43.5±9.4‡</td>
<td>53.8±11.1</td>
</tr>
<tr>
<td>Free cholesterol (mg/dl)</td>
<td>87.0±33.8</td>
<td>72.2±24.8§</td>
<td>59.7±11.6</td>
</tr>
<tr>
<td>FC/leucithin (mol/mol)</td>
<td>1.02±0.29</td>
<td>0.90±0.17</td>
<td>0.83±0.13</td>
</tr>
</tbody>
</table>

Values are means±SD. Before vs. after treatment: †p<0.02, ‡p<0.001.
HbA1c=glycoylated hemoglobin, HDL=high density lipoprotein, FC=free cholesterol.

lipoprotein fractions by using kits from Boehringer Mannheim. Free cholesterol was quantitated in an aliquot of whole plasma and in the same lipoprotein fractions with a kit in which cholesterol ester hydrolase was omitted. Apolipoproteins A-I, A-II, B-14 and E-15 were measured by immunossay. The four major lipoprotein phospholipids, lysolecithin, sphingomyelin (S), lecithin (L), and phosphatidyl ethanolamine (PE), were estimated in whole plasma and in each lipoprotein fraction after their extraction by a thin-layer chromatographic procedure employing activated silica gel plates (0.5 mm in thickness) and a solvent system of chloroform/methanol/acetic acid/water of 25:15:4:2 by volume. Each phospholipid spot was scraped into glass tubes, and the lipid phosphorus was determined by the modified Bartlett procedure.

The reference values for lipoprotein composition displayed in the tables were obtained from 35 healthy nonobese nondiabetic normolipemic hospital and laboratory medical center employees in Chicago and included 15 women and 20 men from 25 to 60 years of age whose weights were within 10% of their ideal body weight determined from a Metropolitan Life Insurance table.

Statistical Analysis

The means for each variable were compared by the paired t test for independent samples, and the linear relationships between variables were estimated by using Spearman rank correlation coefficients. No comparisons were made with the values for the reference group.

Results

Metabolic Parameters

After insulin treatment, all indices of glycemic control improved significantly (Table 1). The average dose of insulin administered to achieve control was 0.75±0.06 U/kg/day. Body weight increased slightly after treatment (75.1±2.4 vs. 76.4±2.3 kg, p<0.05).

Serum Lipids, Lipoproteins, and Lipolytic Enzymes

Since concentrations of plasma neutral and phospholipids and apolipoproteins (apo) were not significantly different in men and women in the reference group or in the NIDDM subjects before or after insulin therapy, all data were expressed as a single pooled group for each of the two groups respectively. After treatment, TG, cholesterol, and free cholesterol (FC) all fell significantly (Table 1). The relative decline in FC exceeded that of L (Table 2) with the result that the FC/L ratio, which was abnormally increased before insulin, fell after therapy but remained higher than that of the reference group.

In VLDL, all core and surface lipids were abnormally increased initially (Tables 2 and 3) and also fell significantly after insulin treatment. Insulin had little effect, however, on the proportion of major lipids present in the lipoprotein core and surface; the TG/cholesteryl ester (CE) and FC/L ratios were decreased before insulin and remained so thereafter. In contrast, the S/L ratio of VLDL, which was low before insulin therapy, was restored to normal.

In LDL, the FC/L and TG/CE ratios were both abnormally increased before rigorous therapy, and neither was significantly altered by it. Thus, these parameters of LDL surface and core remained higher than the values of the reference group despite intensive management. Here, in contrast to VLDL, the S/L ratio was similar to that of the reference group before treatment and was not appreciably affected by insulin (Tables 2 and 3).

In the HDL subfractions, several changes were observed after intensive therapy. Treatment was associated with significant increases in not only HDL cholesterol but also its major phospholipids S and L, FC (Table 2), and apo A-I (Table 4); no net change occurred in HDL apo A-II, or apo E levels. Before insulin treatment, HDL apo A-II levels, however, were lower in men; treatment was associated with an increase only in men (after: men 9.8±5.5 mg/dl, p<0.025; women 11.6±5.8, p=ns). Nevertheless, their HDL apo A-I/A-II ratios were unchanged by insulin therapy. The FC/L ratio, however, which was subnormal before treatment, actually fell further (p<0.001) after insulin treatment. The TG/CE ratio, which was abnormally increased before insulin therapy, fell significantly in HDL2 (−59.8%) but remained elevated.

