Nephrotic syndrome causes upregulation of HDL endocytic receptor and PDZK-1-dependent downregulation of HDL docking receptor

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Abstract

**Background.** Nephrotic syndrome (NS) is associated with dysregulation of lipid/lipoprotein metabolism and impaired high-density lipoprotein (HDL)-mediated reverse cholesterol transport and atherosclerosis. HDL serves as a vehicle for transport of surplus lipids from the peripheral tissues for disposal in the liver via two receptors: (i) scavenger receptor class B type I (SR-BI) which serves as a docking receptor, enabling HDL to unload its lipid cargo and return to circulation to repeat the cycle, and (ii) beta chain ATP synthase which serves as the endocytic receptor mediating removal and catabolism of lipid-poor HDL. SR-BI abundance is regulated by PDZ-containing kidney protein 1 (PDZK1), a multifunctional protein, which prevents SRB-1 degradation at the post-translational level. This study explored the effect of NS on hepatic expression of these important molecules.

**Methods.** Gene expression, protein abundance and immunohistological appearance of the above proteins were measured in the liver of rats with puromycin-induced NS and control rats.

**Results.** The nephrotic animals exhibited severe proteinuria, hyperalbuminemia, hypercholesterolemia, hypertriglyceridemia, reduced HDL/total cholesterol ratio, normal glomerular filtration rate, significant upregulation of the endocytic HDL receptor messenger RNA (mRNA) and protein (P < 0.005) and significant reduction of SR-BI protein (P < 0.002) despite its normal mRNA abundance. The reduction in SR-BI protein abundance in NS animals was accompanied by parallel reductions in PDZK1 mRNA (P = 0.02) and protein abundance (P = 0.012).

**Conclusions.** NS results in elevation of hepatic HDL endocytic receptor and deficiency of HDL docking receptor. The latter is associated with and, in part, mediated by downregulation of PDZK1. Together, these abnormalities can increase catabolism and diminish recycling of HDL and contribute to the defective reverse cholesterol/lipid transport in NS.

**Keywords:** atherosclerosis; cardiovascular disease; dyslipidemia; proteinuria; reverse cholesterol transport

Introduction

Heavy glomerular proteinuria [nephrotic syndrome (NS)] is associated with profound dysregulation of lipid/lipoprotein metabolism and increased risk of atherosclerosis and thromboembolism [1–3]. By mediating reverse cholesterol transport, protecting vascular endothelium and exerting antioxidant, anti-inflammatory and antithrombotic actions, high-density lipoprotein (HDL) confers protection against atherosclerosis and cardiovascular disease [4, 5]. The importance of HDL in protection against atherosclerotic cardiovascular disease is evidenced by the inverse association of coronary atherosclerosis with plasma HDL cholesterol concentration in the general population [6]. In addition to its quantity, functional integrity of HDL is equally important for its antiatherogenic properties. This is clearly illustrated by the occurrence of atherosclerotic cardiovascular disease among patients with normal or even elevated but functionally abnormal HDL [7].

HDL serves as a vehicle for uptake and transport of surplus cholesterol from the peripheral tissues to the liver for disposal in the bile. This process depends on the cell-surface receptors for HDL on hepatocytes of which two have been thus far identified. These include the high-affinity, the HDL docking receptor, scavenger receptor class B type I (SR-BI) and endocytic receptor, beta chain of ATP synthase.

The classical function of SR-BI in the hepatocyte is to mediate selective uptake of cholesteryl esters from the hydrophobic core of HDL without transferring its apolipoprotein component, thus enabling the unloaded HDL to return to circulation and repeat the cycle [8]. SR-BI-knockout mice exhibit marked elevation of plasma cholesterol, abnormally large unesterified cholesterol-rich HDL particles, reduced biliary cholesterol secretion and accelerated atherosclerosis [9–13]. These observations clearly illustrate the central role of SR-BI in reverse cholesterol transport.

