Cross-talk between Thyroid Hormone Receptor and Liver X Receptor Regulatory Pathways Is Revealed in a Thyroid Hormone Resistance Mouse Model*

Received for publication, July 20, 2005, and in revised form, October 28, 2005 Published, JBC Papers in Press, October 31, 2005, DOI 10.1074/jbc.M507877200

Koshi Hashimoto1, Ronald N. Cohen2, Masanobu Yamada3, Kathleen R. Markan5, Tsuyoshi Monden4, Teturo Satoh1, Masatomo Mori4, and Fredric E. Wondisford5

From the 4Department of Medicine and Molecular Science, Graduate School of Medicine, Gunma University, Maebashi, Gunma 371-8511, Japan and the 5Department of Medicine, Section of Endocrinology and Committee on Molecular Metabolism and Nutrition, Pritzker School of Medicine, The University of Chicago, Chicago, Illinois 60637

Hypercholesterolemia is found in patients with hypothyroidism and resistance to thyroid hormone. In this study, we examined cholesterol metabolism in a thyroid hormone receptor β (TR-β) mutant mouse model of resistance to thyroid hormone. Whereas studies of cholesterol metabolism have been reported in TR-β knock-out mice, generalized expression of a non-ligand binding TR-β protein in this knock-in model more fully recapitulates the hypothyroid state, because the hypothyroid effect of TRs is mediated by the unliganded receptor. In the hypothyroid state, a high cholesterol diet increased serum cholesterol levels in wild-type animals (WT) but either did not change or reduced levels in mutant (MUT) mice relative to hypothyroidism alone. 7α-Hydroxylase (CYP7A1) is the rate-limiting enzyme in cholesterol metabolism and mRNA levels were undetectable in the hypothyroid state in all animals. Triiodothyronine replacement restored CYP7A1 mRNA levels in WT mice but had minimal effect in MUT mice. In contrast, a high cholesterol diet markedly induced CYP7A1 levels in MUT but not WT mice in the hypothyroid state. Elevation of CYP7A1 mRNA levels and reduced hepatic cholesterol content in MUT animals are likely because of cross-talk between TR-β and liver X receptor α (LXR-α), which both bind to a direct repeat + 4 (DR+4) element in the CYP7A1 promoter. In transfection studies, WT but not MUT TR-β antagonized induction of this promoter by LXR-α. Electromobility shift analysis revealed that LXR/RXR heterodimers bound to the DR+4 element in the presence of MUT but not WT TR-β. A mechanism for cross-talk, and potential antagonism, between TR-β and LXR-α is proposed.

Thyroid hormone is an important physiological regulator of cholesterol metabolism (1, 2). Hypercholesterolemia is found in patients with hypothyroidism and in those with resistance to thyroid hormone (2–4). Elevated serum cholesterol levels in hypothyroidism are restored to normal levels upon treatment with thyroid hormone (2, 5). In contrast, thyroid hormone treatment occasionally has paradoxical effects on serum cholesterol levels in patients with resistance to thyroid hormone (4). Cholesterol homeostasis is maintained by coordinate regulation of three primary pathways in the liver (Fig. 1) (6, 7). Two of these pathways maintain cholesterol supply by either de novo synthesis, which is largely regulated by hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGC-R),2 or cellular uptake of plasma cholesterol via low density lipoprotein receptor (LDLR). A third pathway involves the elimination of cholesterol through the synthesis of bile acids; 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in this pathway (8).

Thyroid hormone is known to regulate each of these pathways at the transcriptional level, and the predominance thyroid hormone receptor (TR) isoform in the liver is TR-β1 (9–12). Therefore, TR-β knock-out mice (−/−) have been analyzed in detail for changes in cholesterol metabolism (13, 14). Both Weiss et al. (13) and Gullberg et al. (14) reported that serum cholesterol levels did not increase in response to hypothyroidism nor decrease after triiodothyronine (T3) replacement in TR-β knock-out mice (−/−). Gullberg et al. (14) also reported that T3-deficient TR-β−/− mice showed an augmented CYP7A1 response to dietary cholesterol and did not develop hypercholesterolemia to the same extent as wild-type mice. Unfortunately, TR knock-out mice do not recapitulate the findings in either the hypothyroid state or in the syndrome of thyroid hormone resistance as other groups, including our own, have demonstrated that the TR possesses ligand-independent properties (15, 16). We studied, therefore, a mouse model of resistance to thyroid hormone where a point mutation in the ligand-binding domain of TR-β abolished its ability to bind to T3 (A337T, Ref. 15). Here, we characterize an interaction between liver X receptor (LXR) and TR, which bind to a similar DNA-response element, and show a unique role for the unliganded mutant TR-β in cholesterol metabolism.

