Peroxisome Proliferator-activated Receptor-\(\gamma\) Coactivator-1\(\alpha\) Activation of CYP7A1 during Food Restriction and Diabetes Is Still Inhibited by Small Heterodimer Partner*

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Cholesterol 7\(\alpha\)-hydroxylase (CYP7A1) catalyzes the rate-limiting step in the classic pathway of hepatic bile acid biosynthesis from cholesterol. During fasting and in type I diabetes, elevated levels of peroxisome proliferator-activated receptor \(\gamma\)-coactivator-1\(\alpha\) (PGC-1\(\alpha\)) induce expression of the Cyp7A1 gene and overexpression of PGC-1\(\alpha\) in hepatoma cells stimulates bile acid synthesis. Using Ad-PGC-1\(\alpha\)-RNA interference to induce acute disruption of PGC-1\(\alpha\) in mice, here we show that PGC-1\(\alpha\) is necessary for fasting-mediated induction of CYP7A1. Co-immunoprecipitation and promoter activation studies reveal that the induction of CYP7A1 is mediated by direct interaction between PGC-1\(\alpha\) and the AF2 domain of liver receptor homolog-1 (LRH-1). In contrast, the very similar PGC-1\(\beta\) could not substitute for PGC-1\(\alpha\). We also show that transactivation of PGC-1\(\alpha\) and LRH-1 is repressed by the small heterodimer partner (SHP). Treatment of mice with GW4064, a synthetic agonist for farnesoid X receptor, induced SHP expression and decreased both the recruitment of PGC-1\(\alpha\) to the Cyp7A1 promoter and the fasting-induced expression of CYP7A1 mRNA. These data suggest that PGC-1\(\alpha\) is an important co-activator for LRH-1 and that SHP targets the interaction between LRH-1 and PGC-1\(\alpha\) to inhibit CYP7A1 expression. Overall, these studies provide further evidence for the important role of PGC-1\(\alpha\) in bile acid homeostasis and suggest that pharmacological targeting of farnesoid X receptor in vivo can be used to reverse the increase in CYP7A1 associated with adverse metabolic conditions.

Bile acids are synthesized from cholesterol in the mammalian liver and secreted into the gall bladder where they are stored along with phospholipids and cholesterol. In response to food intake, chemoceptive cells of the gastrointestinal tract release cholecystokinin, which stimulates gall bladder contraction releasing bile contents into the small intestine to facilitate digestion and absorption of dietary lipids and fat-soluble vitamins. Under normal conditions, 95% of the bile acids are reabsorbed through the distal intestine and returned to the liver and the remaining 5% are excreted into the feces along with excess cholesterol. Thus, bile acids play a crucial role in regulating mammalian cholesterol and general lipid homeostasis (1, 2). The first and rate-controlling step in the classic pathway for cholesterol conversion to bile acids is catalyzed by cholesterol 7\(\alpha\)-hydroxylase (CYP7A1).2 The activity of CYP7A1 is primarily controlled at the transcriptional level, as the gene is subject to regulation in response to a plethora of hormonal and dietary signals (3–8). Most directly, bile acids themselves have been extensively studied as signaling molecules that regulate Cyp7A1 gene expression and provide a classic negative feedback system of control (2).

The feedback mechanism is composed of several overlapping molecular pathways one of which is initiated through bile acids acting as ligand agonists for the farnesoid X receptor (FXR) (9). Ligand-activated FXR directly binds to the promoter of the small heterodimer partner (SHP) gene and induces expression of the SHP mRNA (10, 11). The SHP protein is an unusual member of the nuclear receptor family because it does not bind DNA directly but rather functions to curtail gene activation mediated by a subset of other DNA binding nuclear receptors through direct protein-protein interactions (12).

The DNA binding monomeric LRH-1 nuclear receptor is the most widely recognized target of SHP repression and LRH-1 stimulation of Cyp7A1 gene expression was the first identified target for SHP in bile acid-dependent feedback regulation (10, 11). Hepatic nuclear factor-4 (HNF-4), another important regulator of CYP7A1 (13), has also been shown to be a target for bile acid-dependent repression of Cyp7A1 (14) and can be a direct target for SHP repression as well (15). However, peptide binding and structural studies reveal there is a significant preference for SHP interacting with LRH-1 over HNF-4 and other nuclear receptors (16). In mice that overexpress SHP constitutively in the liver, CYP7A1 levels are repressed and both LRH-1 and SHP interact with the endogenous Cyp7A1 gene as shown by chromatin immunoprecipitation (ChiP) (17).

