A low saturated fat, low cholesterol diet decreases plasma CETP activity and pre $\beta$-HDL formation but does not affect cellular cholesterol efflux to plasma from type 1 diabetic patients
ABSTRACT

The aim of this study was to evaluate the effect of a low saturated fat, low cholesterol diet on plasma lipoproteins, pre-β-high density lipoprotein (HDL) formation, lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities as well as on the ability of plasma to stimulate cellular cholesterol efflux. Twelve male type 1 diabetic patients with plasma cholesterol >5.0 mmol/L were studied while consuming their usual diet and after 6 weeks of a low fat, low cholesterol diet. Pre-β-HDL formation was measured using crossed immuno-electrophoresis. Plasma LCAT, CETP and PLTP activities were assayed by exogenous substrate methods. The ability of plasma to promote cellular cholesterol efflux out of Fu5AH rat hepatoma cells and out of human skin fibroblasts was also determined. Saturated fat intake was lowered (P = 0.001), due to replacement with carbohydrates, while mono- and polyunsaturated fat intake remained unchanged. Cholesterol intake decreased as well (P = 0.003). The changes in plasma total cholesterol, very low and low density lipoprotein (VLDL+LDL) cholesterol, HDL cholesterol, HDL phospholipids, apolipoprotein (apo) A-I, plasma LCAT activity and PLTP activity were not significant. Plasma CETP activity (P = 0.008) and pre-β-HDL formation (P = 0.008) decreased. The ability of plasma to promote cholesterol efflux out of fibroblasts and Fu5AH cells remained unchanged. Reduction in dietary saturated fat and cholesterol intake does not adversely affect cellular cholesterol efflux to plasma from type 1 diabetic patients, despite a drop in pre-β-HDL formation.

Key words: cellular cholesterol efflux, diet intervention, high density lipoproteins, lipid transfer proteins, Type 1 diabetes mellitus
INTRODUCTION

Coronary heart disease risk is ameliorated by diet intervention aimed to lower plasma cholesterol in non-diabetic populations [1]. Cardiovascular risk is increased in Type 1 diabetes mellitus [2]. Nutrition therapy is an accepted component of diabetes management, and recommendations include the use of a diet low in saturated fat [3]. In case of a high low density lipoprotein (LDL) cholesterol, a reduction in dietary cholesterol intake is thought to be beneficial [3]. However, replacement of saturated fat by carbohydrates not only lowers plasma LDL cholesterol, but may decrease plasma high density lipoprotein (HDL) cholesterol levels as well [3-6]. A reduction in cholesterol intake also decreases plasma HDL cholesterol in non-diabetic [7], as well as in Type 1 diabetic individuals [8].

One of the mechanisms to explain the cardioprotective function of HDL is its role in reverse cholesterol transport (RCT), the process whereby cholesterol is transported from peripheral cells to the liver for metabolism and excretion in the bile [9,10]. The uptake of cell-derived cholesterol by extracellular acceptors including lipid-poor pre $\beta$-HDL particles is considered to represent an important initial step in RCT [9]. Among other factors, the cholesterol esterifying plasma protein, lecithin:cholesterol acyltransferase (LCAT), and the lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phosholipid transfer protein (PLTP) play key roles in HDL metabolism [9,11,12]. Both PLTP and CETP may be involved in pre $\beta$-HDL generation [12]. Of interest, a low cholesterol diet decreases pre $\beta$-HDL in conjunction with a drop in plasma CETP in hypercholesterolaemic subjects [13], raising the possibility that diet intervention may affect RCT.

Several reports indeed suggest that the ability of plasma to promote cellular cholesterol efflux can be affected by diet modification. Cholesterol efflux out of Fu5AH hepatoma model cells is stimulated when cholesterol intake is increased in subjects initially consuming a diet low in saturated fat (NCEP1 diet) [5], whereas the ability of HDL$_3$ particles to promote cholesterol removal from cultured human skin fibroblasts is increased after a diet enriched in monounsaturated fat [14]. On the other hand, diets enriched in saturated fat, monounsaturated fat or n-6 polyunsaturated fat do not affect cholesterol efflux out of mouse L-cell fibroblasts to plasma from African green monkeys [15].