The stability of the SR-BI in the hepatocyte plasma membrane is dependent on its adapter protein, PDZ-containing kidney protein 1 (PDZK1). This is evidenced by the observation that PDZK1-knockout mice exhibit a
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>95% reduction in hepatic SR-BI protein [but not SR-BI messenger (mRNA)] resulting in hypercholesterolemia and large cholesterol-rich HDL particles [14, 15]. In hepatocytes, PDZK1 is predominantly associated with the basolateral plasma membrane where it interacts with the most distal region of the C-terminal cytoplasmic domain of SR-BI [16] via its N-terminal PDZ domain. Thus, as the major adapter protein for SR-BI, PDZK1 plays a critical role in HDL-mediated reverse cholesterol transport. The mechanism(s) by which PDZK1 controls the hepatic and intestinal levels of SR-BI remains unclear. PDZK1 could directly affect the synthesis, posttranslational processing, intracellular transport, subcellular localization, recycling and/or stability of SR-BI.

Several years ago, Martinez et al. [17] showed that the beta chain of ATP synthase (a principal protein complex of the mitochondrial inner membrane) is ectopically expressed in the hepatocyte cell membrane. They further showed that cell-surface-associated beta chain of ATP synthase serves as an apoA-I receptor, mediating endocytosis of the HDL particles including its protein and lipid contents. This process strictly depends on generation of ADP which is produced by the ATPase activity of the beta chain protein after its binding with apoA-I on the cell surface. Unlike SR-BI, which has high affinity for binding cholesterol-rich HDL-2 particles, beta chain ATP synthase mediates removal and catabolism of apo A-1 and lipid-poor HDL particles.

Earlier studies conducted in our laboratories revealed marked reduction of SR-BI protein abundance despite normal SR-BI mRNA expression [18]. The present study was designed to test this hypothesis that the observed disparity in hepatic SR-BI protein and mRNA expression in NS may be due to downregulation of PDZK1. To further explore the effect of nephrotic proteinuria on HDL metabolism, we examined the effect of NS on hepatic expression of the HDL endocytic receptor, beta chain of ATP synthase.

Materials and methods

Animals

Male Sprague–Dawley rats were rendered nephritic by sequential intraperitoneal injections of puromycin aminonucleoside (130 mg/kg on Day 1 and 60 mg/kg on Day 14). Placebo-injected rats served as controls. The rats were observed for 2 weeks following the second puromycin/placebo injection. They were housed in a temperature-controlled facility with 12-h light/dark cycles and were provided free access to regular rat chow and water. At the end of the 2-week observation period, animals were placed in metabolic cages for a 24-h urine collection. They were then anesthetized (pentobarbital 50 mg/kg IP) and euthanized by exsanguination using cardiac puncture. The liver was immediately removed, frozen in liquid nitrogen and stored at −70°C until processed. All experiments were approved by the University of California, Irvine Institutional Committee for the Use and Care of Experimental Animals. Serum creatinine, albumin, total cholesterol, triglyceride, HDL and low-density lipoprotein (LDL) cholesterol concentrations and urinary protein excretion were measured as described in our previous studies [19]. Plasma and hepatic free fatty acid content was measured using a fatty acid assay kit (BioVision Inc., Mountain View, CA) as per manufacturer’s protocol.

