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Mechanisms for increased expression of cholesterol 7α-hydroxylase (Cyp7a1) in lactating rats

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Abstract

Cholesterol 7α-hydroxylase (Cyp7a1) and the bile acid pool size are increased 2–3 fold in lactating postpartum rats. We investigated the interaction of nuclear receptors with the *Cyp7a1* proximal promoter and the expression of regulatory signaling pathways in postpartum rats at day 10 (PPd10) vs female controls to identify the mechanisms of increased expression of Cyp7a1, which is maximal at 16 h. Liver X receptor (LXRα) and RNA Polymerase II (RNA Pol II) recruitment to Cyp7a1 chromatin were increased 1.5- and 2.5-fold, respectively, at 16 h on PPd10. Expression of nuclear receptors farnesoid X receptor (FXR), LXRα, liver receptor homologue (LRH-1), hepatocyte nuclear factor 4α (HNF4α), and short heterodimer partner (SHP) mRNA and co-activator peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α) mRNA was unchanged in PPd10 vs controls at 16 h, while chicken ovalbumin upstream transcription factor II (COUP-TFII) was decreased 40% at 16 h. Investigation of a repressive signaling pathway, the cJun-N-terminal kinase (JNK) signaling pathway in PPd10 vs controls, showed decreased mRNA expression of hepatocyte growth factor (HGF; decreased 60% at 16 h) and tyrosine kinase receptor cMet (decreased 44–50% at 16 h), but these were not accompanied by decreased expression of phosphorylated c-Jun. Importantly, expression of Fibroblast Growth Factor 15 (FGF15) mRNA in the ileum was decreased 70% in PPd10 vs controls, while phosphorylated mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (Erk1/2) protein expression in liver was decreased 88% at 16 h.

Conclusion—The increased recruitment of LXRα, a Cyp7a1 stimulatory pathway, and decreased expression of FGF15 and phosphorylated Erk1/2, a Cyp7a1 repressive pathway, combined to increase Cyp7a1 expression during lactation.

Keywords

bile acids; FXR; JNK/c-Jun; FGF15; LXRα; PGC1α; ERK1/2

Introduction

Cholesterol 7α-hydroxylase (Cyp7a1) catalyzes the rate-limiting step of the classical pathway of bile acid synthesis and is the major mechanism for cholesterol catabolism and removal from the body (1). Bile acids form mixed micelles with phospholipids and cholesterol in bile, which is essential for both the absorption and biliary excretion of cholesterol. Rodent Cyp7a1 expression increases in response to cholesterol-enriched diets (2–4), resulting in increased bile acid synthesis, while feedback inhibition prevents the accumulation of cytotoxic hydrophobic bile acids. The regulation of Cyp7a1 expression is thus critical for maintenance of cholesterol and bile acid homeostasis.

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Several nuclear receptors activate transcription of *Cyp7a1*. LXRα binds to LXR response elements in the proximal rat (5,6) and mouse (7) *Cyp7a1* promoter, following activation by oxysterols. *LXR*$^{-/-}$ mice lack induction of Cyp7a1 in response to cholesterol enriched diets, resulting in the accumulation of cholesterol in the livers of these animals (7). Studies *in vitro* (5) and *in vivo* (8) show that oxysterol activation of LXRα increases expression of Cyp7a1, while LRH-1 serves as an auxiliary factor that promotes LXRα activation of *Cyp7a1* (9). The orphan nuclear receptors HNF4α and COUP-TF II also bind to the proximal region of the rat and human *CYP7A1* promoter and, in transiently transfected HepG2 cells, act synergistically to increase transcription 80-fold (10,11).

Several negative feedback loops within the liver and small intestine down-regulate *Cyp7a1* transcription to prevent accumulation of bile acids to toxic levels. In liver, hydrophobic bile acids activate FXR-mediated transcription of SHP, which binds to LRH-1, decreasing recruitment of co-activators to the proximal promoter and decreasing transcription of *Cyp7a1* (12–14). Treatment of primary human hepatocytes with HGF activates the tyrosine kinase receptor c-Met and the JNK/c-Jun pathway, which leads to decreased transcription of *Cyp7a1* and decreased bile acid synthesis (15). In ileum, FXR activation by bile acids induces expression of FGF15 and the ileal bile acid binding protein (IBABP); the latter serves to maintain a low unbound cellular bile acid concentration (16). FGF15 is secreted into the portal circulation and binds to the hepatic tyrosine kinase FGF receptor 4 (FGFR4), which activates the JNK signaling pathway, causing loss of coactivator binding to the proximal *Cyp7a1* promoter (16–18). The Erk1/2 pathway in human hepatocytes was also recently shown to be an important factor mediating the FGF15/19 decrease in CYP7A1 expression (19). In this manner, increased intestinal bile acids inhibit hepatic bile acid synthesis via a tightly controlled negative feedback loop.

