

**INCREASED CHOLESTEROL EFFLUX FROM
CULTURED FIBROBLASTS TO PLASMA
FROM HYPERTRIGLYCERIDEMIC TYPE 2
DIABETIC PATIENTS: ROLES OF PRE β -HDL,
PHOSPHOLIPID TRANSFER PROTEIN AND
CHOLESTEROL ESTERIFICATION**

R. de Vries¹, A.K. Groen², F.G. Perton¹, G.M. Dallinga-Thie³, M.J.A. van Wijland²,
L.D. Dikkeschei³, B.H.R. Wolffenbuttel¹, A. van Tol^{1,4}, R.P.F. Dullaart^{1,*}

¹ Department of Endocrinology, University Medical Centre Groningen,
University of Groningen, The Netherlands

² Department of Experimental Hepatology, Academic Medical Centre, Amsterdam, The Netherlands

³ Laboratory of Vascular Medicine, ⁴ Department of Cell Biology & Genetics,
Erasmus University Medical Centre, Rotterdam, The Netherlands

⁵ Department of Clinical Chemistry, Isala Clinics, location Weezenlanden, Zwolle, The Netherlands

ABSTRACT

We tested whether hypertriglyceridemia associated with type 2 diabetes mellitus is accompanied by alterations in pre β -HDL, which are considered to be initial acceptors of cell-derived cholesterol, and by changes in the ability of plasma to promote cellular cholesterol efflux. In 28 hypertriglyceridemic and 56 normotriglyceridemic type 2 diabetic patients, and in 56 control subjects, we determined plasma lipids, HDL cholesterol and phospholipids, plasma pre β -HDL and pre β -HDL formation, phospholipid transfer protein (PLTP) activity, plasma cholesterol esterification (EST) and cholesteryl ester transfer (CET) and the ability of plasma to stimulate cholesterol efflux out of cultured human fibroblasts. HDL cholesterol and HDL phospholipids were lower, whereas plasma PLTP activity, EST and CET were higher in hypertriglyceridemic diabetic patients than in the other groups. Pre β -HDL levels and pre β -HDL formation were unaltered, although the relative amount of pre β -HDL (expressed as % of total plasma apo A-I) was increased in hypertriglyceridemic diabetic patients. Cellular cholesterol efflux to plasma from hypertriglyceridemic diabetic patients was increased compared to efflux to normotriglyceridemic diabetic and control plasma, but efflux to normotriglyceridemic diabetic and control plasma did not differ. Multiple linear regression analysis demonstrated that cellular cholesterol efflux to plasma was positively and independently related to pre β -HDL formation, PLTP activity and EST (multiple $r=0.48$), but not to the diabetic state. In conclusion, cholesterol efflux from fibroblasts to normotriglyceridemic diabetic plasma is unchanged. Efflux to hypertriglyceridemic diabetic plasma is enhanced, in association with increased plasma PLTP activity and cholesterol esterification. Unaltered pre β -HDL formation in diabetic hypertriglyceridemia, despite low apo A-I, could contribute to maintenance of cholesterol efflux.

Keywords: ATP-binding cassette transporter type 1, cholesterol efflux, human fibroblasts, pre β -HDL, reverse cholesterol transport, triglycerides, type 2 diabetes mellitus

INTRODUCTION

Efflux of cholesterol from peripheral cells to extracellular acceptors is considered to be an important early step in the anti-atherogenic reverse cholesterol transport (RCT) system, whereby excess cholesterol is transported from the arterial wall back to the liver for metabolism and excretion in the bile [1-3]. A number of pathways, including aqueous diffusion, transport of cholesterol via the ATP-binding cassette transporters (ABCA1 and ABCG1) and scavenger receptor class B type 1 (SR-BI)-mediated efflux are supposed to play a role in cellular cholesterol removal [4-8]. The importance of the ABCA1 system for protection against the development of atherosclerosis is underscored by the observation that cardiovascular risk is elevated in subjects with genetic defects in ABCA1 [9,10]. Small lipid poor and lipid free apolipoprotein (apo) A-I-containing particles, designated pre β -HDL, are recognized to be initial acceptors of cell-derived cholesterol via the ABCA1 system [11], while larger sized HDL may promote cellular cholesterol efflux via ABCG1 and SR-BI [12-14].

