Control of ACAT2 Liver Expression by HNF4α Lesson From MODY1 Patients

C. Pramfalk, E. Karlsson, L. Groop, L.L. Rudel, B. Angelin, M. Eriksson, P. Parini

Objective—ACAT2 is thought to be responsible for cholesteryl ester production in chylomicron and VLDL assembly. Recently, we identified HNF1α as an important regulator of the human ACAT2 promoter. Thus, we hypothesized that MODY3 (HNF1α gene mutations) and possibly MODY1 (HNF4α, upstream regulator of HNF1α, gene mutations) subjects may have lower VLDL esterified cholesterol.

Methods and Results—Serum analysis and lipoprotein separation using size-exclusion chromatography were performed in controls and MODY1 and MODY3 subjects. In vitro analyses included mutagenesis and cotransfections in HuH7 cells. Finally, the relevance in vivo of these findings was tested by ChIP assays in human liver. Whereas patients with MODY3 had normal lipoprotein composition, those with MODY1 had lower levels of VLDL and LDL esterified cholesterol, as well as of VLDL triglyceride. Mutagenesis revealed one important HNF4 binding site in the human ACAT2 promoter. ChIP assays and protein-to-protein interaction studies showed that HNF4α, directly or indirectly (via HNF1α), can bind to the ACAT2 promoter.

Conclusions—We identified HNF4α as an important regulator of the hepatocyte-specific expression of the human ACAT2 promoter. Our results suggest that the lower levels of esterified cholesterol in VLDL- and LDL-particles in patients with MODY1 may—at least in part—be attributable to lower ACAT2 activity in these patients. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: ACAT2 ■ MODY ■ HNF1α ■ HNF4α ■ VLDL

Acylo-CoA: cholesterol acyltransferases (ACAT) 1 and 2 are integral membrane proteins located in the rough endoplasmatic reticulum that catalyze the formation of cholesteryl esters from cholesterol and saturated and monounsaturated fatty acids.1 There are 2 genes encoding ACAT1 and ACAT2, known by international convention as steroyl O-acyltransferases ie, Soat1 and Soat2, respectively. Whereas ACAT1 is ubiquitously expressed, ACAT2 expression is confined to enterocytes and hepatocytes.2 The precise role(s) of ACAT1 and ACAT2 in the homeostasis of cholesterol commence to be revealed. It has been suggested3 that cholesteryl esters formed by ACAT2, but not by ACAT1,4 are incorporated into hepatic and intestinal apoB-containing lipoproteins and secreted into plasma. In mice, ACAT2-derived cholesteryl esters have been shown to promote atherosclerosis, independently of dietary fats.5

Hepatic nuclear factors (HNFs) 1 and 4 are expressed in various organs, including the liver, pancreas, and kidney.6,7 HNF1α and HNF1β share strong homologies which enable them to bind to the same DNA sequence.8 HNF4α is an upstream regulator of the expression of HNF1α, but not of HNF1β.9 Maturity-onset diabetes of the young (MODY) is a group of syndromes characterized by autosomal dominant inheritance, early onset diabetes, and pancreaticβ-cell dysfunction.10 Mutations of the TCF1 (transcription factor 1) gene, which encodes HNF1α, causes MODY3,11 and mutations in the TCF14 gene, which encodes HNF4α, causes MODY1.9

Our recent finding that HNF1α and HNF1β are important regulators of the human ACAT2 promoter12 led us to postulate that MODY3 patients may have alterations in the cholesteryl ester content of apoB-containing lipoproteins. In the present study, we tested this hypothesis by characterizing the lipoprotein lipid composition in MODY1 and MODY3

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patients. Unexpectedly, we found that MODY1, but not MODY3, was associated with reduced cholesteryl esters. This prompted us to characterize the role of HNF4α in the transcriptional regulation of ACAT2.

Methods

The subjects studied included 9 healthy individuals, 6 MODY1, and 5 MODY3 patients (Table). Human ACAT2 promoter studies were performed in HuH7 and HEK293 cells. Chromatin immunoprecipitation (ChIP) assay and protein-to-protein interaction studies were performed in HuH7 and HEK293 cells. Chromatin immunoprecipitation (ChIP) assay and protein-to-protein interaction studies were performed using human livers. Please see supplemental Methods (available online at http://atvb.ahajournals.org) for the details on patients and plasma analysis, cell experiments, mutagenesis, transfections, ChIP assays, protein-to-protein interaction studies, and statistics.