We recently demonstrated an increase in the size and hydrophobicity of the bile acid pool in lactating rats from days 10–21 postpartum (PP) (20). The increase in the bile acid pool coincided with a two-fold increase in the mRNA and protein expression and enzyme activity of Cyp7a1 that occurred at PPd10 and at 16 h (10 h of light) (20). The maximal expression at 16 h represents a shift in the diurnal rhythm of Cyp7a1 in lactation, since maximal Cyp7a1 expression is normally observed late in the dark cycle. Neither the concentration of bile acids nor the expression of SHP in the liver were changed at 16 h, most likely due to increased expression of both Ntcp, the Na^+-dependent bile acid uptake transporter on the basolateral domain of the hepatocyte (21), and Bsep (Abcb11), the ATP-dependent bile acid efflux transporter on the canalicular domain of the hepatocyte (22). In order to determine the mechanisms by which Cyp7a1 expression might be increased in lactation, we utilized chromatin immunoprecipitation (ChIP) to investigate the recruitment of transcription factors to the *Cyp7a1* promoter, and focused on the 16 h time point as the time of increased expression of Cyp7a1. Furthermore, we measured mRNA and protein expression levels of selected transcription factors and relevant signaling pathways in liver and ileum, and observed increased recruitment of LXRα and significantly decreased expression of a negative regulatory factor, phosphorylated Erk1/2, in lactating vs. control female rat liver that coincided with decreased expression of FGF15 in ileum. The decreased expression of the FGF15 inhibitory signaling pathway provides a mechanism by which the bile acid mediated feedback-inhibition of Cyp7a1 is attenuated in lactating rats.

Material and Methods

Chemicals and reagents

TRIzol Reagent and SuperScript III First-Strand Synthesis System for RT-PCR were purchased from Invitrogen Life Technologies (Carlsbad, CA); LightCycler (version 3.5), LightCycler DNA Master SYBR Green I, LightCycler 480, master mix/universal probes,

Complete EDTA-free, Protease Inhibitor Cocktail Tablets from Roche Diagnostics (Indianapolis, IN); EZ ChIP kit and RNA Pol II antibody(17–371) from Millipore (Billerica, MA), and RNeasy Mini kit from Qiagen Inc. (Valencia, CA). Antibodies were obtained as follows: HNF4α (sc-8987X), PGC-1α (sc-13067X), CBP (sc-583), COUP-TF II/ARP-1 (sc-6576), LXR α (sc-1202X), c-Jun (sc-1694), c-p-Jun (sc-7981), GAPDH (sc-25778), and anti-goat IgG horseradish peroxidase (HRP) (sc-2020) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-rabbit IgG HRP (NA934V) and ECL-Plus Reagent from GE Healthcare (Piscataway, NJ); p44/42 MAPK (Erk1/2) (137F5) and Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) from Cell Signaling Technology (Danvers, MA).

Animal care

Female Sprague-Dawley rats (Harlan Industries; Indianapolis, IN) were maintained in a temperature-controlled environment on a 12 hour light/dark cycle (6 am lights on/6 pm lights off). Animals had free access to Teklad Global Diet 2018 (Harlan Laboratories; Cincinnati, OH) and water. For determination of mRNA expression, female virgin rats (control group, C) and post-partum (PP) animals were sacrificed at 4 h (10 h of dark), 10 h (4 h of light), 16 h (10 h of light), and 22h (4 h of dark). For all experiments, litter size was culled within 24 h of birth to 8–11 pups. All protocols involving animals were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Chromatin Immunoprecipitation Assays (ChIP)