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2 The abbreviations used are: CYP7A1, cholesterol 7\(\alpha\)-hydroxylase; FXR, farnesoid X receptor; LRH-1, liver receptor homologue 1; SHP, small heterodimer partner; PGC-1, peroxisome proliferator-activated receptor-\(\gamma\) coactivator-1; HNF-4, hepatic nuclear factor-4; FGF, fibroblast growth factor; STZ, streptozotocin; PEPCK, phosphoenolpyruvate carboxykinase; ChiP, chromatin immunoprecipitation; CYP8B1, cholesterol 12\(\alpha\)-hydroxylase; GST, glutathione S-transferase; ERRE, estrogen receptor-related receptor responsive element; RNAi, RNA interference; CMV, cyto-megalovirus; WT, wild type; Pipes, 1,4-piperazinediethanesulfonic acid; LXR, liver X receptor.
CYP7A1 Activation by LRH-1 and PGC-1α in Fasting and Diabetes

In a complementary regulatory process, bile acids induce activation of the c-Jun NH₂-terminal kinase (JNK) (18), which also plays an important role in inhibition of Cyp7A1 gene expression. Additional studies suggest that bile acids modulate other intracellular signaling systems, such as protein kinase C (19), extracellular signal-regulated kinase (ERK) (20), and phosphatidylinositol 3-kinase (21). However, how these additional pathways directly impact CYP7A1 gene expression has not been fully revealed.

More recent studies have demonstrated that bile acids also induce expression of fibroblast growth factor (FGF) 19 in primary cultures of human hepatocytes (22) or the mouse orthologue FGF15 in the mouse intestine through an FXR-dependent process (23). The secreted FGF 19/15 in turn signals through FGFR4 in the liver to inhibit expression of the hepatic CYP7A1 gene.

PGC-1α was originally identified as a transcriptional co-activator that was induced in brown adipose tissue in response to hypothermic stress (24). PGC-1α is also induced in liver by fasting and diabetes where it regulates several hepatic gluconeogenic genes acting as a transcriptional co-activator for DNA binding nuclear receptor family members (25, 26). In our previous studies, we showed that CYP7A1 was induced during fasting and by streptozotocin (STZ)-induced diabetes and that a recombinant adenovirus expressing PGC-1α induced Cyp7A1 gene expression and bile acid biosynthesis in HepG2 cells (29).

In the current studies, we have extended these earlier observations to show directly that the fasting induced expression of CYP7A1 is attenuated by an RNAi targeted to PGC-1α. We also show that full-length LRH-1 is a direct target for co-activation by PGC-1α, and that a functional interaction between LRH-1 and PGC-1α is inhibited by SHP in both co-transfection studies in cultured cells and chromatin immunoprecipitation assays from mouse liver. Based on these observations we reasoned that a targeted increase of SHP in animals would prevent the fasting and diabetes-dependent induction of CYP7A1. Consistent with this prediction, we also show that treating animals with the FXR synthetic agonist GW4064 to induce SHP expression also prevented the induction of CYP7A1 associated with fasting and diabetes. These results document the effectiveness of pharmacologically targeting FXR and SHP to limit the increased Cyp7A1 gene expression during stressful and pathological metabolic conditions.

MATERIALS AND METHODS

Plasmids—3x(LRH-1)pSynTATA-Luc was generated by inserting synthetic oligonucleotides containing three copies of the LRH-1 binding site, TCAAGGCCA, of the human CYP7A1 promoter into the Sstl and NheI sites, immediately followed by TATA of the pSynTATA-Luc (27). The Sstl and NheI sites were added to the 5’ and 3’ oligonucleotides, respectively, for cloning purposes. ERRE-Luc and pcDNA3.1-PGC-1β were generously provided by Dr. B. Blumberg (University of California, Irvine) and Dr. B. Spiegelman (Harvard Medical School), respectively. pGL3R7α-342 and 3X(LXRE) were gifts from Dr. G. Gil (Virginia Commonwealth University) and Dr. Darryl Granner (Vanderbilt University). CMV-based expression constructs for SRC-1 and CBP were obtained from Dr. Chris Glass (University of California, San Diego).

