Chapter 10

Cellular cholesterol efflux to plasma from proteinuric patients is elevated and remains unaffected by antiproteinuric treatment

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Background—Lipid derangements are assumed to contribute to the elevated cardiovascular risk in proteinuric patients. The impact of proteinuria on reverse cholesterol transport (RCT) is unknown. The first step in RCT, cellular cholesterol efflux to plasma, may be altered in proteinuria, consequent to changes in pre-β HDL formation and plasma phospholipid transfer protein (PLTP) activity. Methods—In 6 non-diabetic male patients with nephrotic-range proteinuria and 12 matched healthy men plasma (apo)lipoproteins, pre-β HDL formation, PLTP activity as well as the ability of plasma to promote cholesterol efflux out of cultured human skin fibroblasts were determined. These variables were also measured in response to antiproteinuric treatment, consisting of single and dual RAAS blockade by losartan and lisinopril. Results—Plasma total cholesterol (p < 0.05), triglycerides (p < 0.05), apolipoprotein (apo) A-I (p < 0.001), apo B (p < 0.001), PLTP activity (p < 0.005) and pre-β HDL formation (p < 0.001) were higher in proteinuric patients. Cellular cholesterol efflux to plasma from proteinuric patients was 41% higher than to plasma from healthy subjects (p < 0.001). Reduction of proteinuria from 5.0 to 1.4 g/d by dual RAAS blockade was associated with a 23 % reduction in plasma apo B levels (p < 0.05). Pre-β HDL formation and plasma PLTP activity did not significantly change. Combined antiproteinuric treatment did not reduce the elevated cellular cholesterol efflux. Conclusion—Cellular cholesterol efflux to plasma from patients with nephrotic-range proteinuria is enhanced, in conjunction with elevated pre-β HDL formation and plasma PLTP activity. These changes may attenuate the cardiovascular risk associated with proteinuria-associated hyperlipidemia. Antiproteinuric therapy lowers plasma apo B, but does not affect cell-derived cholesterol efflux, suggesting that this therapy beneficially affects cardiovascular risk in proteinuric patients.

In proteinuric patients, plasma total cholesterol, very low density lipoprotein (VLDL), low density lipoprotein (LDL) cholesterol and triglycerides (TG) are usually elevated (1,2). These proteinuria-associated abnormalities in apolipoprotein (apo) B-containing lipoproteins may well contribute to the elevated cardiovascular risk in proteinuric patients (3). The impact of proteinuria on HDL metabolism, relevant as this may be to cardiovascular risk, is incompletely understood.

HDL plays an important role in the reverse cholesterol transport (RCT) pathway (4), which provides transport of excess cellular cholesterol from peripheral cells to the liver for metabolism and excretion in the bile. Thus, the RCT pathway plays a protective role in cardiovascular risk. Among other factors, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) are involved in HDL remodelling and metabolism. CETP transfers cholesteryl esters from HDL to apo B-containing lipoproteins, whereas PLTP transfers phospholipids between lipoproteins and is able to
convert HDL in smaller and larger HDL particles (5). During this process small apo A-I containing pre-β HDL particles are generated (6). These particles are initial acceptors of cell-derived cholesterol and are likely to be important for removal of cholesterol from the vessel wall.

The effect of nephrotic syndrome on HDL metabolism in humans, and more specifically on these processes of the RCT pathway, is not fully understood. Plasma HDL cholesterol and its most abundant apolipoprotein, apo A-I, were reported to be low, unaltered or even elevated (1,2,7) in proteinuric patients. Plasma CETP levels and cholesteryl ester transfer rates are elevated in nephrotic patients (2,8), contributing to a low cholesterol content in HDL. Plasma PLTP activity is increased in hypertriglyceridemic subjects (5), but no data are available with respect to plasma PLTP activity and pre-β HDL in proteinuric patients. The effect of proteinuria on the ability of plasma to promote cellular cholesterol efflux, representing a functional measure to evaluate the effectiveness of early steps in the RCT pathway with respect to the constellation of extracellular cholesterol acceptors, is also unknown. Antiproteinuric treatment results in reduction of plasma apo B-containing lipoproteins, irrespective of the mode of antiproteinuric intervention (3,8-10). HDL cholesterol may also drop in response to proteinuria reduction (9,10), but it is unknown whether cell-derived cholesterol efflux is affected by antiproteinuric treatment.

