The Orphan Nuclear Receptor LRH-1 Potentiates the Sterol-mediated Induction of the Human CETP Gene by Liver X Receptor

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The human cholesteryl ester transfer protein (CETP) transfers cholesteryl esters from high density lipoproteins to triglyceride-rich lipoproteins, indirectly facilitating cholesteryl esters uptake by the liver. Hepatic CETP gene expression is increased in response to dietary hypercholesterolemia, an effect that is mediated by the activity of liver X receptor (LXR/RXR) on a direct repeat 4 element in the CETP promoter. In this study we show that the orphan nuclear receptor LRH-1 also transactivates the CETP promoter by binding to a proximal promoter element distinct from the DR4 site. LRH-1 potentiates the sterol-dependent regulation of the wild type CETP promoter by LXR/RXR. Small heterodimer partner, a repressor of LRH-1, abolishes the potentiation effect of LRH-1 but not its basal transactivation of the CETP promoter. Since this mode of regulation of CETP is very similar to that recently reported for the bile salt-mediated repression of Cyp7a1 (encoding the rate-limiting enzyme for conversion of cholester to bile acid in the liver), we examined the effects of bile salt feeding on CETP mRNA expression in human CETP transgenic mice. Hepatic CETP mRNA expression was repressed by a diet containing 1% cholic acid in male mice but was induced by the same diet in female mice. Microarray analysis of hepatic mRNA showed that about 1.5% of genes were repressed, and 2.5% were induced by the bile acid diet. However, the sexually dimorphic regulatory pattern of the CETP gene was an unusual response. Our data provide further evidence for the regulation of CETP and Cyp7a1 genes by similar molecular mechanisms, consistent with coordinate transcriptional regulation of sequential steps of reverse cholesterol transport. However, differential effects of the bile salt diet indicate additional complexity in the response of these two genes.

The cholesteryl ester transfer protein (CETP) catalyzes the transfer of cholesterol ester from HDL to triglyceride-rich lipoproteins (1). CETP is expressed in liver, intestine, and a number of peripheral tissues, such as adipose (1). In humans and animals, plasma CETP and tissue mRNA levels are increased in response to high fat, high cholesterol diets or endogenous hypercholesterolemia. These increases are due to elevated CETP gene transcription especially in the liver (2, 3). Transgenic mice expressing human CETP, controlled by its natural flanking region, also increase expression of CETP in response to hypercholesterolemia (4). The mechanism of this effect was recently shown to involve the transcription factor LXR, binding as a heterodimer with RXR to a site in the CETP proximal promoter, a direct repeat of a nuclear receptor binding sequence separated by 4 nucleotides (DR4 element, −384 to −399). This response was seen with both LXRα and LXRβ, related nuclear receptors that bind and are activated by specific hydroxylated sterols at physiological concentrations (5, 6).

LXRα also transactivates the Cyp7a1 gene, encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in the pathway converting cholester to bile acids (7). Furthermore, disruption of LXRα in mice abolished the induction of Cyp7a1 expression by dietary cholesterol (8). Based on these data, we proposed that LXRs may coordinate the regulation of genes involved in different steps of reverse cholesterol transport (9). This idea was further supported by the demonstration that ABCA1 is up-regulated by sterols in an LXR-dependent fashion, due to the interaction of LXR with a DR4 element in the proximal promoter of the ABCA1 gene (10). ABCA1, the gene that is mutated in Tangier disease, mediates phospholipid and cholesterol efflux from macrophages to apoA-I (11–15). Recently, additional LXR-regulated ABC transporters were shown to be mutated in sitosterolemia, implying a role for these molecules in excretion of sitosterol and cholesterol from intestinal cells (16). In addition to stimulating reverse cholesterol transport and cholesterol excretion, LXRs activate the promoter of SREBP-1c, indicating a role in the regulation of fatty acid synthesis (17, 18).

Liver receptor homologue-1 (LRH-1) is a mouse homologue of the orphan nuclear receptor fushi tarazu F1 (Ptz-F1) from Drosophila. CYP7A promoter binding factor (CPF), the human homologue of LRH-1, was found to transactivate the human CYP7A promoter (19). LRH-1 and CPF bind to an extended nuclear hormone receptor-binding site as monomers. LRH-1 has also been shown to act as a competence factor, enhancing the ability of LXRα/RXRα to mediate a sterol response on the Cyp7a1 gene (20). This interaction is abolished by small heterodimer partner 1 (SHP-1), just as SHP-1 negates the interaction of LXRα/RXRα with the Cyp7a1 gene.