EXPERIMENTAL PROCEDURES

Animals—TR-β knock-in mice have a mutation that deletes three nucleotides in exon 6 of the TR-β gene, resulting in loss of a threonine residue in the receptor, which prevents ligand binding (17–20). The genetic background of the TR-β knock-in mice is a hybrid of two strains (129/Sv x C57/BL6). Four-week-old male mice were employed for the study. The number of mice for each study is indicated in the figure legends. All aspects of animal care were approved by the Institutional Animal Care and Use Committee of Gunma University Graduate School of Medicine (Maebashi, Gunma, Japan) and The University of Chicago (Chicago, IL). Animals were maintained on a 12-h light/12-h dark schedule (light on at 06:00 h) and fed laboratory chow as indicated

2 The abbreviations used are: HMGC-R, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; TR, thyroid hormone receptor; RXR, retinoid X receptor; LXR, liver X receptor; CYP7A1, cholesterol 7α-hydroxylase; LDLR, low density lipoprotein receptor; DR+4, direct repeat + 4 bp. S’DI, type 1 S’ deiodinase; MMI, methimazole; PTU, propylthiouracil; WT, wild type; MUT, mutant; LDL, low density lipoprotein; T3, triiodothyronine.
and water *ad libitum*. A 2% cholesterol diet (21) was purchased from Oriental Bioservice (Tokyo, Japan). Mice were rendered hypothyroid by inclusion of 0.1% methimazole (MMI) in the drinking water and 1% (w/w) propylthiouracil (PTU) in chow for 21 days (22, 23). Then, the mice were injected daily with 5 μg/100 g body weight T₃ for an additional 5-day period to render mice euthyroid. A high cholesterol diet was begun during induction of hypothyroidism with MMI and PTU and continued through the T₃ dosing period. The total duration that animals were fed either chow or a 2% cholesterol diet was 2 weeks. Serum-free thyroxine and free T₃ levels were determined by double antibody radioimmunoassay kits (Ortho Diagnostics Co., Ltd., Tokyo, Japan). Mice were sacrificed in the morning to obtain either blood or tissue samples.

**Lipid Determination**—Serum total cholesterol levels were measured with a Determiner TC555 kit (Kyowa Medex, Tokyo, Japan). Serum triglyceride levels were determined with an SRL kit (SRL, Tokyo, Japan). Whole livers were removed, weighed, and sections were taken (100 mg) for analysis. The liver samples were homogenized in a Polytron in chloroform/methanol (2:1, v/v) and lipid was extracted as previously described (24) and analyzed for cholesterol content with a Determiner TC555 kit (Kyowa Medex).

**Measurement of Bile Acid Pool Size**—Mice were fed a 2% cholesterol diet for 2 weeks. Pool size was determined as the total bile acid content of small intestine, gallbladder, and the liver, combined as described (25). These organs were homogenized in 20 ml of ethanol (at 60 °C), the extract was filtered and a 1-ml aliquot dried with a centrifuge concentrator at room temperature. The residue was dissolved in 1 ml of methanol and subjected to an enzymatic assay for total bile acid content (Kyokuto Pharmaceutical Co., Tokyo, Japan).

**Northern Blot Analysis**—Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and 5–20 μg of total RNA was subjected to Northern blot analysis as indicated in the figure legends. A rat cDNA probe for rat 5’ deiodinase was a gift from Dr. C. N. Mariash. Rat cDNA templates for CYP7A1 (12), HMG-CoA reductase (11), and LDL receptor (11) were a gift from Dr. G. C. Ness. For CYP7A1, HMG-CoA reductase, and LDL receptor, Northern blot analysis was performed with the use of [α-³²P]UTP-labeled antisense riboprobes. The hybridization bands were quantitatively measured using Adobe Photoshop 4.0 (Adobe Systems Corp., San Jose, CA) and NIH Image (Scion Corp., Frederick, MD) and standardized against cyclophilin controls. RNA samples from at least five individual mice treated in the same way were obtained for each condition and genotype. All Northern blots were repeated at least three times, using individual RNA samples, with similar results. Representative Northern blots are shown.