The ChIP protocol was adapted from previous studies (15,23,24) with the following modifications. Rat liver (~0.24 g) was harvested from control or PPd10 rats, washed in 5 ml of phosphate buffered saline (PBS) containing protease inhibitors (Complete EDTA-free, Protease Inhibitor Cocktail Tablets), minced, and washed an additional three times to remove blood. Cross-linking in a 1%-formaldehyde solution for 30 minutes with gentle mixing was followed by 10-minute incubation with 0.125 M glycine at room temperature. Cross-linked samples were centrifuged at 700×g at 4°C for 4 minutes, washed with PBS, and resuspended in 6 times the packed cell volume of homogenization buffer (10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 2 M sucrose, 10% glycerol) and incubated on ice for 10 minutes. The pellets were collected by centrifugation at 1000xg for 4 minutes, resuspended in 1XSDS lysis buffer (EZ ChIP kit) and incubated on ice for 15 minutes. Samples were sonicated (Fisher Scientific, 550 Sonic Dismembrator, setting 2) to provide DNA fragments between 1500 to 150 base pairs. Insoluble material was removed by centrifugation at 14,000xg for 20 minutes at 4°C. Soluble sonicated material from three animals was pooled for each group and the immunoprecipitation reaction was done with the EZ ChIP kit according to protocol instructions. The following antibodies were used to immunoprecipitate proteins interacting with chromatin: 15 μg of HNF4α, PGC-1α, and CBP; 10 μg of COUP-TF II/ARP-1 and LXRα; and 2μg of RNA Pol II. For negative controls, we performed ChIP assays with an IgG antibody, and also with the COUP-TFII antibody followed by amplification of the intron region (+2485 to +2879) of Cyp7a1 to show immuno-specificity of the antibodies for the Cyp7a1 promoter. Real-time PCR was performed to amplify the −204 to +20 region of the rat *Cyp7a* promoter with a reaction mix containing forward 5′-CTTCAGCTTATCGAGTATTG-’ 3 and reverse 5′-CTAGCAAAGCAAGGCTGTC-’3 oligonucleotides, and cycle conditions − 94°C for 0 sec, 55°C for 30 sec, 72°C for 30 sec in a Roche Light Cycler using SYBR Green Master Mix. For the standard curve, serial dilutions of input DNA (0.1, 0.01, 0.001, and 0.0001) were amplified, and the cycle number vs. the logof the fluorescence measurement at the threshold was plottedto generate a standard curve.

Real-Time PCR

Real-time PCR was performed on a Roche LightCycler with a SYBR Green kit (SHP, PGC-1α, HNF4α, and 18S) and the LightCycler 480 with LightCycler 480 master mix/ universal probes (LXRα, FXRα, LRH-1, COUP-TF II, HGF, c-Met, c-Jun, FGF-15, IBABP). Total RNA from liver and terminal ileum was isolatedwith TRIzol Reagent and purified with the Qiagen RNeasy Mini kit, followed by cDNA synthesis with SuperScript III. Dilutions of cDNA template were amplified, and the cycle number vs. the log of the fluorescence measurement at the threshold plotted to generate a standard curve for semiquantitation of mRNA. Conditions used for amplification using the LightCycler were: denaturation for 30 s at 94°C and 40 cycles of 94°C for 0 s; 57°C for 25 s (SHP), 57°C for 10 s (HNF4α), 55°C for 30 s (PGC-1α), 56°C for 20 s (18S); and 72°C for 30 s (18S, PGC-1α), 25 s (SHP), 15 s (HNF4α). The primers for gene sets amplified with the LightCycler for SHP and 18S have been described (20); HNF4α (forward, 5′-TGGCAAACACTACGGAGCCT-’3; reverse, 5′-CTGAAGAATCCCTTGCAGCC-’3); PGC-1α (forward, 5′-ATGAATGCAGCGGTCTTAGC-’ 3; reverse, 5′-TGGTCAGATACTTGAGAAGC-’3). The real-time PCR reaction for Cyp7a1 amplification was performed as previously described (20). For the LightCycler 480, primers and probe sets were designed using the Roche Universal Probe Library to amplify intronic-spanning regions of the gene. Reactions were as follows: denaturation for 5 min at 95°C, and 40 cycles of 95°C for 15 s; 60°C for 30 s.

Western blot analysis

Whole liver was homogenized in buffer (40 mM Tris pH 7.6, 140 mM NaCl, plus protease inhibitors), centrifuged at 1,000× g, and 100 μg of supernatant protein separated on 6–12% Tris-glycine gel and electrophoretically transferred to nitrocellulose membranes. Membranes were immunoblotted overnight at 4°C with 1:200 c-Jun or p-c-Jun, 1:1000 p44/42 MAPK or p-p44/42 MAPK followed by incubation with 1:10,000 IgG HRP antibodies. Membranes were then immunoblotted with GAPDH as a control for equal loading. Immunoreactive bands were visualized (ECL–Plus Reagent) and exposed to film followed by densitometric analysis.

Bile Acid Measurements

Bile acids were harvested from the duodenum, jejunum, proximal ileum, and terminal ileum and measured as previously described (20).

Statistical analysis

Data are expressed as mean ± S.E.M. for n = 3–10 animals per group. Statistical analysis was performed with Student’s t-test, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, or two-way ANOVA test followed by Bonferroni’s test with GraphPad Prism 4.0 software (San Diego, CA), or as indicated in figure legends.

