Role of PCSK9 and IDOL in the pathogenesis of acquired LDL receptor deficiency and hypercholesterolemia in nephrotic syndrome

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ABSTRACT

Background. Nephrotic syndrome (NS) leads to elevation of serum total and LDL cholesterol. This is largely due to impaired LDL clearance, which is caused by hepatic LDL receptor (LDLR) deficiency despite normal LDLR mRNA expression, pointing to a post-transcriptional process. The mechanism(s) by which NS causes LDLR deficiency is not known. By promoting degradation of LDLR, Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) and inducible degrader of the LDL receptor (IDOL) play a major role in post-translational regulation of LDLR. We, therefore, tested the hypothesis that LDLR deficiency despite its normal gene expression in NS may be due to upregulation of hepatic PCSK9 and IDOL.

Methods. LDLR, IDOL and PCSK9 expressions and nuclear translocation of liver X receptor (LXR) that regulates IDOL expression were determined in the liver of rats with puromycin-induced NS and control (CTL) rats.

Results. Compared with the CTLs, the NS rats showed marked elevation of serum total and LDL cholesterol and a significant reduction in hepatic LDLR protein expression. This was accompanied by marked upregulation of hepatic PCSK9 and IDOL expressions and heightened LXR activation.

Conclusions. LDLR deficiency, hypercholesterolemia and elevated plasma LDL in NS are associated with upregulation of PCSK9 and IDOL. Interventions targeting these pathways may be effective in the management of hypercholesterolemia and the associated cardiovascular and other complications of NS.

Keywords: atherosclerosis, lipid disorders, liver X receptor, lipid disorders, proteinuria
INTRODUCTION

Heavy glomerular proteinuria, otherwise known as nephrotic syndrome (NS), is associated with hypercholesterolemia and marked elevation of serum LDL cholesterol [1–3]. Hypercholesterolemia in NS is largely due to impaired clearance and catabolism of LDL and its principal apoprotein, apoB100 [4, 5]. In a series of earlier studies, we found marked reduction in hepatic tissue LDL receptor (LDLR) protein abundance in the liver of Sprague-Dawley rats with puromycin-induced NS and in the Imai rats with spontaneous focal glomerulosclerosis, heavy proteinuria and severe hypercholesterolemia [6–8]. Interestingly, severe acquired LDLR deficiency in the nephrotic animals was accompanied by normal LDLR mRNA expression [6, 9], which pointed to either post-transcriptional or post-translational nature of LDLR deficiency in the nephrotic animals. By virtue of its capacity to bind and clear LDL from the circulation, LDLR plays a pivotal role in LDL and cholesterol metabolism. LDL bound to the LDLR is internalized into the clathrin-coated pits and subsequently undergoes lysosomal degradation. The LDLR is then returned to the cell membrane to repeat the cycle. The acquired LDLR deficiency shown in the above-mentioned studies [6–9] elucidated the principal cause of impaired LDL clearance, elevation of serum LDL concentration and upregulation of cholesterol production machinery in NS. However, the underlying mechanism(s) by which NS causes LDLR deficiency is not known.

In recent years, two major post-translational regulators of LDLR have been identified that play a critical part in LDL metabolism. These include ‘Proprotein Convertase Subtilisin Kexin type 9 (PCSK9)’ and ‘Inducible Degrader of the LDL receptor (IDOL)’. By facilitating degradation of LDLR, these proteins reduce LDLR abundance that leads to reduction in LDL clearance and elevation of plasma total and LDL-cholesterol levels [10–12]. PCSK9 is a serine protease that is produced and released in the circulation by the liver and to a lesser extent by the intestine and kidney. On the surface of hepatocytes, PCSK9 binds to the LDLR forming a complex, which is internalized, and directs the receptor for intracellular degradation [13]. It should be noted that the ability of PCSK9 to promote degradation of LDLR is not related to its enzymatic activity; instead, PCSK9 acts as a chaperone to facilitate intracellular degradation of LDLR [13, 14]. By promoting degradation of LDLR, PCSK9 prevents the recycling of LDLR to the cell membrane leading to post-translational reduction of LDLR expression [11]. In fact, individuals with loss-of-function mutation of PCSK9 exhibit a very low plasma LDL-cholesterol level and a significant reduction in the risk of coronary heart disease [15]. For this reason, PCSK9 has emerged as a novel therapeutic target for the treatment of hypercholesterolemia. The second LDLR degrader is IDOL, which is an E3 ubiquitin ligase that mediates ubiquitination and degradation of LDLR. IDOL expression is regulated by the liver X receptor (LXR) in response to the rise in cellular oxysterols [10, 16].

