Regulation of Anti-atherogenic Apolipoprotein M Gene Expression by the Orphan Nuclear Receptor LRH-1*

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The orphan nuclear receptor liver receptor homolog-1 (LRH-1, NR5A2) has been reported to play a crucial role in early development, in the control of the hepatic inflammatory response, in intestinal cell crypt renewal as well as in bile acid biosynthesis and reverse cholesterol transport (RCT). Here, we report the identification of apolipoprotein M (APOM) as a novel target gene for LRH-1. Using gene-silencing experiments, adenovirus-mediated overexpression, transient transfection, and chromatin immunoprecipitation (ChIP) assays, it is shown that LRH-1 directly regulates human and mouse APOM transcription by binding to an LRH-1 response element located in the proximal APOM promoter region. In addition, we demonstrate that bile acids suppress APOM expression in a SHP-dependent manner in vitro and in vivo by inhibiting LRH-1 transcriptional activity on the APOM promoter as demonstrated by in vivo ChIP assay. Taken together, our results demonstrate that LRH-1 is a novel regulator of APOM transcription and further extend the role of this orphan nuclear receptor in lipoprotein metabolism and cholesterol homeostasis.

The liver receptor homolog-1 (LRH-1, NR5A2) is a member of the nuclear receptor superfamily (1). Nuclear receptors have central roles in nearly every aspect of development and adult physiology. LRH-1 is the mammalian homolog of the Drosophila fushi tarazu F1 receptor (FTZ-F1; NR5A3) and, like FTZ-F1, binds its cognate target sequence (5’-(Py)CAAGG(Py) C(Pu)-3’) as a monomer (2). It is highly expressed in ovary, liver, intestine, and pancreas (2–4). Several groups have recently shown by x-ray crystallography that the human LRH-1 ligand binding pocket can bind various phospholipids including phosphoinositides and phosphatidylethanolamine linking phospholipid metabolism to gene transcription (5–8). LRH-1 has also been shown to be regulated at the post-translational level via phosphorylation (9) and sumoylation (10).

LRH-1 is involved in the regulation of expression of transcription factors implicated in embryonic development such as Oct4 and the hepatic nuclear factors HNF-3β, HNF4α, and HNF1α (11, 12). Recently, LRH-1 was shown to regulate estrogen production through the control of aromatase (CYP19) gene transcription in ovarian and adipose tissue (12, 13) and adiponectin in adipocytes (14). Furthermore, LRH-1 has recently been reported to be involved in intestinal crypt cell renewal by co-activating β-catenin on the cyclin D1 promoter (15). LRH-1 has recently been found to be a negative regulator of the hepatic acute phase response (16). Ectopic expression of LRH-1 using adenovirus resulted in the inhibition of IL1β- and IL6-mediated induction of acute phase gene expression such as haptoglobin, serum amyloid A, C-reactive protein in cultured hepatocytes. In addition, LRH-1 partial deficiency led to an exacerbated inflammatory response in vitro and in vivo indicating that LRH-1 is a physiological modulator of the hepatic acute phase response. Moreover, molecular studies revealed that LRH-1 negatively interferes with the development of the acute phase response by, at least in part, antagonizing C/EBP transcriptional activity and by inducing interleukin 1 receptor antagonist gene expression (16, 17). In addition, Mueller et al. (18) recently reported the involvement of LRH-1 in extra-adrenal glucocorticoid synthesis in the intestine with likely consequences for immune homeostasis.

In addition to its various functions during development, cell cycle, and inflammation, LRH-1 is believed to be a key player in cholesterol homeostasis (1). LRH-1 is known to play a pivotal role in the transcriptional regulation of CYP7A1, the rate-limiting enzyme of the bile acid biosynthetic pathway (3) and CYP8B1, the oxysterol 12α-hydroxylase required for cholic acid production (19). Moreover, LRH-1 has been reported to regulate expression of APO AI (20), ABCG5/ABCG8 (21), CETP (22), SR-BI (4), and the carboxyl ester lipase (23), thereby, implicating this receptor in HDL remodeling and cholesterol transport. More recently, two groups suggested that LRH-1 may play a role in the hepatic lipogenesis (24, 25). Huang et al. (24) showed that the microsomal triglyceride transfer protein is an LRH-1 target gene whereas Matsukuma described a functional cooperation between LRH-1 and LXR in hepatic fatty acid synthase gene regulation (25).

