The Liver X Receptor (LXR) and Hepatic Lipogenesis

THE CARBOHYDRATE-RESPONSE ELEMENT-BINDING PROTEIN IS A TARGET GENE OF LXR

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The liver X receptors, LXRα (NR1H3) and LXRβ (NR1H2), are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. LXRs play a critical role in cholesterol homeostasis and bile acid metabolism. In addition, oral administration of LXR agonists to mice results in elevated hepatic fatty acid synthesis and steatosis and increased secretion of triglyceride-rich very low density lipoprotein resulting in hypertriglyceridemia. This increased hepatic lipogenesis has been largely attributed to the LXR-dependent up-regulation of sterol regulatory element-binding protein 1c (SREBP-1c) expression. However, it has been reported that treating Srebp-1c null mice with the synthetic LXR agonist T0901317 still results in enhanced expression of many lipogenic genes, suggesting additional mechanisms by which LXR can enhance hepatic lipogenesis. In this report, we identify the carbohydrate response element-binding protein (ChREBP) as a novel LXR target that independently enhances the up-regulation of select lipogenic genes. The ChREBP promoter contains functional LXR-binding sites that confer receptor-dependent binding and transactivation. We show that T0901317 treatment of mice is associated with up-regulation of the ChREBP target gene, liver-type pyruvate kinase. Therefore, activation of LXR not only increases ChREBP mRNA via enhanced transcription but also modulates ChREBP activity. This establishes LXR as a master lipogenic transcription factor, as it directly regulates both SREBP-1c and ChREBP to enhance hepatic fatty acid synthesis.

Members of the nuclear hormone receptor superfamily function as ligand-activated transcription factors to regulate genes critical to development, reproduction, and intermediary metabolism (1). A subgroup of these receptors, the “adopted-orphan” receptors (i.e. PPARs, LXR, FXR, CAR, and PXR), appear to serve as lipid sensors, as they are bound and activated by dietary-derived lipids and ultimately regulate genes to facilitate cellular elimination of their respective ligand lipids (2). LXRα (NR1H3) and LXRβ (NR1H2) are sterol sensors and bind oxysterols to regulate genes critical to cholesterol efflux (ABCA1, ABCG1, and apoE), cholesterol conversion to bile acids (CYP7A1), and cholesterol secretion into bile for excretion (ABCG5/G8) (3). As such, these receptors have been evaluated as potential therapeutic targets to alleviate conditions associated with hypercholesterolemia (e.g. atherosclerosis (4)). Indeed, administration of potent, selective LXR agonists to atherogenic mouse strains results in reduced aortic lesion formation (5), and even lesion regression (6). However, hepatic steatosis and hypertriglyceridemia, due in part to enhanced hepatic fatty acid synthesis and very low density lipoprotein secretion, were observed in these animal studies (7, 8).

The molecular mechanism responsible for LXR-mediated hepatic lipogenesis has been largely attributed to the dramatic increase in expression of the sterol regulatory element-binding protein 1c (SREBP-1c) (8–10). SREBP-1c is a direct target gene of the retinoid X receptor (RXR)/LXR heterodimer, and two LXR-responsive elements (LXREs) critical to RXR/LXR-mediated regulation have been identified in the promoter of this gene (9, 10). SREBP-1c is a transcription factor of the basic helix-loop-helix family, and has been shown to control the expression of nearly all genes integral to fatty acid biosynthesis (11). Deletion of the Srebp-1c gene in mice results in a 50% reduction in hepatic fatty acid synthesis rates. However, treating Srebp-1c null mice with an LXR agonist still results in the up-regulation of a subset of lipogenic genes and a modest increase in fatty acid synthesis, suggesting that additional mechanisms exist to link LXR activity and lipogenesis (12).

