Hepatocyte nuclear factor-4α (HNF4A) is an orphan member of the steroid hormone receptor superfamily, expressed predominantly in the liver, intestine, kidney, and pancreas, and one of the key regulators of hepatocyte differentiation in mammals (reviewed in previous studies). The impact of HNF4A on hepatic gene expression has been studied extensively, in particular with in vitro systems using overexpression of HNF4A as a model. These studies have revealed that HNF4A is important for the regulation of genes involved in several different metabolic pathways, including lipid homeostasis. On the basis of these in vitro studies, a large number of HNF4A target genes have been identified, including those encoding proteins involved in lipoprotein metabolism. However, many genes have regulatory elements for several different transcription factors, and it is difficult to assess which factor predominates in vivo.

The analysis of the role of HNF4A in hepatic gene expression is hampered by the embryonic lethality of the standard gene knockout mouse model. However, mice with liver-specific disruption of HNF4A are viable and are characterized by reduced serum cholesterol and triglyceride concentrations and marked reductions in the hepatic expression of genes related to lipid and lipoprotein metabolism. Nevertheless, it is not clear whether these changes in gene expression and serum lipoprotein concentrations are a direct consequence of the reduced HNF4A levels or are a secondary phenomenon related to the severe liver dysfunction observed in these animals. To circumvent these problems, we used short interfering RNA (siRNA) inhibition and a gentle overexpression method to gain further insight into the role of HNF4A in the regulation of the synthesis and secretion of triglyceride-rich lipoproteins (TRLs) by the liver. It was found that up- and downregulation of HNF4A in human hepatoma Huh7 and HepG2 cells is associated with marked changes in the secretion of TRLs. We therefore searched for the critical protein that mediates this effect and demonstrates that HNF4A-dependent regulation of acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) contributes to the variation in hepatic secretion of TRLs.

Key Words: lipoproteins ■ metabolism ■ gene expression ■ liver ■ transcription

DGAT1 Participates in the Effect of HNF4A on Hepatic Secretion of Triglyceride-Rich Lipoproteins

Sergey Krapivner, Maria Jesus Iglesias, Angela Silveira, Jesper Tegnér, Johan Björkegren, Anders Hamsten, Ferdinand M. van’t Hooft

Objective—Hepatocyte nuclear factor-4α (HNF4A) is a transcription factor that influences plasma triglyceride metabolism via an as of yet unknown mechanism. In this study, we searched for the critical protein that mediates this effect using different human model systems.

Methods and Results—Up- and downregulation of HNF4A in human hepatoma Huh7 and HepG2 cells was associated with marked changes in the secretion of triglyceride-rich lipoproteins (TRLs). Short interfering RNA (siRNA) inhibition of HNF4A influenced the expression of several genes, including acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1). siRNA knockdown of DGAT1 reduced DGAT1 activity and decreased the secretion of TRLs. No additive effects of combined siRNA inhibition of HNF4A and DGAT1 were found on the secretion of TRLs, whereas the increase in TRL secretion induced by HNF4A overexpression was largely abolished by DGAT1 siRNA inhibition. A putative binding site for HNF4A was defined by in silico and in vitro methods. HNF4A and DGAT1 expressions were analyzed in 80 human liver samples, and significant relationships were observed between HNF4A and DGAT1 mRNA levels ($r^2=0.50$, $P<0.0001$) and between DGAT1 mRNA levels and plasma triglyceride concentration ($r^2=0.09$, $P<0.01$).

Conclusion—This study identified DGAT1 as an important protein that participates in the effect of HNF4A on hepatic secretion of TRLs. (Arterioscler Thromb Vasc Biol. 2010;30:962-967.)

