Cholesterol Synthesis Inhibition Elicits an Integrated Molecular Response in Human Livers Including Decreased ACAT2

Paolo Parini, Ulf Gustafsson, Matt A. Davis, Lilian Larsson, Curt Einarsson, Martha Wilson, Mats Rudling, Hiroshi Tomoda, Satoshi Omura, Staffan Sahlin, Bo Angelin, Lawrence L. Rudel, Mats Eriksson

Objective—The purpose of this study was to identify how different degrees of cholesterol synthesis inhibition affect human hepatic cholesterol metabolism.

Methods and Results—Thirty-seven normocholesterolemic gallstone patients randomized to treatment with placebo, 20 mg/d fluvastatin, or 80 mg/d atorvastatin for 4 weeks were studied. Based on serum lathosterol determinations, cholesterol synthesis was reduced by 42% and 70% in the 2 groups receiving statins. VLDL cholesterol was reduced by 20% and 55%. During gallstone surgery, a liver biopsy was obtained and hepatic protein and mRNA expression of rate-limiting steps in cholesterol metabolism were assayed and related to serum lipoproteins. A marked induction of LDL receptors and 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase was positively related to the degree of cholesterol synthesis inhibition (ChSI). The activity, protein, and mRNA for ACAT2 were all reduced during ChSI, as was apoE mRNA. The lowering of HDL cholesterol in response to high ChSI could not be explained by altered expression of the HDL receptor CLA-1, ABCA1, or apoA-I.

Conclusions—Statin treatment reduces ACAT2 activity in human liver and this effect, in combination with a reduced ApoE expression, may contribute to the favorable lowering of VLDL cholesterol seen in addition to the LDL lowering during statin treatment. (Arterioscler Thromb Vasc Biol 2008;28:1200-1206)

Key Words: cholesterol ■ lipoprotein ■ liver ■ receptors ■ apolipoproteins

Lipid-lowering therapy by inhibition of the activity of hydroxyl-methyl-glutaryl CoA (HMG CoA) reductase, the rate-limiting enzyme in cholesterol synthesis, represents a major breakthrough in modern medicine. With cholesterol synthesis inhibition (ChSI), plasma levels of atherogenic LDL particles can be substantially reduced resulting in lower cardiovascular morbidity and mortality both in patients with and without manifest disease. The lipid-lowering effects of this class of drugs (statins) are generally ascribed to the compensatory increase in hepatic LDL receptor expression resulting from the activation of the transcription factor sterol regulatory element binding protein 2 (SREBP-2) which occurs in response to reduced cholesterol availability in the liver after ChSI. At higher degrees of HMG CoA reductase inhibition, the concentrations of VLDL cholesterol and triglycerides are reduced, whereas HDL cholesterol levels may tend to be higher, unchanged, or somewhat lowered depending on the statin that is given. The mechanism(s) behind the latter changes are less well characterized, and their relationship to the more positive clinical effects of high-dose ChSI on cardiovascular disease has been debated.

We have recently demonstrated the presence of a specific enzyme catalyzing the esterification of cholesterol in human hepatocytes: acyl-CoA:cholesterol acyltransferase 2 (ACAT2). On the basis of animal studies (for review see), we have postulated that this enzyme—in contrast to ACAT1 which is the major cholesterol esterifying enzyme in cells other than hepatocytes and enterocytes—is involved in the secretion of cholesteryl esters in VLDL from the liver. To further characterize the function of ACAT2, we have now performed a detailed study of the changes in hepatic cholesterol metabolism induced by low and high degrees of ChSI in human liver using a randomized placebo-controlled design. Our studies demonstrate that ChSI by statins resulted in increasing induction of HMG CoA reductase and LDL receptors and a parallel reduction of ACAT2 and apoE expression. These changes occurred together with reduced plasma levels of VLDL and LDL cholesterol. The slight lowering of HDL
The goal of the study was to elucidate the molecular effects of varying degrees of ChSI on hepatic cholesterol metabolism in nonobese gallstone patients, randomized to treatment with placebo (Placebo), fluvastatin 20 mg/d (Low-ChSI), or atorvastatin 80 mg/d (High-ChSI) for 4 weeks before surgery.