In this report, we identify the carbohydrate response element-binding protein (ChREBP) as a novel LXR target gene. ChREBP is a glucose-sensitive transcription factor that promotes the hepatic conversion of excess carbohydrate to lipid (13, 14). LXR and RXR agonists specifically induce expression of ChREBP in wild-type, but not Lxra/β-double knockout mice. This increase in ChREBP mRNA is dependent on two LXREs in the promoter of the ChREBP gene. In addition to an increase in hepatic ChREBP mRNA, there is a concomitant increase in liver-type pyruvate kinase (L-PK) mRNA indicative

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2 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; AMPK, AMP-activated protein kinase; CAR, constitutive androstane receptor; ChREBP, carbohydrate response element-binding protein; FXR, farnesoid X receptor; L-PK, liver-type pyruvate kinase; LXR, liver X receptor; mpk, milligrams/kilograms body weight; PKX, pregnant X receptor; RXR, retinoid X receptor; SCD-1, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein 1c; LXRE, LXR-responsive element; qRT, quantitative real-time; DKO, double knock-out; ACC, acetyl-CoA carboxylase; ChIP, chromatin immunoprecipitation.
LXR Regulation of Hepatic ChREBP

of increased ChREBP activity. Therefore, future studies aimed at identifying selective LXR modulators, drugs that contain the beneficial cholesterol-lowering properties of LXR agonists with minimal impact on lipogenesis, will need to address the effects of these selective LXR modulators on both SREBP-1c and ChREBP activity.

EXPERIMENTAL PROCEDURES

Materials

T0901317 was purchased from Cayman Chemical Co. (Ann Arbor, MI). LG268 was provided by Richard Heyman and Mark Leibowitz of Ligand Pharmaceuticals (La Jolla, CA). Roger Unger (University of Texas, Southwestern) provided troglitazone. All other nuclear receptor agonists were purchased from Sigma. ChREBP-knock-out mice were kindly provided by Kosaku Uyeda (University of Texas, Southwestern (15)). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Real-time PCR reagents were purchased from Applied Biosystems (Foster City, CA).

Animals

Mice were maintained in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle and all experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Male Agouti 129/SvJ mice at 3 months of age were provided with the meal form of a standard rodent diet (diet number 7001, Harlan Teklad Premier Laboratory Diets, Madison, WI) supplemented with various nuclear receptor agonists, during a 12-h dark cycle. Alternatively, male mice (Agouti 129/SvJ, C57Bl/6, or C57Bl/6J) wild-type and 0.05% Tween 80) by oral gavage each day for 3 to 10 days as described for each study in the figure legends. Male mixed strain (C57Bl/6 and A129/SvJ) wild-type and Lxrα/β-double knockout mice were fed the basal rodent diet supplemented with 0.2% (w/w) cholesterol and either LG268 (RXR agonist, 30 mpk) or T0901317 (LXR agonist, 50 mg/kg body weight (mpk)) or vehicle (0.9% carboxymethylcellulose, 9% polyethylene glycol 400, and 0.05% Tween 80) by oral gavage each day for 3 to 10 days as described for each study in the figure legends. Male mixed strain (C57Bl/6 and A129/SvJ) wild-type and Lxrα/β-double knockout mice were fed the basal rodent diet supplemented with 0.2% (w/w) cholesterol and either LG268 (RXR agonist, 30 mpk) or T0901317 (LXR agonist, 50 mpk) for 12 h or 10 days. At the end of the dietary treatments, mice were anesthetized, exsanguinated via the descending vena cava, and tissues removed and flash-frozen in liquid nitrogen.

RNA Measurement

Total RNA was isolated from tissue samples using RNA STAT-60 (Tel-Test Inc., Friendswood, TX).

Northern Analysis—Equal quantities of total RNA from the samples of each group were pooled and poly(A) + RNA was purified using oligo(dT)-cellulose columns (GE Healthcare). mRNA (5 µg/lane) was size fractionated on a 1% formaldehyde-agarose gel and transferred to nylon membrane (Zetaprobe, Bio-Rad) for detection using 32P-labeled cDNAs. The ChREBP cDNA probe was generated by reverse transcription-polymerase chain reaction (RT-PCR) using primers: forward, 5'-caccgctcaagcactcgaagag) and reverse, 5'-catcagctcaagcactcgaagag). Northern hybridization was performed using nylon membrane (Zetaprobe, Bio-Rad) for detection using 32P-labeled cDNAs. The ChREBP cDNA probe was generated by reverse transcription-polymerase chain reaction (RT-PCR) using primers: forward, 5'-caccgctcaagcactcgaagag). Northern hybridization was performed using a DNA probe radiolabeled with 32P-dCTP. Binding reactions were performed in a total volume of 20 µl consisting of 75 mM KCl, 20 mM Hepes (pH 7.4), 2 mM dithiothreitol, 7.5% (v/v) glycerol, 0.1% Nonidet P-40, 2 µg of poly(dl-dC) (Amersham Biosciences), and 2 µl of nuclear receptor lyses or unprogrammed lyses. Protein-DNA complexes were resolved by electrophoresis on a 6% native polyacrylamide gel and visualized by autoradiography.