Materials and Methods

Culture Conditions and Transfection Studies

Human hepatoma Huh7 and HepG2 cells were obtained from the Health Science Research Resources Bank (cell no. JCRB0403; Osaka, Japan) and American Type Culture Collection (HB-8065; Manassas, Va, http://www.atcc.org), respectively. The cells were cultured in low-glucose DMEM (Gibco) supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Lipo-
fectamine 2000 (Invitrogen) was used as transfection agent. siRNA oligonucleotides specific for HNF4A were designed using the siRNA Target Finder program (Ambion). The siRNA probes for DGAT1 and Sterol O-acyltransferase 2 (SOAT2) were predesigned siRNAs purchased from Ambion (catalog nos. 111782 and 111784 for DGAT1 and 111315 and 111316 for SOAT2). Throughout this study, essentially similar data were obtained for the pairs of siRNA probes for HNF4A, DGAT1, and SOAT2. For additional details on methods, please refer to the supplemental materials (available online at http://atvb.ahajournals.org).

Assays

Triglyceride secretion was measured following a 24-hour incubation of the cells with 14C glycerol (PerkinElmer Life Sciences) at a final concentration of 2.85 mCi/mL. The lipids extracted from the media were separated by thin layer chromatography, and the radioactivity associated with triglycerides was quantified. Total apolipoprotein B (APOB) in the cell medium was quantified by ELISA (ALerCHEK Inc). Septacyl S-300 HR (GE Life Sciences) was used for the chromatographic separation of the (lipoproteins) in the culture medium.

Specific gene expression was analyzed by real-time quantitative polymerase chain reaction (PCR), using 18S as invariant control. All assays and reagents were obtained from Applied Biosystems. Total cellular RNA was isolated with the RNaseasy mini kit (Qiagen).

DGAT activity was measured under apparent Vmax conditions in microsomal membranes from Huh7 cells. The assay was modified for the specific measurement of DGAT1 activity. Electrophoretic mobility shift assay (EMSA) was conducted with nuclear extracts from Huh7 cells. Supershift HNF4A antibodies and all antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology. Chromatin immunoprecipitation was performed as described in the supplemental materials.

Gene Expression in Human Liver Samples

Liver biopsies were obtained from patients undergoing elective coronary artery bypass grafting at the Karolinska University Hospital as part of the Stockholm Atherosclerosis Gene Expression Study. The protocol was approved by the Ethics Committee of the Karolinska University Hospital, and all patients gave informed consent to their participation. All genes were quantified by real-time quantitative PCR in 8 replicates for every liver sample. The normalized expression values for HNF4A, DGAT1, APOC3, and APOE were calculated using the mean values of 3 housekeeping genes as an invariant control.

Statistical Analysis

Logarithmic transformation was performed on all skewed variables to obtain a normal distribution before statistical computations and significance testing was undertaken. Differences in continuous variables between groups were tested by the Student 2-tailed t test. Associations between continuous variables were calculated by simple regression.

Results

Effect of HNF4A Gene Inhibition on the Secretion of TRLs

The impact of 2 different HNF4A siRNA probes on HNF4A mRNA and protein concentrations was analyzed 24 and 48 hours after transfection. The HNF4A mRNA level was reduced by 77%±3% (24 hours) and 73%±3% (48 hours) using HNF4A probe 1 (n=12) and by 65%±2% (24 hours) and 61%±2% (48 hours) using HNF4A probe 2 (n=10). Major reductions in HNF4A protein were observed in Western blot experiments when evaluating the 2 HNF4A siRNA probes 24 hours (not shown) and 48 hours (Figure 1A) after transfection.

The human hepatoma Huh7 and HepG2 cell lines were used to study the effect of HNF4A siRNA inhibition on the secretion of TRLs. In studies to be presented elsewhere, we have demonstrated that the rates of secretion of triglycerides and APOB from these cells are linear over time and that there is no evidence of extracellular hydrolysis or reuptake of the secreted lipoproteins. As shown in Figure 1B, the triglycerides and APOB secreted by the Huh7 cells are recovered in the very low-density lipoprotein–low-density (VLDL-LDL) lipoprotein size range as analyzed by molecular sieve chromatography, indicating that these components are associated with TRLs. As shown in Figure 1C and 1D, significant reductions in the secretion of triglycerides and APOB were observed following HNF4A inhibition in Huh7 and HepG2 cells, as assessed at 2 different time points. Analysis by molecular sieve chromatography confirmed that HNF4A inhibition decreases the secretion of intact TRL particles (data not shown).

