Bile Acid Reduces the Secretion of Very Low Density Lipoprotein by Repressing Microsomal Triglyceride Transfer Protein Gene Expression Mediated by Hepatocyte Nuclear Factor-4*

Received for publication, April 16, 2004, and in revised form, August 13, 2004
Published, JBC Papers in Press, August 26, 2004, DOI 10.1074/jbc.M404255200

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Microsomal triglyceride transfer protein (MTP) is involved in the transfer of triglycerides, cholesterol esters, and phospholipids to newly synthesized apolipoprotein (apo) B. It is therefore essential for lipoprotein synthesis and secretion in the liver and the small intestine. Although several recent experiments have revealed the transcriptional regulation of the MTP gene, little has been revealed to date about hepatocyte nuclear factor-4 (HNF-4)-dependent regulation. We here report that the human MTP gene promoter contains a pair of functional responsive elements for HNF-4 and HNF-1, the latter of which is another target gene of HNF-4. Chromatin immunoprecipitation assays provide evidence that endogenous HNF-4 and HNF-1 can bind these elements in chromatin. In Hep G2 cells overexpression of either a dominant negative form of HNF-4 or small interfering RNAs (siRNAs) against HNF-4 dramatically reduces the activities of both the wild type and the HNF-4 site mutant MTP promoter. This suggests that HNF-4 regulates MTP gene expression either directly or indirectly through elevated HNF-1 levels. When Hep G2 cells were cultured with chenodeoxycholic acid (CDCA), a ligand for the farnesoid X receptor (FXR), mRNA levels for MTP and apo B were reduced because of increased expression of the factor small heterodimer partner (SHP), which factor suppresses HNF-4 activities. Chenodeoxycholic acid, but not a synthetic FXR ligand, attenuated expression of HNF-4, bringing about a further suppression of MTP gene expression. Over time the intracellular MTP protein levels and apo B secretion in the culture medium significantly declined. These results indicate that two nuclear receptors, HNF-4 and FXR, are closely involved in MTP gene expression, and the results provide evidence for a novel interaction between bile acids and lipoprotein metabolism.

MTP, expressed specifically in the liver and the small intestine, plays a critical role in the assembly and secretion of very low density lipoproteins (VLDLs) and chylomicrons. MTP exists in the lumen of the endoplasmic reticulum as a heterodimer with protein-disulfide isomerase and is involved in the transfer of triglycerides, cholesterol esters, and phospholipids to newly synthesized apo B (1, 2). If the apo B protein is not properly folded or if the enrichment of lipids is insufficient, the apo B protein is degraded by a ubiquitin-dependent proteasome process instead of proceeding to the formation of lipoprotein particles (3–5). In human patients with abetalipoproteinemia the absence of functional MTP results in a defect in the assembly and secretion of plasma lipoproteins containing apo B (6). A vital role of MTP in the formation and secretion of apo B is further supported by the fact that conditional gene knock-out mice specifically lacking hepatic MTP are unable to form VLDLs in the liver (7). Moreover, specific inhibitors of MTP lipid transfer have been developed and have lowered plasma cholesterol levels successfully (8). These findings clearly indicate that changes in MTP activities under various physiological conditions can modulate lipoprotein production and secretion in the liver and intestine.