The aim of the present study was to test the effect of a low saturated fat, low cholesterol diet on pre $\beta$-HDL formation and on the ability of plasma to promote cellular cholesterol efflux in moderately hypercholesterolaemic Type 1 diabetic patients.
PATIENTS AND METHODS

The study was approved by the local medical ethics committee, and written informed consent was obtained from all participants. Twelve non-smoking male Type 1 diabetic patients (ketosis-prone diabetes; age at onset <35 years; body mass index (BMI) <30 kg/m²; fasting plasma cholesterol ranging from 5.0 to 8.0 mmol/L and triglycerides (TG) <2.5 mmol/L) were selected from the outpatient clinic. Clinically manifest cardiovascular disease, hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure >85 mmHg), diabetic nephropathy, thyroid dysfunction, liver function abnormalities and alcohol use >3 beverages/day were exclusion criteria. If lipid-lowering drugs were taken, this medication was stopped at least 6 weeks before the start of the study. The participants only used insulin during the study. Age of the participants was 47±13 years and diabetes duration was 26±15 years.

Participants were studied while using their habitual diet and after 6 weeks of diet intervention. They were instructed not to change their daily physical activity during the study. A complete dietary history was obtained at each occasion, using the recall technique which covered a one-week period as described [16]. The composition of the diets was analyzed with a computer assisted Nutrient Database (Nevo Foundation, year table 1996).

Dietary advice during the intervention period was given according to the national recommendations of the Dutch Diabetes Federation for dietary treatment in diabetes, which closely resembles the ADA guidelines [3]. The diet consisted of a low fat intake (≤30 Energy%) which was achieved by a reduction in saturated fat to ≤10 Energy%. Monounsaturated fat intake was ≤12 Energy% and polyunsaturated fat intake was ≤8 Energy%. In addition, the participants were instructed to reduce cholesterol intake to <150 mg/day. For each patient it was advised to use an isocaloric diet during the study.

Laboratory methods

Blood was collected after an overnight fast before the morning insulin injections into ethylene diaminetetraacetic acid (EDTA)-containing tubes (1.5 mg/ml) and placed on ice immediately. Plasma was obtained within 30 min by centrifugation at 3000 rpm for 15 min at 4°C. Total cholesterol, TG and choline-containing phospholipids were measured enzymatically. HDL was separated in fresh plasma by precipitation of apolipoprotein (apo) B-containing lipoproteins using sodium phosphotungstate/MgCl₂. Very low and low density lipoprotein (VLDL+LDL) cholesterol was calculated as the difference between plasma total cholesterol and HDL cholesterol. Apo A-I and B were assayed by immunoturbidimetry using kits from Serapak, Bayer, Leverkusen, Germany.
Dietary influence on pre β-HDL and cholesterol efflux in type 1 diabetes

Plasma total cholesterol, TG and HDL cholesterol were assayed shortly after blood collection. For further measurements samples were kept frozen at -80˚C until analysis.

Plasma LCAT activity was measured using excess exogenous substrate containing [3H]cholesterol as described [17]. Plasma CETP activity levels were determined after precipitation of endogenous VLDL+LDL with sodium phosphotungstate/MgCl₂ [17,18]. The isotope assay measures the transfer of radioactive cholesteryl ester between excess exogenous [1,14C-oleate]-cholesteryl ester-labelled LDL and an excess of pooled normal HDL, while LCAT is inhibited with dithiobis-2-nitrobenzoic acid. Plasma CETP activity was calculated as the bi-directional transfer between labelled LDL and HDL [19], and reflects the amount of active CETP in plasma. Plasma PLTP activity was assayed using a phospholipid vesicles-HDL system, as previously described [17,18]. Small plasma samples (1 μl) were incubated with [3H]-phosphatidylcholine-labelled phosphatidylcholine vesicles and excess pooled normal HDL for 45 min at 37˚C. The vesicles were then precipitated using a mixture of NaCl, MgCl₂ and heparin (final concentrations: 230 mmol/L, 92 mmol/L and 200 IU/ml, respectively). The PLTP activity levels vary linearly with the amount of plasma added to the incubation system. This method is specific for PLTP activity and the phospholipid transfer promoting properties of CETP do not interfere with the assay [18].