Several intravascular mechanisms are involved in HDL metabolism, and may affect the ability of plasma to stimulate cellular cholesterol efflux. Phospholipid transfer protein (PLTP) facilitates transfer of phospholipids towards HDL during lipolysis of triglyceride-rich lipoproteins, and is able to convert HDL into larger and smaller particles, thereby giving rise to pre β -HDL [15,16]. Esterification of free cholesterol by lecithin:cholesterol acyltransferase (LCAT) contributes to HDL maturation, increases HDL particle size and decreases pre β -HDL levels [1]. Subsequently, cholesteryl ester transfer protein (CETP) action enables transfer of cholesteryl ester from HDL to lipoproteins of lower density, making HDL particles to become cholesteryl ester depleted and triglyceride enriched [17-23]. Such particles are more suitable for hydrolysis by HL. Combined CETP and hepatic lipase (HL) activity may also contribute to the generation of pre β -HDL [19].

High plasma triglycerides and low HDL cholesterol are well known features of dyslipidemia in type 2 diabetes mellitus, and it is increasingly appreciated that high plasma PLTP activity, elevated plasma cholesterol esterification as well as cholesteryl ester transfer contribute to abnormalities in HDL metabolism in diabetes-associated hypertriglyceridemia as well [17,20-23]. Some studies that were carried out mainly in non-diabetic subjects have suggested that pre β -HDL, either expressed as its concentration or expressed as percentage of total plasma apo A-I, may be increased in hypertriglyceridemia [24-26], but little is known about pre β -HDL in type 2 diabetic patients with or without hypertriglyceridemia.

Using Fu5AH rat hepatoma cells, which have a high expression of SR-BI but

lack functional ABCA1, it has been documented that the ability of type 2 diabetic plasma to stimulate cellular cholesterol efflux is decreased or unaltered [27,28]. An early study with cultured human fibroblasts, a cell system which abundantly expresses ABCA1 [29], has shown that the capacity of plasma from severely hypertriglyceridemic type 2 diabetic patients to promote cellular cholesterol efflux is diminished [30], but it is uncertain whether glycation of HDL could impair its cellular cholesterol efflux-stimulating ability [31,32]. On the other hand, the ability of plasma from non-diabetic hypertriglyceridemic subjects to promote ABCA1-mediated cholesterol efflux out of J774 macrophages [26] and of type 1 diabetic patients to stimulate cholesterol efflux out of both Fu5AH cells and human fibroblasts is enhanced [29]. Thus, evidence concerning abnormalities in the ability of diabetic plasma to stimulate cell-derived cholesterol removal is limited, and it is unclear at present whether this early step in the RCT process is affected by hypertriglyceridemia.

In the present study we questioned whether, in type 2 diabetes, hypertriglyceridemia is not only accompanied by decreased HDL but also by alterations in pre β -HDL, and in the ability of plasma to stimulate cellular cholesterol efflux. Therefore, we compared the ability of plasma from hypertriglyceridemic and normotriglyceridemic type 2 diabetic patients and control subjects to promote cholesterol efflux out of human cultured fibroblasts. Moreover, we assessed the relationships of cellular cholesterol efflux with plasma pre β -HDL, HDL cholesterol and phospholipids, PLTP activity, cholesterol esterification and cholesteryl ester transfer.

RESEARCH DESIGN, SUBJECTS AND METHODS

The medical ethics committee of the University Medical Center Groningen approved the study protocol, and written informed consent was obtained from each participant. Three groups of participants were recruited: type 2 diabetic patients with plasma triglycerides > 2.0 mmol/l, type 2 diabetic patients with plasma triglycerides ≤ 2.0 mmol/l and non-diabetic control subjects with plasma triglycerides ≤ 2.0 mmol/l. Type 2 diabetes mellitus was previously diagnosed using blood glucose cut-off values as defined by the WHO, and patients were treated with diet alone or in combination with oral glucose lowering agents. Insulin treatment was an exclusion criterion. All participants were aged > 18 years. Current or previous smoking and the use of lipid lowering drugs were exclusion criteria. Participants did not have a history of cardiovascular disease or proliferative diabetic retinopathy. None of the participants had liver function abnormalities or thyroid dysfunction. Subjects with micro- or macroalbuminuria

defined as urinary albumin > 20 mg/l were also excluded. Maximal alcohol intake was 3 beverages per day. All participants were evaluated after an overnight fast. BMI was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured after 15 min rest at the left arm in sitting position using a sphygmomanometer. Mean arterial pressure (MAP) was calculated as $1/3 * \text{systolic blood pressure} + 2/3 * \text{diastolic blood pressure}$.