Given the central role of PCSK9 and IDOL in promoting LDLR degradation, the present study was undertaken to test the hypothesis that depletion of LDLR despite its normal gene expression in NS may be, in part, due to upregulation of PCSK9 and IDOL in the liver. To this end, liver tissue LDLR, IDOL and PCSK9 expressions and nuclear LXR contents were determined in rats with puromycin-induced NS and placebo-treated normal control (CTL) rats. The study revealed a significant upregulation of PCSK9 and IDOL expressions in the nephrotic rats’ liver compared with those found in the CTL animals. These findings unraveled the role of PCSK9 and IDOL in the pathogenesis of LDLR deficiency, hypercholesterolemia and elevated plasma LDL level in NS. Based on these observations, interventions targeting these novel pathways may be effective in the management of hypercholesterolemia and the associated cardiovascular and other complications.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were rendered nephrotic by sequential intra-peritoneal injections of puromycin aminonucleoside (PAN, 130 mg/kg on Day 1 and 60 mg/kg on Day 14). Placebo-injected rats served as CTLs. The rats were observed for 2 weeks following the second puromycin or placebo injection. They were housed in a temperature-controlled facility with 12-hour 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. During the evening prior to sacrifice, food was withheld for 14 h. The next morning (between 8 and 10 a.m.) the animals were anesthetized (pentobarbital 50 mg/kg IP) and euthanized by exsanguinations 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 LDL-cholesterol concentrations, urinary protein excretion and kidney tissue total cholesterol and triglyceride were measured as described in our previous studies [17].

RT–PCR

RNA from the liver was isolated using TRIzol® reagent (15596-026, Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s protocol. First-strand cDNA was made from 5 μg of the isolated total RNA primed with oligo (dT) using the Superscript III reverse transcriptase (18080-051, Invitrogen). Gene transcript levels of IDOL and PCSK9 were quantified by TaqMan® gene expression assays using RT–PCR. Gene-specific, exon-spanning primers and TaqMan probes were factory-designed and optimized by the manufacturer. The reference assay ID for each gene is as follows: IDOL (Rn01435259_m1) and PCSK9 (Rn01416753_m1). 18S rRNA was used as the internal control (Rn03928990-1). TaqMan PCR was performed using the MX3000P Detection System (Agilent Technologies, Palo Alto, CA, USA), using the standard cycling conditions (50°C for 2 min, 95°C for 10 min,
followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Wells contained 25 μL PCR mixtures, containing 12.5 μL of TaqMan® gene expression master mix (2x, 4369016, Applied Biosystems, Carlsbad, CA, USA), 1.25 μL TaqMan® gene expression assays (20x), 6.25 μL H2O and 5 μL cDNA. The cDNA of a CTL rat liver specimen was used to create the standard curves and calculate the amplification efficiency. Threshold cycle value and final quantification relative to the amount of 18s were calculated by the instrument software (version 4.10) automatically.

Western blot analyses

The tissues were homogenized on ice in Tissue Extraction Reagent I (Invitrogen) containing 50 mM Tris–HCl (pH 7.4), 250 mM NaCl, 5 mM ethylenediaminetetra-acetic acid, 2 mM sodium orthovanadate, 1 mM sodium fluoride, 20 mM sodium pyrophosphate tetrabasic, 0.02% sodium azide, 1% NP-40, 0.1% sodium dodecyl sulfate, proprietary detergent and Protease Inhibitor Cocktail (Sigma–Aldrich, St. Louis, MO, USA). Protein concentration in the tissue homogenates was determined by using DC protein assay kit (Bio-Rad, Hercules, CA, USA), and 100 μg of total protein per sample was fractionated on 4–12% Novex® Tris-Glycine gel (Invitrogen) at 120 V for 2 h and transferred to nitrocellulose membrane (Invitrogen). The membranes were incubated for 1 h in blocking buffer (1 x TBS, 0.05% Tween-20 and 5% nonfat milk) and then overnight in the same buffer containing the primary antibodies against: IDOL (SAB4501317, Sigma–Aldrich), at 1:500 LDLR (10c-CR1024M1, Fitzgerald Inc. Cambridge, UK) at 1 μg/mL and beta-actin (ab6276, Abcam, Cambridge, UK) at 1:5000. The membrane was washed three times for 10 min in 1 x TBST before a 2-h incubation in a buffer (1x TBST) containing horseradish peroxidase-conjugated anti-rabbit (1:3000) (ab6721, Abcam) and anti-mouse (1:2000) (NA931 V, GE Healthcare, Waukesha, WI, USA) secondary antibodies. The membrane was washed three times, then visualized with ECLTM prime western blot detection reagent (RPN2232, GE Healthcare) and developed by autoluminography. Band densities were quantified using the free ImageJ software (version 10.2) from the National Institutes of Health (www.imagej.nih.gov/ij/).

Data analysis

Student’s t-test and regression analysis were used in statistical analysis of the data, which are presented as mean ± SD. P-values of ≤0.05 were considered significant.