Here, we show by combining gene-silencing experiments and overexpression studies that the recently described apolipoprotein M gene is regulated by LRH-1 at the transcriptional level. Off note, ApoM is believed to be a novel player in HDL metabolism and atherosclerosis (see Ref. 37 for review). Moreover, we identified a novel LRH-1 response element within the APOM gene promoter to which LRH-1 binds in vitro and in
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**Materials and Methods**

**Cell Culture**—HepG2 cells (ATCC) were maintained in Basic Eagle’s medium (BME) supplemented with 2 mM glutamine, 1% non-essential amino acids and 10% (v/v) fetal calf serum in an atmosphere of 5% CO2 at 37 °C. HepG2 cells stably expressing short hairpin RNA (shRNA) targeting LRH-1 expression or lacZ as control were previously described (16). Mouse primary hepatocytes were provided by Biopredic (Rennes, France) and cultured in William’s E medium supplemented with 1% fetal calf serum, 10 mM dexamethasone, 100 units/ml penicillin, 100 μg/ml streptomycin, and ITS-G.

**Adenovirus**—The GFP and LRH-1-FLAG expressing adenoviruses were previously described (16). Cells were infected, in most of the experiments, at a multiplicity of infection (MOI) of 10 particles per cell, by adding virus stocks directly to the cell culture medium.

**Plasmids**—The plasmids, pSG5-LRH-1, pSG5-LRH-1 ΔH12, and pSG5-SHP have been previously described (20). The pSG5 plasmid was purchased from Stratagene (La Jolla, CA). The human APOM promoter construct (−1001; +1) was obtained by PCR amplification using human genomic DNA (Clontech) as template (5′′-GGTACCGCTCATGCCTGAATCCC-3′′ and 5′′-CTCGAGAGCTTTTGGTGGAACATCTT-GGTACCGCTCATGCCTGTAATCCC-3′′). The resulting PCR product was inserted as a KpnI/HindIII fragment into pGL3 basic vector (Promega) yielding hAPOM-Luc. The mutation of the LRH-1 binding site within the human APOM promoter was obtained by site-directed mutagenesis (Stratagene) using the following oligonucleotides: 5′′-CAGGTGAAAGGGTAAAGGGTCGAC-GCAAGGGA-3′′. All constructs were verified by DNA sequence analysis.

**Transient Transfection Assays**—HepG2 cells, plated in 24-well plates at 50–60% confluence in BME supplemented with 10% fetal calf serum, were transiently transfected with reporter and receptor expression plasmids using the Fugene 6 reagent (Roche Applied Science) as indicated in the figure legends. The pSEAP2 expression plasmid (Clontech) was co-transfected to assess transfection efficiency. 48-h post-transfection, cells were collected and assayed for luciferase and receptor expression plasmids using the Fugene 6 reagent (Roche Applied Science) as indicated in the figure legends.

**RNA Analysis**—Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer’s instructions. The RNA was treated with DNase I (Ambion Inc., Austin, TX) at 37 °C for 30 min, followed by inactivation at 75 °C for 5 min. Real time quantitative PCR (RT-QPCR) assays were performed using an Applied Biosystems 7900 sequence detector. Total RNA (1 μg) was reverse-transcribed with random hexamers using Taqman reverse-transcription reagents kit (Applied Biosystems) following the manufacturer’s protocol. RNA expression levels were determined by Sybr green assays as described (20). Cyclophilin transcript was used as an internal control to normalize the variations for RNA amounts. Gene expression levels are expressed relative to cyclophilin mRNA levels. All the primers used in this study are available upon request.

**Western Blot Analysis**—Protein extracts were fractionated on 10% polyacrylamide gel under reducing conditions (sample buffer containing 10 mM dithiothreitol), and transferred onto nitrocellulose membranes. Apoproteins were visualized by probing the membrane with the following antibodies: ApoAl (Biodiagnostic), ApoB100 (Biodiagnostic), and ApoM (Abnova). LRH-1 antibodies were provided by Abcam. After incubation with a secondary peroxidase-conjugated antibody, signals were detected by chemiluminescence (Amersham Biosciences).

**Electrophoretic Mobility Shift Assay (EMSA)**—Double-stranded oligonucleotides (5′′-AAAGGGTCAAGGGTCGAC-3′′) were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to standard procedures. In vitro-translated proteins (2 μl) were incubated with 100,000 cpm of labeled probe for 20 min at room temperature in 20 μl of buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.3 μg bovine serum albumin, and 2 μg of poly(di-dC). For supershift experiments, in vitro translated proteins were incubated 10 min with anti-LRH-1 antibodies (2 μg) before adding the probe. DNA/protein complexes were analyzed by electrophoresis in a 5% non-denaturing polyacrylamide gel with 0.5 × TBE buffer. The gel was then dried and exposed at −80 °C for autoradiography.

**Chromatin Immunoprecipitation Assay (ChIP)**—ChIP assays were performed using the EpiQuick ChIP kit (Epigentek, P-2002) following the manufacturer’s instructions. LRH-1 recruitment to human APOM promoter was determined using LRH-1 polyclonal antibodies (Abcam) or IgGs as control (Santa Cruz Biotechnology). Human APOM promoter occupancy was then assessed by PCR-amplification of its regions using the following oligonucleotides: ApoMChIP-F 5′′-GGGCGCCGCTCATGCCTGAATCCC-3′′ and ApoMChIP-R 5′′-CTCGAGAGCTTTTGGTGGAACATCTT-GGTACCGCTCATGCCTGTAATCCC-3′′. The resulting PCR product was exposed at 80 °C for autoradiography.