Results

Determination of Transcription Factor Binding to the *Cyp7a1* Promoter (−204 to +20) in Control and PPd10 Rats

The bile acid pool size is increased 2.7-fold in PPd10 rats and corresponds with a shift in the diurnal rhythm and a 2-fold increased expression of Cyp7a1 at 16 h (10 h of light, on 12/12 light dark cycle) (Fig 1A) (20). To determine if the recruitment of transcription factors to the *Cyp7a1* promoter were changed at this time, we performed ChIP analyses with antibodies specific for transcription factors known to up-regulate the transcription of rat *Cyp7a1*. Negative controls showed that the immunoprecipitations were specific for the indicated

antibodies (Figure 1B). We found a 2.5-fold increased (P<0.005) recruitment of RNA Pol II to the *Cyp7a1* promoter (Figure 1C) at PPd10, consistent with increased transcription of *Cyp7a1*. Recruitment of LXRα was also increased 1.5-fold at PPd10 relative to controls at 16 h (P<0.05), while increased COUP-TF II binding to the promoter did not reach statistical significance (P=0.0571, Mann-Whitney U-test) (Figure 1C). Neither binding of the orphan nuclear receptor HNF4α nor the co-activators PGC-1α or CBP were changed at PPd10 relative to controls (Figure 1C), nor was chromatin binding of the acetyl-histone H3 or the targets of bile acid mediated repression (9), acetyl-histone H4 and the histone deacetylase H7 (data not shown).

Expression of Nuclear Receptors in PPd10 Rats

The hepatic mRNA expression levels of LXRα, LRH-1, and FXR at 16 h showed no significant differences between groups, although COUP-TF II mRNA expression was decreased 40% (P<0.05) in PPd10 rats (Figure 2). Because PGC-1α (25) and HNF4α (26) have a circadian rhythm, we measured their mRNA expression throughout the light/dark cycle. A significant circadian rhythm was observed for both HNF4α mRNA (Figure 3a) and PGC-1α mRNA (Figure 3b). There was no significant difference in the mRNA levels for HNF4α between control and PPd10 rats, however, the mRNA expression of PGC-1α was significantly increased 4.3-fold at 10 h (4 h of light) in PPd10 rats relative to controls.

Decreased Activation of Bile Acid-Induced Repressive Signaling Pathways

Bile acids induce the expression of SHP by activation of both FXR and the JNK signaling pathways (15,18,27), which decrease Cyp7a1 expression (12,28). Measurement of hepatic mRNA expression of HGF and the HGF target genes c-Met, c-Jun, and SHP throughout the light/dark cycle showed an overall change in HGF mRNA expression in lactation and a significant 60% decrease at PPd10 relative to controls at both 16 h (10 h of light) and 22 h (4 h of dark) (Figure 4a). c-Met mRNA expression was changed in lactation and showed significant decreases at 16 h (44%; 10 h of light) and 22 h (50%; 4 h of dark) in PPd10 rats relative to controls (Figure 4b). The mRNA expression of c-Jun was changed in lactation (lactation effect, P<0.01; Two-Way ANOVA) and showed a significant 62% decrease at 22 h relative to controls (Figure 4c). Similar to previous findings (29,30), SHP mRNA expression exhibited a significant circadian rhythm and lactation altered its expression pattern (Figure 4d). In agreement with our previous study (20), SHP mRNA was not changed at 16 h when Cyp7a1 expression is increased, but was markedly decreased 72% at 22 h in PPd10 rats relative to controls (Figure 4d).

Intestinal bile acids are important in regulating hepatic Cyp7a1 expression (31). We therefore measured the concentrations of intestinal bile acids and the mRNA expression of FXR, IBABP, and FGF15 in the terminal ileum. The bile acid concentrations peaked in the jejunum and proximal ileum in both control and lactating rats (PP days 7–12) (Figure 5a) and did not differ significantly per g tissue weight, so that we did not anticipate changes in IBABP or FGF15 mRNA expression. Surprisingly, FGF15 mRNA expression was markedly decreased (70%) at 16 h in PPd10, while neither FXR nor IBABP mRNA expression levels were significantly different (Figure 5b).

