Acyl-Coenzyme A:Cholesterol Acyltransferase Inhibition Ameliorates Proteinuria, Hyperlipidemia, Lecithin-Cholesterol Acyltransferase, SRB-1, and Low-Density Lipoprotein Receptor Deficiencies in Nephrotic Syndrome

N.D. Vaziri, MD; K.H. Liang, MD

Background—Nephrotic syndrome (NS) is associated with hyperlipidemia, altered lipid regulatory enzymes and receptors, and increased risk of progressive renal and cardiovascular diseases. Acyl-coenzyme A:cholesterol acyltransferase (ACAT) catalyzes intracellular esterification of cholesterol and plays an important role in production of apolipoprotein B–containing lipoproteins, regulation of cholesterol-responsive proteins, and formation of foam cells. Because hepatic ACAT-2 is markedly upregulated in NS, we tested the hypothesis that inhibition of ACAT may improve cholesterol metabolism in NS.

Methods and Results—Rats with puromycin-induced NS were treated with either the ACAT inhibitor CI-976 or placebo for 2 weeks. Normal rats served as controls. Plasma lipids, renal function, and key lipid regulatory factors were measured. Untreated NS rats showed heavy proteinuria; hypoalbuminemia; elevated plasma cholesterol, triglyceride, LDL, VLDL, and total cholesterol–to–HDL cholesterol ratio; increased hepatic ACAT activity, ACAT-2 mRNA, and ACAT-2 protein; and reduced LDL receptor, HDL receptor, otherwise known as scavenger receptor B-1 (SRB-1) and plasma lecithin-cholesterol acyltransferase (LCAT). ACAT inhibitor reduced plasma cholesterol and triglycerides, normalized total cholesterol–to–HDL cholesterol ratio, and lowered hepatic ACAT activity without changing ACAT-2 mRNA or protein. This was accompanied by near normalizations of plasma LCAT, hepatic SRB-1, and LDL receptor and a significant amelioration of proteinuria and hypoalbuminemia.

Conclusions—Pharmacological inhibition of ACAT reverses NS-induced LDL receptor, HDL receptor, and LCAT deficiencies; improves plasma lipid profile; and ameliorates proteinuria in nephrotic animals. Further studies are needed to explore the effect of ACAT inhibition in nephrotic humans. (Circulation. 2004;110:419-425.)

Key Words: ACAT inhibitor ■ nephrotic syndrome ■ hyperlipidemia ■ cholesterol ■ proteinuria

Heavy glomerular proteinuria, known as nephrotic syndrome (NS), is associated with hyperlipidemia, increased risk of cardiovascular disease, and deterioration of renal function. Nephrotic dyslipidemia is marked by hypercholesterolemia; hypertriglyceridemia; elevated plasma concentration and impaired clearance of LDL, VLDL, and IDL; impaired maturation and diminished clearance of HDL; and increased plasma lipoprotein (a). These abnormalities are largely a result of dysregulation of the key enzymes and receptors involved in lipid metabolism: (1) LDL receptor deficiency, which, in part, accounts for elevated plasma level and impaired clearance of LDL; two mechanisms: (2) lecithin-cholesterol acyltransferase (LCAT) deficiency; (3) elevated plasma cholesterol ester transfer protein (CETP), and diminished hepatic SRB-1 (HDL receptor) which collectively account for abnormal composition, impaired maturation, and defective clearance of HDL; (3) dysregulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and cholesterol 7α-hydroxylase (rate-limiting enzyme in cholesterol conversion to bile acid), leading to development and maintenance of hypercholesterolemia; (4) upregulations of hepatic biosynthesis and diminished catabolism of apolipoprotein (apo) B-100 and increased production of lipoprotein (a), which contribute to their elevated plasma levels in NS; (5) downregulations of lipoprotein lipase (LPL) VLDL receptor coupled with upregulations of hepatic acylcoenzyme A:diacylglycerol acyltransferase (DGAT), the final step in triglyceride biosynthesis), coupled with upregulations of hepatic acylcoenzyme A carboxylase, and fatty acid synthase (key enzymes in fatty acid production). These abnormalities account for increased hepatic synthesis, impaired clearance, and elevated plasma triglycerides, VLDL, and IDL in NS (reviewed in Reference 1).
We have recently demonstrated a marked upregulation of acyl-coenzyme A:cholesterol acyltransferase (ACAT) in nephrotic rats. ACAT catalyzes esterification of cholesterol for incorporation into apoB-containing lipoproteins and intracellular storage. Two distinct ACATs have been identified thus far: ACAT-1, which is expressed in all mammalian cells, and ACAT-2, which is expressed primarily in the liver and white fat. In addition, esterification of cholesterol by ACAT can potentially modify the expression and/or activity of cholesterol-responsive proteins. Given the important role of ACAT in lipid metabolism and its marked upregulation in nephrotic rats, we sought to test the hypothesis that pharmacological inhibition of ACAT may improve plasma lipids and the underlying abnormalities of the lipid regulatory enzymes and receptors.