**ChIP assays using mouse liver tissue were performed** as described (49). Briefly, liver tissue was minced to small pieces in phosphate-buffered saline and after addition of formaldehyde to a 1% final concentration immediately subjected to 10 strokes of dounce homogenization. Cross-linking was continued for 10 min and stopped by the addition of glycine at 0.125 × final concentration. Cross-linked nuclei were purified by centrifugation through a sucrose gradient and after extraction were sonicated to obtain small length DNA fragments ranging from 200 to 800 bp. The antibodies used for immunoprecipitations were the polyclonal antibodies against SHP, HNF-1, and LRH-1 described in Refs. 10, 29, 50 and antibodies from Santa Cruz Biotechnology against RNA pol-II (H-224). The specificity of each antibody was verified by IP-Western blot assays using cross-linked extracts. Each assay was performed with three different pools of five liver tissues. Quantitative real-time PCR reactions were performed as described (51) using primers amplifying the mouse APOM upstream region: 5′′-ATCATCATCATCGTGGTTCAGTG-3′′ and 5′′-CTTGAGTGGATGGATGGATG-3′′. The PCR reactions were analyzed by electrophoresis on a 1% agarose gel.

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as fold enrichment over those obtained with immunoprecipitations using a non-immune serum.

**Animal Models—**Experimental protocols were approved by the GlaxoSmithKline Institutional Animal Care and Use Committee. The SHP transgenic mouse model has been previously described (29). C57Bl6 mice (males, 10-weeks old; n = 5/group) were maintained on a chow diet or chow diet supplemented with 0.1% cholic acid (Sigma) for 7 days. After 7 days of treatment, animals were sacrificed and blood was recovered for serum preparation, the liver was quickly removed, frozen in liquid nitrogen and used for RNA extraction. Lipoproteins from plasma were separated by FPLC analysis. Total cholesterol (Roche Applied Science) and triglycerides (Biomerieux) were measured by enzymatic colorimetric assays.

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

**RESULTS AND DISCUSSION**

Several studies suggested that LRH-1 plays a crucial role in lipid homeostasis and more specifically in reverse cholesterol transport (RCT) (1). To identify new LRH-1 target genes supporting its involvement in cholesterol homeostasis, we performed gene-silencing experiments in the liver-derived cell line, HepG2 cells. The expression of a number of genes implicated in lipoprotein metabolism and RCT was determined in HepG2 cells stably expressing short hairpin RNA (shRNA) targeting LRH-1 expression or lacZ as control (Fig. 1A). In this cell line, LRH-1 mRNA is dramatically reduced (~80%) (Fig. 1A and Ref. 16). In line with previous reports (3, 19, 20, 26), the expression of well-established LRH-1 target genes including APOAI, CYP7A1, CYP8B1 was significantly reduced in shLRH-1 HepG2 compared with control cells (Fig. 1A). By contrast, SR-B1 mRNA levels were not affected by LRH-1 gene knockdown suggesting that LRH-1 does not participate to the control of the basal expression of this gene. Among the various apolipoprotein genes studied, only APOM expression was significantly lowered in response to LRH-1 inhibition (~60%). Interestingly, APOA2, APOA4, APOA5, APOB, and APOE gene expression levels were not modified in shLRH-1 compared with control cells suggesting that LRH-1 inhibition is specific. To verify whether these results obtained by RNA interference were not due to off-target effects, we performed LRH-1 overexpression in HepG2 cells using adenovirus (Fig. 1B). Ectopic expression of LRH-1 in HepG2 cells resulted in a significant increase in all the well-established target genes including APOAI, CYP7A1, CYP8B1, SRP, and SR-B1 (Fig. 1B). The lack of effects of LRH-1 overexpression on most of the apolipoprotein genes demonstrates the specificity of those findings. A 4-fold increase in APOM transcripts was noticed in response to LRH-1 overexpression. This latter result and the gene-silencing data (Fig. 1A) strongly suggest that APOM may be a novel LRH-1 target gene in HepG2 cells.

We next focused our attention in dissecting out the molecular mechanism by which LRH-1 regulates APOM expression. First, infection of both HepG2 cells and mouse primary hepatocytes with increasing amounts of virus encoding LRH-1 resulted in a robust and dose-dependent increase in APOM mRNA levels (Fig. 2A and B). Western blot analysis confirmed that this up-regulation also occurs at the protein level (Fig. 2C). Furthermore, ApoM secretion was sharply reduced in shLRH-1 HepG2 compared with shLacZ cells (Fig. 2D) in line with our previous gene expression data (Fig. 1A). As expected, LRH-1 protein levels were significantly lower in shLRH-1 HepG2 compared with shLacZ cells. Those results suggest that LRH-1 regulates APOM expression in both human and mouse hepatocytes.