Fifty-six normotriglyceridemic control subjects, 56 normotriglyceridemic type 2 diabetic patients and 28 hypertriglyceridemic diabetic patients participated in the study. Gender distribution ($p = 0.79$) and age ($p = 0.16$) were not significantly different between the 3 groups. Sixteen normolipidemic control women, 22 normotriglyceridemic diabetic women and 9 hypertriglyceridemic diabetic women were postmenopausal ($p = 0.17$). Of the postmenopausal women, only 2 in the control group, 1 in the normotriglyceridemic diabetic group and none in the hypertriglyceridemic diabetic group used hormonal replacement therapy. In addition to diet, 77 % of the normotriglyceridemic diabetic patients used one or more oral glucose lowering drugs (sulfonylurea: 33%, biguanides: 23% or the combination of these two: 44%). Seventy nine % of the hypertriglyceridemic patients used one or more oral glucose lowering drugs (sulfonylurea: 27%, biguanides: 45% or the combination: 27%). Four normotriglyceridemic diabetic patients and 3 hypertriglyceridemic patients used a thiazolidinedione derivative and 1 patient in each group used acarbose, an α -glucosidase inhibitor. Forty five % of the normotriglyceridemic and 43 % of the hypertriglyceridemic diabetic patients used an antihypertensive drug. Seventeen normotriglyceridemic patients used an ACE-inhibitor or angiotensin-2 antagonist alone or in combination with other antihypertensive medication, 3 patients used a diuretic and 4 patients used a beta blocking agent. Eleven hypertriglyceridemic patients used an ACE-inhibitor or angiotensin-2 antagonist alone or in combination with other antihypertensive drugs. Three patients used a β -blocking agent, of which one in combination with a calcium antagonist. None of the normolipidemic control subjects used antihypertensive drugs.

Laboratory measurements

EDTA-containing plasma samples for measurement of lipids, lipoproteins, pre β -HDL, PLTP activity, plasmas cholesterol esterification (EST) and cholesteryl ester transfer (CET) as well as for cellular cholesterol efflux studies were obtained by centrifugation at 1400 g for 15 min at 4 °C. Directly thereafter, samples were frozen at -80°C until analysis.

The HDL fraction was separated from plasma by precipitation of apolipoprotein B-containing lipoproteins with polyethylene glycol-6000. Cholesterol in plasma and

the HDL fraction and plasma triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11876023 and 11875540 respectively, Roche Diagnostics GmbH, Mannheim, Germany). In the HDL fraction, choline-containing phospholipids were measured as described [29]. Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total plasma cholesterol. Apolipoprotein (apo) A-I and B were assayed by immunoturbidimetry (Roche/Cobas Integra Tina-quant cat no. 03032566 and 033032574, respectively, Roche Diagnostics).

Plasma pre β -HDL was measured as described [33], except that anti-human apo A-I was used. In short, plasma samples were thawed while kept on ice and 0.9 $\mu\text{mol/l}$ Pefabloc SC (Boehringer-Roche, Penzberg, Germany) and 1.8 $\mu\text{g/l}$ Trasylol (Bayer, Mijdrecht, the Netherlands) were added to inhibit proteolysis (both final concentrations). 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 (0.66%, vol/vol; Midland Bioproducts Corporation, Boone, Iowa, USA, cat no 71101), was used to quantitatively precipitate apo A-I. The antiserum was monospecific for human apo A-I using an immunodiffusion assay. Lipoprotein electrophoresis was carried out in 1% (weight/vol) agarose gels in Tris (80 mmol/l) - tricine (24 mmol/l) buffer, 5% (vol/vol) polyethylene glycol 300 (pH 8.6) and run in an LKB 2117 system (4°C for 3 h, 210 V). Plasma was applied at 3 $\mu\text{l/well}$. The track of the first agarose gel was excised and annealed with melted agarose to a gel containing 0.66% (vol/vol) goat anti-human apo A-I anti-serum (Midland Bioproducts corporation, Boone Iowa) and 0.01% m/V tween20, that was cast on GelBond film (Amersham, Uppsala, Sweden). The plate was run in an LKB 2117 system (4°C for 20 h, 50 V) in Tris-tricine buffer. Unreacted antibody was removed by extensive washing in saline. The gel was stained with Coomassie brilliant blue R250 and subsequently dried. The gels were scanned with a HP scanjet 5470c. Areas under the pre β -HDL and α -HDL peaks were calculated using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from <ftp://maxrad6.uthscsa.edu>). The pre β -HDL area was expressed as the percentage of the sum of apo A-I in the pre β -HDL and the α -HDL areas. Pre β -HDL formation, i.e. the ability of plasma to generate pre β -HDL, was assessed using the same procedure, but after 24 h incubation of plasma at 37°C under conditions of LCAT inhibition. To this end, iodoacetate (final concentration 1.0 mmol/l) was added directly after thawing the samples. Pre β -HDL and pre β -HDL formation were calculated using the total plasma apo A-I concentration and were expressed in apo A-I, g/l. Pre β -HDL and pre β -HDL formation were also expressed in relative terms (% of apo A-I), to