pGEX-4T1.PGC-1α was constructed by PCR-based amplification of full-length PGC-1α using the following primers: forward primer, 5’-ATGGCTTGAGCATGTGCAGCCAGAGAC-3’; reverse primer, 5’-TTACCTGGCAAGCTTCTGAGC-3’. The PCR product was digested with BamHI and NotI and inserted into the corresponding restriction enzyme sites of the multiple cloning site of pGEX-4T1.

pcDNA3.1.PGC-1α was a gift from Dr. D. Kelly (Washington University, St. Louis, MO). pcDNA3.1.LRH-1/WT and pcDNA3.1.LRH-1/AF2 were described previously (28). CMV-SHP was a gift from Dr. D. Mangelsdorf (University of Texas Southwestern Medical Center).

Cell Culture and Transient Transfection Assay—293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. Transient transfection was performed by the calcium phosphate co-precipitation method, as described previously (29). Briefly, cells were seeded in 6-well dishes at a density of 3.5 × 10⁴ cells/well 1 day before transfection. 24 h later, cells were transfected with 2 μg/well of the indicated luciferase-reporter, 1 μg/well of cytomegalovirus β-galactosidase plasmid as an internal control for transfection efficiency, and the indicated amount of expression vectors as described in the figure legends. Equal amounts of DNA were used for all transfection reactions by adding empty vector DNA. After 16 h, cells were washed three times with phosphate-buffered saline, re-fed with fresh medium, and cultured for an additional 24 h before harvest. Luciferase and β-galactosidase assays and normalization were described previously (29). Values represent the mean of duplicates ± S.D. Each experiment was repeated at least three times with similar results.

Animal Treatments—Four-week-old B6129 male mice were obtained from Taconic and maintained on a 12-h light/dark cycle with free access to food and water. The mice were allowed to adapt to new environments for at least 1 week before experiments. At 8 weeks of age, mice were administered GW4064 (30 mg/kg body weight) by oral gavage twice a day and sacrificed in the following morning, 8 h after the second treatment. Streptozotocin (STZ) was injected into mice as described previously (29) to induce type I diabetes. Replication defective recombinant adenoviruses, PGC-1α RNAi or a control nonspecific RNAi (gifts from Dr. M. Montminy, Salk Institute), were propagated in 293 cells and purified by CsCl gradient centrifugation. A total of 1 × 10⁹ plaque-forming units of each adenovirus was administered into mice by intravenous injection. 7 days after adenovirus infections, mice were sacrificed for RNA analysis. For fasting experiments, mice were fasted for 24 h, as described previously (29). All mice were sacrificed between 8:00 and 10:00 a.m. Livers were removed and ~20% was frozen in liquid nitrogen and stored at −80 °C until RNA was isolated. The remaining 80% was used directly for chromatin isolation as described below for the chromatin immunoprecipitation assays.

RNA Isolation and Analysis—Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. 20 µg of total RNA was subjected to Northern analysis or qPCR analyses, as described (6, 29). An 80-base HindIII/EcoRI
fragment of rat ribosomal protein L32 cDNA was used as a control to normalize the total amount of RNA per lane. Other cDNA probes used were also described previously (29).

**Chromatin Immunoprecipitation Assays**—Mouse livers were harvested, minced with a razor blade, and cross-linked for 10 min with a final concentration of 1% formaldehyde. The cross-linking was stopped by adding glycine to a final concentration of 0.125 M. Samples were centrifuged and supernatants were removed. Cell pellets were homogenized in a Dounce homogenizer with a B pestle in buffer (5 mM Pipes, pH 7.6, 25 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM sucrose, 10% glycerol, 0.15 mM spermine, and protease inhibitors). The homogenates were layered over buffer A (10 mM Hepes, pH 7.6, 10 mM EDTA, 1% SDS, and protease inhibitors) and sonicated to reduce the size of chromatin to ~500 bases in length. Chromatin was immunoprecipitated with an anti-PGC-1α (Santa Cruz sc-H13067) and analyzed as described previously (6). The following primers were used for quantitative PCR: Cyp7A1, forward, 5′-TGGAAAGCTTCTGCCTGTTT-3′; reverse, 5′-TCCCCAGTCTGTCAAGATGAAGATC-3′ and reverse, 5′-TTGTTTTGGTCCTCCTCGCAG-3′; Pepck, forward, 5′-GTGGAAGTGACACTCAACGC-3′; reverse, 5′-AGGCGAGGCTAGCCGAGACG-3′.