HNF-4 is a highly conserved member of the nuclear receptor superfamily. It is a liver-enriched transcription factor that, together with other factors, plays a key role in the tissue-specific expression of a large number of genes involved in lipid and glucose metabolism. The active form of HNF-4 is a homodimer, and it does not appear to heterodimerize with other members of the nuclear receptor family. Recent investigations have shown that coenzyme A derivatives of certain fatty acids activate the receptor, and these derivatives thus have been characterized as endogenous ligands for HNF-4 (9, 10). A crucial role for HNF-4 in metabolic homeostasis was demonstrated by the finding that mutations in the HNF-4 gene cause the disorder known as maturity onset diabetes of the young (11). Conditional HNF-4 gene knock-out mice, which were produced using the Cre-loxP method with an albumin-Cre transgene, exhibit a great reduction in serum cholesterol and triglycerides because of the decreased levels of MTP and several apolipoproteins (12). Although this result indicates that MTP gene expression is under the control of HNF-4, little is known about the specific step. It has been shown that the transcriptional activity of HNF-4 is regulated by interaction with small heterodimer partner (SHP), an atypical negative nuclear receptor lacking a DNA-binding domain (13, 14). SHP, induced by FXR together with protein kinase; CDCA, chenodeoxycholic acid; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum; DN, dominant negative; siRNA, small interfering RNA; I-BABP,intestinal bile acid-binding protein; ChIP, chromatin immunoprecipitation; ERK, extracellular signal-regulated kinase; DR1, direct repeat 1.

References:

1. The abbreviations used are: MTP, microsomal triglyceride transfer protein; VLDL, very low density lipoprotein; apo, apolipoprotein; HNF, hepatocyte nuclear factor; SHP, small heterodimer partner; FXR, farnesoid X receptor; RXR, retinoid X receptor; MAPK, mitogen-activated protein kinase; CDCA, chenodeoxycholic acid; LPDS, lipoprotein-deficient serum; FBS, fetal bovine serum; DN, dominant negative; siRNA, small interfering RNA; I-BABP, intestinal bile acid-binding protein; ChIP, chromatin immunoprecipitation; ERK, extracellular signal-regulated kinase; DR1, direct repeat 1.
bile acids, controls the transcriptional activity of several other nuclear receptors including the constitutive androstane receptor, thyroid receptor, retinoid X receptor (RXR), retinoic acid receptor, estrogen receptors, peroxisome proliferator-activated receptors, the liver X receptor, and the liver receptor homolog-1 (14–19). Moreover, recent findings have provided evidence that bile acids activate a MAPK pathway (20, 21) and reduce the transactivation potential of HNF-4 (22). These findings prompted us to examine the effect of bile acids, which activate FXR and eventually induce SHP gene expression, on the HNF-4-dependent transcription of the MTP gene.

We here show that MTP gene expression is regulated by HNF-4 and HNF-1, which bind to the individual responsive element in the promoter of the human MTP gene. We also demonstrate that bile acids can down-regulate MTP transcription by impairing the transactivation potential of HNF-4 through the interaction with SHP and suppressing HNF-4 gene expression. In response to attenuated HNF-4 activity the transcription of other HNF-4-responsive genes including HNF-1 and apo B is also reduced, leading to decreased VLDL secretion. Taken together, this evidence suggests that bile acids are able to control lipoprotein synthesis and secretion via the FXR- and HNF-4-mediated pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—CDC20, anti-FLAG M2 antibody, and lipoprotein-deficient serum (LPSD) were purchased from Sigma. An FXR ligand, GW4064, was custom synthesized.

**Cell Culture**—Hep G2 and HEK293 cells were cultured with a medium containing 10% fetal bovine serum (FBS) in collagen-coated dishes as described previously (23, 24).

**Construction of Plasmids**—Expression constructs of human HNF-4, and human HNF-1, were described previously (25). A consensus HNF-4 binding sequence (28), containing a consensus HNF-1 binding sequence, was constructed by inserting the fragment encoding human HNF-4 (upstream and downstream elements of the human MTP gene, 204 bp) for MTP (upstream and downstream, 640 bp fragment from human apo B, 1900-bp fragment of human HNF-1, and 700-bp fragment from 36B4 were used as templates for 32P-labeled probes.