To determine the molecular mechanism by which LRH-1 regulates APOM expression, a 1-kb fragment of the human APOM promoter (28) was isolated by PCR and inserted upstream of a luciferase reporter gene. Transient transfection experiments performed in HepG2 cells revealed that LRH-1 dose-dependently increased APOM promoter activity (up to 4.5-fold) (Fig. 3A) demonstrating that APOM regulation occurs at the transcriptional level. Interestingly, transfection of a dominant negative form of LRH-1 (LRH-1ΔH12) reduced basal APOM promoter activity (Fig. 3B). Bioinformatic analysis revealed the presence of several putative LRH-1 binding sites within the human promoter. However, only one site located within the proximal region (90-bp upstream of the transcrip-

![Figure 1. Inhibition of LRH-1 expression results in down-regulation of APOM gene expression in HepG2 cells.](image-url)
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FIGURE 2. Ectopic expression of LRH-1 induces APOM gene expression in both HepG2 cells and mouse primary hepatocytes. Panels A and B, HepG2 cells (A) or mouse primary hepatocytes (B) were infected with Ad-LRH-1 or Ad-GFP (MOI as indicated). 24-h later, total RNA was extracted and APOM mRNA levels were measured by quantitative RT-QPCR. Results were normalized against Ad-GFP effects. Panels C and D, Western blot analysis of ApoM in the culture medium of HepG2 cells infected with Ad-LRH-1 or Ad-GFP for 24 h (panel C) or in HepG2 cells stably expressing short hairpin RNA (shRNA) targeting LRH-1 expression or lacZ as control (panel D). NS, nonspecific band as loading control. LRH-1 expression was also quantified by Western blot analysis in the intracellular compartment of shLRH-1 and shLacZ cells as described under “Materials and Methods.”

FIGURE 3. LRH-1 regulates APOM expression at the transcriptional level via a response-element located in the proximal promoter. HepG2 cells (panel A) were transfected with increasing amounts of a LRH-1 expression plasmid (0, 50, 100, or 200 ng) and the human APOM promoter (100 ng). Panel B, HepG2 were transfected with the human APOM promoter (100 ng) and pSG5-LRH-1 (200 ng) or pSG5-LRH-1ΔH12 or empty vector (pSG5, 200 ng). Panel C, HepG2 cells were transfected with the human wild-type (wt) or LRH-RE mutated (Mut) promoter construct (100 ng) and LRH-1 (200 ng) or empty vector (pSG5, 200 ng). *p < 0.05 LRH-1 versus empty vector.

LRH-1-mediated repression of LRH-1 transcriptional activity (20, 29–33), we next tested the influence of FXR agonists on APOM expression (Fig. 5). Treatment of primary hepatocytes with a synthetic (GW4064 1 μM) or a natural (chenodeoxycholic acid 50 μM) FXR ligand reduced APOM and increased SHP gene expression (Fig. 5). As a control, CYP7A1 mRNA levels were strongly suppressed by both compounds in line with previous reports (31, 32). The inverse correlation between APOM and
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FIGURE 4. LRH-1 binds to the APOM promoter in vitro and in vivo. The ability of LRH-1 to bind the putative response element in the APOM gene was examined by electrophoretic mobility-shift assay (EMSA) as outlined under "Materials and Methods." Panel A, sequence of the oligonucleotide probes (sense strand only) corresponding to the putative LRH-1 response element from the human and mouse APOM genes (apoM-83). Panel B, EMSA was performed with recombinant human LRH-1 as indicated and radiolabeled apoM-83. Competition EMSA was performed using in vitro synthesized LRH-1 and the increasing (1×, 10×, and 50×) molar excess of unlabeled apoM-83 or apoM-83mut. The position of the shifted LRH-1 complex and free probes are indicated. Panel C, HepG2 cells were subjected to ChIP assay using an LRH-1 antibody or control IgGs as described under "Materials and Methods." In vivo APOM promoter occupancy was assessed by PCR amplification of the proximal promoter region.

FIGURE 5. Natural and synthetic FXR agonists suppress APOM gene expression in primary hepatocytes. Primary human hepatocytes were maintained as described under "Materials and Methods" and treated with chenodeoxycholic acid (CDCA, 50 μM) or GW4064 (1 μM) or vehicle (Me2SO, 0.1%) for 48 h prior to harvest and determination of APOM, CYP7A1, LRH-1, and SHP mRNA levels by RT-QPCR. *p < 0.05 treated versus untreated cells.

