ACAT inhibition reverses LCAT deficiency and improves plasma HDL in chronic renal failure

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Submitted 28 April 2004; accepted in final form 21 July 2004

Vaziri, N. D., and K. Liang. ACAT inhibition reverses LCAT deficiency and improves plasma HDL in chronic renal failure. Am J Physiol Renal Physiol 287: F1038–F1043, 2004. First published July 27, 2004; doi:10.1152/ajprenal.00150.2004.—Chronic renal failure (CRF) is associated with increased risk of arteriosclerotic cardiovascular disease and profound alteration of plasma lipid profile. Uremic dyslipidemia is marked by increased plasma concentration of ApoB-containing lipoproteins and impaired high-density lipoprotein (HDL)-mediated reverse cholesterol transport. These abnormalities are, in part, due to acquired LCAT deficiency and upregulation of hepatic acyl-CoA:cholesterol acyltransferase (ACAT). ACAT catalyzes intracellular esterification of cholesterol, thereby promoting hepatic production of ApoB-containing lipoproteins and conserving HDL-mediated cholesterol uptake in the peripheral tissues. In view of the above considerations, we tested the hypothesis that pharmacological inhibition of ACAT may ameliorate CRF-induced dyslipidemia. 5/6 Nephrectomized rats were treated with either ACAT inhibitor IC-976 (30 mg·kg⁻¹·day⁻¹) or placebo for 6 wk. Sham-operated rats served as controls. Key cholesterol-regulating enzymes, plasma lipids, and creatinine clearance were measured. The untreated CRF rats exhibited increased plasma low-density lipoprotein (LDL) and very LDL (VLDL) cholesterol, unchanged plasma HDL cholesterol, elevated total cholesterol-to-HDL cholesterol ratio, reduced liver microsomal free cholesterol, and diminished creatinine clearance. This was accompanied by reduced plasma LCAT, increased hepatic ACAT-2 mRNA, ACAT-2 protein and ACAT activity, and unchanged hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase. ACAT inhibitor raised plasma HDL cholesterol, lowered LDL and VLDL cholesterol, and normalized total cholesterol-to-HDL cholesterol ratio without changing total cholesterol concentration (hence, a shift from ApoB-containing lipoproteins to HDL). This was accompanied by normalizations of hepatic ACAT activity and plasma LCAT. In conclusion, inhibition of ACAT reversed LCAT deficiency and improved plasma HDL level in CRF rats. Future studies are needed to explore the efficacy of ACAT inhibition in humans with CRF.

high-density lipoprotein: hyperlipidemia; lecithin:cholesterol acyltransferase; cholesterol; acyl-CoA: cholesterol acyltransferase; progression of renal disease; atherosclerosis; cardiovascular disease; end-stage renal disease

ACCELERATED ATHEROSCLEROTIC cardiovascular disease is a major complication of chronic renal failure (CRF) and the main cause of mortality among dialysis-dependent and dialysis-independent patients (3, 10). The atherogenic diathesis in this population is, in part, due to profound abnormalities of high-density lipoprotein (HDL) and triglyceride-rich Apo-B-containing lipoproteins (2, 7, 13, 27). The CRF-associated HDL abnormalities are marked by a reduction of plasma HDL cholesterol relative to the non-HDL cholesterol concentration and impaired maturation of cholesterol ester-poor HDL-3 to cholesterol ester-rich cardioprotective HDL-2 (2, 27). This abnormality is largely due to marked downregulation of lecithin:cholesterol acyltransferase (LCAT) (12, 23, 33), which is essential for optimal cholesterol uptake by HDL. The CRF-induced abnormalities of triglyceride-rich lipoprotein metabolism are manifested by hypertriglyceridemia, elevated level and impaired clearance of very-low-density lipoprotein (VLDL), increased plasma intermediate-density lipoprotein (IDL) concentration, and depressed clearance of chylomicrons (2, 7, 13, 26, 27). These abnormalities are primarily due to downregulation of adipose tissue and skeletal muscle lipoprotein lipase (29, 36) and VLDL receptor (20, 30), as well as hepatic triglyceride lipase deficiency (16).