**Western Blot Analysis**—Hep G2 cells were cultured as described above. On day 1, the cells were refed with medium A containing 5% LPDS supplemented with 100 and 200 μg/ml CDCA. On day 5, the cells were harvested, and Western blot analysis was carried out using a polyclonal antibody against human MTP (RS001, Ref. 25). To analyze apo B secretion, on day 4, the cells were refed with the same medium, and the culture medium was collected on day 5. Western blot analysis was performed using a polyclonal antibody against human apo B (Chemicon International Inc.). As described previously (20, 27), Chromatin Immunoprecipitation (ChIP) Assay—Hep G2 cells were fixed with 1% formaldehyde in phosphate-buffered saline at 37 °C for 10 min, lysed, and sonicated (29). Soluble chromatin prepared with a ChIP assay kit (Upstate Biotechnology) was immunoprecipitated with antibodies against acetyl histone H3 (Upstate Biotechnology), human HNF-4α, (Santa Cruz Biotechnology, sc-9897), human HNF-1α (Santa Cruz Biotechnology, sc-8986), or the Gal4 DNA-binding domain (Santa Cruz Biotechnology, sc-575). Purified samples were used as templates for PCR performed for 44 cycles. Oligonucleotide primers composed of the sequences 5′-GTGAGAGACTGAAAACTGCAGC-3′ and 5′-CATCGGCGGACGAGGAGAC-3′ (205 bp) for MTP (upstream and downstream) were used for PCR.

**RESULTS**

**HNF-4-dependent Regulation of the Human MTP Promoter**—As it has been reported that all of the putative positive and negative response elements of the liver-specific MTP gene expression are localized within the human MTP promoter (−142 bp region (30), we focused on the promoter activity of the first 200 bp 5′ to the transcription start site to identify the cis-acting element for HNF-4. We found three putative HNF-4-responsive elements in this region (Fig. 1) and performed luciferase assays using wild type or mutant versions of reporter genes to confirm certain functional element(s) among them. When Hep G2 cells, which endogenously express HNF-4, were transfected with one of the reporter genes, the luciferase activities were significantly decreased only by disruption of the B site (Fig. 2A). When HEK293 cells were transfected, the pro-
To determine whether HNF-1 can bind to the putative HNF-1-responsive element, gel shift assays were performed with a nucleotide probe covering this element. A specific protein-DNA complex, which was not detected with the nuclear extracts from mock-transfected cells (Fig. 3, lanes 4–6), a complex of FLAG-HNF-4 and was supershifted by the addition of anti-FLAG antibodies (lane 1). Competition was performed using 100- and 300-fold excess amounts of unlabeled MTP A oligonucleotide (5′-CTATTAAATGTGACCGCTTCAGTGAACT-3′) or a 300-fold excess amount of unlabeled MTP mut A oligonucleotide (lanes 4–6, respectively); as described for A, competition also was performed for B (5′-GATTGTGAGTTGGCTGACCCTCTGCTTC-3′) (lanes 7–9) and for C (5′-ACAGTTGTTGGCTGACCCTCTGCTTC-3′) (lanes 10–12). The mutant sequences are shown in Fig. 1.

Endogenous HNF-4 Plays a Crucial Role for MTP Gene Expression—Although Figs. 2 and 3 clearly show that the MTP promoter activity requires both the HNF-4 and HNF-1 functions, we do not know how these factors coordinately regulate MTP gene expression. HNF-1 has been shown to be a direct transcriptional target of HNF-4 in liver (31). Therefore, one reasonable hypothesis is that HNF-4 stimulates MTP gene expression indirectly by increasing HNF-1 levels, which in turn activates the MTP promoter. To confirm the role of HNF-4, we constructed an expression plasmid for DN-HNF-4 that possesses a functional domain but lacks a DNA-binding domain and therefore suppresses the activity of endogenous HNF-4. As shown in gel mobility shift assays (Fig. 5A), a complex of HNF-4 and a probe containing the B site in the MTP promoter (lane 1) was replaced by the addition of excess amounts of DN-HNF-4 (lane 4), suggesting that this dominant negative form suppresses the activity of HNF-4. To distinguish a direct action of HNF-4 from an indirect action mediated through HNF-1, we compared the activity of wild type and mutant promoters in the presence or absence of DN-HNF-4 in Hep G2 cells (Fig. 5B). The activities of wild type and ΔHNF1 promoters significantly declined by expression of DN-HNF-4 through the intact B site. Furthermore, expression of DN-HNF-4 led to reduction in the activity of the promoter with the mutation in the HNF-4 site, confirming that HNF-4 also stimulates MTP gene expression through an indirect effect via HNF-1. Alternatively, endogenous HNF-4 functions were repressed by specific siRNAs, which had already been shown to be effective in our previous report (24). It turns out that quite similar results were obtained by both methods, i.e. weakening of endogenous HNF-4 activities (Fig. 5, B and C). Although in these assay systems it is difficult to compare quantitatively the direct with the indirect action of HNF-4, the significant effects of DN-HNF-4 and siRNAs clearly indicate that both actions are substantial.