**Co-immunoprecipitation Assays**—293T cells were plated in 100-mm dishes at a density of 2.5 × 10⁴ cells/dish the day before transfection. After an overnight incubation, cells were transfected with the indicated vectors for 16 h. The next day, cells were washed with phosphate-buffered saline, refed with fresh normal medium, and cultured for an additional 48 h. Cells were harvested in cell lysis buffer (50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, and protease inhibitors) and placed on ice for 30 min. Samples were centrifuged at 14,000 g for 10 min at 4 °C. After pre-clearing cell lysates with protein A beads at 4 °C for 2 h, samples were centrifuged and supernatants were incubated with an anti-PGC-1α overnight at 4 °C. After an additional incubation of the samples with protein A beads at 4 °C for 2 h, protein A beads were washed and immune complexes were eluted by SDS sample buffer and analyzed on 8% SDS-PAGE by immunoblotting with an anti-PGC-1α or an anti-FLAG M2 mouse monoclonal (Sigma).

**GST-binding Assays**—GST-PGC-1α fusion protein or GST protein alone was expressed in *Escherichia coli* BL21. Equal amounts of GST-PGC-1α or GST were bound to glutathione-agarose beads (Sigma) for 2 h at 4 °C, followed by extensive washes to remove unbound GST protein. Full-length and ΔAF2 LRH-1 proteins were produced and labeled with 35S by the TNT-coupled transcription/translation system (Promega). 35S-Labeled LRH-1/WT or LRH-1/ΔAF2 was incubated with GST-PGC-1α-bound beads for 2 h at 4 °C in HEGN buffer containing 50 mM Hepes, 0.1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 100 mM KCl, 5 mM dithiothreitol, and 0.5% nonfat dry milk, followed by three washes to remove nonspecifically bound proteins. Specific protein bound to GST-PGC-1α was eluted and subject to 8% SDS-PAGE. The gel was dried and visualized by autoradiography.

**Protein Isolation and Blotting**—Total cell lysates from 293T cells transfected with LRH-1/WT or LRH-1/ΔAF2 were fractionated on 8% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed first by Ponceau S staining to confirm that equal amounts of total protein were both loaded and transferred in each lane. Then, the blot was incubated with an anti-FLAG M2 mouse monoclonal (Sigma) followed by a secondary antibody conjugated to horseradish peroxidase. Reactivity was then detected with the ECL kit (Pierce).

**RESULTS**

**Ad-PGC-1α RNAi Reduced Fasting-induced Cyp7A1 Gene Expression**—In a previous report, we showed that PGC-1α induced Cyp7A1 gene expression in cultured cells and that Cyp7A1 was co-induced with PGC-1α during fasting and streptozotocin-induced diabetes in mice (29). To directly evaluate whether PGC-1α is necessary for fasting-induced expression of Cyp7A1, we decreased PGC-1α expression in liver by delivering an adenovirus construct expressing a PGC-1α RNAi into mice by intravenous injection. Cyp7A1 and PGC-1α mRNAs were induced by 10- and 3.5-fold, respectively, in response to fasting in control mice (Fig. 1). Hepatic PGC-1α mRNA levels were effectively reduced by the PGC-1α RNAi and this was accompanied by a significant blunting of the fasting induction of CYP7A1 mRNA (p < 0.05). Expression of PEPCK was also induced by fasting and PGC-1α RNAi treat-
CYP7A1 Activation by LRH-1 and PGC-1α in Fasting and Diabetes

FIGURE 2. PGC-1α is a co-activator for LRH-1. A, HepG2 cells were co-transfected with the indicated luciferase reporters (2 μg) along with expression vectors for PGC-1α (3 μg) or PGC-1β (3 μg) as detailed in the figure. pCMV-β-galactosidase (2 μg) was added to each transfection as an internal control for transfection efficiency. Luciferase activities were measured as described under "Materials and Methods" and were corrected for β-galactosidase activity. Results are expressed as -fold change relative to those of cells transfected with luciferase reporter alone. B and C, 293T cells were co-transfected with the indicated luciferase reporters (2 μg) along with expression vectors for PGC-1α (0.5 μg), PGC-1β (0.5 μg), or LRH-1 (0.5 μg) as detailed in the figure. pCMV-β-galactosidase (1 μg) was added to each transfection as an internal control for transfection efficiency. Luciferase activities were measured and data were analyzed as described in A.