**Cell Culture and Cotransfection Assays**—Transient transfections in HepG2 cells were performed using a standard calcium phosphate method. The cells were maintained in 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Receptor expression plasmids encoding mouse LXR-α, and human TR-β₁, MUT TR-β₁ (Δ337T), and WT RXR-α in pSG5 vectors were cotransfected with a luciferase reporter plasmid (pGL3Luc) carrying mouse CYP7A1 promoter (−607 to +26 bp). CYP7A1 mutant promoter was generated with PCR mutagenesis as it has the mutant DR₁ consensus: ACCATGG, 5'-ACCTCAGGTCACAGGAGGTCAGACAA-3’. Rat cDNA probes were synthesized nuclear receptors. Double-stranded oligonucleotides were synthesized from constructs in the pSG5 expression vector using the TnT T7 Quick-coupled Transcription/Translation System (Promega). Binding reactions contained 20 mM HEPES (pH 7.6), 50 mM KCl, 12% glycerol, 1 mM dithiothreitol, 1 μg of poly(dI-dC)-poly(dI-dC), and 4 μl of each of the synthesized nuclear receptor receptors. Double-stranded oligonucleotides (DR+4 mouse Cyp7A1, −78 bp 5’-TGCTCTGTCACCACGTTCTCGAGCCCCACCATGGAGTAA-3’ −53 bp; mutant DR+4 mouse Cyp7A1: −78 bp 5’-TGCTCTGTCACCACGTTCTCGAGCCCCACCATGGAGTAA-3’ −53 bp; DR+4 consensus: 5’-ACCTCAGGTCACAGGAGGTCAGACAA-3’).
TABLE 1
Free thyroxine (ng/dl) at baseline (regular chow) and after induction of hypothyroidism (MMI/PTU chow)

| Mouse genotype | WT/WT | MUT/WT | MUT/MUT |
|----------------|-------|--------|---------|
| Regular chow   | 2.5 ± 0.1 (20) | 10.1 ± 1.5 (20) | 32.2 ± 5.2 (20) |
| MMI/PTU chow  | 0.08 ± 0.072 (6)* | 0.07 ± 0.05 (6)* | 0.37 ± 0.16 (6)* |

* Statistical differences between values obtained before treatment (regular chow) and induction of hypothyroidism, p < 0.001. Numbers in parentheses are animals in each group. Results are expressed as mean ± S.E.

32P by T4 polynucleotide kinase. Binding reactions were performed at room temperature for 20 min, and the protein-DNA complexes were resolved on a 5% polyacrylamide gel and analyzed by autoradiography.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed on liver extracts using previously described procedures (27). A PCR primer set was designed to amplify a 417-bp fragment of the proximal CYP7A1 promoter, which contained the LXRE (forward primer: −391 bp, GGACAGCCAGTATTTAAAGGCGAGTTG, −366 bp; reverse primer: +25 bp, TGCTTACGAAACGAAGGCGTCAGA, −1 bp). The following antibodies were used in the assay: mouse anti-TR-β1 antibody (sc-738, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-LXR-α antibody (sc-1201, Santa Cruz Biotechnology), rabbit anti-RXR-α antibody (sc-774, Santa Cruz Biotechnology), and as a negative control, normal mouse IgG antibody (sc-2025, Santa Cruz Biotechnology).

Statistical Analyses—Values are expressed as mean ± S.E. The significance of differences between mean values was evaluated using the unpaired Student’s t test. Sample groups showing heterogeneity of variance were appropriately transformed before analysis.

RESULTS
Serum Cholesterol Levels in the TR-β Knock-in Mice—To confirm the dominant negative effects of the mutant TR in the liver, we measured total cholesterol levels in wild-type (WT) and TRβ knock-in mice. Because TR-β mutant (MUT) animals have significantly higher TH levels, we considered that excess TH (Table 1) may overcome some of the dominant negative effects of the mutant TR-β. To eliminate this effect, TR-β WT and MUT mice were rendered hypothyroid with a MMI/PTU diet (22), then euthyroid by T3 administration. As shown in Table 1, TR-β heterozygous (WT/MUT) and especially homozygous (MUT/MUT) mutant mice have extremely high baseline thyroxine levels, which were reduced to hypothyroid levels by MMI/PTU treatment. A MMI/PTU diet followed by T3 administration returned free T3 levels in all mice to euthyroid levels (Table 2).

We next measured serum cholesterol in hypothyroid mice before treatment or after administration of a high cholesterol diet, T3, or both. As shown in Fig. 2A, we found no significant differences in serum cholesterol levels in all genotypes in the hypothyroid state (first set of bars). When we administered a high cholesterol diet (2%) to hypothyroid animals (second set of bars), serum cholesterol levels were appropriately increased in WT animals. Paradoxically, serum cholesterol levels were decreased in MUT/MUT mice after cholesterol administration; WT/MUT mice had an intermediate value. This phenomenon was also decreased in MUT/MUT mice after cholesterol administration; increased in WT animals. Paradoxically, serum cholesterol levels were decreased in MUT/MUT mice after cholesterol administration; increased in WT animals.