In view of high plasma triglycerides in proteinuric patients, we hypothesized that proteinuria is accompanied by changes in plasma PLTP activity and pre-β HDL, which may affect the ability of plasma to stimulate cellular cholesterol efflux. This hypothesis was tested in untreated proteinuric patients. Moreover, we investigated the effect of antiproteinuric treatment on these components of RCT.

**Patients and methods**

*Patients and protocol*

The study was approved by the local medical ethics committee and all participants provided written informed consent. Patients with proteinuric nephropathies were selected from our renal outpatient department and eligibility for participation in the study was considered after a wash-out period from all antihypertensive medication (at least 6 weeks) and all lipid-lowering agents (at least 8 weeks). All patients had to fulfil the following inclusion criteria after wash-out: proteinuria ≥ 2 g/d after wash-out, diastolic blood pressure between 80 and 110 mmHg, creatinine clearance ≥ 30 mL/min/1.73 m², and age between 18 and 70 years. Patients with nephrotic syndrome consequent to a non-primary renal disorder, as well as patients with systemic diseases, cardiovascular disorders or diabetes mellitus were excluded. Only men participated to avoid effects of sex-related differences in HDL cholesterol. For baseline comparisons,
each male patient was matched with 2 male healthy subjects with respect to race (Caucasian), age (within 5 yrs) and body mass index (BMI, calculated as weight divided by height squared; within 2 kg/m\(^2\)). Six patients were identified for study participation and completed the protocol. All patients had proteinuria of biopsy proven glomerular non-diabetic origin (focal segmental glomerular sclerosis \((n = 3)\), membranous glomerulopathy \((n = 2)\), and IgA nephropathy \((n = 1)\). None of the participants received any immunosuppressive treatment.

Patients were treated according to a prospective open-label study protocol designed to obtain individualized maximal antiproteinuric response. In short, patients were treated with losartan (subsequently 50, 100 and 150 once daily) and lisinopril (subsequently 10, 20 and 40 once daily) in random order, each preceded by a baseline period without medication. After these single drug periods, all patients received combined treatment, using the optimal individual antiproteinuric doses for each drug (usually lisinopril 40 mg and losartan 100 mg), in order to obtain the maximal antiproteinuric effect. Each treatment period lasted 6 weeks. At the end of the initial baseline period and at the end of the dual RAAS blockade period, patients visited the hospital after an overnight fast. Data obtained from these visits were used for this study. Blood pressure was measured by an automatic device (Dinamap\textsuperscript{\textregistered}). Mean arterial pressure (MAP) was calculated as: 

\[ \text{MAP} = \frac{2}{3} \times \text{diastolic blood pressure} + \frac{1}{3} \times \text{systolic blood pressure}. \]

The values shown represent the mean value of three readings after 15 min of supine rest.

\textit{Laboratory measurements}

Urinary protein was determined with the pyrogallol red-molybdate method (mean of two 24-h urine collections). Serum creatinine and albumin were determined using an automated multi-analyzer (MEGA\textsuperscript{®}, Merck, Darmstadt, Germany). Venous blood was collected in tubes containing 1.5 mg/mL ethylenediaminetetraacetic acid and was directly placed on ice. Plasma was obtained within 30 min by centrifugation at 3000 rpm for 15 min at 4 °C and was kept frozen at -20 °C until analysis.

Plasma total cholesterol and triglycerides were measured enzymatically. HDL cholesterol was assayed by a homogeneous method using a commercially available assay system (Abbott Inc., Cat. \# 30-3064/R3, Abbott Park, Ill, USA). 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 commercially available kits (Serapak, Bayer, Leverkusen, Germany, Cat. \# 682, and 6822, respectively).