take account for changes in apo A-I associated with diabetes or hypertriglyceridemia [34]. Plasma PLTP activity was assayed using a phospholipid vesicles-HDL system, as previously described [35], using [¹⁴C]-labeled dipalmitoyl phosphatidylcholine. In short, small plasma samples (1 μl) were incubated with [¹⁴C]-phosphatidylcholine-labeled phosphatidylcholine vesicles and excess freshly isolated 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 activity varies linearly with the amount of plasma added to the incubation system. This method is specific for PLTP activity and the phospholipid transfer promoting property of CETP does not interfere with the assay [35]. Plasma PLTP activity is related to the activity in human reference pool plasma and is expressed in arbitrary units (AU; 100 AU corresponds to 13.6 μmol phosphatidylcholine transferred. ml⁻¹. h⁻¹).

Plasma cholesteryl ester transfer (CET) was determined as described previously [36]. Briefly, [³H]cholesterol was equilibrated for 24 h at 4 °C with plasma cholesterol followed by incubation of plasma at 37 °C. Thereafter, apo B-containing lipoproteins were precipitated and the labeled cholesteryl esters were separated from labeled unesterified cholesterol on silica columns. Plasma cholesterol esterification (EST) was measured as formation of cholesteryl esters after addition of [³H]cholesterol to plasma as described [36]. Equilibration of added [³H] cholesterol was obtained after incubation at 4 °C for 24 h. Subsequently, the plasma was incubated at 37 °C.

Cholesterol efflux to plasma was determined using human fibroblasts. Fibroblasts were obtained from a normolipidemic control person 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 [9,29]. The cells were cultured in DMEM supplemented with 10% vol/vol FCS. After washing them with DMEM, they were loaded with [³H]-cholesterol (0.5 μCi/ml) during 24 h in the presence of added unlabelled cholesterol (30 μg/ml) in order to induce ABCA-1 in the fibroblasts [29]. [³H]-cholesterol and unlabelled cholesterol were solubilized in ethanol and diluted into the efflux medium. After cholesterol loading, the cells were washed 3 times with PBS/BSA 0.2% (weight/vol). The efflux assay was started by adding the plasma diluted to 1% in efflux medium. 1.25 U/ml heparin was added to prevent clotting. Independent control experiments demonstrated that this addition of heparin did not influence cholesterol efflux rates (7.3 ± 1.7 %/4 h without heparin vs 7.4 ± 1.6 %/4 h with heparin (n = 8), NS). After 4 h incubation at 37°C the medium was collected and centrifuged. Thereafter, [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 plasma samples were analyzed in duplicate and values were corrected for radioactivity appearing in the culture medium in the absence of plasma. To be able to normalize results between series of experiments and to correct for day-to-day variation, efflux to a human plasma pool was determined in quadruplo. Since this assay system estimates outward flux of labelled cholesterol from the fibroblasts, the influence of back flux of cholesterol from plasma into the cells was evaluated using double isotope labelling experiments. To this end cholesterol loaded cells was also incubated with [^{14}C]-cholesterol (0.2 $\mu\text{Ci/ml}$) enriched 1% vol/vol diluted plasma in a procedure that was otherwise similar to that described above. The percentage back flux was calculated by dividing the [^{14}C]-cholesterol radioactive counts in the cells after incubation by the counts in the medium before incubation. The percentage of back flux of [^{14}C]-cholesterol from plasma to cells was only $5.2 \pm 1.0\%$ (triplicate experiments in 3 different plasma samples). Thus, under the present assay conditions transport of cholesterol was almost exclusively from cells to the medium.

Glucose was measured shortly after blood collection with an APEC glucose analyzer (APEC Inc., Danvers, MA). Glycated hemoglobin (HbA_{1c}) was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands; normal range 4.6-6.1%).