Hepatic Cholesterol Levels in the TR-β Knock-in Mice—Hepatic cholesterol levels were then compared in WT and MUT animals after administration of a high cholesterol diet. Under hypothyroid conditions, hepatic cholesterol levels were significantly lower in WT/MUT and MUT/MUT versus WT mice after a high cholesterol diet (Fig. 2B, first set of bars). Correction of the hypothyroid state reduced hepatic cholesterol levels in WT animals but had no additional significant effect in MUT animals (Fig. 2B, second set of bars). In summary, we found a paradoxical reduction in both serum and hepatic cholesterol levels in mutant mice after administration of a high cholesterol diet. We also performed Oil Red O staining on liver sections from these mice. As shown in Fig. 2C, the liver histology of the WT animals showed increased hepatic Oil Red O staining versus the mutant animals. Measurement of bile acid pools in WT and MUT animals indicates that the decrease in hepatic cholesterol content and staining in MUT animals is associated with a significant increase in their bile acid pool (Fig. 2D).

The Expression of Thyroid Hormone-regulated Genes Involved in Cholesterol Metabolism in the TR-β Knock-in Mice—To understand the molecular mechanism by which cholesterol metabolism is altered in WT mice, the expression of thyroid hormone-regulated genes involved in cholesterol metabolism was surveyed in MUT animals (Fig. 3). The 5′ deiodinase type 1 (5′DI) gene is positively regulated by thyroid hormone in the liver (28) and is a good indicator of thyroid status in mice. During hypothyroidism, 5′DI mRNA levels were undetectable in all genotypes regardless of whether the animals were fed a high cholesterol diet. However, 5′DI mRNA levels were induced by T3 in WT but induction was reduced in MUT animals. In fact, 5′DI induction was totally absent in homozygous mutant animals. These data indicate that the MUT TR-β has a significant dominant negative effect in the liver.

Cholesterol 7α-hydroxylase, CYP7A1, the rate-limiting enzyme involved in cholesterol metabolism to bile acids, is a central regulator of cholesterol homeostasis (29, 30). It is known that thyroid hormone increases CYP7A1 mRNA levels and that this effect is critically important for the hypocholesterolemic action of thyroid hormone (29, 30). As shown in Fig. 3, mRNA levels for CYP7A1 were immeasurable in all genotypes during hypothyroidism. After T3 replacement, CYP7A1 mRNA levels were increased in WT mice; however, this was not the case in mutant mice, where the maximal induction of mRNA levels was 30% in MUT/MUT animals as compared with WT mice. In contrast, administration of a cholesterol diet had a minimal effect on CYP7A1 mRNA levels in WT animals, whereas a large induction of CYP7A1 levels was observed in MUT animals. For example, in MUT/MUT mice, CYP7A1 mRNA expression was dramatically induced by cholesterol administration (280% compared with 15% in WT mice).

HMGC-R is the rate-limiting enzyme in cholesterol biosynthesis (31) and its expression is increased by thyroid hormone. We noted that HMGC-R mRNA levels were increased in WT animals after T3 replacement (defined as 100%, after T3 administration in WT animals, see Fig. 3). In contrast, HMGC-R mRNA levels were reduced by cholesterol treatment in T3-treated WT animals as expected. Interestingly, HMGC-R mRNA levels were increased in the hypothyroid state in MUT animals compared with WT animals, suggesting that the unliganded mutant TR may activate expression of certain genes in the liver.

Cross-talk between TR and LXR Signaling Pathways

TABLE 2
Free T3 (pg/ml) after T3 treatment to hypothyroid animals (MMI/PTU chow)

| Mouse genotype | WT/WT | WT/MUT | MUT/MUT |
|----------------|-------|--------|---------|
| MMI/PTU + T3   | 1.96 ± 0.03 (6) | 1.98 ± 0.27 (7) | 2.06 ± 0.34 (7) |
WT mice) because of the increase in expression in the hypothyroid state. The effect of \( T_3 \) treatment was further reduced in MUT/MUT animals (1.5-fold increase versus the hypothyroid state). Like WT animals, cholesterol administration also decreased HMGC-R mRNA levels in MUT animals.