Tissue preparation and western blot analyses

Frozen liver tissue was processed for determination of SR-BI, PDZK1, beta chain ATP synthase and beta chain protein abundance. Briefly, the liver was homogenized in 1 mL of 20 mM Tris–HCl (pH 7.5) buffer containing 2 mM MgCl2, 0.2 M sucrose and protease inhibitor cocktail (Sigma Aldrich Inc. Saint Louis, MO). The crude extract was centrifuged at 2000 g at 4°C for 15 min to remove tissue debris. The supernatant was used for western blot analyses. Protein concentration was measured prior to each western blot analysis using a BCA Protein Assay Kit purchased from Pierce Biotechnology (Rockford, IL) following the manufacturer’s protocol. Aliquots containing 20–100 μg of protein were fractionated on 4–20%; Tris–glycine (Invitrogen, Carlsbad, CA) at 120 V for 2 h. After electrophoresis, proteins were transferred to Hybond enhanced chemiluminescence (ECL) membrane (Amersham Life Science, Arlington Heights, IL). The membrane was incubated for 1 h in blocking buffer [1× Tris-buffered saline (TBS), 0.1%; Tween 20, 5%; nonfat dry milk] and then overnight in the same buffer containing the primary antibody. The membrane was then washed four times for 5 min in 1× TBS, 0.1%; Tween 20 before a 2-h incubation in blocking buffer (1× TBS, 0.1%; Tween 20, 5%; non-fat dry milk) plus diluted horseradish peroxidase-linked anti-mouse or rabbit IgG (Amersham Life Science). The washing procedures were repeated before the membranes were developed with chemiluminescent agents (ECL; Amersham Life Science) and subjected to autoradiography for 10 s to 5 min. SR-BI and PDZK1 abundance were measured using polyclonal rabbit IgG antibodies obtained from Novus Biologicals, Inc. (Littleton, CO) at 1/1000 to/5000 dilution. Beta chain ATP synthase protein abundance was measured using a polyclonal mouse IgG antibody obtained from BD biosciences (Franklin Lakes, NJ) at 1/2000 dilution. Beta actin was used as housekeeping protein and its abundance was measured using an antibody purchased from Sigma Inc. (St Louis, MO). Horseradish peroxidase-linked anti-rabbit and anti-mouse IgG secondary antibody (Amersham Life Science) was used at 1/3000 dilution.

Immunohistochemical analysis

Briefly, cryopreserved liver tissues were cut at 8-μm thickness, mounted on slides, air dried and fixed in 4% paraformaldehyde and washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was removed using 3% hydrogen peroxide in water. The sections were incubated overnight at 4°C with rabbit anti-PDZK1 (Epitomics, Inc., Burlingame, CA), rabbit anti-SR-B1 (Novus Biologicals Inc., Littleton, CO) or mouse anti-ATP synthase beta (BD Transduction Laboratories, Sparks, MD) antibody, diluted at 1:100 or 1:50 in a PBS buffer containing 1% bovine serum albumin. Antibody binding was amplified using an ABC kit (Vector Laboratories, Burlingame, CA) and the complex visualized using diaminobenzidine. Nuclei were lightly stained with Mayer’s hematoxylin.

The specificity of PDZK1, SR-B1 and ATP synthase beta antibodies was evaluated by western blot in liver tissues.

Real-time polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed using the Bio-Rad iCycler and a Qiagen Quantitect SYBR green PCR kit (Valencia, CA). RNA from 100 μg of (five controls to five nephrotic) rat tissues was isolated using Trizol (Invitrogen) following the manufacturer’s procedure. The RNA was DNase treated and first-strand complementary DNA (cDNA) was made from 5 μg of the isolated total RNA primed with oligo-dT using an Invitrogen Superscript synthesis system. Primers used in the qPCR were specific for the following: rat SR-BI (forward, 5′-GGTGCCAGACCCTGTA-3′ and reverse, 5′-CCTTGCGACCTGTTGA-3′), rat PDZK1 (forward, 5′-ACGGTGAGCAGCAGACT-3′ and reverse, 5′-CTGATGCTGTCCTCCTT-3′) and rat beta chain ATP synthase (forward, 5′-GTGAGGACCCGAGCAG-3′ and reverse, 5′-GGATCCTGCGCCTAAGAAGG-3′). Gene-specific primers corresponding to the PCR targets were designed by using the specifications given by the vendors (Bio-Rad). The length of amplicons were as follows: rat SR-BI (235-bp long), PDZK1 (237-bp long) and beta chain ATP synthase (267-bp long). The amplicons chosen were specifically located inside the open reading frame of the specific rat genes to allow use of plasmid DNA to generate standard curves. Each SYBR green reaction (20 μL total volume) contained 2 μL of diluted cDNA as a template. The amplification program consisted of one cycle of 95°C with a 60-s hold (hot start) followed by 40 cycles of 95°C for 1 min, specified annealing temperature with 30-s hold, 72°C for 1-min hold for extension and data acquisition. Amplification was followed by melting curve analysis program run for one cycle at 95°C with 0-s hold, 65°C with 10-s hold and 95°C with 0-s hold at the step-acquisition mode. A negative control without cDNA template was run with every assay to assess the overall specificity. The final quantization was achieved by a relative standard curve.