In view of the decreased expression of HGF and its target genes c-Met, c-Jun, and SHP in liver, and decreased FGF15 in ileum, we investigated expression of their relevant signaling pathways in liver, i.e., total and phosphorylated c-Jun and Erk1/2. Measurement of protein expression of total and phosphorylated c-Jun showed that total c-Jun was significantly decreased (Figure 6a), whereas phosphorylated c-Jun was not different at 16 h (Fig 6b), but were decreased at 22 h (data not shown), consistent with decreased expression of c-Jun and SHP mRNA at 22 h. However, expression of total Erk1/2 was decreased about 40% (Figure

6c), while expression of phosphorylated Erk1/2 was markedly decreased by 88% at 16 h in PPd10 rats relative to controls (Figure 6d). Expression of total and phosphorylated Erk1/2 had returned to control levels at 22 h (data not shown). The decreased expression of the FGF15 signaling pathways in liver at 16 h is consistent with increased expression of Cyp7a1 at this time.

Discussion

Lactation in rats is accompanied by a 2–3-fold increase in the size and hydrophobicity of the bile acid pool that coincides with a 2-fold increased expression and activity of Cyp7a1 (20). The increase in Cyp7a1 expression occurs in mid- to late lactation and at 16 h (10 of light), rather than 22 h (4 h of dark) as seen in control animals (20). In the present studies we examined the mechanisms by which Cyp7a1 expression is increased and demonstrated a 2.5-fold increased binding of RNA Pol II to the *Cyp7a1* promoter at 16 h, indicative of increased recruitment of transcription machinery essential for increased Cyp7a1 mRNA expression. Recruitment of LXRα to the *Cyp7a1* promoter in PPd10 rats relative to controls at 16 h was also significantly increased, consistent with increased *Cyp7a1* transcription. Increased activation of LXRα could be facilitated by increased ligand availability due to the well-established increases in HMG-CoA reductase activity (32) and cholesterol synthesis (32–34) demonstrated in liver in rats at mid-lactation. We did not detect significant differences in the recruitment of other transcription factors or co-activators (HNF4α, COUP-TFII, CBP, PGC1-α) to the *Cyp7a1* promoter at 16 h, even though mRNA expression of COUP-TFII was decreased at 16 h, and expression of PGC1-α was increased at 10 h.

We also examined the signaling pathways known to inhibit Cyp7a1 expression, and found that hepatic expression of HGF and cMet mRNA was significantly decreased at 16 and 22 h, while mRNA expression of c-Jun and SHP was decreased at 22 h. However, expression of phosphorylated c-Jun protein was not decreased at 16 h, making it unlikely that downregulation of this signaling pathway played a significant role in the increased expression of Cyp7 at 16 h. Importantly, we also found a marked decrease in the ileal expression of FGF15 at 16 h, and this coincided with a profound 88% decrease in phosphorylated Erk1/2 at 16 h. Song et al (19) recently showed that treatment of human hepatocytes with FGF19 did not activate phosphorylation of JNK or alter SHP expression, but did induce rapid phosphorylation of ERK 1/2; these authors concluded that the MAPK/Erk1/2 pathway plays a major role in mediating FGF19 inhibition of CYP7A1. The present data strongly support the hypothesis that decreased expression of FGF15 and decreased activation FGFR4/βKlotho and phosphorylation of Erk1/2 is the predominant mechanism for increasing Cyp7a1 expression in the rat in lactation. These data also support the hypothesis that SHP is not important in regulating Cyp7a1 under normal physiological conditions (35), consistent with the finding that deletion of the *Shp* gene in mice does not prevent bile acid inhibition of Cyp7a1 mRNA expression (28,36). Interestingly, FGF15, acting via FGFR4/β-Klotho in an autocrine manner, decreases expression of Asbt in the enterocyte (37). In earlier studies, we showed a two-fold increase in Asbt protein expression, together with increased Na^+-dependent taurocholate transport in brush border membrane vesicles and increased taurocholate uptake in an intestinal loop model in the proximal ileum in middle and late lactation rats (38). Thus, the loss of FGF15 expression appears to play a critically important role in increasing the size of the bile acid pool in lactation by permitting increased Cyp7a1 expression and activity in the liver, and increasing Asbt expression in the terminal ileum.

FGF15 is an FXR target gene, and bile acids are thereby known to increase its expression. However, mechanisms responsible for decreased FGF15 expression in lactation are not known. Lactation is a physiological state of negative energy balance due to the 4–5-fold

increased energy demand generated by the suckling pups (39). Adaptations to the state of negative energy balance in the lactating rat include hypothyroidism, hypoinsulinemia and hypoleptinemia (39). The latter is thought to facilitate the hyperphagia observed in lactation, since leptin decreases appetite, but leptin also increases energy expenditure (40). FGF19 also increases the metabolic rate and fatty acid oxidation (40), suggesting a rationale, although not a mechanism, for a decrease in its expression in lactation. We postulate that the hyperphagia, together with the increased hydrophobicity and size of the bile acid pool in lactation, are likely to enhance the absorption of lipids and lipid-soluble nutrients, thus compensating for the negative energy balance in lactation. The mechanism for decreased HGF expression is also not known, but HGF stimulates glucose uptake and metabolism in skeletal muscle (41), again providing a possible rationale for its decreased expression in lactation. HGF is secreted in milk, and is thought to act as a trophic factor in the intestine of the newborn (42); further, HGF and c-Met expression are markedly decreased in the lactating mammary gland, possibly by prolactin (43).