Results

Reduced VLDL and LDL Esterified Cholesterol Levels in MODY1 Patients

Contrary to our initial prediction, analysis of lipids in lipoprotein fractions from patients with MODY3 did not differ from controls (Figure 1A through 1D). Instead, MODY1 patients exhibited pronounced differences in lipoprotein lipids compared to controls. Patients with MODY1 had lower VLDL (≈50%, P<0.05) and LDL (≈30%, P<0.01) total cholesterol levels compared to controls (Figure 1A) and dramatically lower VLDL triglyceride levels compared to controls (P<0.01) and to MODY3 (P<0.05; Figure 1C). Also, the VLDL esterified cholesterol levels were ≈50% lower compared to controls (P<0.05) and to MODY3 patients (P<0.05). The LDL esterified cholesterol levels were ≈50% lower in MODY1 compared to controls (P<0.01; Figure 1D). In addition, MODY1 patients had higher HDL free cholesterol compared to controls (≈40%, P<0.05) and to MODY3 patients (≈60%, P<0.05; Figure 1B).

The findings in MODY1 were in accordance with those we had predicted as a consequence of reduced ACAT2 expression. We therefore hypothesized that HNF4α could control

Table. Clinical Characteristics of the Patients

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>MODY1</th>
<th>MODY3</th>
</tr>
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<tr>
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<td>9 (3/6)</td>
<td>6 (0/6)</td>
<td>5 (2/3)</td>
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<tr>
<td>Age, y</td>
<td>40±6</td>
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<td>46±6</td>
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<tr>
<td>Age at diabetes onset, y</td>
<td>Not applicable</td>
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<tr>
<td>BMI, kg/m²</td>
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<td>21.3±0.8</td>
<td>26±2.5</td>
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<tr>
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<tr>
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<td>4.0±0.4</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
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<td>0.5±0.1</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>HbA1c, %</td>
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<td>6.8±1.0</td>
</tr>
<tr>
<td>fS-Glucose, mmol/L</td>
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<td>6.4±0.9</td>
<td>10.0±2.7</td>
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<tr>
<td>fS-C peptid, mmol/L</td>
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<td>0.31±0.04</td>
<td>0.15±0.06</td>
</tr>
<tr>
<td>fS-Insulin</td>
<td>4±0.7</td>
<td>3±0.9</td>
<td>3±1.7</td>
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Values are presented as means±SEM. Ctrl indicates controls without diabetes; BP, blood pressure.

HNF4α Regulates the Human ACAT2 Promoter Activity

In the human hepatoma HuH7 cell line, cotransfections with HNF4α expression vector, along with the human ACAT2 full-length promoter, revealed a strong dose-dependent regulation by HNF4α on the activity of the human ACAT2 promoter (Figure 2A). To identify the region(s) that confer this strong regulatory effect, HuH7 cells were cotransfected with both the HNF4α expression vector (0.5 μg) and different deletion constructs of the human ACAT2 promoter. As shown in Figure 2B, the p-1044 construct conferred the most pronounced induction by HNF4α. Also, the induction increased when comparing the p-1196 and the p-1044 constructs, suggesting the presence of potential repressor element(s) as also observed in previous studies. However, the strong induction by HNF4α pertained to the p-269 construct. To investigate the hepatocyte specificity of these findings, we cotransfected the human kidney cell line HEK293 with the different deletion constructs of the human ACAT2 promoter region and the HNF4α expression vector (0.5 μg). As shown in Figure 2C, HNF4α did not induce human ACAT2 promoter activity as efficiently as in the hepatic cell line (≈4-fold in HEK293 versus >50-fold in HuH7).
Studies on the HNF4 Binding Sites in the Human ACAT2 Promoter

To search for putative HNF4 binding sites in the human ACAT2 promoter region, the sequence was analyzed using the TESS database (www.cbil.upenn.edu at University of Pennsylvania, Philadelphia). Two HNF4 cis-elements were found, one located −247 bp and the other −311 bp upstream of the ATG start codon. We performed mutagenesis on these HNF4 cis-elements, with or without mutation of the previously identified HNF1 binding site (located −866 bp upstream of the ATG start codon). These mutated constructs, along with the HNF4α expression vector (0.1 μg), were used for cotransfection experiments in HuH7 cells. As shown in Figure 2D, deletion of the −247 bp HNF4 binding site only modestly decreased the induction by HNF4α (P<0.05), whereas deletion of the −311 bp HNF4 binding site decreased the induction by HNF4α (≈30%, P<0.05; Figure 2E). The decrease was of greater magnitude (≈50%, P<0.01) when the −866 bp HNF1 binding site also was deleted (Figure 2E), suggesting an interaction between HNF4α and HNF1α. Simultaneous mutation of the two HNF4 sites (−247 and −311 bp) had the same effect on the induction as deletion of solely the −311 bp HNF4 binding site (data not shown), suggesting the importance of this HNF4 binding site in the regulation of the human ACAT2 promoter by HNF4α.