Plasma pre β-HDL formation was measured using crossed immuno-electrophoresis as described [20], except that anti-human apo A-I and an incubation time of 24 h was used. In short, plasma samples were thawed while kept on ice and 0.9 μmol/L Pefabloc SC (Boehringer-Roche) and 1.8 μg/L Trasylol (Bayer 10000 IU/ml) were added to inhibit proteolysis (both final concentrations). Subsequently, an LCAT inhibitor was added (iodoacetate, final concentration 1.0 mmol/L) and the samples were incubated at 37˚C for 24 h to measure the formation of pre β-HDL. The crossed immuno-electrophoresis consisted of agarose electrophoresis in the first dimension for separation of lipoproteins with pre-β and α mobility. Antigen migration from the first agarose gel into the second agarose gel, containing goat anti-human apo A-I antiserum (3%, volume/volume), was used to quantitatively precipitate apo A-I. The antiserum was monospecific for human apo A-I [21]. Lipoprotein electrophoresis was carried out in 1% (weight/volume) agarose gels in barbital buffer (50 mmol/L, pH 8.6) and run in an LKB 2117 system (4˚C for 2 h, 250 V). Plasma was applied at 3 μl/well. The track of the first agarose gel was excised and annealed with melted agarose to a gel containing 3% (volume/volume) goat anti-human apo A-I anti-serum that was cast on GelBond film (Pharmacia). The plate was run in an LKB 2117 system (4˚C for 20 h, 50 V) in barbital buffer. Unreacted antibody was removed by extensive washing in phosphate buffered
saline. The gel was stained with Coomassie brilliant blue R250 and subsequently dried. Areas under the pre-β-HDL and α-HDL peaks were scanned and calculated using Scion software. Pre-β-HDL was expressed in apo A-I concentration (mg/L).

Cholesterol efflux to plasma was determined using both Fu5AH cells (generous gift from Dr N. Fournier, Chatenay-Malabry, France) and human fibroblasts as cholesterol donor. Fibroblasts were obtained from normolipidaemic control persons by explant culture from a 3 mm punch biopsy at a 1 mm skin thickness and were cultured (until passage 5-15) in 24 wells culture plates to full confluency, essentially as described earlier [22]. Both cell types were treated identically with respect to the efflux measurement procedure. The cells were cultured in Dulbecco’s modified Eagle’s solution (DMEM) supplemented with 10% volume/volume fetal calf serum (FCS). After washing them with DMEM, they were loaded with [³H]-cholesterol (38 Ci/mmol) during 24 h in the presence of added unlabelled cholesterol (30 μg/ml) in order to induce ATP-binding cassette transporter A1 (ABCA1) in the fibroblasts [23,24]. Under these experimental conditions ABCA1 expression was confirmed at the protein level by Western blotting analysis using mouse monoclonal anti ABCA1 antibodies kindly provided by Dr M. Hayden, Vancouver, Canada (data not shown). [³H]-cholesterol and unlabelled cholesterol was solubilized in ethanol and diluted into the efflux medium. The cells were then washed 5 times with phosphate buffered saline (PBS)/bovine serum albumin (BSA) 0.2% (weight/volume). The efflux assay was subsequently started by adding individual patient’s plasma to the efflux medium to reach a dilution of 1% in the final incubation. Incubations were carried out in triplicate. After 4 h incubation at 37°C the medium was collected and centrifuged. Subsequently, [³H]-cholesterol was quantified by liquid scintillation counting. Total cellular [³H]-cholesterol was determined after extraction of the cells with 2-propanol. The percentage efflux was calculated by dividing the radioactive counts in the efflux medium by the sum of the counts in the medium and the cell extract. All values were corrected for radioactivity appearing in the culture medium in the absence of plasma. A 1% plasma dilution and an incubation time of 4 h were chosen because under these circumstances cellular cholesterol efflux was in the linear part of the dose-response curve for both cell systems (data not shown). To be able to normalize results between series of experiments and to correct for day-to-day variation, efflux to 50 μg protein/mL HDL (Calbiochem, San Diego, CA, USA) was determined in triplicate. This HDL preparation was from one batch and was stored at 4°C following the instructions of the manufacturer.

Glycated haemoglobin (HbA₁c) was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands, normal range 4.6 - 6.1%).
**Statistical Analysis**

Data are expressed in mean ± standard deviation (SD). Changes in variables are expressed in mean (95% confidence intervals (CI)) and are evaluated by paired t-tests. A two-sided $P$-value <0.05 was considered significant. Assuming that the SD of cellular cholesterol efflux is 8% of the group mean [25] and that the correlation coefficient between repeated measurements is as low as 0.50, it can be calculated that 12 subjects are required to demonstrate a relative change in cholesterol efflux of 8% with a power of 0.85 at a two-sided $P$-value <0.05.