Table 1. Baseline Clinical Characteristics and Plasma Lipid Levels

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Low ChSI</th>
<th>High ChSI</th>
<th>Placebo</th>
<th>Low ChSI</th>
<th>High ChSI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F/F/F</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>50.6±3.9</td>
<td>48.7±3.9</td>
<td>54.3±4.1</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.6±1.3</td>
<td>29.5±1.6</td>
<td>26.7±1.1</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma total cholesterol, mmol/L</td>
<td>5.38±0.46</td>
<td>5.37±0.35</td>
<td>5.75±0.33</td>
<td>NS</td>
<td>5.30±0.44</td>
<td>4.38±0.31</td>
<td>3.13±0.18</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>1.41±0.24</td>
<td>1.48±0.14</td>
<td>1.23±0.14</td>
<td>NS</td>
<td>1.29±0.22</td>
<td>1.51±0.33</td>
<td>0.94±0.13</td>
</tr>
<tr>
<td>VLDL cholesterol, mmol/L</td>
<td>0.80±0.14</td>
<td>0.95±0.12</td>
<td>0.80±0.09</td>
<td>NS</td>
<td>0.83±0.12</td>
<td>0.76±0.08</td>
<td>0.36±0.04</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.69±0.29</td>
<td>2.79±0.24</td>
<td>3.05±0.21</td>
<td>NS</td>
<td>2.61±0.30</td>
<td>2.12±0.18</td>
<td>1.22±0.10</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.89±0.09</td>
<td>1.63±0.09</td>
<td>1.90±0.10</td>
<td>NS</td>
<td>1.86±0.10</td>
<td>1.51±0.10</td>
<td>1.54±0.10</td>
</tr>
<tr>
<td>Apolipoprotein B, g/L</td>
<td>1.07±0.12</td>
<td>1.08±0.08</td>
<td>1.17±0.12</td>
<td>NS</td>
<td>1.02±0.10</td>
<td>0.90±0.06</td>
<td>0.50±0.04</td>
</tr>
<tr>
<td>Apolipoprotein A1, g/L</td>
<td>1.26±0.05</td>
<td>1.27±0.08</td>
<td>1.47±0.07</td>
<td>NS</td>
<td>1.24±0.04</td>
<td>1.22±0.07</td>
<td>1.34±0.10</td>
</tr>
<tr>
<td>Lathosterol/cholesterol, μg/mg</td>
<td>1.02±0.06</td>
<td>1.12±0.09</td>
<td>0.93±0.12</td>
<td>NS</td>
<td>0.84±0.09</td>
<td>0.65±0.08</td>
<td>0.29±0.03</td>
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M indicates male; FF, fertile female; PF, postmenopausal female. Data show mean±SEM. NS indicates not significant, ANOVA.

A tendency to a reduction in total cholesterol was observed with Placebo (Figure 1A), whereas Low-ChSI and High-ChSI resulted in 18% (P<0.05) and 44% (P<0.001) reductions, respectively. Similarly, a nonsignificant reduction in the lathosterol/cholesterol ratio was observed with Placebo (Figure 1B), whereas Low-ChSI and High-ChSI induced significant reductions of 42% (P<0.05) and 70% (P<0.001), respectively. Hence, 3 different levels of cholesterol synthesis were clearly apparent after treatment in the three experimental groups.

Analysis of LDL receptor protein expression in pooled hepatic membranes showed an increase which was inversely related to the degree of ChSI (Figure 1C). Measurement of the LDL receptor gene expression showed a significant (2.7-fold) induction of the mRNA levels only in the High-ChSI group (P<0.05; Figure 1D). To further verify the expected transcriptional effects induced by the treatments, hepatic mRNA levels of HMG CoA reductase were determined. Corresponding to the LDL receptor changes, HMG CoA reductase mRNA was induced in the High-ChSI group (P<0.05; Figure 1E) whereas no significant change was observed in the Low-ChSI group. Accordingly, the expression of SREBP-2 mRNA showed a trend toward an increase that was related to ChSI (Figure 1F). Similar results were also observed for proprotein convertase subtilisin kexin (PCSK)-9, the expression of which showed a nonsignificant increase related to the degree of ChSI (supplemental Table II).

Separation of plasma lipoproteins by size exclusion chromatography demonstrated a reduction in the LDL cholesterol concentration that was inversely related to the degree of LDL receptor induction (Figure 2). Low-ChSI and High-ChSI showed 23% (P<0.01) and 60% (P<0.001) reductions in plasma LDL-cholesterol from baseline, respectively. Similarly, the magnitude of VLDL cholesterol reduction was related to the degree of ChSI (Figure 2). VLDL cholesterol was reduced by 19% in the Low-ChSI (P<0.001) and by 55% (P<0.001) in the High-ChSI group. A significant decrease in HDL cholesterol (~25%; P<0.01) was also observed in the High-ChSI group.
Another major aim of our study was to characterize the hepatic activity and gene expression of the cholesteryl ester-forming enzymes, ACAT2 and ACAT1. When compared to the controls, patients in the high-ChSI group had a 50% reduction in microsomal ACAT2 activity, whereas those in the Low-ChSI group only had a minor decrease (Figure 3A). The decrease in ACAT2 activity in the High-ChSI group was paralleled by a decrease in ACAT2 protein expression (Figure 3B). Measurements of ACAT2 mRNA levels also showed a significant decrease (Figure 3C). No effects were observed for the microsomal activity or for the mRNA expression of ACAT1 (data not shown).

