Regulation of Human Apolipoprotein M Gene Expression by Orphan and Ligand-dependent Nuclear Receptors

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Apolipoprotein M (apoM) plays an important role in the biogenesis and the metabolism of anti-atherogenic HDL particles in plasma and is expressed primarily in the liver and the kidney. We investigated the role of hormone nuclear receptors in apoM gene regulation in hepatic cells. Overexpression via adenovirus-mediated gene transfer and siRNA-mediated gene silencing established that hepatocyte nuclear factor 4 (HNF-4) is an important regulator of apoM gene transcription in hepatic cells. apoM promoter deletion analysis combined with DNA affinity precipitation and chromatin immunoprecipitation assays revealed that HNF-4 binds to a hormone-response element (HRE) in the proximal apoM promoter (nucleotides −33 to −21). Mutagenesis of this HRE decreased basal hepatic apoM promoter activity to 10% of control and abolished the HNF4-mediated transactivation of the apoM promoter. In addition to HNF-4, homodimers of retinoid X receptor and heterodimers of retinoid X receptor with receptors for retinoic acid, thyroid hormone, fibrates (peroxisome proliferator-activated receptor), and oxysterols (liver X receptor) were shown to bind with different affinities to the proximal HRE in vitro and in vivo. Ligands of these receptors strongly induced human apoM gene transcription and apoM promoter activity in HepG2 cells, whereas mutations in the proximal HRE abolished this induction. These findings provide novel insights into the role of apoM in the regulation of HDL by steroid hormones and into the development of novel HDL-based therapies for diseases such as diabetes, obesity, metabolic syndrome, and coronary artery disease that affect a large proportion of the population in Western countries.

Numerous epidemiological studies have shown that levels of high density lipoprotein (HDL) cholesterol in plasma are inversely related to atherosclerosis susceptibility in humans (1–4). The atheroprotective properties of HDL involve cholesterol removal from macrophages of the arterial wall, anti-oxidation, anti-inflammation, and anti-thrombotic functions as well as protection of endothelial cells from apoptosis (5–8). Biogenesis of HDL involves the interaction of apolipoprotein A-I (apoA-I), which is synthesized and secreted by the liver, with the cholesterol and phospholipid membrane transporter ATP-binding cassette transporter A1 (ABCA1) (9–14). The premature HDL particles thus formed (called pre-β-HDL) are subsequently remodeled by various plasma enzymes to form the mature spherical αHDL particles that are catabolized by membrane receptors such as the scavenger receptor class B, type I (9–14).

Apolipoprotein M (apoM)² has been shown recently to participate in HDL maturation in plasma. ApoM is a 26-kDa glycoprotein that belongs to the lipocalin protein superfamily and has been shown to bind lipophilic ligands in its hydrophobic binding pocket (15–18). ApoM is secreted by the liver and associates with HDL through its retained N-terminal signal peptide (19, 20). ApoM is also secreted by the kidney and is involved in the recycling of small lipophilic ligands via the multiple ligand receptor megalin (15).

Studies in humans and in mice overexpressing or lacking apoM have shown a positive association between plasma apoM levels and total as well as HDL and LDL cholesterol concentrations (21–23). Wolfrum et al. (24) demonstrated that the lack of apoM expression in transplantation factor-1α/hepatocyte nuclear factor-1α (TCF-1α/HNF-1α) knock-out mice or in apoM small interfering RNA-injected mice leads to formation of larger size HDL1 particles and the disappearance of pre-β-HDL particles in plasma suggesting that apoM may play a role in HDL remodeling, particularly with regard to metabolism of pre-β-HDL. Adenoviral apoM overexpression in LDL receptor-deficient mice (animal model for premature atherosclerosis) or hepatic overexpression of apoM in apoM transgenic mice led to a reduction in atherosclerosis development (23, 24). ApoM was also found to be positively associated with pre-β-HDL formation in type 2 diabetes subjects (22). ApoM-containing HDL particles isolated from human plasma and apoM transgenic mice have been shown to be more resistant to oxidation and more efficient in protecting against LDL oxidation as well as stimulating cholesterol efflux from macrophage foam cells (23, 25).

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² The abbreviations used are: apoM, apolipoprotein M; SHP, small heterodimer partner; DNAP, DNA-affinity precipitation; HRE, hormone-response element; RXR, retinoid X receptor; RAR, retinoid acid receptor; TR, thyroid hormone receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; 9-cis RA, 9-cis-retinoic acid; FXR, farnesoid X receptor; m.o.i., multiplicity of infection; T₃, triiodothyronine; 22(Ο)HC, 22(Ο)-hydroxycholesterol.

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Genetic association studies in Chinese populations have associated two single-nucleotide polymorphisms located in the apoM proximal promoter region (single-nucleotide polymorphisms T-778C and SNP T-855C) with the development of coronary artery disease (26, 27). The T-778C polymorphism was also found to be associated with type 1 and 2 diabetes in Chinese populations supporting the possible involvement of apoM in the pathogenesis of diabetes as suggested by the reduced apoM expression levels found in animal models of diabetes and some patients with diabetes and metabolic syndrome (22, 28–34).