Chromatin Immunoprecipitation (ChIP) Assay

Frozen, powdered liver tissue was fixed by addition of warmed formaldehyde (37 °C, 1% final concentration), then

by David Russell, University of Texas Southwestern Medical Center.

Quantitative Real-time PCR—qRT-PCR was performed using an Applied Biosystems Prism 7900HT sequence detection system as described (16). Primers for each gene were designed using Primer Express Software (PerkinElmer Life Sciences) and validated by analysis of template titration and dissociation curves. Primer sequences are provided in supplementary Table 1. Results of qRT-PCR were evaluated by the comparative Ct method (user bulletin No. 2, PerkinElmer Life Sciences) using cyclophilin as the invariant control gene. RNA levels are expressed relative to those of the wild-type mice fed the basal diet, and reflect the average ± S.E. for 4–6 mice/group.

Identification and Characterization of the ChREBP Promoter

RNA ligase-mediated rapid amplification of cDNA ends was performed using the GeneRacer kit (Invitrogen) to define the transcriptional start site. Briefly, total RNA isolated from T0901317-treated mouse liver was used in this procedure in conjunction with a reverse ChREBP-specific primer 1 (5'-gccaggctcaagcactcgaagag).

Plasmid Construction

Various lengths of the 5'-flanking region of mouse ChREBP were amplified using PCR using mouse genomic DNA and subcloned into the luciferase reporter plasmid thereby replacing the thymidine kinase promoter (17). Oligonucleotides containing putative LXRE sites were annealed and subcloned into the HindIII site upstream of the thymidine kinase promoter of the luciferase plasmid. Site-directed mutagenesis of the ChBP-3801/+9 plasmid was accomplished using the QuickChange mutagenesis kit (Stratagene). The integrity of each plasmid was verified by DNA sequencing.

Transactivation Assay

Transient transfection of HepG2 or human embryonic kidney 293 cells was performed using FuGENE 6 transfection reagent (Roche). 14 h after introducing DNA, cells were exposed to T0901317 (1 µM), LG268 (100 nM), or vehicle for 18–24 h. Cells were lysed and luciferase and ß-galactosidase activities were determined as previously described (18).

Gel Shift Assays

RXRα and LXRα protein-containing reticulocyte lysates were generated using the TnT Quick Coupled Transcription/Translation system (Promega, Madison, WI). Annealed oligonucleotide probes were labeled with Klenow enzyme (Roche) in the presence of [32P]dCTP. Binding reactions were performed in a total volume of 20 µl consisting of 75 mM KCl, 20 mM Hepes (pH 7.4), 2 mM dithiothreitol, 7.5% (v/v) glycerol, 0.1% Nonidet P-40, 2 µg of poly(dl-dC) (Amersham Biosciences), and 2 µl of nuclear receptor lyses or unprogrammed lyses. Protein-DNA complexes were resolved by electrophoresis on a 6% native polyacrylamide gel and visualized by autoradiography.
incubated 10 min at room temperature. The cross-linking was stopped by addition of 125 mM glycine for 5 min at room temperature, followed by centrifugation and washing the pellet once with ice-cold phosphate-buffered saline. The pellet was resuspended in hypotonic solution (containing 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2% Nonidet P-40, 0.2 mM sodium orthovanadate, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 5% sucrose, 1 mM dithiothreitol, and protease inhibitors), Dounce homogenized, and layered onto a cushion buffer (10 mM Tris/HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 10% sucrose, and protease inhibitors) for centrifugation. The pellet was washed once with ice-cold phosphate-buffered saline and resuspended in SDS lysis buffer (Upstate), and DNA was sheared by sonication to an average length of ~800 bp. Cell debris was removed by centrifugation and protein concentration measured by BCA kit (Pierce). Immunoprecipitation was performed using the Upstate chromatin immunoprecipitation assay kit. 2400 μg (200 μl) of sample was diluted into 2.4 ml with immunoprecipitation dilution buffer and precleared with protein A-agarose beads. 60 μl was withdrawn for input DNA. The supernatant (600 μl) was incubated with 20 μg of LXRα/β (S-20) antibody (Santa Cruz, SC-1000) at 4 °C, overnight. Antibody-protein-DNA complex was pulled down with protein A-agarose beads, and washed sequentially with low-salt, high-salt, and lithium washing buffers, then twice with 10 mM Tris-EDTA (pH 8.0). Protein-DNA complex was obtained by incubation in elution buffer (0.1 M NaHCO₃, 1% SDS) at room temperature for 15 min. DNA-protein cross-links of the eluate were reversed in 200 mM NaCl at 65 °C for 16 h. Proteins were digested by proteinase K (Roche) at 45 °C for 2 h. Immunoprecipitated DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation in the presence of glyco-gen. The DNA pellet was resuspended in 70 μl of H₂O, and 3 μl were used as template for PCR.