Analysis of the gene expression of the apolipoproteins involved in VLDL secretion revealed a significant decrease in apoE mRNA in the High ChSI group (−34%; P<0.05; Figure 3D), whereas no effects on apoB mRNA abundance were observed on ChSI (supplemental Table II). We also determined the apoB and apoE content in the VLDL before and during the different treatments. Interestingly, similar decreases (≈30%) in apoB and apoE were observed both in Low-ChSI and High-ChSI groups. No effects on the mRNA expression of the microsomal triglyceride transfer protein (MTP) were observed in response to ChSI (supplemental Table II).

As mentioned above, HDL cholesterol levels were slightly reduced by High-ChSI treatment. This was independent of changes in apoA-I (% difference from baseline; Control, 0.70±2.81; Low-ChSI, 21.6±12.2; High-ChSI, 3.74±6.94). Because animal experiments indicate that the hepatic expression of the HDL receptors, scavenger receptor class B type I (SR-BI), may partly regulate plasma HDL cholesterol levels, we measured the protein expression of its human counterpart, CLA-I. Unexpectedly, Western blot analysis did not show any change of this protein in response to ChSI (Figure 4A), nor was there any change in its mRNA levels (supplemental Table II). Other hepatic factors involved in the formation of plasma HDL, such as apoA-I, ABCA1, and CETP were not influenced by ChSI, at least not at the mRNA level (supplemental Table II).

Finally, we assessed the effect of ChSI on biliary lipid composition in gallbladder bile after an overnight fast. The absolute concentrations of all biliary lipids (cholesterol, bile acids, and phospholipids) were reduced by High-ChSI treatment (Table 2). This was associated with a reduction of the relative proportion of cholesterol (−40%; P<0.05), resulting in a decreased saturation of gallbladder bile with cholesterol (−38%; P<0.05). The mRNA expression of the biliary export pumps for cholesterol, ABCG5 and ABCG8, were not influenced by ChSI treatment (supplemental Table II); neither was the protein expression of ABCG8 (Figure 4B). No effect of ChSI was seen on the composition of individual bile acids or on bile acid production assayed by measurement of the plasma levels of C4/cholesterol (supplemental Table II). The plasma plant sterols, campesterol and sitosterol, were increased during High-ChSI treatment (Table 2). The change in plant sterol/cholesterol ratios was inversely correlated to molar % cholesterol in bile (R=−0.44 for campesterol and R=−0.40 for sitosterol, respectively; both P<0.05), it may be more plausible to ascribe this relative change as reflection of the reduced secretion of biliary cholesterol.

**Discussion**

In this controlled single-blind parallel-group study, we were able to achieve our goal to obtain 2 widely different degrees of cholesterol synthesis inhibition (ChSI) by using a high dose of a potent statin (atorvastatin) and a low dose of a less...
potent statin (fluvastatin). The data were not intended to
differentiate specific characteristics of either drug. Although
the use of two statins with different structure and metabolism
may somewhat limit the interpretation of our results, some
new as well as established molecular effects of low and high
ChSI on hepatic cholesterol metabolism in humans could be
identified.

In the present study, especially High-ChSI was associated
with a decrease in plasma VLDL cholesterol concentration,
suggesting that a lesser amount of cholesteryl esters is
secreted from the liver into nascent VLDL. We have recently
demonstrated the hepatocyte-specific expression of ACAT2
and its significance in hepatic cholesterol esterification.6,10
Our new findings corroborate the hypothesis that ACAT2 is
a key enzyme in the formation and secretion of cholesteryl
esters in lipoproteins in humans.11 Accordingly, a clear
decrease in hepatic ACAT2 activity and expression was
observed in our patients in response to ChSI, whereas no
treatment effects were identified for ACAT1 activity. In
mice, hepatic ACAT2 has been shown to be essential for the
incorporation of cholesteryl esters in the core of VLDL.12
Hence, our present findings suggest a key role for ACAT2 in
hepatic VLDL cholesterol secretion also in humans.