Effect of HNF4A Gene Inhibition on the Expression of Genes Involved in Lipid and Lipoprotein Metabolism

The impact of HNF4A siRNA inhibition on the expression of genes involved in lipid and lipoprotein metabolism was analyzed in an attempt to delineate the factor(s) mediating the reduced secretion of TRLs. The TaqMan Low Density Array system was used to screen the expression of 37 genes at 2 different time points (Supplemental Table I). A number of genes were subsequently selected for more detailed study by single gene analysis using TaqMan assays. The effects of HNF4A siRNA inhibition in Huh7 cells on the expression of genes with putative roles in secretion of TRLs, analyzed at 2 different time points, are shown in Figure 2. As expected, a significant reduction in the expres-
secretion of APOC3, an established HNF4A target gene, was observed following 24 hours of HNF4A siRNA inhibition. This effect was more pronounced following 48 hours of HNF4A siRNA inhibition, at which time point other known targets for HNF4A (for example APOB) also showed significant reductions in expression (see Supplemental Table I). However, the most noteworthy reductions in expression were observed for DGAT1 and SOAT2, 2 genes with putative roles in the regulation of secretion of TRLs. In contrast, no effects of HNF4A siRNA inhibition were observed on the expression of DGAT2, SOAT1, microsomal triglyceride transfer protein (MTTP), and APOE.

Effect of HNF4A Gene Overexpression on Triglyceride Secretion and Gene Expression

Overexpression studies were performed to determine whether up- and downregulation of HNF4A have opposing effects on triglyceride secretion and gene expression. For these overexpression studies, we developed a gentle transfection technique, thereby avoiding the toxic effects of conventional adenovirus transfection. As shown in Supplemental Figure IA and IB, overexpression of HNF4A led to a significant, 2.3-fold increase in HNF4A protein. This increase was paralleled by a 2.7-fold increase in triglyceride secretion and comparable increases in the expression of DGAT1, SOAT2, and APOC3, whereas no change was observed in the expression of APOE (Supplemental Figure IC).

Effect of DGAT1 and SOAT2 Inhibition on the Secretion of TRLs

The effects of siRNA inhibition of DGAT1 and SOAT2 on the secretion of TRLs were analyzed to test the possible roles of these enzymes in mediating the effect of HNF4A siRNA inhibition on the secretion of TRLs. As shown in Figure 3A, inhibition of DGAT1 led to a profound reduction in the secretion of both triglycerides and APOB, whereas no changes were observed in the secretion of TRLs following inhibition of SOAT2. In agreement with data presented in Figure 3A, HNF4A siRNA inhibition markedly reduced DGAT1 enzymatic activity in microsomal membranes from HepG2 cells (Figure 3B).

The results of the siRNA experiments indicate that DGAT1 is primarily responsible for the HNF4A dependent secretion of TRL. To test this hypothesis directly, the effects of combined siRNA inhibition of HNF4A and DGAT1 were compared with siRNA inhibition of DGAT1 alone. As shown in Figure 3C, no evidence was found for an additional effect of siRNA inhibition of HNF4A on the secretion of TRLs. To test the hypothesis in a different fashion, the effect of combined DGAT1 and HNF4A overexpression on the secretion of TRLs was evaluated. In agreement with data shown in Figure 3A and Supplemental Figure I, it was found that DGAT1 siRNA inhibition and HNF4A overexpression were associated with reduced and enhanced secretion of TRLs, respectively (Figure 3D).