Primers for ChIP assays for conventional PCR and gel-based evaluation of ChIP results are as follows: LXRE-sense, 5′-ttctggactggtaagccatttt, LXRE-antisense 5′-ttctggctctggctgtaacctga; NS-sense, 5′-atctgtgtaacctgctgctg, NS-antisense 5′-taatgtcatatatgtgcatcaagtgt. For quantitative real-time PCR evaluation of ChIP results: LXRE-sense, 5′-ttctggatcgtcagccatttt, LXRE-antisense, 5′-ttctggacttcctgcctgcctg; NS-sense, 5′-acagggccatgagttg, NS-antisense, 5′-aggtataacggttgcagaggt.

Antibodies and Immunoblot Assay—Livers were lysed in buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and phosphatase inhibitor mixture (Calbiochem) and briefly centrifuged. Protein concentration was measured by the Bradford method with bovine serum albumin as a standard. 60 μg of protein were separated in 10% SDS-PAGE and transferred to nitrocellulose membrane. Rabbit polyclonal antibodies to AMP-dependent kinase (AMPK) (number 2532) and phospho-AMPKα/Thr172 (number 2531) were purchased from Cell Signaling Technology and used with ECL reagents (Amersham Biosciences).

**Statistical Analysis**

All results are expressed as the mean ± S.E. for each treatment group. GraphPad Prism software (GraphPad, San Diego, CA) was used to perform all statistical analyses. If unequal variance was indicated by Bartlett’s test, log transformation was performed prior to statistical analysis. As fully described in each figure legend, one of the following statistical tests was used: one-way analysis of variance followed by Dunnett’s post hoc comparison of treatment groups to control (Fig. 1); Student’s t test for comparison of only 2 groups (Fig. 1B); or two-way analysis of variance (factors: genotype, drug in Fig. 6) and if a significant interaction was evident, post hoc analysis by Neuman-Keuls comparison, *, p < 0.05; †, p < 0.01; and ‡‡‡, p < 0.001.

**RESULTS**

**Hepatic ChREBP mRNA Levels Are Increased in Mice Treated with Agonists of RXR and LXR—LXR agonist administration to Srebp-1c null mice results in the up-regulation of selected lipogenic enzymes (12). These same lipogenic genes are known to be directly regulated by ChREBP (19), so we tested the hypothesis that ChREBP could be a target gene of the RXR/LXR heterodimer.** Mice were fed diets containing synthetic agonists specific to various nuclear hormone receptors for 12 h (Fig. 1A). The efficacy and specificity of these agonists at doses used in these studies have been previously established (20). Hepatic RNA levels of ChREBP were increased only in mice receiving the RXR ligand (3.7-fold), the LXR ligand (3.3-fold), or a combination of RXR and LXR agonists (6.3-fold). In an independent experiment, it was also observed that the PPARγ-specific agonist GW0742 administered to mice at 5 mpk for 14 h had no effect on hepatic ChREBP RNA levels (data not shown). These RNA measurements were performed using livers from mice that were provided dietary LXR agonist and analyzed in the post-prandial state in which serum glucose and/or insulin would be expected to be elevated. However, similar increases in hepatic ChREBP RNA expression have been observed in mice given a single equivalent dose of LXR drug by oral gavage and lasted for 14 h (data not shown).