**Methods**

**Animals**

Male Sprague-Dawley rats were rendered nephrotic by sequential injections of puromycin aminonucleoside (130 mg/kg on day 1 and 60 mg/kg on day 14). Placebo-injected rats served as controls. On day 14, the nephrotic animals were randomized to the ACAT inhibitor–treated and untreated subgroups. The treated group received CI-976 compound (Pfizer Global Research and Development) 60 mg/kg on day 14. Placebo-injected rats served as controls. On day 14, animals were housed in a temperature-controlled facility with 12-hour light/dark cycles. At the end of the 2-week treatment period, animals were placed in metabolic cages for a 24-hour urine collection. They were then anesthetized (pentobarbital 50 mg/kg IP) and euthanized by exsanguination by 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.

**Reverse Transcription–Polymerase Chain Reaction**

RNA was isolated from frozen liver using TRIzol reagent (Invitrogen) and purified by RNeasy kit (Qiagen). One microgram total RNA from each sample was reverse transcribed to cDNAs by using Superscript II RT (Invitrogen) with a mixture of oligo(dT) (200 nmol/L reaction) and random primers (200 ng/reaction) in a 20-μL volume at 45°C for 50 minutes. The reaction was stopped by heating at 90°C for 5 minutes.

Expression of ACAT-2, LCAT, cholesterol 7α-hydroxylase, HMG-CoA reductase, SRB-1 (HDL receptor), and LDL receptor mRNAs was assessed by reverse transcription–polymerase chain reaction (RT-PCR) using 18S as internal control. The primer sequences are depicted in Table 1. ACAT-2, LCAT, cholesterol 7α-hydroxylase, HDL receptor, and LDL receptor primers were designed with Primer3 program purchased from Invitrogen. For 18S amplification, we used alternate 18S (Ambion), which yields a 324-bp product. In each PCR reaction, 18S ribosomal RNA was coamplified with the target cDNA (mRNA). The primers were tested for their compatibility with the alternate 18S primer. The cDNAs were amplified using standard PCR buffer, 0.2 mmol/L dNTP, 1 μmol/L specific primer set, 0.5 μmol/L 18S competitor mix, 0.5 μmol/L 18S primer/competitor mix, and 0.75 U of Taq DNA polymerase (Invitrogen) in 25 μL of total volume for 26 to 32 cycles. Each cycle consisted of 3 minutes denaturation at 94°C, 45 seconds of annealing at 57°C, and

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**TABLE 1. Sense and Antisense Primers Used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank No.</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAT-2</td>
<td>AB075946</td>
<td>GCTGAAAGTGAACACTACCCCTT</td>
<td>GACCCATGGCTCTAGATGCT</td>
<td>212</td>
</tr>
<tr>
<td>Ch-7a</td>
<td>X17595</td>
<td>GCTGGAGCATCCTGACATAC</td>
<td>GACCAATGTCACACGTCT</td>
<td>208</td>
</tr>
<tr>
<td>HDLr</td>
<td>U76205</td>
<td>GAGATCCCTGGTCCTCCCTCTCTCT</td>
<td>TAGACACTCTGAGCCCTCTGT</td>
<td>212</td>
</tr>
<tr>
<td>HMG-R</td>
<td>X55286</td>
<td>GCCTGAGCATCCTGACATAC</td>
<td>GACCAATGTCACACGTCT</td>
<td>208</td>
</tr>
<tr>
<td>LCAT</td>
<td>NM-017024</td>
<td>CTCTACATTGACCGAGCAAG</td>
<td>CATCTTCATAGTGAGCC</td>
<td>232</td>
</tr>
<tr>
<td>LDLr</td>
<td>X13722</td>
<td>CGAGTTTCACTGTAGAGACG</td>
<td>GGAGACGTAATGTCATAC</td>
<td>236</td>
</tr>
</tbody>
</table>