An HNF4A Binding Site Located in the DGAT1 Promoter

The effect of HNF4A knockdown on the expression of DGAT1 prompted us to search for potential HNF4A binding sites in the DGAT1 promoter. Using the MatInspector®

Figure 2. Effect of HNF4A silencing on the expression of genes involved in the secretion of TRLs. mRNA concentrations were measured 24 and 48 hours after transfection with HNF4A-specific siRNA. The values are expressed as a percentage of the control experiments (indicated with a dotted line) and represent mean ± SEM of 4 or 5 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 3. DGAT1 mediates the effect of HNF4A on the secretion of TRLs. A, Effects of SOAT2 and DGAT1 silencing on the secretion of triglycerides (TG) and APOB, analyzed 24 hours after transfection of HepG2 cells with gene-specific siRNA. The values represent mean ± SEM of 4 independent experiments. C, Effects of DGAT1 silencing and a combination of HNF4A and DGAT1 silencing on the secretion of TG and APOB, analyzed 24 hours after transfection of HepG2 cells with gene-specific siRNAs. D, Effects of HNF4A overexpression and/or DGAT1 silencing on the secretion of TG and APOB by HepG2 cells, analyzed 48 hours after transfection with HNF4A-overexpression plasmid (HNF4A) and 48 hours after transfection with HNF4A-specific siRNA (siRNA). The values in A, C, and D are expressed as a percentage of the control experiments (indicated with a dotted line) and represent mean ± SEM of 5 or 6 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. NS indicates not significant.
program, a putative HNF4A binding site was identified in the −695 to −675 region of the promoter. The CONSITE program was used to compare the human and mouse promoter sequences. The putative HNF4A binding site was located in a short section of the promoter with a high degree of conservation (Figure 4A).

Binding characteristics of a 19-bp DNA fragment that contained the putative HNF4A binding site in the DGAT1 promoter (Figure 4B) were evaluated by EMSA using nuclear extracts derived from Huh7 cells (Figure 4C, left panel). Increasing concentrations of a major DNA-protein complex were observed with increasing nuclear extract concentrations. The DNA-protein complex had similar electrophoretic characteristics as the DNA-protein complexes generated by 19-bp DNA fragments containing the HNF4A consensus 1 sequence (Figure 4C) and the HNF4A binding sites in the promoters of DGAT1 and APOC3. A supershift was noted when a specific HNF4A antibody was added in the EMSA studies (Figure 4C, right panels).

Transient transfection studies were conducted to assess the physiological significance of the putative HNF4A binding site. As shown in Figure 4D, significantly increased luciferase activities were observed for the construct with the HNF4A binding site compared with the control construct or the construct with the mutated HNF4A binding site, as analyzed under basal cell culture conditions. This effect was more pronounced when HNF4A was overexpressed in the cells, whereas no differences in luciferase activities were observed following HNF4A siRNA inhibition.

Chromatin immunoprecipitation experiments were performed to evaluate the binding of HNF4A to the putative HNF4A binding site in the promoter of DGAT1. The HNF4A binding site in the promoter of APOC3 was used as a positive control. As shown in Figure 4E, PCR products of the expected size were detectable in HNF4A immunoprecipitates obtained in 2 separate experiments, whereas no PCR products were detectable following immunoprecipitation with control IgG or PCR amplification in the absence of template.