ApoM is expressed mainly in the liver and kidney (35), but the regulatory mechanisms that control human apoM gene transcription are not well understood. In the liver, apoM transcription has been shown to be controlled mainly by TCF-1α/HNF-1α, liver receptor homolog-1 (LRH-1), and Forkhead box A2 (FOXA2) transcription factors (33, 36, 37). Small heterodimer partner (SHP) has been shown to inhibit apoM gene expression in response to bile acids by interfering with the function of LRH-1, which binds to the proximal apoM promoter (36). Treatment of cells with lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or interleukin 1 (IL-1) decreased apoM mRNA levels and the secretion of apoM, although in humans with acute bacterial infections or chronic HIV infection, serum apoM levels were decreased (38).

In this study, we demonstrate that hepatocyte nuclear factor-4α (HNF-4α) positively regulates apoM gene expression via a hormone-response element in the proximal promoter adjacent to the previously characterized HNF-1α-binding site. HNF-4α is an orphan member of the hormone nuclear receptor gene superfamily (39, 40) and a key transcription factor in the liver, regulating numerous target genes involved in lipoprotein metabolism, including apolipoproteins, cholesterol synthesis enzymes, and bile acid transporters (41). Mice lacking HNF-4α expression in the liver have altered lipid metabolism exhibiting lipid accumulation in the liver and greatly reduced plasma total and HDL cholesterol levels (42). Recent genome-wide association studies identified a common variant in the coding region of HNF-4α gene (rs1899861) associated with low HDL concentrations in humans (43). A cDNA microarray analysis of HNF-4α-induced genes in human hepatoma cells showed a 4.5-fold increase in apoM expression levels (44). Using siRNA-mediated gene silencing and adenovirus-mediated gene transfer, we show here that HNF-4 is an important regulator of apoM gene expression in hepatic cells. HNF-4 binds to a hormone-response element (HRE) present in the proximal apoM promoter. The same HRE mediates induction of apoM gene transcription by ligands of other members of the hormone nuclear receptor superfamily such as homodimers of retinoid X receptor α (RXRα) and heterodimers of RXRα with retinoic acid receptor (RAR), thyroid hormone receptor β (TRβ), peroxisome proliferator-activated receptor (PPAR), and liver X receptor (LXR).

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, and trypsin/EDTA for cell culture were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Charcoal-stripped serum was prepared after treatment of FBS with charcoal and dextran. Restriction enzymes and T4 DNA ligase were purchased from Minotech (Heraklion, Greece) or New England Biolabs (Beverly, MA). Go TaqDNA polymerase, dNTPs, the luciferase assay system, and the Wizard SV gel and PCR cleanup system were purchased from Promega Corp. (Madison, WI). 9-cis-Retinoic acid (9-cis-RA), fenofibrate, (22R)-hydroxycholesterol, Tnα, poly(di/dc), o-nitrophenyl β-d-galactopyranoside, PMSF, aprotinin, and benzamidine were purchased from Sigma. T0901317 was purchased from Cayman Chemicals (Ann Arbor, MI). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Protein G-Sepharose were purchased from GE Healthcare. TRizol reagent for RNA extraction, SuperScript RNase H-reverse transcriptase, random hexamers, Opti-MEM, Lipofectamine 2000, and Dynabeads M-280 streptavidin were purchased from Invitrogen. The SuperSignal West Pico chemiluminescent substrate was purchased from Pierce. Anti-HNF-4α (C-19), anti-RXRα (D-20), anti-RARα (C-20), anti-LXR (H-144), anti-TRβ1 (J51), and anti-PPARα (H-98) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Myc (9E-10), anti-FLAG M2 (F-3165), and the anti-goat peroxidase-conjugated secondary antibody were purchased from Sigma. Anti-LRH-1 (ab18293) antibody was purchased from Abcam (Cambridge, MA). Anti-apoM (NB100-57088) was purchased from Novus Biologicals (Littleton, CO). Anti-actin and antishoe or anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Chemicon International Inc. (Temecula, CA). Biotinylated oligonucleotides were synthesized at VBC Biotech (Vienna, Austria). All other oligonucleotides were synthesized at the microchemical facility of the Institute of Molecular Biology and Biotechnology (Heraklion, Greece).

Plasmid Construction—The human apoM promoter constructs (~950/+42, −642/+42, −402/+42, −241/+42, −105/+42, and −49/+42)hapoM-luc were generated by PCR amplification of the corresponding fragments using human genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector (Promega Corp.). The apoM promoter construct (~20/+8)hapoM-luc was generated by ligation of a double-stranded oligonucleotide corresponding to the −20/+8 region of the human apoM promoter into the KpnI-HindIII sites of the pGL3basic vector. The (~950/+42)mut hapoluc, which bears mutations in the HRE, was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The mouse apoM promoter construct (~761/−7)mapoM-luc was generated by PCR amplification of the corresponding fragment using mouse genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector. The sh-HNF-4− and sh-control-producing vectors were generated by ligation of a double-stranded oligonucleotide that contained the siRNA-expressing sequence targeting HNF-4 or a scrambled sequence (si-control) into the BglII-HindIII sites of the pSuper.GFP.neo vector (Oligoengine, Seattle). The sequence of all oligonucleotides is shown in supplemental Table 1. The expression vectors pcDNA3-myc-LRH-1 and pCMV-SHP were kindly provided by Dr. Ioannis
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FIGURE 1. HNF-4α is a positive regulator of human apom gene expression. A, human hepatoma HepG2 cells were transiently transfected with increasing amounts (2.0 and 3.0 μg) of sh-HNF-4α or sh-control producing vectors. 48 h later, total RNA was extracted, and apom, HNF-4α, and apoC-III mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apom, HNF-4α, and apoC-III genes were quantified and are shown as a histogram. Each value represents the average from three independent experiments. B, HepG2 cells were infected with a control adenovirus expressing GFP (Ad-GFP) or with a recombinant adenovirus expressing HNF-4α (Ad-HNF-4) at an m.o.i. of 10. 24 h later, total RNA was extracted, and the mRNA levels of apom, HNF-4α, and apoC-III were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apom, HNF-4α, and apoC-III genes were quantified and are shown as a histogram. Each value represents the average from three independent experiments. C, HepG2 cells were transiently transfected with 3.0 μg of sh-HNF-4α or sh-control-producing vector, and the intracellular protein levels of apom, HNF-4α, and actin (loading control) as well as of the secreted apom were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are presented. **, p < 0.01; ***, p < 0.001.