To further substantiate the role of LXR in the agonist-mediated induction of ChREBP, experiments were performed in mice lacking both LXRα and LXRβ (Fig. 1B). The Lxrα/β-double knock-out (LXR-DKO) and matched wild-type mice received synthetic agonists for RXR or LXR overnight (12 h, left panel) or for 10 days (right panel). Increased hepatic ChREBP mRNA levels were not observed in LXR-DKO mice receiving the LXR ligand, demonstrating the receptor-dependent nature of this gene regulation. The basal diet used in these studies (Fig. 1B) contained additional cholesterol to a level of 0.2% (w/w). Therefore, a modest LXR-mediated induction of ChREBP in vehicle-treated wild-type mice by the dietary cholesterol may account for the less pronounced induction by T0901317 seen in these experiments (2–2.4-fold) compared with the study shown in Fig. 1A, and the significant reduction in ChREBP mRNA levels seen in vehicle-treated LXR-DKO mice. The RXR agonist continued to elicit a significant, although reduced, induction of hepatic ChREBP in the LXR-DKO mice, suggesting that an
additional RXR-mediated pathway may exist to regulate expression of ChREBP.

Several distinct ChREBP transcripts have been identified in mouse tissues by Northern analysis (15, 21). Further evaluation of liver samples from mice treated with LXR and RXR agonists by Northern analysis revealed that all four ChREBP mRNA species (2.0, 3.9, 5.4, and 7.3 kb) show similar changes in steady-state levels (Fig. 1C). These results confirm those obtained by quantitative real-time PCR analysis. Although the exact character of the multiple ChREBP mRNA species is currently unknown, these data strongly suggest that the RXR/LXR heterodimer transcriptionally regulates the expression of all mouse ChREBP mRNA species.

**Identification and Characterization of the Mouse ChREBP Gene Promoter**—The transcriptional start site for the mouse ChREBP gene was determined using RNA ligase-mediated rapid amplification of cDNA ends, and revealed that the annotated nucleotide sequence described in the NCBI data base (NM_021455) indeed contains the full RNA sequence. Therefore, the transcriptional start site corresponds to nucleotide 451245 of the mouse genomic contig NT_080526.3 in the GenBank data base.

A nearly 4-kb fragment of the 5'-flanking region of the mouse gene was cloned and ligated into a luciferase reporter plasmid (ChBP3801, Fig. 2) and transfected into HepG2 hepatoma cells along with expression vectors for LXRα and RXRα. The full-length native promoter exhibited increased transcriptional activity in cells exposed to the RXR ligand (1.8-fold), the LXR ligand (2-fold), and a combination of RXR and LXR agonists.
In silico analysis of this promoter fragment using algorithms specific for the identification of nuclear hormone receptor binding sites (23, 24) revealed three putative RXR/LXR-responsive elements (Fig. 2A, box B).

Testing various truncation mutants of the ChREBP promoter suggested that the majority of LXR-mediated transcription required elements contained in the sequence flanked by nucleotides −2761 and −2167 (ChBP600, Fig. 2A and C). Analysis of this sequence, when placed before a minimal thymidine kinase promoter in a luciferase-reporter plasmid, confirmed that ligand addition to receptor-transfected cells resulted in a 9-fold increase in activity (Fig. 2C, ChBP600), entirely due to sequences contained within the central 250 bp of this promoter fragment (Fig. 2C, box B).