**Figure 4.** Characterization of the HNF4A binding site in the DGAT1 promoter. A, Graphic representation of the percentage of conservation between the human and mouse sequences of the DGAT1 promoter, as determined by the ConSite program. B, Alignment of the HNF4A binding sites in the promoters of DGAT1, APOC3, and APOB; the HNF4A consensus sequence used in the EMSA studies (consensus 1); and the consensus sequence derived from reference 7 (consensus 2). C, Characterization of the HNF4A binding site by EMSA using 19-bp DNA fragments containing the HNF4A binding site in the DGAT1 promoter and the HNF4A binding site from consensus-1. The effects of increasing nuclear extract (NE) concentration were evaluated in the left panel, and the results of supershift analysis using HNF4A antibody (ab) are presented in the right panels. The arrows indicate the protein-DNA complex generated by addition of the antibody. D, Evaluation of the HNF4A binding sites in transient transfection studies. Luciferase activities of constructs harboring 21-bp fragments of the HNF4A binding site in the DGAT1 promoter (N), a mutated form of the HNF4A binding site in the DGAT1 promoter (M), and a control construct (control) were measured under basal cell culture conditions (basal), 60 hours after transfection with HNF4A-overexpression plasmid (HNF4A) and 48 hours after transfection with HNF4A-specific siRNA (siRNA). E, Chromatin immunoprecipitation analysis of HNF4A binding sites in the promoters of DGAT1 and APOC3. MWM indicates molecular weight marker; HNF4A, HNF4A-specific immunoprecipitate obtained from 2 independent cell-preparations; IgG, IgG control; NTC, nontemplate control.

**Table 5.** Relationships between Hepatic HNF4A and DGAT1 mRNA Levels and Plasma Triglyceride Concentration

Normalized mRNA levels for selected genes were measured by real-time quantitative PCR in 80 human liver biopsies. As shown in Figure 5A, a significant relationship was observed between the normalized HNF4A and DGAT1 mRNA levels ($r^2=0.50$, $P<0.0001$). As a positive control, we analyzed the relationships between the normalized APOC3 and DGAT1 mRNA levels ($r^2=0.47$, $P<0.001$). No relationship was observed between the normalized APOE and DGAT1 mRNA levels, in line with the absence of an effect of HNF4A siRNA inhibition on APOE mRNA level shown in Figure 2.

A significant relationship ($r^2=0.09$, $P<0.01$) was found between the normalized DGAT1 mRNA level and the plasma triglyceride concentration (Figure 5B). In contrast, no relationships were found between the normalized DGAT1 mRNA level and other plasma lipoprotein measurements (low-density lipoprotein–cholesterol, high-density lipoprotein–cholesterol [LDL-cholesterol]), fasting plasma glucose concentration and body mass index.
hepatic gene expression using the siRNA technique. Overall, the results from our siRNA inhibition studies are in agreement with previous reports on studies in cell culture systems and mouse models, with a marked impact of HNF4A on the expression of the classical HNF4A target genes, such as APOC3. Nevertheless, some differences between the siRNA analysis and the conditional knockout data are noteworthy. For example, Hayhurst et al reported a marked reduction in the expression of MTTP in the livers of HNF4A conditional knockout mice, whereas no change in the expression of MTTP was observed following siRNA knockdown in human hepatoma cells. Similarly, it was reported by Naiki et al that overexpression of HNF4A was associated with a marked increase in APOE mRNA level, whereas in the present study, no effects of up- or downregulation of HNF4A were found on the expression of APOE. These discrepancies point to possible differences in the regulation of expression of MTTP and APOE in mouse and human hepatocytes.

Several proteins have been implicated in the regulation of the secretion of TRLs, most notably MTTP (reviewed in Shelness and Ledford), SOAT1, SOAT2, DGAT1, and DGAT2. No changes in the expressions of MTTP, SOAT1, and DGAT2 were found following HNF4A inhibition in Huh7 cells, indicating that these proteins are not involved in HNF4A-dependent regulation of secretion of TRLs. However, the expression of DGAT1, encoding a key enzyme in the synthesis of triglycerides (reviewed in Yu and Ginsberg), and the expression of SOAT2, coding for a critical enzyme in cellular cholesterol homeostasis, were markedly decreased following HNF4A inhibition. Conversely, overexpression of HNF4A was associated with markedly increased DGAT1 and SOAT2 mRNA levels. siRNA experiments demonstrated that selective inhibition of DGAT1 was associated with substantial reductions in the secretion of TRLs, whereas no changes in the secretion of TRLs were observed following selective inhibition of SOAT2. In addition, no additive effects of combined inhibition of HNF4A and DGAT1 were found on the secretion of TRLs. Taken together, these studies indicate that DGAT1 may mediate the effect of HNF4A on the secretion of TRLs.