FIGURE 3. The AF2 domain of LRH-1 is required for PGC-1α-mediated co-activation. A, a schematic view of LRH-1 wild type and LRH-1 ΔAF2 mutant is shown. B, each construct was transfected in 293T cells where total cell lysates were prepared. Protein expression levels of each construct were measured by immunoblotting with the cell lysates using an antibody against FLAG (inset). 293T cells were co-transfected with a luciferase reporter (2 μg) containing three copies of the LRH-1 binding site along with expression vectors for LRH-1-WT (0.5 μg), LRH-1-ΔAF2 (0.5 μg), and PGC-1α (0.5, 1.5, or 3.0 μg), as indicated. pCMV-β-galactosidase (1 μg) was added to each transfection as an internal control for transfection efficiency. Luciferase activities were measured and data were analyzed as described in the legend to Fig. 2A.

ment seemed to blunt the response but the effect did not reach statistical significance (p = 0.069). These data extend our previous studies and document that PGC-1α plays a key role in the induction of CYP7A1 during fasting.

PGC-1β Does Not Stimulate CYP7A1 Promoter Activity—PGC-1β is very similar to PGC-1α and is involved in many of the same physiological responses (30). However, there are differences in target gene and pathway specificity so we investigated whether the Cyp7A1 promoter was also activated by PGC-1β. When the two co-activators were compared only PGC-1α displayed appreciable co-activation for the Cyp7A1 promoter (Fig. 2A, left panel). Although, an estrogen receptor-related receptor responsive promoter (ERRE) was co-activated by both PGC-1 family members indicating the failure to co-activate Cyp7A1 was not simply due to inefficient expression of PGC-1β (Fig. 2A, right panel).

The PGC-1α responsive region of the murine Cyp7A1 gene harbors cis-acting binding sites for several transcription factors including HNF-4, LXR, and LRH-1. Although a Gal4-LRH-1 fusion protein has been shown to activate a Gal4 reporter (31), PGC-1α-dependent activation by the full-length LRH-1 protein through LRH-1 binding sites has not been specifically examined. To evaluate the ability of LRH-1 to activate transcription through an LRH-1 binding site along with PGC-1α or other co-activators, we constructed a reporter plasmid that contains three copies of the LRH-1 binding site from the human CYP7A1 promoter attached to a minimal TATA box containing the luciferase reporter gene (32). Because this reporter contains only the LRH-1 binding sites and a minimal promoter, it provides a direct readout for the transcriptional activity through LRH-1 recognition elements. As shown in Fig. 2B, addition of an LRH-1 expression construct stimulated the triple response element reporter 6-fold and inclusion of the PGC-1α expression vector greatly enhanced LRH-1-mediated transactivation. As expected, the PGC-1α construct had no activity in the absence of co-transfected LRH-1. Expression from the control promoter construct lacking the LRH-1 recognition sites was not significantly affected by either LRH-1 or PGC-1α. These results clearly indicate that PGC-1α stimulated the transcriptional potential of LRH-1 through the LRH-1 target sites in the promoter. In contrast, PGC-1β did not function as a co-activator for the LRH-1 reporter either (Fig. 2C).

The AF2 Domain of LRH-1 Is Required for PGC-1α-mediated Co-Activation—Because the carboxyl-terminal AF2 domain of nuclear receptors is important for recruitment of other co-activators, we examined whether the AF2 domain of LRH-1 might be required for PGC-1α-mediated co-activation. As shown in Fig. 3, PGC-1α enhanced transcriptional activity of the WT LRH-1 construct in a dose-dependent manner, but it had no effect when included with a construct containing a deletion of the AF2 domain. Importantly, deletion of the AF-2 domain did not alter protein expression as measured by an immunoblot detecting the FLAG epitope present in both proteins (Fig. 3B, inset).