SHP expression in response to FXR agonists indirectly supports the existence of a SHP-dependent mechanism of APOM repression. To further investigate this potential signaling cascade leading to APOM gene repression in response to bile acids, transient transfection experiments were performed (Fig. 6). Co-transfection of low amounts of a SHP-expressing vector led to the inhibition LRH-1-induced APOM promoter activity (Fig. 6A). Basal promoter activity was also decreased in response to SHP overexpression but to a lesser extent. The low amounts of SHP expressing vector used in this setting are compatible with the high affinity of SHP for LRH-1 (34). Basal repression of APOM transcription is likely due to the inhibition of endogenous LRH-1 bound to APOM promoter in HepG2 cells. To demonstrate this, increasing concentrations of SHP expressing vector were co-transfected with the wild-type or LRH-1 response element mutated promoter construct in HepG2 cells. Whereas the wild-type promoter activity was decreased in a dose-dependent manner in response to SHP, the mutated construct containing the disrupted LRH-1 binding site was not affected suggesting that promoter repression occurs in a LRH-1-dependent manner (Fig. 6B). Altogether, these findings suggest that the suppression of APOM by bile acids and the synthetic FXR agonist is most likely mediated by a FXR-SHP-LRH-1 cascade at least in vitro.

To further test the relevance of those observations in vivo, C57BL6 mice were fed with a diet supplemented with a low dose of cholic acid (0.1%) for 7 days. At the end of the protocol after an overnight fast, mice were sacrificed and liver gene expressions were determined by RT-QPCR (Fig. 7A). As expected, bile acid supplementation led to a strong inhibition of both CYP7A1 and CYP8B1 gene expressions whereas SHP was significantly up-regulated (5-fold) (Fig. 7A). Interestingly, both APOAI and APOM mRNA levels were decreased in response to bile acids which is consistent with our in vitro generated data (Fig. 5). This inhibition also occurred at the protein level as demonstrated by the Western blot analysis performed on plasma derived from those mice. ApoM levels were significantly reduced in response to the cholic acid supplementation (~53% as demonstrated by densitometric analysis and normalized against the NS control). Off note, ApoB100 protein levels were not affected by bile acid treatment (Fig. 7B). Those results indicate that bile acids suppress APOM expression in vitro and in vivo.

Finally, to address the question of the role of SHP in bile acid-induced APOM suppression in vivo, we studied APOM regulation in transgenic mice constitutively expressing SHP in the liver (29). As a matter of fact, those mice have significantly lower HDL-cholesterol levels (-40%) (29). Off note, mice treated with bile acids do also display lower HDL-cholesterol levels (data not shown, Refs. 35, 36). APOM but not APOB mRNA levels were significantly reduced in SHP-Transgenic mice compared with wild-type mice as measured by RT-QPCR (Fig. 8A). As a control, both CYP7A1 and CYP8B1 gene expression levels were significantly lowered in response to SHP overexpression as previously described (Fig. 8A) (29). Those results are very reminiscent of what was obtained in C57BL6 mice fed with the cholic acid-supplemented diet (Fig. 7). Finally, the recruitment of the various transcription factors as well as the RNA polymerase 2 to APOM promoter was analyzed in vivo. Soluble formaldehyde-crosslinked chromatin was prepared from SHP-Transgenic mice, wild-type mice and mice treated for 5 days with the bile acid-supplemented diet. LRH-1, SHP, HNF1, and RNA polymerase 2 promoter occupancies were determined using specific antibodies (see "Materials and Meth-
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The expression of ApoM is suppressed by bile acids, leading to a decrease in its levels. This suppression is mediated by LRH-1, which acts as an orphan nuclear receptor. LRH-1 binds to the APOM promoter, recruiting the transcriptional co-repressor SHP to inhibit gene expression. Consequently, bile acids suppress ApoM expression, which has been shown to be associated with a reduced risk of atherosclerosis.

FIGURE 6. SHP inhibits APOM promoter activity in a LRH-1-dependent manner. Panel A, LRH-1-mediated APOM promoter activation is abolished by SHP overexpression. HepG2 cells were transfected with the human APOM promoter (100 ng) and different combinations of LRH-1 (200 ng) and SHP (2–10 ng) or empty vector (pSG5). Panel B, HepG2 cells were transfected with the wild type or the LRH-1 response element-mutated (LRH-RE mut) APOM promoter (100 ng) and increasing amounts of SHP (50, 100, and 200 ng) or empty vector (pSG5).

FIGURE 7. Bile acids suppress APOM expression in vivo. Panel A, C57Bl6 mice were fed with a diet supplemented with cholic acid (0.1%) or chow for 7 days. After an overnight fast, livers were removed and gene expression levels were measured by RT-QPCR. Panel B, ApoA1, ApoB100, and ApoM plasma levels were determined by Western blot analysis. *p < 0.05 0.1% CA versus chow.

ods”). Under basal conditions, both LRH-1 and HNF1 transcription factors were found to be associated to the mouse APOM gene promoter (Fig. 8B). Hepatic SHP overexpression as well as bile acid supplementation (which leads to SHP mRNA induction) did not modify LRH-1 recruitment but strongly increased SHP association to the APOM promoter similarly to what was described for the CYP7A1 gene (29) (Fig. 8B). As expected, RNA polymerase 2 recruitment was decreased in response to an increase in SHP expression, which is consistent with its repressive function on gene transcription. Finally, HNF1 recruitment, as a control, was not modified in all the tested conditions. Taken together these results strongly suggest that bile acids suppress APOM expression in vivo by inhibiting LRH-1 transcriptional activity via the recruitment of SHP to the promoter.