**Results**

BMI was 26.0±3.2 kg/m$^2$ at baseline and 26.1±3.2 kg/m$^2$ at follow-up ($P = 0.70$). HbA$_1c$ also remained unchanged (7.6±1.0% at baseline and 7.4±1.1% at follow-up, $P = 0.19$). Total energy intake decreased slightly from 8.75±1.60 to 8.32±1.62 Mjoules/day after diet intervention ($P = 0.03$). A reduction in total fat intake from 36±6 to 29±2 Energy% ($P = 0.001$) was achieved by lowering saturated fat intake (14±3 Energy% at baseline and 8±1 Energy% at follow-up, $P = 0.001$). Carbohydrate intake increased from 43±5 Energy% at baseline to 50±3 Energy% at follow-up ($P = 0.003$). Monounsaturated fat intake (12±3 Energy% at baseline and 11±1 Energy% at follow-up, $P = 0.15$) and polyunsaturated fat intake remained unchanged (8±2 Energy% at baseline and 8±1 Energy% at follow-up, $P = 0.31$), as did protein intake ($P = 0.37$, data not shown). Dietary cholesterol decreased from 188±47 to 127±35 mg/day ($P = 0.003$).

Plasmacholesterol,VLDL+LDL cholesterol,HDL cholesterol and phospholipids, plasma TG, as well as plasma apo A-I and apo B levels did not change significantly in response to dietary intervention (Table 1). Pre β-HDL formation decreased by 24% (95% CI 1 to 47%) (Table 1). Plasma CETP activity decreased by 12% (95% CI 4 to 22%), whereas the changes in plasma LCAT and PLTP activity were not significant (Table 1).

Cholesterol efflux out of Fu5AH cells was 9.0±1.1% per 4 h at baseline and 8.8±1.1% after diet intervention (relative change –1.7% (95% CI –6.5 to 2.9%); $P = 0.39$; Fig. 1A). Cholesterol efflux out of human fibroblasts was also unaffected (16.0±1.2% per 4 h before and 15.5±1.1% after diet intervention; relative change –2.9% (95% CI –7.7 to 2.0%); $P = 0.20$; Fig. 1B).
Table 1. Plasma (apo)lipoproteins, pre β-HDL formation, plasma lecithin: cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) activities in response to low saturated fat, low cholesterol (LSF, LC) diet in 12 Type 1 diabetic patients.

<table>
<thead>
<tr>
<th></th>
<th>Usual diet</th>
<th>LSF, LC diet</th>
<th>Change (95% confidence intervals)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma total cholesterol (mmol/L)</td>
<td>6.06±1.03</td>
<td>5.64±1.11</td>
<td>-0.41 (-0.96 to 0.13)</td>
<td>0.12</td>
</tr>
<tr>
<td>VLDL+LDL cholesterol (mmol/L)</td>
<td>4.99±1.05</td>
<td>4.66±1.10</td>
<td>-0.33 (-0.84 to 0.18)</td>
<td>0.19</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.07±0.21</td>
<td>0.98±0.13</td>
<td>-0.09 (-0.20 to 0.03)</td>
<td>0.12</td>
</tr>
<tr>
<td>HDL phospholipids (mmol/L)</td>
<td>1.35±0.21</td>
<td>1.28±0.20</td>
<td>-0.07 (-0.17 to 0.03)</td>
<td>0.17</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>1.12±0.47</td>
<td>1.08±0.39</td>
<td>-0.03 (-0.15 to 0.08)</td>
<td>0.54</td>
</tr>
<tr>
<td>Apolipoprotein A-I (g/L)</td>
<td>1.42±0.21</td>
<td>1.31±0.15</td>
<td>-0.11 (-0.26 to 0.05)</td>
<td>0.16</td>
</tr>
<tr>
<td>Apolipoprotein B (g/L)</td>
<td>0.97±0.25</td>
<td>0.98±0.28</td>
<td>0.01 (-0.11 to 0.13)</td>
<td>0.86</td>
</tr>
<tr>
<td>Pre β-HDL formation (apo A-I, g/L)</td>
<td>0.20±0.07</td>
<td>0.15±0.07</td>
<td>-0.05 (-0.09 to -0.02)</td>
<td>0.008</td>
</tr>
<tr>
<td>Plasma LCAT activity (AU)</td>
<td>93±15</td>
<td>89±13</td>
<td>-5 (-13 to 4)</td>
<td>0.26</td>
</tr>
<tr>
<td>Plasma CETP activity (AU)</td>
<td>110±22</td>
<td>96±22</td>
<td>-14 (-24 to -4)</td>
<td>0.008</td>
</tr>
<tr>
<td>Plasma PLTP activity (AU)</td>
<td>127±34</td>
<td>122±33</td>
<td>-5 (-13 to 4)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Data in mean ± SD and in mean (95% confidence intervals).