In silico analysis of this promoter fragment using algorithms specific for the identification of nuclear hormone receptor binding sites (23, 24) revealed three putative RXR/LXR-responsive elements (Fig. 2A, box B). Similar results were obtained when LXRβ was provided instead of LXRα, or when the human embryonic kidney cell line HEK 293 was used rather than HepG2 cells (data not shown). Merely providing the RXR and LXR receptors to cells resulted in enhanced basal expression of the native promoter (3-fold). Similar results were obtained when LXRα was provided instead of LXRα, or when the human embryonic kidney cell line HEK 293 was used rather than HepG2 cells (data not shown). Merely providing the RXR and LXR receptors to cells resulted in enhanced basal expression of the native promoter (3-fold). Similar results were obtained when LXRα was provided instead of LXRα, or when the human embryonic kidney cell line HEK 293 was used rather than HepG2 cells (data not shown).

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LXR Regulation of Hepatic ChREBP

A.

Insulin → LXR → SREBP-1c → ACCβ, AcCS, ACL, GPAT

Glucose → LXR → ChREBP → L-PK

ME, FAS, ACCα, SCD1

B.

LXRα

WT Chrebp/-

SREBP-1c

WT Chrebp/-

ChREBP

MLX

WT Chrebp/-

Relative mRNA level

WT Chrebp/-

L-PK

WT Chrebp/-

ME

WT Chrebp/-

FAS

WT Chrebp/-

SCD-1

WT Chrebp/-

ACCβ

WT Chrebp/-

AcCS

WT Chrebp/-

ACL

WT Chrebp/-

GPAT

WT Chrebp/-
sive elements of the DR4 type (see supplemental Fig. 1). Two of these LXREs (LXRE1 and LXRE2, Fig. 3A) were able to confer receptor/ligand-specific transactivation by a heterologous promoter in a cell reporter assay, with LXRE1 exhibiting greater activity than LXRE2 (Fig. 3B). The relevance of these short nucleotide motifs was demonstrated by evaluating their requirement in the context of the native ChREBP promoter (Fig. 3C): introducing mutations into the LXRE1 motif resulted in a greater than 50% loss in LXR-mediated transactivation; mutations within LXRE2 had a modest effect on the native promoter, but mutation of both LXREs eliminated nearly all RXR/LXR-mediated transcription of the luciferase reporter gene.

ChREBP promoter occupancy by the RXR/LXR heterodimer was tested by electrophoretic mobility shift assay, an in vitro biochemical method (Fig. 4A). Both RXR and LXR proteins were required to detect a higher order complex containing the radiolabeled LXRE probes of the ChREBP promoter, similar to that observed for the positive control LXRE of the rat cholesterol 7α-hydroxylase gene. The binding of RXR/LXR to LXRE1 is stronger than that observed for LXRE2, which correlates with the transactivation potential of these response elements as revealed by a cell reporter assay (Fig. 3B). Excess unlabeled LXRE1 or a positive-control DR4-oligonucleotide could specifically displace radiolabeled LXRE1 from this complex, but a mutated version, mLXRE1, was unable to compete for binding.

In vivo association of the RXR/LXR heterodimer to the ChREBP promoter in mouse liver was assessed by ChIP assay (Fig. 4, B and C). An αLXR antibody was capable of pulling down the LXRE-containing DNA fragment of the ChREBP gene from wild-type mouse liver, but not from liver samples of mice lacking LXR. This difference was evident, although starting (input) genomic DNA, as measured by PCR of the LXRE fragment in the absence of antibody (Fig. 4B, upper panel), was similar for the precipitations. As negative controls, 1) a nonspecific (NS) PCR assay was run that amplifies a genomic sequence located over 4 kb upstream of the mouse ChREBP transcriptional start site (and unlikely to be present in an LXRE1-containing fragment based on our 800-bp shearing size), and 2) precipitation was also performed with a nonspecific IgG in lieu of LXR antibody. A more quantitative assessment of these results was obtained by determining the abundance of precipitated genomic sequences by real-time PCR (Fig. 4C), and confirmed the observations seen with gel-based assay (Fig. 4B). In summary, these series of experiments demonstrate that the RXR/LXR heterodimer binds specifically to promoter elements to regulate transcription of ChREBP.