In this study, we identified APOM as a novel target gene of the orphan nuclear receptor LRH-1. To our knowledge, LRH-1 is the first nuclear receptor described to regulate APOM transcription. ApoM is believed to be a novel player in HDL metabolism and atherosclerosis (see Ref. 37 for review). Interestingly, ApoM was initially isolated from triglyceride-rich lipoproteins (38) but has been found to be mainly present in HDL particles and in chylomicrons to a lesser extent (39). Two independent studies revealed that ApoM is indeed associated with a small HDL subpopulation (27, 39). In vivo APOM gene knock-down in mice using small interfering RNA led to the appearance of larger HDL and the absence of preβ-migrating HDL particles in the plasma (27). Preβ-HDL particles are lipid-poor lipoproteins that serve as key-acceptors for peripheral cellular cholesterol and are crucial mediators of the RCT (40). As a consequence, ApoM-deficient HDL induced significantly less cholesterol efflux from macrophages compared with normal HDL in vitro (27). Furthermore, adeno viral ApoM overexpression in LDLr KO mice was shown to slow down the progression of atherosclerosis, at least in part, by increasing total HDL-cholesterol and presumably RCT (27). More recently, Christoffersen et al. (39) demonstrated that ApoM is part of an HDL sub-population that protects against LDL oxidation. Those anti-oxidative properties may also contribute to the overall atherothrombogenic effects of ApoM overexpression. Whether LRH-1 protects against the development of the atherosclerotic plaque by regulating the expression of an array of proteins involved in lipoprotein and RCT including ApoM remains to be determined. Current investigations in our laboratory using LRH-1 conditional knock-out mice aim at delineating the function of LRH-1 in cholesterol homeostasis in vivo. Nevertheless, the identification of APOM as a novel LRH-1 target gene extents previous reports suggesting its implication in lipid homeostasis.

Bile acids were also found to regulate APOM gene expression in vitro and in vivo. Molecular studies performed in HepG2 cells (Figs. 5 and 6) as well as in vivo investigations carried out in mice fed with a diet supplemented with cholic acid or in SHP-transgenic mice (Figs. 7 and 8) strongly suggest that bile acids suppress APOM expression by inhibiting LRH-1 transcriptional activity via SHP recruitment to APOM promoter. This molecular mechanism has been shown to be operative for a number of LRH-1 target genes including CYP7A1 (31, 32), CYP8B1 (30), APOAI (20), ASBT (41) or SR-B1 (33). In a recent article, Huang et al. (24) showed that APOM mRNA levels were higher in livers from SHP-deficient mice. Moreover, in leptin-deficient mice, they found that APOM gene expression was completely absent (in agreement with a previous report, Ref.
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FIGURE 8. SHP negatively regulates liver APOM gene expression via its recruitment to the APOM promoter. Panel A, RT-QPCR assays were performed with liver RNA prepared from 60-day-old SHP-Transgenic or wild-type mice as control (n = 5/group). Panel B, analysis of transcription factors recruited to the mouse APOM promoter in wild-type, SHP-Transgenic, and mice-fed with a diet supplemented with cholic acid (0.5%) for 5 days. Each assay was performed with three different pools of five liver tissues. All of the amplification data were first normalized to input (non-immunoprecipitated chromatin) and expressed as fold enrichment over those obtained with immunoprecipitations using a non-immune serum. *, p < 0.05 SHP-Tg or WT+CA versus WT.

42) but could be rescued by back-crossing the animals with the SHP KO mice. Those results strongly suggest an important role for SHP in repressing APOM gene transcription in the liver and are in line with the present study. Nevertheless, we cannot rule out the involvement of SHP-independent mechanisms in APOM gene regulation, as suggested for CYP7A1 (43). Bile acids are known to dramatically affect HDL metabolism and RCT. A number of clinical studies have documented an inverse correlation between circulating levels of HDL and bile acids (45). Thus, patients receiving the enteric bile acid sequestrant, cholestyramine, had elevated levels of HDL-cholesterol (46). Therefore, patients receiving enteric bile acid sequestrant, cholestyramine, had elevated levels of HDL-cholesterol (46). Conversely, patients with progressive familial intrahepatic cholestasis (PFIC) or biliary atresia, conditions that result in systemic and hepatic accumulation of bile acids, exhibit reduced HDL-cholesterol levels compared with healthy individuals (47). Interestingly, cholestatic patients have reduced preβ-migrating HDL particles compared with healthy individuals (48). It is tempting to speculate that ApoM (which is mainly found in the preβ HDL subpopulations, Ref. 27) levels are reduced in those patients. This hypothesis should have to be verified in future studies.