CDCA Affects MTP Gene Expression—It has been shown that
CDCA induces SHP gene expression through activation of FXR and that SHP in turn inactivates HNF-4 functions (14). Taking into account the fact that apo B is also one of the target genes for HNF-4, it might be that bile acids reduce MTP-mediated secretion of apo B-containing lipoproteins from hepatocytes. To investigate this possibility, Hep G2 cells were cultured with a medium containing CDCA for 24 h, and Northern blot analyses were carried out. Because of a long cascade from FXR to MTP through SHP and HNF-4, we analyzed changes in mRNA levels for several genes up to 24 h so as not to overlook any effects of CDCA (Fig. 6). In addition, because we had demonstrated previously that CDCA activates the MAPK pathway (20), the cells were cultured with the indicated CDCA concentration to stimulate this pathway. As shown in Fig. 6, after a 6-h incubation, only mRNA levels for SHP, a target gene of FXR, were up-regulated, whereas the others were unaffected. MTP mRNA levels were reduced after 12 h and longer incubation with 100 μM CDCA, and the levels were reduced more robustly with 200 μM CDCA. Similar patterns were observed in terms of apo B, HNF-1, and HNF-4, which are all direct transcriptional targets of HNF-4. These results imply that the FXR-mediated activation of SHP might lead to suppression of HNF-4 target gene expression. It is likely that SHP mRNA levels were reduced after 24 h because of a self-regulatory mechanism (32).

SHP Suppresses Transcription of the MTP Gene—To investigate the direct effect of SHP on MTP promoter activity, luciferase assays were performed. When Hep G2 cells were transfected with an SHP expression plasmid, the MTP promoter activity was reduced in a dose-dependent manner (Fig. 7A). It appears that overexpressed SHP directly inhibits HNF-4 activities. To confirm an SHP-mediated reduction of HNF-4 activities, the transcriptional activity of a Gal4-HNF-4 fusion protein was examined in the presence of SHP (Fig. 7B). The overexpression of SHP resulted in inhibition of the activity of the fusion protein. This inhibitory pattern is in good accord with the pattern in Fig. 7A, suggesting that the inhibitory effect observed in Fig. 7A is mainly caused by the repression of HNF-4 activities mediated by SHP. Next, we tested whether SHP might directly suppress HNF-4 gene expression. The HNF-4 promoter containing 1.0 kb of the 5′-flanking region of the human HNF-4 gene did not respond to SHP (Fig. 7C). A previous investigation demonstrated that this 1.0-kb upstream region exhibits full promoter activity and contains functional binding sites for several transcription factors including HNF-1, HNF-4, HNF-6, GATA-6, and Sp1 (33). These results indicate that CDCA-mediated suppression of MTP gene expression is partly attributable to the functions of SHP. Moreover, it appears that HNF-4 gene expression might be regulated by an alternative mechanism rather than the FXR-SHP pathway.