**TABLE 2. General Laboratory Data in the Control, Nephrotic (NS), and ACAT Inhibitor–Treated Nephrotic (NS-AI) Rats**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NS</th>
<th>NS-AI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>59 ± 2.2</td>
<td>264 ± 17*</td>
<td>105 ± 5.9*†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Free cholesterol, mg/dL</td>
<td>16.9 ± 2</td>
<td>71 ± 8.1*</td>
<td>43 ± 3.5*†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>27 ± 2.7</td>
<td>173 ± 16*</td>
<td>46 ± 1.6†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VLDL cholesterol, mg/dL</td>
<td>9.3 ± 0.9</td>
<td>33 ± 3*</td>
<td>18 ± 1.4*†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol/HDL ratio</td>
<td>2.7 ± 0.3</td>
<td>4.8 ± 0.5*</td>
<td>2.7 ± 0.1†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>47 ± 4.5</td>
<td>167 ± 15*</td>
<td>91 ± 7†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Free fatty acids, mEq/L</td>
<td>0.6 ± 0.004</td>
<td>1.1 ± 0.06*</td>
<td>0.8 ± 0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>3.8 ± 0.1</td>
<td>2.3 ± 0.2*</td>
<td>3.0 ± 0.1†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>2.6 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Urine protein, mg/24 h</td>
<td>11 ± 1.3</td>
<td>217 ± 25*</td>
<td>96 ± 12†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>346 ± 8</td>
<td>329 ± 8*</td>
<td>308 ± 9*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>116 ± 3</td>
<td>130 ± 4*</td>
<td>136 ± 4*</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*P < 0.05 vs NL; †P < 0.05 vs NS; n = 6 in each group.
45 seconds of extension at 72°C. PCR products were separated on a 2.5% agarose gel with ethidium bromide by electrophoresis. Signal intensity was determined by laser scanning densitometry. ACAT-2, LCAT, cholesterol 7α-hydroxylase, HDL receptor, and LDL receptor mRNAs were normalized to their corresponding 18S values.

**Western Blot Analysis and Activity Assays**

LDL receptor protein abundance in the liver tissue was measured by Western blot analysis using a mouse anti-bovine LDL receptor antibody (Cortex Biochem Inc). Microsomal HMG-CoA reductase and activity were determined as described previously. Plasma LCAT was quantified by ELISA using a rabbit anti-human LCAT antibody (generously provided by Professor John S. Parks, Wake Forest University, Winston-Salem, NC) as described previously. ACAT-2 protein was determined by Western analysis using a polyclonal ACAT-2 antibody (generously provided by Professor Lawrence Rudel, Wake Forest University) as described previously. Hepatic microsomal ACAT activity was determined by Western analysis using an antibody generously supplied by Professor John Y.L. Chiang (Northeastern Ohio University, Rootstown, Ohio) as described previously.

**Data Analysis**

ANOVA and multiple-range test were used in statistical evaluation of the data, which are shown as mean±SEM. Probability values of \( P<0.05 \) were considered significant.

**Results**

**General Data**

The untreated nephrotic animals exhibited heavy proteinuria; hypoalbuminemia; normal creatinine clearance; marked elevations of plasma total cholesterol, LDL, VLDL, triglyceride, free cholesterol and free fatty acid; and increased total cholesterol–to–HDL cholesterol ratio (Table 2). Administration of ACAT inhibitor resulted in marked reductions of plasma total cholesterol, free cholesterol, VLDL, LDL, triglycerides, and free fatty acids; normalization of plasma total cholesterol–to–HDL cholesterol ratio; and amelioration of proteinuria and hypoalbuminemia.