PGC-1α Interacts with the AF-2 Domain of LRH-1—To determine whether PGC-1α might directly interact with LRH-1 through the AF-2 domain, we performed both co-immunoprecipitation and in vitro GST binding assays (Fig. 4). In the GST binding assay, the recombinant GST-PGC-1α fusion protein
CYP7A1 Activation by LRH-1 and PGC-1α in Fasting and Diabetes

FIGURE 4. LRH-1 AF2 domain interacts with PGC-1α. A, GST binding assay: GST-PGC-1α fusion protein or GST alone was expressed in E. coli and cell lysates were prepared. GST binding assays were performed using [35S]methionine-labeled full-length LRH-1 or LRH-1-ΔAF2 with GST-PGC-1α fusion protein or GST. 35S bound proteins were eluted and analyzed on 8% SDS-PAGE. Input represents 10% of the synthesis reaction used in each pull down. B, co-immunoprecipitation assay: 293T cells were transfected with different combinations of PGC-1α, 2XFLAG-LRH-1-WT, as indicated. Cell lysates were immunoprecipitated with an anti-PGC-1α antibody (Fig. 4A) along with full-length FLAG-LRH-1. The arrowhead at the left denotes a nonspecific band (NS). WB, Western blot.

FIGURE 5. PGC-1α is a preferred co-activator for LRH-1. A and B, 293T cells were co-transfected with the indicated luciferase reporters (2 µg) along with expression vectors for PGC-1α (1.5 µg), SRC-1 (1.5 µg), CBP (1.5 µg), LRα (0.05 µg), RXRα (0.05 µg), or LRH-1 (0.5 µg) as detailed in the figure. Where indicated in B, 293T cells were treated with the LXR agonist GW3965 (500 nM) for 24 h. PCMV-β-galactosidase (1 µg) was added to each transfection as an internal control for transfection efficiency. Luciferase activities were measured and data were analyzed as described in the legend to Fig. 2A. The p values for the difference between LRH-1 and PGC-1α co-transfections are 0.0021 for PGC-1α, 0.0026 for SRC-1, and 0.0037 for CBP.

directly interacted with 35S-labeled LRH-1-WT (Fig. 4A). However, this strong interaction was not observed when the AF2 deletion construct was analyzed in parallel. Co-transfection of PGC-1α along with full-length FLAG-LRH-1 resulted in co-precipitation of LRH-1 by the PGC-1α antibody (Fig. 4B). Taken together, these results indicate that PGC-1α enhances transcription through a direct protein-protein interaction with the AF2 domain of LRH-1.

PGC-1α Is a Preferred Co-activator for LRH-1—In addition to PGC-1α, other transcriptional co-activators typified by SRC-1 and CBP also interact functionally with the AF2 domain of nuclear receptors (33). Therefore, we evaluated whether these two co-activators could also function with LRH-1 (Fig. 5). Although both were similarly effective as PGC-1α in providing modest co-activation of agonist-stimulated LXR (Fig. 5B), neither one provided appreciable stimulation to LRH-1 (Fig. 5A).

SHP Inhibits PGC-1α/LRH-1 Transactivation—Upon bile acid binding, FXR induces expression of SHP, which in turn inhibits the CYP7A1 promoter. SHP does not bind directly to DNA, and its inhibitory effect requires the conserved LRH-1 binding site (10, 11). Based on this prior observation and our results described above, we reasoned that SHP might interfere with the PGC-1α-dependent co-activation of LRH-1. To test this hypothesis, we first performed studies in 293T cells where expression vectors for LRH-1, SHP, and PGC-1α were co-transfected with the LRH reporter vector analyzed above. As shown in Fig. 6A, SHP inhibited the PGC-1α-dependent stimulation of the LRH-1 reporter in a potent dose-dependent manner. This indicates that PGC-1α-dependent co-activation of LRH-1 is subject to regulation by SHP. A similar result was observed when LRH-1 stimulation of the native CYP7A1 promoter was analyzed in parallel (Fig. 6B).

To determine whether these molecular mechanistic studies might be predictive of what occurs in a more physiologic setting, we analyzed the effects of pharmacologically elevated SHP on the recruitment of PGC-1α to the CYP7A1 promoter by ChIP of mouse liver extracts (Fig. 6C). In lanes 1–4 we show that PGC-1α binding to the endogenous hepatic CYP7A1 promoter is stimulated by fasting in mice, which further documents the important role of PGC-1α in CYP7A1 induction during fasting. When mice were administered the GW4064 synthetic FXR ligand to induce SHP expression prior to fasting, the recruitment of PGC-1α was blunted. This is consistent with our model that SHP interferes with PGC-1α recruitment to the Cyp7A1 promoter. In control experiments, PGC-1α binding to the PEPCK promoter was induced by fasting but not repressed by GW4064 administration and PGC-1α binding to the Fgf15 gene was not affected by fasting or GW4064.