Although a decreased ACAT2 activity in part could ac-
count for the reduction in VLDL cholesterol secretion, it
cannot be excluded that the secretion of apoB-containing
lipoproteins from the liver is also decreased in High-ChSI by
the higher LDL receptor expression. These receptors have
been proposed to mediate the degradation of apoB before its
secretion and to be responsible for the reuptake and degra-
dation of newly secreted apoB.13 We could indeed confirm
and extend previous reports that the hepatic LDL receptor

Figure 2. Effects of ChSI on plasma lipoprotein cholesterol profiles. Gray solid line, lipoprotein cholesterol pro-
files before treatment; black solid line, cholesterol lipoprotein profiles after
treatment. Insets show % variation
from baseline levels of the area under the curve for each lipoprotein fraction.
Data show mean±SEM **P<0.01;
***P<0.001. For details please see
supplemental materials.

Figure 3. Effects of ChSI on hepatic ACAT2 and Apo E expres-
sion. A, ACAT2 activity in pooled liver microsomes. B, ACAT2
protein expression in pooled liver microsomes. C, Hepatic
ACAT2 mRNA levels in individual mRNA preparations. D, ApoE
mRNA in individual mRNA preparations. Data show mean±SEM
when individual samples were assayed. *P<0.05. For details
please see supplemental materials.

Figure 4. Effects of ChSI on hepatic CLA-I and ABCG8 protein
expression. Liver membrane proteins were loaded and sepa-
rated on SDS/PAGE. After transfer onto nitrocellulose filter,
samples were incubated with either (A) anti-mouse SR-BI, reac-
tive to human CLA-I, or (B) with antihuman ABCG8 antibody.
Data show mean±SEM. For details please see supplemental materials.
Table 2. Bile Composition and Plasma 7α-Hydroxy-4-Cholesten-3-One (C4) and Plant Sterols

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Low ChSI</th>
<th>High ChSI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biliary cholesterol, mmol/L</strong></td>
<td>13±1.7</td>
<td>15±2.1</td>
<td>4.2±1.2*</td>
</tr>
<tr>
<td><strong>Biliary bile acids, mmol/L</strong></td>
<td>10±1.9</td>
<td>83±13</td>
<td>51±17*</td>
</tr>
<tr>
<td><strong>Biliary phospholipids, mmol/L</strong></td>
<td>34±5.0</td>
<td>34±4.2</td>
<td>21.6±2.6*</td>
</tr>
<tr>
<td><strong>Cholesterol % molar</strong></td>
<td>9.6±1.1</td>
<td>12±1.5</td>
<td>5.8±0.8*</td>
</tr>
<tr>
<td><strong>Bile acid % molar</strong></td>
<td>65±2.8</td>
<td>62±2.1</td>
<td>66±2.7</td>
</tr>
<tr>
<td><strong>Phospholipids % molar</strong></td>
<td>25±1.8</td>
<td>27±1.4</td>
<td>28±2.1</td>
</tr>
<tr>
<td><strong>Saturation index, %</strong></td>
<td>134±18</td>
<td>152±17</td>
<td>83±12*</td>
</tr>
<tr>
<td><strong>Cholic acid, %</strong></td>
<td>37±2.3</td>
<td>31±2.3</td>
<td>36±2.5</td>
</tr>
<tr>
<td><strong>Chenodeoxycholic acid, %</strong></td>
<td>35±1.9</td>
<td>37±3.1</td>
<td>41±2.1</td>
</tr>
<tr>
<td><strong>Deoxycholic acid, %</strong></td>
<td>25±2.8</td>
<td>28±4.7</td>
<td>20±3.1</td>
</tr>
<tr>
<td><strong>Ursodeoxycholic acid, %</strong></td>
<td>1.3±0.4</td>
<td>3.0±1.5</td>
<td>2.7±0.9</td>
</tr>
<tr>
<td><strong>Lithocholic acid, %</strong></td>
<td>1.5±0.3</td>
<td>1.5±0.3</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td><strong>Plasma C4/cholesterol, ng/mmol/L</strong></td>
<td>2.8±0.3</td>
<td>2.5±0.3</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td><strong>Campesterol/cholesterol, % variation from baseline</strong></td>
<td>33±53</td>
<td>-5±12</td>
<td>117±28***</td>
</tr>
<tr>
<td><strong>Sitosterol/cholesterol, % variation from baseline</strong></td>
<td>19±23</td>
<td>5±15</td>
<td>151±33**</td>
</tr>
</tbody>
</table>

Mean±SEM; *vs Placebo, P<0.05; **vs Placebo, P<0.01; ***vs Placebo, P<0.001.