Expression of LDLR is positively regulated by thyroid hormone (10–12) and this receptor promotes the uptake of LDL into the cell. As shown in Fig. 3, WT mice demonstrate a significant increase in LDLR mRNA levels from the hypothyroid to the \( T_3 \) treated state (15–100%). Similar data were seen in animals rendered euthyroid by \( T_3 \) administration. \( T_3 \), hepatic cholesterol levels (mean ± S.E.) from 4-week-old male mice initially rendered hypothyroid and given a 2% cholesterol diet, in the absence (−) or presence (+) of \( T_3 \) replacement. Wild-type (WT), heterozygous knock-in (WT/MUT), or homozygous knock-in (MUT/MUT) were used (\( n = 6 \) for each group). C, Oil Red O staining of liver from mice rendered hypothyroid and supplied with a 2% cholesterol diet in the absence (−) or presence (+) of \( T_3 \) replacement. D, bile acid pool size was measured in all three animal groups fed a 2% cholesterol diet during hypothyroidism.

Finally, we measured LXR-α mRNA levels in these mice (Fig. 3). This receptor is essential for CYP7A1 expression and regulation by serum cholesterol levels (33). LXR-α mRNA levels in all genotypes displayed a similar pattern of gene expression. \( T_3 \) treatment resulted in a 2-fold increase in gene expression in both WT and MUT mice, suggesting a potential role for TRα or a non-TR mediated pathway in this process. Cholesterol treatment had no significant effect on gene expression.

Cross-talk of TR-β and LXR-α on the DR +4 Element in the CYP7A1 Gene Promoter—We next determined whether high cholesterol treatment could induce CYP7A1 expression in the presence of WT and MUT TR-β. We employed a co-transfection study using HepG2 cells, a hepatocyte cell line. In rodents, the CYP7A1 gene promoter contains a single DR +4 element in the proximal promoter, which functions as a positive LXRE (34, 35). We, therefore, investigated a possible interaction between LXR-α and TR-β on a mouse CYP7A1 gene as illustrated in the model (Fig. 4A). As shown in Fig. 4B, LXR-α co-transfection stimulated the mouse CYP7A1 reporter activity about 2-fold after treatment with its ligand, 22(R)-hydroxycholesterol (36). Co-transfection of WT TR-β1, in contrast, reduced both basal and ligand-stimulated reporter activity in a dose-dependent manner. Co-transfection of the MUT TR-β, however, had no significant effect on LXR-mediated transactivation by 22(R)-hydroxycholesterol. In data not shown, the reporter
was stimulated ~3-fold by 10 nM T3 after cotransfection with the WT but not MUT TR-β and responses to both ligands were eliminated when the LXRE/thyroid hormone response element was mutated as described. These data indicate that WT antagonized LXR-α action, whereas the MUT TR-β did not.

Next, we performed a gel shift analysis using in vitro translated TR-β, RXR-α, and LXR-α proteins and radiolabeled mouse CYP7A1 and consensus DR1-4 probes. As shown in Fig. 4C, the WT TR/RXR heterodimer migrated more rapidly than the LXR/RXR heterodimer (compare lanes 2 and 4). T3 treatment dissociated the WT TR homodimer (compare lanes 2 and 3) but had no effect on the MUT TR homodimer (compare lanes 8 and 9). The LXR ligand, 22(R)-hydroxycholesterol, had no effect on LXR/RXR heterodimer binding (data not shown). Indicating that DNA binding of the LXR/RXR heterodimer, like the TR/RXR heterodimer, was not dependent on ligand. When WT TR, LXR, and RXR were included in the binding reaction, only WT TR/RXR heterodimer formation was observed (lane 5), and this effect was not altered by the addition of T3 (compare lanes 5 and 6) or by 22(R)-hydroxycholesterol (data not shown). In contrast, when MUT TR, LXR, and RXR were included in the binding reaction, both MUT TR/RXR and LXR/RXR heterodimers were observed (lane 10). Western blot analysis for the TR-β protein confirmed that both the WT and MUT proteins were synthesized equally in an in vitro coupled transcription/translation system (data not shown).

Finally, we evaluated the occupancy of the proximal LXRE in the CYP7A1 gene in WT and MUT mice using a ChIP assay. As shown in Fig. 4D, TR-β1 binding to the CYP7A1 promoter in the liver of WT mice was significant (lane 4), whereas LXR-α binding to that same fragment was minimal (lane 7). In contrast, LXR-α binding to this fragment was significant in MUT/MUT mice (lane 9), whereas TR-β1 binding to this fragment was minimal (lane 6). The WT/MUT animals displayed an intermediate pattern. As controls in this experiment, a nonspecific
IgG did not precipitate a protein-DNA complex in any mice (lanes 1–3) and a RXR-α specific antibody showed an equivalent pattern in all mouse genotypes (lanes 10–12).