Cell Culture, Transient Transfection, and Reporter Assays—Human hepatoma HepG2 cells and human embryonic kidney cells (HEK293T) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO2 atmosphere. For the treatment of cells with 9-cis-retinoic acid, fibrates, 22cis-retinoic acid, and 9-cis-retinoic acid for PCR with the primers shown in supplemental Table 2. For the normalization of the samples, the cDNA of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was also amplified by PCR. Gene expression levels are expressed relative to GAPDH mRNA levels. The quantification of the results was performed by measuring the intensity of the bands using the Tinascan version 2 software of Raytest (Straubenhardt, Germany).

Immunoblot Analysis—For the purification of protein extracts, cells were washed with ice-cold PBS, collected by centrifugation at 5000 rpm for 5 min at 4 °C, and resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitors. Lysates were allowed to rotate at 4 °C for 30 min and purified by centrifugation at 13,000 rpm for 5 min at 4 °C. Protein concentration was measured using the Bio-Rad DC protein assay kit, and equal amounts were loaded on SDS, 10.5% (w/v) polyacrylamide gels followed by electrotransfer onto nitrocellulose membranes. Proteins were visualized by probing the membrane with appropriate monoclonal or polyclonal antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence. To normalize the variations for protein amounts, membranes were stripped and re-probed with an antibody to β-actin.

Chromatin Immunoprecipitation (ChIP) Assays—Chromatin immunoprecipitation was performed as described previously (47) using chromatin from HepG2 cells and antibodies against HNF-4α, RXRα, RARα, PPARα, LXRα, and TRβ1. Immunoprecipitated chromatin was analyzed by PCR using primers shown in supplemental Table 3. PCR products were analyzed by agarose gel electrophoresis.

DNA Affinity Precipitation (DAP)—For the purification of nuclear extracts, HepG2 or transfected HEK293T cells were washed with ice-cold PBS, collected by centrifugation at 4000 rpm for 10 min at 4 °C, and resuspended in a hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.2 mM EDTA) supplemented with protease inhibitors. After a 10-min
incubation on ice and homogenization with a 27-gauge syringe, nuclei were collected by centrifugation at 6000 rpm for 15 min at 4 °C and resuspended in a low salt buffer (20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 1.5 mM MgCl₂, 0.02 M KCl, and 0.2 mM EDTA) and a high salt buffer (20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 1.5 mM MgCl₂, 1.2M KCl, and 0.2 mM EDTA) supplemented with protease inhibitors followed by rotation on a rotating platform for 30 min at 4 °C. Nuclear extracts were purified by centrifugation at 13,000 rpm for 40 min at 4 °C. For protein-DNA interactions, Dynabeads were washed once with 1/100B&W buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 mM DTT) and mixed with 0.58 μg of the biotinylated PCR fragment or oligonucleotide, and incubated at room temperature (25 °C) for 15 min. The oligonucleotide-coupled beads were washed twice with 1× B&W buffer and once with D buffer (20 mM Hepes (pH 7.9), 10% (v/v) glycerol, 40 mM KCl, and 0.5 mM DTT). The protein-DNA binding interactions were allowed to proceed for 30 min on ice in a buffer containing 10% (v/v) glycerol, 20 mM Hepes (pH 7.9), 40 mM KCl, 20 mM MgCl₂, 40 mM spermidine, 1 mg/ml BSA, 0.2 mM zinc acetate, 0.5% Nonidet P-40, and 0.5 mM DTT. Each reaction mixture included 30 μg of nuclear extracts, 3 μg of competitor poly(dI/dC), and the biotinylated oligonucleotide-coupled Dynabeads or uncoupled Dynabeads as controls in a total reaction volume of 50 μl. The sequence of the primers utilized for the isolation of the biotinylated PCR promoter fragments or the oligonucleotides used for DNA affinity precipitation assays are shown in supplemental Table 4. Nuclear receptor bound to the oligonucleotides was detected by SDS-PAGE and immunoblotting using the corresponding antibodies.

**Statistical Analysis**—Results are shown as means ± S.D. Statistical significance was determined using the Student’s t test. Differences with p < 0.05 were considered to be statistically significant.

**RESULTS**

**HNF-4 Is a Positive Regulator of Human apoM Gene Expression in Hepatic Cells**—To investigate the role of HNF-4 in apoM gene regulation in the liver, RNA interference by shRNAs was employed. For this purpose, an HNF-4 shRNA (sh-HNF-
A 4)-producing vector was constructed along with a scrambled shRNA (sh-control)-producing vector and used in transient transfection assays in the human hepatoblastoma-derived cell line HepG2. Transient expression of increasing amounts of sh-HNF-4 dose-dependently reduced HNF-4 mRNA levels up to 70% (Fig. 1A) and HNF-4 protein levels by ~50% compared with cells expressing sh-control (Fig. 1C). Decreased HNF-4 expression in sh-HNF-4-transfected cells was associated with a 50% reduction in apoM mRNA levels (Fig. 1A) similar to the reduction observed in the mRNA levels of the apoC-III gene, a well established target gene of HNF-4 (48–50). Both the intracellular and the secreted levels of apoM protein were also decreased in sh-HNF-4-treated cells compared with sh-control-treated cells (Fig. 1C).