STATISTICAL ANALYSIS

Data are shown as mean \pm SD or in case of a skewed distribution as median (interquartile range). Chi-square analysis was used to evaluate differences in proportions among the groups. Between group differences were evaluated using Kruskal-Wallis analysis of variance. If significant differences were found, Mann-Whitney U tests were performed with Duncan's tests to correct for multiple comparisons. Univariate correlations were assessed by linear regression analysis, using Pearson's correlation coefficients. Multiple stepwise linear regression analysis was used to reveal independent relationships between variables. When variables had a skewed distribution, logarithmically transformed values were used. Two-sided p -values <0.05 were considered to be statistically significant.

RESULTS

As shown in Table 1, MAP and BMI were higher in the normo- and hypertriglyceridemic diabetic groups than in the control group. Among normotriglyceridemic and hypertriglyceridemic diabetic patients, MAP was not significantly different between subjects using and not using antihypertensive medication. Plasma total cholesterol, non-HDL cholesterol and apo B levels were highest in hypertriglyceridemic diabetic patients, but were similar in normotriglyceridemic diabetic patients and control subjects. Plasma apo A-I was lowest in the hypertriglyceridemic diabetic group but was not different in the normotriglyceridemic diabetic and control groups. HbA_{1c} was similarly elevated in both diabetic groups compared to control subjects (Table 1).

Table 1. Clinical characteristics, plasma lipids and apolipoprotein (apo) levels in hypertriglyceridemic and normotriglyceridemic type 2 diabetic patients and in control subjects

	<i>I</i> <i>Control subjects</i> <i>n = 56</i>	<i>II</i> <i>Type 2 diabetic</i> <i>patients</i> <i>Tg ≤ 2.0 mmol/l</i> <i>n = 56</i>	<i>III</i> <i>Type 2 diabetic</i> <i>patients</i> <i>Tg > 2.0 mmol/l</i> <i>n = 28</i>	<i>ANOVA</i> <i>p-value</i>
Sex F/M	26/30	26/30	11/17	
Age (years)	55 ± 10	58 ± 9	58 ± 8	0.16
BMI (kg/m ²)	25.2 ± 3.4	28.7 ± 5.2 ^a	30.2 ± 4.8 ^a	< 0.001
MAP (mmHg)	99 ± 13	107 ± 11 ^a	107 ± 13 ^a	< 0.001
Glucose (mmol/l)	5.7 ± 0.7	8.4 ± 1.9 ^a	9.0 ± 2.0 ^a	< 0.001
HbA _{1c}	5.3 ± 0.4	6.7 ± 0.9 ^a	7.0 ± 1.0 ^a	< 0.001
Plasma total cholesterol (mmol/l)	5.3 ± 0.7	5.3 ± 0.8	5.6 ± 1.0 ^{ab}	0.02
Plasma triglycerides (mmol/l)	1.1 (0.8 – 1.5)	1.3 (0.9 – 1.7)	2.6 (2.2 – 2.8) ^{ab}	< 0.001
Non-HDL cholesterol (mmol/l)	3.6 ± 0.7	3.8 ± 0.8	4.5 ± 0.9 ^{ab}	< 0.001
Plasma apo A-I (g/l)	1.46 ± 0.23	1.39 ± 0.25	1.25 ± 0.21 ^{ab}	0.001
Plasma apo B (g/l)	0.85 ± 0.19	0.88 ± 0.19	1.04 ± 0.21 ^{ab}	< 0.001

Data in mean ± SD or in median (interquartile range). Tg: triglycerides; BMI: body mass index; MAP: mean arterial pressure.

^a p<0.05 vs group I; ^b p<0.05 vs group II

HDL cholesterol was lowest in hypertriglyceridemic diabetic patients, intermediate in normotriglyceridemic diabetic patients and highest in control subjects (Table 2). HDL phospholipids were somewhat lower in hypertriglyceridemic diabetic

patients compared to control subjects. Plasma pre β -HDL levels and pre β -HDL formation were similar in the 3 groups. However, when expressed in % of apo A-I, both pre β -HDL and pre β -HDL formation were found to be increased in hypertriglyceridemic diabetic patients.