In a recent study, we found marked upregulation of hepatic acyl-CoA:cholesterol acyltransferase-2 (ACAT-2) mRNA, ACAT-2 protein, and total ACAT activity in rats with CRF induced by 5/6 nephrectomy (22) and advanced spontaneous focal glomerulosclerosis (35). ACAT catalyzes intracellular esterification of cholesterol and formation of cholesterol ester. This process is essential for intestinal absorption of cholesterol, production and secretion of apolipoprotein B-containing lipoproteins, regulation of cholesterol-responsive proteins, as well as foam cell formation, which plays an integral part in atherogenesis (1, 5, 6, 8, 9, 15, 24). HDL-mediated retrieval of surplus cholesterol from the peripheral tissues depends on hydrolysis of cholesterol esters contained in the intracellular fat droplets (by neutral cholesterol ester hydrolase) and the consequent migration of free cholesterol to the cell membrane (11). It is, therefore, intuitive that by promoting intracellular esterification of cholesterol, ACAT can hinder HDL-mediated uptake of free cholesterol from the peripheral tissues.

In view of the critical role of ACAT in lipid metabolism and its marked upregulation in CRF (22), we hypothesized that pharmacological inhibition of ACAT may ameliorate the associated lipid disorders. The present study was designed to test this hypothesis.

MATERIALS AND METHODS

Animal models. Male Sprague-Dawley rats, weighing 225–250 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were housed in a climate-controlled, light-regulated facility with 12:12-h day-night cycles. The animals were fed regular rat chow (Purina Mills, Brentwood, MO) and water ad libitum and randomly assigned to the CRF and control groups. The animals assigned to the CRF group were subjected to 5/6 nephrectomy by surgical resection
using a dorsal incision, as described previously (34). The animals assigned to the control group were subjected to sham operation. All surgical procedures were carried out under general anesthesia (Nembutal 50 mg/kg ip). Strict hemostasis and aseptic techniques were observed. After a 2-wk recovery period, the CRF rats were randomly assigned to the ACAT inhibitor-treated and untreated groups. The ACAT inhibitor-treated group received the IC-976 compound (30 mg·kg\(^{-1}\)·day\(^{-1}\); Pfizer Global Research and Development, Groton, CT) mixed in powdered rat chow for 6 wk. The untreated CRF and sham-operated control rats received drug-free powdered food. The ACAT inhibitor-treated group received the IC-976 compound (30 mg·kg\(^{-1}\)·day\(^{-1}\)) assigned to the ACAT inhibitor-treated and untreated groups. The animals were placed in metabolic cages for a 24-h urine collection. They were then killed by exsanguination via cardiac puncture between the hours of 9 and 11 AM. The liver was removed immediately, snap-frozen in liquid nitrogen, and stored at -70°C until processed. Serum cholesterol, triglyceride, LDL, VLDL, HDL, and creatinine concentrations were determined by standard automated methods. The cholesterol concentration was quantified in plasma by using an ELISA kit (BIOCHIM, Northville, MI) and determined in a manner which was identical to that described in our previous studies (31, 35). The polyclonal anti-HMG-CoA reductase antibody used for Western analysis was generous gift from Prof. G. C. Ness, Department of Biochemistry and Molecular Biology, University of South Florida, Tampa, FL. The protocol employed in this study was approved by the Institutional Committee for Care and Use of Animals at the University of California, Irvine.

Preparation of liver microsomes. Four hundred milligrams of frozen liver were homogenized in ice-cold buffer A containing 50 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, and 1 mM PMSF, pH 7.4. Microsomes were then isolated by differential centrifugation as described previously (22). The microsomal membranes were then resuspended in buffer A, divided into aliquots, and stored at -70°C until used.

RNA isolation and RT–PCR. RNA was isolated from frozen liver using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified by RNeasy kit (Qiagen, Valencia, CA). One microgram total RNA from each sample was reverse transcribed to cDNA by using Superscript II RT (Invitrogen) with a mixture of oligo(dT) (200 ng/reaction) and random primers (200 ng/reaction) in a 20-µl reaction with Superscript RT (Invitrogen). cDNAs were amplified using standard PCR buffer, 0.2 mM dNTP, 1 µM specific primer set, 0.5 µM 18S primer/competimer mix, 2 mM MgCl\(_2\), and 0.75 unit of Taq DNA polymerase (Invitrogen) in 25 µl of total volume for 26 to 32 cycles. Each cycle consisted of 3-min denaturation at 94°C and 45-s annealing at 57°C and 45-s 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, and HMG-CoA reductase mRNAs were normalized to their corresponding 18S ribosomal RNA.