In agreement with these findings, overexpression in HepG2 cells of a dominant negative mutant of HNF-4 (HNF-4 DN) that lacks the activation function 2 (AF-2) (51) reduced significantly both apoM mRNA levels to 40% of control (supplemental Fig. 1A) and protein levels (supplemental Fig. 1B). The mRNA levels of the apoC-III gene that was used as a positive control showed a comparable reduction in the presence of the HNF-4 DN mutant (supplemental Fig. 1A).

The effect of HNF-4 on apoM gene expression was next examined using adenovirus-mediated gene transfer. For this purpose, a recombinant adenovirus expressing wild type HNF-4 (Ad-HNF-4) was generated and used to infect HepG2 cells. As a control, a recombinant adenovirus expressing the green fluorescent protein (Ad-GFP) was used. Ad-HNF-4 infection in HepG2 cells led to a 2.4-fold increase in HNF-4 mRNA levels (Fig. 1B) and protein levels (Fig. 1D) compared with control Ad-GFP-infected cells. Importantly, HNF-4 overexpression was associated with a 1.9-fold increase in apoM mRNA levels (Fig. 1B) and a significant increase in both intracellular and the secreted apoM protein levels (Fig. 1D). In summary, the combined findings of Fig. 1 and supplemental Fig. 1 indicate that HNF-4 is an important regulator of apoM gene expression in hepatic cells.

**Transactivation of the Human apoM Promoter by HNF-4 Requires the Proximal −49/−20 Region**—To investigate further the mechanism of apoM gene regulation by HNF-4, a 1-kb genomic fragment bearing the human apoM promoter from nucleotide −950 to +42 was amplified by PCR using human genomic DNA as a template and cloned upstream of the luciferase reporter gene (Fig. 2A). Transactivation assays in HepG2 cells showed that HNF-4 transactivated the human apoM promoter in a dose-dependent manner (Fig. 2B).

To identify the minimal regulatory region of the apoM promoter that is required for the HNF-4-mediated transactivation, a series of luciferase reporter plasmids containing consecutive 5’ deletions of the apoM promoter were constructed (−642/−42, −402/−42, −241/−42, −105/−42, −49/−42, and −20/−48) as shown in Fig. 2A, and their transcriptional activity in the presence of HNF-4 was analyzed by transient transfection assays in human embryonic kidney HEK293T cells that do not express endogenously HNF-4 or other hepatocyte-specific nuclear factors. As shown in Fig. 2C, HNF-4 overexpression in HEK293T cells was associated with a 10-fold increase in the −950/−42 apoM promoter activity. Using the deletion mutants of the apoM promoter, it was shown that the proximal apoM promoter between nucleotides −49 and +42 was the shortest apoM promoter fragment that was responsive to HNF-4 overexpression (5.7-fold, see “Discussion”) because further deletion to nucleotide −20 completely abolished the HNF-4-mediated transactivation (Fig. 2C). In agreement with these findings, overexpression of the dominant negative form of HNF-4 in HepG2 cells was associated with the reduction of the activity of all apoM promoter fragments tested (to 20–35% relative to the control) except for the −20/+8 apoM promoter that remained unaffected (Fig. 2D). Taken together, the findings of Fig. 2 indicated that the proximal apoM promoter region between nucleotides −49 and −20 is required for transactivation by the orphan nuclear receptor HNF-4 in hepatic cells.

**HNF-4 Regulates apoM Gene Expression via a Hormone-response Element Located in the −33 to −21 Region of the apoM Promoter**—Binding of HNF-4 to the human apoM promoter in vivo was established using chromatin immunoprecipitation assays in HepG2 cells. As shown in Fig. 3B, an antibody against endogenous HNF-4 could efficiently immunoprecipitate the proximal −241/+42 apoM promoter but not the distal −950/−616 region of the apoM promoter. In control reactions, HNF-4 was found to be associated with the proximal promoter of the human apolipoprotein C-III gene (region −256/−45) but not with the unrelated proximal promoter of the small GTPase RHOB gene (region −313/−185) (52) confirming the specificity of the chromatin immunoprecipitation experiment.

Binding of HNF-4 to the proximal apoM promoter in vitro was established using the DNAP assay. For this purpose, two biotinylated overlapping PCR fragments covering the proximal apoM promoter region between nucleotides −241 and +42 were generated (−241/−81 and −105/+42) (Fig. 4A). As