HNF4α Binds to the Human ACAT2 Promoter In Vivo

To verify whether HNF4α can bind to the ACAT2 promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assays using human liver. We used specific antibodies against HNF1α and HNF4α to also assess whether the 2 nuclear receptors interact with each other when binding to the ACAT2 promoter because mutagenesis indicated this possibility. An IgG antibody was used as baseline control to compare the levels of specific DNA fragments. Also, a small aliquot of chromatin was saved and used as an input control. Primers designed to target the −866 bp HNF1, −247 bp HNF4, or −311 bp HNF4 binding sites in the ACAT2 promoter were used in PCR analyses. To verify that the HNF1 and HNF4 binding sites were physically separated by the sonication of the chromatin, we performed PCR reactions in which the forward primers and the reverse primers were all paired to each others (ie, forward primers for −866 bp HNF1 site with reverse primers for −247 or −311 bp HNF4 site, and so on). If physical separation was achieved, no amplification would occur. No amplifications were obtained confirming the physical separation of the −866 bp HNF1, −247 bp HNF4, or −311 bp HNF4 binding sites in the ACAT2 promoter were used in PCR analyses. To verify that the HNF1 and HNF4 binding sites were physically separated by the sonication of the chromatin, we performed PCR reactions in which the forward primers and the reverse primers were all paired to each others (ie, forward primers for −866 bp HNF1 site with reverse primers for −247 or −311 bp HNF4 site, and so on). If physical separation was achieved, no amplification would occur. No amplifications were obtained confirming the physical separation of the −866 bp HNF1, −247 bp HNF4, or −311 bp HNF4 binding sites (data not shown). Immunoprecipitation with HNF1α and HNF4α antibodies led to: (1) 10-fold and 8-fold enrichment of the −866 bp HNF1 site, respectively (Figure 3A); (2) 14-fold and 11-fold enrichment of the −247 bp HNF4 site, respectively (Figure 3B); and (3) 15-fold and 26-fold enrichment of the −311 bp HNF4 site, respectively (Figure 3C). Collectively, these data show that HNF1α and HNF4α interact when binding to the HNF1 and to the 2 HNF4 cis-elements present in the human ACAT2 promoter in vivo. Because both HNF1α and HNF1β could regulate and bind to the
human ACAT2 gene, we also wanted to test whether an interaction between HNF1β and HNF4α may occur when binding to the ACAT2 promoter in vivo. Using a specific antibody for HNF1β we performed ChIP assays as described above. No enrichment of either the −247 bp HNF4 or the −311 bp HNF4 site was detected (Figure 3D).

HEK293 cells do not express HNF1α, HNF4α, or ACAT2 as verified by measuring mRNA expressions by real-time RT-PCR (data not shown). These cells were thus used as negative controls for the ChIP assay (supplemental Figure 1). HuH7 cells, on the other hand, express high levels of ACAT2, HNF1α and HNF4α. Accordingly, the specificity of the HNF1α and the HNF4α antibody used in the ChIP assay and for the protein-to-protein interaction experiments (see below) were tested using nuclear extract from HEK293 and from HuH7 cells (supplemental Figure 1).

**Protein-to-Protein Interaction Between HNF1α and HNF4α in Human Liver**

To further strengthen the evidence of a protein-to-protein interaction between HNF1α and HNF4α, nuclear extracts from human liver were immunoprecipitated with antibodies raised against either HNF1α or HNF4α, and immunoblotted using primary antibodies against HNF1α and HNF4α (Figure 3E). Lanes 1 and 2 represent samples immunoprecipitated with HNF1α whereas lanes 3 and 4 represent samples immunoprecipitated with HNF4α. The blot incubated with HNF1α antibody gave clear bands at ~79 kDa for both HNF1α and HNF4α, indicating that a protein-to-protein interaction between these two occurs. The blot incubated with HNF4α antibody gave clear bands at ~54 kDa for both HNF1α and HNF4α, which indicates that a protein-to-protein interaction occurs.