Baseline values: Campesterol/cholesterol (µg/mmol): Placebo, 643±157; Low ChSi, 654±140; High ChSi, 662±99. Sitosterol/cholesterol (µg/mmol): Placebo, 438±72; Low ChSi, 492±81; High ChSi, 452±57.

activity is increased after statin treatment in humans.14–16 Further, our data support the concept that also in humans a feed-forward regulation of the SREBP-2 gene expression is present, although the increase in SREBP-2 mRNA at High-ChSI was associated with a reduced expression of apoE mRNA. Because apoE is a ligand for the LDL receptor, it might compete with apoB for binding to the LDL receptors within the secretory pathway of VLDL or at the cell surface. Hence, apoE has been predicted to have an opposite regulatory role on the secretion of VLDL in humans.13 Consistent with this hypothesis, genetically modified mice deficient in apoE have a reduced VLDL secretion19,20 and mice overexpressing apoE have enhanced VLDL secretion.21 In contrast, we did not observe any changes in other genes involved in VLDL assembly and secretion, such as MTP or apoB. Thus, the upregulation of LDL receptors, the downregulation of apoE expression, and the decrease in HMG CoA Red and ACAT2 activity could all contribute to the decrease in VLDL cholesterol levels that follows ChSi in humans.

In animal models of atherosclerosis, disruption of the ACAT2 gene leads to prevention of disease22; this occurs despite elevations in plasma apoB. Consequently, not only the number of apoB-containing lipoproteins, but also the amount of ACAT2-derived cholesteryl esters present in the core of these lipoproteins seems to be a critical factor in the development of atherosclerosis. All these observations indirectly suggest that a less atherogenic composition of the apoB-containing lipoproteins attributable to a decreased ACAT2 activity in the liver may convey an additional benefit after High-ChSI in humans. In mice, disruption of ACAT2 may result in a substitution of triglycerides for cholesteryl esters in the core of VLDL particles, resulting in increased plasma triglyceride levels.12,22 At the level of ACAT2 inhibition achieved by ChSi in humans, this exchange does not seem to occur because the group of patients on High-ChSi presented the lowest level of ACAT2 activity and had the lowest plasma triglyceride levels. Furthermore, it has been shown that inhibition of cholesteryl ester production from ACAT2 by dietary polyunsaturated fat in nonhuman primates leads to a reduced LDL particle size.23 How ACAT2 activity may modify LDL particle size in humans still remains to be studied. However, in contrast to humans, the size of LDL particles in nonhuman primates is mainly determined by the cholesteryl ester content because the extremely low levels of triglycerides do not allow for particle remodeling by triglyceride for cholesteryl ester exchange. Irregardless of possible effects of ACAT2 inhibition on LDL particle size, a decreased content of atherogenic cholesteryl esters (synthesized by ACAT2) in apoB containing lipoproteins should also be beneficial in humans. Studies in humans have also demonstrated that the amount of cholesterol per apoB in LDL particles, and not their size, is a strong risk factor for developing CVD.24

In contrast to what has been observed in nonhuman primates—where the primary regulation of ACAT2 expression in the liver is not transcriptional25—the decreased mRNA expression of ACAT2 after ChSi in human beings indicates that sterols may also act as regulators of ACAT2 gene expression. In line with this, analysis of the transcriptional regulation of the ACAT2 gene in human hepatoma cell lines suggested a clear negative sterol regulation for this gene.26

When High-ChSI was achieved by 80 mg/d atorvastatin, HDL cholesterol was reduced by 25% (Figure 2). In the present study, the cholesterol concentrations of lipoprotein fractions were calculated by integration of chromatograms obtained by size-exclusion analysis, a technique that has been shown to correlate well with ultracentrifugation/precipitation techniques.27 However, some degree of overestimation of the decrease in HDL and VLDL may result from the reduction of the LDL peak. We hypothesized that the reduction in HDL cholesterol might be linked to an increased CLA-I expression, which in turn could mediate increased HDL cholesterol uptake by the liver.8 However, CLA-I protein and gene expression were not changed after atorvastatin treatment, suggesting that other mechanisms explain the reduction in HDL cholesterol. Interestingly, evidence that the liver is the major source of cholesterol for lipidation of circulating HDL has been produced by disrupting hepatic ABCA1 in mice.28 If the liver is also the source of much of the cholesterol in plasma HDL in humans, it may be speculated that the pool of cholesterol targeted for secretion into HDL may be reduced by a significant inhibition of cholesterol synthesis, as a partial explanation for the decrease in HDL cholesterol during high dose atorvastatin treatment.
High-ChSI resulted in a decrease in the cholesterol saturation of bile attributable to reduced molar % cholesterol, an effect that has been attributed to a decrease in biliary cholesterol secretion.29 ABCG5 and ABCG8 have been identified as the essential mediators of biliary cholesterol secretion from the canalicular membrane of the hepatocyte, and we hypothesized that changes in their expression might explain the reduction in biliary cholesterol. However, there were no effects of ChSI on mRNA levels for ABCG5 or ABCG8, nor on the protein level of ABCG8. This suggests that the reduced availability of hepatic cholesterol may more likely be the critical factor for biliary cholesterol secretion in our experimental model.