To address the underlying mechanisms by which CDCA affects HNF-4 gene expression, the effects of CDCA were compared with those of a synthetic FXR ligand, GW4064. When Hep G2 cells were treated with either CDCA or GW4064 for 18 h, MTP mRNA levels were reduced more powerfully by the addition of CDCA than by the addition of GW4064 (Fig. 8A). SHP mRNA levels, which appeared to be declining already from the peak levels observed at −6 h (Fig. 6), were slightly increased by both compounds almost equally. HNF-4 gene expression was not affected by the addition of a synthetic ligand but was suppressed by CDCA.

To investigate further the difference between CDCA and GW4064, we carried out reporter assays with these FXR ligands. The MTP promoter activity was reduced by CDCA in a dose-dependent manner (Fig. 8B). Although GW4064 also repressed the MTP promoter activity, its effect was weaker than the CDCA effect (a 20% decrease at 10 μM GW4064 versus a 50% decrease at 200 μM CDCA). This result is consistent with the result of MTP mRNA levels in Fig. 8A. On the other hand, the human L-BABP promoter, which contains a functional FXR-responsive element (20), was almost equally regulated by both ligands (Fig. 8C). These results further support the notion that CDCA exerts its effect on transcription of the HNF-4 gene by an FXR-independent mechanism.

CDCA Treatment Reduces the MTP Protein Levels and ApoB Lipoprotein B Secretion—To further confirm that CDCA-mediated suppression of MTP gene expression leads to a decrease in
the intracellular MTP protein levels and in turn a reduction of apo B secretion, Western blot analyses were performed using Hep G2 cells treated with CDCA and their culture medium. The intracellular MTP protein levels indeed did decline with the addition of CDCA in a dose-dependent manner (Fig. 9), consistent with the results of a decline in their mRNA levels (Fig. 6). Concomitantly, the apo B protein levels in the culture medium were also reduced by CDCA treatment. Taken together, these results make it likely that the suppression of apo B secretion is attributable to the combined effects of the decreased MTP activity and the reduced apo B synthesis.

**DISCUSSION**

In the current study we demonstrate that the human MTP gene promoter contains a pair of functional HNF-4 and HNF-1 binding sites. The finding that disruption of one of these sites led to a dramatic suppression of the activity of the promoter in Hep G2 cells (Figs. 2A and 4A) implies the importance of these transcription factors in the tissue-specific MTP gene expression. This notion is further supported by the observation that overexpression of HNF-4 in HEK293 cells remarkably stimulated promoter activity (Fig. 2B). The luciferase activities of pMTP-204 when transfected with 0.8 μg of pSi are considered as “1.” The values given are the averages of data from more than three experiments performed in triplicate. Data are expressed as the means ± S.D.

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MTP mRNA levels were undetectably low in the HNF-4-null mouse liver (12), whereas HNF-1α-deficient mice exhibited normal hepatic levels for HNF-4 and MTP (35). SHP is an unusual orphan nuclear receptor that contains a putative ligand-binding domain but lacks a conserved DNA-binding domain (13). It has been reported that SHP can negatively regulate the transcriptional activity of a number of nuclear receptors. Because SHP is one of the FXR target genes, bile acids can exert a modulation of lipid metabolism through the actions of SHP. Therefore, we hypothesized that bile acids might be able to regulate expression of HNF-4 target genes, MTP and apo B, by modulating HNF-4 functions and in turn lead to reduction of VLDL secretion from hepatocytes. The current data clearly demonstrate that CDCA treatment stimulates SHP gene expression, which suppresses the MTP promoter activity, decreases MTP and apo B mRNA levels, and eventually elicits reduced apo B secretion from Hep G2 cells (Fig. 10). While we prepared this paper, several investigators reported that bile acid can regulate the transcription of various genes involved in blood pressure, gluconeogenesis, and triglyceride metabolism through the SHP-dependent pathway (36–38). In the current study we provided evidence that an alternative pathway as well as the SHP-dependent process might participate in the regulation of MTP gene expression by bile acid. A synthetic FXR ligand as well as CDCA was able to increase SHP mRNA levels, but its inhibitory effect on MTP gene expression was weaker than that of CDCA (Fig. 8, A and B). This suggests that the FXR-independent activity must be responsible for the CDCA effects. Two previous reports on SHP-null mice demonstrated that the repression of cholesterol 7α-hydroxylase gene expression, which is partly caused by SHP, is retained in SHP-null mice fed bile acids, demonstrating the existence of compensatory pathways of bile acid signaling...
FIG. 9. Effects of CDCA on the intracellular MTP protein levels and the apo B secretion in the medium. Hep G2 cells were cultured in a medium containing 5% LPDS with 100 or 200 μM CDCA for 4 days. Total cellular protein (50 μg/ lane) and the culture medium for the last 24 h (8 μl/ lane) were subjected to SDS-PAGE and Western blotting using antibodies against human MTP or human apo B. The signals were quantified with a LuminoImager (LAS-3000, Fujifilm).