GW4064 Inhibits Induction of CYP7A1 mRNA during Fasting and in Diabetes—The results in Fig. 6B indicate that induction of SHP by FXR represses Cyp7A1 by decreasing the recruitment of PGC-1α to the promoter. This observation coupled with our previous studies and co-induction of PGC-1α and CYP7A1 by fasting and STZ-induced diabetes (29) predicted that GW4064 treatment might prevent the fasting dependent induction of CYP7A1. To test this prediction, we treated control, fasted, or STZ-induced diabetic mice (development of diabetes by STZ was confirmed as blood glucose levels in the mice increased to over 400 mg/dl data not shown) for 24 h with GW4064. Consistent with our earlier work, CYP7A1 mRNA levels were increased by STZ and fasting by 2- and 5.5-fold, respectively (Fig. 7), and mRNA levels for PGC-1α were also induced as expected. The mRNA for PEPCK, a key hepatic gluconeogenic enzyme analyzed as a control, was also induced by fasting and in diabetic mice. GW4064-induced SHP expression in Chow-fed control mice (p < 0.035) concomitant with a significant decrease in expression of CYP7A1 (p < 0.0083). SHP expression was also induced 3-fold by GW4064 in mice treated with STZ (p < 0.006) or 4-fold in the fasted group (p < 0.013). In agreement with our hypothesis, induction of CYP7A1 by either STZ or fasting was significantly blocked by GW4064 treatment (p < 0.013 and 0.0001, respectively). PGC-1α mRNA levels in
mRNA levels by GW4064 treatment ($p < 0.005$). PEPCK mRNA was induced by STZ and fasting and interestingly, GW4064 treatment resulted in a small but statistically significant increase in PEPCK mRNA in the chow, STZ, and fasting groups ($p < 0.001$, 0.05, and 0.01, respectively) consistent with a recent report demonstrating that PEPCK is a direct FXR target gene (34). These effects occurred without appreciable changes in LRH-1 expression.

**DISCUSSION**

Our earlier work demonstrated that PGC-1α was a key co-regulator for CYP7A1 and our current experiments utilizing RNAi knockdown and ChIP studies performed in mice significantly extend these original observations. Another important observation here is that LRH-1 is a selective and direct target for PGC-1α co-activation and we also demonstrate that SHP can disrupt the productive association between LRH-1 and PGC-1α.

Germ line disruption of LRH-1 in mice results in embryonic lethality but a recent report described the initial characterization of a mouse line with a tissue-specific deficiency of LRH-1 in hepatocytes (35). It was surprising that in the chow-fed mutant mice the basal CYP7A1 mRNA levels were similar to control animals, whereas expression of two other well characterized LRH-1 target genes, Shp and Cyp8B1, was significantly lower. At first glance, this result suggests that Cyp7A1 is not a true LRH-1 target gene. However, promoter activation (10, 11, 36) and *in vivo* ChIP studies (17) indicate the LRH-1 site is important for expression and LRH-1 binds to the Cyp7A1 promoter in liver, respectively. It is also well established that CYP7A1 expression is highly regulated by a complex physiological mechanism (1, 2).

For example, SHP negatively regulates CYP7A1 through bile acid feedback and CYP8B1 controls production of cholic acid, which is the major physiological bile acid-dependent regulator of CYP7A1 (37). The bile acid pool size is reduced in the hepatic Lrh-1 knock-out suggesting that the animal cannot increase CYP7A1 sufficiently to compensate for bile acid deficiency. In contrast, in a line of Cyp8B1 knock-out mice, there is a similar decrease in the cholic acid level but the overall bile acid pool size is actually increased (37). Interestingly, CYP7A1 mRNA levels are dramatically elevated in Cyp8B1 knock-out probably due to a misregulatory overshoot in response to the impaired bile acid feedback mechanism, which is compromised due to the loss of cholic acid (37).
The major difference between Cyp8B1 and Lrh-1 knockouts is the loss of LRH-1 only in the latter model. The decrease in cholic acid in both models would trigger bile acid-dependent de-repression but the loss of LRH-1 would not allow the full de-repression and dramatic elevation of CYP7A1 mRNA coupled with the increase in bile acid pool that occurs in Cyp8B1 knock-out. Taken together with promoter activation and ChIP studies, these observations are consistent with a key role for LRH-1 in activation of CYP7A1.