Data analysis

Student’s t-test was used in statistical evaluation of the data which are shown as mean ± SEM. P-values <0.05 were considered significant.
Results

General data

Data are shown in Table 1. The nephrotic group exhibited severe proteinuria, hypoalbuminemia, normal plasma creatinine and creatinine clearance. Compared with the control group, the nephrotic animals had marked elevation of plasma free and total cholesterol, LDL and HDL cholesterol, triglyceride and free fatty acid concentrations and increased plasma total cholesterol-to-HDL cholesterol ratio.

SR-BI and PDZK1 data

In confirmation of our earlier study [18], SR-BI protein abundance was significantly reduced, whereas SR-BI mRNA abundance was unchanged in the liver of nephrotic group as compared to the corresponding values found in the control animals (Figure 1). The reduction of SR-B1 protein abundance observed by western blot analysis was confirmed by immunohistological analysis performed on the frozen liver tissue sections (Figure 2).

The reduction in SR-BI protein abundance in the nephrotic liver was accompanied by a parallel reduction in PDZK1 protein abundance (Figure 3). The reduction of PDZK1 protein abundance observed by western blot analysis was confirmed by immunohistological analysis.

Table 1. Plasma concentrations of cholesterols, albumin, creatinine, triglycerides, HDL cholesterol, free fatty acid, hepatic free fatty acid and urinary protein excretion in the NS and control (CTL) groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>NS</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>95.5 ± 5.56</td>
<td>496.6 ± 28.3a</td>
</tr>
<tr>
<td>Free cholesterol, mg/dL</td>
<td>43.4 ± 5.56</td>
<td>217.6 ± 28.33a</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>46.3 ± 6.07</td>
<td>243.6 ± 7.99a</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>36.0 ± 3.74</td>
<td>169.8 ± 19.97a</td>
</tr>
<tr>
<td>Total HDL cholesterol ratio</td>
<td>2.72 ± 0.26</td>
<td>3.02 ± 0.22</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>65.7 ± 8.73</td>
<td>416.1 ± 73.82a</td>
</tr>
<tr>
<td>Plasma albumin, g/dL</td>
<td>3.54 ± 0.09</td>
<td>1.89 ± 0.13a</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dL</td>
<td>0.75 ± 0.04</td>
<td>0.74 ± 0.06</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>1.24 ± 0.16</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Urine protein, mg/day</td>
<td>68.3 ± 12.66</td>
<td>646.5 ± 70.79a</td>
</tr>
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*P < 0.005 versus CTL.

Fig. 1. (A) Bar graphs depicting SR-B1/beta actin mRNA ratio in hepatic tissues of nephrotic and control rats. (B) Representative western blots and group data depicting protein abundance of hepatic tissue SR-B1/beta actin protein ratio in the NS and control (CTL) groups. n = 5 in each group. *P < 0.03.
performed on the frozen liver tissue sections (Figure 4). A direct correlation was found between SR-BI and PDZK1 protein abundance in the study groups.

ATP synthase beta chain data

In contrast to SR-BI and PDZK1, protein abundance of the beta chain of ATP synthase in the hepatic tissue was significantly increased in the nephrotic rats compared with the normal control group. This was accompanied by a parallel increase in the ATP synthase beta chain mRNA abundance in the liver of NS animals (Figure 5). The upregulation of beta chain ATP synthase mRNA and protein abundance was confirmed by immunohistological analysis performed on the frozen liver tissue sections (Figure 6).

Discussion

NS results in profound dysregulation of lipid metabolism which is marked by hypercholesterolemia, hypertriglyceridemia, elevated plasma LDL and lipoprotein (a) [LP(a)], impaired clearance of very low-density lipoprotein (VLDL) and chylomicrons and accumulation of their atherogenic remnants in the plasma [3, 20–23]. In addition, NS significantly increases the ratios of cholesterol-to-triglycerides, free cholesterol-to-cholesterol esters and phospholipids to proteins in all lipoprotein fractions [22]. Elevation of plasma cholesterol and LDL levels in NS is primarily caused by increased biosynthesis and reduced catabolism of Apo B and cholesterol [23–25]. These events are occasioned by upregulation of hepatic Apo B [23], 3-hydroxy-3-methylglutaryl-coenzyme A reductase [26, 27] and acyl-CoA cholesterolacyltransferase [ACAT-2] [28] coupled with reduced catabolism caused by acquired LDL receptor deficiency [29] and inappropriately low cholesterol to bile acids conversion capacity [30].