**Liver Cholesterol**

No significant difference was found in liver total cholesterol among the untreated nephrotic (21±0.6 mg/g protein), the ACAT inhibitor–treated nephrotic (25±0.7 mg/g protein), and the normal control groups (22±0.8 mg/g protein). Free cholesterol was significantly lower \( (P<0.05) \) in untreated nephrotic rats \( (14±0.3 \text{ mg/g protein}) \) compared with those in the normal control \( (18±0.3 \text{ mg/g protein}) \) and ACAT inhibitor–treated groups \( (17±0.2 \text{ mg/g protein}) \). Microsomal total cholesterol in the untreated nephrotic rats \( (31±2 \text{ mg/g protein}) \) was comparable to that in the control groups \( (33±0.8 \text{ mg/g protein}) \). ACAT inhibition raised microsomal total cholesterol in the nephrotic animals \( (39±1.2 \text{ mg/g protein}, P<0.05) \). In contrast, free microsomal cholesterol was significantly lower in the untreated nephrotic rats \( (11±0.9 \text{ mg/g protein}) \) than in the control group \( (15.8±1.1 \text{ mg/g protein}, P<0.05) \) and was nearly normalized by ACAT inhibitor \( (13±1.7 \text{ mg/g protein}) \).

**ACAT Data**

In confirmation of our earlier studies, hepatic ACAT-2 mRNA and protein abundance were markedly increased in the untreated nephrotic animals (Figure 1). ACAT inhibitor normalized ACAT activity without changing ACAT-2 mRNA or protein.

**LCAT Data**

As reported previously, the untreated nephrotic animals showed a significant reduction in plasma LCAT despite normal hepatic LCAT mRNA (Figure 2). ACAT inhibition nearly normalized plasma LCAT without altering LCAT mRNA abundance.

**LDL Receptor Data**

The untreated nephrotic animals showed a marked reduction in hepatic LDL receptor protein despite normal LDL receptor
mRNA, confirming our previous studies (Figure 3). Inhibition of ACAT normalized LDL receptor protein without altering its mRNA abundance.

**HDL Receptor (SRB-1) Data**

In confirmation of our previous study, the untreated nephrotic rats exhibited a marked reduction in the hepatic HDL receptor protein despite normal HDL receptor mRNA (Figure 4). Treatment with ACAT inhibitor normalized HDL receptor protein without affecting its mRNA abundance.

**HMG-CoA Reductase Data**

Despite a 4.5-fold increase in plasma cholesterol, liver tissue HMG-CoA reductase mRNA, protein, and activity were unchanged in untreated nephrotic rats (Figure 5). ACAT inhibition significantly reduced plasma cholesterol without altering hepatic HMG-CoA reductase expression or activity. Thus, the treatment improved the cholesterol biosynthetic capacity relative to plasma cholesterol in the nephrotic animals.

**Cholesterol 7α-Hydroxylase Data**

Despite severe hypercholesterolemia, hepatic tissue cholesterol 7α-hydroxylase mRNA and protein were unchanged in the untreated nephrotic animals, confirming our earlier studies (Figure 6). Hepatic cholesterol 7α-hydroxylase remained unchanged in the ACAT-inhibitor–treated nephrotic rats despite marked reduction of plasma cholesterol, pointing to improved cholesterol catabolic capacity relative to plasma cholesterol.