TNFa, tumor necrosis factor α; EST, expressed sequence tag; SHP, small heterodimer partner.
action of DAX1 with SF-1 (21) and represses the activity of several nuclear receptors (22–24). Since CETP and Cyp7a1 are both regulated by LXR/RXR, we investigated the role of LRH-1 and SHP-1 in the regulation of CETP gene expression. Our study shows a marked similarity between the effects of these factors on the CETP and Cyp7a1 promoters. This supports the idea of coordinate regulation of the sequential metabolic steps mediated by CETP and CYP7A1. However, divergent responses of these two genes to a cholic acid-containing diet indicate additional complexity in the in vivo response to bile acids.

Materials and Methods

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C. 70–80% confluent cells were transfected using Lipofectamine transfection reagent (Life Technologies, Inc.) as described previously (10). 0.2 μg of reporter DNA, 25 ng of pCMV-Rl (Resella) (Promega), and 100 ng of receptors (CMX-hRXRα, CMX-hLXRα, CMX-LXRβ, CMV-LRH, and CMV-SH) were used in each transfection experiment. Empty pcDNA3.1 expression vector (Invitrogen) was used as control and to maintain equal amounts of DNA (0.625 μg per well in a 24-well plate) for each transfection. The transfected cells were cultured in 5% LPDS medium (consists of Dulbecco’s modified Eagle’s medium with 5.1 lipoprotein deficiency) in the presence of 2 μg/ml 22R-hydroxy cholesterol (Sigma) or vehicle alone for 24 h. The luciferase activities were measured using Promega Dual Luciferase assay system. Reporter constructs were constructed as described previously (9). Each experiment was carried out in duplicate.

Plasmid Construction—LRH-1 and SHP-1 were cloned by reverse transcription-polymerase chain reaction from mouse liver RNA and subcloned into pcDNA3.1 (Invitrogen). The 10-amino acid epitope from human c-Myc (EQKLLISEDDL), which can be recognized by the monoclonal antibody 9E10, was used for tagging the LRH-1 gene to produce Myc-LRH fusion protein. Myc-LRH was subcloned into the pcDNA3.1 expression vector.

Dietary and mRNA Studies in Human CETP Transgenic Mice—Human CETP transgenic mice (C57BL/6J background, 10–15 weeks old), expressing CETP controlled by its native flanking region (3.4-kb CA) were used for competitive suppression of CETP expression. Four female or four male mice for each diet. Poly(A) mRNA was prepared using Qiagen Oligotex mRNA purification Kit. Northern blotting was carried out as described (25). The blots were exposed to a PhosphorImager screen and visualized with Molecular Dynamics PhosphorImager system. The intensity of the bands was quantified using an ImageQuant tool IQuant version 1.2.

Microarray Printing and Hybridization—The Echerichia coli bacteriophage lambda DNA consisting of 1248 unique cDNA or EST clones were individually grown in 96-well microplates (Corning Costar Co.). The cDNA inserts were polymerase chain reaction-amplified with vector-derived primers directly using 5'-3' dIII overhangs, corresponding to wild type CETP binding site (LRHBS) (5'-aggagaagctttgca3') and mutated LRHBS (5'-aggagaagctttgc3') or Cyp7a1 LRH element (LRHE) (20), were used in gel shift experiments. 2 μl of lysates expressing Myc-LRH or luciferase (control) were mixed with ~50 fmol of 32P-end-labeled LRHBS fragment in a volume of 20 μl of binding buffer (75 mM KCl, 20 mM Hepes, pH 7.9, 2.5 mM diethiothreitol, 10% glycerol, 2 μg of poly(dI-dC), 30 pmol of nonspecific single-stranded oligonucleotides). Reactions were incubated at room temperature for 20 min. Protein–DNA complexes were resolved on 5% polyacrylamide gels at 140 V for 1 h. For competition experiments, ~50-fold molar excess of unlabeled competitor DNA relative to labeled DNA were added to the reaction mixture before the addition of the labeled probe. In antibody experiments, the protein lysates were first incubated with 0.4 μg of Myc epitope antibody 9E10 (sc-40, Santa Cruz Biotechnology) to control monoclonal anti-actin antibody (sc-8432, Santa Cruz Biotechnology) for 10 min without the labeled DNA, followed by 20 min of incubation in the presence of labeled DNA.