Hypertriglyceridemia in NS is largely due to impaired clearance of VLDL and chylomicrons and accumulation of their atherogenic remnants in the plasma [23–25]. These events are primarily caused by downregulation of adipose tissue and skeletal muscle lipoprotein lipase and VLDL receptor [31, 32], hepatic lipase [33] and cholesteryl ester acyltransferase [34]. In addition, NS results in upregulation of hepatic diacylglyceride acyltransferase [35], which is the rate-limiting enzyme in triglyceride biosynthesis pointing to increased endogenous triglyceride production capacity. Together, these alterations in lipid and lipoprotein metabolism result in a highly atherogenic plasma lipid profile in NS.

Via its central role in reverse cholesterol transport and its antioxidant, anti-inflammatory and antithrombotic actions, HDL confers protection against atherosclerosis and cardiovascular disease [4, 5]. NS adversely affects HDL metabolism in ways that limits reverse cholesterol transport. For instance, NS results in significant urinary losses of lecithin-cholesterol aclytransferase and consequent reduction of plasma LCAT concentration [36]. The associated LCAT deficiency, in turn, limits transformation of cholesterol ester-poor HDL to cholesterol ester-rich HDL-2 particle. The effect of LCAT deficiency in NS is compounded by upregulation of ACAT [28, 37], which limits free cholesterol efflux in peripheral tissues for uptake by HDL. The final step in reverse cholesterol transport is disposal of the
HDL’s lipid cargo in the liver. As noted in the introduction, SR-BI is the principal portal for unloading of the HDL lipid cargo in the liver. Previous studies by our group revealed marked reduction of SR-BI protein abundance despite normal SR-BI mRNA expression. Given the critical role of PDZK in stability of SR-BI, this study was undertaken to determine if the observed disparity in hepatic SR-BI protein and mRNA expression may be due to downregulation of PDZK1. In confirmation of our earlier study, we found significant reduction of SR-BI protein but not mRNA abundance in liver tissue of the nephrotic animals compared to that of a control (CTL) rat, confirming the result obtained by Western blot analysis.

Nakamura et al. have shown that C-terminal region of PDZK1 is essential for upregulating SR-BI protein expression. They have further demonstrated that PDZK1 is phosphorylated at Ser-509 via a cyclic adenosine monophosphate-dependent protein kinase A and that phosphorylation of PDZK1 plays an important part in the regulation of hepatic SR-BI expression [38]. Due to the lack of commercially available antibody to phosphorylated PDZK1, we were not able to determine the effect of NS on PDZK1 phosphorylation. Further studies are required to explore this issue.

To further explore the effect of nephrotic proteinuria on HDL metabolism, we examined the effect of NS on hepatic expression of the HDL endocytic receptor, beta chain ATP synthase. The study revealed significant upregulation of this endocytic receptor in the nephrotic liver. As noted above, unlike SR-BI which serves as the docking receptor for cholesterol-rich HDL-2 particles, beta chain ATP synthase mediates removal and catabolism of apo A-1 and lipid-poor HDL particles. The negative impact of NS on HDL maturation occasioned by LCAT deficiency and upregulation of ACAT can render the circulating HDL particles increasingly susceptible for uptake and degradation by the endocytic receptor which can further compromise reverse cholesterol transport.

In conclusion, NS results in elevation of hepatic HDL endocytic receptor and deficiency of HDL docking receptor. The latter is associated with and, in part, mediated by downregulation of PDZK1. Together, these abnormalities can increase catabolism and diminish recycling of HDL and contribute to the defective reverse cholesterol/lipid transport in NS.

Conflict of interest statement. None declared.

References

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