The lactating dam provides an important model system to characterize the regulation of Cyp7a1 and bile acid synthesis. A number of novel adaptations contribute to the increase in Cyp7a1 expression despite an increased size of the bile acid pool that are not seen in mice deficient in various regulatory genes, and/or in animals fed bile acids. Thus, prolactin acts early in lactation (within 48 h) to increase expression of Ntcp and Bsep (21,22), preventing an increase in the intracellular concentration of bile acids and activation of FXR and SHP (20) that would inhibit bile acid synthesis. During mid-lactation when the energy demand generated by the growing pups increases significantly (39), the expression and activity of Asbt and Cyp7a1 increase together with the size of the bile acid pool (20,38). The increasing size of the intestine (44) accommodates the increased bile acid pool size, so that again the intracellular concentration of bile acids does not increase and FXR is not activated in the terminal ileum. Prolactin and FGF15 are two hormones critical to this adaptation that is essential to facilitate the absorption of sufficient lipids, cholesterol and important lipid-soluble nutrients to ensure the survival of the dam and an adequate production of milk for survival of the pups. An important question we are currently investigating is the mechanism for the decreased expression of FGF15 in the ileum of lactating rats.

In summary, we have demonstrated increased recruitment of RNA Pol II and LXR to the *Cyp7a1* promoter, consistent with the significantly increased mRNA and protein expression, as well as enzymatic activity of Cyp7a1 at 16 h in lactating rats. Importantly, the expression of a Cyp7a1 repressive pathway, i.e., expression of FGF15 in the ileum, was decreased in lactation, together with a marked decrease in expression of phosphorylated Erk1/2 in the liver at the time of maximal expression of Cyp7a1. These findings strongly support the hypothesis that the decreased feed-back inhibitory mechanisms for Cyp7a1 permit the recruitment of nuclear receptors and activation of *Cyp7a1* transcription at mid-lactation, the time of maximal increases in the size of the bile acid pool. These data also indicate that FGF15 acting via FGFR4/βKlotho in the liver is an important regulator of bile acid synthesis in the rat.

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List of Abbreviations

CBP	cAMP response element binding protein (CREB)-binding protein

COUP-TF II	chicken ovalbumin upstream transcription factor II
ChIP	chromatin immunoprecipitation assay
Cyp7a1	cholesterol 7α-hydroxylase
ERK1/2	mitogen-actiavated protein kinase/extracellular signal-regulated kinase 1/2
FGF15	fibroblast growth factor 15
FGFR4	fibroblast growth factor receptor 4
FXR	farnesoid X receptor
HGF	hepatocyte growth factor
HNF4α	hepatocyte nuclear factor 4α
IBABP	ileal bile acid binding protein
JNK	c-Jun N-terminal kinase
LRH-1	liver receptor homologue 1
LXR	liver X receptor
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1α
RNA Pol II	RNA Polymerase II
SHP	short heterodimer partner

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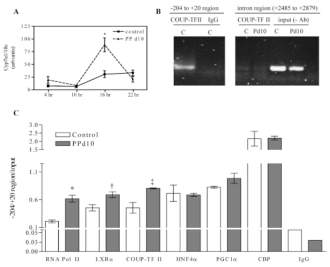

Figure 1.

Increased expression of Cyp7a1 and recruitment of RNA Pol II and LXRα to Cyp7a1 chromatin at 16 h of PPd10 rats. (**A**) Cyp7a1 mRNA analysis was performed by real-time PCR as described (20). ChIP analysis was performed on chromatin samples from the livers of control and PPd10 rats killed at 16 h and with antibodies against RNA Pol II, LXRα, COUP-TF II, HNF-4α, PGC-1α, CBP, and IgG. **(B)** PCR of ChIP Assay Controls**. Left panel,** PCR products of the −204 to +20 region of the rat *Cyp7a1* promoter of ChIP assays using extracts from control rats and COUP-TF II and IgG antibodies and **Right panel**, PCR products of the +2485 to +2879 region of the rat intron region of *Cyp7a1* of ChIP assays using extracts from control and PPd10 rats and COUP-TF II antibody or input samples (chromatin extract, pre-immunoprecipation). **(C)** The −204 to +20 region of the rat Cyp7a1 promoter was amplified by real-time PCR. The data were normalized to input values for each sample and show quantitation of the −204/+20 region in control and PPd10 rats. Each bar represents the mean ± S.E.M of 3–4 rats per group. *P<0.005, † P<0.05, vs. control rats as determined by Student's t-test; ‡ P=0.0571, PPd10 vs control rats (Mann-Whitney U-test).