Table 2. HDL lipids and plasma pre β -HDL parameters in hypertriglyceridemic and normotriglyceridemic type 2 diabetic patients and in control subjects

	<i>I</i> <i>Control subjects</i> <i>n=56</i>	<i>II</i> <i>Type 2 diabetic</i> <i>patients</i> <i>Tg \leq 2.0 mmol/l</i> <i>n=56</i>	<i>III</i> <i>Type 2 diabetic</i> <i>patients</i> <i>Tg > 2.0 mmol/l</i> <i>n=28</i>	<i>ANOVA</i> <i>p-value</i>
HDL cholesterol (mmol/l)	1.68 \pm 0.41	1.48 \pm 0.37 ^a	1.17 \pm 0.29 ^{a,b}	< 0.001
HDL phospholipids (mmol/l)	1.43 \pm 0.29	1.44 \pm 0.32	1.30 \pm 0.37 ^a	0.04
Pre β -HDL (apo A-I g/l)	0.048 \pm 0.018	0.049 \pm 0.020	0.054 \pm 0.016	0.45
Pre β -HDL formation (apo A-I g/l)	0.296 \pm 0.066	0.294 \pm 0.071	0.304 \pm 0.059	0.80
Pre β -HDL (% apo A-I)	3.4 \pm 1.0	3.6 \pm 1.5	4.4 \pm 1.3 ^{a,b}	0.004
Pre β -HDL formation (% apo A-I)	20.4 \pm 3.4	21.2 \pm 4.3	24.5 \pm 4.1 ^{a,b}	< 0.001

Data in mean \pm SD. Tg: triglycerides.

^a p<0.05 vs group I; ^b p<0.05 vs group II

As shown in Table 3, plasma PLTP activity was highest in hypertriglyceridemic diabetic patients and lowest in control subjects. Plasma EST and CET were also highest in the hypertriglyceridemic diabetic groups and lowest in the control group. The ability of plasma from hypertriglyceridemic diabetic patients to stimulate cholesterol efflux out of fibroblasts was higher compared to plasma from normotriglyceridemic diabetic patients and control subjects, but efflux to plasma from normotriglyceridemic diabetic patients and control subjects was similar (Table 3).

To evaluate the relationships of cellular cholesterol efflux with HDL-related variables was related, univariate correlation analysis was performed in diabetic patients (n = 84), control subjects (n = 56) and in all subjects combined (Table 4). Cholesterol efflux from fibroblasts to plasma from diabetic patients was positively correlated with HDL phospholipids, plasma pre β -HDL formation, PLTP activity, EST and CET, but not with HDL cholesterol, pre β -HDL levels and plasma apo A-I. Cellular cholesterol efflux to plasma from control subjects was significantly correlated with plasma CET only. In all subjects combined, cellular cholesterol efflux was positively correlated with HDL

phospholipids, pre β -HDL formation, plasma PLTP activity, EST and CET. Multiple linear regression analysis was performed in the combined subjects in order to disclose those variables with which cellular cholesterol efflux was independently associated.

Table 3. Plasma phospholipid transfer protein (PLTP) activity, cholesterol esterification (EST), cholesteryl ester transfer (CET), and cholesterol efflux from cultured fibroblasts to plasma from hypertriglyceridemic and normotriglyceridemic type 2 diabetic patients and control subjects

	<i>I</i> <i>Control subjects</i> <i>n = 56</i>	<i>II</i> <i>Type 2 diabetic</i> <i>patients</i> <i>Tg \leq 2.0 mmol/l</i> <i>n = 56</i>	<i>III</i> <i>Type 2 diabetic</i> <i>patients</i> <i>Tg > 2.0 mmol/l</i> <i>n = 28</i>	<i>ANOVA</i> <i>p-value</i>
Plasma PLTP activity (AU)	92.8 \pm 9.7	101.9 \pm 12.3 ^a	106.5 \pm 8.8 ^{a,b}	< 0.001
Plasma EST (nmol/ml/h)	50.9 \pm 11.6	59.5 \pm 14.0 ^a	73.3 \pm 18.8 ^{a,b}	< 0.001
Plasma CET (nmol/ml/h)	18.1 \pm 5.4	20.9 \pm 5.1 ^a	29.0 \pm 9.7 ^{a,b}	< 0.001
Cholesterol efflux from fibroblasts to plasma (% / 4 h)	8.3 \pm 0.9	8.4 \pm 0.8	9.0 \pm 0.7 ^{a,b}	< 0.001

Data in mean \pm SD. Tg: triglycerides.