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**FIGURE 3.** Chromatin immunoprecipitation assays establishing the recruitment of HNF-4 to the proximal human apoM promoter in HepG2 cells. A, a schematic representation of the human apoM promoter region, with arrows showing the location of the oligonucleotide primer sets (distal region, −950/−616; proximal region, −241/+42), that were utilized in the chromatin immunoprecipitation assays. B, HepG2 cells were subjected to chromatin immunoprecipitation in the absence (2nd lane) or presence (3rd lane) of an anti-HNF-4 antibody using primers corresponding to the proximal or the distal region of the apoM promoter, the proximal region (−256/−45) of the apoC-III promoter harboring a DRI HRE (positive control), or the proximal region (−313/−185) of the RHOB promoter (unrelated region, negative control). Nonimmunoprecipitated chromatin was included as a positive control (1st lane, input). The experiment was performed three times, and representative images are presented.
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A

\[
\begin{align*}
-341 & \\
-241 & \\
-183 & \\
-124 & \\
-68 & \\
-11 & \\
\end{align*}
\]

\[\text{HNF-4} \quad \text{HRE (DR-1)} \]

B

C

D

E

shown in Fig. 4B, endogenous HNF-4 present in nuclear extracts from HepG2 cells bound to the \(-105/+42\) biotinylated promoter fragment (4th lane) but not to the \(-241/-81\) biotinylated promoter fragment (3rd lane). In control experiments it was shown that HNF-4 did not bind to the streptavidin Dynabeads (Fig. 4B, 2nd lane, no probe) or to a double-stranded biotinylated oligonucleotide corresponding to the \(-76/-43\) region of the human \(RHOB\) promoter that contains a previously characterized CAAT box (Fig. 4B, 5th lane) (52). In competition DNAP assays, it was shown that binding of HNF-4 to the \(-105/+42\) biotinylated promoter fragment could be competed out by increasing amounts of a nonbiotinylated PCR promoter fragment corresponding to the \(-49/+42\) region of the \(apoM\) promoter (Fig. 4C). This finding, taken together with the transactivation data of Fig. 2, strongly suggested that the HNF-4-binding site is located in the region defined by nucleotides \(-49\) and \(-20\).

The sequence of the proximal human \(apoM\) promoter contains a putative DR1 (direct repeat with one nucleotide spacing) HRE spanning nucleotides \(-33\) to \(-21\) (Fig. 4A). As DR-1 elements are preferred binding sites for HNF-4 (53), we investigated whether HNF-4 binds to the \(apoM\) promoter via this DR-1 HRE. For this purpose, DNAP experiments were performed using endogenous HNF-4 from HepG2 nuclear extracts and a double-stranded biotinylated oligonucleotide corresponding to the \(-40/-14\) region of the \(apoM\) promoter that includes the putative HRE. As shown in Fig. 4D (top), HNF-4 bound efficiently to the \(-40/-14\) biotinylated oligonucleotide probe (3rd lane). In line with a previous study (36), the \(-40/-14\) apoM-biotinylated probe bound the liver receptor homolog 1 (LRH-1) (Fig. 4D, bottom, 3rd lane). LRH-1 recognizes a 5’-CAAGG-3’ motif present in the \(-29/-25\) region of the \(apoM\) promoter, and LRH-1 binding as well as LRH-1-mediated transactivation were abolished by a C/T substitution at the first position of this motif (36). To characterize this HRE further, a biotinylated oligonucleotide probe was used bearing three nucleotide substitutions in the two half-repeats and the flanking region. As shown in Fig. 4D (top, 4th lane), these mutations completely abolished the binding of HNF-4 to this oligonucleotide, whereas they had no effect on binding of LRH-1 (Fig. 4, bottom, 4th lane) (see “Discussion”).

To exclude the presence of additional HNF-4-binding sites in the proximal \(apoM\) promoter, the same HRE mutations were introduced into the \(-105/+42\) \(apoM\) promoter fragment \((-105/+42\)mut). As shown in Fig. 4E (top, 4th lane), no binding of HNF-4 to the mutated \(-105/+42\) \(apoM\) promoter fragment was observed. As expected, the mutations had no effect on the binding of LRH-1 to this promoter fragment (Fig. 4E, bottom, 4th lane).

To investigate the functional importance of the proximal HRE for the HNF-4-mediated transactivation of the \(apoM\) promoter, the same mutations in the HRE that abolished the binding of HNF-4 (Fig. 4) were introduced into the \((-950/+42)\) apoM luciferase reporter plasmid (Fig. 5A). As shown in Fig. 5B,
increasing amounts of shRNA targeting HNF-4 in HepG2 cells dose-dependently reduced the activity of the wild type promoter to 43% of control, although it did not affect the activity of the mutant promoter (Fig. 5D). In agreement with the findings of Fig. 4, the HRE mutations did not abolish the transactivation of the apoM promoter by LRH-1 in HEK293T cells (supplemental Fig. 2A) or HepG2 cells (data not shown). The combined findings of Figs. 4 and 5 and supplemental Fig. 2 indicated that HNF-4 directly regulates apoM gene expression via a novel HRE located in the −33 to −21 region.

Novel HRE on the Proximal apoM Promoter Binds Ligand-dependent Nuclear Receptors—Previous studies have shown that hormone-response elements present in the promoters of various apolipoprotein genes such as apoC-III, apoA-I, apoC-II, apoA-IV, or apoA-II could be shared by many hormone nuclear receptors, including HNF-4 and homodimers or heterodimers of RXRα (54, 55). Initially, the association of RXRα homodimers and its heterodimers with RARα, PPARα, LXRα, TRβ, FXR, as well as of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor I (COP1-TFI or Ear-3) with the novel HRE in the proximal apoM promoter was examined by DNAP assays. As shown in Fig. 6A, RXRα homodimers (1st lane) and its heterodimers with RARα (2nd lane), PPARα (3rd lane), LXRα (4th lane), and TRβ1 (5th lane) bound to the wild type promoter (~40×14 oligonucleotide). Binding of all heterodimers except RXRα/TRβ was abolished by mutations in the HRE (~40×14 mut).