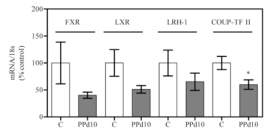

Figure 2.

The expression of LXRα, FXR, LRH-1, and COUP-TFII mRNA at mid-lactation at 16 h. Real-time PCR was performed in duplicate on cDNA synthesized from liver total RNA from control female rats and PPd10 rats at 16 h for n=4–9 animals per group. Each bar represents the mean ± S.E.M. *P<0.05 vs. control, Student's t-test.

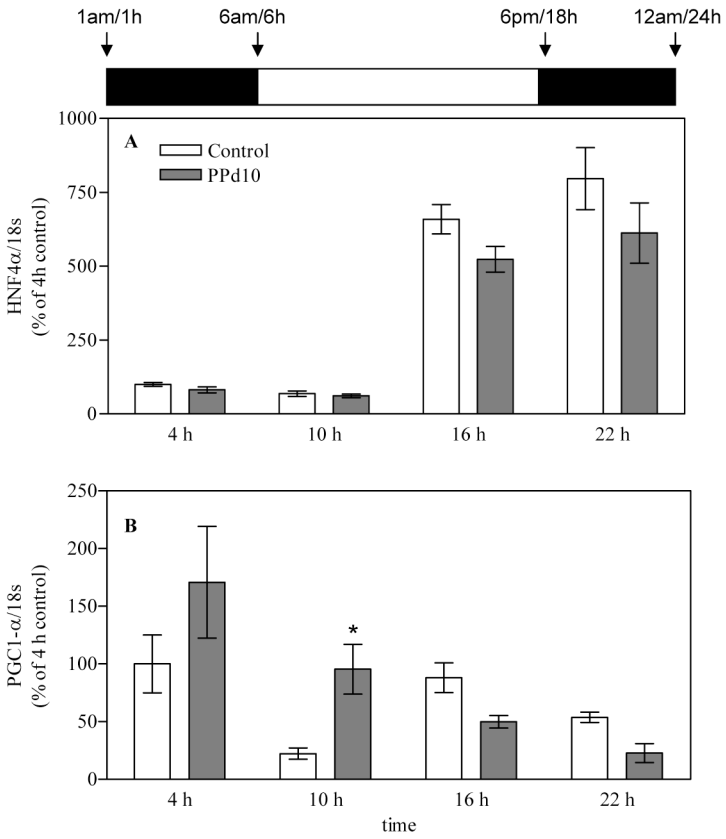

Figure 3.

The expression of HNF4α and PGC-1α mRNA at mid-lactation. Real-time PCR was performed in duplicate on cDNA synthesized from liver total RNA from control female rats and PPd10 rats at 4, 10, 16, and 22 h. Each bar represents the mean ± S.E.M. (n=3–8 animals per group) **(A)** HNF4α mRNA expression was unchanged between groups; however a significant circadian rhythm was found (time effect, $P<0.0001$; Two-Way ANOVA, $P<0.0001$) **(B)** PGC-1α mRNA expression showed a significant interaction between treatment and time (interaction, $P<0.05$; time effect, $P<0.005$; Two-Way ANOVA, $P<0.05$). The protected Fisher's LSD test (Student's t-test with the Satterthwaite correction for unequal variances) showed significantly increased expression of PGC-1α mRNA at 10 h (* $P=0.0258$) vs control.