DISCUSSION

Thyroid hormone has important effects on cholesterol metabolism. Previous investigators have used TR knock-out animals to analyze cholesterol metabolism and have concluded that TR has both ligand-independent and ligand-dependent functions in cholesterol metabolism (13, 37). Given this knowledge, analysis of cholesterol metabolism in animals lacking TR may lead to limited or perhaps erroneous conclusions. In this study, therefore, the TR-β allele was mutated in mice so that the resulting receptor was unable to bind to T₃. This mutation (Δ337T) was found in a family with thyroid hormone resistance and acts as a dominant negative receptor in tissues (15). Because it constitutively binds to corepressor molecules, it closely mimics the TR in the hypothyroid state.

Paradoxically, we found that animals bearing this mutation were protected against hypercholesterolemia induced by a high cholesterol diet (Fig. 2).

To determine the mechanism of this effect, we chose to examine three major cholesterol metabolism-related genes whose gene expressions are regulated by thyroid hormone: CYP7A1, HMGC-R, and LDL-R. We confirmed that the mutant TR functioned as a dominant negative in the liver by first measuring 5β-DI expression (Fig. 3). A progressive reduction in T₃-stimulated 5β-DI mRNA expression activity was found comparing WT to WT/MUT and MUT/MUT animals. Next, thyroid hormone is known to modulate serum cholesterol levels principally by regulating CYP7A1 mRNA levels (29, 30). CYP7A1 mRNA levels were undetectable in the hypothyroid state in all mice. T₃ treatment induced CYP7A1 mRNA levels in WT mice, whereas a high cholesterol diet had minimal effect on CYP7A1 mRNA expression (Fig. 3). In contrast, a high cholesterol diet markedly induced CYP7A1 levels in MUT mice, and T₃ treatment had a minimal effect (Fig. 3). Gullberg et al. (14) also reported that hypothyroidism reduced CYP7A1 mRNA levels and activity in TR-β knock-out mice (14). The degree of suppression of CYP7A1 mRNA levels during hypothyroidism in the TR-β knock-out animals (~70%) appears to be less than that observed in our MUT animals, which indicates that the mutant TR-β functioned as a
dominant negative inhibitor on the CYP7A1 gene in our studies. In summary, our results support a dominant negative effect of the mutant TR-β on CYP7A1 gene expression.

On the other hand, HMGC-R and LDL-R mRNA levels, which are also positively regulated by thyroid hormone, were not reduced in MUT mice in the basal state when compared with WT mice. In fact, the mutant TR-β functioned as a ligand-independent activator on these genes (Fig. 3). In all animals, HMGC-R and LDL-R gene expression was inhibited by a high cholesterol diet after T₃ replacement, although expression from both genes was significantly higher after T₃ replacement in MUT versus WT mice (Fig. 3). These results point to differences in regulation of these gene products versus CYP7A1. Thus, the dominant negative effect of the mutant TR-β is variable and may depend on the nature of the regulatory pathway.

Clearly, hepatic cholesterol levels were reduced in MUT animals after treatment with a high cholesterol diet or after treatment with both T₃ and a high cholesterol diet (Fig. 2B). The most likely explanation for the decreased hepatic cholesterol levels is an increase in CYP7A1 activity. The increase in bile acid pool size in the MUT animals indicates that the LXRE in the CYP7A1 promoter is primarily occupied by TR/RXR heterodimers, whereas the same element (Fig. 4C) regardless of the presence of its ligand (22(R)-OH-cholesterol, data not shown). Interestingly, co-transfection of LXR-α and WT TR-β, but not MUT TR-β, antagonized LXR-α activity on the CYP7A1 promoter (Fig. 4B).

To determine the mechanism of this phenomenon, gel mobility shift assays were performed. As expected and as reported previously, the WT TR-β formed both TR homodimers and TR/RXR heterodimers and the former was dissociated by T₃ (Fig. 4C). In contrast, the mutant TR-β homodimer was not dissociated by T₃ as previously reported (Fig. 4C, Ref. 15). LXR/RXR heterodimers were readily detected on the DR4 element (Fig. 4C) regardless of the presence of its ligand (22(R)-OH-cholesterol, data not shown). However, WT TR/RXR heterodimers were preferred over LXR/RXR heterodimers on the DR4 element when tested in competition (Fig. 4C). In contrast, MUT TR-β was a much less effective competitor than WT TR-β for the heterodimerization with RXR-α (Fig. 4C) and LXR/RXR heterodimers were observed in this competition. Consistent with this finding, CHIP assay of liver tissue from WT mice indicates that the LXRE in the CYP7A1 promoter is primarily occupied by TR/RXR heterodimers, whereas the same element in MUT/MUT mice is primarily occupied by LXR/RXR heterodimers (Fig. 4D).