Similar, though not identical, changes in HDL composition followed insulin treatment. Here, total and esterified cholesterol both increased (p<0.001, Table 3). Although HDL2 FC showed no net change, the FC/L ratio increased significantly (p<0.05) to normal levels. In contrast, the S/L ratio, which was increased before treatment, was unchanged by it. As noted in HDL2, TG declined and cholesterol rose, with the result that the TG/CE ratio fell significantly (p<0.001), remaining, however, elevated after therapy (Table 3). Although HDL2 phospholipid and apo A-I levels were not altered by insulin therapy, apo A-II and apo E both decreased significantly (p<0.005).

Mass post-heparin LPL was unchanged after insulin therapy (before, 21.8±5.9; after, 22.5±5.9 umol FFA/ml/hour). On the other hand, post-heparin HL fell (before, 25.2±12.6; after, 20.7±9.3umol FFA/ml/hour, p<0.01), but the result did not reach statistical significance. There was no demonstrable correlation between either HbA1c or glucose and 1) hepatic or adipose tissue lipase activities and 2) the FC/L ratios in whole plasma or VLDL before or after...
Table 2. Effects of Intensive Diabetic Management on Whole-Plasma and Lipoprotein Phospholipid and Free Cholesterol Composition In 18 Nonlnsulin-dependent Diabetic Patients

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Before</th>
<th>After</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>87.0±33.8</td>
<td>72.2±24.9§</td>
<td>59.7±11.6</td>
</tr>
<tr>
<td>HDL</td>
<td>2.5±3.2±0.02</td>
<td>0.187±0.05</td>
<td>1.25±0.30</td>
</tr>
</tbody>
</table>

Table 3. Effects of Intensive Diabetic Management on Lipoprotein Core Lipid Composition in 18 Nonlnsulin-dependent Diabetic Patients

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Before</th>
<th>After</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>126.8±55.4</td>
<td>67.6±33.3§</td>
<td>44.0±26.3</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>26.6±13.2</td>
<td>20.7±11.6</td>
<td>8.9±5.9</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>15.0±7.2</td>
<td>8.6±4.9</td>
<td>3.7±3.1</td>
</tr>
<tr>
<td>TG/CE</td>
<td>9.6±3.9</td>
<td>6.3±2.1</td>
<td>17.1±10.8</td>
</tr>
<tr>
<td>LDL</td>
<td>54.4±8.5</td>
<td>48.9±7.4</td>
<td>31.2±7.7</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>136.3±33.4</td>
<td>122.6±27.4</td>
<td>112.9±29.0</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>78.3±24.5</td>
<td>64.7±17.9</td>
<td>74.9±24.4</td>
</tr>
<tr>
<td>TG/CE</td>
<td>0.77±0.26</td>
<td>0.84±0.25</td>
<td>0.56±0.24</td>
</tr>
</tbody>
</table>

The values are means±SD. The LDL fraction is d=1.019 to 1.063. Before vs. after treatment: *p<0.05, tp<0.02, +p<0.01, §p<0.001.

Abbreviations are explained in the legend to Table 2.
treatment. Nor was any significant relationship found between HL and HDL cholesterol, L, or FC/L or S/L ratios. Although no group correlation was demonstrable between HL and HDU cholesterol, L, or FC/L or S/L ratios.