^a p<0.05 vs group I; ^b p<0.05 vs group II

Table 4. Univariate linear regression analysis of cellular cholesterol efflux with HDL cholesterol, HDL phospholipids, plasma apo A-I, pre β -HDL levels and pre β -HDL formation, phospholipid transfer protein (PLTP) activity, plasma cholesterol esterification (EST) and cholesteryl ester transfer (CET) in type 2 diabetic patients, control subjects and in all subjects combined

	<i>type 2 diabetic patients</i> <i>(n = 84)</i>	<i>control subjects</i> <i>(n = 56)</i>	<i>all subjects</i> <i>(n = 140)</i>
HDL cholesterol (mmol/l)	0.13	0.08	0.03
HDL phospholipids (mmol/l)	0.26 ^a	0.13	0.19 ^a
Plasma apo A-I (g/l)	0.12	0.06	0.05
Pre β -HDL (apo A-I g/l)	0.19	0.08	0.15
Pre β -HDL formation (apo A-I g/l)	0.35 ^b	0.17	0.27 ^b
Plasma PLTP activity (AU)	0.41 ^c	0.21	0.37 ^c
Plasma EST (nmol/ml/h)	0.38 ^c	0.25	0.37 ^c
Plasma CET (nmol/ml/h)	0.24 ^a	0.28 ^a	0.29 ^c

Pearson correlation coefficients are shown. ^ap<0.05; ^bp<0.01; ^cp<0.001

In this model only variables which were significantly related to cholesterol efflux in univariate analysis (Table 4) were included. As shown in Table 5, cellular cholesterol efflux was independently related to pre β -HDL formation, plasma PLTP activity and EST (multiple $r = 0.48$), but not significantly with HDL phospholipids ($p = 0.13$) and plasma CET ($p = 0.41$). In this model, there was no independent contribution of the presence of diabetes ($p = 0.76$). Moreover, cholesterol efflux from cultured fibroblasts to patient plasma was not affected by the use of sulfonylurea ($p = 0.16$), biguanides ($p = 0.46$) or thiazolidinedione derivatives ($p = 0.98$).

Table 5. Multiple linear regression model showing HDL-related variables which independently contribute to cellular cholesterol efflux in all subjects combined

	<i>B (SE)</i>	<i>Partial r</i>	<i>p</i>
Constant	5.22 (0.59)		<0.001
Pre β -HDL formation	2.64 (1.06)	0.209	0.014
Plasma PLTP activity	0.0164 (0.006)	0.216	0.011
Plasma EST	0.0149 (0.004)	0.276	0.001

Multiple $r = 0.48$; B: coefficient or constant; SE: standard error; PLTP: phospholipid transfer protein; EST: cholesterol esterification.

DISCUSSION

This study shows for the first time that the plasma levels of the pre β -migrating HDL fraction, as well as the ability of plasma to generate such particles during *in vitro* incubation, is unchanged in normo- and hypertriglyceridemic type 2 diabetic patients compared to control subjects. Of note, elevated levels of pre β -HDL as well as enhanced pre β -HDL formation were documented in hypertriglyceridemic diabetic patients when these parameters were expressed as percentage of apo A-I, which takes into account the lower apo A-I levels in association with high triglyceride levels. Furthermore, our study clearly demonstrates that the ability of normotriglyceridemic diabetic plasma to stimulate cholesterol efflux out of cultured human fibroblasts is not decreased, whereas the ability of hypertriglyceridemic diabetic plasma to promote cellular cholesterol efflux is even increased. We conclude from these experiments that, as far as the ability of plasma to remove excess cellular cholesterol from cultured fibroblasts is involved, there is no primary defect in this early process in the RCT pathway in type 2 diabetes.

The reported plasma concentrations of pre β -HDL vary considerably between studies. In the present study pre β -HDL was assayed by crossed immuno-electrophoresis. Our method of pre β -HDL measurement does not discern between various pre β -HDL

subspecies, making it likely that not only free apo A-I, but also particles already containing some lipids were co-measured in our assay. Nonetheless, the levels currently found in control subjects are closely comparable to those of small pre β -HDL documented by Ishida et al. using electrophoretic transfer followed by solid phase radioimmunoassay [24]. In that early report, pre β -HDL was elevated in hypertriglyceridemic individuals, both when expressed in absolute and in relative amounts, but only a few diabetic patients were studied [24]. In non-diabetic subjects, the relative amount of pre β -HDL was reported to be higher than those measured in our study, and was found to be elevated with hypertriglyceridemia and hypercholesterolemia [26]. In another study, a positive correlation of plasma pre β -HDL levels with triglycerides was found but only in subjects with familial low HDL cholesterol [25]. Using a different methodological approach, it was observed recently that the relative amount of pre β 1-HDL is increased in hypertriglyceridemic type 2 diabetic patients [37]. In this kinetic study, the *in vivo* conversion of α -HDL into pre β 1-HDL was increased in the diabetic patients [37]. This finding appears to be consistent with our results, at least when the relative distribution of pre β -HDL and α -HDL are compared. It is also relevant to reconcile that both absolute and relative pre β -HDL formation were unchanged in normotriglyceridemic diabetic patients, although we did not extensively document the time course of pre β -HDL increment under conditions of LCAT inhibition. Both HDL cholesteryl ester depletion and triglyceride enrichment consequent to CETP action [15,17,38] and the ability of PLTP to mediate the release of lipid-poor apo A-I during HDL remodeling [15,16,38,39], a process which is enhanced when HDL are enriched with triglycerides [40] contribute to pre β -HDL generation. In the current study, plasma PLTP activity and CETP-mediated cholesteryl ester transfer were particularly elevated in hypertriglyceridemic diabetic patients as expected [17,20]. Therefore, it is plausible to propose that increased plasma PLTP and CETP action may act in concert in the higher relative pre β -HDL levels found in hypertriglyceridemic diabetic plasma.