Plasma PLTP activity was assayed in a liposome vesicles-HDL system using a previously described method, which is not affected by the phospholipid transfer
promoting action of CETP (11). Plasma PLTP activity is linearly related to the amount of plasma used in the incubations and is not influenced by the endogenous lipoproteins in plasma. Plasma PLTP activity is expressed in arbitrary units (AU), corresponding to the percentages of the activities in normal human pool plasma.

Plasma pre-β HDL formation was measured by crossed immuno-electrophoresis using frozen plasma essentially as described (12). In brief, plasma samples were thawed while kept on ice with addition of proteolysis inhibitors. Subsequently, iodoacetate, an LCAT inhibitor was added and the samples were incubated at 37 °C for 24 h to measure the formation of pre-beta HDL. At the end of the procedure, 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. The pre-β HDL area was calculated as the percentage of the sum of the pre-β HDL and the α HDL areas. Pre-β HDL was expressed in apo A-I concentration (g/L).

Cholesterol efflux to plasma was determined using human fibroblasts, as described (12). Fibroblasts were obtained from normolipidemic control persons by explant culture from a 3 mm punch biopsy at a 1 mm skin thickness. The cells were then cultured in DMEM and subsequently were loaded with [3H]-cholesterol and unlabelled cholesterol during 24 h. Unlabelled cholesterol was added to induce ATP-binding cassette transporter-AI (ABCA1) expression in fibroblasts. Subsequently, the cells were extensively washed and the efflux assay was started by adding plasma diluted to 1% in efflux medium. At this concentration the plasma dose-response curve for cholesterol efflux is in the linear range. An incubation time of 4 h at 37 °C was chosen to minimize analytical errors. [3H]-cholesterol was quantified by liquid scintillation counting after collection of the medium. Total cellular [3H]-cholesterol was determined after extraction of the cells with 2-propanol and the percentage efflux was calculated. All values were corrected for radioactivity appearing in the culture medium in the absence of plasma. To be able to normalize between series of experiments and to correct for between-day variation, efflux to 50 μg protein/mL HDL (Calbiochem, San Diego, CA, USA) was determined in triplicate.

Statistical analysis
Results are expressed as mean and range. Data from patients before, at baseline, and after the three treatment periods were compared with data from healthy subjects by one-way ANOVA. Where appropriate, one-way ANOVA with post-hoc Duncan correction for multiple comparisons or paired t-tests were used to evaluate the effect of antiproteinuric treatment in these patients. In proteinuric patients, relationships between variables were assessed by Spearman’s rank correlation analysis using four data sets, obtained at baseline and after each treatment period. Z-transformation was applied to
Table 1. Proteinuria, mean arterial pressure, serum albumin and creatinine clearance (mean (range)) at baseline and after losartan 100 mg, lisinopril 40 mg and combined treatment with losartan and lisinopril in 6 non-diabetic proteinuric patients.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Losartan 100 mg</th>
<th>Lisinopril 40 mg</th>
<th>Losartan + Lisinopril</th>
</tr>
</thead>
<tbody>
<tr>
<td>U&lt;sub&gt;prot&lt;/sub&gt; (g/d)</td>
<td>5.0 (2.2-8.2)</td>
<td>3.0 (1.1-6.5)*</td>
<td>1.8 (0.0-6.0)*</td>
<td>1.4 (0.0-5.6)*$</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>105 (90-127)</td>
<td>90 (71-97)*</td>
<td>85 (73-102)*</td>
<td>78 (68-89)*$#</td>
</tr>
<tr>
<td>Se&lt;sub&gt;Alb&lt;/sub&gt; (g/L)</td>
<td>38 (36-42)</td>
<td>41 (35-45)*</td>
<td>40 (34-43)</td>
<td>40 (35-46)*</td>
</tr>
<tr>
<td>Cl&lt;sub&gt;creat&lt;/sub&gt; (mL/min)</td>
<td>78 (40-92)</td>
<td>81 (57-107)</td>
<td>73 (37-99)</td>
<td>67 (40-82)</td>
</tr>
</tbody>
</table>

U<sub>prot</sub>, proteinuria. MAP, mean arterial pressure. Se<sub>Alb</sub>, serum albumin. Cl<sub>creat</sub>, creatinine clearance. * p < 0.05 vs. baseline; $ p < 0.05 vs. losartan; # p<0.05 vs. lisinopril correct for multiple comparisons. A p-value < 0.05 was considered significant.