In conclusion, this study led to the identification of LRH-1 as a novel regulator of the APOM gene and reinforces the concept that LRH-1 plays a crucial role in lipoprotein metabolism and cholesterol homeostasis.

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REFERENCES

1. Fayard, E., Auwerx, J., and Schoonjans, K. (2004) Trends Cell Biol. 14, 250–260
2. Galameath, L., Pare, J. F., Allard, D., Hamel, D., Levesque, L., Tugwood, J. D., Green, S., and Belanger, L. (1996) Mol. Cell. Biol. 16, 3853–3865
3. Nitta, M., Ku, S., Brown, C., Okamoto, A. Y., and Shan, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6660–6665
4. Schoonjans, K., Annicotte, J. S., Huby, T., Botrugno, O. A., Fayard, E., Ueda, Y., Chapman, J., and Auwerx, J. (2002) EMBO Rep. 3, 1181–1187
5. Krylova, I. N., Sablin, E. P., Moore, J., Xu, R. X., Waitt, G. M., Mackay, J. A., Juzumienes, D., Bynum, J. M., Madaus, K., Montanes, L., Lebedeva, L., Suzawa, M., Williams, J. D., Williams, S. F., Guy, R. K., Thornton, I., Fletterick, R. J., Willson, T. M., and Ingraham, H. A. (2005) Cell 120, 343–355
6. Li, Y., Choi, M., Cavey, G., Daugherty, I., Suiño, K., Kovach, A., Bingham, N. C., Kleyer, S. A., and Xu, H. E. (2005) Mol. Cell 17, 491–502
7. Ortlund, E. A., Lee, Y., Solomon, I. H., Hager, J. M., Safi, R., Choi, Y., Gaud, Z., Tripathy, A., Raetz, C. R., McDonnell, D. P., Moore, D. D., and Redinbo, M. R. (2005) Nat. Struct. Mol. Biol. 12, 357–363
8. Wang, W., Zhang, C., Marimuthu, A., Krupka, H. I., Tabrizizad, M., Shelloe, R., Mehra, U., Eng, K., Nguyen, H., Settachatgul, C., Powell, B., Milburn, M. V., and West, B. L. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 7505–7510
9. Lee, Y. K., Choi, Y. H., Chua, S., Park, Y. J., and Moore, D. D. (2006) J. Biol. Chem. 281, 7850–7855
10. Chalkiadaki, A., and Talianidis, I. (2005) Mol. Cell. Biol. 25, 5095–5105
11. Gu, P., Goodwin, B., Chung, A. C., Xu, X., Wheeler, D. A., Price, R. R., Galardi, C., Peng, L., Latour, A. M., Koller, B. H., Gossen, J., Kleyer, S. A., and Cooney, A. J. (2005) Mol. Cell. Biol. 25, 3492–3505
12. Pare, J. F., Malenfant, D., Courtine, C., Jacob-Wagner, M., Roy, S., Allard, J., and Belanger, L. (2004) J. Biol. Chem. 279, 21206–21216
13. Clyne, C. D., Speed, C. J., Zhou, J., and Simpson, E. R. (2002) J. Biol. Chem. 277, 20591–20597
14. Iwaki, M., Matsuda, M., Maeda, N., Funahashi, T., Matsuzawa, Y., Makishima, M., and Shimomura, I. (2003) Diabetes 52, 1655–1663
15. Botrugno, O. A., Fayard, E., Annicotte, J. S., Haby, C., Brennan, T., Wendling, O., Tanaka, T., Kodama, T., Thomas, W., Auwerx, J., and Schoonjans, K. (2004) Mol. Cell 15, 499–509
16. Venteclenf, N., Smith, J. C., Goodwin, B., and Delerive, P. (2006) Mol. Cell. Biol. 26, 6799–6807
17. Venteclenf, N., and Delerive, P. (2007) J. Biol. Chem. 282, 4393–4399
18. Mueller, M., Cima, I., Noti, M., Fuhrer, A., Jakob, S., Dubuquoy, L., Schoonjans, K., and Brunner, T. (2006) J. Exp. Med. 203, 2057–2062
19. Castillo-Olivares, A., and Gil, G. (2000) J. Biol. Chem. 275, 17793–17799
20. Delerive, P., Galardi, C. M., Bisi, J. E., Nicodeme, E., and Goodwin, B. (2004) Mol. Endocrinol. 18, 2378–2387
21. Freeman, L. A., Kennedy, A., Wu, J., Bark, S., Remaley, A. T., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2004) J. Lipid Res. 45, 1197–1206
22. Luo, Y., Liang, C. P., and Tall, A. R. (2001) J. Biol. Chem. 276, 24767–24773