**ChREBP Target Gene Expression Is Increased by LXR Activation**—Although LXR binds the ChREBP promoter, and LXR drugs increase hepatic ChREBP levels, an assessment of ChREBP protein and/or activity would provide evidence that this pathway is important in hepatic lipid metabolism. The ultimate readout for activity of the transcription factor ChREBP is an increase in the amount of RNA for its target genes. However, this analysis is complicated by the fact that the majority of ChREBP target genes identified to date are also directly regulated by LXR itself (e.g. fatty acid synthase (22)) or indirectly regulated by LXR through enhanced activity of the LXR target gene SREBP-1c (e.g. ACC (25) refer to Fig. 6). In contrast, the L-PK gene appears to be uniquely regulated by ChREBP (19), and unaffected directly by LXRs or SREBP-1c. In livers of wild-type mice receiving the LXR agonist T0901317 there is a significant increase in L-PK mRNA, however, this change is not observed in LXR-DKO mice, demonstrating that ChREBP protein activity is increased by LXR activation (Fig. 5A).

The glucose-responsive element of the L-PK gene promoter is bound and regulated by ChREBP/Mlx heterotetramers (26), and has also been reported to be bound by an additional dimer consisting of the proteins TRANSILN and TRAX (27). Therefore to rule out the possibility that LXR is increasing L-PK transcription by up-regulating the expression of these other proteins, we measured their RNA levels in the samples exhibiting increased L-PK transcript levels (Fig. 5A). The LXR agonist had no effect on the expression levels of Mlx, Translin, or Trax mRNA (Fig. 5C). Interestingly, the RXR ligand caused a modest but significant increase in Mlx mRNA, most likely by its actions on an alternate permissive RXR/nuclear receptor heterodimer pair, and could possibly account for the LXR-independent increase in L-PK mRNA observed by RXR ligand in the livers of LXR-DKO mice (Fig. 5B).

In summary, these studies demonstrate that LXR activation not only increases the mRNA levels of ChREBP, but also increases the activity of this glucose-responsive transcription factor.

We also examined the phosphorylation status of AMPK because this kinase regulates the DNA-binding activity of ChREBP protein. AMPK-mediated phosphorylation of Ser566 inhibits DNA binding by ChREBP (28). Protein extracts prepared from livers of mice treated with T0901317 exhibit reduced phosphorylation of AMPK (supplemental Fig. 2) that depends on the presence of LXR. This condition would predict that LXR activation is correlated with enhanced ChREBP binding to its target genes. The molecular mechanism responsible for this reduction of AMPK phosphorylation is still under investigation, however, this observed change is consistent with previous reports in which AMPK phosphorylation is inversely correlated with the expression of SCD-1, another gene up-regulated in liver by LXR (29).

To assess the contribution of up-regulated ChREBP to the lipogenesis induced by LXR agonists, we measured the hepatic RNA levels of key lipogenic enzymes in wild-type and Chrebp null mice (Fig. 6, A and B). The deletion of ChREBP had no...

**FIGURE 6.** The contribution of ChREBP to the increase in hepatic lipogenesis following treatment with an LXR agonist. A, schematic diagram illustrating the central role of LXR in hepatic lipogenesis. AcCoA, acetyl-CoA synthetase; ACL, ATP citrate lyase; GPAT, glycerol-3-phosphate acyltransferase; ME, malic enzyme; FAS, fatty acid synthase (see Refs. 11, 14, 19, 38, and 39). B, hepatic mRNA expression of genes in wild-type (WT) and Chrebp null mice after 7 days of treatment with T0901317 (50 mpk daily by oral gavage). Data are expressed as mean ± S.E., n = 5 per group. Statistical testing was performed by two-way analysis of variance using genotype and drug as factors. No significant differences among groups were observed for LXRα and Mlx. A statistical difference due to genotype (but not drug) was evident for SREBP-1c, ACC, AcCoA, ACL, and GPAT. For the remaining genes, a significant interaction was observed between genotype and drug, demonstrating that the absence of ChREBP results in altered response to T0901317, bars with different letters are significantly different, p < 0.05.
effect on basal expression of LXRA, LXRB (not shown), or SREBP-1c, as previously reported (15). The induction of SREBP-1c by T0901317 was similar regardless of ChREBP status, and target genes thought to be exclusively regulated by SREBP-1c (ACCβ, AcCoS, ACL, and GPAT) showed similar increases by T0901317 in both wild-type and Chrebp null mice. However, those genes previously characterized as direct targets of ChREBP, such as fatty acid synthase, ACCα, malic enzyme, L-PK, and SCD-1 exhibited significantly lower induction by T0901317 in the absence of ChREBP (ACCα RNA levels were 70% of those seen in wild-type mice, data not shown). These results further support the finding that ChREBP is a direct target gene of LXR and that ChREBP serves as an intermediate in the LXR-mediated regulation of lipogenic gene expression.