Results

Potentiation of LXR Function by LRH-1—Whereas LXRα induce robust sterol activation of a deleted or multicyclic version of the CETP promoter (9), activation of the intact, single copy CETP promoter by sterol was either modest (LXRα) or non-existent (LXRβ) (Fig. 1). To see if the competence factor LRH might increase the sterol activation, as reported recently for the Cyp7a1 promoter (20), we co-transfected LXR/RXR and LRH in 293 cells. For both LXRα and LXRβ, this resulted in a significant increase in sterol-dependent activation of promoter activity (Fig. 1). LRH alone increased basal promoter activity (about 3-fold) but did not provide a sterol response (Fig. 1). In contrast to these findings with the CETP promoter, the ABCI promoter was well activated by steroids in the presence of LXR/RXR, and the response was not further increased by co-expression of LRH (Fig. 2), showing that the LRH effect is promoter-specific.

Mutation of the LXR-binding site (DR4) in the CETP promoter abolished both the sterol-dependent increase in promoter activity, and the amplification of this effect by LRH (Fig. 3). However, this mutation did not affect the increase in basal promoter activity attributable to LRH.

LRH Binding to the CETP Promoter Is Required for Potentiation of Sterol Induction: Mapping the LRH-Binding Site—The increase in basal activity by LRH, independent of the DR4 mutation (Fig. 3), suggests that LRH binds to a site distinct from the LXR-binding region. Consistent with this idea, mu-
Regulation of Human CETP Gene by LRH-1 and SHP-1

LRH-1 has been shown to interact with LXR (20). Since LRH-1 also regulates the CETP promoter, we next showed binding of LRH to this site. Gel shift analysis showed specific binding of LRH to a DNA fragment containing the −75 to −83 region (LRHBS) of the CETP promoter (Fig. 7, arrow). In vitro translated, Myc epitope-tagged LRHs were used in the gel shift experiments. The Myc-tagged protein showed full potentiation of the sterol induction of the CETP promoter (not shown) and bound specifically to the CETP LRHBS (Fig. 7, a, lane 2, arrow). This specific band was reduced in competition assays, using either the CETP LRHBS or the Cyp7a LRHRE (20) (Fig. 7, a, lanes 3 and 5), whereas the mutant CETP LRHBS was unable to compete for binding (Fig. 7a, lane 4). This same mutation abolished the functional effects of LRH (Fig. 6b). A factor from reticulocyte lysates also bound specifically to the CETP LRHBS (Fig. 7a, lane 1, indicated by asterisk). However, this gel shift band was not competed by the Cyp7a LRHRE. An antibody to the Myc epitope, but not a control antibody (anti-actin), specifically reduced the intensity of the shifted band resulting from the binding of Myc-LRH to the CETP LRHBS (Fig. 7b, lanes 3 and 4, arrow).

SHP Abolishes the Sterol Potentiation Effect of LRH-1 on LXR in the CETP Promoter—LRH-1 has been shown to interact with and be repressed by small heterodimer partner (SHP), an orphan nuclear receptor (20). Since LRH-1 also regulates CETP gene expression, we analyzed the effect of SHP on the expression of the CETP gene by co-transfecting SHP with RXR, LXR,
CETP transactivation of the A, moter by LRH-1.

The only difference was that expression of LXR regulated similarly by LRH and SHP (Supplemental Fig. 2).

b Value is greater than 0.1 for LXR.

6-fold for LXR, respectively, in the 3.4-kb promoter, a more robust response than seen with the shorter promoter fragment (Fig. 1). LRH further increased the induction fold to about 4-fold for LRXa/RXRα and 6-fold for LRXα/RXRα.

To see if SHP also represses the basal transactivation activity of LRH-1 on the CETP promoter, increasing amounts of SHP plasmid were co-transfected with LRH. However, the LRH-1-dependent increase of the reporter activity was not repressed by expression of SHP (Fig. 9). Therefore, the inhibitory effect of SHP specifically abolished the potentiation effect of LRH-1, while not affecting the increase in basal promoter activity induced by LRH-1 (Fig. 8 and 9). These results indicate that SHP only represses the LRH-1 competence effect on the CETP promoter in a sterol-dependent manner.