FIG. 10. Mechanism by which CDCA reduces the VLDL secretion. Open arrows depict the increase or decrease in the mRNA or protein levels. The activation or inactivation of proteins is indicated by plus or minus signs, respectively. Because the effect of the MAPK pathway on HNF-4 gene expression remains unclear in the current report, the filled arrow from MAPK to HNF-4α is marked by a question mark.

(21, 39). We have shown previously that CDCA treatment activated the extracellular signal-regulated kinase (ERK) 1/2 in Hep G2 cells and stimulated the low density lipoprotein receptor gene expression in an FXR-independent manner (20). It also was reported that the c-Jun N-terminal kinase pathway was activated by bile acid (21, 22). Although we treated Hep G2 cells with several MAPK inhibitors to prove the importance of the MAPK pathway, SHP gene expression was unexpectedly affected by these inhibitors (data not shown). Thus, it was hard to distinguish the effects of the MAPK pathway from the SHP action in the presence of these inhibitors. At present we do not know which pathway participates in an SHP-independent effect of bile acid. It is quite important to elucidate the complex network between bile acid and cholesterol metabolism.

Acknowledgment—We thank Dr. Kevin Boru of Pacific Edit for review of the manuscript.

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Several reports have indicated that MAPK promoter activity is controlled by insulin, sterol-regulatory element-binding proteins, and chicken ovalbumin upstream promoter transcription factor II (COUP-TFI) (25, 30, 43). It has been shown that COUP-TFI can bind to the direct repeat 1 (DR1) sequence (−39 to −50 in Fig. 1) and suppress transcription by competing with RXRα (43). Although this DR1 overlaps with the HNF-4 binding site B by two nucleotides, the current observation that the mutant reporter gene pMTP-BKO, which contains the intact DR1 but lacks the HNF-4 binding motif, did not respond to HNF-4 (Fig. 2) suggests no involvement of this DR1 site in the HNF-4-dependent regulation of the MTP gene expression. Further studies are needed to elucidate the precise mechanisms for the network among these several transcription factors responsible for the regulation of MTP transcription.

In conclusion, our results reveal a novel pathway of regulation of VLDL secretion by bile acid that involves three nuclear receptors, FXR, HNF-4, and SHP. Bile acid impairs transcription of both the MTP and apo B genes, which are regulated mainly by HNF-4, by the inhibitory effect of SHP on the trans-activation potential of HNF-4 and an attenuated expression of HNF-4. This study provides evidence for molecular mechanisms regulating the complicated links between bile acid and cholesterol metabolism.

Acknowledgment—We thank Dr. Kevin Boru of Pacific Edit for review of the manuscript.
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J. Biol. Chem. 2004, 279:45685-45692.
doi: 10.1074/jbc.M404255200 originally published online August 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404255200

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