The risk of accelerated development of atherosclerotic cardiovascular complications is substantially increased in NIDDM, and the presence of hypertension, smoking, and high cholesterol clearly potentiates this risk.4 However, the basis for premature atherosclerosis in treated NIDDM patients who have no risk factors other than diabetes remains unclear. Our data indicate that well-controlled NIDDM patients have potentially atherogenic qualitative abnormalities in lipoprotein surface and core lipid composition, which persist even after their plasma lipid levels are normalized by aggressive treatment with insulin. It should be pointed out, however, that despite the aggressive therapeutic measures, normalization of either glucose or insulin levels was not likely to have been attained. Consequently, despite the remarkable improvement in overall diabetic control and in the plasma lipid profile of these patients, the persisting abnormalities may relate in part to individual metabolic differences among patients, such as obesity or inherited dyslipidemia, or to factors such as persistent hyperglycaemia and the intensive insulin therapy itself.

The specific disturbances in composition that we observed after insulin include an enrichment in FC relative to L in VLDL and LDL, with the result that the FC/L ratio in these lipoproteins and plasma increased compared to our reference group. This observation is significant because Kurkovsky et al.28 found in a Lipid Research Clinics study in Toronto that the FC/L ratio is a potent predictor for cardiovascular risk comparable to HDL cholesterol. This increase in the FC/L ratio is in keeping with our previous findings in normolipidemic insulin-dependent diabetes mellitus (IDDM) subjects.30,31 While the enrichment we observed in lipoprotein FC is consistent with evidence that cholesterol synthesis increases in NIDDM patients,32 its persistence after apparently successful diabetic therapy suggests that it may not be readily reversed.

Precisely how an increased FC/L ratio in plasma lipoproteins adversely affects cardiovascular risk is unknown. It is possible that when lipoproteins are oversaturated with FC, their fluidity and stability may decrease, and their physicochemical properties and behavior may change. Indeed, Fielding33 has presented evidence that several key steps in reverse cholesterol transport may be adversely affected when lipoproteins are enriched in FC. Specifically, FC-enriched VLDL and LDL from NIDDM patients, which have increased FC/phospholipid ratios, were shown to have an impaired capacity to participate in neutral lipid exchange and to accept CE in transfer (CET) from HDL.34 Moreover, these workers found that intact plasma from NIDDM was a poor acceptor of cholesterol from cultured cells.35

In this study, we observed that the TG/CE ratios of both LDL and HDL were increased. This finding is consistent with the enrichment of their core lipids with TG in NIDDM shown earlier.36,37 As we found with the abnormalities in surface lipid composition, these changes also persisted after marked improvement in glycemic control. In contrast to the increase in the TG/CE ratio present in LDL and HDL.
core lipid content, a reciprocal decrease was present in VLDL, which was relatively depleted in TG and enriched in CE. As a result, the TG/CE ratio of NIDDM VLDL before treatment was substantially lower than that of our reference group and remained so after insulin therapy. While LPL, HL, and neutral lipid exchange reactions all contribute to the remodeling of lipoproteins that normally takes place in plasma, a disturbance in the latter system would appear to best explain the composite of altered TG/CE ratios observed. Specifically, if the heteroexchange of CE from HDL for TG from VLDL (i.e., CET) were accelerated, a relative enrichment of VLDL with CE reflected by a decreased TG/CE ratio as we have observed would result. Indeed, our finding that CET was accelerated in both IDDM and NIDDM patients (unpublished observations) suggests that facilitated neutral lipid exchange reactions underlie these changes in VLDL and HDL core lipid composition. Since intensive insulin therapy improved their hepatic and LPL activities, persistence of the abnormal TG/CE ratios in HDL increases the likelihood that accelerated CET was a major mechanism contributing to this compositional disturbance.

The most extensive changes in lipoprotein composition after rigorous treatment were observed in HDL. While surface and core lipid abnormalities were present before insulin therapy in both subfractions, treatment had differing effects on each. Total and esterified cholesterol both increased on an absolute basis and relative to TG in both HDL subfractions. The magnitude of this change, however, and the accompanying reduction in the TG/CE ratio after insulin was greater in HDL2 than in HDL3. Among the abnormalities present in surface lipids in HDL2 before treatment, the elevated S/L ratio declined to normal after insulin; the reduced pretreatment FC/L ratio, on the other hand, fell even further. In HDL3, the S/L ratio was abnormally increased before insulin therapy and was unaffected by it; here the reduced pre-treatment FC/L ratio rose significantly to normal levels.