It is noteworthy that two studies analyzing CYP7A1 in an Hnf-4 liver-specific knock-out also show little change in Cyp7A1 gene expression at least during the light cycle (38), whereas there is overwhelming evidence that HNF-4, like LRH-1, is a key regulator of CYP7A1 as well (1, 2). Thus, a loss of HNF-4, LRH-1, or CYP8B1 likely leads to metabolic adaptations that result in re-balancing the level of hepatic CYP7A1.

Bile acid synthesis from cholesterol is a net oxidative process and a major role for PGC-1α in the liver is to stimulate oxidative metabolism (30). Hepatic PGC-1α is also activated during times of metabolic stress, such as diabetes and energy restriction and interaction between PGC-1α and LRH-1 is important for activation of CYP7A1 under these stressful conditions (29). However, the physiological rationale for increased cholesterol oxidation along with increased mitochondrial oxidative metabolism is not presently clear. Nonetheless, the pathophysiology associated with altered bile acid regulation during food restriction and diabetes is likely to be relevant to important clinical observations. For example, there is an increased risk of gallstone disease associated with rapid weight loss in obese patients (39) and the use of bile acid-binding resins improves insulin signaling and diabetes in both animal models and human diabetic patients (40, 41).

Our animal experiments demonstrate that the pharmacologic application of a synthetic FXR agonist can increase SHP and reverse the fasting and diabetes-dependent increase in CYP7A1 (Fig. 7). The additional mechanistic studies provide a molecular pathway for these effects. SHP has a significantly higher affinity for LRH-1 than for other tested activators including HNF-4 (16), which supports our model that the LRH-1/PGC-1α interaction is key to fasting induction of CYP7A1 and is preferentially sensitive to inhibition at relatively lower absolute levels of SHP. The modest but significant increase in hepatic SHP expression is not likely by itself to fully account for the efficient suppression of CYP7A1 in diabetes and fasting shown in Fig. 7. However, GW4064 also increases production of intestinal FGF15 that is important for hepatic CYP7A1 repression (23) and this pathway is also likely to be important in the suppression of CYP7A1 observed in Fig. 7. These results predict that FXR agonists such as GW4064 might prove beneficial in managing complications associated with rapid weight loss and diabetes.

The closely related PGC-1α and β proteins have overlapping but separate roles in metabolism (30). For example, PGC-1β seems to be more active in stimulating genes associated with oxidative phosphorylation relative to PGC-1α. In this regard, it is important to note that our studies show that PGC-1β does not stimulate CYP7A1 nor does it function as a co-activator for LRH-1 (Fig. 2). Thus, activation of CYP7A1 through LRH-1 using PGC-1α but not PGC-1β is likely an important molecular mechanism separating activation of oxidative pathways that use reducing equivalents to make ATP versus oxidative pathways that use reducing equivalents to directly synthesize important cellular biochemical components.

CYP7A1 is also subject to SHP repression under conditions where PGC-1α is not activated. In this case, the mechanism likely is due to the inhibition of the association of other factors such as HNF-4 or LXR with co-activators along with direct repression of transcription mediated by SHP interacting with transcriptional repressor complexes (42).

The recognition that PGC-1α regulates CYP7A1 first established a molecular linkage between glucose metabolism, diabetes, and bile acid metabolism (29, 43). Subsequent reports have extended these findings suggesting that FXR agonist treatment and bile acid binding resin therapy improves the diabetic phenotype (34, 40, 45). More recently, it was demonstrated that PGC-1α gene expression is directly regulated by SHP (46) providing even more evidence for an important connection between bile acids and glucose metabolism.

It is interesting that along with PGC-1α being regulated by SHP, the Ship gene has also been reported to be activated by PGC-1α (47) and PGC-1α also activates FXR expression (44). Thus the PGC-1 interacting network has evolved with regulatory checks and balances to prevent dramatic changes in gene expression that would result in aberrant metabolic flux.

The blunting of STZ-induced PGC-1α expression by the synthetic FXR agonist treatment in our studies (Fig. 7) is consistent with the direct regulation of PGC-1α by SHP as well. Interestingly, this inhibition was only evident relative to the increased expression in STZ-treated mice and was not observed in either the chow-fed or fasted groups. This suggests that SHP inhibits an activator responsible for PGC-1α induction specifically in diabetes and serves to further highlight the selectivity of the SHP mechanism for repression.

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