23. Fayard, E., Schoonjans, K., Annicotte, J. S., and Auwerx, J. (2003) J. Biol. Chem. 278, 35725–35731
24. Huang, J., Iqbal, J., Li, P. K., Chan, L., Hussain, M. M., Moore, D. D., and Wang, L. (2007) Hepatology 46, 147–157
25. Matsukuma, E. K., Wang, L., Bennett, M. K., and Osborne, T. F. (2007) J. Biol. Chem. 282, 20164–20171
26. Lee, Y. K., Parker, K. L., Choi, H. S., and Moore, D. D. (1999) J. Biol. Chem. 274, 20869–20873
27. Wolfrum, C., Loy, M., and Stoffel, M. (2005) Nat. Med. 11, 418–422
28. Richter, S., Shih, D. Q., Pearson, E. R., Wolfrum, C., Fajans, S. S., Hattersley, A. T., and Stoffel, M. (2003) Diabetes 52, 2989–2995
29. Boulias, K., Katikili, N., Bamberg, K., Underhill, P., Greenfield, A., and Talianidis, I. (2005) EMBO J. 24, 2624–2633
30. Castillo-Olivares, A., Campos, J. A., Pandak, W. M., and Gil, G. (2004) J. Biol. Chem. 279, 16813–16821
31. Goodwin, B., Jones, S. A., Price, R. R., Watson, M. A., McKee, D. D., Moore, L. B., Galardi, C., Wilson, J. G., Lewis, M. C., Roth, M. E., Maloney, P. R., Willson, T. M., and Kleyer, S. A. (2000) Mol. Cell 6, 517–526
32. Lu, T. T., Makishima, M., Repa, J. J., Schoonjans, K., Kerr, T. A., Auwerx, J., and Mangelsdorf, D. J. (2000) Mol. Cell 6, 507–515
33. Malerod, L., Sporstol, M., Juvet, L. K., Mousavi, S. A., Gjoen, T., Berg, T., Roos, N., and Eskild, W. (2005) Biochem. Biophys. Res. Commun. 336, 1096–1105
34. Suzuki, T., Kasahara, M., Yoshioka, H., Umesono, K., and Morohashi, K. (2002) Endocr. Res. 28, 537
35. Claudel, T., Sturm, E., Duez, H., Torra, I. P., Sirvent, A., Kosykh, V., Fruchart, J. C., Daillongeville, J., Hum, D. W., Kuipers, F., and Staels, B. (2002) J. Clin. Investig. 109, 961–971
36. Srivastava, R. A., Srivastava, N., and Averna, M. (2000) Eur. J. Biochem. 267, 4272–4280
37. Dahlback, B., and Nielsen, L. B. (2006) Curr. Opin. Lipidol. 17, 291–295
38. Xu, N., and Dahlback, B. (1999) J. Biol. Chem. 274, 31286–31290
39. Christoffersen, C., Nielsen, L. B., Axler, O., Andersson, A., Johnsen, A. H., and Dahlback, B. (2006) J. Lipid Res. 47, 1833–1843
40. Rader, D. J. (2006) J. Clin. Investig. 116, 3090–3100
41. Chen, F., Ma, L., Dawson, P. A., Sinal, C. J., Sehayek, E., Gonzalez, F. J., Breslow, J., Anantharayanan, M., and Shneider, B. L. (2003) J. Biol. Chem. 278, 19909–19916
42. Xu, N., Nilsson-Ehle, P., Hurtig, M., and Ahren, B. (2004) Biochem. Biophys. Res. Commun. 321, 916–921
43. Holt, I. A., Luo, G., Billin, A. N., Bisi, J., McNeill, Y. Y., Kozarsky, K. F., Donahee, M., Wang, D. Y., Mansfield, T. A., Kliwer, S. A., Goodwin, B., and Jones, S. A. (2003) Genes Dev. 17, 1581–1591
44. Shih, D. Q., Bussen, M., Sehayek, E., Anantharayanan, M., Shneider, B. L., Suchy, F. J., Shefer, S., Bolllileni, J. S., Gonzalez, F. J., Breslow, J. L., and Stoffel, M. (2001) Nat. Genet. 27, 375–382
45. Insull, W., Jr. (2006) South. Med. J. 99, 257–273
46. Bard, J. M., Parra, H. J., Douste-Blazy, P., and Fruchart, J. C. (1990) Metabolism 39, 269–273
47. Melter, M., Rodeck, B., Kardorff, R., Hoyer, P. F., Petersen, C., Ballauf, A., and Brodehl, J. (2000) Am. J. Gastroenterol. 95, 3522–3528
48. Tallet, F., Vasson, M. P., Couderc, R., Lefevre, G., and Raichvarg, D. (1996) Clin. Chim. Acta 244, 1–15
49. Ktistaki, E., and Talianidis, I. (1997) Science 277, 109–112
50. Ktistaki, E., and Talianidis, I. (1997) Science 277, 109–112
51. Kouskouti, A., and Talianidis, I. (2005) EMBO J. 24, 347–357