RESULTS

Age was 47 (39-54) years in proteinuric patients and 51 (45-56) years in control subjects (NS). BMI was also not different between the groups (24.5 (21.9-27.0) kg/m<sup>2</sup> and 24.4 (22.9-25.8) kg/m<sup>2</sup> for proteinuric patients and control subjects, respectively (NS). Renal data and blood pressure from the proteinuric patients are given in table 1. In table 2, further clinical characteristics, plasma lipids, lipoproteins, apolipoproteins, pre-ß HDL formation and PLTP activity levels of the proteinuric and matched healthy control subjects are given. Plasma total cholesterol, triglycerides and apo B levels were higher in proteinuric patients. The difference in plasma VLDL + LDL between patients and healthy subjects did not reach statistical significance. HDL cholesterol was similar in patients and healthy subjects, whereas plasma apo A-I was higher in patients. Plasma PLTP activity was elevated in the patient group, coinciding with an increase in pre-ß HDL formation. As shown in figure 1, cholesterol efflux out of fibroblasts amounted to 18.3 (17.7-18.7) %/4 h to plasma from proteinuric patients vs. 13.0 (12.2-13.8) %/4 h to plasma from healthy subjects (p < 0.001), resulting in a 41% higher efflux using patient plasma.

In patients, proteinuria was lowered in response to losartan and lisinopril treatment (table 1). Dual RAAS blockade, consisting of the combination of the individual optimal antiproteinuric doses of losartan and lisinopril, resulted in the maximal reduction in proteinuria which amounted to 73 (50-97) % compared to baseline. Proteinuria reduction was accompanied by a rise in serum albumin. MAP fell during each treatment period with the lowest values being recorded during dual RAAS blockade. The changes in creatinine clearance did not reach statistical significance.

Table 2 shows that plasma apo B levels dropped in response to antiproteinuric treatment with lisinopril and dual RAAS blockade, whereas the reductions in plasma total cholesterol and VLDL + LDL cholesterol were significant after lisinopril treatment. Plasma apo A-I was lowered after lisinopril and combined treatment, but HDL
Table 2. Lipids profiles, apo(lipoprotein)s, pre-ß HDL formation and PLTP activity (mean (range)) of male non-diabetic proteinuric and healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>Male control subjects (n = 12)</th>
<th>Male proteinuric patients (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Losartan 100 mg</td>
</tr>
<tr>
<td><strong>Total C (mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.65</td>
<td>4.60 #</td>
<td>4.78 #</td>
</tr>
<tr>
<td>(4.27-5.03)</td>
<td>(4.60-7.36)</td>
<td>(4.72-6.85)</td>
</tr>
<tr>
<td><strong>TG (mmol/L)</strong></td>
<td>1.05</td>
<td>1.88 #</td>
</tr>
<tr>
<td>(0.68-1.43)</td>
<td>(1.02-2.73)</td>
<td>(0.86-2.58)</td>
</tr>
<tr>
<td><strong>VLDL + LDL-C (mmol/L)</strong></td>
<td>3.07</td>
<td>4.09</td>
</tr>
<tr>
<td>(2.70-3.45)</td>
<td>(2.48-5.41)</td>
<td>(2.86-5.07)</td>
</tr>
<tr>
<td><strong>HDL-C (mmol/L)</strong></td>
<td>1.11</td>
<td>1.05</td>
</tr>
<tr>
<td>(0.96-1.25)</td>
<td>(0.80-1.31)</td>
<td>(0.70-1.39)</td>
</tr>
<tr>
<td><strong>Plasma Apo A-I (g/L)</strong></td>
<td>1.14</td>
<td>1.46 $</td>
</tr>
<tr>
<td>(1.03-1.25)</td>
<td>(1.29-1.64)</td>
<td>(1.23-1.58)</td>
</tr>
<tr>
<td><strong>Plasma Apo B (g/L)</strong></td>
<td>0.77</td>
<td>1.45 $</td>
</tr>
<tr>
<td>(0.69-0.85)</td>
<td>(1.07-1.82)</td>
<td>(0.98-1.65)</td>
</tr>
<tr>
<td><strong>PLTP activity (AU)</strong></td>
<td>89.8</td>
<td>112.1 $‡</td>
</tr>
<tr>
<td>(81.6-98.1)</td>
<td>(96.2-127.9)</td>
<td>(96.6-142.0)</td>
</tr>
<tr>
<td><strong>Pre-ß HDL formation (apo A-I, g/L)</strong></td>
<td>0.19</td>
<td>0.36 $</td>
</tr>
<tr>
<td>(0.15-0.22)</td>
<td>(0.24-0.47)</td>
<td>(0.15-0.35)</td>
</tr>
</tbody>
</table>