The increased plant sterol/cholesterol ratio in plasma observed after High-ChSI would suggest that intestinal cholesterol absorption may be increased, as proposed previously.9 However, the parallel observation of decreased molar % cholesterol content in bile may instead suggest that other mechanisms are involved. If the amount of biliary cholesterol reaching the intestine is reduced during High-ChSI, less cholesterol will be available for absorption and a relative enrichment of plant sterol would occur, explaining the paradoxical increase in plasma plant sterol/cholesterol ratio. Although speculative, this interpretation is indirectly supported by our finding of an inverse correlation between plasma plant sterol/cholesterol ratio and biliary molar % cholesterol.

In conclusion, this study shows for the first time in humans that a downregulation of hepatic genes modulating cholesterol ester composition and secretion of VLDL particles—ACAT2 and apoE—occurs after cholesterol synthesis inhibition. These effects should complement the beneficial changes in plasma LDL metabolism induced by increased LDL receptors, by acting also on the secretion of LDL-precursor lipoproteins. Statins have been previously shown to decrease plasma LDL cholesterol in patients homozygous for LDL receptor deficiency,31 and our observations may also provide a partial explanation for such LDL cholesterol decrease.

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References


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Material and Methods

Patients, treatments, and operative procedure

Fertile female, post-menopausal female, and male patients (altogether 42), scheduled for elective cholecystectomy because of uncomplicated gallstone disease, were enrolled in the study. For inclusion, the patients had to have a total plasma cholesterol > 3.0 mmol/L, normal or slightly elevated plasma triglycerides (<3.6 mmol/L), and also to be non-obese (BMI<30). Evidence of hepatic, renal, metabolic or endocrine dysfunction and history of concomitant medication interfering with the metabolism of statins were criteria for exclusion, as was compliance to treatment of < 80%. Five patients did not complete the study because of low compliance and thus 12 males, 12 fertile females, and 13 post-menopausal females were evaluated (Table 1). Each of the 3 patient groups was randomized to three treatment arms: placebo, fluvastatin 20 mg/day (Low-ChSI) or atorvastatin 80 mg/day (High-ChSI) for 4 weeks prior to surgery. Fasting blood samples were drawn at randomisation and on the day before surgery. The last dose was administered the day before operation. Surgery was performed under general anesthesia between 09.00 h and 11.00 h after an overnight fast. The patients were all operated with a laparoscopic technique. A biopsy was obtained from the left lobe of the liver and the specimen was immediately frozen in liquid nitrogen. All patients had cholesterol gallstones, as judged from postoperative visual inspection. Except for safety parameters, all analyses were performed after the last patients had completed the study. Informed consent was obtained from all patients prior to inclusion into the study, which was approved by the Human Ethics Committee of Karolinska Institutet and by the Swedish Medical Product Agency.
Chemical analysis of plasma

Plasma analyses of total cholesterol, triglycerides, and apolipoproteins B and AI were performed by certified routine assays. Size-fractionation of lipoproteins was performed on 10 µl of individual plasma samples using a Superose 6 PC 3.2/30 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described 1. The respective lipoprotein fraction lipid concentrations were calculated after integration of the individual chromatograms. SDS/PAGE separation of apolipoproteins in the VLDL fraction was performed using pooled plasma after ultracentrifugation at d<1.006 g/mL as previously described. 2 The bands corresponding to apolipoproteins B and E were quantitated by densitometry.

Unesterified lathosterol, an indirect marker of HMG-CoA reductase activity in the liver 3 and whole body cholesterol synthesis 4, was determined by isotope dilution-mass spectrometry after the addition of deuterium-labeled internal standard 5. 7α-hydroxy-4-cholesten-3-one (C4), an intermediate in bile acid formation that directly correlates to bile acid synthesis, was analysed by HPLC in pooled serum samples as described 6. The plant sterols sitosterol and campesterol were analysed by gas chromatography-mass spectrometry (GC/MS) using D5-campesterol and D5-sitosterol as internal standards 7.

Preparation of hepatic membranes, ligand blot assay of LDL receptors and Western blot assays.