### Table 1. Sense and antisense primers used for quantification of ACAT-2, Ch-7α, and HMG-R mRNA abundance by 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>gcttgagtaactccctct</td>
<td>gacactgcctctagctct</td>
<td>212</td>
</tr>
<tr>
<td>Ch-7α</td>
<td>X17595</td>
<td>gcttgagcatctgagcatac</td>
<td>gacactgcactctagctct</td>
<td>208</td>
</tr>
<tr>
<td>HMG-R</td>
<td>X55286</td>
<td>gcttgagcatctgagcatac</td>
<td>gacactgcactctagctct</td>
<td>208</td>
</tr>
</tbody>
</table>

Ch-7α, cholesterol 7α-hydroxylase; HMG-R, HMG-CoA reductase.

Measurements of HMG-CoA reductase protein and activity. Hepatic microsomal HMG-CoA reductase abundance and activity were determined in a manner which was identical to that described in our previous studies (31, 35). The polyclonal anti-HMG-CoA reductase antibody used for Western analysis was a generous gift from Prof. J. Y. L. Chiang (Northeastern Ohio University) as described in our previous studies (19).

Measurements of plasma LCAT concentration. Plasma LCAT concentration was quantified by ELISA using an antibody generously provided by Prof. J. S. Parks, Wake Forest University, Winston-Salem, NC, as described in our earlier studies (35).

ACAT measurements. ACAT-2 protein abundance in the liver was determined by Western blot analysis using a polyclonal ACAT-2 antibody (generously provided by Prof. L. Rudel, Wake Forest University, Winston-Salem, NC) as described previously (33). Hepatic microsomal ACAT activity was quantified in a manner which was identical to that described in our earlier studies (32).

Data analysis. ANOVA and a multiple range test were used in statistical evaluation of the data, which are shown as means ± SE. P values <0.05 were considered significant.

### RESULTS

General data. Data are summarized in Table 2. As expected, the CRF group exhibited a significant reduction in creatinine clearance, a modest reduction in body weight, and marked elevation of arterial blood pressure. The ACAT inhibitor-treated group showed a significantly higher creatinine clearance than that seen in the untreated CRF group. However, the CRF group exhibited a significant reduction in hepatic cholesterol 7α-hydroxylase and microsomal-free cholesterol levels compared to CRF-AI, with no significant differences in body weight or systolic pressure. The CRF group also showed a significant increase in liver total cholesterol concentration.

### Table 2. General data in untreated rats with CRF, CRF-AI, and normal control rats

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Control</th>
<th>CRF</th>
<th>CRF-AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>2.6±0.2</td>
<td>0.5±0.09*</td>
<td>0.9±0.07†</td>
</tr>
<tr>
<td>Total chol, mg/dl</td>
<td>59±2</td>
<td>124±11*</td>
<td>143±10*</td>
</tr>
<tr>
<td>LDL chol, mg/dl</td>
<td>27±2</td>
<td>69±10*</td>
<td>49±10†</td>
</tr>
<tr>
<td>VLDL chol, mg/dl</td>
<td>9±3</td>
<td>33±3.4*</td>
<td>29±2.6†</td>
</tr>
<tr>
<td>HDL chol, mg/dl</td>
<td>23±2.4</td>
<td>36±4.2</td>
<td>66±6.2†</td>
</tr>
<tr>
<td>Total chol/HDL chol, ratio</td>
<td>2.7±0.3</td>
<td>3.7±0.5*</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>47±4.5</td>
<td>166±16.8*</td>
<td>144±13*</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>3.8±0.1</td>
<td>3.1±0.06*</td>
<td>3.1±0.1*</td>
</tr>
<tr>
<td>Liver total chol, mg/g protein</td>
<td>19±1.2</td>
<td>23±1.3</td>
<td>21±3.1</td>
</tr>
<tr>
<td>Microsomal-free chol, mg/g protein</td>
<td>10.3±1</td>
<td>6.1±0.2*</td>
<td>8.1±0.5</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>389±16</td>
<td>366±8*</td>
<td>368±13*</td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>123±2</td>
<td>165±4*</td>
<td>168±4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 in each group. chol, cholesterol; LDL, low-density lipoprotein; VLDL, very LDL; HDL, high-density lipoprotein; CRF, chronic renal failure; CRF-AI, ACAT inhibitor-treated CRF. *P < 0.05 vs. control. †P < 0.05 vs. CRF.
ACAT inhibition had no effect on either body weight or arterial pressure.