RESULTS

General data

Data are summarized in Table 1. As expected, compared with the CTL group, the nephrotic group had marked proteinuria and hypoalbuminemia. Heavy proteinuria in the nephrotic group was associated with a marked elevation in serum total cholesterol, LDL cholesterol, VLDL cholesterol, and triglyceride concentrations and total cholesterol/HDL cholesterol ratio. No significant difference was found in serum creatinine concentration or creatinine clearance between the nephrotic and CTL groups. Likewise, arterial blood pressure was comparable in the two groups. However, body weight was significantly lower in the nephrotic compared with the CTL group.

Hepatic LDLR, PCSK and IDOL expressions and LXR activity

Data are shown in Figures 1–4. In confirmation of our earlier studies [6–9] compared with the CTL group, the nephrotic animals exhibited marked reduction of hepatic tissue LDLR abundance. Acquired LDLR deficiency in the rats with NS was accompanied by a marked increase in hepatic tissue PCSK9 mRNA abundance. Moreover, compared with the normal CTL group, the nephrotic rats showed a significant increase in IDOL mRNA abundance. Upregulation of the IDOL gene expression in the liver of nephrotic animals was

Table 1. Plasma concentrations of total, LDL and VLDL cholesterol, albumin, creatinine and triglyceride, Total/HDL cholesterol concentration ratio, creatinine clearance (Ccr), urinary protein excretion and body weight in the NS and CTL groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>CTL</th>
<th>NS</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>58 ± 2.0</td>
<td>268 ± 18*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>28 ± 2.5</td>
<td>175 ± 18*</td>
</tr>
<tr>
<td>VLDL cholesterol, mg/dL</td>
<td>9.0 ± 0.9</td>
<td>34 ± 2.8*</td>
</tr>
<tr>
<td>Total/HDL cholesterol ratio</td>
<td>2.7 ± 0.3</td>
<td>4.8 ± 0.5*</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>47 ± 4.5</td>
<td>167 ± 15*</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>3.9 ± 0.1</td>
<td>2.2 ± 0.2*</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.37 ± 0.02</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Ccr, mL/min</td>
<td>2.7 ± 0.2</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Urine protein, mg/24h</td>
<td>11 ± 1.3</td>
<td>217 ± 25*</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>346 ± 8.3</td>
<td>329 ± 7.0</td>
</tr>
</tbody>
</table>

*P < 0.01

FIGURE 1: Representative western blots and group data depicting the LDLR and beta-actin abundance in the liver tissue of NS and CTL rats. *P < 0.05.
accompanied by a marked increase in its protein abundance. Since IDOL expression is regulated by LXR, we determined its nuclear translocation in the study animals. The results showed marked increase in the nuclear LXR abundance in the liver of nephrotic rats compared with that found in the CTL group pointing to its heightened activation in NS.

**Correlations**

A significant inverse correlation was found between serum cholesterol concentration and hepatic LDLR protein abundance \(r = -0.882; \ P < 0.02\) in the study animals. LDLR protein abundance was inversely related to PCSK9 \(r = -0.811; \ P < 0.05\) and IDOL abundance \(r = -0.717; \ P < 0.109\) in the liver of the study animals. Serum LDL cholesterol showed positive correlations with PCSK9 \(r = 0.937; \ P < 0.01\) and IDOL abundance \(r = 0.916; \ P < 0.01\) in the liver of the study animals.

**DISCUSSION**

The nephrotic animals employed in the present study exhibited hypercholesterolemia and elevation of LDL-cholesterol concentration, which were accompanied by and largely due to acquired LDLR deficiency as shown in our earlier studies [6–9]. The associated LDLR deficiency plays a central part in the pathogenesis of hypercholesterolemia by limiting removal of cholesterol-rich LDL particles from the circulation. The reduction in uptake of extracellular cholesterol, in turn, stimulates cholesterol biosynthesis by upregulating HMG-CoA reductase expression and activity in the nephrotic liver [7, 18]. LDLR deficiency in Imai rats with severe spontaneous focal segmental glomerulosclerosis and in SD rats with puromycin-induced NS is associated with normal LDLR mRNA expression pointing to a post-transcriptional etiology [6–9]. Upregulation of hepatic tissue PCSK9 and IDOL found in the present study unraveled the underlying mechanism of acquired LDLR deficiency in NS.