Liver plasma membranes were prepared from pools of liver as described previously 8. For ligand blot assay of LDL receptors, polyacrylamide gels (10% with SDS) were loaded with membrane protein prepared from pooled samples of liver. Filters were incubated with 125I-labeled rabbit β-migrating very low density lipoproteins (β-VLDL), as described 8. The LDL-receptor expression (120 kDa band) was determined using a Fuji Bio-imaging analyzer (BAS
1800, Fuji Photo Film Co, Japan) after subtraction of background. CLA-I receptors were
determined on liver membranes by Western Blot analysis. Reduced samples were separated
on 3-8% Tris-acetate gels (NuPAGE Invitrogen, Carlsbad, CA). Proteins were then
transferred onto nitrocellulose filters. CLA-I protein was detected with rabbit polyclonal
antibodies (1:3000) against mouse SR-B1 (Novus Biologicals, Inc, Littleton, Co) as described
elsewhere. 9 ABCG8 was detected with rabbit polyclonal antibody to human ABCG8
following manufacturer’s instruction (Novus Biologicals, Inc, Littleton, Co). Western blots
were quantified by Image Gauge software, (Fuji Photo Film Co, Japan).

RNA preparation and mRNA determination
Total RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA). One µg total
RNA was transcribed into cDNA using random hexamer priming and Omniscript™ (Qiagen,
Valencia, CA). Quantification of specific mRNAs was performed by SYBR® Green real-time
PCR using an ABI PRISM 7000 thermocycler (PE Applied Biosystems, Foster City, CA). In
order to prevent amplification of genomic DNA, primer sets were designed whenever possible
to cross an exon-exon boundary (Supplementary Table 1). Data are expressed in arbitrary
units and normalized by correction for the signal obtained in the same cDNA preparation for
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Degraded RNA samples and
samples showing >2 cycle difference for GAPDH from average were not used in the final
computations.

Isolation of microsomes, enzymatic analyses, and ACAT2 protein determination
Isolation of hepatic microsomes and determination of total ACAT enzymatic activity were
performed as described previously 10, except that preincubation was with a cholesterol-
saturated solution of β-hydroxypropyl cyclodextrin for 30 min before addition of 14C-oleoyl-
CoA. In order to separately identify ACAT1 and ACAT2 activities, pyripyropene A, a specific ACAT2 inhibitor, was included in the preincubation and reaction mixture at a concentration of 5 µM. For ACAT2 protein analysis by Western blot, 10 or 20 µg of microsomal protein was analyzed. Separation and blotting were as described earlier. Immunoaffinity purified monkey ACAT2 antibody, prepared as previously described, was applied to the nitrocellulose at a concentration of 1 µg/ml in the blocking solution. Western blots were quantified by Image Gauge software (Fuji Photo Film Co, Japan).

**Analysis of biliary lipids and bile acid composition**

Biliary cholesterol, total bile acids and phospholipids in gallbladder bile were measured as previously described. The saturation index was calculated using Carey’s critical tables. For bile acid composition, bile was hydrolyzed in 1M potassium hydroxide at 110ºC for 12 hrs. The deconjugated bile acids were thereafter extracted with diethyl ether after acidification to pH 1 by 8M HCl, preparation of trimethylsilyl ethers and analyzed by gas-liquid chromatograph (Agilent Technologies, Böblingen, Germany) using a HPI column (Agilent Technologies).

**Statistics**

Data are presented as means ± SEM. The significance of differences between groups was tested by one-way ANOVA followed by post-hoc comparisons of group means according to the LSD or the Dunnett methods (Statistica software, Stat Soft, Tulsa OK). Correlations were calculated with the Spearman rank order test.
References


FIGURE LEGENDS

Figure 1
Effects of different degrees of cholesterol synthesis inhibition (ChSI) on hepatic cholesterol metabolism. Normocholesterolemic non-obese gallstone patients were randomized for treatment with placebo (Placebo), fluvastatin 20 mg/day (Low-ChSI), or atorvastatin 80 mg/day (High-ChSI) for 4 weeks. A) Percent reduction from baseline levels (see Table I) in plasma total cholesterol; B) Percent reduction from baseline levels (Table I) in plasma lathosterol/cholesterol ratio – a marker for body cholesterol synthesis; C) LDL receptor activity measured in hepatic membranes by ligand blot technique; D) LDL receptor mRNA; E) HMG CoA reductase mRNA; F) SREBP-2 mRNA. All mRNA determinations were done by real time RT-PCR, expressed in arbitrary units, and normalized by correction for the signal obtained in the same cDNA preparation for GAPDH mRNA. Data show mean + SEM. *, p<0.05; ***, p<0.001.