Measurements of plasma lipids revealed significant elevations of triglycerides, total cholesterol, VLDL, and LDL concentrations in the untreated CRF rats. The elevations of serum total cholesterol, LDL, and VLDL cholesterol values were accompanied by a normal plasma HDL concentration and consequently a high total cholesterol-to-HDL cholesterol concentration ratio in the untreated CRF group. Administration of ACAT inhibitor for 6 wk resulted in a dramatic rise in HDL cholesterol, a modest reduction of VLDL and LDL cholesterol, and no significant change in total cholesterol concentration, pointing to a shift in cholesterol from ApoB-containing lipoproteins to HDL. Consequently, plasma total cholesterol-to-HDL cholesterol ratio was reduced from an abnormally high value to one that was significantly lower than that seen in the control group. Plasma triglyceride concentration was markedly elevated in the CRF group and was not significantly affected by ACAT inhibition.

Hepatic ACAT. Data are illustrated in Fig. 1. In confirmation of our previous studies (22), the untreated CRF animals exhibited significant increases in hepatic tissue ACAT-2 mRNA, ACAT-2 protein, and total ACAT enzymatic activity. Administration of IC-976 for 6 wk resulted in normalization of hepatic ACAT enzymatic activity without changing either ACAT-2 mRNA or protein abundance. This finding confirms the efficacy of the compound employed in inhibiting ACAT activity in vivo.

LCAT. Data are shown in Fig. 2. The untreated CRF group exhibited a significant reduction in plasma LCAT concentration, confirming our earlier studies (33). Administration of ACAT inhibitor resulted in complete normalization of plasma LCAT.

HMG-CoA reductase and cholesterol 7α-hydroxylase. Data are depicted in Figs. 3 and 4. Hepatic tissue HMG-CoA reductase mRNA, protein, and enzymatic activity were unchanged by either CRF or ACAT inhibition. Similarly, cholesterol 7α-hydroxylase mRNA and protein were unchanged in the treated and untreated CRF rats.

DISCUSSION

The untreated CRF animals exhibited marked elevations of plasma triglyceride and VLDL concentrations and modest elevation of total cholesterol and LDL cholesterol levels. However, HDL cholesterol concentration was nearly unchanged and total cholesterol-to-HDL cholesterol ratio was markedly elevated in the CRF animals. Because cellular cholesterol homeostasis is largely determined by the balance between the LDL-mediated influx and HDL-mediated efflux of cholesterol, the observed changes in plasma lipids are consistent with a shift in hepatic ACAT activity in vivo.
In this study, we used 2,2-dimethyl-N-(2,4,6-trimethoxyphenyl) dodecanamide (CI-976), which is a potent ACAT inhibitor (4). Using this compound, we recently found marked amelioration of plasma lipid profile and several lipid-regulatory proteins in rats with nephrotic syndrome (28). The present study showed that pharmacological inhibition of ACAT with CI-976 for 6 wk resulted in a twofold rise in plasma HDL concentration in the treated CRF animals. This was accompanied by modest reductions in LDL and VLDL cholesterol concentrations and no significant change in plasma total cholesterol concentration. Accordingly, administration of ACAT inhibitor led to a shift in plasma cholesterol from apolipoprotein B-containing lipoproteins (LDL and VLDL) to HDL, hence a marked improvement in the corresponding ratio.

The dramatic rise in plasma HDL concentration observed in the ACAT inhibitor-treated rats was accompanied by the reversal of CRF-induced LCAT deficiency. This phenomenon

cholesterol, this unfavorable ratio, consistently found in the CRF humans (and animals), can, in part, contribute to atherosclerosis and possibly glomerulosclerosis by expanding cellular cholesterol burden.

Fig. 3. Representative RT-PCR and group data depicting hepatic HMG-CoA reductase and 18s rRNA abundance, representative Western blot and group data depicting hepatic HMG-CoA reductase protein abundance and HMG-CoA reductase enzymatic activity in the untreated rats with CRF, CRF-AI, and normal CTL group. No significant differences were found among the study groups; n = 6 in each group.