TC, plasma total cholesterol. TG, plasma triglycerides. C, cholesterol. VLDL + LDL, very-low + low-density lipoproteins. HDL, high-density lipoproteins. Apo A-I, apolipoprotein A-I. Apo B, apolipoprotein B. PLTP, plasma phospholipid transfer protein. $ p < 0.001, ‡ p < 0.005, # p < 0.05 vs. control subjects; * p < 0.05 vs. baseline.

cholesterol did not change. Plasma triglycerides and PLTP activity were unaltered after proteinuria lowering, and PLTP activity remained higher compared to control subjects at each treatment period. In the proteinuric patients, there was no relationship of plasma PLTP activity with the degree of proteinuria as measured during the four observation periods (averaged r = -0.17, p = 0.56). In contrast, plasma apo B was positively correlated with proteinuria (averaged r = 0.52, p < 0.05). Neither plasma PTLP activity nor apo B was correlated with creatinine clearance (averaged r = 0.39, p = 0.17; and averaged r = 0.29, p = 0.33, respectively).

Pre-ß HDL formation and cellular efflux were only measured at baseline and during dual RAAS blockade. The changes in pre-ß HDL formation were not significant. Figure 1 shows that the ability of patient plasma to induce cholesterol efflux out of fibroblasts was not affected by dual RAAS blockade and remained elevated compared to plasma from healthy subjects.

**DISCUSSION**

This study shows for the first time that plasma pre-ß HDL formation is enhanced and that the plasma PLTP activity level is higher in proteinuric patients compared to healthy subjects. Another novel finding is that plasma from proteinuric patients has a 41% higher ability to stimulate cholesterol efflux out of human fibroblasts than plasma...
from healthy subjects, without any overlap between proteinuric patients and healthy subjects. As expected (3,8-10), reduction of proteinuria by RAAS-blockade resulted in a drop in plasma apo B levels. Of interest, combined antiproteinuric treatment did not affect the ability of plasma to promote cellular cholesterol efflux.

Several factors may be involved in the increase in pre-ß HDL formation in patients with nephrotic-range proteinuria. Plasma apo A-I was elevated in the presently studied proteinuric patients which may contribute to enhanced pre-ß HDL formation. The mechanism for this plasma apo A-I elevation is uncertain, but protein expression of SR-BI, an important HDL receptor is diminished whereas apo A-I mRNA expression is elevated in the liver of nephrotic rats (13). In vitro studies have shown that both CETP and PLTP are involved in the generation of pre-ß HDL particles (5). We and others have previously demonstrated that the plasma CETP activity level is elevated in proteinuric patients (2,8). This increase in CETP activity, together with higher levels of apo B-containing lipoproteins in proteinuria, may enhance the transport of cholesteryl esters from HDL towards VLDL and LDL, and that of triglycerides towards HDL (5). As a result, HDL particles become cholesterol poor, as presently evidenced by a low concentration of cholesterol in HDL relative to apo A-I, as well as enriched with triglycerides. This HDL enrichment with triglycerides will stimulate PLTP-mediated pre-ß HDL generation (6). Thus, high plasma PLTP and CETP activity levels may act in concert in generating lipid-poor pre-ß HDL particles in proteinuric patients.