No binding to the apoM HRE was detected by TRβ1 homodimers (Fig. 6A, 6th lane), RXRα/FXRα heterodimers (7th lane), or Ear-3 (8th lane). Similar to HNF-4, RXRα homodimers and RXRα/RARα heterodimers bound to a larger apoM promoter fragment (~105×42), but this binding was severely affected by the mutations in the HRE (supplemental Fig. 3). In contrast, binding of RXRα/TRβ1 heterodimers to the above apoM promoter fragment was not affected by the HRE mutations (supplemental Fig. 3) in agreement with the findings of Fig. 6A.

FIGURE 5. Mutations in the proximal HRE reduce basal apoM promoter activity and abolish HNF-4-mediated transactivation. A, schematic representation of the wild type (−950/+42) human apoM-luc promoter construct and the corresponding construct bearing the mutations in the HRE that are shown in Fig. 4A (1.0, −950/+42) human apoM-luc (mut). B, HepG2 cells were transiently transfected with the WT or mutated (−950/+42) human apoM-luc reporter plasmid (1.0 μg) along with the HNF-4α expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg). C, HEK293T cells were transiently transfected with the WT or mutated (−950/+42) human apoM-luc reporter plasmid (1.0 μg) along with the HNF-4α expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg). D, HepG2 cells were transiently transfected with the WT or mutated (−950/+42) human apoM-luc reporter plasmid (1.0 μg) along with increasing concentrations of the sh-control- or sh-HNF-4-producing vector (1.0, 2.0, and 3.0 μg) and a β-galactosidase expression vector (1.0 μg). β-D-galactosidase activity was normalized to β-galactosidase activity and presented with histograms. Each value represents the average (± S.D.) from at least three independent experiments performed in duplicate. **, p < 0.01; ***, p < 0.001.

mutagenesis of this HRE decreased the basal activity of the (−950/+42) apoM promoter in HepG2 cells to 12% of the control indicating the importance of this element for apoM gene regulation in hepatic cells. In contrast, the HRE mutations did not affect the basal activity of the apoM promoter in HEK293T cells that lack endogenous HNF-4 (Fig. 5C). Furthermore, the HRE mutations abolished the HNF-4-mediated transactivation of the apoM promoter in both HepG2 (Fig. 5B) and HEK293T (Fig. 5C) cells. Moreover, expression of

FIGURE 6. Proximal HRE on the apoM promoter binds ligand-dependent nuclear receptors (homo- and heterodimers of RXRα). A, DNA-affinity precipitation experiments using nuclear extracts from HEK293T cells transiently transfected with expression vectors for RXRα and its heterodimer partners or Ear-3 as indicated on the right and biotinylated oligonucleotides corresponding to the wild type −40−14 region of the human apoM promoter, the mutated −40−14 apoM promoter, or the −76−43 region of the human RHOB promoter. Oligonucleotide-bound nuclear receptors were detected by Western blotting (WB) using the corresponding antibodies shown on the left. All experiments were performed three times, and representative images are shown. B, HepG2 cells were subjected to chromatin immunoprecipitation in the absence (−ab) or the presence (+ab) of an anti-RXR, RAR, PPAR, LXR, or TRβ1 antibody as indicated on the right. Promoter occupancy was assessed by PCR amplification using primers corresponding to the proximal region of the apoM promoter (~214×42), the proximal region of the apoC-III promoter (~256×45), and of the apoA-I promoter (~315×22) as positive controls or the proximal region of the RHOB promoter (~313×185) as negative control. Nonimmunoprecipitated chromatin was also included (input).

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The recruitment of the RXRα homodimers and heterodimers to the apoM promoter in vivo was investigated by chromatin immunoprecipitation assays in HepG2 cells using antibodies against the corresponding nuclear receptors. These experiments (Fig. 6B) showed that nuclear receptors RXRα, RARα, PPARα, LXRα, and TRβ are all recruited to the proximal apoM promoter region −241/+42 albeit with different affinities in agreement with the findings from DNAP assays of Fig. 6A (strong binding of RXRα and RARα and less efficient binding of PPARα, LXRα, and TRβ). The specificity of the above nuclear receptors for the apoM HRE was established using primers that amplified the apoC-III and apoA-I promoters (positive controls) as well as the RHOB promoter (negative control). Altogether, the findings of Fig. 6 indicated that in addition to HNF-4, homodimers of RXRα and heterodimers of RXRα with other ligand-dependent nuclear receptors can occupy the same HRE in the proximal apoM promoter with different affinities.

Regulation of apoM Gene Expression by 9-cis-Retinoic Acid—To assess the functional relevance of the association of RXRα homo- and heterodimers with the novel HRE of the proximal apoM promoter, nuclear receptor signaling was stimulated in HepG2 cells by the administration of the corresponding ligands, and their effect on apoM gene expression was evaluated using RT-PCR. As shown in Fig. 7A, treatment of HepG2 cells with 9-cis-RA for different times (2–24 h) resulted in a biphasic mode of apoM gene regulation with an early activation phase (at 2–4 h), which was followed by a repression phase (at 6–24 h). To investigate the mechanism of this biphasic regulation of apoM gene transcription by 9-cis-RA, we determined the mRNA levels of RXRα, HNF-4, and HNF-1, all of which have been shown previously to be regulated by RXR (56–58). HNF-4 and HNF-1 exhibited a similar response to retinoic acid treatment; their mRNA levels were increased at 2–6 h of treatment and HNF-1 exhibited a similar response to retinoic acid treatment (negative control) as well as the RHOB promoter. Altogether, the findings of Fig. 6 indicated that in addition to HNF-4, homodimers of RXRα and heterodimers of RXRα with other ligand-dependent nuclear receptors can occupy the same HRE in the proximal apoM promoter with different affinities.