**Discussion**

The untreated nephrotic rats exhibited a marked upregulation of liver ACAT-2 mRNA and protein and total microsomal ACAT activity, confirming earlier studies. It is of note that the ACAT activity data represent the sum of ACAT-1 and -2 activities. Because ACAT-1 expression is unaffected by NS, the higher value seen in the untreated NS group is because of increased ACAT-2. ACAT-mediated esterification is essential for incorporation of cholesterol into apoB-containing lipoproteins (chylomicrons and VLDL). Moreover, modulation of intracellular free and esterified cholesterol by ACAT contributes to regulation of cholesterol-responsive proteins, such as cholesterol a7α-hydroxylase. Upregulation of hepatic ACAT-2 in the untreated nephrotic group was accompanied by a nearly 6-fold rise in cholesterol content of apoB-containing lipoproteins (LDL plus VLDL) in the fasting plasma. Given the critical role of ACAT in production of VLDL and hence its final byproduct, LDL, upregulation of hepatic ACAT-2 can contribute to this abnormality by compounding the effects of LDL receptor and VLDL receptor deficiencies in NS. Administration of IC-976, an inhibitor of ACAT-1 and -2, normalized microsomal ACAT activity, which led to a rise in microsomal free cholesterol.
cholesterol (diminished ACAT-mediated esterification) and a decline in plasma total, LDL, and VLDL cholesterol levels. This was associated with and, in part, caused by amelioration of LDL receptor deficiency. It thus appears that upregulation of hepatic ACAT may directly or indirectly contribute to dysregulation of LDL receptor in NS. In addition, amelioration of proteinuria must have contributed to improvement of LDL receptor deficiency.

HDL is the principal vehicle for removal of surplus cholesterol from the peripheral tissues for disposal in the liver (reverse cholesterol transport). Esterification of cholesterol by LCAT is critical for optimal cholesterol uptake and maturation of HDL-3 to HDL-2.47 Maturation of HDL-3 to HDL-2 is impaired in NS.1–4,14 This is because of acquired LCAT deficiency, which was nearly corrected by ACAT inhibitor.

SRB-1, otherwise known as HDL receptor, serves as a docking platform for unloading of the HDL cholesterol and triglyceride contents in the liver.48 The untreated nephrotic rats exhibited downregulation of hepatic SRB-1, which accounts for impaired HDL clearance in NS. Administration of ACAT inhibitor normalized SRB-1 abundance. Amelioration of LCAT and SRB-1 deficiencies by ACAT inhibition can enhance reverse cholesterol transport and thus lower the risk of renal-cardiovascular complications in NS. In fact, plasma total cholesterol–to–HDL cholesterol ratio, which was nearly double the control value in the nephrotic animals, was normalized by ACAT inhibition.

Despite marked reductions of plasma cholesterol, hepatic HMG-CoA reductase did not increase and cholesterol 7α-hydroxylase did not decrease in the IC-976–treated nephrotic animals. This is most likely related to the modest rise in microsomal free cholesterol (which regulates expression and activity of these enzymes) and restoration of LDL receptor and SRB-1–mediated cholesterol uptake. ACAT inhibition led to a significant attenuation of hypertriglyceridemia in the nephrotic rats. Several factors may have contributed to this phenomenon: first, reversal of LCAT deficiency and the associated improvement of HDL-3 maturation to HDL-2 can...
enhance LPL-mediated hydrolysis of triglycerides in VLDL. This is because HDL-2 is an efficient donor of apoE (LPL and VLDL receptor ligand) and apoC-II (LPL cofactor) to the nascent VLDL, an event that is critical for optimal lipolysis of VLDL. Second, improvement in HDL metabolism can enhance lipolysis of IDL by hepatic triglyceride lipase via HDL-2-mediated extraction of apoE and apoC, which interfere with the lipolytic action of this enzyme. Third, amelioration of SRB-1 deficiency can enhance hydrolysis of triglyceride content of HDL by hepatic triglyceride lipase. Finally, the reduction in severity of proteinuria must have contributed to the improvements of the lipid regulatory enzymes and receptors and plasma lipids.

As noted above, improvement in lipid profile with pharmacological inhibition of ACAT resulted in a significant amelioration of proteinuria and hypoalbuminemia in the nephrotic animals. Similarly, HMG-CoA reductase inhibitors attenuate proteinuria in nephrotic humans and animals.49–54 These observations point to the existence of a vicious circle involving proteinuria and hyperlipidemia.

In conclusion, pharmacological inhibition of ACAT reverses LDL receptor, HDL receptor, and LCAT deficiencies, improves plasma lipid profile, and ameliorates proteinuria in rats with NS. Further studies are needed to explore the effect of ACAT inhibition in nephrotic humans.

References
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