**DISCUSSION**

The LXRs have been characterized as sterol sensors that regulate intracellular cholesterol levels by increasing sterol efflux into the bloodstream, cholesterol efflux into bile, or cholesterol conversion to bile acids. These properties have received great attention because they would be beneficial in disorders characterized by cholesterol accumulation. Indeed, administration of potent, selective LXR agonists to atherogenic mouse strains results in reduced aortic lesion formation (5), and even lesion regression (6). However, LXR agonists clearly increase hepatic lipogenesis resulting in increased triglyceride production (steatosis) and very low density lipoprotein secretion (hypertriglyceridemia). The cellular benefit to increasing lipogenesis in the face of excess free cholesterol is not yet entirely clear, but has been hypothesized to improve the ratio of cholesterol to other lipids in an effort to maintain plasma membrane fluidity (10). An alternative hypothesis posits that as most mammals do not ingest pure cholesterol, but rather diets containing high fats including cholesterol, the ability to store these excess calories as lipid would be beneficial (30). Either way, this enhanced lipogenesis has dampened the enthusiasm for the development of LXR drugs for therapy and will require the identification of selective LXR modulators to increase beneficial target genes with minimal effect on detrimental, in this case lipogenic targets, a strategy that has been successful for the estrogen receptor (31).

LXR-mediated lipogenesis has been largely attributed to its ability to increase the expression and activity of SREBP-1c. The results provided in this report now demonstrate that this effect is also due to increased ChREBP expression and activity. It is interesting to note that hepatic fatty acid synthesis rates are reduced in Srebp-1c knock-out mice by about 50% (12), in Chrebp-null mice by 60% (15), but in LXR-DKO mice by 80% (10), suggesting that LXR elimination is more severe due to concomitant reductions in both transcription factor targets.

Both SREBP-1c and ChREBP are regulated to a large degree by post-translational mechanisms. SREBP-1c protein is tethered in membranes of the endoplasmic reticulum and nuclear envelope, and released under low-cholesterol conditions by trafficking to the Golgi via SREBP cleavage-activating protein escort, followed by proteolytic cleavage by site 1 protease and site 2 protease to release the transcription factor portion of this protein (reviewed in Ref. 11). ChREBP is sequestered in the cytosol by virtue of PKA-mediated phosphorylation of residues that promote interaction with the protein 14-3-3 (32). High intracellular glucose levels increase the production of xylulose 5-phosphate to increase the activity of protein phosphatase 2A phosphatase to promote ChREBP nuclear localization (reviewed in Refs. 14 and 33). Therefore, it is interesting to find that LXR action, at the level of transcription, can in many circumstances override these post-translational regulatory mechanisms.

LXR agonists have been reported to reduce serum glucose levels, particularly in diabetic animal models (34–36). The capacity of LXR to concomitantly up-regulate an insulin-responsive transcription factor (SREBP-1c (37)) and a glucose-responsive transcription factor (ChREBP (14)) suggests that it enhances glucose conversion to fatty acids thereby improving hyperglycemia (Fig. 6). An LXR-mediated increase in ChREBP will enhance the terminal stages of glycolysis to produce pyruvate (via L-PK), and LXR-mediated up-regulation of SREBP-1c will promote the recycling of pyruvate from citrate-liberated oxaloacetate by malic enzyme to provide reducing equivalents necessary for fatty acid synthesis. Finally LXR-mediated increases in both ChREBP and SREBP-1c will increase expression of lipogenic enzymes to enhance fatty acid synthesis from these glycolytic end products. In total, LXR appears to serve as a master hepatic lipogenic transcriptional regulator to promote glucose conversion to lipid (Fig. 6).

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