We used cholesterol-loaded cultured skin fibroblasts to document the ability of plasma to stimulate cellular cholesterol efflux. These cells abundantly express ABCA1 after cholesterol preloading *in vitro* [29,41]. The importance of ABCA1 for cholesterol efflux from these cells is illustrated by the observation that cholesterol efflux out of fibroblasts from subjects with ABCA1 deficiency to purified apo A-I is almost absent [9]. Fibroblasts express negligible SR-BI [42], but the extent to which other processes contribute to cholesterol efflux from these cells to plasma is still unknown [12,13,42]. With macrophages it has been recently shown that cholesterol efflux is also dependent on mature forms of HDL via ABCG1 [12,43], and that LCAT-mediated cholesterol esterification is important for efflux from these cells to large-sized α -HDL [44]. Fibroblasts

express ABCG1 as well, which like ABCA1, is upregulated by an LXR agonist [45,46]. We evaluated the relationships of fibroblast cholesterol efflux with several HDL-related plasma components, including pre β -HDL, HDL cholesterol, HDL phospholipids, PLTP activity, plasma cholesterol esterification and cholesteryl ester transfer. Multiple regression analysis revealed that cellular cholesterol efflux was positively related to pre β -HDL formation rather than to plasma pre β -HDL level as such. This would raise the possibility that the dynamics of pre β -HDL generation, as reflected by pre β -HDL formation, are important in cellular cholesterol efflux. Our analysis also demonstrated an independent relationship of cholesterol efflux from cultured fibroblasts with plasma PLTP activity, in keeping with its ability to directly stimulate cholesterol removal out of ABCA1 expressing cells *in vitro* [47]. Furthermore, cholesterol efflux was positively associated with plasma cholesterol esterification but not independently with cholesteryl ester transfer. This suggests that LCAT action is important for cholesterol removal from fibroblasts. Of relevance, no independent effect of the diabetic state on the ability of plasma to promote cellular cholesterol efflux was found. It thus appears that in adequately controlled diabetes *in vivo* plasma apolipoprotein glycation does not impair this process to any appreciable extent. Taken together, we interpret the increased ability of plasma from hypertriglyceridemic diabetic patients to promote cellular cholesterol removal to be attributable to elevated plasma PLTP activity and cholesterol esterification. Unaltered pre β -HDL formation is could be important for the maintenance of cholesterol efflux to diabetic plasma.

It is generally accepted that removal of excess cholesterol from peripheral cells to extracellular acceptors and subsequent delivery to the liver provides a mechanism that protects against atherosclerosis development [1,3]. Therefore, the enhanced ability of hypertriglyceridemic diabetic plasma to stimulate cellular cholesterol efflux could be favorable in terms of cardiovascular risk modulation. On the other hand, we did not document whether the *in situ* ability of peripheral cells to transport cholesterol to the extracellular space is altered in the diabetic state. The present study suggests that pre β -HDL may contribute to an anti-atherogenic defense mechanism via stimulation of reverse cholesterol transport. However, CETP and PLTP comprise both atherogenic and anti-atherogenic actions and, at least in patients with type 2 diabetes mellitus, the net effects of high plasma PLTP activity and cholesteryl ester transfer are probably pro-atherogenic [48,49].

In conclusion, this study demonstrates for the first time that cholesterol efflux from cultured fibroblasts to plasma from hypertriglyceridemic type 2 diabetic patients is enhanced in association with increased plasma PLTP activity and cholesterol esterification. Unaltered pre β -HDL formation, despite low plasma apo A-I, could contribute to

maintenance of cholesterol efflux in diabetic hypertriglyceridemia. Cholesterol efflux from fibroblasts to normotriglyceridemic diabetic plasma is unchanged.

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