Regulation of CETP Gene by Bile Salt in CETP Transgenic Mice—It has been proposed that the repression of LRH-1 activity by SHP may mediate the bile acid repression of Cyp7a (20, 27). Since CETP expression seems to be regulated by LRH-1 and SHP in a similar fashion to Cyp7a, we analyzed the regulation of CETP gene expression by bile acid in CETP transgenic mice. CETP transgenic mice expressing CETP controlled
by its native promoter (−3.4 kb) (25) were fed a chow diet with or without 1% cholic acid for 5 days. Northern blot analysis was carried out to analyze the expression of several genes that are regulated by bile acids (Fig. 10). As expected, Cyp7a expression was completely repressed by dietary cholic acid. Cyp8b (encoding 12α-Hydroxylase), also regulated by LRH-1 (28), was repressed by cholic acid feeding as reported (29). The cholic acid-containing diet also led to an increase in SHP mRNA and a decrease in Cyp27 expression (20, 29). For all of these genes a similar response was seen in male and female mice. Intriguingly, however, the response of CETP mRNA was sexually dimorphic. In male mice, CETP mRNA was decreased upon cholic acid feeding, whereas in female mice the bile salt diet led to a 2-fold induction of CETP mRNA.

Microarray Analysis of Hepatic mRNA from Mice Fed a 1% Cholic Acid Diet—In order to gain further insights into the effects of bile acids on gene expression, we carried out microarray analysis using mRNA from male and female mice. The microarrays contained about 1,200 cDNAs and ESTs, enriched for genes expressed in liver. Also, a substantial number of transcription factors expressed in liver were represented. About 2.5% of genes were induced, and 1.5% genes were repressed by dietary cholic acid, with parallel effects in both sexes (Supplemental Table 1). We and others (30) have found that the large majority of genes with altered expression on the microarrays show similar or larger changes in expression when assessed by Northern analysis. As an example, SR-BI, a high density lipoprotein receptor (31), showed 1.7- or 2.6-fold induction by the diet, in male and female mice, respectively. Northern blot analysis confirmed the results (Fig. 10).

Among the 1200 genes analyzed, 11 genes (0.9%) displayed sexually dimorphic changes in mRNA expression, in response to the bile acid-enriched diet (Table 1). The most common response was a higher induction in male than in female mice. The opposite pattern of response, i.e. female greater than male, was only demonstrated by the human CETP gene and by one EST. Notable among the dimorphically induced genes was a TNFα-induced protein, suggesting a possible differential effect of TNFα on target genes. Hepatic TNFα is induced by the bile acid-enriched diet, and both CETP and Cyp7a are repressed by TNFα (32–34).

**DISCUSSION**

We have shown that the orphan nuclear receptor LRH-1 binds and transactivates the human CETP promoter. In 293 cells, the native CETP promoter showed modest sterol induction in the presence of LXRE/RXRα or LXRβ/RXRα (Fig. 1 and Supplemental Fig. 2). The expression of LRH-1 enhanced the response of LXR/RXR to sterols. The principal tissue expressing LRH-1 is the liver, which also is a major site where CETP is expressed (4). Even though CETP is expressed in peripheral tissues, the sterol regulation in peripheral tissue is less pronounced than in liver (4). A requirement for LRH-1 in the sterol induction mediated by LXR might confer tissue specificity for the induction of genes by sterols, since the ubiquitously expressed LXRβ could otherwise mediate sterol induction of CETP gene. Additional physiological significance might be that LRH-1 abolishes the requirement for RXR ligands, such as 9-cis-retinoic acid or docosahexanoic acid (35), in order to obtain significant LXR-mediated sterol induction of gene expression. Thus, in response to endogenous hypercholesterolemia, 24(S),25-epoxycholesterol, which is synthesized from a shunt pathway (36) and has been shown to act as an LXR ligand (6), might bind and activate LXR/RXR without the requirement for additional RXR ligands. This effect is clearly shown in Fig. 8 and Supplemental Fig. 2. The presence of LRH increased the induction of CETP by steroid but had no additional incremental effect as a result of the addition of 9-cis-retinoic acid.