By mediating degradation and limiting recycling of LDLR, the observed upregulation of hepatic PCSK9 can contribute to the pathogenesis of the LDLR deficiency and the associated hypercholesterolemia and elevation of plasma LDL in NS. In fact, several studies have shown a direct correlation between plasma PCSK9 and LDL-cholesterol level in the general population [19–21] and a strong association between elevated plasma PCSK9 levels with recurrent clinical events in statin-treated patients with cardiovascular disease [22]. PCSK9 expression is primarily regulated by transcription factor, sterol-regulatory element-binding protein 2 (SREBP2) [23, 24], which also regulates expressions HMG-CoA reductase and LDLR. SREBP2 is activated by reduction and is inhibited by elevation of intracellular free cholesterol levels. The role of the reduction of hepatocyte cholesterol content in mediating upregulation of PCSK9 is evidenced by increased PCSK9

**FIGURE 2:** Bar graphs depicting mRNA abundance of PCSK9 in liver of NS and CTL groups. *P < 0.05.

**FIGURE 3:** Bar graphs depicting liver tissue IDOL mRNA and protein abundance in the NS and CTL groups. *P < 0.01.
expression and plasma concentration with statin treatment [25–27]. Several factors tend to reduce hepatocyte free cholesterol concentration and, thereby, promote activation of SREBP-2 in NS. Chief among them is acyl-CoA cholesterolacyltransferase-2 (ACAT-2), which catalyzes esterification of free cholesterol in the liver and is markedly upregulated in NS [8, 28]. The potential role of upregulation of ACAT-2 is evidenced by the dramatic amelioration of hypercholesterolemia and marked reduction in plasma LDL-cholesterol level with administration of an ACAT inhibitor in the nephrotic animals [8]. In addition, by limiting the removal of LD and its cholesterol cargo from the circulation, depletion of hepatic LDLR in the nephrotic liver contributes to the reduction of hepatocyte cholesterol, which can raise PCSK9 expression via activation of SREBP-2. Thus, upregulation of PCSK9 and depletion of LDLR can participate in a vicious circuit wherein each begets and amplifies the other.

Upregulation of the PCSK9, in our nephrotic animals, was accompanied by upregulation of IDOL, which is an intracellular mediator of LDLR degradation. IDOL is an E3 ubiquitin ligase that mediates ubiquitination and degradation of LDLR. IDOL expression is regulated by the LXR. Activation of LXR leads to induction of IDOL [10]. In fact, upregulation of hepatic tissue IDOL was accompanied by heightened activation of LXR as evidenced by significant increase in its nuclear content in the liver of our nephrotic rats. It should be noted that the LXR–IDOL driven feedback inhibition of cholesterol uptake is independent of and complementary to that of the SREBP–PCSK9 pathway. In this context, unlike IDOL which operates within the cell, PCSK9 induces the internalization of the LDLR by binding to its extracellular domain [29, 30]. Moreover, PCSK9 can mediate LDLR degradation in cells lacking IDOL, indicating that IDOL and PCSK9 operate via complementary but independent pathways [31].

In addition to the liver, IDOL is expressed in other tissues, including adipose tissue, intestine and macrophages. Recent studies have revealed that besides mediating ubiquitination of the LDLR, IDOL promotes degradation of VLDL receptor and ApoE receptor-2, which are members of the LDLR family [32]. Modulation of LDLR, VLDL receptor and ApoER2 expression by IDOL in these tissues/cells can have significant consequences. Earlier studies conducted in our laboratories have shown marked downregulation of LDLR receptor in adipose tissue, skeletal muscle and myocardium in animals with NS [33, 34]. In addition, recent studies by Wang et al. [35] have shown downregulation of VLDL receptor in the liver of nephrotic rats. The potential role of IDOL in the pathogenesis of VLDL receptor deficiency in NS is presently unknown and requires further investigation.

Earlier studies conducted in patients with NS have shown that elevation of serum LDL is associated with increased LDL synthesis and no change in LDL catabolism [36–38]. The disparity between LDL synthesis and its catabolism shown in the nephrotic patients is consistent with the acquired LDLR deficiency found in experimental animals. This is because by necessity the lack of rise in LDL clearance despite its increased production denotes relative deficiency of the LDL clearing capacity (i.e. LDLR deficiency) in the nephrotic individual.

It should be noted that the circulating PCSK9 levels display a marked diurnal variation that closely parallels that of cholesterol synthesis [39]. In addition, PCSK9 level is profoundly affected by fasting and feeding states [40]. For these reasons, the plasma and tissue specimens were obtained after 14 h fasting and between the hours of 8 and 10 a.m.

In conclusion, LDLR deficiency and the resultant hypercholesterolemia and elevation of plasma LDL cholesterol in NS are associated with and, at least in part, due to upregulation of PCSK9 and IDOL. By promoting degradation and limiting recycling of the LDLR, upregulation of PCSK9 and IDOL accounts for our previously demonstrated post-transcriptional LDLR deficiency in the nephrotic animals. The resulting LDLR deficiency, in turn, heightens the risk of cardiovascular events by raising the concentration and prolonging the residence time of LDL in the circulation.

CONFLICT OF INTEREST STATEMENT

None declared.

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Mechanism of LDL receptor deficiency in nephrotic syndrome

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Received for publication: 21.5.2013; Accepted in revised form: 10.9.2013