Figure 1. Cholesterol efflux to plasma from Type 1 diabetic patients before (open bars) and after (hatched bars) a low saturated fat, low cholesterol (LSF, LC) diet. A: Fu5AH hepatoma cells; B: human skin fibroblasts. Data in mean ± SEM.
DISCUSSION

This study demonstrates for the first time that plasma pre-β-HDL formation is decreased in response to a low saturated fat, low cholesterol diet in Type 1 diabetic patients. Notably, cholesterol efflux out of Fu5AH cells as well as human skin fibroblasts to plasma remained essentially unchanged after dietary intervention. Thus it appears that neither the drop in pre-β-HDL nor the (statistically insignificant) decrease in HDL cholesterol, HDL phospholipids and plasma apo A-I after this diet modification result in a relevant impairment of the reverse cholesterol transport pathway, as far as the ability of plasma to stimulate cell-derived cholesterol removal is concerned.

Cellular cholesterol removal to extracellular acceptors is governed by several mechanisms such as aqueous diffusion, scavenger receptor class B type I (SR-BI)-, ABCA1- and the recently described ABCG1- and ABCG4- mediated efflux [26-28]. Fu5AH cells express SR-BI but hardly any ABCA1 [26], and cholesterol efflux out of this cell system to human plasma is positively correlated with HDL cholesterol, HDL phospholipids and total plasma apo A-I [26,27,29]. Cultured human skin fibroblasts express ABCA1 but little CLA1, the human analogue of SR-BI [22-24]. Cholesterol efflux from these cells is at least partly dependent on lipid-poor acceptors, such as pre-β-HDL [30]. Besides, PLTP may directly promote cholesterol efflux out of fibroblasts [31]. ABCG1 and ABCG4 mediate cholesterol efflux to mature HDL [28], but it is not yet known whether and to what extent these transporters are expressed in Fu5AH cells and human skin fibroblasts. The present observation showing that a low saturated fat, low cholesterol diet does not affect cholesterol efflux with either cell system could indicate that the magnitude of response in HDL parameters is not large enough to diminish the ability of plasma to stimulate cellular cholesterol removal. Further, it could be proposed that diet modification alters HDL lipid composition or other HDL subfractions, possibly counteracting effects of changes in pre-β-HDL on cellular cholesterol efflux. As yet, an increased cholesterol efflux has only been observed when Fu5AH cells are incubated with diluted plasma obtained after a polyunsaturated fat-enriched diet [32] and when fibroblasts are incubated with HDL₃ after a monounsaturated fat-enriched diet [14].

Plasma CETP levels are decreased in response to a low fat diet [6] and increased after cholesterol feeding [7,33], although this has not been found in all studies [13,34]. In the current study, we measured CETP activity using an isotopic assay that reflects the amount of active CETP, and the CETP activity so measured closely correlates with its concentration in plasma [35]. The presently documented drop in plasma CETP activity thus agrees with these earlier findings [6,7,33], and may be due to an inhibitory effect of this diet modification on CETP synthesis as demonstrated in animal experiments.
The lower plasma CETP activity may explain in part the decrease in pre β-HDL formation, as suggested before [12,13]. Besides CETP, other factors including PLTP activity and the HDL triglyceride content are important in pre β-HDL generation [37]. It is of interest that our study shows that a decrease in pre β-HDL formation can occur in the absence of a change in plasma PLTP activity and plasma triglycerides. In addition, a decrease in plasma CETP may be relevant for cardiovascular risk assessment, because high plasma CETP levels could predict the risk of future coronary artery disease [38].

The present study should be considered preliminary because of the relatively small number of participants and the lack of a controlled, randomized study design. It can be argued that the absence of a significant decrease in plasma total cholesterol, VLDL+LDL and HDL cholesterol in response to diet modification is attributable to insufficient power. Nonetheless, the currently observed magnitude of changes in lipoprotein cholesterol is within the expected range when extrapolating results from comparable dietary interventions in non-diabetic subjects [4]. However, the average relative decreases in efflux from Fu5AH cells and human fibroblasts only amounted to 1.7 and 2.9%, with a lower limit of their 95% CI of −6.5% and −7.7%, respectively. This makes a change in cellular cholesterol efflux of more than 8% with either cell system very unlikely.

In conclusion, the commonly recommended low saturated fat, low cholesterol diet does not appear to adversely affect cellular cholesterol efflux to plasma from moderately hypercholesterolaemic Type 1 diabetic patients. These observations suggest that changes in HDL metabolism in response to this diet modification do not to an important extent alter the effect of plasma on this early step in reverse cholesterol transport.

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Dietary influence on pre β-HDL and cholesterol efflux in type 1 diabetes