FIGURE 7. Regulation of apoM gene expression by 9-cis-retinoic acid. A, HepG2 cells were treated with 9-cis-RA (1 μM) for the indicated times or left untreated. Total RNA was extracted, and apoM, HNF-4α, HNF-1α, and RXRα mRNA levels were analyzed by RT-PCR, normalized to the mRNA levels of the GAPDH gene, quantified, and are shown as a histogram. Each value represents the average from three independent experiments. B, HepG2 cells were treated with 9-cis-RA (1 μM) for the indicated times or left untreated, and the protein levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are presented. *, p < 0.05; **, p < 0.01.

Overexpression of RXRα along with its heterodimeric partner RARα in HEK293T cells was associated with a strong (24-fold) transactivation of the wild type (−950/+42)apoM promoter in the presence of 9-cis-RA but not that of the promoter bearing mutations in the HRE (Fig. 8C). Using the apoM promoter deletion constructs, it was established that, similarly to HNF-4, deletion of the promoter region between nucleotides −49 and −20 abolished RXRα- and RARα-dependent transactivation of the apoM promoter (Fig. 8D).

Induction of apoM Gene Expression by Fibrates, Oxysterols, and T3—To assess the functional contribution of the other RXRα heterodimers (RXRα/PPARα, RXRα/TRβ, and RXRα/LXRα) in the regulation of the apoM gene, HepG2 cells were treated with the corresponding ligands for 3 and 24 h, and the expression levels of apoM were monitored by RT-PCR assays and immunoblotting. As shown in Fig. 9A, treatment of HepG2 cells with fibrates, 22(OH)C, and T3 (ligands of PPARα, LXRα, and TRβ) caused a 5.1-, 5.5-, and 3.1-fold increase in apoM mRNA levels, respectively, compared with untreated cells. ApoM protein levels were also significantly increased after treatment of cells with the ligands for 3 h (Fig. 9B). Induction of
apoM mRNA and protein levels by these ligands persisted for 24 h of treatment (Fig. 9, C and D). Significant increase in the protein levels of the secreted apoM was also observed in the media of HepG2 cells that had been treated with 9-cis-RA, fibrates, and 22(OH)C for 24 h (supplemental Fig. 5).

Overexpression of the RXRa along with PPARa, LXRa, or TRβ1 in HEK293T cells that had been treated with the corresponding ligands was associated with a strong transactivation of the wild type (−950/+42)apoM promoter (28.4-, 46.5-, and 38.9-fold, respectively), whereas no significant effect was observed on the activity of the mutated apoM promoter (Fig. 9E). Strong transactivation by the RXR homo- and heterodimers as well as by HNF-4 and LRH-1 was observed using the mouse apoM promoter (−761/−7), which contains the same HRE that is 100% homologous with the human (supplemental Fig. 6, A and B). No transactivation could be observed by RXRa/FXRa heterodimers in the presence of cholesterol in accordance with the DNA binding data of Fig. 6A (data not shown). Finally, deletion analysis of the apoM promoter showed that the RXR/PPAR, RXR/LXR, and RXR/TRβ heterodimers in the presence of their ligands transactivated strongly all apoM promoter fragments tested except the (−20/+8) reporter plasmid that lacks the proximal HRE (Fig. 9F), thus establishing the contribution of the proximal HRE in the stimulation of apoM gene expression by fibrates, oxysterols, and T₃.

**DISCUSSION**

The impact of apoM on HDL metabolism has been studied mainly in mouse models. These studies indicated that apoM might affect the quality rather than the quantity of HDL and that apoM overexpression reduces the development of atherosclerosis in an atherosclerosis-prone setting (23, 24) suggesting that exploring new ways of apoM gene up-regulation may provide new therapeutic tools for the treatment of coronary artery disease.

In this study, we show that the apolipoprotein M gene is under the control of hormone nuclear receptors that have been shown to play pivotal roles in lipid and glucose metabolism such as HNF-4 and the receptors for retinoic acid, thyroid hormone, fibrates, and oxysterols (54, 55, 60). Among these nuclear receptors, HNF-4 seems to play a major role in the regulation of apoM gene expression in the liver. This is in agreement with a previous cDNA microarray analysis of HNF-4α-induced genes in human hepatoma cells that showed a 4.5-fold increase in apoM expression levels by overexpressed HNF-4 (44). HNF-1 knock-out mice are characterized by the complete absence of apoM in plasma and abnormal HDL profiles (24). Previous studies had shown that the HNF-1 promoter contains a binding site for HNF-4 and that the two factors participate in a transcriptional network operating in hepatic cells that control the transcription of many liver-specific genes, including genes involved in lipoprotein metabolism (61–63). The importance of HNF-4 and HNF-1 in lipid homeostasis is also supported by a recent genome-wide association study that showed a statistically significant association between polymorphisms in the two genes and abnormalities in the HDL and LDL levels (43).
A second key finding of this study is that apoM gene expression is subject to regulation by oxysterols that are the natural endogenous ligands of the LXR receptors. Previous studies had provided contradictory results regarding the effect of LXR ligands on apoM regulation. In one study, Zhang et al. (64) showed that oral administration of the synthetic LXR agonist T0901317 in mice was associated with a reduction in basal apoM mRNA levels in the liver. In another study, Calayir et al. (65) showed that T00901317 down-regulated apoM gene expression in mouse liver, but it up-regulated apoM gene expression in mouse intestinal cells. Similar up-regulation was observed when the natural LXR ligand 22(OH)C was used (65). These contradictory findings should be evaluated in light of a previous study showing that T0901317 is a dual LXR/FXR agonist that activates FXR more efficiently than its natural ligand, the bile acid chenodeoxycholic acid (66). Furthermore, Venteclef et al. (36) showed recently that bile acids suppress apoM gene expression in hepatocellular carcinoma cells.