Interesting responses to insulin therapy also were observed in HDL apoprotein composition. In HDL2, apo A-I increased significantly relative to A-II, suggesting that the number of particles containing only apo A-I in the whole group had increased. Differences, however, were present in the absolute levels of apo A-II in HDL2 in men and women before insulin treatment. In contrast to HDL2 apo A-I levels, which were similar in both sexes before treatment and rose after insulin treatment, apo A-II levels were lower in men than in women before insulin, and only rose significantly in men. In men, no change at all was observed in the apo A-I/A-II ratio in HDL2; NIDDM women, this ratio increased in eight out of nine subjects, suggesting that insulin therapy may have increased the proportion of their HDL2 particles containing apo A-I only. This difference implies that despite their menopausal status, their HDL response to insulin therapy was affected by hormonal differences that existed between the middle-aged men and women in this study. The significance of this apparent sex-related response in HDL2 composition with regard to atherosclerosis is unclear.

In contrast to the differences observed in HDL2, in HDL3 the apo A-I was unchanged, and apo A-II actually declined, which is consistent with insulin affecting a reduction in the number of particles containing both apo A-I and A-II and a relative increase in those containing apo A-I only. Since HDL particles containing both A-I and A-II are believed to be less effective promoters of efflux of free cholesterol from cells than those containing A-I only, a relative reduction was found in this subpopulation of HDL particles and an increase in those with only apo A-I is theoretically beneficial, since it would facilitate this important first step in reverse cholesterol transport.

Although only 10% to 15% of circulating HDL particles in humans contain apo E, they are significant because they can bind to cells expressing apo B/E (LDL) receptors with a higher affinity than LDL itself and thus can deliver cholesterol to cells. Since apo E associates with small HDL particles in humans before they acquire cholesterol and enlarge in size, the changes we have observed in HDL apo E concentrations after insulin therapy are of interest. Here, too, the effects of intensive treatment on apo E-containing particles differed in each HDL subfraction. In HDL2, no net change in apo E occurred during insulin treatment. However, since the total number of particles increased, the relative number of those containing apo E appears to have declined. On the other hand, in HDL3 the apo E content actually fell with a significant change in total mass or particle number. Thus, insulin therapy appears to have reduced the number and amount of apo E-containing HDL particles. Our finding that insulin treatment also had disparate effects on both the protein and lipid composition of the HDL subfractions is consistent with evidence that the HDL2 and HDL3 subfractions are subject to differing regulatory influences. Since adipose tissue LPL and HL are both insulin-sensitive enzyme systems, it is likely that they also made some contribution to the changes we observed in HDL composition.

In this collaborative study, neutral lipid measurements were performed on frozen, previously isolated lipoprotein fractions in Helsinki (excluding IDL) and HDL subfractions precipitated from frozen whole plasma in Chicago. Certain discrepancies exist in the actual values obtained for some of the lipoprotein TG and cholesterol values and in the HDL subfractions between our data and that previously published. The directional changes, however, are fully consistent in the two sets of data and do not alter our major conclusions.

Despite the anti-atherogenic profiles achieved in their whole plasma and in some lipoprotein lipid with vigorous insulin therapy in this cohort of Finnish NIDDM patients, a number of potentially significant abnormalities in apoprotein and surface and core lipid composition persisted, which may be helpful for understanding the predisposition of diabetic populations to accelerated atherogenesis. The disturbances we have found may alter gradients normally present on NIDDM lipoproteins, which influence the directional fluxes of their constituents in transfer reactions. In addition, these alterations may not only impair the normal movement of FC between lipoproteins and cells, but they may also alter the sites of catabolism of the particles themselves. If these data can be extrapolated to NIDDM patients elsewhere, they suggest that the persist-
ing compositional derangements of the type we find after treatment may have far ranging clinical consequences in NIDDM patients, in spite of good clinical control.

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pared with low density lipoproteins. Biochemistry 1978; 17:1440–1447


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