**Effects of MODY1 Mutations on the Human ACAT2 Promoter**

To test the functional consequences of MODY1 mutations in the HNF4α gene on the human ACAT2 promoter we introduced the K99fsdelAA, the R154X, and the R303H mutations into the HNF4α expression vector and performed cotransfections of these mutants (0.1 μg) along with the human ACAT2 promoter in HuH7 cells. As shown in Figure 4A, the K99fsdelAA mutation reduced the basal activity (P<0.05) and completely abolished the transactivation potential of HNF4α overexpression (P<0.001) on the ACAT2 promoter activity. The R154X mutation resulted in reduced transactivation compared to HNF4α overexpression (2-fold versus 10-fold, P<0.01) but did not completely abolish the transactivation potential of HNF4α overexpression on the ACAT2 promoter activity. The R303H mutation affected the transactivation potential of HNF4α overexpression amplified (A through D). Nuclear extracts were immunoprecipitated with HNF1α (lane 1 and 2) or HNF4α (lane 3 and 4), run on SDS-PAGE, and blotted using antibodies raised against HNF1α (upper panel) or HNF4α (lower panel; E).
composition observed in MODY3 may be attributable to the presence of HNF1β as supported by the ChIP analysis at the −866 bp site. We also excluded the possibility of an interaction between HNF1β and HNF4α. ChIP analysis showed indeed a lack of enrichment of the −247 bp and −311 bp HNF4 sites, after immunoprecipitation with HNF1β antibody. Thus a clear difference between HNF1α and HNF1β exists in the regulation of the human ACAT2 promoter.

HNF4α is an upstream regulator of HNF1α, but not of HNF1β. The significantly lower levels of esterified cholesterol in VLDL and LDL from MODY1 subjects suggested that HNF4α may have direct effects on human ACAT2 gene expression. Accordingly, we could identify HNF4α as a strong activator of the human ACAT2 promoter, through a direct and indirect—via interaction with HNF1α—binding. We also show that the HNF4α mutations present in our MODY1 patients fail to normally induce the activity of the human ACAT2 promoter, suggesting a decreased expression of this gene in these patients. Contrary to HNF1, we were not able to identify 1 single trans-acting element responsible for the regulation of the ACAT2 promoter by HNF4α. In the ACAT2 promoter region analyzed, 2 HNF4 trans-acting elements were identified (located at −311 bp and −247 bp). Although the HNF4 trans-acting element located at −311 bp seems to be more important, its deletion did not completely abolish the stimulatory effects of HNF4α on the ACAT2 promoter activity. Similar results have been obtained in HepG2 cells when the putative HNF4 binding sites in the promoter region of the fatty acid binding protein-1 (FABP1) is mutated. The residual HNF4α activation of the mutated FABP1 promoter suggested that an interaction between HNF4α and HNF1α takes place. In our ChIP analysis, we could demonstrate direct binding of HNF4α to the 2 HNF4 trans-acting elements in vivo, and also a protein-to-protein interaction between HNF1α and HNF4α. This interaction, which was also confirmed by Western blot analysis, seems to arise when HNF1α and HNF4α bind to both the HNF1 trans-acting element (at −866 bp) and the HNF4 trans-acting elements (at −311 and −247 bp), respectively. Because of the proximity of 2 putative HNF1 binding sites to the HNF4 trans-acting elements at −311 and −247 bp (Figure 4B and 4C), we could not determine whether the interaction between the HNF1α and HNF4α implicate a direct binding of HNF1α to the DNA because ChIP analysis does not allow it. The 2 HNF1 sites (located at −220 bp and −276 bp), although not previously shown to be of importance for the hepatocyte-specific expression of ACAT2, may still take part in the metabolic regulation. Nevertheless, a cooperative interaction between HNF1α and HNF4α seems to exist in the regulation of ACAT2 promoter activity.

Previous studies have shown that MODY1 patients have low triglyceride levels, a finding confirmed in our study. Shih et al. reported that MODY1 patients also have low apoCIII levels. Because apoCIII is an inhibitor of lipoprotein lipase, this may in part be responsible for the lower VLDL triglycerides seen in MODY1. Additionally, Lehto et al.

Figure 4. Effects of MODY1 mutations (K99fsdelAA, R154X, and R303H), introduced into the HNF4α expression vector, on the human ACAT2 promoter (A). Data represent mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 (n=4). Models (B and C) describe protein-to-protein interaction between HNF1α and HNF4α when binding to sites in the human SOAT2 (ACAT2) gene.

(7-fold versus 10-fold) on the ACAT2 promoter activity to a lower extent.