**FIGURE 9.** Induction of apoM gene expression by fibrates, oxysterols, and T3. A and C, HepG2 cells were treated with fibrates (250 μM), 22(OH)C (1 μM), or T3 (1 μM) for 3 h (A) or 24 h (C). Total RNA was extracted, and apoM mRNA levels were analyzed by RT-PCR. The normalized mRNA levels of the apoM gene were quantified and are shown as a histogram. Each value represents the average from three independent experiments. B and D, HepG2 cells were treated with fibrates (250 μM), 22(OH)C (1 μM) or T3 (1 μM) for 3 h (B) or 24 h (D), and the expression levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. Each experiment was performed three times, and representative images are presented. E, HEK293T cells were transiently transfected with the WT or mutated human apoM-luc reporter plasmid (1.0 μg) along with expression vectors for RXRα, PPARγ, LXRα, and TRβ1 (1.0 μg each) at the combinations indicated along with a β-galactosidase expressing plasmid (1.0 μg). Following transfection, cells were treated with the appropriate ligands as in E for 24 h. Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (± S.D.) from at least three independent experiments performed in duplicate. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**FIGURE 10.** Summary of regulatory elements and transcription factors that participate in the control of human apoM gene transcription. See text for details.
hepatic cells via an LRH-1 (liver receptor homologue-1)-binding site in the proximal apoM promoter and that this repression required SHP. SHP is an inhibitory nuclear receptor activated by FXRs that interacts physically with many nuclear receptors, including LRH-1, and interferes with their transactivation potential (67). The LRH-1 site reported by Venteclef et al. (36) is located inside the proximal apoM HRE that was characterized in this study. Interestingly, the C/T substitution in the first base of the LRH-1-binding site that was shown by Venteclef et al. (36) to abolish binding of LRH-1 and to inhibit the LRH-1-mediated transactivation of the apoM promoter was not equally effective in our study. This controversy could possibly be accounted for by the two additional substitutions that we introduced to the apoM HRE, which possibly restored LRH-1 binding to this element and the LRH-1-mediated transactivation as shown in Fig. 4, D and E, and supplemental Fig. 2A.

Based on the above findings, we are tempted to speculate that the negative effect of T0901317 on apoM gene expression in liver cells reported previously (64, 65) could be due to the activation of the FXR/SHP pathway that inhibits LRH-1 in hepatic cells. In agreement with this hypothesis, we found that treatment of HepG2 cells with 1 μM T0901317 for 3 h caused a 2-fold increase in SHP mRNA levels. In contrast, the SHP mRNA levels were not affected by 22(2H)OC (supplemental Fig. 7A). Furthermore, T0901317 treatment or SHP overexpression inhibited the LRH-1-mediated transactivation of the −105/+42 apoM promoter bearing a mutation in the LXR element in HepG2 cells suggesting that in hepatic cells T0901317 suppresses apoM gene transcription via an SHP/LRH-1 pathway (supplemental Fig. 7, C and D). In contrast to HepG2 cells, T0901317 failed to inhibit apoM gene transcription in intestinal Caco-2 cells where LRH-1 is expressed at low levels or in Caco-2 cells overexpressing LRH-1 suggesting that the T0901317/SHP pathway is not operating in these cells. As a result, T0901317 induced apoM gene expression in Caco-2 cells (supplemental Fig. 7B). Although the proximal apoM HRE resembles a DR-1 element (direct repeat with 1 nucleotide spacing), which is not favored by LXR (supplemental Fig. 7, B), it is located inside the proximal apoM HRE, which possibly restored apoM gene expression in the liver. Although it is possible that other key players are missing from this picture, strong evidence indicates that liver apoM gene expression is controlled by the interplay between liver-enriched factors such as HNF-1, HNF-4, LRH-1, and FOXA2/HNF-3β with ligand-dependent nuclear receptors such as homo- and heterodimers of RXR. Thus, apoM is a novel target for ligands shown previously to have a beneficial effect on HDL levels (oxysterols and fibrates). Synergistic interactions between the above factors could be required for optimal apoM gene expression. In support of this hypothesis, we showed that HNF-4 transactivates the apoM promoter synergistically with LRH-1 (supplemental Fig. 2, B and C) and HNF-1 (data not shown).

HDL is an important atheroprotective molecule, and many different HDL-based therapies of diabetes, obesity, metabolic syndrome, and coronary artery disease are currently under development. Having established the importance of apoM for HDL physiology and cholesterol homeostasis, it is anticipated that the detailed understanding of how this gene is regulated in health and disease will provide novel therapeutic and diagnostic tools for the above diseases that affect a large proportion of the population in Western countries.

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