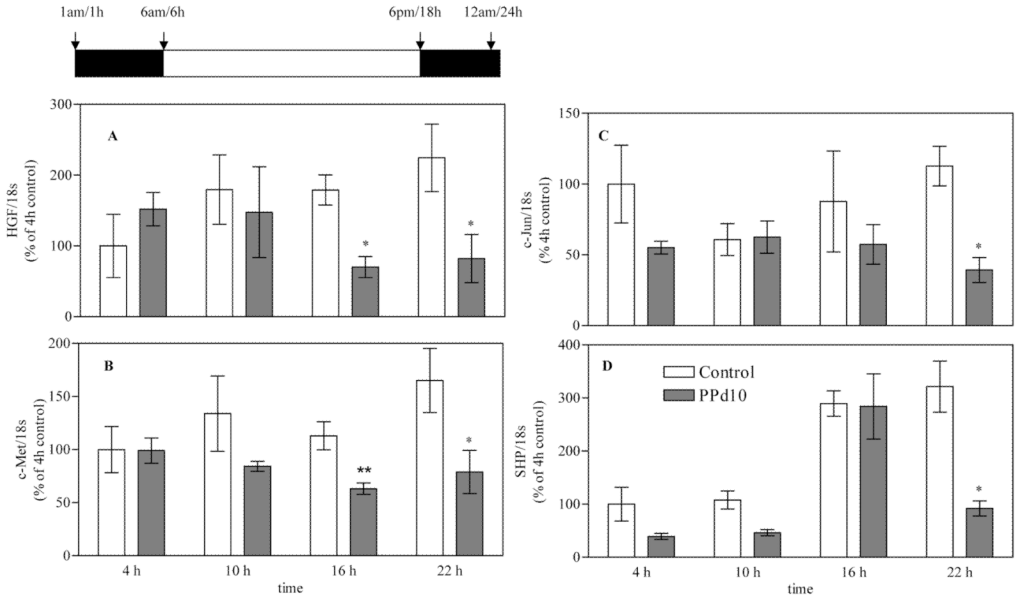

Figure 4.

The expression of HGF, c-Jun, c-Met, and SHP mRNA at mid-lactation. Real-time PCR was performed in duplicate on cDNA synthesized from liver total RNA from control female rats and PPd10 rats at 4, 10, 16, and 22 h. Each bar represents the mean + S.E.M. (n=3–10 animals per group). Statistical differences were determined by Two-Way ANOVA followed by a Bonferroni correction, or as otherwise indicated. **(A)** HGF mRNA expression was decreased at 16 and 22 h in PPd10 rats (* $P<0.05$) and a significant effect of lactation ($P<0.05$). **(B)** c-Met was decreased at 16 h (**$P<0.01$) and 22 h (*$P<0.05$) using the protected Fisher's LSD test (Student's t-test with the Satterthwaite correction for unequal variance) in PPd10 rats and showed a significant lactation effect ($P<0.005$). **(C)** c-Jun mRNA expression was decreased at 22 h in PPd10 rats (*$P<0.05$) with a significant effect of lactation ($P<0.05$) **(D)** SHP mRNA was decreased at 22 h ($P<0.05$) with significant effects of time ($P<0.0001$) and lactation ($P<0.01$).

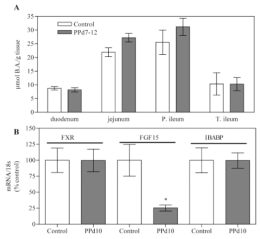

Figure 5.

Bile acid concentration in the small intestine, and FXR, FGF15, and IBABP mRNA expression at 16 h in the terminal ileum of control and PPd7–12 rats. **(A)** Total bile acids (BA) as μmol/g tissue (including contents) in the duodenum, jejunum, and proximal (P) or terminal (T) ileum of control and PPd 7–12 rats. Each bar represents the mean ± S.E.M for n=4 animals per group. **(B)** Real-time PCR was performed in duplicate on cDNA synthesized from ileal total RNA from control female rats and PP rats at 16 h for n=4–15 animals per group. Each bar represents the mean ± S.E.M. * $P<0.05$, PPd10 vs. control, Student's t-test.

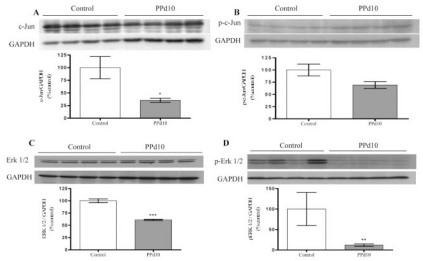

Figure 6.

The expression of c-Jun and phosphorylated-c-Jun (p-c-Jun) and Erk 1/2 and phosphorylated-Erk 1/2 (p-Erk 1/2) at 16 h. The upper panels show the immunoblots of c-Jun (**A**), p-c-Jun (**B**), Erk 1/2 (C) and p-Erk 1/2 (D) in liver homogenate. The bars represent the mean ± SEM of protein intensity normalized to GAPDH intensity for $n = 4$ animals in control and PPd10 rats. ***$P < 0.0001$ and * $P < 0.03$ vs. control as determined by Student's *t*-test and **$P < 0.0286$ vs. control rats as determined Mann Whitney U-test.