A formal promoter analysis of hepatic DGAT1 has to our knowledge not been performed before, and it was not known before the present study whether a functional HNF4A responsive element was present in the DGAT1 promoter. Genome-wide analysis of HNF4A target genes using oligonucleotide microarray or chromatin immunoprecipitation technique did not identify DGAT1 as an HNF4A target gene, but it is not clear whether DGAT1 was included in the screens used in these studies. However, computer-assisted analysis of the human DGAT1 promoter revealed the presence of a sequence that closely resembles the HNF4A responsive element consensus sequence. This sequence was located in a short section of the promoter exhibiting a high degree of conservation, underlining the potential physiological significance of the putative HNF4A binding site. Subsequent EMSA studies, transient transfection assays, and chromatin immunoprecipitation experiments provided further evidence that this section constitutes a functional binding site for HNF4A. On the basis of these observations, it is proposed that this binding site...
mediates the regulatory role of HNF4A on the expression of DGAT1.

The present study provides, to the best of our knowledge, the first analysis of HNF4A and DGAT1 mRNA levels in liver samples obtained from a large group of human subjects. This analysis uncovered a significant association between hepatic HNF4A and DGAT1 mRNA levels. In addition, a significant relationship was observed between the hepatic DGAT1 mRNA level and the plasma triglyceride concentration. These findings are in line with the reduced plasma triglyceride concentrations observed in subjects heterozygous for maturity-onset diabetes of the young 1 (MODY1)-related mutations in HNF4A, a phenomenon that is due to HNF4A haploinsufficiency and not related to the diabetes.17,18

There are conflicting reports in the literature as to the role of DGAT1 in the secretion of TRLs. DGAT1-knockout mice have normal plasma triglyceride concentrations on low-fat as well as high-fat diets,19,20 indicating that DGAT1 is not involved in the regulation of the hepatic secretion of TRLs. However, DGAT1-knockout mice exhibit reduced hepatic triglyceride content19,20 and disturbed chylomicron secretion.21 Moreover, overexpression of human DGAT1 in McA-Rh7777 rat hepatoma cells and long-term overexpression of DGAT1 in mice22,23 increase the secretion of TRLs, whereas small-molecule inhibition of DGAT1 lowers the plasma triglyceride concentration in the Zucker fatty rat and the hyperlipidemic hamster.24 The present study extends these observations to human model systems and provides additional evidence for a regulatory role of DGAT1 in the hepatic secretion of TRLs.

In summary, our data support a role for DGAT1 in the regulation of the plasma triglyceride concentration in humans, suggesting that modulation of DGAT1 activity may be beneficial for the treatment of dyslipidemia.21

Sources of Funding
This study was supported by grants from the Swedish Medical Research Council (8691 and 15142), the European Commission (LSHM-CT-2007-037273), the Knut and Alice Wallenberg Foundation, the Swedish Heart-Lung Foundation, the Foundation for Old Soldiers, the Fredrik and Ingrid Thuring Foundation, and the Stockholm County Council (562183 and 560833). M.J.L. is a recipient of a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (PIEFGA-2008-221346).

Disclosures
None.

References
DGAT1 Participates in the Effect of HNF4A on Hepatic Secretion of Triglyceride-Rich Lipoproteins
Sergey Krapivner, Maria Jesus Iglesias, Angela Silveira, Jesper Tegnér, Johan Björkegren, Anders Hamsten and Ferdinand M. van’t Hooft

Arterioscler Thromb Vasc Biol. 2010;30:962-967; originally published online February 18, 2010;
doi: 10.1161/ATVBAHA.109.201426

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/30/5/962

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/02/18/ATVBAHA.109.201426.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
A

24h after transfection

B

48h after transfection