Fig. 4. Representative RT-PCR and group data depicting hepatic cholesterol 7α-hydroxylase (Ch-7α) and 18s rRNA abundance, and representative Western blots and group data depicting hepatic Ch-7α protein abundance in the untreated rats with CRF, CRF-AI, and normal CTL group. No significant differences were found among the study groups; n = 6 in each group.
must have played an important part in the observed rise in HDL cholesterol concentration. This is because by esterifying free cholesterol on the surface of HDL and consequent sequestration of cholesterol ester in the core of HDL, LCAT helps to maintain a favorable concentration gradient for uptake of free cholesterol by HDL from the peripheral cells (11).

HDL-mediated cholesterol uptake from the peripheral tissues depends on hydrolysis of cholesterol esters contained in the intracellular fat droplets (by neutral cholesterol ester hydrolase) and migration of free cholesterol to the cell membrane (11). To the extent that ACAT promotes intracellular esterification of cholesterol, its biological action hinders HDL-mediated uptake of free cholesterol. Consequently, pharmacological inhibition of ACAT in the extrahepatic tissues can facilitate HDL-mediated reverse cholesterol transport. This phenomenon can, therefore, contribute to the observed rise in plasma HDL cholesterol to supranormal levels in our ACAT inhibitor-treated CRF animals.

The authors wish to point out that, unlike humans, rats lack cholesteryl ester transfer protein (CETP) (25). This protein mediates the transfer of cholesterol ester contained in HDL-2 in exchange for triglyceride contained in IDL within the circulation. The absence of CETP in the rat and its presence in humans are responsible for the higher HDL cholesterol level in rat plasma than that in human plasma. Accordingly, we predict that due to CETP-mediated transport of cholesterol from HDL to ApoB-containing lipoproteins, the magnitude of rise in HDL cholesterol and the corresponding fall in total cholesterol-to-HDL cholesterol ratio in response to ACAT inhibition would be less striking in CRF humans than in the CRF rats.

In confirmation of our earlier studies in CRF rats with 5/6 nephrectomy (22) and advanced spontaneous glomerulosclerosis (35), liver-specific ACAT-2 mRNA and protein abundance as well as total ACAT enzymatic activity were markedly elevated in the untreated CRF group employed in the present study. It is of note that according to our earlier studies (22, 35), the observed increase in hepatic total ACAT activity is sole due to upregulation of liver-specific ACAT-2 isotype because ACAT-1 mRNA and protein are unchanged in the CRF liver (22). Consequently, ACAT-1 mRNA and protein were not measured in this study. Administration of IC-976 resulted in normalization of hepatic ACAT enzymatic activity without altering the corresponding mRNA or protein expression. This observation is consistent with the expected pharmacological action of this agent as an inhibitor of both ACAT-1 and ACAT-2 activity (4).

Hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase, which represent key enzymes in cholesterol biosynthesis and catabolism, were unchanged in the CRF animals. Chronic ACAT inhibition had no discernible impact on either HMG-CoA reductase or cholesterol 7α-hydroxylase levels in the liver of CRF animals.

The ACAT inhibitor-treated CRF rats showed a higher creatinine clearance than that seen in the untreated CRF group observed for 6 wk. This observation suggests that pharmacological inhibition of ACAT may help to directly and/or indirectly (e.g., via improvement of HDL metabolism) retard deterioration of renal function in animals with renal mass reduction. It should be noted that the difference in creatinine clearance between the treated and untreated CRF groups, while statistically significant, was small and the duration of treatment (6 wk) was relatively short. Thus further studies are required to explore the renoprotective action, if any, of an ACAT inhibitor. It is of interest that earlier studies identified diminished HDL cholesterol as an independent risk factor for progression of renal disease in humans (14). In fact, patients with hereditary LCAT deficiency, which leads to profound abnormality of HDL metabolism, frequently exhibit progressive renal disease and premature arteriosclerotic cardiovascular disease (17). Together, these observations support the role of impaired HDL metabolism in the pathogenesis and progression of renal disease. It is, therefore, plausible that reversal of the CRF-induced acquired LCAT deficiency and improvement of HDL metabolism by ACAT inhibition may favorably affect progression of renal disease in CRF animals and humans. Further studies are required to explore this possibility.

In conclusion, pharmacological inhibition of ACAT in rats with renal mass reduction reversed CRF-induced LCAT deficiency and significantly raised plasma HDL cholesterol concentration. Further studies are needed to explore the effect of ACAT inhibition in humans with chronic kidney disease.

REFERENCES