ABCA1-mediated processes are considered to play a key role in cellular cholesterol efflux to extracellular acceptors, such as pre-ß HDL (14). The relevance of ABCA1 for fibroblast cholesterol efflux is illustrated by the observation that efflux out of these cells from patients with genetic ABCA1 deficiency to apo A-I is almost
abrogated (15). In our study, we used normal human skin fibroblasts which, after cholesterol loading, abundantly express ABCA1 (12). Lipid poor acceptors including pre-β HDL particles are considered to be the initial acceptors of cholesterol from these cells (16). Besides, PLTP could also directly promote cholesterol efflux out of fibroblasts (17). Thus, it is likely that enhanced pre-β HDL formation and elevated PLTP activity are involved in the higher cholesterol efflux out of fibroblasts to proteinuric plasma, although effects of other HDL-related variables remain possible. Moreover, it should be realized that we did not document whether the in situ capacity of peripherical cells to transport cholesterol to the extracellular space is abnormal in proteinuric patients. We propose that an enhanced ability of plasma to stimulate cellular cholesterol efflux can be envisaged to favorably modulate the increased cardiovascular risk in proteinuric patients, which is in part attributable to high levels of apo B-containing lipoproteins (3).

Since it has been previously suggested that plasma apo A-I and / or HDL cholesterol can decrease after rigorous antiproteinuric treatment (9,10), we tested whether this intervention would decrease the ability of plasma to stimulate cellular cholesterol removal. In our study, plasma apo A-I decreased in response to lisinopril and dual RAAS blockade, but the changes in HDL cholesterol and pre-β HDL formation were not significant. Plasma PLTP activity did not change after antiproteinuric treatment and remained elevated compared to control subjects. Importantly, cellular cholesterol efflux from fibroblasts was also unaltered after combined antiproteinuric treatment. This suggests that even after pronounced antiproteinuric therapy this early step in the reverse cholesterol transport process remains unaffected, as far as the ability of plasma to stimulate cellular cholesterol efflux is concerned. However, since no data are available concerning effects of lisinopril or losartan on plasma PLTP activity, pre-β HDL and cell-derived cholesterol transport to plasma in subjects without proteinuria, we cannot completely exclude that effects of proteinuria reduction on these variables were masked by opposite effects of these medications per se.

PLTP is a multifacetted lipid transfer protein that, besides its role in stimulating cellular cholesterol efflux via direct and indirect mechanisms in vitro, has atherogenic properties as well. Of interest, in vivo studies in mice transgenic for human PLTP show increased atherosclerosis, which is attributable to decreased HDL levels and a stimulatory effect of PLTP on hepatic VLDL secretion (18). Limited data available so far in humans suggest that plasma PLTP activity may be elevated in patients with cardiovascular disease (19). To which extent high PLTP activity contributes to the cardiovascular risk in proteinuric patients is unknown at present. Finally, in the interpretation of the present findings in terms of cardiovascular risk, it should be realized that HDL also has antiinflammatory and antioxidative properties that were not
evaluated in our study (20).

In conclusion, albeit in a small number of patients, the present study demonstrates that the ability of plasma from proteinuric patients to stimulate cholesterol efflux out of human skin fibroblasts is strongly elevated. This abnormality is probably due to enhanced pre-ß HDL formation and PLTP activity. Thus, with respect to plasma-related acceptors of cell-derived cholesterol, this early step in RCT appears to be favorably altered in proteinuric patients, which may alleviate the cardiovascular consequences of their hyperlipidemia. Remarkably, the elevated cellular cholesterol efflux to plasma persists despite pharmacological reduction of proteinuria—along with a drop in plasma apo B levels. These effects lend support to the possibility that antiproteinuric treatment beneficially affects cardiovascular risk in proteinuric patients.

REFERENCES


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