The effect of LRH-1 was strikingly specific for the LXR-dependent sterol induction of CETP expression. The mechanism of the LRH-1 potentiation effect required the binding of LRH-1 to a site (−75 to −83) (LRHBS) on the CETP promoter, and the DNA binding ability is required for the potentiation function of LRH-1, since mutation of the LRH-1 DNA-binding zinc finger motif abolished this effect (not shown). The activity of LRH-1 on the CETP promoter might enhance ligand binding to LXR or cooperatively work with LXR/RXR to recruit ligand-dependent co-activators. The co-activator SRC-1 (nuclear receptor co-activator) has been shown to interact with LXRβ/RXR upon binding of 22(R)-hydroxycholesterol and/or 9-cis-retinoic acid (37). It is possible that sterol-dependent binding of co-activator is enhanced either by the LRH/LXR activation or by retinoid binding to RXR. This would explain why in the presence of sterols the expression of LRH or activation by 9-cis-retinoic acid achieved similar promoter activity (Fig. 8).

Recently, considerable evidence has been obtained to support the idea that LXR/RXR coordinates the regulation of genes involved in reverse cholesterol transport (9), such as ABCA1

**FIG. 10.** Northern blot analysis of mRNA from male (M) and female (F) mice fed a chow diet or a chow diet supplemented with 1% cholic acid. Mice expressing the human CETP transgene, controlled by its native promoter, were fed with either rodent chow diet or chow diet containing 1% cholic acid for 5 days. Liver poly(A)⁺ mRNA was prepared from total RNA pooled from four mice per diet per sex. 2 μg of poly(A)⁺ RNA was loaded, and gene expression was analyzed by Northern blot, using the cDNA probes shown. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
with LRH-1 and represses LRH-1 activity in cell culture (20, 27). The abolition of LRH facilitation of the LXR-mediated sterol response by SHP was strikingly similar in the CETP and Cyp7a promoters (this study and (20)). A weak or absent effect of SHP on basal promoter activity in the presence of LRH also appears to be similar for CETP and Cyp7a (20). However, in previous studies (4) we observed that a high fat, high cholesterol diet containing bile salts induced hepatic CETP mRNA expression, whereas Cyp7a mRNA is repressed in response to the same diet. In the present study a somewhat different response of CETP and Cyp7a genes to a chow diet supplemented with 1% cholic acid was seen. Whereas Cyp7a mRNA was repressed by the bile salt-supplemented diet in both sexes, CETP mRNA was repressed in males but induced in female mice.

In an attempt to understand these different responses, we characterized the responses of about 1200 different cDNAs and ESTs to the bile salt diet. The majority of differentially expressed genes were either induced or repressed in parallel fashion in both sexes. However, 11 genes gave a sexually dimorphic response, with the majority showing an induction in males but not in females. Thus, the CETP response is unusual and is unlikely to be mediated by a simple mechanism, such as induction of a single transcription factor in one sex but not the other. The array results provide an intriguing hint for how the differential response could be mediated. Thus, the TNFα-induced protein 2 was increased in males and decreased in females, suggesting that there could be a dimorphic effect of TNFα signaling in male versus female mice. Hepatic TNFα expression is induced by the cholic acid diet (it is probably made in Kupffer cells) (33), and TNFα has been shown to repress both CETP (34) and Cyp7a (32). If the effect of TNFα is larger in male mice, this could explain why CETP is repressed in this sex. One has to then hypothesize an additional factor that up-regulates CETP in response to bile acids and only becomes apparent in females because of a lessened TNFα effect. The related gene PLTP is up-regulated by the bile salt diet, through an FXR mechanism (43), and it is conceivable that CETP could be similarly regulated. Induction of TNFα and interleukin-1 by the bile salt diet has also been suggested as a possible mechanism of repression of Cyp7a (33, 45), but this response was not dimorphic. There could be additional mechanisms of repression of Cyp7a, such as that proposed involving FXR, SHP, and LRH, affecting both sexes.

In summary, we have shown that the competence factor, LRH, enhances the LXR-mediated sterol response of the CETP promoter, probably contributes to the moderate tissue specificity of this response (46), and provides a way for the CETP promoter to respond to sterols, independent of RXR agonists.
Both of these effects, i.e. tissue-specific responses and responses to LXR/RXR that are independent of RXR ligands, may be general properties of competence factors in transcriptional responses. The interaction of LXR and LRH in the sterol-dependent induction of the CYP7A1 promoters, and the repression of this effect by SHP, is strikingly similar for CETP and CYP7A, supporting the idea of coordinate regulation of these two genes in the liver (9). However, the divergent responses of these and other genes to diets containing bile salts highlights the complexity of the in vivo response to this challenge.

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