Discussion

Recently, we identified HNF1α as an important liver-specific trans-acting element in the human ACAT2 gene, and showed that HNF1α and HNF1β, which bind to this site both in vivo and in vitro, are important regulators of the human ACAT2 promoter. As ACAT2 is responsible for production of cholesteryl esters in hepatic VLDL assembly, we initially hypothesized that MODY3 and possibly MODY1 (HNF4α is an upstream regulator of HNF1α) subjects may have lower VLDL and LDL esterified cholesterol compared to controls. Surprisingly, serum analysis of the lipoprotein fractions showed that only the MODY1 patients had significantly lower VLDL and LDL esterified cholesterol levels; the MODY3 patients instead had similar lipoprotein composition as controls. Pontoglio et al. have shown that homologous inactivation of the Tcf1 (HNF1α) gene in mice only weakly affects the transcription of most hepatic genes initially thought to be under strict HNF1α control. They suggested that HNF1β, which is present at low levels in hepatocytes, may compensate for the loss of HNF1α function. Accordingly, the apparently normal lipoprotein
reported that MODY1 subjects carrying the K99fsdelAA mutation in exon 3 of the HNF4α gene had lower triglycerides and apoCIII than non-diabetic family members. Four of the MODY1 patients in our study were carriers of this mutation, which results in a frameshift and a premature stop codon leading to truncation of the HNF4α protein to 122 instead of 465 amino acids. In contrast, Lindner et al. reported that MODY1 subjects carrying the R154X mutation in exon 4 of the HNF4α gene do not display lower triglycerides and apoCIII. The R154X mutation results in the synthesis of a truncated protein of 153 amino acids with an intact DNA-binding domain, but lacking the ligand binding and transactivation domain. Conditional liver-specific disruption of the TCF14 (HNF4α) gene in mice resulted in lower serum triglycerides and decreased expression of apoB and microsomal triglyceride transfer protein (MTP), 2 genes involved in hepatic VLDL secretion. Odom et al.21 have shown that both HNF1α and HNF4α regulate the human MTP gene; also that HNF4α regulates apoB and apoCIII. HNF4α may thus influence VLDL secretion from the liver by affecting the expression of several important proteins taking part in VLDL assembly. Studies of functional properties of several MODY1-associated mutations of HNF4α have shown that mutations can have variable effects on the ability of HNF4α to transactivate target genes.22–24 Eeckhoute et al.25 reported that the R154X mutation decreased the transcriptional activity of HNF4α and that the decrease was more pronounced in pancreatic β-cells compared with non-β-cells. This is in line with our results showing that the R154X mutation decreased the transactivation, whereas the K99fsdelAA mutation completely abolished the transactivation potential of HNF4α on the ACAT2 promoter. These results may also indirectly contribute to explaining the differences between the K99fsdelAA and the R154X mutations on triglyceride levels of MODY1 patients. The R303H mutation, in contrast, is less well characterized and results in a G→A substitution in codon 303 of exon 8. This mutation affected the transactivation potential of HNF4α on the ACAT2 promoter to a lower extent than the other mutations.

Although ACAT2 is responsible for the production of cholesteryl esters in hepatic VLDL assembly, we do not know how rate-limiting its activity is for the VLDL production and secretion in humans. Liver biopsies from MODY1 and MODY3 patients are unavailable, and animal models heterozygous for HNF1α or HNF4α do not exhibit any insulin secretion defect or glucose intolerance.26 HNF4α knock-out mice dies before birth,27 and conditional liver-specific disruption results in severe lipid and glucose abnormalities. HNF1α knock-out mice do not die but exhibit hepatic, pancreatic, and renal dysfunctions.28 Thus, appropriate comparative models to study whether HNF1α or HNF4α mutations results in reduced ACAT2 activity and how this might affect the VLDL secretion in vivo are lacking. Thus, we cannot assert that the lower levels of cholesteryl esters seen in MODY1 are solely attributable to a reduced ACAT2 expression. As mentioned above, the lipid phenotype in MODY1 patients rather derives from a general impairment of VLDL assembly and secretion to which a reduced ACAT2 activity should obviously contribute.

The role of the ACAT2-derived cholesteryl esters (cholesteryl palmitate and oleate) in the development of atherosclerosis has been extensively and directly investigated in animals.29,30 However, the recently published ULSAM (Uppsala Longitudinal Study of Adult Men) study by Waren-sjo et al.31 provides strong evidence for the importance of ACAT2-derived cholesteryl esters in coronary heart disease (CHD). In more than 2000 men with 461 cases of death from CHD, a statistically significant positive association between the ACAT2-derived cholesteryl ester content of plasma lipoproteins and CHD mortality was described. The conclusions from the ULSAM study validate the earlier results from the ARIC (Atherosclerosis Risk in Communities) study,32 in which the average carotid intima-media thickness (IMT) was positively associated with the proportion of ACAT2-derived cholesteryl esters in plasma. Thus, it may be speculated that MODY1 patients may have a less atherogenic lipoprotein profile and a lower risk for CHD compared to other forms of diabetes.

In summary, the present study has identified a role for HNF4α in the regulation of human hepatic ACAT2 gene expression.

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References


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