Based on these in vitro data, we offer an explanation for the mechanism of the marked increase in the CYP7A1 mRNA expression in MUT mice fed a high cholesterol diet. In these animals, LXR/RXR heterodimers would be more likely to bind to DR4+ elements making their target genes more responsive to cholesterol. Thus, at least two types of molecular mechanisms are involved in the dominant negative effect by MUT TR-β: 1) thyroid hormone action is impaired by the mutant TR-β itself such as found on the 5’ DI gene; and 2) MUT TR-β affects the function of another nuclear hormone receptors (LXR-α) such as found on the CYP7A1 gene. Furthermore, our in vivo data reinforces the in vitro studies of Kawai et al. (39), who suggested that TR and RXR bind to certain DR4+ elements in common, and showed that unliganded TR represses LXR activation on LXREs derived from the dMTV-LTR and SREBP-1c promoters.

Thus, our data demonstrate a cross-talk between LXR-α and TR-β in vivo, which was uncovered through expression of a MUT TR-β. It may not be possible to extend this finding beyond rodents given that the human CYP7A1 gene is reported to lack this proximal LXRE and be unresponsive to cholesterol-mediated stimulation (32, 40). However, studies designed to evaluate human gene expression in a rodent background have limitations, which include the number and location of the human transgene in the mouse genome, the presence of the regulatory element at distances far from the human gene that are not included within the transgene, and unknown species-specific effects that could be critical for normal regulation. Thus it remains possible that the endogenous human gene is subjected to cross-talk regulation by LXR-α and TR-β.

In the present study, we also found that the role of unliganded TR-β depends on each individual gene promoter. Dominant negative activity was observed on some but not all gene products. Moreover, it was revealed that the unliganded TR-β had considerable effects on cholesterol metabolism via a cross-talk between TR-β and LXR-α and this likely plays a pivotal role in the activation of CYP7A1 gene expression.

**Cross-talk between TR and LXR Signaling Pathways**

**Acknowledgments**—We thank Dr. G. C. Ness (University of South Florida, Tampa, Florida) for providing cDNA probes for rat CYP7A1, HMGC-CoA reductase, and LDL receptor. We also thank Dr. C. N. Mariash (University of Minnesota, Minneapolis, MN) for 5’ DI cDNA probes. We appreciate the technical advice regarding measurement of hepatic cholesterol levels from Dr. M. Kawai (Mitsubishi Pharma Corp., Yokohama, Japan).

**REFERENCES**

1. Ferber, D. (2000) *Science* **289**, 1446–1447

2. Braverman, L., and Utiger, R. D. (2000) *Werner & Ingbar’s The Thyroid*, 8th Ed., Lippincott Williams & Wilkins, Philadelphia, PA.

3. Underwood, A. H., Emmett, J. C., Ellis, D., Flynn, S. B., Lesen, P. D., Benson, G. M., Novelli, R., Pearce, N. J., and Shah, V. P. (1986) *Nature* **324**, 425–429

4. Refetoff, S., Weiss, R. E., and Usala, S. J. (1993) *Endocr. Rev.* **14**, 348–399

5. O’Brien, T., Dinneen, S. F., O’Brien, P. C., and Palumbo, P. J. (1993) *Mayo Clin. Proc.* **68**, 860–866

6. Russell, D. W., and Setchell, K. D. (1992) *Biochemistry* **31**, 4737–4749

7. Lu, T. T., Makishima, M., Repa, J. J., Schoonjans, K., Kerr, T. A., Auwerx, J., and Mangelsdorf, D. J. (2000) *Cell* **101**, 507–515

8. Chiang, Z. J., and Stroup, D. (1994) *J. Biol. Chem.* **269**, 17502–17507

9. Myant, N. R., and Mitropoulos, K. A. (1977) *J. Lipid Res.* **18**, 135–153

10. Bakker, O., Hudig, F., Meijijans, S., and Wiersinga, W. M. (1998) *Biochem. Biophys. Res. Commun.* **249**, 517–521

11. Ness, G. C., and Lopez, D. (1995) *Arch. Biochem. Biophys.* **324**, 404–408

12. Ness, G. C., Pendleton, L. C., Li, Y. C., and Chiang, Z. J. (1996) *Biochem. Biophys. Res. Commun.* **232**, 1150–1156

13. Weiss, R. E., Murata, Y., Cua, K., Hayashi, Y., Seo, H., and Refetoff, S. (1998) *Endocrinology* **139**, 4945–4952

14. Guilberg, H., Rudling, M., Forrest, D., Angelin, B., and Vennstrom, B. (2000) *Mol. Endocrinol.* **14**, 1739–1749

15. Hashimoto, K., Curty, F. H., Berges, P. P., Lee, C. E., Abel, E. D., Elmqist, J. K., Cohen, E. N., and Wondisford, F. E. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3984–4003