Figure 2
Effects of different degrees of cholesterol synthesis inhibition (ChSI) on plasma lipoprotein cholesterol profiles. Lipoproteins were separated from 10 µl of plasma from each patient and cholesterol was determined on-line as described in Methods. The chromatogram averaging the data for all of the patients in each group is shown. Gray solid line, lipoprotein cholesterol profiles before treatment; black solid line, cholesterol lipoprotein profiles after treatment. Insets show % variation from baseline levels of the area-under-the-curve for each lipoprotein fraction. Data show mean ± SEM. **, p<0.01; ***, p<0.001.
**Figure 3**

Effects of different degrees of cholesterol synthesis inhibition (ChSI) on hepatic ACAT2 and Apo E expression. ACAT activity was determined in pooled hepatic microsomes (9-11 patients/group), after preincubation with cholesterol-saturated solution. In separate tubes, pyripyropene A, a specific ACAT2 inhibitor, was included in the preincubation and reaction mixture at a concentration of 5 µM to separately identify ACAT1 (inhibited) and ACAT2 (uninhibited-ACAT1) activities. A) ACAT2 activity in pooled liver microsomes; B) ACAT2 protein expression in pooled liver microsomes. Protein solubilized from microsomes (10 µg and 20 µg/lane) was loaded and electrophoretically separated. Samples were incubated with anti-monkey ACAT2 antibody at a final concentration of 1 µg/mL. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (was used at a 1:15 000 dilution. Prior to separation, liver microsomes were incubated at 37°C for 30 min in 100 mM DTT; C) hepatic ACAT2 mRNA levels in individual mRNA preparations; D) apo E mRNA in individual mRNA preparations. All mRNA determinations were done by RT-PCR as described in Figure 1. Data show mean ± SEM when individual samples were assayed. *, p<0.05.

**Figure 4**

Effects of different degrees of cholesterol synthesis inhibition (ChSI) on hepatic CLA-I and ABCG8 protein expression. Liver membrane proteins (10 µg or 20 µg/lane) were loaded and separated on SDS/PAGE. After transfer onto nitrocellulose filter, samples were incubated with either A) anti-mouse SR-BI, reactive to human CLA-I, or B) with anti-human ABCG8 antibody. Western blots were quantified by Image Gauge software, Fuji Film. Data show mean ± SEM. *, p<0.05.
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<th>Final conc.</th>
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<td>Rev 5’-ACA GGC GAG CCA CAA TGG-3’</td>
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Supplementary Table II. mRNA expression of hepatic genes controlling cholesterol efflux and HDL metabolism normalized for the expression of GAPDH mRNA.

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<th>Gene</th>
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<th>Low ChSI vs. Placebo</th>
<th>High ChSI</th>
<th>High ChSI vs. Placebo</th>
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<td>ABCA1</td>
<td>8.00 ± 0.78</td>
<td>9.35 ± 0.72</td>
<td><em>p = 0.45</em></td>
<td>8.96 ± 1.36</td>
<td><em>p = 0.83</em></td>
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<td>ABCG5</td>
<td>40.3 ± 5.06</td>
<td>36.1 ± 3.46</td>
<td><em>p = 0.80</em></td>
<td>48.1 ± 4.40</td>
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<td>ABCG8</td>
<td>13.2 ± 1.76</td>
<td>11.8 ± 0.96</td>
<td><em>p = 0.83</em></td>
<td>16.0 ± 2.44</td>
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<td>Apo AI</td>
<td>503 ± 60</td>
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<td>CETP</td>
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<td>CLA-I</td>
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<td>MTP</td>
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<td>NPC1L1</td>
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<td>PCSK-9</td>
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<td>50.9 ± 8.50</td>
<td><em>p = 0.78</em></td>
<td>68.8 ± 14.1</td>
<td><em>p = 0.19</em></td>
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ABCA1: ATP-binding cassette A1; ABCG5: ATP-binding cassette G5; ABCG8: ATP-binding cassette G8; APOAI: apolipoprotein AI; CETP: cholesterol ester transfer protein; CLA-I: CD36 and LIMPII Analogous-1; MTP: microsomal triglyceride transfer protein; NPC1L1: Niemann-Pick C1 like 1; SREBP-2: sterol regulatory element binding protein-2.

Data are expressed in arbitrary units that have been normalized by correction for the signal obtained in the same cDNA preparation for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (mean ± SEM; 8-9 patients/group). P-values refer to the post-hoc comparison according to Dunnet method.