16. Kaneshige, M., Kaneshige, K., Nakai, A., Seino, S., Kazlauskaite, R., Pankratz, D. G., Wynshaw-Boris, A., Refetoff, S., Weintraub, B., Willingham, M. C., Barlow, C., and Cheng, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13209–13214

17. Lazar, M. A. (1993) *Endocr. Rev.* **14**, 18–193

18. Usala, S. J., Menke, J. B., Watson, T. L., Wondisford, F. E., Weintraub, B. D., Berard, J., Bradley, W. E., Ono, S., Mueller, O. T., and Bercu, B. B. (2001) *Mol. Endocrinol.* **5**, 327–335

19. Ono, S., Schwartz, I. D., Mueller, O. T., Root, A. W., Usala, S. J., and Bercu, B. B. (1991) *J. Clin. Endocrinol. Metab.* **73**, 990–994

20. Sakurai, A., Takeda, K., Xin, A., Ceccarelli, P., Nakai, A., Seino, S., Bell, G. I., Refetoff, S., and DeGroot, L. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 860–866

21. Theodossiou, C., Skrepnik, N., Roberts, E. G., Prasad, C., Schapira, D. V., and Hunt, J. D. (1999) *Cancer* **86**, 1596–1601

22. Woll, B., Aratan-Spire, S., and Czerneckich, P. (1984) *Endocrinology* **114**, 1334–1337

23. Yokode, M., Hamner, R. E., Iishibashi, S., Brown, M. S., and Goldstein, J. L. (1990) *Science* **250**, 1273–1275
Cross-talk between TR and LXR Signaling Pathways

25. Turley, S. D., Schwarz, M., Spady, D. K., and Dietschy, J. M. (1998) *Hepatology* 28, 1088–1094
26. Cohen, R. N., Cohen, L. E., Botero, D., Yu, C., Sagar, A., Jurkiewicz, M., and Radovick, S. (2003) *J. Clin. Endocrinol. Metab.* 88, 4832–4839
27. Zhou, X. Y., Shibusawa, N., Naik, K., Porras, D., Temple, K., Ou, H., Kaihara, K., Roe, M. W., Brady, M. J., and Wondisford, F. E. (2004) *Nat. Med.* 10, 633–637
28. Toyoda, N., Zavacki, A. M., Maia, A. L., Harney, J. W., and Larsen, P. R. (1995) *Mol. Cell. Biol.* 15, 5100–5112
29. Ness, G. C., Pendelton, L. C., and Zhao, Z. (1994) *Biochim. Biophys. Acta* 1214, 229–233
30. Crestani, M., Karam, W. G., and Chiang, J. Y. (1994) *Biochem. Biophys. Res. Commun.* 198, 546–553
31. Ness, G. C., and Chambers, C. M. (2000) *Proc. Soc. Exp. Biol. Med.* 224, 8–19
32. Chen, J. Y., Levy-Wilson, B., Goodart, S., and Cooper, A. D. (2002) *J. Biol. Chem.* 277, 42588–42895
33. Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J. M., Hammer, R. E., and Mangelsdorf, D. J. (1998) *Cell* 93, 693–704
34. Lehmann, J. M., Kliwer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J. L., Sundseth, S. S., Winegar, D. A., Blanchard, D. E., Spencer, T. A., and Willson, T. M. (1997) *J. Biol. Chem.* 272, 3137–3140
35. Li, Y., Bolten, C., Bhat, B. G., Woodring-Dietz, J., Li, S., Prayaga, S. K., Xia, C., and Lala, D. S. (2002) *Mol. Endocrinol.* 16, 506–514
36. Janowski, B. A., Willy, P. J., Devi, T. R., Falek, J. R., and Mangelsdorf, D. J. (1996) *Nature* 383, 728–731
37. Gullberg, H., Rudling, M., Salto, C., Forrest, D., Angelin, B., and Vennstrom, B. (2002) *Mol. Endocrinol.* 16, 1767–1777
38. Chawla, A., Repa, J. J., Evans, R. M., and Mangelsdorf, D. J. (2001) *Science* 294, 1866–1870
39. Kawai, K., Sasaki, S., Morito, H., Ito, T., Suzuki, S., Misawa, H., and Nakamura, H. (2004) *Endocrinology* 145, 5515–5524
40. Agellon, L. B., Drover, V. A., Cheema, S. K., Gbaguidi, G. F., and Walsh, A. (2002) *J. Biol. Chem.* 277, 20131–20134